US009447401B2

(12) **United States Patent**
Wei et al.

(10) **Patent No.: US 9,447,401 B2**
(45) **Date of Patent: Sep. 20, 2016**

(54) **PH20 POLYPEPTIDE VARIANTS, FORMULATIONS AND USES THEREOF**

(71) Applicants: **Ge Wei**, San Diego, CA (US); **H. Michael Shepard**, San Diego, CA (US); **Qiping Zhao**, San Diego, CA (US); **Robert James Connor**, Oceanside, CA (US)

(72) Inventors: **Ge Wei**, San Diego, CA (US); **H. Michael Shepard**, San Diego, CA (US); **Qiping Zhao**, San Diego, CA (US); **Robert James Connor**, Oceanside, CA (US)

(73) Assignee: **Halozyme, Inc.**, San Diego, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 387 days.

(21) Appl. No.: **13/694,731**

(22) Filed: **Dec. 28, 2012**

(65) **Prior Publication Data**

US 2013/0302275 A1 Nov. 14, 2013
US 2014/0178330 A9 Jun. 26, 2014

Related U.S. Application Data

(60) Provisional application No. 61/796,208, filed on Nov. 1, 2012, provisional application No. 61/631,313, filed on Dec. 30, 2011.

(51) **Int. Cl.**

C12N 9/26	(2006.01)
C12N 15/00	(2006.01)
A61K 38/47	(2006.01)
A61K 38/28	(2006.01)
A61K 45/06	(2006.01)
A61K 9/00	(2006.01)
A61K 47/10	(2006.01)
C12Q 1/34	(2006.01)
A61K 38/00	(2006.01)

(52) **U.S. Cl.**

CPC ***C12N 9/2474*** (2013.01); ***A61K 9/0019*** (2013.01); ***A61K 38/28*** (2013.01); ***A61K 38/47*** (2013.01); ***A61K 45/06*** (2013.01); ***A61K 47/10*** (2013.01); ***C12Q 1/34*** (2013.01); ***C12Y 302/01035*** (2013.01); *G01N 2333/926* (2013.01); *G01N 2333/928* (2013.01)

(58) **Field of Classification Search**

None

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

3,536,809	A		10/1970	Applezweig	424/28
3,598,123	A		8/1971	Zaffaroni	424/435
3,630,200	A		12/1971	Higuchi	424/427
3,710,795	A		1/1973	Higuchi et al.	424/424
3,845,770	A		11/1974	Theeuwes et al.	424/427
3,916,899	A		11/1975	Theeuwes et al.	424/424
4,002,531	A		1/1977	Royer	435/188
4,008,719	A		2/1977	Theeuwes et al.	424/427
4,044,126	A		8/1977	Cook et al.	514/180
4,179,337	A		12/1979	Davis et al.	435/181
4,364,923	A		12/1982	Cook et al.	424/46
4,414,209	A		11/1983	Cook et al.	514/180
4,475,901	A	*	10/1984	Kraegen et al.	604/67
4,769,027	A		9/1988	Baker et al.	424/493
4,952,496	A		8/1990	Studier et al.	435/91.41
4,980,286	A		12/1990	Morgan et al.	435/371
5,033,252	A		7/1991	Carter	53/425
5,052,558	A		10/1991	Carter	206/439
5,059,595	A		10/1991	Le Grazie	424/468
5,073,543	A		12/1991	Marshall et al.	514/21
5,120,548	A		6/1992	McClelland et al.	424/473
5,122,614	A		6/1992	Zalipsky	548/520
5,323,907	A		6/1994	Kalvelage	206/531
5,324,844	A		6/1994	Zalipsky	548/520
5,446,090	A		8/1995	Harris	525/54.1
5,591,767	A		1/1997	Mohr et al.	514/413
5,612,460	A		3/1997	Zalipsky	530/391.9
5,639,476	A		6/1997	Oshlack et al.	424/468
5,643,575	A		7/1997	Martinez et al.	424/194.1
5,672,662	A		9/1997	Harris et al.	525/408
5,674,533	A		10/1997	Santus et al.	424/493
5,721,348	A		2/1998	Primakoff	536/22.1
5,733,566	A		3/1998	Lewis	424/426
5,766,581	A		6/1998	Bartley et al.	424/85.1
5,795,569	A		8/1998	Bartley et al.	424/85.1
5,808,096	A		9/1998	Zalipsky	548/520
5,854,046	A		12/1998	Au-Young et al.	435/201
5,900,461	A		5/1999	Harris	525/54.11
5,919,455	A		7/1999	Greenwald et al.	424/178.1
5,932,462	A		8/1999	Harris et al.	435/188
5,958,750	A		9/1999	Au-Young et al.	435/201
5,985,263	A		11/1999	Lee et al.	424/85.2
5,990,237	A		11/1999	Bentley et al.	525/54.2
6,057,110	A		5/2000	Au-Young et al.	435/6
6,113,906	A		9/2000	Greenwald et al.	424/194.1

(Continued)

FOREIGN PATENT DOCUMENTS

CN	102065886	A	5/2011
EP	0400472		12/1990

(Continued)

OTHER PUBLICATIONS

Have et al. (1998) Cloning and characterization of the cDNA encoding the PH20 protein in the European red fox *Vulpes vulpes*, Reproduct. Fertil. Dev., vol. 10, pp. 165-172.*

(Continued)

Primary Examiner — Manjunath Rao
Assistant Examiner — Samuel Liu
(74) *Attorney, Agent, or Firm* — Dentons US LLP; Stephanie Seidman

(57) **ABSTRACT**

Modified PH20 hyaluronidase polypeptides, including modified polypeptides that exhibit increased stability and/or increased activity, are provided. Also provided are compositions and formulations and uses thereof.

49 Claims, 13 Drawing Sheets

(56) References Cited

U.S. PATENT DOCUMENTS

6,193,963	B1	2/2001	Stern et al.	424/94.6
6,214,966	B1	4/2001	Harris	528/322
6,258,351	B1	7/2001	Harris	424/78.3
6,340,742	B1	1/2002	Burg et al.	530/351
6,413,507	B1	7/2002	Bentley et al.	424/78
6,420,339	B1	7/2002	Gegg et al.	514/12
6,437,025	B1	8/2002	Harris et al.	523/406
6,448,369	B1	9/2002	Bentley et al.	528/425
6,461,802	B1	10/2002	Van Thillo et al.	430/336
6,461,863	B1	10/2002	Jarvis	435/320.1
6,495,659	B2	12/2002	Bentley et al.	528/425
6,737,505	B2	5/2004	Bentley et al.	528/425
6,828,401	B2	12/2004	Nho et al.	526/333
6,858,736	B2	2/2005	Nho et al.	546/290
7,105,330	B2	9/2006	Stern et al.	435/200
7,279,457	B2	10/2007	Pohl	514/3
7,585,940	B2	9/2009	Skerra et al.	530/350
7,767,429	B2	8/2010	Bookbinder et al.	435/201
7,781,397	B2	8/2010	Stern et al.	424/94.62
7,829,081	B2	11/2010	Bookbinder et al.	424/94.62
7,846,431	B2	12/2010	Bookbinder et al.	424/94.62
7,871,607	B2	1/2011	Bookbinder et al.	424/94.62
8,105,586	B2	1/2012	Bookbinder et al.	424/94.3
8,187,855	B2	5/2012	Baker et al.	435/201
8,202,517	B2	6/2012	Bookbinder et al.	424/94.62
8,257,699	B2	9/2012	Bookbinder et al.	424/94.62
8,318,154	B2	11/2012	Frost et al.	424/94.5
8,343,487	B2	1/2013	Baker et al.	424/94.62
8,401,194	B2 *	3/2013	Nierzwick et al.	380/270
8,431,124	B2	4/2013	Bookbinder et al.	424/94.62
8,431,380	B2	4/2013	Bookbinder et al.	435/201
8,450,470	B2	5/2013	Bookbinder et al.	536/23.2
8,568,713	B2	10/2013	Frost et al.	424/94.5
8,580,252	B2	11/2013	Bookbinder et al.	424/85.2
8,765,685	B2	7/2014	Bookbinder et al.	514/20.9
8,772,246	B2	7/2014	Bookbinder et al.	435/200
9,284,543	B2	3/2016	Wei et al.	435/201
2001/0021763	A1	9/2001	Harris	528/75
2001/0044526	A1	11/2001	Shen	530/409
2001/0046481	A1	11/2001	Bentley et al.	424/78.18
2002/0052430	A1	5/2002	Harris et al.	523/406
2002/0072573	A1	6/2002	Bentley et al.	525/409
2002/0156047	A1	10/2002	Zhao	514/58
2003/0017108	A1	1/2003	Zamora et al.	424/1.49
2003/0114647	A1	6/2003	Harris et al.	530/402
2003/0143596	A1	7/2003	Bentley et al.	435/6
2003/0158333	A1	8/2003	Roberts et al.	530/402
2003/0220447	A1	11/2003	Harris	528/322
2004/0013637	A1	1/2004	Bentley et al.	424/78.17
2004/0235734	A1	11/2004	Bossard et al.	514/12
2004/0268425	A1	12/2004	Bookbinder et al.	800/18
2005/0054048	A1	3/2005	Grasso et al.	424/133.1
2005/0114037	A1	5/2005	Desjarlais et al.	702/19
2005/0171328	A1	8/2005	Harris	528/322
2005/0209416	A1	9/2005	Harris	525/523
2005/0260186	A1	11/2005	Bookbinder et al.	424/94.62
2006/0104968	A1	5/2006	Bookbinder et al.	424/94.62
2007/0067855	A1	3/2007	Jarvis et al.	800/13
2007/0189962	A1	8/2007	Pastan	424/133.1
2009/0042785	A1	2/2009	Matschiner et al.	514/12
2009/0123367	A1	5/2009	Bookbinder et al.	424/1.49
2009/0181032	A1	7/2009	Bookbinder et al.	424/141.1
2009/0214505	A1	8/2009	Bookbinder et al.	424/94.1
2009/0253175	A1	10/2009	Bookbinder et al.	435/69.1
2009/0304665	A1	12/2009	Frost et al.	424/94.5
2009/0305982	A1	12/2009	Jensen et al.	435/69.1
2009/0311237	A1	12/2009	Frost et al.	424/94.62
2010/0003238	A1	1/2010	Frost et al.	424/94.62
2010/0074885	A1	3/2010	Schiff et al.	424/130.1
2010/0143457	A1	6/2010	Wei et al.	424/450
2010/0172892	A1	7/2010	Uvarkina et al.	424/94.62
2011/0152359	A1	6/2011	Bookbinder et al.	435/200
2012/0020951	A1	1/2012	Shepard et al.	424/130.1
2012/0148555	A1	6/2012	Bookbinder et al.	435/200
2012/0171153	A1	7/2012	Frost et al.	424/94.62
2012/0213767	A1	8/2012	Wei et al.	424/450
2012/0251517	A1	10/2012	Frost et al.	424/94.62
2012/0251620	A1	10/2012	Bookbinder et al.	424/450
2012/0294830	A1	11/2012	Bookbinder et al.	424/85.2
2013/0011378	A1	1/2013	Yang et al.	424/94.3
2013/0022588	A1	1/2013	Yang et al.	424/94.3
2013/0022592	A1	1/2013	Vaughn et al.	424/94.62
2013/0058893	A1	3/2013	Bookbinder et al.	435/200
2013/0202583	A1	8/2013	Jiang et al.	424/94.62
2013/0251786	A1	9/2013	Li et al.	424/94.62
2013/0302400	A1	11/2013	Maneval et al.	435/195
2014/0037613	A1	2/2014	Bookbinder et al.	424/94.62
2014/0105824	A1	4/2014	Shepard et al.	424/9.2
2014/0135682	A1	5/2014	Frost et al.	424/94.5
2014/0199282	A1	7/2014	Bookbinder et al.	435/200
2015/0010529	A1	1/2015	Wei	424/94.62

FOREIGN PATENT DOCUMENTS

EP	0822199	9/2004
EP	1064951	8/2007
JP	2007-153797	6/2007
WO	WO 92/16640	10/1992
WO	WO 94/28024	12/1994
WO	WO 00/02017	1/2000
WO	WO 01/87925	4/2001
WO	WO 02/49673	6/2002
WO	WO 2004/056312	7/2004
WO	WO 2005/000360	1/2005
WO	WO 2009/128917	10/2009
WO	WO 2009/134380	11/2009
WO	WO 2010/077297	7/2010
WO	WO 2010/149772	12/2010
WO	WO 2012/136768	11/2012
WO	WO 2012/174478	12/2012
WO	WO 2013/102144	7/2013
WO	WO 2015/003167	1/2015

OTHER PUBLICATIONS

Attachment M3WD76 Felca (2015, updated) Hyaluronidase/PH20 from Felis catus, pp. 1-3.*

U.S. Appl. No. 13/999,454, filed Feb. 26, 2014.

U.S. Appl. No. 14/120,224, filed May 7, 2014.

U.S. Appl. No. 14/323,932, filed Jul. 3, 2014.

Letter/Written Disclosure of the Supplemental Information Disclosure Statement for the above-referenced application, mailed Jul. 10, 2014, 2 pages.

Maksimenko et al. "Resistance of dextran-modified hyaluronidase to inhibition by heparin," Biochemistry (Mosc). 66(4):456-463 (2001).

Reitinger et al., "Designed human serum hyaluronidase 1 variant, HYALIDeltaL, exhibits activity up to pH 5.9," J Biol Chem. 284(29):19173-19177 (2009).

Schantéet al., "Improvement of hyaluronic acid enzymatic stability by the grafting of amino-acids," Carbohydrate Polymers, 87(3):2211-2216 (2012).

Jiang et al., "Effects of recombinant human PH20 (rHuPH20) on interstitial matrices: creating a favorable environment for the delivery of cytostatic agents," [abstract]. In: Proceedings of the 96th Annual Meeting of the American Association for Cancer Research; Apr. 16-20, 2005; Anaheim, CA.:AACR; 2005. vol. 46, p. 1198, Abstract No. 5075, XP001525054, Apr. 2005.

Morrow et al., "Human hyaluronidase coinjection accelerates insulin pharmacokinetics and glucodynamics of 3 rapid insulin analogs," American Diabetes Association Scientific Sessions, held on Jun. 28, 2010 in Orlando, FL., Abstract # 353-OR, 2 pages.

Nagy, Z., "Prospective study on rHuPH20," European Society of Human Reproduction & Embryology Annual Meeting, Jun. 19-22, 2005, Copenhagen, Denmark. Abstract O-213 (2005), 1 page.

Partial International Search Report, sent by facsimile Sep. 4, 2013, in connection with corresponding International Patent Application No. PCT/US2012/072182, 6 pages.

International Search Report and Written Opinion, mailed Dec. 17, 2013, in connection with corresponding International Patent Application No. PCT/US2012/072182, 18 pages.

(56) **References Cited**

OTHER PUBLICATIONS

Response, dated Mar. 17, 2014, to Written Opinion, mailed Dec. 17, 2013, in connection with corresponding International Patent Application No. PCT/US2012/072182, 94 pages.
Second Written Opinion, mailed May 21, 2014, in connection with corresponding International Patent Application No. PCT/US2012/072182, 8 pages.
Letter/Written Disclosure of the Supplemental Information Disclosure Statement for the above-referenced application, filed herewith Oct. 20, 2014, 2 pages.
Morrow et al., "Human hyaluronidase coinjection accelerates insulin pharmacokinetics and glucodynamics of 3 rapid insulin analogs," American Diabetes Association Scientific Sessions, held on Jun. 28, 2010 in Orlando, FL, Abstract # 353-OR, 2 pages.
Second Written Opinion, mailed Jul. 30, 2014, in connection with International Patent Application No. PCT/US2012/072182, 9 pages.
Response, dated Sep. 1, 2014, to Second Written Opinion, mailed Jul. 30, 2014, in connection with International Patent Application No. PCT/US2012/072182, 73 pages.
International Preliminary Report on Patentability, mailed Sep. 12, 2014, in connection with International Patent Application No. PCT/US2012/072182, 11 pages.
Letter/Written Disclosure of the Supplemental Information Disclosure Statement for the above-referenced application, filed herewith Sep. 2, 2015, 2 pages.
"PEGPH20: The Science & The Strategy," presented at the J. P. Morgan Healthcare Conference on Jan. 7, 2015, 81 pages.
Torley, H., "Halozyme Therapeutics, Inc. The next chapter begins: creating value through growth," presented at the 32nd Annual J.P. Morgan Healthcare Conference Jan. 7, 2014, 26 pages.
News Release, "Halozyme announces presentation of clinical data at the American Diabetes Association 74th Scientific Sessions," Published on Jun. 15, 2014 [online]Retrieved from:<URL: halozyme.com/Investors/News-Releases/News-Release-Details/2014/Halozyme-Announces-Presentation-Of-Clinical-Data-At-The-American-Diabetes-Association-74th-Scientific-Sessions/default.aspx [retrieved on Aug. 18, 2014], 3 pages.
Response, dated Jul. 21, 2014, to second Written Opinion, mailed May 21, 2014, in connection with International Patent Application No. PCT/US2012/072182, 72 pages.
Office Action, issued May 25, 2015, in connection with Chinese Patent Application No. 201280070954.9 [English language translation and original document in Chinese], 7 pages.
U.S. Appl. No. 13/507,540, filed Jul. 6, 2012.
U.S. Appl. No. 13/694,005, filed Oct. 18, 2012.
U.S. Appl. No. 13/694,071, filed Oct. 24, 2012.
U.S. Appl. No. 13/815,311, filed Feb. 19, 2013.
Letter/Written Disclosure of the Supplemental Information Disclosure Statement for the above-referenced application, filed herewith Apr. 8, 2013, 2 pages.
Adams et al., "The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice," Nature 318:533-538 (1985).
Alexander et al., "Expression of the c-myc oncogene under control of an immunoglobulin enhancer in E mu-myc transgenic mice," Mol. Cell Biol. 7:1436-1444 (1987).
Ansel, H., *Introduction to Pharmaceutical Dosage Forms*, Fourth Edition, Lea & Febiger:Philadelphia, p. 126 (1985).
Anttila et al., "High levels of stromal hyaluronan predict poor disease outcome in epithelial ovarian cancer," Cancer Rearch 60:150-155 (2000).
Arming et al., "In vitro mutagenesis of PH-20 hyaluronidase from human sperm," Eur J Biochem 247(3):810-814 (1997).
Atkinson, M. And E. Leiter, "The NOD mouse model of type 1 diabetes: as good as it gets?" Nature Med. 5:601-604 (1999).
Auvinen, P. "Hyaluronan in peritumoral stroma and malignant cells associates with breast cancer spreading and predicts survival," American Journal of Pathology 156(2):529-536 (2000).
Baumgartner et al., "Phase I study in chemoresistant loco-regional malignant disease with hyaluronidase," Reg. Cancer Treat. 1:55-58 (1988).
Beckenlehner et al., "Hyaluronidase enhances the activity of adriamycin in breast cancer models in vitro and in vivo," J. Cancer Res. Oncol. 118:591-596 (1992).
Benhar et al., "Pseudomonas exotoxin A mutants. Replacement of surface-exposed residues in domain III with cysteine residues that can be modified with polyethylene glycol in a site-specific manner," J. Biol. Chem. 269:13398-13404 (1994).
Bianchi et al., "Synthetic depsipeptide substrates for the assay of human hepatitis C virus protease," Anal. Biochem. 237: 239-244 (1996).
Bitter et al., "Expression and secretion vectors for yeast," Methods in Enzymol 153:516-544 (1987).
Bonner, W. and E. Cantey, "Colorimetric method for determination of serum hyaluronidase activity," Clin. Chim. Acta 13:746-752 (1966).
Bookbinder et al., "A recombinant human enzyme for enhanced interstitial transport of therapeutics," J Control Release, 114:230-241 (2006).
Bordier C., "Phase separation of integral membrane proteins in Triton X-114 solution," J Biol Chem. 256(4):1604-1607 (1981).
Bouffard et al., "An in vitro assay for hepatitis C virus NS3 serine proteinase," Virology 209:52-59 (1995).
Brinster et al., "Regulation of metallothionein—thymidine kinase fusion plasmids injected into mouse eggs," Nature 296:39-42 (1982).
Brown et al., "Codon utilisation in the pathogenic yeast, *Candida albicans*," Nucleic Acids Res. 19(15):4298 (1991).
Brumeanu et al., "Derivatization with monomethoxypolyethylene glycol of Igs expressing viral epitopes obviates adjuvant requirements," J Immunol. 154:3088-3095 (1995).
Caliceti, P. and F. Veronese, "Pharmacokinetic and biodistribution properties of poly(ethylene glycol)-protein conjugates,"Adv. Drug Deliv. Rev. 55(10):1261-1277 (2003).
Carrillo, H. and D. Lipman, "The multiple-sequence alignment problem in biology," SIAM J Applied Math 48:1073-1082 (1988).
Cefalu, W., "Animal models of type 2 diabetes: clinical presentation and pathophysiological relevance to the human condition," ILAR Joumal 47(3):186-198 (2006).
Chapman et al., "Therapeutic antibody fragments with prolonged in vivo half-lives," Nature Biotech. 17:780-783 (1999).
Cheng et al., "PEGylated adenoviruses for gene delivery to the intestinal epithelium by the oral route," Pharm. Res. 20(9):1444-1451 (2003).
Cherr et al., "The dual functions of GPI-anchored PH-20: hyaluronidase and intracellular signaling," Matrix Biol. 20:515-525 (2001).
Cherr et al., "The PH-20 protein in cynomolgus macaque spermatozoa: identification of two different forms exhibiting hyaluronidase activity," Dev. Biol., 175:142-153, 1996.
Cho et al., "Construction of hepatitis C-SIN virus recombinants with replicative dependency on hepatitis C virus serine protease activity," J. Virol. Meth. (65):201-207 (1997).
Chowpongpang et al., "Cloning and characterization of the bovine testicular PH-20 hyaluronidase core domain," Biotechnol Lett. 26(15):1247-1252 (2004).
Colbere-Garapin et al., "A new dominant hybrid selective marker for higher eukaryotic cells," J Mol Biol 150:1-14 (1981).
Conserved domain search from U.S. Appl. No. 10/795,095 of SEQ ID No. 6, Primakoff et al. U.S. Pat. No. 5,721,348, performed on the NCBI website on Aug. 5, 2008.
Csoka et al., "Hyaluronidases in tissue invasion," Invasion Metastasis 17:297-311 (1997).
Csoka et al., "Purification and microsequencing of hyaluronidase isozymes from human urine," FEBS Lett., 417(3):307-310 (1997).
Csoka et al., "The six hyaluronidase-like genes in the human and mouse genomes," Matrix Biol. 20:499-508 (2001).
Czejka et al., "Influence of hyaluronidase on the blood plasma levels of 5-fluorouracil in patients," Pharmazie 45:H.9 (1990).
De Maeyer et al., "The growth rate of two transplantable murine tumors, 3LL lung carcinoma and B16F10 melanoma, is influenced

(56) References Cited

OTHER PUBLICATIONS

by Hyal-1, a locus determining hyaluronidase levels and polymorphism," Int. J. Cancer 51:657-660 (1992).
De Salegui et al., "A comparison of serum and testicular hyaluronidase," Arch. Biochem. Biophys. 121:548-554 (1967).
DeBoer et al., "The tac promoter: a functional hybrid derived from the trp and lac promoters," Proc. Natl. Acad. Sci. USA 80:21-25 (1983).
Delpech et al., "Enzyme-linked hyaluronectin: a unique reagent for hyaluronan assay and tissue location and for hyaluronidase activity detection," Anal. Biochem. 229:35-41 (1995).
Dorfinan, A. and M. Ott, "A turbidimetric method for the assay of hyaluronidase," J. Biol. Chem. 172:367-375 (1948).
D'Souza et al., "In vitro cleavage of hepatitis C virus polyprotein substrates by purified recombinant NS3 protease," J. Gen. Virol. 76:1729-1736 (1995).
Duttaroy et al., "Development of a long-acting insulin analog using albumin fusion technology," Diabetes 54(1):251-258 (2005).
Elder et. al, "Intra-arterial hyaluronidase in severe peripheral arterial disease," Lancet 648-649 (1980).
Ernst et al., "Enzymatic degradation of glycosaminoglycans," Critical Reviews in Biochemistry and Molecular Biology 30(5):387-444 (1995).
European Pharmacopoeia, Chapter 5.1.3, "Efficacy of antimicrobial preservation," 447-449 (2005).
Fankhauser, N. and P.Mäser, "Identification of GPI anchor attachment signals by a Kohonen self-organizing map," Bioinformatics 21(9) 1846-1852 (2005).
Felix et al., "Pegylated peptides. IV. Enhanced biological activity of site-directed pegylated GRF analogs," Int. J. Peptide Res. 46:253-264 (1995).
Few, B., "Hyaluronidase for treating intravenous extravasations," MCN Amer. J. Matern. Child Nurs. 12(1):23 (1987).
Filocamo et al., "Chimeric Sindbis viruses dependent on the NS3 protease of hepatitis C virus," J.Virology 71:1417-1427 (1997).
Forsburg, S., "Codon usage table for Schizosaccharomyces pombe," Yeast. 10(8):1045-1047 (1994).
Fransson et al., "Solvent effects on the solubility and physical stability of human insulin-like growth factor I," Pharm Res. 14(5):606-612 (1997).
Frost, G. and R. Stern, "A microtiter-based assay for hyaluronidase activity not requiring specialized reagents," Anal. Biochem. 251:263-269 (1997).
Gardner et al., "The complete nucleotide sequence of an infectious clone of cauliflower mosaic virus by M13mp7 shotgun sequencing," Nucleic Acids Res. 9:2871-2888 (1981).
GenBank Accession No. AAC60607, PH-20 [*Homo sapiens*], published on Jun. 5, 2000 [online] [retrieved on Dec. 12, 2012][retrieved from the Internet: URL:<ncbi.nlm.nih.gov/protein/AAC60607], 1 page.
Gilbert, W. and L. Villa-Komaroff, "Useful Proteins from Recombinant Bacteria," Scientific American 242(4):74-94 (1980).
Girish et al., "Hyaluronidase inhibitors: a biological and therapeutic perspective," Curr Med Chem 16(18):2261-2288 (2009).
Gmachl et al., "The human sperm protein PH-20 has hyaluronidase activity," FEBS 336(3):545-548 (1993).
Good et al., "Hydrogen ion buffers for biological research," Biochemistry 5(2):467-477 (1966).
Gribskov et al., "Sigma factors from *E. coli, B. subtilis*, phage Sp01, and phage T4 are homologous proteins," Nucl. Acids Res. 14(16):6745-6763 (1986).
Grosschedl et al., "Introduction of a mu immunoglobulin gene into the mouse germ line: specific expression in lymphoid cells and synthesis of functional antibody," Cell 38:647-658 (1984).
Guiotto et al., "An improved procedure for the synthesis of branched polyethylene glycols (PEGs) with the reporter dipeptide Met-betaAla for protein conjugation," Bioorg. Med. Chem. Lett. 12:177-180 (2002).
Guntenhoner et al., "A substrate-gel assay for hyaluronidase activity," Matrix 12:388-396 (1992).
Guo et al., "Protein tolerance to random amino acid change," Proc. Nat'l. Acad. Sci. USA 101:9205-9210 (2004).
Hahm et al., "Generation of a novel poliovirus with a requirement of hepatitis C virus protease NS3 activity," Virology 226:318-326 (1996).
Haller et al., "Escaping the interstitial matrix with enzyme-mediated drug delivery," Drug Delivery Technology, 5(5):1-6 (2005).
Hamatake et al., "Establishment of an in vitro assay to characterize hepatitis C virus NS3-4A protease trans-processing activity ," Intervirology 39:249-258 (1996).
Hammer et al., "Diversity of alpha-fetoprotein gene expression in mice is generated by a combination of separate enhancer elements," Science 235:53-58 (1987).
Hanahan, D., "Heritable formation of pancreatic beta-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes," Nature 315(6015):115-122 (1985).
Harris, J. and R. Chess, "Effect of pegylation on pharmaceuticals," Nat Rev Drug Discov 2(3):214-221 (2003).
Hartman S. and R. Mulligan, "Two dominant-acting selectable markers for gene transfer studies in mammalian cells," Proc Natl Acad Sci 85:8047-8051 (1988).
Herrera-Estrella et al., "Expression of chimaeric genes transferred into plant cells using a ti-plasmid-derived vector,"Nature 303:209-213 (1984).
Herrera-Estrella et al., "Light-inducible and chloroplast-associated expression of a chimaeric gene introduced into Nicotiana tabacum using a Ti plasmid vector," Nature 310(5973):115-120 (1984).
Hibi et al., "Chondroitinase C activity of *Streptococcus intermedius*," FEMS-Microbiol-Lett. 48(2):121-124 (1989).
Hofmger et al., "Isoenzyme-specific differences in the degradation of hyaluronic acid by mammalian-type hyaluronidases," Glycoconj J 25:101-109 (2008).
Holash et al., "VEGF-Trap: a VEGF blocker with potent antitumor effects," Proc Nati Acad Sci U S A. 99(17):11393-11398 (2002).
Hooper et al., "Determination of glycosyl-phosphatidylinositol membrane protein anchorage" Proteomics 1:748-755 (2001).
Horn et al., "Intravesical chemotherapy of superficial bladder tumors in a controlled trial with cis-platinum versus cis-platinum plus hyaluronidase," J. Surg. Oncol. 28:304-307 (1985).
Huang, X. and M. Miller. "A time-efficient, linear-space local similarity algorithm." Adv. Appl. Math. 12:337-357 (1991).
Hunnicut et al., "Structural relationship of sperm soluble hyaluronidase to the sperm membrane protein PH-20," Biol Reprod. 54(6):1343-1349 (1996).
Ito et al., "Cultivation of hepatitis C virus in primary hepatocyte culture from patients with chronic hepatitis C results in release of high titre infectious virus," J. Gen. Virol. 77:1043-1054 (1996).
IUPAC-IUB Commission on Biochemical Nomenclature, "A One-Letter Notation for Amino Acid Sequences: Tentative Rules," J. Biol. Chem. 243:3557-3559 (1968).
IUPAC-IUB, "Abbreviated nomenclature of synthetic poypeptides-polymerized amino acids-revised recommendations," Commission on Biochemical Nomenclature, Biochemistry 11:1726-1731 (1972).
Jay et al., "Construction of a general vector for efficient expression of mammalian proteins in bacteria: use of a synthetic ribosome binding site," Proc. Natl. Acad. Sci. USA 78:5543-5548 (1981).
Karvinen et al., "Hyaluronan, CD44 and versican in epidermal keratinocyte tumors," British Journal of Dermatology 148:86-94 (2003).
Kelsey et al., "Species- and tissue-specific expression of human alpha 1-antitrypsin in transgenic mice," Genes and Devel. 1:161-171 (1987).
Kim et al., "Sperm penetration through cumulus mass and zona pellucida," Int J Dev Biol. 52(5-6):677-682 (2008).
Kodukula et al., "Biosynthesis of phosphatidylinositol glycan-anchored membrane proteins" J. Biol. Chem. 266:4464-4470 (1991).
Kohno et al., "Effects of hyaluronidase on doxorubicin penetration into squamous carcinoma multicellular tumor spheroids and its cell lethality," J. Cancer Res. Oncol. 120:293-297 (1994).
Kollias et al., "Regulated expression of human A gamma-, beta-, and hybrid gamma beta-globin genes in transgenic mice: manipulation of the developmental expression patterns," Cell 46:89-94 (1986).

(56) **References Cited**

OTHER PUBLICATIONS

Krumlauf et al., Developmental regulation of alpha-fetoprotein genes in transgenic mice, Mol. Cell. Biol. 5:1639-1648 (1985).
Krupers et al., "Complexation of poly(ethylene oxide) with poly(acrylic acid-co-hydroxyethyl methacrylate)s," Eur. Polym J. 32(6):785-790 (1996).
Kyte et al., "A Simple Method for Displaying the Hydropathic Character of a Protein" J. Mol. Biol. 157:105-132 (1982).
Lalancette et al, "Characterization of an 80-kilodalton bull sperm protein identified as PH-20," Biol Reprod. 65(2):628-636 (2001).
Lam et al., "The effect of benzyl alcohol on recombinant human interferon-gamma," Pharm Res. 14(6):725-729 (1997).
Lammerts van Bueren et al., "The antibody zalutumumab inhibits epidermal growth factor receptor signaling by limiting intra- and intermolecular flexibility," Proc Natl Acad Sci U S A. 105(16):6109-6114 (2008). Retraction also included, Proc Natl Acad Sci U S A. 109(14):5548 (2008).
Lathrop et al., "cDNA cloning reveals the molecular structure of a sperm surface protein, PH-20, involved in sperm-egg adhesion and the wide distribution of its gene among mammals," J Cell Biol. 111(6 Pt 2):2939-2949 (1990).
Laurent, T. and J. Fraser, "Hyaluronan," FASEB J 6:2397-2404 (1992).
Leder et al., "Consequences of widespread deregulation of the c-myc gene in transgenic mice: multiple neoplasms and normal development," Cell 45:485-495 (1986).
Li et al., "Importance of glycosylation and disulfide bonds in hyaluronidase activity of macaque sperm surface PH-20," J. Androl. 23:211-219 (2002).
Li et al., "Structural basis for EGF receptor inhibition by the therapeutic antibody IMC-11F8," Structure. I6(2):216-227 (2008).
Liang et al., "Prediction of antigenic epitopes on protein surfaces by consensus scoring," BMC Bioinformatics. 10:302, 10 pages (2009).
Lin et al., "A hyaluronidase activity of the sperm plasma membrane protein PH-20 enables sperm to penetrate the cumulus cell layer surrounding the egg," J Cell Biol. 125(5):1157-1163 (1994).
Lin et al., "Molecular cloning of the human and monkey sperm surface protein PH-20," Proc. Natl. Acad. Sci. USA 90:10071-10075 (1993).
Lipponen et al "High stromal hyaluronan level is associated with poor differentiation and metastasis in prostate cancer," European Journal of Cancer 37:849-856 (2001).
Louveau, I. and F. Gondret, "GH and insulin affect fatty acid synthase activity in isolated porcine adipocytes in culture without any modifications of sterol regulatory element binding protein-1 expression," J Endocrin. 181:271-280 (2004).
Lowy et al., "Isolation of transforming DNA: cloning the hamster aprt gene," Cell 22:817-823 (1980).
Lu, H. and E. Wimmer., "Poliovirus chimeras replicating under the translational control of genetic elements of hepatitis C virus reveal unusual properties of the internal ribosomal entry site of hepatitis C virus," Proc. Natl. Acad. Sci. USA 93:1412-1417 (1996).
Lu, Y. and A. Felix, "Pegylated peptides I: Solid-phase synthesis of N alpha-pegylated peptides using Fmoc strategy," Peptide Res 6:140-146 (1993).
Lu, Y. and A. Felix, "Pegylated peptides. II. Solid-phase synthesis of amino-, carboxy- and side-chain pegylated peptides," Int. J. Peptide Protein Res. 43:127-138 (1994).
Ma et al., "Fucosylation in prokaryotes and eukaryotes" Glycobiology 16(12):158R-184R (2006).
Maa and Hsu, "Aggregation of recombinant human growth hormone induced by phenolic compounds" Int. J. Pharm. 140(2):155-168 (1996).
MacDonald, R., "Expression of the pancreatic elastase I gene in transgenic mice," Hepatology 7:42S-51S(1987).
Magram et al., "Developmental regulation of a cloned adult beta-globin gene in transgenic mice," Nature 315:338-340 (1985).
Mammalian Gene Collection (MGC) Program Team, "Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences," Proc. Natl. Acad. Sci. USA 99:16899-16903 (2002).
McIlvaine, WB, "A buffer solution for colorimetric comparison," J. Biol. Chem. 49:183-186 (1921).
Mehvar et al., "Modulation of the pharmacokinetics and pharmacodynamics of proteins by polyethylene glycol conjugation," J. Pharm. Pharmaceut. Sci. 3(1):125-136 (2000).
Menzel, E. and C. Farr, "Hyaluronidase and its substrate hyaluronan: biochemistry, biological activities and therapeutic uses," Cancer Lett., 131:3-11 (2003).
Merrifield, J., "Solid phase peptide synthesis. I. The synthesis of a tetrapeptide," J Am Chem Soc 85:2149-2154 (1963).
Meyer et al., "The soluble hyaluronidase from bull testes is a fragment of the membrane-bound PH-20 enzyme." FEBS Letters 413(2):385-388 (1997).
Michelacci et al., "Chondroitinase C from Flavobacterium heparinum," J. Biol. Chem. 251:1154-1158 (1976).
Miller et al., "Use of retroviral vectors for gene transfer and expression," Meth. Enzymol. 217:581-599 (1993).
Mizutani et al., Characterization of hepatitis C virus replication in cloned cells obtained from a human T-cell leukemia virus type 1-infected cell line, MT-2, J.Virol. 70:7219-7223 (1996).
Mizutani et al., "Inhibition of hepatitis C virus replication by antisense oligonucleotide in culture cells," Biochem. Biophys. Res. Commun. 212:906-911 (1995).
Molineux, G., "Pegylation: engineering improved biopharmaceuticals for oncology," Pharmacotherapy 23 (8 Pt 2):3S-8S (2003).
Monfardini et al, "A branched monomethoxypoly(ethylene glycol) for protein modification," Bioconjugate Chem. 6:62-69 (1995).
Nadjsombati et al., "Dose-range developmental toxicity of rHuPH20 in mice," Matrix Biology, 27:23 (2008).
Nakayama et al., "CD15 expression in mature granulocytes is determined by alpha 1,3-fucosyltransferase LX, but in promyelocytes and monocytes by alpha 1,3-fucosyltransferase IV," J. Biol. Chem., 276(19):16100-16106 (2001).
Needleman, S. and C. Wunsch "A general method applicable to the search for similarities in the amino acid sequence of two proteins," J Mol Biol. 48:443-453 (1970).
Omaetxebarria et al., "Computational approach for identification and characterization of GPI-anchored peptides in proteomics experiments," Proteomics 7(12):1951-1960 (2007).
Omitz et al., "Elastase I promoter directs expression of human growth hormone and SV40 T antigen genes to pancreatic acinar cells in transgenic mice," Cold Spring Harbor Symp. Quant. Biol. 50:399-409 (1986).
Ostresh et al., "Peptide libraries: determination of relative reduction rates of protected amino acids in competitive couplings," Biopolymers 34:1681-1689 (1994).
Padavattan, S. "Crystal structure determination of hyaluronidase : a major bee venom allergen, in complex with an IgG Fab fragment and purification and biophysical characterization of bovine testes hyaluronidase," 2006, PhD Thesis, University of Basel, Faculty of Science. DOI: 10.5451/unibas-004349798. 98 pages.
Paul, A. and D. Sochart, "Improving the results of ganglion aspiration by the use of hyaluronidase," J Hand Surg 22(2):219-221 (1997).
Paulick, M. and C. Bertozzi, "The glycosylphosphatidylinositol anchor: A complex membrane-anchoring structure for proteins," Biochemistry 47:6991-7000 (2008).
Pawlowski et al., "The effects of hyalurodinase upon tumor formation in BALB/c mice painted with 7,12-dimethylbenz-(a)anthracene," Int. J. Cancer 23:105-109 (1979).
Pedersen et al., "Sym004: a novel synergistic anti-epidermal growth factor receptor antibody mixture with superior anticancer efficacy," Cancer Res. 70(2):588-597 (2010).
Pham et al., "Large-scale transient transfection of serum-free suspension-growing HEK293 EBNA1 cells: peptone additives improve cell growth and transfection efficiency," Biotechnology and Bioengineering 84:332-342 (2003).

(56) **References Cited**

OTHER PUBLICATIONS

Phelps et al., "Restricted lateral diffusion of PH-20, a PI-anchored sperm membrane protein," Science 240:1780-1782 (1988).
Pierleoni et al., PredGPI: a GPI-anchor predictor, BMC Bioinformatics 9:392 (2008).
Pinkert et al., "An albumin enhancer located 10 kb upstream functions along with its promoter to direct efficient, liver-specific expression in transgenic mice," Genes and Devel. 1:268-276 (1987).
Pirinen et al "Prognostic value of hyaluronan expression in non-small cell lung cancer: Increased stromal expression indicated unfavorable outcome in patients with adenocarcinoma" Int. J. Cancer 95, 12-17 (2001).
Primakoff et al., "Fully effective contraception in male and female guinea pigs immunized with the sperm protein PH-20," Nature 335:543-546 (1988).
Readhead et al., "Expression of a myelin basic protein gene in transgenic shiverer mice: correction of the dysmyelinating phenotype," Cell 48:703-712 (1987).
Remmele et al., "Interleukin-1 receptor (IL-IR) liquid formulation development using differential scanning calorimetry," Pharm Res. 15(2):200-208 (1998).
Rhodes et al., "Transformation of maize by electroporation of embryos," Methods Mol Biol 55:121-131 (1995).
Richmond, T., "Precompiled codon-usage tables," Genome Biology 1(1):reports241, 2 pages (2000).
Ripka et al., "Two Chinese hamster ovary glycosylation mutants affected in the conversion of GDP-mannose to GDP-fucose," Arch. Biochem. Biophys. 249(2):533-545 (1986).
Roberts et al., "Chemistry for peptide and protein PEGylation," Advanced Drug Delivery Review 54:459-476 (2002).
Ropponen et al "Tumor Cell-associated Hyaluronan as an Unfavorable Prognostic Factor in Colorectal Cancer" Cancer Research 58:342-347 (1998).
Sato et al., "Cloning and expression in *Escherichia coli* of the gene encoding the Proteus vulgaris chondroitin ABC lyase," Appl. Microbiol. Biotechnol. 41(1):39-46 (1994).
Sato, H., "Enzymatic procedure for site-specific pegylation of proteins," Adv. Drug Deliv. Rev. 54:487-504 (2002).
Sawhney et al., "Bioerodible hydrogels based on photopolymerized poly(ethylene glycol)-co poly(hydroxyl acid) Diacrylate Macromers," Macromolecules 26:581-587 (1993).
Scharf et al., "Heat stress promoters and transcription factors," Results Probl Cell Differ 20:125-162 (1994).
Scheithauer et al., "In vitro evaluation of the anticancer drug modulatory effect of hyaluronidase in human gastrointestinal cell lines," Anticancer Res. 8:391-396 (1988).
Schuller et al., "Pharmacokinetics of intrahepatic 5-fluorouracil + preinjected hyaluronidase," Proc. Amer. Assoc. Cancer Res. 32:173, abstract No. 1034 (1991).
Schwartz and Dayhoff, eds., Atlas of Protein Science and Structure, National Biomedical Research Foundation, pp. 353-358 (1979).
Schwartzman, J., "Hyaluronidase: A Review in Its Therapeutic Use in Pediatrics," J. Pediat. 39:491-502 (1951).
Seaton et al., "Rat sperm 2B1 glycoprotein (PH20) contains a C-terminal sequence motif for attachment of a glycosyl phosphatidylinositol anchor. Effects of endoproteolytic cleavage on hyaluronidase activity," Biol Reprod. 62(6):1667-1676 (2000).
Shani, M., "Tissue-specific expression of rat myosin light-chain," Nature 314:283-286 (1985).
Sharp et al., "Codon usage patterns in *Escherichia coli, Bacillus subtilis, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Drosophila melanogaster* and *Homo sapiens*; a review of the considerable within-species diversity," Nucleic Acids Res. 16(17):8207-8211 (1988).
Sharp, P. and E. Cowe, "Synonymous codon usage in *Saccharomyces cerevisiae*," Yeast. 7(7):657-678 (1991).
Shekhar et al., "The matrix reloaded: halozyme's recombinant enzyme helps injected drugs spread faster," Chem. Biol. 14:603-604 (2007).
Shimizu, Y. and H. Yoshikura, "Multicycle infection of hepatitis C virus in cell culture and inhibition by alpha and beta interferons," J. Virol. 68:8406-8408 (1994).
Singh et al., "Mechanisms of m-cresol-induced protein aggregation studied using a model protein cytochrome c," J Pharm Sci. 100(5):1679-1689 (2011) Epub date Jan. 12, 2011.
Smith, T. and M. Waterman, "Comparison of biosequences," Advances in Applied Mathematics 2:482-489 (1981).
St Croix et al., "Reversal of intrinsic and acquired forms of drug resistance by hyaluronidase treatment of solid tumors," Cancer Lett 131(1):35-44 (1998).
Steinlcuhler et al., "Product inhibition of the hepatitis C virus NS3 protease," Biochem. 37:8899-8905 (1998).
Stem, R., "Devising a pathway for hyaluronan catabolism: are we there yet?" Glycobiology 13:105R-115R (2003).
Sturla et al., "Core fucosylation of N-linked glycans in leukocyte adhesion deficiency/congenital disorder of glycosylation IIc fibroblasts," Glycobiology I5(10):924-935 (2005).
Sudo et al., "Establishment of an in vitro assay system for screening hepatitis C virus protease inhibitors using high performance liquid chromatography," Antiviral Res. 32:9-18 (1996).
Sutton, S. and D. Porter, "Development of the antimicrobial effectiveness as USP chapter <51>," PDA J. Pharm. Sci Technol., 56(6):300-311 (2002).
Swift et al., "Tissue-specific expression of the rat pancreatic elastase I gene in transgenic mice," Cell 38(3):639-646 (1984).
Takahashi et al., "A fluorimetric Morgan-Elson assay method for hyaluronidase activity," Anal. Biochem. 322:257-263 (2003).
Takeshita et al., "An enzyme-linked immunosorbent assay for detecting proteolytic activity of hepatitis C virus proteinase," (1997) Anal. Biochem. 247:242-246.
Taliani et al., "A continuous assay of hepatitis C virus protease based on resonance energy transfer depsipeptide substrates," Anal. Biochem. 240:60-67 (1996).
ten Have et al., "Cloning and characterization of the cDNA encoding the PH20 protein in the European red fox *Vulpes vulpes*," Reprod Fertil Dev. 10(2):165-172 (1998).
Tkalec et al., "Isolation and expression in *Escherichia coli* of cs1A and cs1B, genes coding for the chondroitin sulfate-degrading enzymes chondroitinase AC and chondroitinase B, respectively, from Flavobacterium heparinum," Applied and Environmental Microbiology 66(1):29-35 (2000).
Tsubery et al., "Prolonging the action of protein and peptide drugs by a novel approach of reversible polyethylene glycol modification.," J Biol. Chem 279(37):38118-38124 (2004).
Tsuda et al., "Substrate specificity studies of flavobacterium chondroitinase C and heparitinases towards the glycosaminoglycan-protein linkage region. Use of a sensitive analytical method developed by chromophore-labeling of linkage glycoserines using dimethylaminoazobenzenesulfonyl chloride," Eur. J. Biochem. 262:127-133 (1999).
Tung et al., "Mechanism of infertility in male guinea pigs immunized with sperm PH-20," Biol. Reprod. 56(5):1133-1141 (1997).
Udenfriend, S. and K. Kodukula, "Prediction of omega site in nascent precursor of glycosylphosphatidylinositol protein," Methods Enzymol. 250:571-582 (1995).
UniProt Murine PH20 sequence, Retrieved from the Internet: <URL: uniprot.org/uniprot/P48794 [retrieved on Sep. 1, 2010] [5 pages].
United States Pharmacopeia. USP <51>. Antimicrobial effectiveness testing. United States Pharmacopeia Convention, Inc, Rockville, MD., 5 pages.
USP XXII-NF XVII, United States Pharmacopeia Convention, Inc, Rockville, MD., pp. 644-645 (1990).
Varela et al., "Exome sequencing identifies frequent mutation of the SWI/SNF complex gene PBRM1 in renal carcinoma,"Nature. 469(7331):539-542 (2011).
Veronese et al., "Branched and Linear Poly(Ethylene Glycol): Influence of the Polymer Structure on Enzymological, Pharmacokinetic, and Immunological Properties of Protein Conjugates," J. Bioactive Compatible Polymers 12:197-207 (1997).

(56) **References Cited**

OTHER PUBLICATIONS

Wagner et al., "Nucleotide sequence of the thymidine kinase gene of herpes simplex virus type 1," Proc. Natl. Acad. Sci. USA 78:1441-1445 (1981).

Wang et al., "The molecular physiology of hepatic nuclear factor 3 in the regulation of gluconeogenesis," J. Biol Chem. 275(19):14717-14721 (2000).

Watson et al., Molecular Biology of the Gene, 4th Edition, The Benjamin/Cummings Pub. Co., p. 224 (1987).

Weiss et al., "Activities of monomeric insulin analogs at position A8 are uncorrelated with their thermodynamic stabilities," J. Biol. Chem. 276(43):40018-40024 (2001).

White et al., "Comparison of the glycosyl-phosphatidylinositol cleavage/attachment site between mammalian cells and parasitic protozoa," J. Cell Sci. 113(Pt.4):721-727 (2000).

Wigler et al., "Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells," Cell 11:223-232 (1977).

Wigler et al., "Transformation of mammalian cells with an amplifiable dominant-acting gene," Proc Natl Acad Sci. USA 77:3567-3570 (1980).

Yamamoto et al., "Identification of a functional promoter in the long terminal repeat of Rous sarcoma virus," Cell 22:787-797 (1980).

Yamane-Ohnuki et al., "Establishment of FUT8 knockout Chinese hamster ovary cells: an ideal host cell line for producing completely defucosylated antibodies with enhanced antibody-dependent cellular cytotoxicity," Biotech. Bioeng. 87:614-622 (2004).

Yang, X. and X. Yu, "An introduction to epitope prediction methods and software," Rev Med Virol. 19(2):77-96 (2009).

Yocum et al., "Assessment and implication of the allergic sensitivity to a single dose of recombinant human hyaluronidase injection: A double-blind placebo-controlled clinical trial," J Infus Nursing 30:293-299 (2007).

Yudin et al., "Characterization of the active site of monkey sperm hyaluronidase," Reproduction. 121(5):735-743 (2001).

Zalipsky, S., "Chemistry of polyethylene glycol conjugates with biologically active molecules," Adv. Drug Del. Rev. 16:157-182 (1995).

Zanker et al., "Induction of response in previous chemotherapy resistant patients by hyaluronidase," Proc. Amer. Assoc. Cancer Res. 27:390 Abstract 1550 (1986).

Zhao, X. and J. Harris, "Novel degradable poly(ethylene glycol) esters for delivery," in Poly(ethylene glycol), Chemistry and Biological Applications, ACS Symposium Series 680, Hams, J. and S. Zalipsky, (eds), 458-472 (1997).

Bee et al., "Recombinant human PH20 is well tolerated at higher intravenous and subcutaneous doses in cynomolgus monkeys," EUFEPS 2008, Munich, Germany. Abstract, 2 pages.

Bee et al., "Recombinant human PH20 is well tolerated at higher intravenous and subcutaneous doses in cynomolgus monkeys " EUFEPS 2008, Munich, Germany. Poster, 1 page.

Bookbinder et al., "Biochemical characterization of recombinant human PH20 (SPAM1) hyaluronidase," Hyaluronan (ISHAS) 2007, Charleston, SC. Abstract, 1 page.

Bookbinder et al., "Biochemical characterization of recombinant human PH20 (SPAM1) hyaluronidase," Hyaluronan (ISHAS) 2007, Charleston, SC. Poster, 1 page.

Bookbinder et al., "Enhancing drug transport through temporary matrix depolymerization," Keystone Symposia 2005. (poster—12 pages) (together poster and abstract—13 pages).

Bookbinder et al., "Enhancing drug transport through temporary matrix depolymerization," Keystone Symposia 2005, Abstract, 1 page.

Bookbinder et al., "Evaluation of the compatibility and pharmacokinetics of co-formulated biologics with recombinant human hyaluronidase: Dose Response," American Association of Pharmaceutical Scientists Conference, Nov. 2006. Abstract, 2 pages.

PinIcstaff et al., "Evaluation of the compatibility and pharmacokinetics of co-formulated biologics with recombinant human hyaluronidase: Dose Response," American Association of Pharmaceutical Scientists Conference, Nov. 2006. Poster, 1 page.

Byerley et al., "'Cutting out the bull'. Recombinant human hyaluronidase: Moving to an animal-free system," Association of Clinical Embryologists, 2006, Dublin, Ireland. Abstract published in Human Fertility, 9(2):110 (2006).

Frost et al., "Punctuated equilibrium: The evolution of recombinant human hyaluronidase," Ophthalmic Anesthesia Society, 2006, Chicago, IL, abstract, 1 page.

Frost et al., "Punctuated equilibrium: The evolution of recombinant human hyaluronidase," Ophthalmic Anesthesia Society, 2006, Chicago, IL, presentation, 35 pages.

Haller et al., "Enhanze technology—a revolution in drug dispersion," Biotechnology Industry Organization (BIO) Annual Meeting, Jun. 19-22, 2005, Philadelphia, PA. Abstract, 3 pages.

Haller et al., "Recombinant human hyaluronidase for the interstitial transport of therapeutics," American Association of Pharmaceutical Scientists Conference, Jun. 2006, San Antonio, TX, Abstract, 2 pages.

Haller et al., "Recombinant human hyaluronidase for the interstitial transport of therapeutics," Controlled Release Society Conference, Vienna, Austria, 2006, Abstract, 2 pages.

Haller et al., "Revolutionizing drug dispersion with enhanze technology," American Association of Pharmaceutical Scientists Annual Meeting, Nov 6-10, 2005, Nashville, TN, Poster 1 page.

Haller et al., "Revolutionizing drug dispersion with enhanze technology," Biotechnology Industry Organization (BIO) Annual Meeting, Jun. 19-22, 2005, Philadelphia, PA. Poster, 1 page.

Haller et al., "The effects of recombinant human hyaluronidase on drug dispersion," American Association of Pharmaceutical Scientists Annual Meeting, Abstract in AAPS Journal 7(S2) May 5, 2005; 3 pages.

Haller, "Enhanze technology—an enzymatic drug delivery system (DDS)," Japanese Export Trade Organization, Nov. 2005, Santa Clara, CA. Abstract, 2 pages.

Haller, M., "Focus on enhanced and innovative recombinant human enzymes," Japanese Export Trade Organization, Sep. 2004, Chicago, IL., Presentation, 16 pages.

Haller, M., "Halozyme's enhanze technology for the enhanced dispersion of co-injected pharmaceuticals," Japanese Export Trade Organization, Sep. 2004, Chicago, IL, Abstract, 2 pages.

Haller, M., "Enzyme-facilitated parenteral drug transport," Strategic Research Institute's 10th Anniversary Drug Delivery Technology and Deal-making Summit 2005, New Brunswick, NJ. Presentation, 24 pages.

Hofer et al., "Human recombinant hyaluronidase increases the convection of molecules up to 0.2 μm in athymic nude mice," American Association for Laboratory Animal Science, 2006, Salt Lake City, UT. Abstract published in J. Am. Assoc. Lab. Animal Sci., 45:120, Abstract P97 (2006), 1 page.

Hofer et al., "Human Recombinant Hyaluronidase Increases the Convection of Molecules up to 0.2 μm in Athymic Nude Mice," American Association for Laboratory Animal Science, 2006, Salt Lake City, UT. Poster published in J. Am. Assoc. Lab. Animal Sci., 45:120, Poster P97 (2006), 1 page.

Keller et al., "Pharmacokinetic, pharmacodynamic and toxicologic effects of a recombinant human hyaluronidase (rHuPH20) in rodent and non-human primate models," Hyaluronan (ISHAS) 2007, Charleston, SC, Abstract, 1 page.

Keller et al., "Pharmacokinetic, pharmacodynamic and toxicologic effects of a recombinant human hyaluronidase (rHuPH20) in rodent and non-human primate models," Hyaluronan (ISHAS) 2007, Charleston, SC, Poster, 1 page.

Kodandapani et al., "Diverse structural and functional roles of N-glycosylations on rHuPH20," Glycan Abstract, 1 page (2009).

Nadjsombati et al., "Evaluation of developmental and prenatal/postnataal reproduction toxicity of rHuPH20 in mice," American College of Toxicology 30th annual meeting, Nov. 1-4, 2009, Palm Springs, CA, Abstract, 1 page.

Nagy et al., "Prospective, randomized study on bovine and recombinant human (Cumulase®) Hyaluronidases," American Society of Reproductive Medicine, 2006, New Orleans, LA, 06-A-886-ASRM, 2 pages.

(56) **References Cited**

OTHER PUBLICATIONS

Nagy, Z., "Prospective study on rHuPH20," European Society of Human Reproduction & Embryology Annual Meeting, Jun. 19-22, 2005, Copenhagen, Denmark. Abstract O-213, 1 page.
Pinkstaff et al., "Recombinant human hyaluronidase for drug and fluid dispersion," American Association of Pharmaceutical Scientists Annual Meeting, Nov. 2006, Boston, MA, Abstract, 2 pages.
Pinkstaff et al., "Recombinant human hyaluronidase for drug and fluid dispersion," American Association of Pharmaceutical Scientists Annual Meeting, Nov. 2006, Boston, MA, Poster, 1 page.
Wei et al., "Functions of N-linked glycans on human hyaluronidase PH20," presented at San Diego Glycobiology Symposium 2009. Poster 83, 1 page.
Wei et al., "Structure function analysis of the human hyaluronidase enzymes," Matrix Biology 27 (Supplement 1):S41, Dec. 2008, American Society for Matrix Biology Biennial Meeting, San Diego, CA, (available on-line Nov. 17, 2008), Abstract 132, 1 page.
Wei et al., "Structure function analysis of the human hyaluronidase enzymes," Presented at American Society for Matrix Biology Biennial Meeting, San Diego, CA, Dec. 9, 2008, Poster B4, 1 page.
Wilson, M., "Enhanze technology—an enzymatic drug delivery system (DDS)," Japanese Export Trade Organization, Nov. 2005, Santa Clara, CA. Oral presentation, 22 pages.
Letter/Written Disclosure of the Supplemental Information Disclosure Statement for the above-referenced application, filed herewith Apr. 20, 2016, 2 pages.
Derwent English abstract for Japanese Publication No. JP 2007-153797, published Jun. 21, 2007, entitled "Therapeutic Agent and Preventive Agent of Autoimmune Disease, Inflammation, and Nervous Disease," 3 pages.
Eskens et al., "Enzymatic glycocalyx treatment impairs insulin-mediated recruitment of microvascular blood volume and decreases insulin sensitivity in rats," FASEB Journal 25: 1023.13 (2011) (Abstract).
M3WD76 Felca (2015, updated) Hyaluronidase/PH20 from Felis catus, 3 pages.
Wells et al., "Additivity of Mutational Effects in Proteins" Biochem 29: 8509-8517 (1990).
News Release, Halozyme Therapeutics, Inc., "Halozyme to Present Data from Five Preclinical Studies at American Association of Cancer Research Annual Conference," Published Mar. 17, 2016 [online], Retrieved from: <halozyme.com/investors/news-releases/news-release-details/2016/Halozyme-To-Present-Data-From-Five-Preclinical-Studies-At-American-Association-Of-Cancer-Research-Annual-Conference/defaultaspx [retrieved on Mar. 28, 2016], 5 pages.
Examiner's Report, issued Mar. 1, 2016, in connection with Canadian Patent Application No. 2861919, 7 pages.
Response, filed Oct. 9, 2015, to Office Action, issued May 25, 2015, in connection with Chinese Patent Application No. 201280070954.9 [English instructions and document as filed in Chinese], 37 pages.
Official Action, issued Jan. 19, 2016, in connection with Japanese Patent Application No. 2014-550526 [English translation and original document in Japanese], 8 pages.
Search Report and Written Opinion, dated Dec. 4, 2015, in connection with Singaporean Patent Application No. 11201403714T, 7 pages.
Office Action, issued Feb. 3, 2016, in connection with Chinese Patent Application No. 201280070954.9 [English language translation and original document in Chinese], 6 pages.

* cited by examiner

FIGURE 1

LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSLFSFIGSPRINATGQGVTIFYVDR 60

LGYYPYIDSITGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGMAVIDWEEWRPTWA 120

RNWKPKDVYKNRSIELVQQQNVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRPNHL 180

WGYYLFPDCYNHHYKKPGYNGSCENVEIKRNDDLSWLWNESTALYPSIYLNTQQSPVAAT 240

LYVRNRVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLSQDELVYTFGETVALGASG 300

IVIWGTLSIMRSMKSCLLLDNYMETILNPYIINVTLAAKMCSQVLCQEQGVCIRKNWNSS 360

DYLHLNPDNFAIQLEKGGKFTVRGKPTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV 420

DVCIADGVC**IDAFLKPPMETEEPQIFYNASPSTLSATMFIVSILF**LIISSVASL 474

FIGURE 2A

```
SEQIDNO_3           LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSLFSFIGSPRINATGQGVTIFYVDR 60
chimp_SEQIDNO_10_   LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSLFSFIGSPRINVTGQDVTIFYVDR 60
                    ***********************************************.***.********

SEQIDNO_3           LGYYPYIDSITGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGMAVIDWEEWRPTWA 120
chimp_SEQIDNO_10_   LGYYPYIDSITGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGMAVIDWEEWRPTWA 120
                    ************************************************************

SEQIDNO_3           RNWKPKDVYKNRSIELVQQQNVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRPNHL 180
chimp_SEQIDNO_10_   RNWKPKDVYKNRSIELVQQQNVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRPNHL 180
                    ************************************************************

SEQIDNO_3           WGYYLFPDCYNHHYKKPGYNGSCFNVEIKRNDDLSWLWNESTALYPSIYLNTQQSPVAAT 240
chimp_SEQIDNO_10_   WGYYLFPDCYNHHYKKPGYNGSCFNVEIKRNDDLSWLWNESTALYPSIYLNTQQSPVAAT 240
                    ************************************************************

SEQIDNO_3           LYVRNRVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLSQDELVYTFGETVALGASG 300
chimp_SEQIDNO_10_   LYVRNRVQEAIRVSKIPDAKSPLPVFVYTRIVFTDQVLKFLSQDELVYTFGETVALGASG 300
                    *******:******************.*********************************

SEQIDNO_3           IVIWGTLSIMRSMKSCLLLDNYMETILNPYIINVTLAAKMCSQVLCQEQGVCIRKNWNSS 360
chimp_SEQIDNO_10_   IVIWGTLSIMRSMKSCLLLDNYMETILNPYIINVTLAAKMCSQVLCQEQGVCIRKNWNSS 360
                    ************************************************************

SEQIDNO_3           DYLHLNPDNFAIQLEKGGKFTVRGKPTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV 420
chimp_SEQIDNO_10_   DYLHLNPDNFAIQLEKGGKFTVRGKPTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV 420
                    ************************************************************

SEQIDNO_3           DVCIADGVCIDAFLKPPMETEEPQIFY--------------------------- 447
chimp_SEQIDNO_10_   DVCIADGVCIDAFLKPPMETEESQIFYNASPSTLSATMFIVSILFLIISSVASL 474
                    **********************.****
```

FIGURE 2B

```
SEQIDNO_3             LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSLFSFIGSPRINATGQGVTIFYVDR 60
Rhesus_SEQIDNO_12_    LNFRAPPIIPNVPFLWAWNAPSEFCLGKFNEPLDMSLFTLMGSPRINITGQGVTIFYVDR 60
                      *******:*********************:*********:::****** ***********

SEQIDNO_3             LGYYPYIDSITGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGMAVIDWEEWRPTWA 120
Rhesus_SEQIDNO_12_    LGYYPYIDLTTGVTVHGGIPQKVSLQDHLDKSKQDILFYMPVDNLGMAVIDWEEWRPTWA 120
                      ********  *****:******:********:*:** ***********************

SEQIDNO_3             RNWKPKDVYKNRSIELVQQQNVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRPNHL 180
Rhesus_SEQIDNO_12_    RNWKPKDVYKNRSIELVQQQNVQLSLPQATDKAKQEFEKAGKDFMLETIKLGRSLRPNHL 180
                      **************************.:**:*************::******: ******

SEQIDNO_3             WGYYLFPDCYNHHYKKPGYNGSCFNVEIKRNDDLSWLWNESTALYPSIYLNTQQSPVAAT 240
Rhesus_SEQIDNO_12_    WGYYLFPDCYNHHYRKPGYNGSCFDVEIKRNDDLSWLWNESTALYPSIYLNTQQSVVVAT 240
                      **************:*********:****************************** *.**

SEQIDNO_3             LYVRNRVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLSQDELVYTFGETVALGASG 300
Rhesus_SEQIDNO_12_    LYVRNRVREAIRVSKIPDAKNPLPVFVYARLVFTDQVLKFLSREELVSTLGETVALGASG 300
                      ********************.*****.*:*:***********::*** *:**********

SEQIDNO_3             IVIWGTLSIMRSMKSCLLLDNYMETILNPYIINVTLAAKMCSQVLCQEQGVCIRKNWNSS 360
Rhesus_SEQIDNO_12_    IVIWGSLSITRSMKSCLLLDTYMETILNPYIINVTLAAKMCSQVLCQEQGVCIRKDWNSS 360
                      *****:*** **********.**********************************:****

SEQIDNO_3             DYLHLNPDNFAIQLEKGGKFTVRGKPTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV 420
Rhesus_SEQIDNO_12_    DYLHLNPDNFDIRLEKGGKFTVHGKPTVEDLEEFSEKFYCSCYTNLSCKEKADVKDTDAV 420
                      ********** *:*********:****:****:**********:.***************

SEQIDNO_3             DVCIADGVCIDAFLKPPMETE-EPQIFY----------------------------- 447
Rhesus_SEQIDNO_12_    DVCIADGVCIDASLKPPVETEGSPPIFYNTSSSTVSTTMFIWRLEVWDQGISRIGFF 477
                      ************ ****:*** .* ***
```

FIGURE 2C

```
SEQIDNO_3           LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSLFSFIGSPRINATGQGVTIFYVDR 60
Cyno_SEQIDNO_14_    LNFRAPPIIPNVPFLWAWNAPSEFCLGKFNEPLDMSLFTLMGSPRINVTGQGVTIFYVDR 60
                    *******:*********************:********:::******.************

SEQIDNO_3           LGYYPYIDSITGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGMAVIDWEEWRPTWA 120
Cyno_SEQIDNO_14_    LGYYPYIDLTTGVTVHGGIPQKVSLQDHLDKSKQDILFYMPVDNLGMAVIDWEEWRPTWA 120
                    ********  *****:******:********:*:** ***********************

SEQIDNO_3           RNWKPKDVYKNRSIELVQQQNVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRPNHL 180
Cyno_SEQIDNO_14_    RNWKPKDVYKNRSIELVQQQNVQLSLPQATDKAKQEFEKAGKDFMLETIKLGRSLRPNHL 180
                    **************************.:**:*************::******: ******

SEQIDNO_3           WGYYLFPDCYNHHYKKPGYNGSCFNVEIKRNDDLSWLWNESTALYPSIYLNTQQSPVAAT 240
Cyno_SEQIDNO_14_    WGYYLFPDCYNHHYRKPGYNGSCFDVEIKRNDDLSWLWNESTALYPSIYLNTQQSVVVAT 240
                    **************:*********:****************************** *.**

SEQIDNO_3           LYVRNRVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLSQDELVYTFGETVALGASG 300
Cyno_SEQIDNO_14_    LYVRNRVREAIRVSKIPDAKNPLPVFVYARLVFTDQVLKFLSREELVSTLGETVALGASG 300
                    ********************.*****.*:*:***********::*** *:**********

SEQIDNO_3           IVIWGTLSIMRSMKSCLLLDNYMETILNPYIINVTLAAKMCSQVLCQEQGVCIRKNWNSS 360
Cyno_SEQIDNO_14_    IVIWGSLSITRSMKSCLLLDTYMETILNPYIINVTLAAKMCSQVLCQEQGVCIRKDWNSS 360
                    *****:*** **********.**********************************:****

SEQIDNO_3           DYLHLNPDNFAIQLEKGGKFTVRGKPTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV 420
Cyno_SEQIDNO_14_    DYLHLNPDNFDIRLEKGGKFTVHGKPTVEDLEEFSEKFYCSCYTNLSCKEKADVKDTDAV 420
                    ********** *:*********:****:****:**********:.***************

SEQIDNO_3           DVCIADGVCIDAFLKPPMETE-EPQIFY--------------------------- 447
Cyno_SEQIDNO_14_    DVCIADGVCIDASLKPPVETEGSPPIFYNTSSSTVSTTMFIVNILFLIISSVASL 475
                    ************ ****:*** .* ***
```

FIGURE 2D

```
SEQIDNO_3               LNFRAPPVIPNVPFLWAWNAPSEFCLG-KFDEPLDMSLFSFIGSPRINATGQGVTIFYVD 59
bovine_SEQIDNO_16_      LDFRAPPLISNTSFLWAWNAPVERCVNRRFQLPPDLRLFSVKGSPQKSATGQFITLFYAD 60
                        *:*****:*.*..******** * *:. :*: * *: ***. ***: .**** :*:**.*

SEQIDNO_3               RLGYYPYIDSITGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGMAVIDWEEWRPTW 119
bovine_SEQIDNO_16_      RLGYYPHIDEKTGKTVFGGIPQLGNLKSHMEKAKNDIAYYIPNDSVGLAVIDWENWRPTW 120
                        ******:**. ** ** *****  .*:.*::***:**::*:* *.:*:******:*****

SEQIDNO_3               ARNWKPKDVYKNRSIELVQQQNVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRPNH 179
bovine_SEQIDNO_16_      ARNWKPKDVYRDESVELVLQKNPQLSFPEASKIAKVDFETAGKSFMQETLKLGKLLRPNH 180
                        **********::.*:*** *:* ***:.**:: ** :**.***.*: **:**********

SEQIDNO_3               LWGYYLFPDCYNHHYKKPGYNGSCENVEIKRNDDLSWLWNESTALYPSIYLNT-QQSPVA 238
bovine_SEQIDNO_16_      LWGYYLFPDCYNHNHNQPTYNGNCPDVEKRRNDDLEWLWKESTALFPSVYLNIRLKSTQN 240
                        *************::::* ***.* :** :*****.***:*****:**:***   :*.

SEQIDNO_3               ATLYVRNRVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLSQDELVYTFGETVALGA 298
bovine_SEQIDNO_16_      AALYVRNRVQEAIRLSKIASVESPLPVFVYARPVFTDGSSTYLSQGDLVNSVGEIVSLGA 300
                        *:*******:****:***...:******.*:* ****   .:***.:** :.** *:***

SEQIDNO_3               SGIVIWGTLSIMRSMKSCLLLDNYMETILNPYIINVTLAAKMCSQVLCQEQGVCIRKNWN 358
bovine_SEQIDNO_16_      SGIIMWGSLNLSLSMQSCMNLGTYLNTTLNPYIINVTLAAKMCSQVLCHNEGVCTRKHWN 360
                        ***::**:*.:  **:**: *..*::* ********************:::*** **:**

SEQIDNO_3               SSDYLHLNPDNFAIQLEKGGKFTVRGKPTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTD 418
bovine_SEQIDNO_16_      SSDYLHLNPMNFAIQTGEGGKYTVPGTVTLEDLQKFSDTFYCSCYANIHCKKRVDIKNVH 420
                        ********* *****  :***:** *. *****::**:.******:.: **::.*:*:..

SEQIDNO_3               AVDVCIADGVCIDAFLKP ------------------------------------------ 436
bovine_SEQIDNO_16_      SVNVCMAEDICIDSPVKL QPSDHSSSQEASTTTFSSISPSTTTATVSPCTPEKHSPECLK 480
                        :*:**:*:.:***: :*

SEQIDNO_3               ---------------------------PMETEEPQIFY 447
bovine_SEQIDNO_16_      VRCSEVIPNVTQKACQSVKLKNISYQSPIQNIKNQTTY 518
                                                   *::. : *  *
```

FIGURE 2E

```
SEQIDNO_3           LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSLFSFIGSPRINATGQGVTIFYVDR 60
Mouse_SEQIDNO_20_   VDYRAAPILSNTTFLWIWNVPTERCVGNVNDPIDLSFFSLIGSPRKTATGQPVTLFYVDR 60
                    :::**.*::.*..*** **.*:* *:*:.::*:*:*:**:***** .**** **:*****

SEQIDNO_3           LGYYPYIDSITGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGMAVIDWEEWRPTWA 120
Mouse_SEQIDNO_20_   LGLYPHIDANQAEHY-GGIPQRGDYQAHLRKAKTDIEHYIPDDKLGLAIIDWEEWRPTWL 119
                    ** **:**:  .    *****: . * ** ***.** .*:* *:**:*:**********

SEQIDNO_3           RNWKPKDVYKNRSIELVQQQNVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRPNHL 180
Mouse_SEQIDNO_20_   RNWKPKDNYRNKSIELVQSTNPGLSITEATQKAIQQFEEAGRKFMEGTLHLGKFLRPNQL 179
                    ******* *:*:******. *  **:****:** *:**:**:.*:  *::***:****:*

SEQIDNO_3           WGYYLFPDCYNHHYKKPGYNGSCENVEIKRNDDLSWLWNESTALYPSIYLNTQQ-SPVAA 239
Mouse_SEQIDNO_20_   WGYYLFPDCYNNKFQDPKYDGQCPAVEKKRNDNLKWLWKASTGLYPSVYLKKDLKSNRQA 239
                    ***********::::.* *:*.*  ** ****:*.***: **.****:**:.:  *   *

SEQIDNO_3           TLYVRNRVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLSQDELVYTFGETVALGAS 299
Mouse_SEQIDNO_20_   TLYVRYRVVEAIRVSKVGNASDPVPIFVYIRLVFTDRTSEYLLEDDLVNTIGEIVALGTS 299
                    ***** ** *******: :*..*:*:*.* *:****:. ::* :*:** *:** ****:*

SEQIDNO_3           GIVIWGTLSIMRSMKSCLLLDNYMETILNPYIINVTLAAKMCSQVLCQEQGVCIRKNWNS 359
Mouse_SEQIDNO_20_   GIIIWDAMSLAQRAAGCPILHKYMQTTLNPYIVNVTLAAKMCSQTLCNEKGMCSRRKESS 359
                    **:**.::*: :   .* :*.:**:* *****:***********.**:*:*:* *:: .*

SEQIDNO_3           SDYLHLNPDNFAIQLEKGGKFTVRGKPTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDA 419
Mouse_SEQIDNO_20_   DVYLHLNPSHFDIMLTETGKYEVLGNPRVGDLEYFSEHFKCSCFSRMTCKETSDVKNVQD 419
                    . ******.:* * * : **: * *:* : *** ***:* ***:* ::***.:***:.:

SEQIDNO_3           VDVCIADGVCIDAFLKPP------------METEEPQIFY------------------ 447
Mouse_SEQIDNO_20_   VNVCVGDNVCIKAKVEPNPAFYLLPGKSLLFMTTLGHVLYHLPQDIFVFPRKTLVSTP 477
                    *:**:.*.***.* ::*             : *   :::*
```

FIGURE 2F

```
SEQIDNO_3          LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSLFSFIGSPRINATGQGVTIFYVDR 60
Rat_SEQIDNO_22_    VDYRATPVLSDTTFVWVWNVPTEACVENVTEPIDLSFFSLIGSPRKTAIGQPVTLFYVDR 60
                   :::**.**:.:..*:*.**.*:* *: :. **:*:*:**:***** .* ** **:*****

SEQIDNO_3          LGYYPYIDSITGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGMAVIDWEEWRPTWA 120
Rat_SEQIDNO_22_    LGNYPHIDAQQ-TEHHGGIPQKGDLTTHLVKAKEDVERYIPTDKLGLAIIDWEEWRPTWM 119
                   ** **:**:   .  :****** .*  ** ***:*:  *:*.*:**:*:**********

SEQIDNO_3          RNWKPKDVYKNRSIELVQQQNVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRPNHL 180
Rat_SEQIDNO_22_    RNWTPKDIYRNKSIELVQAADPAINITEATVRAKAQFEGAAKEFMEGTLKLGKHIRPKHL 179
                   ***.***:*:*:******  :  :.:**** :** :** *.*:*:  *:**** :**:**

SEQIDNO_3          WGYYLFPDCYNHHYKKPGYNGSCFNVEIKRNDDLSWLWNESTALYPSIYLNTQQ-SPVAA 239
Rat_SEQIDNO_22_    WGFYLFPDCYNNKFQVDNYDGQCPDVEKKRNDDLDWLWKESTGLYPSVYLKKDLKSSRKA 239
                   **:********::::  .*:*.* :** ******.***:***.****:**:.:  *.  *

SEQIDNO_3          TLYVRNRVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLSQDELVYTFGETVALGAS 299
Rat_SEQIDNO_22_    TLYVRYRVLESIRVSKVSDESNPVPIFVYIRLVFTDHVSEYLLEDDLVNTIGEIVAQGTS 299
                   ***** ** *:*****:.* ..*:*:*.* *:****:* ::* :*:** *:** ** *:*

SEQIDNO_3          GIVIWGTLSIMRSMKSCLLLDNYMETILNPYIINVTLAAKMCSQVLCQEQGVCIRKNWNS 359
Rat_SEQIDNO_22_    GIIIWDAMSLAQRSAGCPILRQYMKTTLNPYIVNVTLAAKMCSQTLCKEKGMCSRKTESS 359
                   **:**.::*: :   .* :* :**:* *****:***********.**:*:*:* **. .*

SEQIDNO_3          SDYLHLNPDNFAIQLEKGGKFTVRGKPTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDA 419
Rat_SEQIDNO_22_    DAYLHLDPSSFSINVTEAGKYEVLGKPEVKDLEYFSEHFKCSCFSKMTCEETSDMRSIQD 419
                   . ****:*..*:*:: :.**: * *** ::*** ***:* ***:*.::*:*.:*::. :

SEQIDNO_3          VDVCIADGVCIDAFLKPP------------METEEPQIFY------------------ 447
Rat_SEQIDNO_22_    VNVCMGDNVCIKATLGPNSAFHLLPGKGLLLMTTLAHILHHLPHDIFVFPWKMLVSTP 477
                   *:**:.*.***.* * *             : *  .:*::
```

FIGURE 2G

```
SEQIDNO_3             LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSLFSFIGSPRINATGQGVTIFYVDR 60
Rabbit_SEQIDNO_24_    ANFRAPPVIPNVPFLWAWNAPTEFCLGKSGEPLDMSLFSLFGSPRKNKTGQGITIFYVDR 60
                       ********************:****** .*********::**** * ****:*******

SEQIDNO_3             LGYYPYIDSITGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGMAVIDWEEWRPTWA 120
Rabbit_SEQIDNO_24_    LGYYPYIDPHTGAIVHGRIPQLGPLQQHLTKLRQEILYYMPKDNVGLAVIDWEEWLPTWL 120
                      ********. **. *:* ***  .**:** * :::* :*** **:*:******** ***

SEQIDNO_3             RNWKPKDVYKNRSIELVQQQNVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRPNHL 180
Rabbit_SEQIDNO_24_    RNWKPKDIYRIKSIELVKSQHPQYNHSYATEKAKRDFEKAGKDFMEETLKLGRLLRPNHL 180
                      *******:*: :*****:.*: *  . : ******::********: **:***:*******

SEQIDNO_3             WGYYLFPDCYNHHYKKP-GYNGSCENVEIKRNDDLSWLWNESTALYPSIYLNTQQ---SP 236
Rabbit_SEQIDNO_24_    WGYYLFPDCYNHHYDKPNLYKGSCEDIEKKRNDDLSWLWKESTALFPSVYLTSRARSATA 240
                      **************.**  *:****::* **********:*****:**:**.::     :.

SEQIDNO_3             VAATLYVRNRVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLSQDELVYTFGETVAL 296
Rabbit_SEQIDNO_24_    LSKLYVVRNRVHEAIRVSKIPDDKSPLPNFVYTRLVFTDQIFQFLSHHDLVYTIGEIVAL 300
                      ::    *****:********** ***** *.***:*****:::***:.:****:** ***

SEQIDNO_3             GASGIVIWGTLSIMRSMKSCLLLDNYMETILNPYIINVTLAAKMCSQVLCQEQGVCIRKN 356
Rabbit_SEQIDNO_24_    GASGIVVWGSQSLARSMKSCLHLDNYMKTILNPYLINVTLAAKMCNQVLCQEQGVCTRKN 360
                      ******:**: *: ******* *****:******:**********.********** ***

SEQIDNO_3             WNSSDYLHLNPDNFAIQLEKGGKFTVRGKPTLEDLEQFSEKFYCSCYSTLSCKEKADVKD 416
Rabbit_SEQIDNO_24_    WNPNDYLHLNPGNFAIQLGSNGTYKVDGKPTLTDLEQFSKNFQCSCYTNLNCKERTDMNN 420
                      **..*******.****** ..*.:.* ***** ******::* ****:.*.***::*:::

SEQIDNO_3             TDAVDVCIADGVCIDAFLKPPMETEEPQ-------------------------------- 444
Rabbit_SEQIDNO_24_    VRTVNVCAVENVCIDTNVGPQAVTYAPKEKKDVAHILSNTTSINSSTTMSLPFPRKHVSG 480
                      . :*:** .:.****: : *   *  *:

SEQIDNO_3             -------------IFY-------------- 447
Rabbit_SEQIDNO_24_    CLLVLCMYSQYLNICYRLVAIGIQHGYYLK 510
                                   * *
```

FIGURE 2H

```
SEQIDNO_3                     LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSLFSFIGSPRINATG 50
GuineaPig_SEQIDNO_29_         -DKRAPPLIPNVPLLWVWNAPTEFCIGGTNQPLDMSFFSIVGTPRKNITG 49
                               : ****:*****:**.****:***:*  ::*****:**::*:** * **

SEQIDNO_3                     QGVTIFYVDRLGYYPYIDSITGVTVNGGIPQKISLQDHLDKAKKDITFYM 100
GuineaPig_SEQIDNO_29_         QSITLYYVDRLGYYPYIDPHTGAIVHGGLPQLMNLQQHLRKSRQDILFYM 99
                              *.:*::************. **. *:**:** :.**:** *:::** ***

SEQIDNO_3                     PVDNLGMAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNVQLSLTEAT 150
GuineaPig_SEQIDNO_29_         PTDSVGLAVIDWEEWRPTWTRNWRPKDIYRNKSIELVKSQHPQYNHSYAV 149
                              *.*.:*:************:***:***:*:*:*****:.*: * . : *.

SEQIDNO_3                     EKAKQEFEKAGKDFLVETIKLGKLLRPNHLWGYYLFPDCYNHHYKKPGYN 200
GuineaPig_SEQIDNO_29_         AVAKRDFERTGKAFMLETLKLGKSLRPSSLWGYYLFPDCYNTHFTKPNYD 199
                                **::**::** *::**:**** ***. ************ *:.**.*:

SEQIDNO_3                     GSCFNVEIKRNDDLSWLWNESTALYPSIYLNTQQ-SPVAATLYVRNRVRE 249
GuineaPig_SEQIDNO_29_         GHCPPIELQRNNDLQWLWNDSTALYPSVYLTSRVRSSQNGALYVRNRVHE 249
                              * *  :*::**:**.****:*******:**.::  *.  .:*******:*

SEQIDNO_3                     AIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLSQDELVYTFGETVALGAS 299
GuineaPig_SEQIDNO_29_         SIRVSKLMDDKNPLPIYVYIRLVFTDQTTTFLELDDLVHSVGEIVPLGVS 299
                              :*****: * *.***::.* *:*****. .**. *:**::.** *.**.*

SEQIDNO_3                     GIVIWGTLSIMRSMKSCLLLDNYMETILNPYIINVTLAAKMCSQVLCQEQ 349
GuineaPig_SEQIDNO_29_         GIIIWGSLSLTRSLVSCIGLENYMKGTLLPYLINVTLAAKMCGQVLCKNQ 349
                              **:***:**: **: **: *:***:  * **:**********.****::*

SEQIDNO_3                     GVCIRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPTLEDLEQFSEKFY 399
GuineaPig_SEQIDNO_29_         GICTRKDWNTNTYLHLNATNFDIELQQNGKFVVHGKPSLEDLQEFSKNFH 399
                              *:* **:**:. *****. ** *:*::.***.*:***:****::**::*:

SEQIDNO_3                     CSCYSTLSCKEKADVKDTDAVDVCIADGVCIDAFL---------KPPMET 440
GuineaPig_SEQIDNO_29_         CSCYTNVACKDRLDVHNVRSVNVCTANNICIDAVLNFPSLDDDDEPPITD 449
                              ****:.::**:: **::. :*:** *:.:****.*         :**:

SEQIDNO_3                     EEPQ-------------------------------IFY------- 447
GuineaPig_SEQIDNO_29_         DTSQNQDSISDITSSAPPSSHILPKDLSWCLFLLSIFSQHWKYLL 494
                              : .*                               **
```

FIGURE 2I

```
SEQIDNO_3      LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSLFSFIGSPRINATGQGVTIFYVDR 60
FOX            QEFRAPPFIPNVSFLWGWNAPTELCAKRFNVQLDLNLFSLIGSPLKTVVGQGIAIFYADR 60
                :*****.****.***.****:*:*  :*:  **:.***:****  ...***::***.**

SEQIDNO_3      LGYYPYIDSITGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGMAVIDWEEWRPTWA 120
FOX            LGYYPHINKTTGKHVNGGIPQLGSLKKHLDKAKKDISHYIETDSMGLAVIDWDSWRPNWA 120
               *****:*:. **  *******  **:.*********:.*: .*.:*:*****:.***.**

SEQIDNO_3      RNWKPKDVYKNRSIELVQQQNVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRPNHL 180
FOX            RNWRPKHIYKEQSIDLAQQQHIHLNLTEVTQIAQADFEKAARCFMQETLKLGKFLRPNYL 180
               ***:**.:**::**:*.***:::*.***.*: *: :****.: *: **:****:****:*

SEQIDNO_3      WGYYLFPDCYNHHYKKPGYNGSCFNVEIKRNDDLSWLWNESTALYPSIYLNTQQ-SPVAA 239
FOX            WGFYLYPDCYNYNYKNPNYNGSCYDIEERRNDEIDWLWKESTALFPSIYLKSKLKSSPFT 240
               **:**:*****::**:*.*****:::* :***::.***:*****:*****:::  *.  :

SEQIDNO_3      TLYVRNRVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLSQDELVYTFGETVALGAS 299
FOX            ALYVRNRVLEAIRVSKVKDIKHPLPIFVYARPVFTDVLLTYLTEDDLVNTIGESVSLGVS 300
               :******* *******: * * ***:*.*:* **** :*.:*::*:** *:**:*:**.*

SEQIDNO_3      GIVIWGTLSIMRSMKSCLLLDNYMETILNPYIINVTLAAKMCSQVLCQEQGVCIRKNWNS 359
FOX            GIVMWGSLNLTENVQICTELDTYIKNKLNPYIINVTLAAKMCSQVLCQDEGVCIRKHWNS 360
               ***:**:*.: ..:: *  **.*::. *********************::******:***

SEQIDNO_3      SDYLHLNPDNFAIQLEKGGKFTVRGKPTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDA 419
FOX            NDYLHLNPVNFAIQLERSGRYTVQGKPTLEDLQQFSKKFYCACYANTHCRERVDMTDIHT 420
               .******* *******:.*::**:********:***:****:**:.  *:*:.*:.* .:

SEQIDNO_3      VDVCIADGVCIDAFLKPP----------METEEPQIFY---------------------- 447
FOX            IKVCVGEDVCIDVYLNLVPSGHLPVWKGKYVTSSNIFSVMPPATGPPCVPGRDLNRCLKA 480
               :.**:.:.****.:*:              . ..:**

SEQIDNO_3      -------------------------
FOX            RFIVEDNSKTTQTGYQSIYIKNKKQ 505
```

FIGURE 2J

```
SEQIDNO_3               LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSLFSFIGSPRINATGQGVTIFYVDR 60
GIBBON_SEQIDNO_857      LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSLFSLTGSPRINVTGQGVTIFYVDR 60
                        ***************************************: ******.************

SEQIDNO_3               LGYYPYIDSITGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGMAVIDWEEWRPTWA 120
GIBBON_SEQIDNO_857      LGYYPYIDSITGVTVNGGIPQKISLQDHLDKAKQDITFYMPVDNLGMAVIDWEEWRPTWA 120
                        *********************************:**************************

SEQIDNO_3               RNWKPKDVYKNRSIELVQQQNVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRPNHL 180
GIBBON_SEQIDNO_857      RNWKPKDVYKNRSIELVQQQNVQLSLAEATEKAKQEFEKAGKDFMVETIKLGKLLRPNHL 180
                        **************************:*****************:***************

SEQIDNO_3               WGYYLFPDCYNHHYKKPGYNGSCFNVEIKRNDDLSWLWNESTALYPSIYLNTQQSPVAAT 240
GIBBON_SEQIDNO_857      WGYYLFPDCYNHHYKKPGYNGSCFNVEIKRNDDLSWLWNESTALYPSIYLNTQQSPVAAT 240
                        ************************************************************

SEQIDNO_3               LYVRNRVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLSQDELVYTFGETVALGASG 300
GIBBON_SEQIDNO_857      LYVRNRVREAIRVSKIPDAKSPLPVFVYARIVFTDQVLKFLSRDELVYTLGETVALGASG 300
                        **************************.*:*************:******:**********

SEQIDNO_3               IVIWGTLSIMRSMKSCLLLDNYMETILNPYIINVTLAAKMCSQVLCQEQGVCIRKNWNSS 360
GIBBON_SEQIDNO_857      IVIWGSLSIVRSMKSCLLLDNYMETILNPYIINVTLAAKMCSQVLCQEQGVCIRKDWNSS 360
                        *****:***:*********************************************:****

SEQIDNO_3               DYLHLNPDNFAIQLEKGGKFTVRGKPTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV 420
GIBBON_SEQIDNO_857      DYLHLNPDNFAIQLEKGGKFTVRGKPTPEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV 420
                        *************************** ********************************

SEQIDNO_3               DVCIADGVCIDAFLKPPMETEEPQIFY--------------------------- 447
GIBBON_SEQIDNO_857      DVCIADGVCIDAFLKPPKETEESQIFYNASPSTLSATMFIVSILFLIISSVVSL 474
                        ***************** ****.****
```

FIGURE 2K

```
SEQIDNO_3             LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSLFSFIGSPRINATGQGVTIFYVDR 60
MARMOSET_SEQIDNO_859  LNFRAPPIIPNVPFLWAWNAPSEFCLGKFDEPLDMSLFSLIGSPRINVTGQGVTIFYVDR 60
                      *******:*******************************:*******.************

SEQIDNO_3             LGYYPYIDSITGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGMAVIDWEEWRPTWA 120
MARMOSET_SEQIDNO_859  LGYYPYIDPTTGAVVNGGIPQKIALQDHLDKVRKDIIFYMPVDNLGMGVIDWEEWRPTWA 120
                      ********. **..*********:*******.:*** **********.************

SEQIDNO_3             RNWKPKDVYKNRSIELVQQQNVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRPNHL 180
MARMOSET_SEQIDNO_859  RNWKPKDIYKNKSIEMVQQRNVQLNLTQATDIAKQEFEKAAKDFMLETIKLGKALRPNHL 180
                      *******:***:***:***:****.**:**: ********.***::******* ******

SEQIDNO_3             WGYYLFPDCYNHHYKKPGYNGSCFNVEIKRNDDLSWLWNESTALYPSIYLNTQQSPVAAT 240
MARMOSET_SEQIDNO_859  WGYYLFPDCYNHHYKKPDYNGSCFNIEIKRNNDLSWLWNESTALYPSIYLNTQQSAVAAM 240
                      *****************.*******:*****:***********************.***

SEQIDNO_3             LYVRNRVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLSQDELVYTFGETVALGASG 300
MARMOSET_SEQIDNO_859  LYVRNRVQEAIRVSKTPNANSPLPVFVYARLVFTDQVLRFLSQDELVYTLGETVALGASG 300
                      *******:******* *:*:******.*:*:*******:**********:**********

SEQIDNO_3             IVIWGTLSIMRSMKSCLLLDNYMETILNPYIINVTLAAKMCSQVLCQEQGVCIRKNWNSS 360
MARMOSET_SEQIDNO_859  IVIWGSLSIMRSMKSCLLLDTYMETVLNPYIINTTLAAKMCSQVLCQEQGVCIRKDWNSS 360
                      *****:**************.****:*******.*********************:****

SEQIDNO_3             DYLHLNPDNFAIQLEKGGKFTVRGKPTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV 420
MARMOSET_SEQIDNO_859  DYLHLNPDNFAIETEKGGKFTVRGKPTYEDLEQFSEKFYCSCYTSLSCKVKADVKDTDAV 420
                      ************: ************* ***************::**** **********

SEQIDNO_3             DVCIADGVCIDAFLKPPMETEEP-QIFY-------------------------- 447
MARMOSET_SEQIDNO_859  DVCIADGVCIDASLKPPKETEESSQIFYNPSSSTPSAAIFIVAILFFISCVVSL 474
                      ************ **** ****. ****
```

FIGURE 2L

```
SEQIDNO_3              LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSLFSFIGSPRINATGQGVTIFYVDR 60
ORANGUTAN_SEQIDNO_861  LNFRAPPIIPNMPFLWAWNAPSEFCLGKFDEPLDMSLFSLIGSPRINVTGQAVTIFYVDR 60
                       *******:***:***************************:*******.***.********

SEQIDNO_3              LGYYPYIDSITGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGMAVIDWEEWRPTWA 120
ORANGUTAN_SEQIDNO_861  LGYYPYIDSITGVTVNGGIPQKISLQDHLDKAKKDILFYMPVDNLGMAVIDWEEWRPTWA 120
                       ************************************ ***********************

SEQIDNO_3              RNWKPKDVYKNRSIELVQQQNVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRPNHL 180
ORANGUTAN_SEQIDNO_861  RNWKPKDVYKNRSIELVQQQNVQLNLTEATEKAKQEFEKAGKDFMVETIKLGKLLRPNHL 180
                       ************************.*******************:***************

SEQIDNO_3              WGYYLFPDCYNHHYKKPGYNGSCFNVEIKRNDDLSWLWNESTALYPSIYLNTQQSPVAAT 240
ORANGUTAN_SEQIDNO_861  WGYYLFPDCYNHHYKKPGYNGSCFNVEIKRNDDLSWLWNESTALYPSIYLNTQQSPVAAT 240
                       ************************************************************

SEQIDNO_3              LYVRNRVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLSQDELVYTFGETVALGASG 300
ORANGUTAN_SEQIDNO_861  LYVRNRVREAIRVSKIPDAKSPLPVFVYARIVFTDQVLKFLSQDELVYTFGETVALGASG 300
                       **************************.*:*******************************

SEQIDNO_3              IVIWGTLSIMRSMKSCLLLDNYMETILNPYIINVTLAAKMCSQVLCQEQGVCIRKNWNSS 360
ORANGUTAN_SEQIDNO_861  IVIWGSLSIMRSMKSCLLLDNYMETILNPYIINVTLAAKMCSQVLCQEQGVCIRKDWNSS 360
                       *****:*************************************************:****

SEQIDNO_3              DYLHLNPDNFAIQLEKGGKFTVRGKPTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV 420
ORANGUTAN_SEQIDNO_861  DYLHLNPDNFAIQLEKGGKFTVRGKPTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV 420
                       ************************************************************

SEQIDNO_3              DVCIADGVCIDAFLKPPMETEEPQIFY----------------------------- 447
ORANGUTAN_SEQIDNO_861  DVCIADGVCIDAFLKPPMETEESQIFYNASPSTLSATMFIWRLEVWDQGISRMGFF 476
                       **********************.****
```

PH20 POLYPEPTIDE VARIANTS, FORMULATIONS AND USES THEREOF

RELATED APPLICATIONS

Benefit of priority is claimed to U.S. Provisional Application No. 61/631,313, filed Dec. 30, 2011, and to U.S. Provisional Application No. 61/796,208 filed Nov. 1, 2012, each entitled "PH20 Polypeptide Variants, Formulations and Uses Thereof."

This application is related to International PCT Application Serial No. PCT/US2012/072182, filed the same day herewith, entitled "PH20 Polypeptide Variants, Formulations and Uses Thereof," which claims priority to U.S. Provisional Application No. 61/631,313 and U.S. Provisional Application No. 61/796,208.

The subject matter of each of the above-noted related applications is incorporated by reference in its entirety.

INCORPORATION BY REFERENCE OF SEQUENCE LISTING PROVIDED ON COMPACT DISCS

An electronic version on compact disc (CD-R) of the Sequence Listing is filed herewith in duplicate (labeled Copy #1 and Copy #2), the contents of which are incorporated by reference in their entirety. The computer-readable file on each of the aforementioned compact discs, created on Dec. 28, 2012, is identical, 3.48 megabytes in size, and titled 3087seq.001.txt. A substitute Sequence Listing, incorporated by reference in its entirety, is provided on identical compact discs (labeled Copy 1 Replacement Mar. 20, 2013, Copy 2 Replacement Mar. 20, 2013). The computer-readable file on each of the aforementioned compact discs, created on Mar. 20, 2013, is identical, 3.50 megabytes in size, and titled 3087seq.002.txt. A substitute Sequence Listing, incorporated by reference in its entirety, is provided on identical compact discs (labeled Copy 1 Replacement Apr. 18, 2013, Copy 2 Replacement Apr. 18, 2013). The computer-readable file on each of the aforementioned compact discs, created on Apr. 18, 2013, is identical, 3.50 megabytes in size, and titled 3087seq.003.txt.

FIELD OF THE INVENTION

Modified PH20 hyaluronidase polypeptides, including modified polypeptides that exhibit increased stability and/or increased activity, are provided. Also provided are compositions and formulations and uses thereof.

BACKGROUND

Hyaluronan (hyaluronic acid; HA) is a polypeptide that is found in the extracellular matrix of many cells, especially in soft connective tissues. HA also is found predominantly in skin, cartilage, and in synovial fluid in mammals. Hyaluronan also is the main constituent of the vitreous of the eye. HA has a role in various physiological processes, such as in water and plasma protein homeostasis (Laurent T C et al. (1992) *FASEB J* 6:2397-2404). Certain diseases are associated with expression and/or production of hyaluronan. Hyaluronan-degrading enzymes, such as hyaluronidases, are enzymes that degrade hyaluronan. By catalyzing HA degradation, hyaluronan-degrading enzymes (e.g., hyaluronidases) can be used to treat diseases or disorders associated with accumulation of HA or other glycosaminoglycans. Also, since HA is a major component of the interstitial barrier, hyaluronan-degrading enzymes (e.g., hyaluronidase) increase tissue permeability and therefore can be used to increase the dispersion and delivery of therapeutic agents. Various hyaluronidases have been used therapeutically (e.g., Hydase™, Vitrase™ and Wydase™), typically as dispersing and spreading agents in combination with other therapeutic agents. Many of these are ovine or bovine forms, which can be immunogenic for treatment of humans. Improved hyaluronan-degrading enzymes, such as hyaluronidases, and compositions thereof that can be used for treatment are needed.

SUMMARY

Provided are modified PH20 polypeptides that have an altered property or properties compared to the PH20 polypeptide that do not have the modification(s). The modifications include amino acid replacement, deletion and/or insertions. Detailed structure/function of virtually each amino acid in a PH20 polypeptide is provided herein, as well as the identification of residues and loci that contribute to alteration of a property, such as stability in particular conditions, is provided. Hence, provided are modified PH20 polypeptides that contain one or more amino acid replacements that result in a PH20 polypeptide that retains activity and/or exhibits increased or altered stability under a variety of conditions. Activity retained can be, for example, hyaluronidase activity that is as least about 40% or more of the PH20 polypeptide that does not include the replacement. Exemplary modifications are amino acid replacements. For purposes herein, amino acid replacements are denoted by the single amino acid letter followed by the corresponding amino acid position in SEQ ID NO:3 in which the replacement occurs. Single amino acid abbreviations for amino acid residues are well known to a skilled artisan (see e.g. Table 1), and are used herein throughout the description and examples. For example, replacement with P at a position corresponding to position 204 in a PH20 polypeptide with reference to amino acid residue positions set forth in SEQ ID NO:3 means that the replacement encompasses F204P in a PH20 polypeptide set forth in SEQ ID NO:3, or the same replacement at the corresponding position in another PH20 polypeptide.

Provided are modified PH20 polypeptides that contain at least one amino acid replacement in a PH20 polypeptide, whereby the modified PH20 polypeptide exhibits increased stability compared to the PH20 polypeptide not containing the amino acid replacement. Increased stability can be manifested as increased resistance to one or more protein conditions that are denaturing to proteins. The stability of modified and unmodified PH20 is compared under the same conditions. Exemplary protein denaturation (or denaturing, used interchangeably herein) conditions include, but are not limited to, elevated temperature greater than 30° C. or about 30° C., agitation, low salt, including essentially or substantially or no salt, and presence of excipients that tend to denature proteins. Exemplary of such excipients are antiadherent(s), binders), coating(s), fillers) and diluent(s), flavors), color(s), lubricant(s), glidant(s), preservative(s), detergent(s), sorbent(s) and combinations thereof.

The modified PH20 polypeptide can be one in which the unmodified form thereof has at least about 68% sequence identity to SEQ ID NO: 3 and further contains modifications that alter stability and/or can be a PH20 polypeptide that includes as many as about up to 100, 110, 120, 130, 150 amino acid differences from PH20 but retains enzymatic activity, particularly, at least about 40% of the activity of the unmodified PH20 polypeptide and exhibits increased stability, such as stability under denaturing conditions. Thus, included are modified PH20 polypeptides that have at least 68% or about 68% amino acid sequence identity to the sequence of amino acids set forth in SEQ ID NO:3. Included are modified PH20 polypeptides that have at least 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%), 95%, 96%, 97%, 98% or 99% amino acid sequence identity to the sequence of amino acids set forth in SEQ ID NO:3. Exemplary of such modified PH20 polypeptides are polypeptides that contain amino acid replacement(s) in a PH20 polypeptide that contains the sequence of amino acid residues as set forth in any of SEQ ID NOS: 3, 7, 10, 12, 14, 24, 32-66, 69, 72, 857, 859, 861, 870 or a sequence of amino acids that is at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identical to any of SEQ ID NOS: 3, 7, 10, 12, 14, 24, 32-66, 69, 72, 857, 859, 861, or 870.

For example, provided herein is a modified PH20 polypeptide that exhibits increased stability containing an amino acid replacement in a PH20 polypeptide that confers the increased stability, wherein increased stability is manifested as increased resistance to denaturation in the presence of one or more protein denaturation conditions, stability is increased compared to the PH20 polypeptide not containing the amino acid replacement, and the unmodified PH20 polypeptide consists of the sequence of amino acids set forth in SEQ ID NO: 7 or is a C-terminal truncated fragment thereof that is a soluble PH20 polypeptide or has at least 85% sequence identity thereto. As above, the modified PH20 polypeptide that exhibits increased stability exhibits increased stability to a denaturation condition that is temperature greater than or about 30° C.; agitation; low or no salt; or presence of an excipient or a denaturing agent, such as an antiadherent(s), binder(s), coating(s), filler(s) and diluent(s), flavor(s), color(s), lubricant(s), glidant(s), preservative(s), detergent(s), sorbent(s) or sweetener(s) and a combination thereof, and in particular a preservative. In some examples of such modified PH20 polypeptides that exhibit increased stability, the denaturation condition is temperature greater than 30° C., and the modified PH20 polypeptide exhibits greater hyaluronidase activity at the temperature compared to the unmodified PH20 polypeptide not containing the amino acid replacement(s) where the activities are compared under the same conditions. In other examples, the protein denaturation condition is the presence of low concentrations of salt of less than 100 mM, and the modified PH20 polypeptide exhibits increased hyaluronidase activity in the presence of low concentrations of salt compared to the unmodified PH20 polypeptide not containing the amino acid replacement(s) where the activities are compared under the same conditions.

In any of the above examples of a modified PH20 polypeptide that exhibits increased stability, stability can be assessed based on a variety of parameters including hyaluronidase activity, solubility, aggregation and/or crystallization. Stability can be assessed in the presence of a denaturing condition. When stability of two or more polypeptides is compared, stability is assessed under the same conditions. In some instances, among the PH20 polypeptides provided herein, the modified PH20 polypeptide exhibits at least 120%, 130%, 135%, 140%, 145%, 150%, 160%, 170%, 180%, 200%, 250%, 300%, 350%, 400%, 500%, 1500%, 2000%, 3000%, 4000%, 5000% or more of the hyaluronidase activity of the PH20 polypeptide not containing the amino acid replacement(s).

In any of the above examples of a modified PH20 polypeptide that exhibits increased stability, denaturing conditions include the presence of excipients that denature proteins. Exemplary of such conditions is the presence of a preservative, such as a phenolic preservative. Provided are modified PH20 polypeptides that exhibit increased stability in the presence of an anti-microbial effective amount of one or more phenolic preservatives. An anti-microbial effective amount is the total amount of one or more phenolic preservative agents, which can be expressed as a percentage (%) of mass concentration (w/v) that is or is between (or at least about or at about) 0.05% to 0.6%, 0.1% to 0.4%, 0.1% to 0.3%, 0.15% to 0.325%, 0.15% to 0.25%, 0.1% to 0.2%, 0.2% to 0.3% or 0.3% to 0.4%, inclusive. Exemplary phenolic preservatives include, but are not limited to, phenol, metacresol (m-cresol), benzyl alcohol, and a paraben, such as methylparaben propylparaben, m-cresol, phenol or m-cresol and phenol. Exemplary of the stability achieved by provided modified PH20 polypeptides are those that exhibit at least 15% or about 15% of the hyaluronidase activity for at least 4 hours in the presence of preservative(s) compared to the modified PH20 polypeptide in absence of preservative. Activity is compared under the same conditions except for the presence of preservative(s). For example, provided are modified PH20 polypeptides that exhibit at least (or at least about) 16%, 17%, 18%, 19%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more of the hyaluronidase activity in the presence of a phenolic preservative(s) compared to absence of the same preservative(s). Thus, provided, among the modified PH20 polypeptides provided herein, are PH20 polypeptides that, by virtue of amino acid replacement(s), are phenophilic compared to PH20 polypeptides without such replacement. Included are modified PH20 polypeptides where the hyaluronidase activity is exhibited after at least 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 24 hours, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 3 weeks, 4 weeks or more in the presence of the preservative(s) compared to the hyaluronidase activity of the modified PH20 polypeptide in the absence of preservative for the same time period and under the same conditions except for the presence of preservative(s).

In examples of a modified PH20 polypeptide that exhibits increased stability to a phenolic preservative, increased stability in a phenolic preservative can be exhibited under temperature conditions that include any temperature between, for example, 0° C. and 40° C., such as between or about between 0° C. to 40° C., 2° C. to 6° C., 24° C. to 32° C. and 35° C. to 40° C. Exemplary polypeptides exhibit increased stability at temperatures of between or about between 30° C. to 45° C., 35° C. to 45° C., 30° C. to 37° C., 35° C. to 37° C. or 37° C. to 42° C., each inclusive. The particular modified PH20 polypeptide and conditions depend upon the intended formulation, conditions to which the formulation will be exposed and/or intended application.

Particular and exemplary modified PH20 polypeptides that exhibit increased stability, such as increased stability to a phenolic preservative, include those that contain a single amino acid modification, such as a replacement, and combinations of modifications, such as at least or 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70, 80, 90, 100 and more modifications. These include modified PH20 polypeptides that contain one or more amino acid replacements, where at least one replacement is at an amino acid position corresponding (i.e., by alignment) to a position selected from among 10, 12, 20, 22, 26, 34, 36, 46, 50, 52, 58, 68, 70, 74, 82, 83, 84, 86, 97, 127, 131, 138, 142, 143, 144, 166, 169, 174, 193, 195, 196, 204, 205, 206, 213, 219, 234, 237, 238, 240, 249, 261, 267, 277, 279, 291, 309, 310, 314, 315, 317, 318, 347, 367, 375, 376, 399, 401, 407, 416, 419, 421, 431, 433, 439, 440, 443 or 445 with reference to amino acid positions set forth in SEQ ID NO:3, wherein corresponding amino acid positions are identified by alignment of the PH20 polypeptide with the polypeptide set forth in SEQ ID NO:3. Exemplary of such modifications are at least one amino acid replacement selected from among replacement with: glycine (G) at a position corresponding to position 10; K at a position corresponding to position 12; S at a position corresponding to position 20; T at a position corresponding to position 22; M at a position corresponding to position 26; W at a position corresponding to position 34; N at a position corresponding to position 36; L at a position corresponding to position 46; M at a position corresponding to position 50; T at a position corresponding to position 52; S at a position corresponding to position 52; C at a position corresponding to position 58; K at a position corresponding to position 58; R at a position corresponding to position 58; N at a position corresponding to position 58; Y at a position corresponding to position 58; P at a position corresponding to position 58; H at a position corresponding to position 58; P at a position corresponding to position 68; V at a position corresponding to position 70; E at a position corresponding to position 74; L at a position corresponding to position 82; N at a position corresponding to position 82; V at a position corresponding to position 83; Q at a position corresponding to position 83; S at a position corresponding to position 83; G at a position corresponding to position 83; N at a position corresponding to position 84; A at a position corresponding to position 86; K at a position corresponding to position 86; E at a position corresponding to position 97; L at a position corresponding to position 97; R at a position corresponding to position 127; R at a position corresponding to position 131; L at a position corresponding to position 138; K at a position corresponding to position 142; N at a position corresponding to position 142; P at a position corresponding to position 142; S at a position corresponding to position 142; T at a position corresponding to position 142; G at a position corresponding to position 143; K at a position corresponding to position 143; T at a position corresponding to position 144; Q at a position corresponding to position 166; T at a position corresponding to position 166; L at a position corresponding to position 169; G at a position corresponding to position 174; N at a position corresponding to position 174; Q at a position corresponding to position 193; T at a position corresponding to position 195; N at a position corresponding to position 195; E at a position corresponding to position 196; R at a position corresponding to position 196; P at a position corresponding to position 204; A at a position corresponding to position 205; E at a position corresponding to position 205; I at a position corresponding to position 206; A at a position corresponding to position 213; I at a position corresponding to position 219; M at a position corresponding to position 234; T at a position corresponding to position 237; H at a position corresponding to position 238; Q at a position corresponding to position 240; V at a position corresponding to position 249; A at a position corresponding to position 261; K at a position corresponding to position 261; T at a position corresponding to position 267; K at a position corresponding to position 277; H at a position corresponding to position 279; V at a position corresponding to position 279; V at a position corresponding to position 291; E at a position corresponding to position 309; Q at a position corresponding to position 310; Y at a position corresponding to position 314; Y at a position corresponding to position 315; N at a position corresponding to position 317; W at a position corresponding to position 317; D at a position corresponding to position 318; G at a position corresponding to position 347; A at a position corresponding to position 367; R at a position corresponding to position 375; R at a position corresponding to position 376; V at a position corresponding to position 399; E at a position corresponding to position 401; A at a position corresponding to position 407; L at a position corresponding to position 416; K at a position corresponding to position 419; H at a position corresponding to position 421; E at a position corresponding to position 431; T at a position corresponding to position 433; V at a position corresponding to position 433; C at a position corresponding to position 439; P at a position corresponding to position 440; G at a position corresponding to position 443; N at a position corresponding to position 445, with reference to amino acid residue positions set forth in SEQ ID NO:3. For example, the modified PH20 polypeptide can contain at least one amino acid replacement selected from among replacement with: T at a position corresponding to position 52, K at a position corresponding to position 58, R at a position corresponding to position 58, P at a position corresponding to position 68, V at a position corresponding to position 83, P at a position corresponding to position 204, A at a position corresponding to position 261, T at a position corresponding to position 267, K at a position corresponding to position 277 and H at a position corresponding to position 421, with reference to amino acid residue positions set forth in SEQ ID NO:3. An exemplary modified PH20 polypeptide is one that includes P (or a conservative amino acid thereto) at a position corresponding to position 204 in a PH20 polypeptide with reference to amino acid residue positions set forth in SEQ ID NO:3.

Thus, provided herein are modified PH20 polypeptides that exhibit increased stability in the presence of a phenolic preservative containing an amino acid replacement in a PH20 polypeptide that confers the increased stability, wherein stability is increased compared to the unmodified polypeptide without the amino acid replacement, and the unmodified PH20 polypeptide has the sequence of amino acids set forth in SEQ ID NO: 7 or is a C-terminal truncated fragment thereof that is a soluble PH20 polypeptide or has at least 85% sequence identity thereto. For example, the unmodified PH20 polypeptide is a soluble PH20 polypeptide that has the sequence of amino acids set forth in any of SEQ ID NOS: 3 or 32-66. In particular examples, the modified PH20 polypeptide has at least 85% sequence identity to SEQ ID NO:3. In any of such examples of a modified PH20 polypeptide, the polypeptide contains 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75 or more amino acid replacements. In examples herein, the modified PH20 polypeptide is a human PH20. The modified PH20 polypeptide exhibits stability in the presence of phenolic preservatives if it exhibits at least 15% of the hyaluronidase activity in the presence of a preservative(s) for at least 4 hours compared to the hyaluronidase activity in the absence of the phenolic preservative(s), wherein the activity is compared under the same conditions except for the presence of the phenolic preservative(s). In any of the above examples, the modified PH20 polypeptide is stable in the presence of an of an anti-microbial effective amount of one or more phenolic preservatives, such as a total amount of one or more phenolic preservative agents as a percentage (%) of mass concentration (w/v) that is from or from about 0.05% to 0.6%, 0.1% to 0.4%, 0.1% to 0.3%, 0.15% to 0.325%, 0.15% to 0.25%, 0.1% to 0.2%, 0.2% to 0.3% or 0.3% to 0.4%, inclusive. The phenolic preservative can be a phenol, metacresol (m-cresol), benzyl alcohol or a paraben, such as m-cresol, phenol, or m-cresol and phenol. The amino acid replacement can be at amino acid residue 204, 58, 10, 12, 20, 22, 26, 34, 36, 46, 50, 52, 68, 70, 74, 82, 83, 84, 86, 97, 127, 131, 138, 142, 143, 144, 166, 169, 174, 193, 195, 196, 205, 206, 213, 219, 234, 237, 238, 240, 249, 261, 267, 277, 279, 291, 309, 310, 314, 315, 317, 318, 347, 367, 375, 376, 399, 401, 407, 416, 419, 421, 431, 433, 439, 440, 443 or 445 with reference to amino acid positions set forth in SEQ ID NO:3, wherein corresponding amino acid positions are identified by alignment of the PH20 polypeptide with the polypeptide set forth in SEQ ID NO:3. For example, the amino acid replacement is G at a position corresponding to position 10; K at a position corresponding to position 12; S at a position corresponding to position 20; T at a position corresponding to position 22; M at a position corresponding to position 26; W at a position corresponding to position 34; N at a position corresponding to position 36; L at a position corresponding to position 46; M at a position corresponding to position 50; T at a position corresponding to position 52; S at a position corresponding to position 52; C at a position corresponding to position 58; K at a position corresponding to position 58; R at a position corresponding to position 58; N at a position corresponding to position 58; Y at a position corresponding to position 58; P at a position corresponding to position 58; H at a position corresponding to position 58; P at a position corresponding to position 68; V at a position corresponding to position 70; E at a position corresponding to position 74; L at a position corresponding to position 82; N at a position corresponding to position 82; V at a position corresponding to position 83; Q at a position corresponding to position 83; S at a position corresponding to position 83; G at a position corresponding to position 83; N at a position corresponding to position 84; A at a position corresponding to position 86; K at a position corresponding to position 86; E at a position corresponding to position 97; L at a position corresponding to position 97; R at a position corresponding to position 127; R at a position corresponding to position 131; L at a position corresponding to position 138; K at a position corresponding to position 142; N at a position corresponding to position 142; P at a position corresponding to position 142; S at a position corresponding to position 142; T at a position corresponding to position 142; G at a position corresponding to position 143; K at a position corresponding to position 143; T at a position corresponding to position 144; Q at a position corresponding to position 166; T at a position corresponding to position 166; L at a position corresponding to position 169; G at a position corresponding to position 174; N at a position corresponding to position 174; Q at a position corresponding to position 193; T at a position corresponding to position 195; N at a position corresponding to position 195; E at a position corresponding to position 196; R at a position corresponding to position 196; P at a position corresponding to position 204; A at a position corresponding to position 205; E at a position corresponding to position 205; I at a position corresponding to position 206; A at a position corresponding to position 213; I at a position corresponding to position 219; M at a position corresponding to position 234; T at a position corresponding to position 237; H at a position corresponding to position 238; Q at a position corresponding to position 240; V at a position corresponding to position 249; A at a position corresponding to position 261; K at a position corresponding to position 261; T at a position corresponding to position 267; K at a position corresponding to position 277; H at a position corresponding to position 279; V at a position corresponding to position 279; V at a position corresponding to position 291; E at a position corresponding to position 309; Q at a position corresponding to position 310; Y at a position corresponding to position 314; Y at a position corresponding to position 315; N at a position corresponding to position 317; W at a position corresponding to position 317; D at a position corresponding to position 318; G at a position corresponding to position 347; A at a position corresponding to position 367; R at a position corresponding to position 375; R at a position corresponding to position 376; V at a position corresponding to position 399; E at a position corresponding to position 401; A at a position corresponding to position 407; L at a position corresponding to position 416; K at a position corresponding to position 419; H at a position corresponding to position 421; E at a position corresponding to position 431; T at a position corresponding to position 433; V at a position corresponding to position 433; C at a position corresponding to position 439; P at a position corresponding to position 440; G at a position corresponding to position 443; or N at a position corresponding to position 445, with reference to amino acid residue positions set forth in SEQ ID NO:3. In particular, the amino acid replacement is T at a position corresponding to position 52, K at a position corresponding to position 58, R at a position corresponding to position 58, P at a position corresponding to position 68, V at a position corresponding to position 83, P at a position corresponding to position 204, A at a position corresponding to position 261, T at a position corresponding to position 267, K at a position corresponding to position 277 or H at a position corresponding to position 421, with reference to amino acid residue positions set forth in SEQ ID NO:3, such as replacement with P at a position corresponding to position 204 or R at a position corresponding to position 58. The modified PH20 polypeptide that exhibits increased stability to phenolic preservatives can be substantially purified or isolated. The modified PH20 polypeptide that exhibits increased stability to phenolic preservatives can be modified by glycosylation, sialation, albumination, farnysylation, carboxylation, hydroxylation and phosphorylation, and generally is glycosylated, whereby the polypeptide contains at least an N-acetylglucosamine moiety linked to each of at least three asparagine (N) residues, such as at amino acid residues corresponding to amino acid residues 200, 333 and 358 of SEQ ID NO:3. The modified PH20 polypeptide that exhibits increased stability to phenolic preservatives can be conjugated to a polymer, such as PEG or dextran and/or can be conjugated to a moiety that is a multimerization domain, a toxin, a detectable label or a drug.

Among modified PH20 polypeptides provided herein that exhibit increased stability are those that exhibit increased hyaluronidase activity at the elevated temperature compared to the PH20 polypeptide not containing the amino acid replacement(s), such as at least 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 300%, 400%, 500% or more hyaluronidase activity for at least 4 hours compared to the PH20 polypeptide not containing the amino acid replacement(s). Also among the polypeptides are those that exhibit activity, but also typically exhibit increased stability or other property at elevated temperatures, such as a modified PH20 polypeptide that exhibits at least 95%, 96%, 97%, 98%, 99%, 100%, 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 300%, 400%, 500% of the hyaluronidase activity for at least 4 hours at a temperature of between or about between 32° C. to 37° C. compared to the hyaluronidase activity of the modified PH20 polypeptide at a temperature of between or about between 2° C. to 8° C., where activity is compared under the same conditions except for the differences in temperature. The hyaluronidase activity can be exhibited after at least 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 24 hours, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 3 weeks, 4 weeks or more at elevated temperatures of between or about between 32° C. to 37° C. compared to the hyaluronidase activity of the modified PH20 polypeptide at a temperature between or about between 2° C. to 8° C., where activity is compared for the same time period and under the same conditions except for the difference in temperature. Exemplary of such modified polypeptides are those that contain at least one amino acid replacement at an amino acid position corresponding to a position selected from among 1, 11, 12, 14, 20, 26, 29, 34, 50, 58, 70, 82, 83, 84, 86, 87, 140, 142, 143, 147, 152, 166, 167, 172, 174, 178, 193, 195, 206, 212, 213, 219, 233, 237, 240, 267, 277, 291, 292, 309, 313, 314, 317, 318, 347, 367, 368, 371, 374, 389, 392, 395, 396, 406, 419, 421, 439 and 443 with reference to amino acid positions set forth in SEQ ID NO:3, wherein corresponding amino acid positions are identified by alignment of the PH20 polypeptide with the polypeptide set forth in SEQ ID NO:3. Exemplary mutations include, for example, replacement with R at a position corresponding to position 1; S at a position corresponding to position 11; I at a position corresponding to position 12; V at a position corresponding to position 14; S at a position corresponding to position 20; M at a position corresponding to position 26; with R at a position corresponding to position 29; W at a position corresponding to position 34; M at a position corresponding to position 50; K at a position corresponding to position 58; Q at a position corresponding to position 58; Q at a position corresponding to position 58; V at a position corresponding to position 70; L at a position corresponding to position 82; Q at a position corresponding to position 83; R at a position corresponding to position 84; A at a position corresponding to position 86; S at a position corresponding to position 87; K at a position corresponding to position 140; S at a position corresponding to position 142; T at a position corresponding to position 142; K at a position corresponding to position 143; S at a position corresponding to position 147; T at a position corresponding to position 152; T at a position corresponding to position 166; D at a position corresponding to position 167; A at a position corresponding to position 172; G at a position corresponding to position 174; N at a position corresponding to position 174; R at a position corresponding to position 178; Q at a position corresponding to position 193; T at a position corresponding to position 195; I at a position corresponding to position 206; S at a position corresponding to position 212; A at a position corresponding to position 213; I at a position corresponding to position 219; G at a position corresponding to position 233; T at a position corresponding to position 237; A at a position corresponding to position 240; Q at a position corresponding to position 240; T at a position corresponding to position 267; E at a position corresponding to position 277; S at a position corresponding to position 291; H at a position corresponding to position 292; V at a position corresponding to position 292; S at a position corresponding to position 309; H at a position corresponding to position 313; S at a position corresponding to position 314; I at a position corresponding to position 317; T at a position corresponding to position 317; W at a position corresponding to position 317; R at a position corresponding to position 318; G at a position corresponding to position 347; A at a position corresponding to position 367; R at a position corresponding to position 368; S at a position corresponding to position 371; P at a position corresponding to position 374; A at a position corresponding to position 389; V at a position corresponding to position 392; A at a position corresponding to position 395; H at a position corresponding to position 396; N at a position corresponding to position 406; H at a position corresponding to position 419; K at a position corresponding to position 419; R at a position corresponding to position 421; S at a position corresponding to position 421; A at a position corresponding to position 439; C at a position corresponding to position 439; and G at a position corresponding to position 443, with reference to amino acid positions set forth in SEQ ID NO:3. In particular examples provided herein, any of such modified PH20 polypeptides contain a single amino acid modification, such as a replacement, and combinations of modifications, such as at least or 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70, 80, 90, 100 and more modifications. The modification, such as replacement, can be in an unmodified PH20 polypeptide that has the sequence of amino acids set forth in SEQ ID NO: 7 or is a C-terminal truncated fragment thereof that is a soluble PH20 polypeptide, such as is set forth in any of SEQ ID NOS: 3 or 32-66, or has at least 85% sequence identity thereto. For example, any of such modified PH20 polypeptides has at least 85% sequence identity to SEQ ID NO:3.

Also provided are modified PH20 polypeptides that exhibit increased stability in low salt conditions, such as, for example, concentrations of NaCl of less than 100 mM, such as, but not limited to concentrations of NaCl less than 90 mM, 80 mM, 70 mM, 60 mM, 50 mM, 40 mM, 30 mM, 25 mM, 20 mM, 15 mM, 10 mM, 5 mM or less. Among the modified PH20 polypeptides are those that exhibit increased hyaluronidase activity at lower concentrations of salt compared to the PH20 polypeptide not containing the amino acid replacement(s). Such activity includes, for example, at least more than 100%, or at least 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 300%, 400%, 500% or more hyaluronidase activity compared to the PH20 polypeptide not containing the amino acid replacement(s). Exemplary of such modified PH20 polypeptides are those that exhibit at least 60% of the hyaluronidase activity in low concentrations of salt, such as between or about between 10 mM NaCl and 100 mM NaCl, inclusive (or comparable concentrations of other salts or mixtures of salts), compared to the hyaluronidase activity of the modified PH20 polypeptide in 150 mM NaCl, where activities are compared under the same conditions except for the difference in salt concentration. In particular examples provided herein, any of such modified PH20 polypeptides contain a single amino acid modification, such as a replacement, and combinations of modifications, such as at least or 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70, 80, 90, 100 and more modifications. The modification, such as replacement, can be in an unmodified PH20 polypeptide that has the sequence of amino acids set forth in SEQ ID NO: 7 or is a C-terminal truncated fragment thereof that is a soluble PH20 polypeptide, such as is set forth in any of SEQ ID NOS: 3 or 32-66, or has at least 85% sequence identity thereto. For example, any of such modified PH20 polypeptides has at least 85% sequence identity to SEQ ID NO:3.

Also provided are modified PH20 polypeptides that contain at least one amino acid replacement in a PH20 polypeptide, where the modified PH20 polypeptide exhibits increased hyaluronidase activity compared to the PH20 polypeptide not containing the amino acid replacement. When comparing activity among polypeptides, activity is compared under the same conditions. Among these are polypeptides, where the unmodified PH20 exhibits at least 68%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% amino acid sequence identity to the sequence of amino acids set forth in SEQ ID NO:3, or the resulting modified PH20 exhibits such sequence identity to the sequence of amino acids set forth in SEQ ID NO:3. Exemplary of such modified PH20 polypeptides are any that contain an amino acid replacement(s) in the sequence of amino acids set forth in any of SEQ ID NOS: 3, 7, 10, 12, 14, 24, 32-66, 69, or 72, or a sequence of amino acids that is at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identical to any of SEQ ID NOS: 3, 7, 10, 12, 14, 24, 32-66, 69, or 72. The amino acid replacement(s) also can be made in the sequence of amino acids set forth in any of SEQ ID NOS: 857, 859, 861 or 870, or a sequence of amino acids that is at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identical to any of SEQ ID NOS: 857, 859, 861 or 870. In particular, provided are modified PH20 polypeptides that contain an amino acid replacement in the sequence of amino acids set forth in SEQ ID NOS: 3, 7, 32-66, 69 or 72. Among the modified PH20 polypeptides are those that that exhibit at least 120%, 130%, 135%, 140%, 145%, 150%, 160%, 170%, 180%, 200%, 250%, 300%, 350%, 400%, 500%, 1500%, 2000%, 3000%, 4000%, 5000% or more of the hyaluronidase activity of the PH20 polypeptide not containing the amino acid replacement. Activity can be assessed at any temperature, in particular such activity is present when the hyaluronidase is exposed to a temperature that is at a temperature between or about between 2° C. to 8° C. These modified PH20 polypeptides contain at least one amino acid replacement at an amino acid position corresponding to a position selected from among 1, 12, 15, 24, 26, 27, 29, 30, 31, 32, 33, 37, 39, 46, 48, 52, 58, 63, 67, 68, 69, 70, 71, 72, 73, 74, 75, 84, 86, 87, 92, 93, 94, 97, 118, 120, 127, 131, 135, 141, 142, 147, 148, 150, 151, 152, 155, 156, 163, 164, 165, 166, 169, 170, 174, 198, 206, 209, 212, 213, 215, 219, 233, 234, 236, 238, 247, 257, 259, 260, 261, 263, 269, 271, 272, 276, 277, 278, 282, 291, 293, 305, 308, 309, 310, 313, 315, 317, 318, 320, 324, 325, 326, 328, 347, 353, 359, 371, 377, 380, 389, 392, 395, 399, 405, 407, 409, 410, 418, 419, 421, 425, 431, 433, 436, 437, 438, 439, 440, 441, 442, 443, 445, 446 and 447 with reference to amino acid positions set forth in SEQ ID NO:3, wherein corresponding amino acid positions are identified by alignment of the PH20 polypeptide with the polypeptide set forth in SEQ ID NO:3. Exemplary modifications include at least one amino acid replacement selected from among replacement with: histidine (H) at a position corresponding to position 1; Q at a position corresponding to position 1; E at a position corresponding to position 12; T at a position corresponding to position 12; V at a position corresponding to position 15; E at a position corresponding to position 24; H at a position corresponding to position 24; E at a position corresponding to position 26; K at a position corresponding to position 26; K at a position corresponding to position 27; R at a position corresponding to position 27; E at a position corresponding to position 29; I at a position corresponding to position 29; L at a position corresponding to position 29; M at a position corresponding to position 29; P at a position corresponding to position 29; S at a position corresponding to position 29; V at a position corresponding to position 29; G at a position corresponding to position 30; H at a position corresponding to position 30; K at a position corresponding to position 30; M at a position corresponding to position 30; R at a position corresponding to position 30; S at a position corresponding to position 30; A at a position corresponding to position 31; C at a position corresponding to position 31; H at a position corresponding to position 31; I at a position corresponding to position 31; K at a position corresponding to position 31; L at a position corresponding to position 31; P at a position corresponding to position 31; R at a position corresponding to position 31; S at a position corresponding to position 31; T at a position corresponding to position 31; V at a position corresponding to position 31; F at a position corresponding to position 32; G at a position corresponding to position 32; H at a position corresponding to position 32; W at a position corresponding to position 33; F at a position corresponding to position 37; N at a position corresponding to position 39; T at a position corresponding to position 39; R at a position corresponding to position 46; F at a position corresponding to position 48; H at a position corresponding to position 48; N at a position corresponding to position 48; Q at a position corresponding to position 52; K at a position corresponding to position 58; Q at a position corresponding to position 58; W at a position corresponding to position 63; V at a position corresponding to position 67; H at a position corresponding to position 68; Q at a position corresponding to position 68; A at a position corresponding to position 69; C at a position corresponding to position 69; F at a position corresponding to position 69; G at a position corresponding to position 69; I at a position corresponding to position 69; L at a position corresponding to position 69; M at a position corresponding to position 69; P at a position corresponding to position 69; R at a position corresponding to position 69; W at a position corresponding to position 69; Y at a position corresponding to position 69; A at a position corresponding to position 70; C at a position corresponding to position 70; F at a position corresponding to position 70; G at a position corresponding to position 70; H at a position corresponding to position 70; K at a position corresponding to position 70; L at a position corresponding to position 70; N at a position corresponding to position 70; P at a position corresponding to position 70; R at a position corresponding to position 70; S at a position corresponding to position 70; T at a position corresponding to position 70; V at a position corresponding to position 70; R at a position corresponding to position 71; S at a position corresponding to position 71; M at a position corresponding to position 72; Q at a position corresponding to position 72; H at a position corresponding to position 73; L at a position corresponding to position 73; W at a position corresponding to position 73; A at a position corresponding to position 74; C at a position corresponding to position 74; G at a position corresponding to position 74; N at a position corresponding to position 74; P at a position corresponding to position 74; R at a position corresponding to position 74; S at a position corresponding to position 74; V at a position corresponding to position 74; W at a position corresponding to position 74; F at a position corresponding to position 75; L at a position corresponding to position 75; R at a position corresponding to position 75; T at a position corresponding to position 75; G at a position corresponding to position 84; R at a position corresponding to position 84; A at a position corresponding to position 86; C at a position corresponding to position 87; T at a position corresponding to position 87; Y at a position corresponding to position 87; C at a position corresponding to position 92; I at a position corresponding to position 93; L at a position corresponding to position 93; R at a position corresponding to position 93; T at a position corresponding to position 93; R at a position corresponding to position 94; G at a position corresponding to position 97; Q at a position corresponding to position 118; F at a position corresponding to position 120; V at a position corresponding to position 120; Y at a position corresponding to position 120; H at a position corresponding to position 127; N at a position corresponding to position 127; G at a position corresponding to position 131; R at a position corresponding to position 131; V at a position corresponding to position 131; D at a position corresponding to position 135; G at a position corresponding to position 135; R at a position corresponding to position 135, with H at a position corresponding to position 141; Y at a position corresponding to position 141; R at a position corresponding to position 142; R at a position corresponding to position 147; V at a position corresponding to position 147; K at a position corresponding to position 148; G at a position corresponding to position 150; K at a position corresponding to position 151; L at a position corresponding to position 151; M at a position corresponding to position 151; Q at a position corresponding to position 151; R at a position corresponding to position 151; R at a position corresponding to position 152; G at a position corresponding to position 155; K at a position corresponding to position 155; D at a position corresponding to position 156; A at a position corresponding to position 163; E at a position corresponding to position 163; K at a position corresponding to position 163; R at a position corresponding to position 163; M at a position corresponding to position 164; D at a position corresponding to position 165; N at a position corresponding to position 165; A at a position corresponding to position 166; F at a position corresponding to position 166; H at a position corresponding to position 166; L at a position corresponding to position 166; Q at a position corresponding to position 166; R at a position corresponding to position 166; T at a position corresponding to position 166; Y at a position corresponding to position 166; L at a position corresponding to position 169; R at a position corresponding to position 170; K at a position corresponding to position 174; D at a position corresponding to position 198; K at a position corresponding to position 206; L at a position corresponding to position 206; N at a position corresponding to position 212; M at a position corresponding to position 213; N at a position corresponding to position 213; M at a position corresponding to position 215; S at a position corresponding to position 219; K at a position corresponding to position 233; R at a position corresponding to position 233; M at a position corresponding to position 234; R at a position corresponding to position 236; E at a position corresponding to position 237; S at a position corresponding to position 238; I at a position corresponding to position 247; T at a position corresponding to position 257; P at a position corresponding to position 259; Y at a position corresponding to position 260; K at a position corresponding to position 261; N at a position corresponding to position 261; K at a position corresponding to position 263; R at a position corresponding to position 263; A at a position corresponding to position 269; L at a position corresponding to position 271; M at a position corresponding to position 271; T at a position corresponding to position 272; D at a position corresponding to position 276; S at a position corresponding to position 276; Y at a position corresponding to position 276; K at a position corresponding to position 277; R at a position corresponding to position 277; T at a position corresponding to position 277; H at a position corresponding to position 278; K at a position corresponding to position 278; N at a position corresponding to position 278; R at a position corresponding to position 278; S at a position corresponding to position 278; T at a position corresponding to position 278; Y at a position corresponding to position 278; M at a position corresponding to position 282; V at a position corresponding to position 291; A at a position corresponding to position 293; C at a position corresponding to position 293; F at a position corresponding to position 293; M at a position corresponding to position 293; P at a position corresponding to position 293; Q at a position corresponding to position 293; V at a position corresponding to position 293; E at a position corresponding to position 305; G at a position corresponding to position 308; N at a position corresponding to position 308; E at a position corresponding to position 309; L at a position corresponding to position 309; N at a position corresponding to position 309; Q at a position corresponding to position 309; R at a position corresponding to position 309; T at a position corresponding to position 309; A at a position corresponding to position 310; G at a position corresponding to position 310; K at a position corresponding to position 313; R at a position corresponding to position 313; H at a position corresponding to position 315; I at a position corresponding to position 317; K at a position corresponding to position 317; R at a position corresponding to position 317; M at a position corresponding to position 318; H at a position corresponding to position 320; K at a position corresponding to position 320; R at a position corresponding to position 320; R at a position corresponding to position 324; A at a position corresponding to position 325; D at a position corresponding to position 325; E at a position corresponding to position 325; G at a position corresponding to position 325; H at a position corresponding to position 325; K at a position corresponding to position 325; M at a position corresponding to position 325; N at a position corresponding to position 325; Q at a position corresponding to position 325; S at a position corresponding to position 325; V at a position corresponding to position 326; I at a position corresponding to position 328; K at a position corresponding to position 328; L at a position corresponding to position 328; S at a position corresponding to position 328; Y at a position corresponding to position 328; G at a position corresponding to position 347; S at a position corresponding to position 347; V at a position corresponding to position 353; with T at a position corresponding to position 359; R at a position corresponding to position 371; P at a position corresponding to position 377; T at a position corresponding to position 377; W at a position corresponding to position 380; Y at a position corresponding to position 380; K at a position corresponding to position 389; M at a position corresponding to position 392; R at a position corresponding to position 395; M at a position corresponding to position 399; T at a position corresponding to position 399; W at a position corresponding to position 399; G at a position corresponding to position 405; D at a position corresponding to position 407; Q at a position corresponding to position 407; A at a position corresponding to position 409; Q at a position corresponding to position 409; T at a position corresponding to position 410; P at a position corresponding to position 418; F at a position corresponding to position 419; I at a position corresponding to position 419; K at a position corresponding to position 419; R at a position corresponding to position 419; S at a position corresponding to position 419; H at a position corresponding to position 421; K at a position corresponding to position 421; N at a position corresponding to position 421; Q at a position corresponding to position 421; R at a position corresponding to position 421; S at a position corresponding to position 421; K at a position corresponding to position 425; A at a position corresponding to position 431; H at a position corresponding to position 431; K at a position corresponding to position 431; Q at a position corresponding to position 431; R at a position corresponding to position 431; S at a position corresponding to position 431; V at a position corresponding to position 431; L at a position corresponding to position 433; R at a position corresponding to position 433; T at a position corresponding to position 433; V at a position corresponding to position 433; K at a position corresponding to position 436; I at a position corresponding to position 437; M at a position corresponding to position 437; T at a position corresponding to position 438; V at a position corresponding to position 439; H at a position corresponding to position 440; R at a position corresponding to position 440; F at a position corresponding to position 441; R at a position corresponding to position 442; A at a position corresponding to position 443; M at a position corresponding to position 443; M at a position corresponding to position 445; P at a position corresponding to position 445; A at a position corresponding to position 446; D at a position corresponding to position 447; N at a position corresponding to position 447; and/or with Q at a position corresponding to position 447, with reference to amino acid positions set forth in SEQ ID NO:3.

Among the polypeptides that exhibit increased hyaluronidase activity are those that exhibit at least 2.0-fold of the hyaluronidase activity of the PH20 polypeptide not containing the amino acid replacement. For example, among these are modified PH20 polypeptides that contain at least one amino acid replacement at an amino acid position corresponding to a position selected from among 24, 29, 31, 48, 58, 69, 70, 75, 84, 97, 165, 166, 271, 278, 317, 320, 325 and 326 with reference to positions set forth in SEQ ID NO:3, wherein corresponding amino acid positions are identified by alignment of the PH20 polypeptide with the polypeptide set forth in SEQ ID NO:3, such as modified PH20 polypeptides that contain at least one amino acid replacement selected from among replacement with: E at a position corresponding to position 24; E at a position corresponding to position 29; V at a position corresponding to position 31; N at a position corresponding to position 48; K at a position corresponding to position 58; Q at a position corresponding to position 58; A at a position corresponding to position 69; F at a position corresponding to position 69; G at a position corresponding to position 69; P at a position corresponding to position 69; R at a position corresponding to position 69; A at a position corresponding to position 70; F at a position corresponding to position 70; G at a position corresponding to position 70; H at a position corresponding to position 70; H at a position corresponding to position 70; N at a position corresponding to position 70; R at a position corresponding to position 70; T at a position corresponding to position 70; V at a position corresponding to position 70; L at a position corresponding to position 75; T at a position corresponding to position 75; G at a position corresponding to position 84; G at a position corresponding to position 97; D at a position corresponding to position 165; L at a position corresponding to position 166; R at a position corresponding to position 166; T at a position corresponding to position 166; L at a position corresponding to position 271; H at a position corresponding to position 278; R at a position corresponding to position 278; K at a position corresponding to position 317; K at a position corresponding to position 320; E at a position corresponding to position 325, with G at a position corresponding to position 325; K at a position corresponding to position 325; N at a position corresponding to position 325; Q at a position corresponding to position 325; and V at a position corresponding to position 326; with reference to amino acid positions set forth in SEQ ID NO:3.

Among any of the polypeptides provided herein that exhibit increased hyaluronidase activity, any of such modified PH20 polypeptides contain a single amino acid modification, such as a replacement, and combinations of modifications, such as at least or 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70, 80, 90, 100 and more modifications. The modification, such as replacement, can be in an unmodified PH20 polypeptide that has the sequence of amino acids set forth in SEQ ID NO: 7 or is a C-terminal truncated fragment thereof that is a soluble PH20 polypeptide, such as is set forth in any of SEQ ID NOS: 3 or 32-66, or has at least 85% sequence identity thereto. For example, any of such modified PH20 polypeptides has at least 85% sequence identity to SEQ ID NO:3.

Also provided are modified PH20 polypeptides that contain at least one amino acid replacement in the PH20 polypeptide whose sequence is set forth in SEQ ID NO:7, a C-terminally truncated fragment thereof, a soluble fragment thereof, or in a PH20 polypeptide that has a sequence of amino acids that is at least 91% identical to the sequence of amino acids set forth in SEQ ID NO:7, where at least one amino replacement(s) is at an amino acid position corresponding to a position selected from among 1, 2, 3, 4, 5, 6, 8, 9, 10, 11, 12, 13, 14, 15, 20, 22, 23, 24, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 54, 58, 59, 60, 61, 63, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 77, 79, 81, 82, 83, 84, 85, 86, 87, 89, 90, 91, 92, 93, 94, 96, 97, 98, 99, 102, 103, 104, 105, 106, 107, 108, 110, 114, 117, 118, 119, 120, 122, 124, 125, 127, 128, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 186, 192, 193, 195, 196, 197, 198, 200, 202, 204, 205, 206, 208, 209, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 224, 226, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 242, 245, 247, 248, 251, 253, 255, 256, 257, 258, 259, 260, 261, 263, 264, 265, 266, 267, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 297, 298, 300, 301, 302, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 320, 321, 323, 324, 325, 326, 327, 328, 331, 334, 335, 338, 339, 342, 343, 347, 348, 349, 351, 353, 356, 357, 358, 359, 360, 361, 367, 368, 369, 371, 373, 374, 375, 376, 377, 378, 379, 380, 381, 383, 385, 387, 388, 389, 391, 392, 393, 394, 395, 396, 397, 398, 399, 401, 403, 404, 405, 406, 407, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 425, 426, 427, 428, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446 and 447 with reference to amino acid positions set forth in SEQ ID NO:3 or 7, where corresponding amino acid positions are identified by alignment of the PH20 polypeptide with the polypeptide set forth in SEQ ID NO:3; and provided that if the modified PH20 polypeptide contains an amino acid replacement at a position corresponding to position 13, 47, 131, or 219 the replacement is not replacement with an Alanine (A). Among these modified PH20 polypeptides are those that exhibit at least 40% of the hyaluronidase activity of the PH20 polypeptide not containing the amino acid replacement, where, as in all instances herein activity is compared under the same conditions.

Included among these polypeptides are those that contain an amino acid replacement in the sequence of amino acids set forth in any of SEQ ID NOS: 3, 7, 32-66, 69 and 72, or in a sequence of amino acids that exhibits at least 91% sequence identity to any of SEQ ID NOS: 3, 7, 32-66, 69, or 72. In particular, the modified PH20 polypeptide contains amino acid replacements in SEQ ID NO: 3, 7, 32-66, 69; or 72, which are polypeptides that are a C-terminally truncated fragment of SEQ ID NO:7, or a PH20 polypeptide that has a sequence of amino acids that is at least 91% identical to the sequence of amino acids set forth in SEQ ID NO: 7. In particular, among any of such modified PH20 polypeptides provided herein are any including those in which the amino acid replacement is an amino acid replacement set forth in Table 3 below. For example, such modified PH20 polypeptides include those that have at least one amino acid replacement at an amino acid position corresponding to a position selected from among 1, 6, 8, 9, 10, 11, 12, 14, 15, 20, 22, 24, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 46, 47, 48, 49, 50, 52, 58, 59, 63, 67, 68, 69, 70, 71, 72, 73, 74, 75, 79, 82, 83, 84, 86, 87, 89, 90, 92, 93, 94, 97, 102, 104, 107, 114, 118, 120, 127, 128, 130, 131, 132, 135, 138, 139, 140, 141, 142, 143, 144, 146, 147, 148, 149, 150, 151, 152, 155, 156, 158, 160, 162, 163, 164, 165, 166, 167, 169, 170, 172, 173, 174, 175, 178, 179, 193, 195, 196, 198, 204, 205, 206, 209, 212, 213, 215, 219, 220, 221, 222, 232, 233, 234, 235, 236, 237, 238, 240, 247, 248, 249, 257, 258, 259, 260, 261, 263, 267, 269, 271, 272, 273, 274, 276, 277, 278, 279, 282, 283, 285, 287, 289, 291, 292, 293, 298, 305, 307, 308, 309, 310, 313, 314, 315, 317, 318, 320, 321, 324, 325, 326, 328, 335, 347, 349, 351, 353, 356, 359, 367, 368, 369, 371, 373, 374, 375, 376, 377, 380, 381, 383, 385, 389, 392, 393, 395, 396, 399, 401, 404, 405, 406, 407, 409, 410, 412, 416, 418, 419, 421, 425, 427, 428, 431, 433, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446 or 447 with reference to amino acid positions set forth in SEQ ID NO:3. Exemplary of such replacements are those that contain at least one amino acid replacement selected from among replacement with: histidine (H) at a position corresponding to position 1; A at a position corresponding to position 1; E at a position corresponding to position 1; G at a position corresponding to position 1; K at a position corresponding to position 1; Q at a position corresponding to position 1; R at a position corresponding to position 1; A at a position corresponding to position 6; M at a position corresponding to position 8; Q at a position corresponding to position 9; G at a position corresponding to position 10; H at a position corresponding to position 10; S at a position corresponding to position 11; E at a position corresponding to position 12; I at a position corresponding to position 12; K at a position corresponding to position 12; T at a position corresponding to position 12; V at a position corresponding to position 14; V at a position corresponding to position 15; M at a position corresponding to position 15; S at a position corresponding to position 20; T at a position corresponding to position 22; E at a position corresponding to position 24; H at a position corresponding to position 24; R at a position corresponding to position 24; A at a position corresponding to position 26; E at a position corresponding to position 26; K at a position corresponding to position 26; M at a position corresponding to position 26; Q at a position corresponding to position 26; R at a position corresponding to position 26; D at a position corresponding to position 27; K at a position corresponding to position 27; R at a position corresponding to position 27; R at a position corresponding to position 28; E at a position corresponding to position 29; I at a position corresponding to position 29; K at a position corresponding to position 29; L at a position corresponding to position 29; M at a position corresponding to position 29; P at a position corresponding to position 29; R at a position corresponding to position 29; S at a position corresponding to position 29; T at a position corresponding to position 29; V at a position corresponding to position 29; G at a position corresponding to position 30; H at a position corresponding to position 30; K at a position corresponding to position 30; L at a position corresponding to position 30; M at a position corresponding to position 30; R at a position corresponding to position 30; S at a position corresponding to position 30; A at a position corresponding to position 31; C at a position corresponding to position 31; G at a position corresponding to position 31; H at a position corresponding to position 31; I at a position corresponding to position 31; K at a position corresponding to position 31; L at a position corresponding to position 31; P at a position corresponding to position 31; R at a position corresponding to position 31; S at a position corresponding to position 31; T at a position corresponding to position 31; V at a position corresponding to position 31; W at a position corresponding to position 31; C at a position corresponding to position 32; F at a position corresponding to position 32; G at a position corresponding to position 32; H at a position corresponding to position 32; W at a position corresponding to position 33; G at a position corresponding to position 33; W at a position corresponding to position 34; Q at a position corresponding to position 35; V at a position corresponding to position 35; H at a position corresponding to position 36; N at a position corresponding to position 36; F at a position corresponding to position 37; M at a position corresponding to position 37; Y at a position corresponding to position 38; A at a position corresponding to position 39; L at a position corresponding to position 39; N at a position corresponding to position 39; T at a position corresponding to position 39; L at a position corresponding to position 40; T at a position corresponding to position 41; L at a position corresponding to position 46; R at a position corresponding to position 46; D at a position corresponding to position 47; F at a position corresponding to position 47; T at a position corresponding to position 47; W at a position corresponding to position 47, with F at a position corresponding to position 48; H at a position corresponding to position 48; K at a position corresponding to position 48; N at a position corresponding to position 48; R at a position corresponding to position 49; D at a position corresponding to position 50; S at a position corresponding to position 50; M at a position corresponding to position 50; N at a position corresponding to position 52; Q at a position corresponding to position 52; R at a position corresponding to position 52; S at a position corresponding to position 52; T at a position corresponding to position 52; C at a position corresponding to position 58; K at a position corresponding to position 58; L at a position corresponding to position 58; P at a position corresponding to position 58; Q at a position corresponding to position 58; R at a position corresponding to position 58; H at a position corresponding to position 58; N at a position corresponding to position 58; Y at a position corresponding to position 58; N at a position corresponding to position 59; K at a position corresponding to position 63; L at a position corresponding to position 63; M at a position corresponding to position 63; R at a position corresponding to position 63; W at a position corresponding to position 63; V at a position corresponding to position 67; H at a position corresponding to position 68; P at a position corresponding to position 68; Q at a position corresponding to position 68; A at a position corresponding to position 69; C at a position corresponding to position 69; E at a position corresponding to position 69; F at a position corresponding to position 69; G at a position corresponding to position 69; I at a position corresponding to position 69; L at a position corresponding to position 69;

M at a position corresponding to position 69; P at a position corresponding to position 69; R at a position corresponding to position 69; T at a position corresponding to position 69; W at a position corresponding to position 69; Y at a position corresponding to position 69; A at a position corresponding to position 70; C at a position corresponding to position 70; F at a position corresponding to position 70; G at a position corresponding to position 70; H at a position corresponding to position 70; K at a position corresponding to position 70; L at a position corresponding to position 70; N at a position corresponding to position 70; P at a position corresponding to position 70; R at a position corresponding to position 70; S at a position corresponding to position 70; T at a position corresponding to position 70; V at a position corresponding to position 70; Y at a position corresponding to position 70; G at a position corresponding to position 71; N at a position corresponding to position 71; R at a position corresponding to position 71; S at a position corresponding to position 71; K at a position corresponding to position 72; M at a position corresponding to position 72; Q at a position corresponding to position 72; A at a position corresponding to position 73; H at a position corresponding to position 73; K at a position corresponding to position 73; L at a position corresponding to position 73; Q at a position corresponding to position 73; R at a position corresponding to position 73; T at a position corresponding to position 73; W at a position corresponding to position 73; A at a position corresponding to position 74; C at a position corresponding to position 74; E at a position corresponding to position 74; F at a position corresponding to position 74; G at a position corresponding to position 74; H at a position corresponding to position 74; K at a position corresponding to position 74; L at a position corresponding to position 74; M at a position corresponding to position 74; N at a position corresponding to position 74; P at a position corresponding to position 74; R at a position corresponding to position 74; S at a position corresponding to position 74; V at a position corresponding to position 74; W at a position corresponding to position 74; F at a position corresponding to position 75; L at a position corresponding to position 75; M at position corresponding to position 75; R at a position corresponding to position 75; T at a position corresponding to position 75; L at a position corresponding to position 79; L at a position corresponding to position 82; N at a position corresponding to position 82; V at a position corresponding to position 83; Q at a position corresponding to position 83; S at a position corresponding to position 83; G at a position corresponding to position 83; E at a position corresponding to position 84; F at a position corresponding to position 84; G at a position corresponding to position 84; N at a position corresponding to position 84; R at a position corresponding to position 84; A at a position corresponding to position 86; H at a position corresponding to position 86; K at a position corresponding to position 86; N at a position corresponding to position 86; S at a position corresponding to position 86; T at a position corresponding to position 86; W at a position corresponding to position 86; C at a position corresponding to position 87; G at a position corresponding to position 87; L at a position corresponding to position 87; M at a position corresponding to position 87; R at a position corresponding to position 87; S at a position corresponding to position 87; T at a position corresponding to position 87; V at a position corresponding to position 87; Y at a position corresponding to position 87; C at a position corresponding to position 89; A at a position corresponding to position 90; E at a position corresponding to position 90; H at a position corresponding to position 90; K at a position corresponding to position 90; N at a position corresponding to position 90; R at a position

corresponding to position 90; C at a position corresponding to position 92; L at a position corresponding to position 92; I at a position corresponding to position 93; L at a position corresponding to position 93; Q at a position corresponding to position 93; R at a position corresponding to position 93; S at a position corresponding to position 93; T at a position corresponding to position 93; D at a position corresponding to position 94; Q at a position corresponding to position 94; R at a position corresponding to position 94; A at a position corresponding to position 97; C at an amino acid residue corresponding to position 97; D at a position corresponding to position 97; E at a position corresponding to position 97; G at a position corresponding to position 97; L at a position corresponding to position 97; S at a position corresponding to position 97; S at a position corresponding to position 102; T at a position corresponding to position 102; R at a position corresponding to position 104; L at a position corresponding to position 107; A at a position corresponding to position 114; Q at a position corresponding to position 118; H at a position corresponding to position 120; F at a position corresponding to position 120; I at a position corresponding to position 120; S at a position corresponding to position 120; V at a position corresponding to position 120; Y at a position corresponding to position 120; E at a position corresponding to position 127; H at a position corresponding to position 127; N at a position corresponding to position 127; Q at a position corresponding to position 127; R at a position corresponding to position 127; I at a position corresponding to position 128; R at a position corresponding to position 130; G at a position corresponding to position 131; I at a position corresponding to position 131; M at a position corresponding to position 131; Q at a position corresponding to position 131; R at a position corresponding to position 131; V at a position corresponding to position 131; N at a position corresponding to position 132; L at a position corresponding to position 132; D at a position corresponding to position 135; G at a position corresponding to position 135; R at a position corresponding to position 135, with L at a position corresponding to position 138; T at a position corresponding to position 139; K at a position corresponding to position 140; H at a position corresponding to position 141; R at a position corresponding to position 141; S at a position corresponding to position 141; W at a position corresponding to position 141; Y at a position corresponding to position 141; D at a position corresponding to position 142; G at a position corresponding to position 142; K at a position corresponding to position 142; N at a position corresponding to position 142; P at a position corresponding to position 142; Q at a position corresponding to position 142; R at a position corresponding to position 142; S at a position corresponding to position 142; T at a position corresponding to position 142; G at a position corresponding to position 143; K at a position corresponding to position 143; R at a position corresponding to position 144; T at a position corresponding to position 144; P at a position corresponding to position 146; R at a position corresponding to position 146; A at a position corresponding to position 147; F at a position corresponding to position 147; L at a position corresponding to position 147; R at a position corresponding to position 147; S at a position corresponding to position 147; V at a position corresponding to position 147; H at a position corresponding to position 148; K at a position corresponding to position 148; Q at a position corresponding to position 148; T at a position corresponding to position 149; V at a position corresponding to position 149; A at a position corresponding to position 150; D at a position corresponding to position 150; G at a position corresponding to position 150; N at a position corresponding to position 150; S at a position corresponding to position 150; W at a position corresponding to position 150; Y at a position corresponding to position 150; A at a position corresponding to position 151; H at a position corresponding to position 151; K at a position corresponding to position 151; L at a position corresponding to position 151; M at a position corresponding to position 151; Q at a position corresponding to position 151; R at a position corresponding to position 151; S at a position corresponding to position 151; T at a position corresponding to position 151; V at a position corresponding to position 151; W at a position corresponding to position 151; Y at a position corresponding to position 151; R at a position corresponding to position 152; T at a position corresponding to position 152; W at a position corresponding to position 152; D at a position corresponding to position 155; G at a position corresponding to position 155; K at a position corresponding to position 155; R at a position corresponding to position 155; D at a position corresponding to position 156; Q at a position corresponding to position 158; S at a position corresponding to position 158; S at a position corresponding to position 160; E at a position corresponding to position 162; A at a position corresponding to position 163; E at a position corresponding to position 163; K at a position corresponding to position 163; Q at a position corresponding to position 163; R at a position corresponding to position 163; S at a position corresponding to position 163; M at a position corresponding to position 164; V at a position corresponding to position 164; D at a position corresponding to position 165; F at a position corresponding to position 165; N at a position corresponding to position 165; S at a position corresponding to position 165; V at a position corresponding to position 165; A at a position corresponding to position 166; E at a position corresponding to position 166; F at a position corresponding to position 166; H at a position corresponding to position 166; L at a position corresponding to position 166; Q at a position corresponding to position 166; R at a position corresponding to position 166; T at a position corresponding to position 166; W at a position corresponding to position 166; Y at a position corresponding to position 166; D at a position corresponding to position 167; L at a position corresponding to position 169; R at a position corresponding to position 170; A at a position corresponding to position 172; R at a position corresponding to position 173; G at a position corresponding to position 174; K at a position corresponding to position 174; N at a position corresponding to position 174; R at a position corresponding to position 174; T at a position corresponding to position 174; T at a position corresponding to position 175; K at a position corresponding to position 178; R at a position corresponding to position 178; K at a position corresponding to position 179; Q at a position corresponding to position 193; T at a position corresponding to position 195; N at a position corresponding to position 195; with E at a position corresponding to position 196; R at a position corresponding to position 196; with D at a position corresponding to position 198; P at a position corresponding to position 204; A at a position corresponding to position 205; E at a position corresponding to position 205; L at a position corresponding to position 205; T at a position corresponding to position 205; I at a position corresponding to position 206; K at a position corresponding to position 206; L at a position corresponding to position 206; R at a position corresponding to position 206; R at a position corresponding to position 209; N at a position corresponding to position 212; S at a position corresponding to position 212; A at a position corresponding to position 213; M at a position corresponding to position 213; N at a position corresponding to position 213; H at a position corresponding to position 215; M at a position corresponding to position 215; I at a position corresponding to position 219; K at a position corresponding to position 219; S at a position corresponding to position 219; H at a position corresponding to position 220; I at a position corresponding to position 220; L at a position corresponding to position 220; V at a position corresponding to position 220; Q at a position corresponding to position 221; G at a position corresponding to position 222; F at a position corresponding to position 232; G at a position corresponding to position 233; K at a position corresponding to position 233; R at a position corresponding to position 233; M at a position corresponding to position 234; A at a position corresponding to position 235; R at a position corresponding to position 236; C at a position corresponding to position 237; E at a position corresponding to position 237; H at a position corresponding to position 237; Q at a position corresponding to position 237; T at a position corresponding to position 237; E at a position corresponding to position 238; H at a position corresponding to amino acid position 238; S at a position corresponding to position 238; A at a position corresponding to position 240; Q at a position corresponding to position 240; I at a position corresponding to position 247; A at a position corresponding to position 248; V at a position corresponding to position 249; G at a position corresponding to position 257; T at a position corresponding to position 257; R at a position corresponding to position 257; N at a position corresponding to position 258; S at a position corresponding to position 258; P at a position corresponding to position 259; M at a position corresponding to position 260; Y at a position corresponding to position 260; A at a position corresponding to position 261; K at a position corresponding to position 261; N at a position corresponding to position 261; K at a position corresponding to position 263; R at a position corresponding to position 263; T at a position corresponding to position 267; A at a position corresponding to position 269; L at a position corresponding to position 271; M at a position corresponding to position 271; D at a position corresponding to position 272; T at a position corresponding to position 272; H at a position corresponding to position 273; Y at a position corresponding to position 273; F at a position corresponding to position 274; D at a position corresponding to position 276; H at a position corresponding to position 276; M at a position corresponding to position 276; R at a position corresponding to position 276; S at a position corresponding to position 276; Y at a position corresponding to position 276; A at a position corresponding to position 277; E at a position corresponding to position 277; H at a position corresponding to position 277; K at a position corresponding to position 277; M at a position corresponding to position 277; N at a position corresponding to position 277; Q at a position corresponding to position 277; R at a position corresponding to position 277; S at a position corresponding to position 277; T at a position corresponding to position 277; E at a position corresponding to position 278; F at a position corresponding to position 278; G at a position corresponding to position 278; H at a position corresponding to position 278; K at a position corresponding to position 278; N at a position corresponding to position 278; R at a position corresponding to position 278; S at a position corresponding to position 278; T at a position corresponding to position 278; Y at a position corresponding to position 278; H at a position corresponding to position 279; M at a position corresponding to position 282; S at a position corresponding to position 283; H at a position corresponding to position 285; T at a position corresponding to position 287; S at a position corresponding to position 289; S at a position corresponding to position 291; V at a position corresponding to position 291; C at a position corresponding to position 292; F at a position corresponding to position 292; H at a position corresponding to position 292; K at a position corresponding to position 292; R at a position corresponding to position 292; V at a position corresponding to position 292; A at a position corresponding to position 293; C at a position corresponding to position 293; D at a position corresponding to position 293; F at a position corresponding to position 293; K at a position corresponding to position 293; M at a position corresponding to position 293; P at a position corresponding to position 293; Q at a position corresponding to position 293; V at a position corresponding to position 293; Y at a position corresponding to position 293; G at a position corresponding to position 298; E at a position corresponding to position 305; G at a position corresponding to position 307; D at a position corresponding to position 308; G at a position corresponding to position 308; K at a position corresponding to position 308; N at a position corresponding to position 308; R at a position corresponding to position 308; E at a position corresponding to position 309; G at a position corresponding to position 309; H at a position corresponding to position 309; L at a position corresponding to position 309; M at a position corresponding to position 309; N at a position corresponding to position 309; Q at a position corresponding to position 309; R at a position corresponding to position 309; S at a position corresponding to position 309; T at a position corresponding to position 309; V at a position corresponding to position 309; A at a position corresponding to position 310; G at a position corresponding to position 310; Q at a position corresponding to position 310; S at a position corresponding to position 310; A at a position corresponding to position 313; G at a position corresponding to position 313; H at a position corresponding to position 313; K at a position corresponding to position 313; P at a position corresponding to position 313; R at a position corresponding to position 313; T at a position corresponding to position 313; Y at a position corresponding to position 313; with S at a position corresponding to position 314; Y at a position corresponding to position 314; A at a position corresponding to position 315; H at a position corresponding to position 315; Y at a position corresponding to position 315; A at a position corresponding to position 317; I at a position corresponding to position 317; K at a position corresponding to position 317; N at a position corresponding to position 317; Q at a position corresponding to position 317; R at a position corresponding to position 317; S at a position corresponding to position 317; T at a position corresponding to position 317; W at a position corresponding to position 317; D at a position corresponding to position 318; H at a position corresponding to position 318; K at a position corresponding to position 318; M at a position corresponding to position 318; R at a position corresponding to position 318; H at a position corresponding to position 320; K at a position corresponding to position 320; R at a position corresponding to position 320; R at a position corresponding to position 321; S at a position corresponding to position 321; N at a position corresponding to position 324; R at a position corresponding to position 324; A at a position corresponding to position 325; D at a position corresponding to position 325; E at a position corresponding to position 325; G at a position corresponding to position 325; H at a position corresponding to position 325; K at a position corresponding to position 325; M at a position corresponding to position 325; N at a position corresponding to position 325; Q at a position corresponding to position 325; S at a position corresponding to position 325; V at a position corresponding to position 325; L at a position corresponding to position 326; V at a position corresponding to position 326; C at a position corresponding to position 328; G at a position corresponding to position 328; I at a position corresponding to position 328; K at a position corresponding to position 328; L at a position corresponding to position 328; S at a position corresponding to position 328; Y at a position corresponding to position 328; S at a position corresponding to position 335; A at a position corresponding to position 347; G at a position corresponding to position 347; S at a position corresponding to position 347; M at a position corresponding to position 349; R at a position corresponding to position 349; S at a position corresponding to position 351; V at a position corresponding to position 353; with H at a position corresponding to position 356; S at a position corresponding to position 356; E at a position corresponding to position 359; H at a position corresponding to position 359; T at a position corresponding to position 359; A at a position corresponding to position 367; G at a position corresponding to position 367; K at a position corresponding to position 367; S at a position corresponding to position 367; A at a position corresponding to position 368; E at a position corresponding to position 368; K at a position corresponding to position 368; L at a position corresponding to amino acid position 368; M at a position corresponding to amino acid position 368; R at a position corresponding to position 368; T at a position corresponding to amino acid position 368; H at a position corresponding to position 369; R at a position corresponding to position 369; F at a position corresponding to position 371; H at a position corresponding to position 371; K at a position corresponding to position 371; L at a position corresponding to position 371; R at a position corresponding to position 371; S at a position corresponding to position 371; M at a position corresponding to position 373; H at a position corresponding to position 374; P at a position corresponding to position 374; A at a position corresponding to position 375; G at a position corresponding to position 375; K at a position corresponding to position 375; R at a position corresponding to position 375; D at a position corresponding to position 376; E at a position corresponding to position 376; Q at a position corresponding to position 376; R at a position corresponding to position 376; T at a position corresponding to position 376; V at a position corresponding to position 376; Y at a position corresponding to position 376; D at a position corresponding to position 377; E at a position corresponding to position 377; H at a position corresponding to position 377; K at a position corresponding to position 377; P at a position corresponding to position 377; R at a position corresponding to position 377; S at a position corresponding to position 377; T at a position corresponding to position 377; W at a position corresponding to position 380; Y at a position corresponding to position 380; S at a position corresponding to position 381; I at a position corresponding to position 383; K at a position corresponding to position 383; L at a position corresponding to position 383; S at a position corresponding to position 383; A at a position corresponding to position 385; Q at a position corresponding to position 385; V at a position corresponding to position 385; A at a position corresponding to position 389; G at a position corresponding to position 389; L at a position corresponding to position 389; K at a position corresponding to position 389; Q at a position corresponding to position 389; S at a position corresponding to position 389; A at a position corresponding to position 392; F at a position corresponding to position 392; M at a position corresponding to position 392; Q at a position corresponding to position 392; R at a position corresponding to position 392; V at a position corresponding to position 392; F at a position corresponding to position 393; M at a position corresponding to position 393; A at a position corresponding to position 395; H at a position corresponding to position 395; R at a position corresponding to position 395; A at a position corresponding to position 396; H at a position corresponding to position 396; Q at a position corresponding to position 396; S at a position corresponding to position 396; K at a position corresponding to position 399; M at a position corresponding to position 399; T at a position corresponding to position 399; V at a position corresponding to position 399; W at a position corresponding to position 399; A at a position corresponding to position 401; E at a position corresponding to position 401; A at a position corresponding to position 404; G at a position corresponding to position 405; F at a position corresponding to position 406; N at a position corresponding to position 406; A at a position corresponding to position 407; D at a position corresponding to position 407; E at a position corresponding to position 407; F at a position corresponding to position 407; H at a position corresponding to position 407; Q at a position corresponding to position 407; P at a position corresponding to position 407; A at a position corresponding to position 409; Q at a position corresponding to position 409; T at a position corresponding to position 410; Q at a position corresponding to position 412; R at a position corresponding to position 412; V at a position corresponding to position 412; L at a position corresponding to position 416; E at a position corresponding to position 418; L at a position corresponding to position 418; P at a position corresponding to position 418; R at a position corresponding to position 418; V at a position corresponding to position 418; F at a position corresponding to position 419; H at a position corresponding to position 419; I at a position corresponding to position 419; K at a position corresponding to position 419; R at a position corresponding to position 419; S at a position corresponding to position 419; Y at a position corresponding to position 419; A at a position corresponding to position 421; H at a position corresponding to position 421; K at a position corresponding to position 421; N at a position corresponding to position 421; Q at a position corresponding to position 421; R at a position corresponding to position 421; S at a position corresponding to position 421; G at a position corresponding to position 425; K at a position corresponding to position 425; Q at a position corresponding to position 427; T at a position corresponding to position 427; L at a position corresponding to position 428; A at a position corresponding to position 431; G at a position corresponding to position 431; E at a position corresponding to position 431; H at a position corresponding to position 431; K at a position corresponding to position 431; L at a position corresponding to position 431; N at a position corresponding to position 431; Q at a position corresponding to position 431; R at a position corresponding to position 431; S at a position corresponding to position 431; V at a position corresponding to position 431; A at a position corresponding to position 433; H at a position corresponding to position 433; I at a position corresponding to position 433; K at a position corresponding to position 433; L at a position corresponding to position 433; R at a position corresponding to position 433; T at a position corresponding to position 433; V at a position corresponding to position 433; W at a position corresponding to position 433; K at a position corresponding to position 436; I at a position corresponding to position 437; M at a position corresponding to position 437; A at a position corresponding to position 438; D at a position corresponding to position 438; E at a position corresponding to position 438; L at a position corresponding to position 438; N at a position corresponding to position 438; T at a position corresponding to position 438; A at a position corresponding to position 439; C at a position corresponding to position 439; K at a position corresponding to position 439; P at a position corresponding to position 439; Q at a position corresponding to position 439; T at a position corresponding to position 439; V at a position corresponding to position 439; D at a position corresponding to position 440; H at a position corresponding to position 440; M at a position corresponding to position 440; P at a position corresponding to position 440; R at a position corresponding to position 440; S at a position corresponding to position 440; A at a position corresponding to position 441; F at a position corresponding to position 441; C at a position corresponding to position 442; G at a position corresponding to position 442; R at a position corresponding to position 442; A at a position corresponding to position 443; E at a position corresponding to position 443; F at a position corresponding to position 443; G at a position corresponding to position 443; M at a position corresponding to position 443; N at a position corresponding to position 443; E at a position corresponding to position 444; H at a position corresponding to position 444; V at a position corresponding to position 444; H at a position corresponding to position 445; M at a position corresponding to position 445; N at a position corresponding to position 445; P at a position corresponding to position 445; Q at a position corresponding to position 445; S at a position corresponding to position 445; T at a position corresponding to position 445; V at a position corresponding to position 445; W at a position corresponding to position 445; A at a position corresponding to position 446; M at a position corresponding to position 446; W at a position corresponding to position 446; D at a position corresponding to position 447; E at a position corresponding to position 447; G at a position corresponding to position 447; I at a position corresponding to position 447; N at a position corresponding to position 447; P at a position corresponding to position 447; Q at a position corresponding to position 447; T at a position corresponding to position 447, and/or replacement with V at a position corresponding to position 447, each with reference to amino acid positions set forth in SEQ ID NO:3. Among these modified PH20 polypeptides are those that exhibit at least 40% of the activity of the PH20 that does not contain the particular amino acid replacement. Activity can vary between, for example, 40% to 5000%, 40% to 2000%, 40% to 1000%, 40% to 500%, 40% to 100%, 80% to 2000%, 80% to 600%, 80% to 200%, 80% to 300%, of the hyaluronidase activity of the PH20 polypeptide not containing the amino acid replacement. Such activity includes at least 50%, 60%, 70%, 80%, 90%, 100%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, 1000%, 2000%, 3000% or more of the hyaluronidase activity of the PH20 polypeptide not containing the amino acid replacement, where, as in all instances herein, the activities are compared under the same conditions.

In particular, provided are modified PH20 polypeptides that contain at least one amino acid replacement in a PH20 polypeptide set forth in SEQ ID NO:7, a C-terminally truncated fragment thereof, or in a PH20 polypeptide that has a sequence of amino acids that is at least 91% identical to the sequence of amino acids set forth in SEQ ID NO: 7 or a corresponding truncated fragment, where: the modified PH20 polypeptides exhibit less than 20% of the hyaluronidase activity of the PH20 polypeptide not containing the amino acid replacement, where activities are compared under the same conditions; the amino acid replacement(s) is at an amino acid position corresponding to a position selected from among 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 25, 27, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 94, 95, 96, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 143, 144, 145, 149, 150, 152, 153, 154, 155, 156, 157, 158, 159, 161, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 197, 198, 199, 200, 201, 202, 203, 204, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 278, 279, 280, 282, 283, 284, 285, 286, 287, 288, 289, 290; 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 331, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 408, 410, 411, 412, 413, 414, 415, 416, 417, 419, 420, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 434, 437, 438, 439, 440, 441, 442, 443, 444, or 447 with reference to amino acid positions set forth in SEQ ID NO:3 or 7; corresponding amino acid positions are identified by alignment of the PH20 polypeptide with the polypeptide set forth in SEQ ID NO:3; and provided that:

(i) if the modified PH20 polypeptide contains an amino acid replacement at a position corresponding to position 200, 333, 358 or 393 the replacement is not replacement with an Alanine (A).

(ii) if the modified PH20 polypeptide contains an amino acid replacement at a position corresponding to position 111 or 249 the replacement is not replacement with an asparagine (N);

(iii) if the modified PH20 polypeptide contains an amino acid replacement at a position corresponding to position 113 the replacement is not replacement with a glutamine (Q);

(iv) if the modified PH20 polypeptide contains an amino acid replacement at a position corresponding to position 176 the replacement is not replacement with a glycine (G); and

(v) if the modified PH20 polypeptide contains an amino acid replacement at a position corresponding to position 252 the replacement is not replacement with a threonine (T).

Exemplary of such modified PH20 polypeptides are any that contain amino acid replacement(s) in a PH20 polypeptide that has the sequence of amino acids set forth in any of SEQ ID NOS: 3, 7, 32-66, 69, or 72, or in a sequence of amino acids that exhibits at least 91% sequence identity to any of SEQ ID NOS: 3, 7, 32-66, 69, or 72. For example, the modified PH20 polypeptide contains amino acid replacement(s) in SEQ ID NOS: 3, 7, 32-66, 69, or 72, which are polypeptides that are a C-terminally truncated fragment of SEQ ID NO:7, or a PH20 polypeptide that has a sequence of amino acids that is at least 91% identical to the sequence of amino acids set forth in SEQ ID NO:7. In examples of such modified PH20 polypeptides provided herein, the modified PH20 polypeptides can exhibit similar or the same activity as the PH20 without the modification, or can exhibit increased activity or activity that is less than 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, 0.1%, 0.05% or less of the hyaluronidase activity of the PH20 polypeptide not containing the amino acid replacement. Exemplary of such modified PH20 polypeptides are any set forth in Table 5.

Among any and all of the modified PH20 polypeptides provided herein and above, the modified PH20 polypeptide is one that does not consist of the sequence of amino acids set forth in any of SEQ ID NOS: 3, 6-66, 69-72, 856-861, 869 or 870. In particular, among any of the modified PH20 polypeptides provided herein above or elsewhere herein are any that contain an amino acid replacement(s) in a PH20 polypeptide having the sequence of amino acids set forth any of SEQ ID NO: 3, 7, 69 or 72 provided that: (i) where the modified PH20 polypeptide includes only a single amino acid replacement the replacement does not corresponds to amino acid replacements V12A, N47A, D111N, E113Q, N131A, R176G, N200A, N219A, E249Q, R252T, N333A or N358A, with reference to amino acid positions set forth in SEQ ID NO:3; (ii) where the modified PH20 polypeptide includes only two amino acid replacements the replacements do not correspond to amino acid replacements P13A/L464W, N47A/N131A, N47A/N219A, N131A/N219A or N333A/N358A with reference to positions set forth in SEQ ID NO:3; and (iii) where the modified PH20 polypeptide includes only three amino acid replacements the replacements do not correspond to amino acid replacements N47A/N131A/N219A, with reference to amino acid positions set forth in SEQ ID NO:3.

Any of the above modified PH20 polypeptides and any provided herein and described above and below can contain 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, or more of the amino acid replacements. The modified PH20 polypeptides can include a signal sequence, including the native sequence or a heterologous sequence or a modified sequence, and also include a mature PH20 polypeptide that lacks the signal sequence.

Among any of the modified PH20 polypeptides provided herein above or described below are modified PH20 polypeptides that contain or have the sequence of amino acids set forth in any of SEQ ID NOS: 73-855 or a sequence of amino acids that exhibits at least 75%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to a sequence of amino acids set forth in any of SEQ ID NOS: 73-855 and that contains at least one amino acid replacement, such as any described above or elsewhere herein, with reference to positions compared to the sequence of amino acids set forth in SEQ ID NO:3. In any of the examples of the modified PH20 polypeptides provided herein, the modified PH20 polypeptide does not have or contain the sequence of amino acids set forth in any of SEQ ID NOS: 8-31, 69-72, 856-861, 869 or 870.

The modified PH20 polypeptides provided herein can be substantially purified or isolated, can exhibit catalytic activity at neutral pH, can be secreted upon expression from cells and are soluble in the supernatant, and/or can include modified amino acids, such as a modification selected from among glycosylation, sialation, albumination, farnysylation, carboxylation, hydroxylation, conjugation to a polymer, such as PEGylation or conjugation to dextran, conjugation to another moiety, such as a multimerization domain, toxin, detectable label or drug, and phosphorylation. The modified PH20 polypeptide can be glycosylated, such as by containing at least an N-acetylglucosamine moiety linked to each of at least three asparagine (N) residues, where, for example, the three asparagine residues correspond to amino acid residues 200, 333 and 358 of SEQ ID NO:3. Multimerization domains include Fc domains.

Also provided are nucleic acid molecules that encode any of the modified PH20 polypeptides provided herein. Vectors, eukaryotic and prokaryotic, that contain the nucleic acid molecules are provided. The vectors include expression vectors and include mammalian vectors, including viral vectors. Viral vectors include adenovirus vectors, retrovirus vectors, vaccinia virus vectors, herpes simplex virus and cytomegalovirus vectors, and other such viral vectors. Of interest are oncolytic vectors that accumulate in or are targeted to tumors. Also provided are cells that contain the nucleic acid molecules and cells that contain the vectors. The cells can be prokaryotic or eukaryotic, particularly mammalian cells, such as Chinese Hamster Ovary (CHO) cells.

Also provided herein is a modified PH20 polypeptide that is produced by any of the provided cells. Thus, provided herein are methods of producing a modified PH20 polypeptide by culturing any of the cells provided herein under conditions whereby an encoded modified PH20 polypeptide is produced and secreted by the cell, and recovering the expressed polypeptide. Also provided herein is a method of producing a modified PH20 polypeptide by introducing any of the nucleic acids provided herein or any of the vectors provided herein into a cell capable of incorporating N-linked sugar moieties into the polypeptide, culturing the cell under conditions whereby an encoded modified PH20 polypeptide is produced and secreted by the cell, and recovering the expressed polypeptide. In such examples, the nucleic acid is operably linked to a promoter. The cultured cell can be a eukaryotic cell, such as a mammalian cell, for example, a Chinese hamster ovary (CHO) cell.

Also provided are pharmaceutical compositions that contain any of the modified PH20 polypeptides provided herein or any of the nucleic acids or vectors provided herein. The compositions can be formulated with other agents and/or with other components, such as preservatives. The compositions can be formulated so that the components, particularly the PH20 and any other active agent, remain active or are stable under preselected conditions. In addition, as described herein, the PH20 polypeptides are modified so that they exhibit increased stability under various conditions. For example, provided are compositions in which the modified PH20 polypeptide is stable (i.e., retains activity as described herein) at a temperature from or from about 2° C. to 8° C., inclusive, for at least 1 month or is stable at a temperature from or from about 30° C. to 42° C., inclusive, for at least 3 days. Provided are compositions in which the modified PH20 polypeptide in the composition is stable at a temperature from or from about 2° C. to 8° C., inclusive, for at least 2 months, 3 months, 4 months, 5 months, 6 months, 7 months, at least 8 months, at least 9 months, at least 10 months, at least 11 months, at least 12 months, 13 months, 14 months, 15 months, 16 months, 17 months, 18 months, 19 months, 20 months, 21 months, 22 months, 23 months, 24 months, months, 26 months, 27 months, 28 months, 29 months or 30 months. Also provided are compositions in which the modified PH20 polypeptide in the composition is stable at a temperature from or from about 30° C. to 42° C., inclusive, for at least 3 days, at least 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, days, 35 days, 40 days, 45 days, 50 days, 60 days or more. The pharmaceutical compositions can contain a pharmaceutically acceptable excipient.

The conditions, formulations, components, and modified PH20 polypeptide are chosen to achieve a desired stability. The pharmaceutical compositions can be formulated for direct administration or can require dilution. They can be formulated for multiple or single dosage administration. Exemplary compositions include concentrations of modified PH20 between or about between 0.1 μg/mL to 100 μg/mL, 1 μg/mL to 50 μg/mL or 1 μg/mL to 20 μg/mL, or 10 U/mL to 5000 U/mL, 50 U/mL to 4000 U/mL, 100 U/mL to 2000 U/mL, 300 U/mL to 2000 U/mL, 600 U/mL to 2000 U/mL, or 100 U/mL to 1000 U/mL. Exemplary salts include NaCl at a concentration, for example, of less than or about or 200 mM, 180 mM, 150 mM, 140 mM, 130 mM, 120 mM, 110 mM, 100 mM, 90 mM, 80 mM, 70 mM, 60 mM, 50 mM, 40 mM, 30 mM, 25 mM, 20 mM, 15 mM, 10 mM, 5 mM or less, or between or about between 0.1 mM to 200 mM, 0.1 mM to 100 mM, 120 mM to 200 mM, 10 mM to 50 mM, 10 mM to 90 mM, 80 mM to 200 mM, 80 mM to 140 mM, 50 mM to 100 mM, 80 mM to 100 mM, 50 mM to 80 mM, 100 mM to 140 mM or 120 mM to 140 mM.

The pharmaceutical compositions can contain an antimicrobially effective amount of a preservative or mixture of preservatives, such as one, two, three, four or more of a phenolic preservative(s), a non-phenolic preservative(s) or a phenolic preservative(s) and a non-phenolic preservative(s), such as, but not limited to, phenol, m-cresol, methylparaben, benzyl alcohol, thimerosal, benzalkonium chloride, 4-chloro-1-butanol, chlorhexidine dihydrochloride, chlorhexidine digluconate, L-phenylalanine, EDTA, bronopol, phenylmercuric acetate, glycerol, imidurea, chlorhexidine, sodium dehydroacetate, o-cresol, p-cresol, chlorocresol, cetrimide, benzethonium chloride, ethyl paraben, propylparaben, butylparaben and any combinations thereof. Phenols include, for example, phenol, metacresol (m-cresol), benzyl alcohol, and parabens, such as methylparaben or propylparaben. Anti-microbial effective concentrations of one or more preservative agents (as a percentage (%) of mass concentration (w/v)) can be between 0.05% to 0.6%, 0.1% to 0.4%, 0.1% to 0.3%, 0.15% to 0.325%, 0.15% to 0.25%, 0.1% to 0.2%, 0.2% to 0.3% or 0.3% to 0.4% inclusive. Examples thereof are pharmaceutical compositions where the preservatives are phenol, m-cresol or phenol and m-cresol and the amount as a % of mass concentration (w/v) in the formulation is between or about between 0.1% to 0.25% phenol and between or about between 0.05% to 0.2% m-cresol, is between or about between 0.10% to 0.2% phenol and between or about between 0.6% to 01.8% m-cresol, between or about between 0.1% to 0.15% phenol and 0.8% t 0.15% m-cresol, is between or about between 0.10% to 0.15% phenol and between or about between 0.06

to 0.09% m-cresol or is between or about between 0.12% to 0.18% phenol and between or about between 0.14 to 0.22% m-cresol.

The pharmaceutical compositions can contain a further therapeutically active agent. The active agent can be formulated in the composition or provided as a combination with the PH20-containing composition, but in a separate composition for administration separately, sequentially, intermittently, simultaneously or together. Therapeutically active agents include, for example, an agent selected from among a chemotherapeutic agent, an analgesic agent, an anti-inflammatory agent, an antimicrobial agent, an amoebicidal agent, a trichomonacidal agent, an anti-parkinson agent, an anti-malarial agent, an anticonvulsant agent, an anti-depressant agent, and antiarthritics agent, an anti-fungal agent, an antihypertensive agent, an antipyretic agent, an anti-parasite agent, an antihistamine agent, an alpha-adrenergic agonist agent, an alpha blocker agent, an anesthetic agent, a bronchial dilator agent, a biocide agent, a bactericide agent, a bacteriostat agent, a beta adrenergic blocker agent, a calcium channel blocker agent, a cardiovascular drug agent, a contraceptive agent, a decongestant agent, a diuretic agent, a depressant agent, a diagnostic agent, a electrolyte agent, a hypnotic agent, a hormone agent, a hyperglycemic agent, a muscle relaxant agent, a muscle contractant agent, an ophthalmic agent, a parasympathomimetic agent, a psychic energizer agent, a sedative agent, a sympathomimetic agent, a tranquilizer agent, an urinary agent, a vaginal agent, a viricide agent, a vitamin agent, a non-steroidal anti-inflammatory agent, an angiotensin converting enzyme inhibitor agent, a polypeptide, a protein, a nucleic acid, a drug, an organic molecule and a sleep inducer. Exemplary of such agents are antibodies, particularly monoclonal antibodies, an Immune Globulin preparation, a bisphosphonate, a cytokine, a chemotherapeutic agent, a coagulation factor and an insulin. Insulins include, for example, basal insulins and fast-acting insulin, such as regular insulin, particularly recombinant human insulin, and insulin analogs, such as insulin lispro, insulin aspart or insulin glulisine. Particular fast-acting insulins are those with an A chain having a sequence of amino acids set forth in SEQ ID NO:862 and a B chain having a sequence of amino acids set forth in SEQ ID NO:863 or an insulin with an A chain with a sequence of amino acids set forth as amino acid residue positions 88-108 of SEQ ID NO:864 and a B chain with a sequence of amino acids set forth as amino acid residue positions 25-54 of SEQ ID NO:864 or an insulin analog that is selected from among an insulin having an A chain with a sequence of amino acids set forth in SEQ ID NO:862 and a B chain having a sequence of amino acids set forth in any of SEQ NOS:865-867. The amount of fast-acting insulin in the compositions can be empirically determined, but typically can be 10 U/mL to 1000 U/mL, 50 U/mL to 500 U/mL, 100 U/mL to 1000 U/mL or 500 U/mL to 1000 U/mL, inclusive.

In particular examples, provided herein is a pharmaceutical composition containing any of the modified PH20 polypeptides provided herein that exhibit increased stability to a phenolic preservative and an insulin, such as a fast-acting insulin. The modified PH20 polypeptides and insulin can be provided in therapeutically effective amounts. For example, provided herein is a pharmaceutical composition that contains any of the modified PH20 polypeptides provided herein that exhibits increased stability to a phenolic preservative in an amount that is from or from about 100 U/mL to 1000 U/mL and a fast-acting insulin in an amount that is from or from about 10 U/mL to 1000 U/mL. For example, the fast-acting insulin can be an insulin analog, such as insulin lispro, insulin aspart or insulin glulisine or other analog. Any of such pharmaceutical compositions can be formulated at a pH that is from or from about 7.0 to 7.6. Any of such pharmaceutical compositions also can be formulated to contain salt, such as NaCl, at a concentration that is from or from about 0.1 mM to 200 mM and/or an anti-microbial effective amount of at least one preservative where the composition generally contains at least one phenolic preservative. The anti-microbial effective amount is a total amount of one or more preservative agents as a percentage (%) of mass concentration (w/v) that is or is between 0.05% to 0.6%. The phenolic preservative(s) can be a phenol, metacresol (m-cresol), benzyl alcohol, or a paraben. In any of the above examples of a pharmaceutical composition, the composition also can contain a surfactant, such as a polypropylene glycol, polyethylene glycol, glycerin, sorbitol, poloxamer or polysorbate, in an amount as a % of mass concentration (w/v) in the formulation that is at least or at least about 0.001%; a buffering agent that is a non-metal binding agent or is a metal binding agent, such as Tris, histidine, phosphate or citrate, wherein the concentration of the buffering agent is between or between about 1 mM to 100 mM; glycerin in a concentration less than 60 mM; an antioxidant, such as cysteine, tryptophan or methionine, at a concentration between or from about between 2 mM to 50 mM, inclusive; and/or zinc at a concentration of between or about between 0.001 to 0.1 mg per 100 units of insulin (mg/100U). Also provided herein are closed loop systems, insulin pumps including continuous subcutaneous infusion insulin (CSII) pumps and insulin pens that contain any of the pharmaceutical compositions. The pharmaceutical compositions can be used in methods or uses for treating diabetes, such as type 1 diabetes mellitus, type 2 diabetes mellitus or gestational diabetes.

Other therapeutic agents in any of the pharmaceutical compositions provided herein include, but are not limited to Adalimumabs, Agalsidase Betas, Alefacepts, Ampicillins, Anakinras, Antipoliomyelitic Vaccines, Anti-Thymocytes, Azithromycins, Becaplermins, Caspofungins, Cefazolins, Cefepimes, Cefotetans, Ceftazidimes, Ceftriaxones, Cetuximabs, Cilastatins, Clavulanic Acids, Clindamycins, Darbepoetin Alfas, Daclizumabs, Diphtheria, Diphtheria antitoxins, Diphtheria Toxoids, Efalizumabs, Epinephrines, Erythropoietin Alphas, Etanercepts, Filgrastims, Fluconazoles, Follicle-Stimulating Hormones, Follitropin Alphas, Follitropin Betas, Fosphenyloins, Gadodiamides, Gadopentetates, Gatifloxacins, Glatiramers, GM-CSF's, Goserelins, Goserelin acetates, Granisetrons, *Haemophilus Influenza* B's, Haloperidols, Hepatitis vaccines, Hepatitis A Vaccines, Hepatitis B Vaccines, Ibritumomab Tiuxetans, Ibritumomabs, Tiuxetans, Immunoglobulins, *Hemophilus influenza* vaccines, Influenza Virus Vaccines, Infliximabs, Insulin lispro, 75% neutral protamine lispro (NPL)/25% insulin lispro, 50% neutral protamine Hagedorn (NPH)/50% regular insulin, 70% NPH/30% regular insulin; Regular insulin, NPH insulin, Ultra insulin, Ultralente insulin, and Insulin Glargines, Interferons, Interferon alpha, Interferon Betas, Interferon Gammas, Interferon alpha-2a, Interferon alpha 2-b, Interferon Alphacon, Interferon alpha-n, Interferon Betas, Interferon Beta-1a's, Interferon Gammas, Interferon alpha-con, Iodixanols, Iohexyls, Iopamidols, Ioversols, Ketorolacs, Laronidases, Levofloxacins, Lidocaines, Linezolids, Lorazepams, Measles Vaccines, Measles virus, Mumps viruses, Measles-Mumps-Rubella Virus Vaccines, Rubella vaccines, Medroxyprogesterones, Meropenems, Methylprednisolones, Midazolams, Morphines, Octreotides, Omalizumabs, Ondansetrons, Palivizumabs, Pantoprazoles, Pegaspargases, Pegfilgrastims, Peg-Interferon Alpha-2a's, Peg-Interferon Alpha-2b's, Pegvisomants, Pertussis vaccines, Piperacillins, Pneumococcal Vaccines and Pneumococcal Conjugate Vaccines, Promethazines, Reteplases, Somatropins, Sulbactams, Sumatriptans, Tazobactams, Tenecteplases, Tetanus Purified Toxoids, Ticarcillins, Tositumomabs, Triamcinolones, Triamcinolone Acetonides, Triamcinolone hexacetonides, Vancomycins, Varicella Zoster immunoglobulins, Varicella vaccines, other vaccines, Alemtuzumabs, Alitretinoins, Allopurinols, Altretamines, Amifostines, Anastrozoles, Arsenics, Arsenic Trioxides, Asparaginases, *Bacillus* Calmette-Guerin (BCG) vaccines, BCG Live, Bexarotenes, Bleomycins, Busulfans, Busulfan intravenous, Busulfan orals, Calusterones, Capecitabines, Carboplatins, Carmustines, Carmustines with Polifeprosans, Celecoxibs, Chlorambucils, Cisplatins, Cladribines, Cyclophosphamides, Cytarabines, Cytarabine liposomals, Dacarbazines, Dactinomycins, Daunorubicin liposomals, Daunorubicins, Daunomycins, Denileukin Diftitoxes, Dexrazoxanes, Docetaxels, Doxorubicins, Doxorubicin liposomals, Dromostanolone propionates, Elliott's B Solutions, Epirubicins, Epoetin alfas, Estramustines, Etoposides, Etoposide phosphates, Etoposide VP-16s, Exemestanes, Floxuridines, Fludarabines, Fluorouracils, 5-Fluorouracils, Fulvestrants, Gemcitabines, Gemtuzumabs, Ozogamicins, Gemtuzumab ozogamicins, Hydroxyureas, Idarubicins, Ifosfamides, Imatinib mesylates, Irinotecans, Letrozoles, Leucovorins, Levamisoles, Lomustines, CCNUs, Mechlorethamines, Nitrogen mustards, Megestrols, Megestrol acetates, Melphalans, L-PAMs, Mercaptopurines, 6-Mercaptopurines, Mesnas, Methotrexates, Methoxsalens, Mitomycins, Mitomycin C's, Mitotanes, Mitoxantrones, Nandrolones, Nandrolone Phenpropionates, Nofetumomabs, Oprelvekins, Oxaliplatins, Paclitaxels, Pamidronates, Pegademases, Pentostatins, Pipobromans, Plicamycins, Mithramycins, Porfimers, Porfimer sodiums, Procarbazines, Quinacrines, Rasburicases, Rituximabs, Sargramostims, Streptozocins, Talcs, Tamoxifens, Temozolomides, Teniposides, Testolactones, Thioguanines, 6-Thioguanines, Triethylenethiophosphoramides (Thiotepas), Topotecans, Toremifenes, Trastuzumabs, Tretinoins, Uracil Mustards, Valrubicins, Vinblastines, Vincristines, Vinorelbines, Zoledronates, Acivicins, Aclarubicins, Acodazoles, Acronines, Adozelesins, Aldesleukins, Retinoic Acids, Alitretinoins, 9-Cis-Retinoic Acids, Alvocidibs, Ambazones, Ambomycins, Ametantrones, Aminoglutethimides, Amsacrines, Anaxirones, Ancitabines, Anthramycins, Apaziquones, Argimesnas, Asperlins, Atrimustines, Azacitidines, Azetepas, Azotomycins, Banoxantrones, Batabulins, Batimastats, Benaxibines, Bendamustines, Benzodepas, Bicalutamides, Bietaserpines, Biricodars, Bisantrenes, Bisnafide Dimesylates, Bizelesins, Bortezomibs, Brequinars, Bropirimines, Budotitanes, Cactinomycins, Canertinibs, Caracemides, Carbetimers, Carboquones, Carmofurs, Carubicins, Carzelesins, Cedefingols, Cemadotins, Chlorambucils, Cioteronels, Cirolemycins, Clanfenurs, Clofarabines, Crisnatols, Decitabines, Dexniguldipines, Dexormaplatins, Dezaguanines, Diaziquones, Dibrospidiums, Dienogests, Dinalins, Disermolides, Dofequidars, Doxifluridines, Droloxifenes, Duazomycins, Ecomustines, Edatrexates, Edotecarins, Eflornithines, Elacridars, Elinafides, Elsamitrucins, Emitefurs, Enloplatins, Enpromates, Enzastaurins, Epipropidines, Eptaloprosts, Erbulozoles, Esorubicins, Etanidazoles, Etoglucids, Etoprines, Exisulinds, Fadrozoles, Fazarabines, Fenretinides, Fluoxymesterones, Fluorocitabines, Fosquidones, Fostriecins, Fotretamines, Galarubicins, Galocitabines, Geroquinols, Gimatecans, Gimeracils, Gloxazones, Glufosfamides, Ilmofosines, Ilomastats, Imexons, Improsulfans, Indisulams, Inproquones, Interleukins, Interleukin-2s, recombinant Interleukins, Intoplicines, Iobenguanes, Iproplatins, Irsogladines, Ixabepilones, Ketotrexates, L-Alanosines, Lanreotides, Lapatinibs, Ledoxantrones, Leuprolides, Leuprorelins, Lexacalcitols, Liarozoles, Lobaplatins, Lometrexols, Lonafarnibs, Losoxantrones, Lurtotecans, Mafosfamides, Mannosulfans, Marimastats, Masoprocols, Maytansines, Mechlorethamines, Melengestrols, Melphalans, Menogarils, Mepitiostanes, Metesinds, Metomidates, Metoprines, Meturedepas, Miboplatins, Miproxifenes, Misonidazoles, Mitindomides, Mitocarcins, Mitocromins, Mitoflaxones, Mitogillins, Mitoguazones, Mitomalcins, Mitonafides, Mitoquidones, Mitospers, Mitozolomides, Mivobulins, Mizoribines, Mofarotenes, Mopidamols, Mubritinibs, Mycophenolic Acids, Nedaplatins, Neizarabines, Nemorubicins, Nitracrines, Nocodazoles, Nogalamycins, Nolatrexeds, Nortopixantrones, Ormaplatins, Ortataxels, Oteracils, Oxisurans, Oxophenarsines, Patupilones, Peldesines, Peliomycins, Pelitrexols, Pemetrexeds, Pentamustines, Peplomycins, Perfosfamides, Perifosines, Picoplatins, Pinafides, Piposulfans, Pirfenidones, Piroxantrones, Pixantrones, Plevitrexeds, Plomestanes, Porfiromycins, Prednimustines, Propamidines, Prospidiums, Pumitepas, Puromycins, Pyrazofurins, Ranimustines, Riboprines, Ritrosulfans, Rogletimides, Roquinimexs, Rufocromomycins, Sabarubicins, Safingols, Satraplatins, Sebriplatins, Semustines, Simtrazenes, Sizofirans, Sobuzoxanes, Sorafenibs, Sparfosates, Sparfosic Acids, Sparsomycins, Spirogermaniums, Spiromustines, Spiroplatins, Squalamines, Streptonigrins, Streptovarycins, Sufosfamides, Sulofenurs, Tacedinalines, Talisomycins, Tallimustines, Tariquidars, Tauromustines, Tecogalans, Tegafurs, Teloxantrones, Temoporfins, Teroxirones, Thiamiprines, Tiamiprines, Tiazofurins, Tilomisoles, Tilorones, Timcodars, Timonacics, Tirapazamines, Topixantrones, Trabectedins, Ecteinascidin 743, Trestolones, Triciribines, Trilostanes, Trimetrexates, Triplatin Tetranitrates, Triptorelins, Trofosfamides, Tubulozoles, Ubenimexs, Uredepas, Valspodars, Vapreotides, Verteporfins, Vinblastines, Vindesines, Vinepidines, Vinflunines, Vinformides, Vinglycinates, Vinleucinols, Vinleurosines, Vinrosidines, Vintriptols, Vinzolidines, Vorozoles, Xanthomycin A's, Guamecyclines, Zeniplatins, Zilascorbs [2-H], Zinostatins, Zorubicins, Zosuquidars, Acetazolamides, Acyclovirs, Adipiodones, Alatrofloxacins, Alfentanils, Allergenic extracts, Alpha 1-proteinase inhibitors, Alprostadils, Amikacins, Amino acids, Aminocaproic acids, Aminophyllines, Amitriptylines, Amobarbitals, Amrinones, Analgesics, Anti-poliomyelitic vaccines, Anti-rabic serums, Anti-tetanus immunoglobulins, tetanus vaccines, Antithrombin IIIs, Antivenom serums, Argatrobans, Arginines, Ascorbic acids, Atenolols, Atracuriums, Atropines, Aurothioglucoses, Azathioprines, Aztreonams, Bacitracins, Baclofens, Basiliximabs, Benzoic acids, Benztropines, Betamethasones, Biotins, Bivalirudins, Botulism antitoxins, Bretyliums, Bumetanides, Bupivacaines, Buprenorphines, Butorphanols, Calcitonins, Calcitriols, Calciums, Capreomycins, Carboprosts, Carnitines, Cefamandoles, Cefoperazones, Cefotaximes, Cefoxitins, Ceftizoximes, Cefuroximes, Chloramphenicols, Chloroprocaines, Chloroquines, Chlorothiazides, Chlorpromazines, Chondroitinsulfuric acids, Choriogonadotropin alfas, Chromiums, Cidofovirs, Cimetidines, Ciprofloxacins, Cisatracuriums, Clonidines, Codeines, Colchicines, Colistins, Collagens, Corticorelin ovine triflutates, Corticotrophins, Cosyntropins, Cyanocobalamins, Cyclosporines, Cysteines, Dacliximabs, Dalfopristins, Dalteparins, Danaparoids, Dantrolenes, Deferoxamines, Desmopressins, Dexamethasones, Dexmedetomidines, Dexpanthenols, Dextrans, Iron dextrans, Diatrizoic acids, Diazepams, Diazoxides, Dicyclomines, Digibinds, Digoxins, Dihydroergotamines, Diltiazems, Diphenhydramines, Dipyridamoles, Dobutamines, Dopamines, Doxacuriums, Doxaprams, Doxercalciferols, Doxycyclines, Droperidols, Dyphyllines, Edetic acids, Edrophoniums, Enalaprilats, Ephedrines, Epoprostenols, Ergocalciferols, Ergonovines, Ertapenems, Erythromycins, Esmolols, Estradiols, Estrogenics, Ethacrynic acids, Ethanolamines, Ethanols, Ethiodized oils, Etidronic acids, Etomidates, Famotidines, Fenoldopams, Fentanyls, Flumazenils, Fluoresceins, Fluphenazines, Folic acids, Fomepizoles, Fomivirsens, Fondaparinuxs, Foscarnets, Fosphenyloins, Furosemides, Gadoteridols, Gadoversetamides, Ganciclovirs, Gentamicins, Glucagons, Glucoses, Glycines, Glycopyrrolates, Gonadorelins, Gonadotropin chorionics, *Haemophilus* B polysaccharides, Hemins, Herbals, Histamines, Hydralazines, Hydrocortisones, Hydromorphones, Hydroxocobalamins, Hydroxyzines, Hyoscyamines, Ibutilides, Imiglucerases, Indigo carmines, Indomethacins, Iodides, Iopromides, Iothalamic acids, Ioxaglic acids, Ioxilans, Isoniazids, Isoproterenols, Japanese encephalitis vaccines, Kanamycins, Ketamines, Labetalols, Lepirudins, Levobupivacaines, Levothyroxines, Lincomycins, Liothyronines, Luteinizing hormones, Lyme disease vaccines, Mangafodipirs, Manthtols, Meningococcal polysaccharide vaccines, Meperidines, Mepivacaines, Mesoridazines, Metaraminols, Methadones, Methocarbamols, Methohexitals, Methyldopates, Methylergonovines, Metoclopramides, Metoprolols, Metronidazoles, Minocyclines, Mivacuriums, Morrhuic acids, Moxifloxacins, Muromonab-CD3s, Mycophenolate mofetils, Nafcillins, Nalbuphines, Nalmefenes, Naloxones, Neostigmines, Niacinamides, Nicardipines, Nitroglycerins, Nitroprussides, Norepinephrines, Orphenadrines, Oxacillins, Oxymorphones, Oxytetracyclines, Oxytocins, Pancuroniums, Panthenols, Pantothenic acids, Papaverines, Peginterferon alpha 2As, Penicillin Gs, Pentamidines, Pentazocines, Pentobarbitals, Perflutrens, Perphenazines, Phenobarbitals, Phentolamines, Phenylephrines, Phenyloins, Physostigmines, Phytonadiones, Polymyxin, Pralidoximes, Prilocalnes, Procainamides, Procaines, Prochlorperazines, Progesterones, Propranolols, Pyridostigmine hydroxides, Pyridoxines, Quinidines, Quinupristins, Rabies immunoglobulins, Rabies vaccines, Ranitidines, Remifentanils, Riboflavins, Rifampins, Ropivacaines, Samariums, Scopolamines, Seleniums, Sermorelins, Sincalides, Somatrems, Spectinomycins, Streptokinases, Streptomycins, Succinylcholines, Sufentanils, Sulfamethoxazoles, Tacrolimuses, Terbutalines, Teriparatides, Testosterones, Tetanus antitoxins, Tetracaines, Tetradecyl sulfates, Theophyllines, Thiamines, Thiethylperazines, Thiopentals, Thyroid stimulating hormones, Tinzaparins, Tirofibans, Tobramycins, Tolazolines, Tolbutamides, Torsemides, Tranexamic acids, Treprostinils, Trifluoperazines, Trimethobenzamides, Trimethoprims, Tromethamines, Tuberculins, Typhoid vaccines, Urofollitropins, Urokinases, Valproic acids, Vasopressins, Vecuroniums, Verapamils, Voriconazoles, Warfarins, Yellow fever vaccines, Zidovudines, Zincs, Ziprasidone hydrochlorides, Aclacinomycins, Actinomycins, Adriamycins, Azaserines, 6-Azauridines, Carzinophilins, Chromomycins, Denopterins, 6-Diazo 5 Oxo-L-Norleucines, Enocitabines, Floxuridines, Olivomycins, Pirarubicins, Piritrexims, Pteropterins, Tegafurs, Tubercidins, Alteplases, Arcitumomabs, bevacizumabs, Botulinum Toxin Type A's, Botulinum Toxin Type B's, Capromab Pendetides, Daclizumabs, Dornase alphas, Drotrecogin alphas, Imciromab Pentetates, Iodine-131's, an antibiotic agent; an angiogenesis inhibitor; anti-cataract and anti-diabetic retinopathy substances; carbonic anhydrase inhibitors; mydriatics; photodynamic therapy agents; prostaglandin analogs; growth factor; anti-neoplastics; anti-metabolites; anti-viral; amebicides and anti-protozoals; anti-tuberculosis and anti-leprotic; antitoxins and antivenins; antihemophilic factor, anti-inhibitor coagulant complex, antithrombin III, coagulations Factor V, coagulation Factor IX, plasma protein fraction, von Willebrand factor; anti-platelet agent a colony stimulating factor (CSF); an erythropoiesis stimulator; hemostatics and albumins; Immune Globulins; thrombin inhibitors; anticoagulants; a steroidal anti-inflammatory drug selected from among alclometasones, algestones, beclomethasones, betamethasones, budesonides, clobetasols, clobetasones, clocortolones, cloprednols, corticosterones, cortisones, cortivazols, deflazacorts, desonides, desoximetasones, dexamethasones, diflorasones, diflucortolones, difluprednates, enoxolones, fluazacorts, flucloronides, flumethasones, flunisolides, fluocinolones, fluocinonides, fluocortins, fluocortolones, fluorometholones, fluperolones, fluprednidenes, fluprednisolones, flurandrenolides, fluticasones, formocortals, halcinonides, halobetasols, halometasones, halopredones, hydrocortamates, hydrocortisones, loteprednol etabonate, mazipredones, medrysones, meprednisones, methylprednisolones, mometasone furoate, paramethasones, prednicarbates, prednisolones, prednisones, prednivals, prednylidenes, rimexolones, tixocortols and triamcinolones; Docosanols, prostaglandins, prostaglandin analogs, anti-prostaglandins and prostaglandin precursors; miotics, cholinergics and anti-cholinesterase; and anti-allergenics.

The compositions and modified PH20 polypeptides can be used to treat any condition normally treated by the PH20 polypeptide or the therapeutically active agent. These include, for example, conditions in which hyaluronan plays a role or is associated with the etiology of the disease due to, for example, accumulation or overproduction of hyaluronan. Hence provided are methods, uses of the compositions and modified PH20 polypeptides for treating a hyaluronan-associated disease or condition by administering any of the modified PH20 polypeptides or compositions provided herein. Hyaluronan-associated diseases and conditions include, for example, inflammatory disease and tumors or cancers, including a late-stage cancer, metastatic cancers and undifferentiated cancers, such as ovarian cancer, in situ carcinoma (ISC), squamous cell carcinoma (SCC), prostate cancer, pancreatic cancer, non-small cell lung cancer, breast cancer and colon cancer. The PH20 polypeptide can be modified to exhibit increased half-life for such treatments. For example, the PH20 polypeptide can be modified with a polymer such as a PEG moiety for such treatments.

Also provided are methods for increasing delivery of a therapeutic agent to a subject by: administering to a subject any of the modified PH20 polypeptides or compositions provided herein, and administering the therapeutic agent. The therapeutic agent can be administered in the same composition or separately, and can be administered before or after, simultaneously, or intermittently, with administration of the PH20 polypeptide(s). Administration includes any route, including intravenous and subcutaneous administration, such as simultaneously with, intermittently with, or subsequent to administration of the therapeutic agent. The therapeutic agents include any of those set forth above, elsewhere herein and/or known to those of skill in the art.

Also provided are methods for treating an excess of glycosaminoglycans; for treating a tumor; for treating glycosaminoglycan accumulation in the brain; for treating a cardiovascular disorder; for treating an ophthalmic disorder; for treating pulmonary disease; for increasing penetration of chemotherapeutic agents into solid tumors; for treating cellulite; for treating a proliferative disorder; or for increasing bioavailability of drugs and other therapeutic agents by administering the modified PH20 polypeptides or compositions provided herein.

Also provided are pharmaceutical compositions for use in treating a hyaluronan-associated disease or disorder; for use in delivering a therapeutic agent to a subject; for treating an excess of glycosaminoglycans; for treating a tumor; for treating glycosaminoglycan accumulation in the brain; for treating a cardiovascular disorder; for treating an ophthalmic disorder; for treating pulmonary disease; for increasing penetration of chemotherapeutic agents into solid tumors; for treating cellulite; for treating a proliferative disorder; or for increasing bioavailability of drugs and other therapeutic agents; and for any other use of compositions containing PH20 polypeptides.

Provided herein is a method for identifying or selecting a modified hyaluronan-degrading enzyme that exhibits stability under a denaturation condition that includes the steps of: a) testing the activity of a modified hyaluronan-degrading enzyme in a composition containing a denaturing agent and/or under a denaturing condition; b) testing the activity of the modified hyaluronan-degrading enzyme in the same composition and/or under the same conditions as a) except absent the denaturing agent or condition; and c) selecting or identifying a modified hyaluronan-degrading enzyme that exhibits activity in a) that is at least 5% of the activity in b). In such an example, the activity is hyaluronidase activity. In some examples of the methods, a modified hyaluronan-degrading enzyme is selected or identified if the activity in a) is at least 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more of the activity in b), for example, a modified hyaluronan-degrading enzyme is selected or identified if the activity in a) is at least 40% or more of the activity in b). The method also can include steps of: d) comparing the activity of the modified hyaluronan-degrading enzyme in a) to the activity of the unmodified hyaluronan-degrading enzyme tested under the same conditions; and e) identifying or selecting a modified hyaluronan-degrading enzyme that exhibits at least 120%, 130%, 135%, 140%, 145%, 150%, 160%, 170%, 180%, 200%, 250%, 300%, 350%, 400%, 500%, 1500%, 2000%, 3000%, 4000%, 5000% or more of the hyaluronidase activity compared to the unmodified hyaluronan-degrading enzyme.

Also provide herein is a method for identifying or selecting a modified hyaluronan-degrading enzyme that exhibits stability, such as increased stability, under a denaturation condition, that includes the steps of: a) testing the activity of a modified hyaluronan-degrading enzyme in a composition containing a denaturing agent and/or under a denaturing condition; b) testing the activity of the corresponding unmodified hyaluronan-degrading enzyme in a composition containing the same denaturing agent and/or under the same denaturing condition as a), whereby the activity is tested under the same conditions as a); and c) selecting or identifying a modified hyaluronan-degrading enzyme that exhibits greater activity than the unmodified hyaluronan-degrading enzyme, thereby identifying or selecting a modified hyaluronan-degrading enzyme that exhibits increased stability under a denaturation condition. In such an example, the activity can be a hyaluronidase activity. In examples of the method, a modified hyaluronan-degrading enzyme is selected or identified if the activity is at least 120%, 130%, 135%, 140%, 145%, 150%, 160%, 170%, 180%, 200%, 250%, 300%, 350%, 400%, 500%, 1500%, 2000%, 3000%, 4000%, 5000% or more of the activity compared to the unmodified hyaluronan-degrading enzyme. In such an example, the method also can include additional steps of: d) testing the activity of the selected or identified modified hyaluronan-degrading enzyme in a composition containing a denaturing agent and/or under a denaturing condition; e) testing the activity of the same selected or identified modified hyaluronan-degrading enzyme in the same composition and/or under the same conditions as d) except absent the denaturing agent or condition; and f) selecting or identifying a modified hyaluronan-degrading enzyme that exhibits activity in d) that is at least 5% of the activity in e). In such an example, the activity is hyaluronidase activity. In some examples of the methods, a modified hyaluronan-degrading enzyme is selected or identified if the activity in d) is at least 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more of the activity in e), for example, a modified hyaluronan-degrading enzyme is selected or identified if the activity in d) is at least 40% or more of the activity in e).

In any of the methods provided herein for identifying or selecting a modified hyaluronan-degrading enzyme, the denaturing agent or condition is caused by temperature, agitation, no or low salt or the presence of an excipient. For example, the denaturing agent or condition is caused by elevated temperature that is from or from about 30° C. to 42° C., such as greater than or greater than about 30° C., 31° C., 32° C., 33° C., 34° C., 35° C., 36° C., 37° C., 38° C., 39° C., 40° C., 41° C. or 42° C. In other examples, the denaturing agent or condition is the absence of salt or low salt less than 100 mM, such as low salt less than 90 mM, 80 mM, 70 mM, 60 mM, 50 mM, 40 mM, 30 mM, 25 mM, 20 mM, 15 mM, 10 mM, 5 mM. In further examples, the denaturing agent or condition is a denaturing excipient selected from among an antiadherents, binders, coatings, fillers and diluents, flavors, colors, lubricants, glidants, preservatives, sorbents and sweeteners.

In particular examples of any of the methods provided herein for identifying or selecting a modified hyaluronan-degrading enzyme, the denaturing agent or condition is a preservative(s), for example, a phenolic preservative(s). The phenolic preservative(s) can be a phenol, metacresol (m-cresol), benzyl alcohol, or a paraben. For example, the denaturing agent or condition is a preservative(s) that is phenol and/or m-cresol. In such examples, the total amount of phenolic preservative in the composition, as a percentage (%) of mass concentration (w/v), is from or from about 0.05% to 0.6%, 0.1% to 0.4%, 0.1% to 0.3%, 0.15% to 0.325%, 0.15% to 0.25%, 0.1% to 0.2%, 0.2% to 0.3% or 0.3% to 0.4% inclusive.

In any of the methods provided herein for identifying or selecting a modified hyaluronan-degrading enzyme, prior to testing the activity of a hyaluronan-degrading enzyme composition in a) and/or b), the hyaluronan-degrading enzyme is exposed to the denaturation condition or denaturing agent for a predetermined time. The predetermined time is a time period that is user selected depending on the particular hyaluronan-degrading enzyme that is being evolved or selected, the particular denaturation condition or denaturing agent, the amount or extent of the denaturation condition or denaturing agent, the application or use of the hyaluronan-degrading enzyme and other similar factors. For example, the predetermined time can be from or from about 1 minute to 1 month, 1 minute to 3 weeks, 1 minute to 2 weeks, 1 minute to 1 week, 1 minute to 24 hours, 1 minute to 12 hours, 30 minutes to 6 hours or 1 hour to 4 hours, such as at least or about at least 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 24 hours, two days, three days, four days, five days, six days, 7 days, two weeks or one month.

In any of the methods provided herein for identifying or selecting a modified hyaluronan-degrading enzyme, the modified hyaluronan-degrading enzyme is one that contains an amino acid replacement, insertion or deletion of amino acids compared to an unmodified hyaluronan-degrading enzyme. For example, the modified hyaluronan-degrading enzyme contains an amino acid replacement, such as a single amino acid replacement or two, three, four, five, six, seven, eight, nine or more amino acid replacements compared to an unmodified form of the hyaluronan-degrading enzyme. In particular aspects of the method, a library or collection of modified hyaluronan-degrading enzymes are screened in order to evolve or identify or select a modified hyaluronan-degrading enzyme that exhibits stability, such as increased stability, under a denaturation condition. Thus, in examples of the methods herein, a plurality of modified hyaluronan-degrading enzymes are tested in a) and/or b). In such examples, the plurality of modified hyaluronan-degrading enzymes are modified compared to the corresponding unmodified hyaluronan-degrading enzyme to generate a collection of modified hyaluronan-degrading enzymes, whereby each modified protein in the collection is tested in each of a) and/or b). In the collection or library, each modified hyaluronan-degrading enzyme contains a single amino acid replacement compared to the unmodified form of the hyaluronan-degrading enzyme, such that the plurality of modified enzymes are such that the amino acid at each modified position is replaced by up to 1-19 other amino acids other than the original amino acid at the position, whereby each modified hyaluronan-degrading enzyme contains a different amino acid replacement, and every amino acid along the length of the hyaluronan-degrading enzyme, or a selected portion thereof, is replaced.

In any of the methods provided herein, the modified hyaluronan-degrading enzyme is modified compared to an unmodified hyaluronan-degrading enzyme by insertion, deletion or replacement of an amino acid(s). The unmodified hyaluronan-degrading enzyme can be a chondroitinase or can be a hyaluronidase. In examples herein, the unmodified hyaluronidase is a PH20 hyaluronidase or truncated form thereof lacking a C-terminal glycosylphosphatidylinositol (GPI) anchor attachment site or a portion of the GPI anchor attachment site, whereby the truncated form exhibits hyaluronidase activity. PH20 hyaluronidase can be a human, monkey, bovine, ovine, rat, fox, mouse or guinea pig PH20. In particular examples, the PH20 hyaluronidase is a human PH20 or a C-terminal truncated form thereof. For example, the unmodified hyaluronan-degrading enzyme is one that has the sequence of amino acids set forth in any of SEQ ID NOS: 3, 7, 10, 12, 14, 24, 32-66, 69, 72, 857, 859, 861, 870 or a sequence of amino acids that is at least 80% sequence identity to any of SEQ ID NOS: 3, 7, 10, 12, 14, 24, 32-66, 69, 72, 857, 859, 861, 870, such as at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% sequence identity to any of SEQ ID NOS: 3, 7, 10, 12, 14, 24, 32-66, 69, 72, 857, 859, 861, or 870. In particular examples, the unmodified hyaluronan-degrading enzyme is a PH20 hyaluronidase having the sequence of amino acids set forth in any of SEQ ID NOS: 3, 7, 32-66, 69 or 72, or a sequence of amino acids that exhibits at least 85% sequence identity to any of SEQ ID NOS: 3, 7, 32-66, 69 or 72, such as a sequence of amino acids that exhibits at least 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 3, 7, 32-66, 69 or 72.

In any of the methods provided herein for identifying or selecting a modified hyaluronan-degrading enzyme that exhibits stability, the method is performed in vitro. Also provided are any of the methods that are iterative, whereby the steps of the method are repeated a plurality of times, wherein in each repetition, further modified hyaluronan-degrading enzymes of a selected modified hyaluronan-degrading enzyme are generated and tested, whereby the modified hyaluronan-degrading enzyme is evolved to exhibit increased stability under a denaturation condition. Also provided herein is a modified hyaluronan-degrading enzyme identified by any of the methods provided herein.

BRIEF DESCRIPTION OF THE FIGURES

FIG. **1** depicts the amino acid sequence of full-length human PH20 (set forth in SEQ ID NO:7) and soluble C-terminal truncated variants thereof. The C-terminal amino acid residue of exemplary C-terminal truncated variants of full-length PH20 are indicated by bold font. The complete amino acid sequences of exemplary C-terminal truncated variants of full-length PH20 also are provided in SEQ ID NOS: 3 and 32-66. The C-terminal amino acid residue of an exemplary soluble PH20, whose complete sequence is set forth in SEQ ID NO:3, also is indicated by underline. Exemplary, non-limiting, positions for amino acid replacements are indicated by highlighting. Corresponding positions can be identified by alignment of a sequence of interest with any of SEQ ID NOS: 3, 7 or 32-66, and in particular with SEQ ID NO:3.

FIG. **2** (A-L) depicts exemplary alignments of human soluble PH20 set forth in SEQ ID NO:3 with other PH20 polypeptides. A "*" means that the aligned residues are identical, a ":" means that aligned residues are not identical, but are similar and contain conservative amino acids residues at the aligned position, and a "." means that the aligned residues are similar and contain semi-conservative amino acid residues at the aligned position. Exemplary, non-limiting, corresponding positions for amino acid replacements are indicated by highlighting. For example, FIG. **2**A depicts the alignment of a human soluble PH20 set forth in SEQ ID NO:3 with chimpanzee PH20 set forth in SEQ ID NO:10. FIG. **2**B depicts the alignment of a human soluble PH20 set forth in SEQ ID NO:3 with Rhesus monkey PH20 set forth in SEQ ID NO:12. FIG. **2**C depicts the alignment of a human soluble PH20 set forth in SEQ ID NO:3 with Cynomolgus monkey PH20 set forth in SEQ ID NO: 14. FIG. **2**D depicts the alignment of human soluble PH20 set forth in SEQ ID NO:3 with bovine PH20 set forth in SEQ ID NO:16. FIG. **2**E depicts the alignment of a human soluble PH20 set forth in SEQ ID NO:3 with mouse PH20 set forth in SEQ ID NO:20. FIG. **2**F depicts the alignment of a human soluble PH20 set forth in SEQ ID NO:3 with rat PH20 set forth in SEQ ID NO:22. FIG. **2**G depicts the alignment of a human soluble PH20 set forth in SEQ ID NO:3 with rabbit PH20 set forth in SEQ ID NO:24. FIG. **2**H depicts the alignment of a human soluble PH20 set forth in SEQ ID NO:3 with guinea pig PH20 set forth in SEQ ID NO:29. FIG. **2**I depicts the alignment of a human soluble PH20 set forth in SEQ ID NO:3 with Fox PH20 set forth in SEQ ID NO:31. FIG. **2**J depicts the alignment of a human soluble PH20 set forth in SEQ ID NO:3 with Gibbon PH20 set forth in SEQ ID NO:857. FIG. **2**K depicts the alignment of a human soluble PH20 set forth in SEQ ID NO:3 with Marmoset PH20 set forth in SEQ ID NO: 859. FIG. **2**L depicts the alignment of a human soluble PH20 set forth in SEQ ID NO:3 with Orangutan PH20 set forth in SEQ ID NO:861.

DETAILED DESCRIPTION

Outline
- A. Definitions
- B. PH20 Hyaluronidase
  - 1. Structure
  - 2. Function
  - 3. Soluble PH20 Polypeptides
- C. Modified PH20 Polypeptides
  - 1. Active Mutants
    - a. Increased Activity
    - b. Increased Stability
      - i. Phenophiles
      - ii. Thermophiles
      - iii. Absence of Salt
  - 2. Inactive Mutants
  - 3. Additional Modifications
    - a. Decreased Immunogenicity
    - b. Conjugation to Polymers
- D. Methods for Identifying Modified Hyaluronan-Degrading Enzymes with Altered properties or Activities
  - 1. Hyaluronan-Degrading Enzymes and Libraries of Modified Hyaluronan-Degrading Enzymes
  - 2. Screening or Testing for a Desired Activity or Property
  - 3. Selection or Identification
  - 4. Iterative Methods
- E. Production of Modified Polypeptides and Encoding Nucleic Acid Molecules
  - 1. Isolation or Preparation of Nucleic Acids Encoding PH20 Polypeptides
  - 2. Generation of Mutant or Modified Nucleic Acid and Encoding Polypeptides
  - 3. Vectors and Cells
  - 4. Expression
    - a. Prokaryotic Cells
    - b. Yeast Cells
    - c. Insects and Insect Cells
    - d. Mammalian expression
    - e. Plants and plant cells
  - 5. Purification
  - 6. Modification of Polypeptides by PEGylation
- F. Pharmaceutical Compositions and Formulations, Dosages and Administration
  - 1. Formulations (liquids, injectables, solutions and emulsions)
    - a. Lyophilized
    - b. Exemplary Formulations
      - i. NaCl
      - ii. pH and Buffer
      - iii. Preservatives
      - iv. Stabilizers
  - 2. Compositions for Other Routes of Administration
  - 3. Dosages and Administration
  - 4. Exemplary PH20-Insulin Co-Formulations
  - 5. Packaging, Articles of Manufacture and Kits
- G. Methods of Assessing Hyaluronidase Activity
  - 1. Hyaluronidase Activity
  - 2. Solubility
  - 3. Purity, Crystallization or Aggregation
  - 4. Pharmacodynamics/Pharmacokinetics

- H. Methods of Treatment and Combination Therapy
  - 1. Methods of Delivering Therapeutic Agents Delivery of Insulin
  - 2. Methods of Treating Hyaluronan-Associated Disease and Conditions
  - 3. Other Uses
  - 4. Contraception
- I. Examples

A. Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the invention(s) belong. All patents, patent applications, published applications and publications, GenBank sequences, databases, websites and other published materials referred to throughout the entire disclosure herein, unless noted otherwise, are incorporated by reference in their entirety. In the event that there are a plurality of definitions for terms herein, those in this section prevail. Where reference is made to a URL or other such identifier or address, it understood that such identifiers can change and particular information on the internet can come and go, but equivalent information can be found by searching the internet. Reference thereto evidences the availability and public dissemination of such information.

As used herein, a hyaluronan-degrading enzyme refers to an enzyme that catalyzes the cleavage of a hyaluronan polymer (also referred to as hyaluronic acid or HA) into smaller molecular weight fragments. Exemplary hyaluronan-degrading enzymes are hyaluronidases, and particular chondroitinases and lyases that have the ability to depolymerize hyaluronan. Exemplary chondroitinases that are hyaluronan-degrading enzymes include, but are not limited to, chondroitin ABC lyase (also known as chondroitinase ABC), chondroitin AC lyase (also known as chondroitin sulfate lyase or chondroitin sulfate eliminase) and chondroitin C lyase. Chondroitin ABC lyase contains two enzymes, chondroitin-sulfate-ABC endolyase (EC 4.2.2.20) and chondroitin-sulfate-ABC exolyase (EC 4.2.2.21). An exemplary chondroitin-sulfate-ABC endolyases and chondroitin-sulfate-ABC exolyases include, but are not limited to, those from *Proteus vulgaris* and *Pedobacter heparinus* (the *Proteus vulgaris* chondroitin-sulfate-ABC endolyase is set forth in SEQ ID NO:922; Sato et al. (1994) *Appl. Microbiol. Biotechnol.* 41(1):39-46). Exemplary chondroitinase AC enzymes from bacteria include, but are not limited to, those from *Pedobacter heparinus*, set forth in SEQ ID NO: 923, *Victivallis vadensis*, set forth in SEQ ID NO:924, and *Arthrobacter aurescens* (Tkalec et al. (2000) *Applied and Environmental Microbiology* 66(1):29-35; Ernst et al. (1995) *Critical Reviews in Biochemistry and Molecular Biology* 30(5):387-444). Exemplary chondroitinase C enzymes from bacteria include, but are not limited to, those from *Streptococcus* and *Flavobacterium* (Hibi et al. (1989) *FEMS-Microbiol-Lett.* 48(2): 121-4; Michelacci et al. (1976) *J. Biol. Chem.* 251:1154-8; Tsuda et al. (1999) *Eur. J. Biochem.* 262:127-133).

As used herein, hyaluronidase refers to a class of enzymes that degrade hyaluronan. Hyaluronidases include, but are not limited to, bacterial hyaluronidases (EC 4.2.2.1 or EC 4.2.99.1), hyaluronidases from leeches, other parasites and crustaceans (EC 3.2.1.36), and mammalian-type hyaluronidases (EC 3.2.1.35). Hyaluronidases include any of non-human origin including, but not limited to, murine, canine, feline, leporine, avian, bovine, ovine, porcine, equine, piscine, ranine, bacterial, and any from leeches, other parasites, and crustaceans. Exemplary human hyaluronidases include HYAL1, HYAL2, HYAL3, HYAL4, and PH20. Also included amongst hyaluronidases are soluble hyaluronidases, including, ovine and bovine PH20, and soluble PH20. Exemplary hyaluronidases include any set forth in SEQ ID NOS: 6, 7-31, 69, 70, 71, 72, 856-861, 869-921, mature forms thereof (lacking the signal sequence), or allelic or species variants thereof. Hyaluronidases also include truncated forms thereof that exhibit hyaluronidase activity, including C-terminal truncated variants that are soluble.

As used herein, PH20 refers to a type of hyaluronidase that occurs in sperm and is neutral-active. PH-20 occurs on the sperm surface, and in the lysosome-derived acrosome, where it is bound to the inner acrosomal membrane. PH20 includes those of any origin including, but not limited to, human, chimpanzee, Cynomolgus monkey, Rhesus monkey, murine, bovine, ovine, guinea pig, rabbit and rat origin. Exemplary PH20 polypeptides, including precursor and mature forms, include those from human (SEQ ID NO:6 and 7), chimpanzee (SEQ ID NO:8, 9, 10, 869 and 870), Rhesus monkey (SEQ ID NO:11 and 12), Cynomolgus monkey (SEQ ID NO:13 and 14), cow (e.g., SEQ ID NOS:15-18); mouse (SEQ ID NO:19 and 20); rat (SEQ ID NO:21 and 22); rabbit (SEQ ID NO:23 and 24); sheep (SEQ ID NOS:25-27), guinea pig (SEQ ID NO:28 and 29); fox (SEQ ID NO: 30 and 31); Gibbon (SEQ ID NO:856 and 857), Marmoset (SEQ ID NO:858 and 859) and orangutan (SEQ ID NO:860 and 861). Reference to PH20 includes precursor PH20 polypeptides and mature PH20 polypeptides (such as those in which a signal sequence has been removed), truncated forms thereof that have activity, and includes allelic variants and species variants, variants encoded by splice variants, and other variants, including polypeptides that have at least 40%, 45%, 50%, 55%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the precursor polypeptides set forth in SEQ ID NO:7, or the mature forms thereof. PH20 polypeptides also include those that contain chemical or posttranslational modifications and those that do not contain chemical or posttranslational modifications. Such modifications include, but are not limited to, PEGylation, albumination, glycosylation, farnysylation, carboxylation, hydroxylation, phosphorylation, and other polypeptide modifications known in the art. Examples of commercially available bovine or ovine soluble hyaluronidases are Vitrase® hyaluronidase (ovine hyaluronidase) and Amphadase® hyaluronidase (bovine hyaluronidase).

As used herein, a soluble PH20 refers to a polypeptide characterized by its solubility under physiological conditions. Generally, a soluble PH20 lacks all or a portion of a glycophosphatidyl anchor (GPI) attachment sequence, or does not otherwise sufficiently anchor to the cell membrane. For example, a soluble PH20 can be a C-terminally truncated variant of a PH20 lacking a contiguous sequence of amino acids that corresponds to all or a portion of a glycophosphatidyl anchor (GPI) attachment sequence. Hence, upon expression from a cell, a soluble PH20 is secreted into the medium. Soluble PH20 proteins can be distinguished, for example, by its partitioning into the aqueous phase of a Triton X-114 solution warmed to 37° C. (Bordier et al., (1981) *J. Biol. Chem.,* 256:1604-7). Membrane-anchored, such as lipid anchored hyaluronidases, will partition into the detergent rich phase, but will partition into the detergent-poor or aqueous phase following treatment with Phospholipase-C. Included among soluble PH20 hyaluronidases are membrane anchored hyaluronidases in which one or more regions associated with anchoring of the hyaluronidase to the membrane has been removed or modified, where the soluble form retains hyaluronidase activity. Soluble hyaluronidases include recombinant soluble hyaluronidases and those contained in or purified from natural sources, such as, for example, testes extracts from sheep or cows. Exemplary of such soluble hyaluronidases are soluble human PH20 (SEQ ID NO: 3 or 32-66). Other soluble hyaluronidases include ovine (SEQ ID NO:25-27) and bovine (SEQ ID NO:16 or 18) PH20.

As used herein, soluble human PH20 (sHuPH20) includes human PH20 polypeptides that lack a contiguous sequence of amino acids from the C-terminus of human PH20 that includes all or a portion of the glycosylphosphatidylinositol (GPI) anchor sequence (C-terminally truncated PH20 polypeptides) such that upon expression, the polypeptides are soluble under physiological conditions. For example, soluble human PH20 polypeptides are C-terminally truncated polypeptides of human PH20 set forth as SEQ ID NO:6 in its precursor form or in SEQ ID NO:7 in its mature form lacking the signal sequence, or allelic variants thereof (e.g. set forth in any of SEQ ID NOS: 68-72). Solubility can be assessed by any suitable method that demonstrates solubility under physiologic conditions. Exemplary of such methods is the Triton® X-114 assay, that assesses partitioning into the aqueous phase and that is described above. In addition, a soluble human PH20 polypeptide is, if produced in CHO cells, such as CHO—S cells, a polypeptide that is expressed and is secreted into the cell culture medium. Soluble human PH20 polypeptides, however, are not limited to those produced in CHO cells, but can be produced in any cell or by any method, including recombinant expression and polypeptide synthesis. Reference to secretion in CHO cells is definitional. Hence, if a polypeptide could be expressed and secreted in CHO cells and is soluble in the media, i.e., partitions into the aqueous phase when extracted with Triton® X-114, it is a soluble PH20 polypeptide whether or not it is so-produced. The precursor polypeptides for sHuPH20 polypeptides can include a signal sequence, such as a heterologous or non-heterologous (i.e., native) signal sequence. Exemplary of the precursors are those that include a signal sequence, such as the native 35 amino acid signal sequence at amino acid positions 1-35 (see, e.g., amino acids 1-35 of SEQ ID NO:6).

As used herein, "native" or "wildtype" with reference to a PH20 polypeptide refers to a PH20 polypeptide encoded by a native or naturally occurring PH20 gene, including allelic variants, that is present in an organism, including a human and other animals, in nature. Reference to wild-type PH20 without reference to a species is intended to encompass any species of a wild-type PH20. Included among wild-type PH20 polypeptides are the encoded precursor polypeptide, fragments thereof, and processed forms thereof, such as a mature form lacking the signal peptide as well as any pre- or post-translationally processed or modified forms thereof. Also included among native PH20 polypeptides are those that are post-translationally modified, including, but not limited to, those that are modified by glycosylation, carboxylation and/or hydroxylation. The amino acid sequences of exemplary wild-type human PH20 are set forth in SEQ ID NOS: 6 and 7 and those of allelic variants, including mature forms thereof, are set forth in SEQ ID NOS:68-72. Other animals produce native PH20, including, but not limited to, native or wildtype sequences set forth in any of SEQ ID NOS: 8-31, 856-861, 869 or 870.

As used herein, modification is in reference to modification of a sequence of amino acids of a polypeptide or a sequence of nucleotides in a nucleic acid molecule and includes deletions, insertions, and replacements of amino acids and nucleotides, respectively. Modifications also can include post-translational modifications or other changes to the molecule that can occur due to conjugation or linkage, directly or indirectly, to another moiety. Methods of modifying a polypeptide are routine to those of skill in the art, such as by using recombinant DNA methodologies.

As used herein, a "modified hyaluronan-degrading enzyme" refers to a hyaluronan-degrading enzyme that contains a modification compared to a reference or unmodified hyaluronan-degrading enzyme. The modification can be an amino acid replacement (substitution), insertion (addition) or deletion of one or more amino acid residues. The amino acid residue can be a natural or non-natural amino acid. In some cases, the modification can be a post-translational modification. A modified hyaluronan-degrading enzyme can have up to 150 amino acid differences compared to a reference or unmodified hyaluronan-degrading enzyme, so long as the resulting modified hyaluronan-degrading enzyme exhibits hyaluronidase activity. Typically, a modified hyaluronan-degrading enzyme contains 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 amino acid modifications.

As used herein, an unmodified hyaluronan-degrading enzyme refers to a starting polypeptide that is selected for modification as provided herein. The starting polypeptide can be a naturally-occurring, wild-type form of a polypeptide. In addition, the starting polypeptide can be altered or mutated, such that it differs from a native wild type isoform but is nonetheless referred to herein as a starting unmodified polypeptide relative to the subsequently modified polypeptides produced herein. Thus, existing proteins known in the art that have been modified to have a desired increase or decrease in a particular activity or property compared to an unmodified reference protein can be selected and used as the starting unmodified polypeptide. For example, a protein that has been modified from its native form by one or more single amino acid changes and possesses either an increase or decrease in a desired property, such as a change in an amino acid residue or residues to alter glycosylation, can be selected for modification, and hence referred to herein as unmodified, for further modification. An unmodified hyaluronan-degrading enzyme includes human and non-human hyaluronan-degrading enzymes, including hyaluronan-degrading enzymes from non-human mammals and bacteria. Exemplary unmodified hyaluronan-degrading enzyme are any set forth in SEQ ID NOS: 2, 3, 6, 7-66, 68-72, 856-861, 869-924 or mature, C-terminally truncated forms thereof that exhibit hyaluronidase activity, or a hyaluronan-degrading enzyme that exhibits at least 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to any of SEQ ID NOS: 2, 3, 6, 7-66, 68-72, 856-861, 869-924. It is understood that an unmodified hyaluronan-degrading enzyme generally is one that does not contain the modification(s), such as amino acid replacement(s) of a modified hyaluronan-degrading enzyme.

As used herein, "modified PH20 polypeptide" or "variant PH20 polypeptide" refers to a PH20 polypeptide that contains at least one amino acid modification, such as at least one amino acid replacement as described herein, in its sequence of amino acids compared to a reference unmodified PH20 polypeptide. A modified PH20 polypeptide can have up to 150 amino acid replacements, so long as the resulting modified PH20 polypeptide exhibits hyaluronidase activity. Typically, a modified PH20 polypeptide contains 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 amino acid replacements. It is understood that a modified PH20 polypeptide also can include any one or more other modifications, in addition to at least one amino acid replacement as described herein.

As used herein, an unmodified PH20 polypeptide refers to a starting PH20 polypeptide that is selected for modification as provided herein. The starting polypeptide can be a naturally-occurring, wild-type form of a polypeptide. In addition, the starting polypeptide can be altered or mutated, such that it differs from a native wild type isoform but is nonetheless referred to herein as a starting unmodified polypeptide relative to the subsequently modified polypeptides produced herein. Thus, existing proteins known in the art that have been modified to have a desired increase or decrease in a particular activity or property compared to an unmodified reference protein can be selected and used as the starting unmodified polypeptide. For example, a protein that has been modified from its native form by one or more single amino acid changes and possesses either an increase or decrease in a desired property, such as a change in an amino acid residue or residues to alter glycosylation, can be selected for modification, and hence referred to herein as unmodified, for further modification. Exemplary unmodified PH20 polypeptides is a human PH20 polypeptide or allelic or species variants thereof or other variants, including mature and precursor polypeptides. For example, exemplary reference PH20 polypeptides is a mature full length PH20 polypeptide set forth in SEQ ID NOS: 7, 69 or 72, or in C-terminally truncated forms thereof such as set forth in any of SEQ ID NOS: 3 and 32-66, or in a PH20 polypeptide that exhibits at least 68%, 69%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to any of SEQ ID NOS: 3, 7, 32-66, 69 or 72. A reference PH20 polypeptide also can include the corresponding precursor form such as set forth in any of SEQ ID NOS: 2, 6, 68, 70, 71 or other precursor forms, or in a PH20 polypeptide that exhibits at least 68%, 69%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to any of SEQ ID NOS: 2, 6, 68, 70, 71. It is understood that an unmodified hyaluronan-degrading enzyme generally is one that does not contain the modification(s), such as amino acid replacement(s) of a modified hyaluronan-degrading enzyme.

As used herein, an N-linked moiety refers to an asparagine (N) amino acid residue of a polypeptide that is capable of being glycosylated by post-translational modification of a polypeptide. Exemplary N-linked moieties of human PH20 include amino acids N47, N131, N200, N219, N333, N358 and N365 of the sequence of amino acids set forth in SEQ ID NO: 3 or 7 (corresponding to amino acid residues N82, N166, N235, N254, N368, N393 and N490 of human PH20 set forth in SEQ ID NO: 6).

As used herein, an N-glycosylated polypeptide refers to a PH20 polypeptide containing oligosaccharide linkage of at least three N-linked amino acid residues, for example, N-linked moieties corresponding to amino acid residues N200, N333 and N358 of SEQ ID NO:3 or 7. An N-glycosylated polypeptide can include a polypeptide where three, four, five and up to all of the N-linked moieties are linked to an oligosaccharide. The N-linked oligosaccharides can include oligomannose, complex, hybrid or sulfated oligosaccharides, or other oligosaccharides and monosaccharides.

As used herein, an N-partially glycosylated polypeptide refers to a polypeptide that minimally contains an N-acetylglucosamine glycan linked to at least three N-linked moieties. A partially glycosylated polypeptide can include various glycan forms, including monosaccharides, oligosaccharides, and branched sugar forms, including those formed by treatment of a polypeptide with EndoH, EndoF1, EndoF2 and/or EndoF3.

As used herein, "conditions" refers to any parameter that can influence the activity or properties of a protein or agent. For purposes herein, conditions generally refer to the presence, including amount, of excipients, carriers or other components in a formulation other than the active agent (e.g., modified PH20 hyaluronidase); temperature; time (e.g., time of storage or exposure); storage vessel; properties of storage (e.g., agitation) and/or other conditions associated with exposure or use.

As used herein, "denaturation" or "denaturing" or grammatical variations thereof with reference to a protein refers to a biochemical change in a protein so that a property or activity of the protein is diminished or eliminated. The biochemical change can be a change in the tertiary structure of the protein to unfold. The property or activity can be completely abolished or can be reduced by 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or more.

As used herein, property refers to a physical or structural property, such as the three-dimensional structure, pi, half-life, conformation and other such physical characteristics. For example, a change in a property can be manifested as the solubility, aggregation or crystallization of a protein.

As used herein, activity refers to a functional activity or activities of a polypeptide or portion thereof associated with a full-length (complete) protein. Functional activities include, but are not limited to, biological activity, catalytic or enzymatic activity, antigenicity (ability to bind or compete with a polypeptide for binding to an anti-polypeptide antibody), immunogenicity, ability to form multimers, and the ability to specifically bind to a receptor or ligand for the polypeptide.

As used herein, hyaluronidase activity refers to the ability to enzymatically catalyze the cleavage of hyaluronic acid. The United States Pharmacopeia (USP) XXII assay for hyaluronidase determines hyaluronidase activity indirectly by measuring the amount of higher molecular weight hyaluronic acid, or hyaluronan, (HA) substrate remaining after the enzyme is allowed to react with the HA for 30 min at 37° C. (USP XXII-NF XVII (1990) 644-645 United States Pharmacopeia Convention, Inc, Rockville, Md.). A Reference Standard solution can be used in an assay to ascertain the relative activity, in units, of any hyaluronidase. In vitro assays to determine the hyaluronidase activity of hyaluronidases, such as PH20, including modified PH20 polypeptides, are known in the art and described herein. Exemplary assays include the microturbidity assay described herein that measures cleavage of hyaluronic acid by hyaluronidase indirectly by detecting the insoluble precipitate formed when the uncleaved hyaluronic acid binds with serum albumin. Reference Standards can be used, for example, to generate a standard curve to determine the activity in Units of the hyaluronidase being tested.

As used herein, neutral active refers to the ability of a PH20 polypeptide to enzymatically catalyze the cleavage of hyaluronic acid at neutral pH, such as at a pH between or about between pH 6.0 to pH 7.8.

As used herein, "increased activity" with reference to a modified PH20 hyaluronidase means that, when tested under the same conditions, the modified PH20 hyaluronidase exhibits greater hyaluronidase activity compared to an unmodified PH20 hyaluronidase not containing the amino acid replacement(s). For example, a modified PH20 hyaluronidase exhibits at least or about at least 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 250%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, 1000% or more of the activity of the unmodified or reference PH20 hyaluronidase.

As used herein, "solubility" with reference to a protein refers to a protein that is homogenous in an aqueous solution, whereby protein molecules diffuse and do not sediment spontaneously. Hence a soluble protein solution is one in which there is an absence of a visible or discrete particle in a solution containing the protein, such that the particles cannot be easily filtered. Generally, a protein is soluble if there are no visible or discrete particles in the solution. For example, a protein is soluble if it contains no or few particles that can be removed by a filter with a pore size of 0.22 μm.

As used herein, aggregation or crystallization with reference to a protein refers to the presence of visible or discrete particles in a solution containing the protein. Typically, the particles are greater than 10 μm in size, such as greater than 15 μm, 20 μm, 25 μm, 30 μm, 40 μm, 50 μm or greater. Aggregation or crystallization can arise due to reduced solubility, increased denaturation of a protein or the formation of covalent bonds.

As used herein, "denaturing condition" or "denaturation condition" refers to any condition or agent that, when exposed to a protein, affects or influences the degradation or denaturation of the protein, generally as a result of a loss or partial loss of the tertiary or secondary structure of the protein. Denaturing conditions can result in effects such as loss or reduction in activity, loss or reduction of solubility, aggregation and/or crystallization. The denaturing condition need not be one that is completely deadly to the protein, but nevertheless is one that leads to a reduction in the activity of the protein over time. Thus, a condition is denaturing if the activity of the protein is reduced by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%), 90%, 95% or more in the presence of the condition than in its absence. A denaturing condition can be due to an external stress or physical condition (e.g., agitation, temperature, time of storage, absence of a stabilizer) or can be due to the presence of a denaturing agent. For example, the denaturing condition can be caused by heat, acid or a chemical denaturant. Exemplary denaturing conditions include, but are not limited to, the presence of a strong acid or base, a concentrated inorganic salt, an organic solvent (e.g., alcohol or chloroform), urea, high or low pH (extremes of pH), elevated temperature (e.g., heat), the presence of excipients that can be denaturing (e.g., phenolic preservatives or detergent), and low or substantially no stabilizing agent that otherwise is required for stability of the protein (e.g., NaCl).

As used herein, "denaturing agent" or "denaturant" refers to any substance, molecule or compound that causes denaturation. For example, a denaturing agent can include a strong acid or base, a concentrated inorganic salt, an organic solvent (e.g., alcohol or chloroform), a preservative, detergent or other excipient.

As used herein, "resistance to a denaturation condition" refers to any amount of decreased reduction or elimination of a property or activity of the protein associated with or caused by denaturation. For example, denaturation is associated with or causes increased crystallization or aggregation, reduced solubility or decreased activity. Hence, resistance to denaturation means that the protein exhibits decreased aggregation or crystallization, increased solubility or increased or greater activity (e.g., hyaluronidase activity) when exposed to a denaturing condition compared to a reference protein (e.g. unmodified enzyme). The resistance to a denaturation condition need not be absolute or permanent, but can be achieved because the denaturation of the modified hyaluronan-degrading enzyme occurs more slowly than the unmodified enzyme in the denaturation condition such that an activity or property of the modified hyaluronan-degrading enzyme is achieved for longer. For example, a modified hyaluronan-degrading enzyme, such as a modified PH20 hyaluronidase, exhibits resistance to a denaturation condition if it exhibits, for example, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, . . . 20%, . . . 30%, . . . 40%, . . . 50%, . . . 60%, 70%, . . . 80%, . . . 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) more resistance to denaturation in the presence of a denaturation condition or denaturing agent than an unmodified polypeptide. In some instances, a modified polypeptide exhibits 105%, 110%, 120%, 130%, 140%), 150%, 200%, 300%), 400%, 500%, or more increased resistance to denaturation compared to an unmodified polypeptide.

As used herein, stability of a modified PH20 hyaluronidase means that it exhibits resistance to denaturation caused by a denaturation condition or denaturing agent. A modified PH20 polypeptide exhibits stability if it retains some activity in the presence of a denaturation condition or denaturing agent, such as at least 20%, 30%), 40%), 50%, 60%, 70%, 80%, 90% or more of the original or initial hyaluronidase activity prior to exposure to the denaturing condition(s). Generally, a modified PH20 hyaluronidase is stable if it retains at least 50% or more of the hyaluronidase activity under a denaturation condition compared to the absence of the denaturation condition. Assays to assess hyaluronidase activity are known to one of skill in the art and described herein. It is understood that the stability of the enzyme need not be permanent or long term, but is manifested for a duration of time in which activity is desired. For example, a modified PH20 hyaluronidase is stable if it exhibits an activity for at least 2 hours, 3 hours, 4 hours, 6 hours, 12 hours, 24 hours, one day, two days, three days, four days, five days, six days, one week, one month, six months or one year upon exposure, or during exposure, to one or more denaturing condition(s) or agent(s) (e.g., presence of a denaturing excipient such as a preservative). For example, a modified PH20 hyaluronidase is stable if it exhibits an activity upon or during exposure to one or more denaturing condition(s) or agent(s) (e.g., presence of a denaturing excipient such as a preservative) for at least 1 month at temperatures from or from about 2° C. to 8° C., inclusive or for at least 3 days at a temperature from or from about 30° C. to 42° C., inclusive.

Hence, "stable" or "stability," with reference to a formulation or a co-formulation provided herein, refers to one in which a modified hyaluronan-degrading enzyme, such as a modified PH20 hyaluronidase, therein is stable upon exposure to one or more denaturing condition(s) or agent(s) therein (e.g., presence of a denaturing excipient such as a preservative) for at least 1 month at temperatures from or from about 2° C. to 8° C., inclusive or for at least 3 days at a temperature from or from about 30° C. to 42° C., inclusive.

As used herein, "increased stability" with reference to a modified PH20 hyaluronidase means that, in the presence of the same denaturing or denaturation condition(s) (e.g., presence of a denaturing excipient such as a preservative), the modified PH20 hyaluronidase exhibits greater hyaluronidase activity compared to an unmodified PH20 hyaluronidase not containing the amino acid replacements). For example, a modified PH20 hyaluronidase exhibits increased stability if it exhibits at least or about at least 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 250%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, 1000% or more of the activity of the unmodified or reference PH20 hyaluronidase in the presence of a denaturing or denaturation condition(s) (e.g., in the presence of a denaturing excipient such as a preservative).

As used herein, "elevated temperatures" refers to temperatures that are greater than room temperature or ambient temperature. Generally, an elevated temperature is a temperature that is at least, greater than, or about 30° C., such as 30° C. to 42° C., and generally 32° C. to 37° C. or 35° C. to 37° C., inclusive.

As used herein, room temperature refers to a range generally from about or at to 18° C. to about or at 32° C. Those of skill in the art appreciate that room temperature varies by location and prevailing conditions. For example, room temperatures can be higher in warmer climates such as Italy or Texas.

As used herein, recitation that proteins are "compared under the same conditions" means that different proteins are treated identically or substantially identically such that any one or more conditions that can influence the activity or properties of a protein or agent are not varied or not substantially varied between the test agents. For example, when the hyaluronidase activity of a modified PH20 polypeptide is compared to an unmodified PH20 polypeptide any one or more conditions such as the amount or concentration of the polypeptide; presence, including amount, of excipients, carriers or other components in a formulation other than the active agent (e.g., modified PH20 hyaluronidase); temperature; time of storage; storage vessel; properties of storage (e.g., agitation) and/or other conditions associated with exposure or use are identical or substantially identical between and among the compared polypeptides.

As used herein, "predetermined time" refers to a time that is established or decided in advance. For example, the predetermined time can be a time chosen in advance that is associated with the desired duration of activity of a hyaluronan-degrading enzyme depending on the desired application or use of the protein. A predetermined time can be hours, days, months or years. For example, a predetermined time can be at least about or about 2 hours, 3 hours, 4 hours, five hours, six hours, 12 hours, 24 hours, 2 days, three days, four days, five days, six days, one week, two weeks, three weeks, one month, six months, one year or more.

As used herein, "storage" means that a formulation is not immediately administered to a subject once prepared, but is kept for a period of time under particular conditions (e.g., particular temperature; time, and/or form (e.g., liquid or lyophilized form)) prior to use. For example, a liquid formulation can be kept for days, weeks, months or years, prior to administration to a subject under varied temperatures such as refrigerated (0° C. to 10° C., such as 2° to 8° C.), room temperature (e.g., temperature up to 32° C., such as 18° C. to about or at 32° C.), or elevated temperature (e.g., 30° C. to 42° C., such as 32° C. to 37° C. or 35° C. to 37° C.).

As used herein, an "excipient" refers to a compound in a formulation of an active agent that does not provide the biological effect of the active agent when administered in the absence of the active agent. Exemplary excipients include, but are not limited to, salts, buffers, stabilizers, tonicity modifiers, metals, polymers, surfactants, preservatives, amino acids and sugars.

As used herein, a stabilizing agent refers to compound added to the formulation to protect the modified PH20 polypeptide or other active agent from degradation, if necessary, such as due to denaturation conditions to which a formulation herein is exposed when handled, stored or used. Thus, included are agents that prevent proteins from degradation from other components in the compositions. Exemplary of such agents are amino acids, amino acid derivatives, amines, sugars, polyols, salts and buffers, surfactants, inhibitors or substrates and other agents as described herein.

As used herein, an antimicrobial effectiveness test or preservative effectiveness test (PET) demonstrates the effectiveness of the preservative system in a product. A product is inoculated with a controlled quantity of specific organisms. The test then compares the level of microorganisms found on a control sample versus the test sample over a period of 28 days. Generally, target markets have differing PET requirements. For example, the PET requirements of the United States Pharmacopoeia (USP) and the European Pharmacopoeia (EP) differ. Parameters for performing an antimicrobial effectiveness test, including in different markets, are known to one of skill in the art as described herein.

As used herein, an anti-microbially or anti-microbial effective amount of a preservative refers to an amount of the preservative that kills or inhibits the propagation of microbial organisms in a sample that may be introduced from storage or use. For example, for multiple-dose containers, an anti-microbially effective amount of a preservative inhibits the growth of microorganisms that may be introduced from repeatedly withdrawing individual doses. USP and EP (EPA and EPB) have anti-microbial requirements that determine preservative effectiveness, and that vary in stringency. For example, an anti-microbial effective amount of a preservative is an amount such that at least a 1.0 $\log_{10}$ unit reduction in bacterial organisms occurs at 7 days following inoculation in an antimicrobial preservative effectiveness test (APET). In a particular example, an anti-microbial effective amount of a preservative is an amount such that at least a 1.0 $\log_{10}$ unit reduction in bacterial organisms occurs at 7 days following inoculation, at least a 3.0 $\log_{10}$ unit reduction of bacterial organisms occurs at 14 days following inoculation, at least no further increase in bacterial organisms occurs after 28 days following inoculation, and at least no increase in fungal organisms occurs after 7 days following inoculation. In a further example, an anti-microbial effective amount of a preservative is an amount such that at least a 1.0 $\log_{10}$ unit reduction of bacterial organisms occurs at 24 hours following inoculation, at least a 3.0 $\log_{10}$ unit reduction of bacterial organisms occurs at 7 days following inoculation, no further increase in bacterial organisms occurs after 28 days following inoculation, at least a 1.0 $\log_{10}$ unit reduction of fungal organisms occurs at 14 days following inoculation, and at least no further increase in fungal organisms occurs after 28 days following inoculation. In an additional example, an anti-microbial effective amount of a preservative is an amount such that at least a 2.0 $\log_{10}$ unit reduction of bacterial organisms occurs at 6 hours following inoculation, at least a 3.0 $\log_{10}$ unit reduction of bacterial organisms occurs at 24 hours following inoculation, no recovery of bacterial organisms occurs after 28 days following inoculation of the composition with the microbial inoculum, at least a 2.0 $\log_{10}$ unit reduction of fungal organisms occurs at 7 days following inoculation, and at least no further increase in fungal organisms occurs after 28 days following inoculation.

As used herein, "preservative" refers to a naturally occurring or synthetically or recombinantly produced substance that, when added to a molecule or protein composition, prevents microbial growth, including bacterial or fungal growth, in the composition.

As used herein, a "phenolic preservative" refers to a preservative that contains one hydroxyl group attached to an aromatic carbon ring, such as a benzene ring. Exemplary phenolic preservatives, include but are not limited to, phenol, m-cresol, p-hydroxybenzoic acid, methylparaben, ethylparaben, and propylparaben. For example, cresols, including meta-cresol (m-cresol), has a methyl group substituted onto the benzene ring of a phenol molecule.

As used herein, a "phenophile" refers to a protein, such as a modified PH20 polypeptide, that exhibits stability in the presence of an anti-microbially effective amount of a preservative(s). The term "phenolphile" can be used interchangeably herein with "phenophile" and has the same meaning. For example, a modified PH20 polypeptide that is a phenophile or phenolphile typically exhibits increased stability compared to an unmodified PH20 hyaluronidase not containing the amino acid replacements) when tested under the same denaturing condition(s) containing a phenolic preservative(s). For example, a modified PH20 hyaluronidase exhibits at least or about at least 110%, 120%, 130%, 140%, 150%, 160%, 170%), 180%, 190%), 200%, 250%, 300%, 400%, 500%, 600%), 700%, 800%, 900%, 1000% or more of the activity of the unmodified or reference PH20 hyaluronidase in the presence of a phenolic preservative(s).

As used herein, a "thermophile" refers to a protein, such as a modified PH20 polypeptide, that exhibits stability under elevated temperatures greater than or about 30° C., such as 30° C. to 42° C., and generally 32° C. to 37° C. or 35° C. to 37° C. For example, a modified PH20 polypeptide that is a thermophile typically exhibits increased stability compared to an unmodified PH20 hyaluronidase not containing the amino acid replacement(s) when tested under the same elevated temperature denaturing condition(s). For example, a modified PH20 hyaluronidase exhibits at least or about at least 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 250%, 300%, 400%, 500%, 600%, 700%, 800%, 900%), 1000% or more of the activity of the unmodified or reference PH20 hyaluronidase under elevated temperatures.

As used herein, the term "detergent" is used interchangeably with the term "surfactant" or "surface acting agent." Surfactants are typically organic compounds that are amphiphilic, i.e., containing both hydrophobic groups ("tails") and hydrophilic groups ("heads"), which render surfactants soluble in both organic solvents and water. A surfactant can be classified by the presence of formally charged groups in its head. A non-ionic surfactant has no charge groups in its head, whereas an ionic surfactant carries a net charge in its head. A zwitterionic surfactant contains a head with two oppositely charged groups. Some examples of common surfactants include: Anionic (based on sulfate, sulfonate or carboxylate anions): perfluorooctanoate (PFOA or PFO), perfluorooctane sulfonate (PFOS), sodium dodecyl sulfate (SDS), ammonium lauryl sulfate, and other alkyl sulfate salts, sodium laureth sulfate (also known as sodium lauryl ether sulfate, or SLES), alkyl benzene sulfonate; cationic (based on quaternary ammonium cations): cetyl trimethylammonium bromide (CTAB) a.k.a. hexadecyl trimethyl ammonium bromide, and other alkyltrimethylammonium salts, cetylpyridinium chloride (CPC), polyethoxylated tallow amine (POEA), benzalkonium chloride (BAC), benzethonium chloride (BZT); Zwitterionic (amphoteric): dodecyl betaine; cocamidopropyl betaine; coco ampho glycinate;

nonionic: alkyl poly(ethylene oxide), alkylphenol poly(ethylene oxide), copolymers of poly(ethylene oxide) and poly (propylene oxide) (commercially known as Poloxamers or Poloxamines), alkyl polyglucosides, including octyl glucoside, decyl maltoside, fatty alcohols (e.g., cetyl alcohol and oleyl alcohol), cocamide MEA, cocamide DEA, polysorbates (Tween 20, Tween 80, etc.), Triton detergents, and dodecyl dimethylamine oxide.

As used herein, a "buffer" refers to a substance, generally a solution, that can keep its pH constant, despite the addition of strong acids or strong bases and external influences of temperature, pressure, volume or redox potential. A buffer prevents change in the concentration of another chemical substance, e.g., proton donor and acceptor systems that prevent marked changes in hydrogen ion concentration (pH). The pH values of all buffers are temperature and concentration dependent. The choice of buffer to maintain a pH value or range can be empirically determined by one of skill in the art based on the known buffering capacity of known buffers. Exemplary buffers include but are not limited to, bicarbonate buffer, cacodylate buffer, phosphate buffer or Tris buffer. For example, Tris buffer (tromethamine) is an amine based buffer that has a pKa of 8.06 and has an effective pH range between 7.9 and 9.2. For Tris buffers, pH increases about 0.03 unit per ° C. temperature decrease, and decreases 0.03 to 0.05 unit per ten-fold dilution.

As used herein, the residues of naturally occurring α-amino acids are the residues of those 20 α-amino acids found in nature which are incorporated into protein by the specific recognition of the charged tRNA molecule with its cognate mRNA codon in humans.

As used herein, nucleic acids include DNA, RNA and analogs thereof, including peptide nucleic acids (PNA) and mixtures thereof. Nucleic acids can be single or double-stranded. When referring to probes or primers, which are optionally labeled, such as with a detectable label, such as a fluorescent or radiolabel, single-stranded molecules are contemplated. Such molecules are typically of a length such that their target is statistically unique or of low copy number (typically less than 5, generally less than 3) for probing or priming a library. Generally a probe or primer contains at least 14, 16 or 30 contiguous nucleotides of sequence complementary to or identical to a gene of interest. Probes and primers can be 10, 20, 30, 50, 100 or more nucleic acids long.

As used herein, a peptide refers to a polypeptide that is from 2 to 40 amino acids in length.

As used herein, the amino acids which occur in the various sequences of amino acids provided herein are identified according to their known, three-letter or one-letter abbreviations (Table 1). The nucleotides which occur in the various nucleic acid fragments are designated with the standard single-letter designations used routinely in the art.

As used herein, an "amino acid" is an organic compound containing an amino group and a carboxylic acid group. A polypeptide contains two or more amino acids. For purposes herein, amino acids include the twenty naturally-occurring amino acids, non-natural amino acids and amino acid analogs (i.e., amino acids wherein the α-carbon has a side chain).

As used herein, "amino acid residue" refers to an amino acid formed upon chemical digestion (hydrolysis) of a polypeptide at its peptide linkages. The amino acid residues described herein are presumed to be in the "L" isomeric form. Residues in the "D" isomeric form, which are so designated, can be substituted for any L-amino acid residue as long as the desired functional property is retained by the polypeptide. NH_2 refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxyl terminus of a polypeptide. In keeping with standard polypeptide nomenclature described in *J. Biol. Chem.,* 243:3557-3559 (1968), and adopted 37 C.F.R. §§1.821-1.822, abbreviations for amino acid residues are shown in Table 1:

TABLE 1

Table of Correspondence

SYMBOL		
1-Letter	3-Letter	AMINO ACID
Y	Tyr	Tyrosine
G	Gly	Glycine
F	Phe	Phenylalanine
M	Met	Methionine
A	Ala	Alanine
S	Ser	Serine
I	Ile	Isoleucine
L	Leu	Leucine
T	Thr	Threonine
V	Val	Valine
P	Pro	Proline
K	Lys	Lysine
H	His	Histidine
Q	Gln	Glutamine
E	Glu	Glutamic Acid
Z	Glx	Glu and/or Gln
W	Trp	Tryptophan
R	Arg	Arginine
D	Asp	Aspartic Acid
N	Asn	Asparagine
B	Asx	Asn and/or Asp
C	Cys	Cysteine
X	Xaa	Unknown or Other

It should be noted that all amino acid residue sequences represented herein by formulae have a left to right orientation in the conventional direction of amino-terminus to carboxyl-terminus. In addition, the phrase "amino acid residue" is broadly defined to include the amino acids listed in the Table of Correspondence (Table 1) and modified and unusual amino acids, such as those referred to in 37 C.F.R. §§1.821-1.822, and incorporated herein by reference. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino acid residues, to an amino-terminal group such as NH_2 or to a carboxyl-terminal group such as COOH.

As used herein, "naturally occurring amino acids" refer to the 20 L-amino acids that occur in polypeptides.

As used herein, "non-natural amino acid" refers to an organic compound that has a structure similar to a natural amino acid but has been modified structurally to mimic the structure and reactivity of a natural amino acid. Non-naturally occurring amino acids thus include, for example, amino acids or analogs of amino acids other than the 20 naturally-occurring amino acids and include, but are not limited to, the D-stereoisomers of amino acids. Exemplary non-natural amino acids are described herein and are known to those of skill in the art.

As used herein, an isokinetic mixture is one in which the molar ratios of amino acids has been adjusted based on their reported reaction rates (see, e.g., Ostresh et al., (1994) Biopolymers 34:1681).

As used herein, suitable conservative substitutions of amino acids are known to those of skill in this art and can be made generally without altering the biological activity of the resulting molecule. Those of skill in the art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (see, e.g., Watson et al. Molecular Biology of the Gene, 4th Edition, 1987, The Benjamin/Cummings Pub. co., p. 224). Such substitutions can be made in accordance with those set forth in TABLE 2 as follows:

TABLE 2

Original residue	Exemplary conservative substitution
Ala (A)	Gly; Ser
Arg (R)	Lys
Asn (N)	Gln; His
Cys (C)	Ser
Gln (Q)	Asn
Glu (E)	Asp
Gly (G)	Ala; Pro
His (H)	Asn; Gln
Ile (I)	Leu; Val
Leu (L)	Ile; Val
Lys (K)	Arg; Gln; Glu
Met (M)	Leu; Tyr; Ile
Phe (F)	Met; Leu; Tyr
Ser (S)	Thr
Thr (T)	Ser
Trp (W)	Tyr
Tyr (Y)	Trp; Phe
Val (V)	Ile; Leu

Other substitutions also are permissible and can be determined empirically or in accord with known conservative substitutions.

As used herein, a DNA construct is a single or double stranded, linear or circular DNA molecule that contains segments of DNA combined and juxtaposed in a manner not found in nature. DNA constructs exist as a result of human manipulation, and include clones and other copies of manipulated molecules.

As used herein, a DNA segment is a portion of a larger DNA molecule having specified attributes. For example, a DNA segment encoding a specified polypeptide is a portion of a longer DNA molecule, such as a plasmid or plasmid fragment, which, when read from the 5' to 3' direction, encodes the sequence of amino acids of the specified polypeptide.

As used herein, the term polynucleotide means a single- or double-stranded polymer of deoxyribonucleotides or ribonucleotide bases read from the 5' to the 3' end. Polynucleotides include RNA and DNA, and can be isolated from natural sources, synthesized in vitro, or prepared from a combination of natural and synthetic molecules. The length of a polynucleotide molecule is given herein in terms of nucleotides (abbreviated "nt") or base pairs (abbreviated "bp"). The term nucleotides is used for single- and double-stranded molecules where the context permits. When the term is applied to double-stranded molecules it is used to denote overall length and will be understood to be equivalent to the term base pairs. It will be recognized by those skilled in the art that the two strands of a double-stranded polynucleotide can differ slightly in length and that the ends thereof can be staggered; thus all nucleotides within a double-stranded polynucleotide molecule cannot be paired. Such unpaired ends will, in general, not exceed 20 nucleotides in length.

As used herein, "at a position corresponding to" or recitation that nucleotides or amino acid positions "correspond to" nucleotides or amino acid positions in a disclosed sequence, such as set forth in the Sequence listing, refers to nucleotides or amino acid positions identified upon alignment with the disclosed sequence to maximize identity using a standard alignment algorithm, such as the GAP algorithm. For purposes herein, alignment of a PH20 sequence is to the amino acid sequence set forth in any of SEQ ID NOS: 3, 7 or 32-66, and in particular SEQ ID NO:3. Hence, reference herein that a position or amino acid replacement corresponds to positions with reference to SEQ ID NO:3 also means that the position or amino acid replacement corresponds to positions with reference to any of SEQ ID NOS: 7 or 32-66, since the sequences therein are identical to the corresponding residues as set forth in SEQ ID NO:3. By aligning the sequences, one skilled in the art can identify corresponding residues, for example, using conserved and identical amino acid residues as guides. In general, to identify corresponding positions, the sequences of amino acids are aligned so that the highest order match is obtained (see, e.g.: *Computational Molecular Biology*, Lesk, A. M., ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*, Smith, D. W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data, Part I*, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; and *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; Carrillo et al. (1988) *SIAM J Applied Math* 48:1073). FIG. **2** (A-L) exemplifies exemplary alignments and identification of exemplary corresponding residues for replacement.

As used herein, "sequence identity" refers to the number of identical or similar amino acids or nucleotide bases in a comparison between a test and a reference polypeptide or polynucleotide. Sequence identity can be determined by sequence alignment of nucleic acid or protein sequences to identify regions of similarity or identity. For purposes herein, sequence identity is generally determined by alignment to identify identical residues. Alignment can be local or global, but for purposes herein alignment is generally a global alignment where the full-length of each sequence is compared. Matches, mismatches and gaps can be identified between compared sequences. Gaps are null amino acids or nucleotides inserted between the residues of aligned sequences so that identical or similar characters are aligned. Generally, there can be internal and terminal gaps. Sequence identity can be determined by taking into account gaps as the number of identical residues/length of the shortest sequence×100. When using gap penalties, sequence identity can be determined with no penalty for end gaps (e.g., terminal gaps are not penalized). Alternatively, sequence identity can be determined without taking into account gaps as the number of identical positions/length of the total aligned sequence×100.

As used herein, a "global alignment" is an alignment that aligns two sequences from beginning to end, aligning each letter in each sequence only once. An alignment is produced, regardless of whether or not there is similarity or identity between the sequences. For example, 50% sequence identity based on "global alignment" means that in an alignment of the full sequence of two compared sequences each of 100 nucleotides in length, 50% of the residues are the same. It is understood that global alignment also can be used in determining sequence identity even when the length of the aligned sequences is not the same. The differences in the terminal ends of the sequences will be taken into account in determining sequence identity, unless the "no penalty for end gaps" is selected. Generally, a global alignment is used on sequences that share significant similarity over most of their length. Exemplary algorithms for performing global alignment include the Needleman-Wunsch algorithm (Needleman et al. *J. Mol. Biol.* 48:443 (1970). Exemplary programs for performing global alignment are publicly available and include the Global Sequence Alignment Tool available at the National Center for Biotechnology Information (NCBI) website (ncbi.nlm.nih.gov/), and the program available at deepc2.psi.iastate.edu/aat/align/align.html.

As used herein, a "local alignment" is an alignment that aligns two sequence, but only aligns those portions of the sequences that share similarity or identity. Hence, a local alignment determines if sub-segments of one sequence are present in another sequence. If there is no similarity, no alignment will be returned. Local alignment algorithms include BLAST or Smith-Waterman algorithm (*Adv. Appl. Math.* 2:482 (1981)). For example, 50% sequence identity based on "local alignment" means that in an alignment of the full sequence of two compared sequences of any length, a region of similarity or identity of 100 nucleotides in length has 50% of the residues that are the same in the region of similarity or identity.

For purposes herein, sequence identity can be determined by standard alignment algorithm programs used with default gap penalties established by each supplier. Default parameters for the GAP program can include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non identities) and the weighted comparison matrix of Gribskov et al. *Nucl. Acids Res.* 14:6745 (1986), as described by Schwartz and Dayhoff, eds., *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, pp. 353-358 (1979); (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps. Whether any two nucleic acid molecules have nucleotide sequences or any two polypeptides have amino acid sequences that are at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% "identical," or other similar variations reciting a percent identity, can be determined using known computer algorithms based on local or global alignment (see e.g., wikipedia.org/wiki/Sequence_alignment_software, providing links to dozens of known and publicly available alignment databases and programs). Generally, for purposes herein sequence identity is determined using computer algorithms based on global alignment, such as the Needleman-Wunsch Global Sequence Alignment tool available from NCBI/BLAST (blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&Page_TYPE=BlastHome); LAlign (William Pearson implementing the Huang and Miller algorithm (*Adv. Appl. Math.* (1991) 12:337-357)); and program from Xiaoqui Huang available at deepc2.psi.iastate.edu/aat/align/align.html. Generally, when comparing nucleotide sequences herein, an alignment with penalty for end gaps is used. Local alignment also can be used when the sequences being compared are substantially the same length.

Therefore, as used herein, the term "identity" represents a comparison or alignment between a test and a reference polypeptide or polynucleotide. In one non-limiting example, "at least 90% identical to" refers to percent identities from 90 to 100% relative to the reference polypeptide or polynucleotide. Identity at a level of 90% or more is indicative of the fact that, assuming for exemplification purposes a test and reference polypeptide or polynucleotide length of 100 amino acids or nucleotides are compared, no more than 10% (i.e., 10 out of 100) of amino acids or nucleotides in the test polypeptide or polynucleotide differs from that of the reference polypeptides. Similar comparisons can be made between a test and reference polynucleotides. Such differences can be represented as point mutations randomly distributed over the entire length of an amino acid sequence or they can be clustered in one or more locations of varying length up to the maximum allowable, e.g., 10/100 amino acid difference (approximately 90% identity). Differences also can be due to deletions or truncations of amino acid residues. Differences are defined as nucleic acid or amino acid substitutions, insertions or deletions. Depending on the length of the compared sequences, at the level of homologies or identities above about 85-90%, the result can be independent of the program and gap parameters set; such high levels of identity can be assessed readily, often without relying on software.

As used herein, an allelic variant or allelic variation references any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and can result in phenotypic polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or can encode polypeptides having altered amino acid sequence. The term "allelic variant" also is used herein to denote a protein encoded by an allelic variant of a gene. Typically the reference form of the gene encodes a wildtype form and/or predominant form of a polypeptide from a population or single reference member of a species. Typically, allelic variants, which include variants between and among species typically have at least 80%, 90% or greater amino acid identity with a wildtype and/or predominant form from the same species; the degree of identity depends upon the gene and whether comparison is interspecies or intraspecies. Generally, intraspecies allelic variants have at least about 80%, 85%, 90% or 95% identity or greater with a wildtype and/or predominant form, including 96%, 97%, 98%, 99% or greater identity with a wildtype and/or predominant form of a polypeptide. Reference to an allelic variant herein generally refers to variations in proteins among members of the same species.

As used herein, "allele," which is used interchangeably herein with "allelic variant" refers to alternative forms of a gene or portions thereof. Alleles occupy the same locus or position on homologous chromosomes. When a subject has two identical alleles of a gene, the subject is said to be homozygous for that gene or allele. When a subject has two different alleles of a gene, the subject is said to be heterozygous for the gene. Alleles of a specific gene can differ from each other in a single nucleotide or several nucleotides, and can include modifications such as substitutions, deletions and insertions of nucleotides. An allele of a gene also can be a form of a gene containing a mutation.

As used herein, species variants refer to variants in polypeptides among different species, including different mammalian species, such as mouse and human. Exemplary of species variants provided herein are primate PH20, such as, but not limited to, human, chimpanzee, macaque, cynomolgus monkey, gibbon, orangutan, or marmoset. Generally, species variants have 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% sequence identity. Corresponding residues between and among species variants can be determined by comparing and aligning sequences to maximize the number of matching nucleotides or residues, for example, such that identity between the sequences is equal to or greater than 95%, equal to or greater than 96%, equal to or greater than 97%, equal to or greater than 98% or equal to greater than 99%. The position of interest is then given the number assigned in the reference nucleic acid molecule. Alignment can be effected manually or by eye, particularly where sequence identity is greater than 80%.

As used herein, substantially pure means sufficiently homogeneous to appear free of readily detectable impurities, as determined by standard methods of analysis, such as thin layer chromatography (TLC), gel electrophoresis and high performance liquid chromatography (HPLC), used by those of skill in the art to assess such purity, or sufficiently pure such that further purification would not detectably alter the physical and chemical properties, such as enzymatic and biological activities, of the substance. Methods for purification of the compounds to produce substantially chemically pure compounds are known to those of skill in the art. A substantially chemically pure compound can, however, be a mixture of stereoisomers or isomers. In such instances, further purification might increase the specific activity of the compound.

As used herein, isolated or purified polypeptide or protein or biologically-active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or tissue from which the protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. Preparations can be determined to be substantially free if they appear free of readily detectable impurities as determined by standard methods of analysis, such as thin layer chromatography (TLC), gel electrophoresis and high performance liquid chromatography (HPLC), used by those of skill in the art to assess such purity, or sufficiently pure such that further purification would not detectably alter the physical and chemical properties, such as enzymatic and biological activities, of the substance. Methods for purification of the compounds to produce substantially chemically pure compounds are known to those of skill in the art. A substantially chemically pure compound, however, can be a mixture of stereoisomers. In such instances, further purification might increase the specific activity of the compound.

Hence, reference to a substantially purified polypeptide, such as a substantially purified PH20 polypeptide refers to preparations of PH20 proteins that are substantially free of cellular material, includes preparations of proteins in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly-produced. In one embodiment, the term substantially free of cellular material includes preparations of enzyme proteins having less than about 30% (by dry weight) of non-enzyme proteins (also referred to herein as contaminating proteins), generally less than about 20% of non-enzyme proteins or 10% of non-enzyme proteins or less than about 5% of non-enzyme proteins. When the enzyme protein is recombinantly produced, it also is substantially free of culture medium, i.e., culture medium represents less than about or at 20%, 10% or 5% of the volume of the enzyme protein preparation.

As used herein, the term substantially free of chemical precursors or other chemicals includes preparations of enzyme proteins in which the protein is separated from chemical precursors or other chemicals that are involved in the synthesis of the protein. The term includes preparations of enzyme proteins having less than about 30% (by dry weight), 20%, 10%, 5% or less of chemical precursors or non-enzyme chemicals or components.

As used herein, synthetic, with reference to, for example, a synthetic nucleic acid molecule or a synthetic gene or a synthetic peptide refers to a nucleic acid molecule or polypeptide molecule that is produced by recombinant methods and/or by chemical synthesis methods.

As used herein, production by recombinant means or using recombinant DNA methods means the use of the well known methods of molecular biology for expressing proteins encoded by cloned DNA.

As used herein, vector (or plasmid) refers to discrete elements that are used to introduce a heterologous nucleic acid into cells for either expression or replication thereof. The vectors typically remain episomal, but can be designed to effect integration of a gene or portion thereof into a chromosome of the genome. Also contemplated are vectors that are artificial chromosomes, such as yeast artificial chromosomes and mammalian artificial chromosomes. Selection and use of such vehicles are well known to those of skill in the art.

As used herein, an expression vector includes vectors capable of expressing DNA that is operatively linked with regulatory sequences, such as promoter regions, that are capable of effecting expression of such DNA fragments. Such additional segments can include promoter and terminator sequences, and optionally can include one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, and the like. Expression vectors are generally derived from plasmid or viral DNA, or can contain elements of both. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome.

As used herein, vector also includes “virus vectors” or “viral vectors.” Viral vectors are engineered viruses that are operatively linked to exogenous genes to transfer (as vehicles or shuttles) the exogenous genes into cells. Viral vectors include, but are not limited to, adenoviral vectors, retroviral vectors and vaccinia virus vectors.

As used herein, “operably” or “operatively linked” when referring to DNA segments means that the segments are arranged so that they function in concert for their intended purposes, e.g., transcription initiates downstream of the promoter and upstream of any transcribed sequences. The promoter is usually the domain to which the transcriptional machinery binds to initiate transcription and proceeds through the coding segment to the terminator.

As used herein, a conjugate refers to a modified PH20 polypeptide linked directly or indirectly to one or more other polypeptides or chemical moieties. Such conjugates include fusion proteins, those produced by chemical conjugates and those produced by any other method whereby at least one modified PH20 polypeptide is linked, directly or indirectly to another polypeptide or chemical moiety so long as the conjugate retains hyaluronidase activity. Exemplary of conjugates provided herein include PH20 polypeptides linked directly or indirectly to a multimerization domain (e.g. an Fc moiety), a toxin, a label or a drug.

As used herein, a fusion protein refers to a polypeptide encoded by a nucleic acid sequence containing a coding sequence from one nucleic acid molecule and the coding sequence from another nucleic acid molecule in which the coding sequences are in the same reading frame such that when the fusion construct is transcribed and translated in a host cell, the protein is produced containing the two proteins. The two molecules can be adjacent in the construct or separated by a linker polypeptide that contains, 1, 2, 3, or more, but typically fewer than 10, 9, 8, 7, or 6 amino acids.

The protein product encoded by a fusion construct is referred to as a fusion polypeptide. Examples of fusion polypeptides include Fc fusions.

As used herein, a polymer that is conjugated to a modified PH20 polypeptide refers to any polymer that is covalently or otherwise stably linked, directly or via a linker, to such polypeptide. Such polymers, typically increase serum half-life, and include, but are not limited to, sialic moieties, polyethylene glycol (PEG) moieties, dextran, and sugar and other moieties, such as for glycosylation.

As used herein, the term assessing or determining is intended to include quantitative and qualitative determination in the sense of obtaining an absolute value for the activity of a product, and also of obtaining an index, ratio, percentage, visual or other value indicative of the level of the activity. Assessment can be direct or indirect.

As used herein, a "composition" refers to any mixture of two or more products or compounds. It can be a solution, a suspension, liquid, powder, a paste, aqueous, non-aqueous, or any combination thereof.

As used herein, a formulation refers to a composition containing at least one active pharmaceutical or therapeutic agent and one or more excipients.

As used herein, a co-formulation refers to a composition containing two or more active or pharmaceutical or therapeutic agents and one or more excipients. For example, a co-formulation of a fast-acting insulin and a hyaluronan degrading enzyme contains a fast-acting insulin, a hyaluronan degrading enzyme, and one or more excipients.

As used herein, "a combination" refers to any association between two or among more items or elements. Exemplary combinations include, but are not limited to, two or more pharmaceutical compositions, a composition containing two or more active ingredients, such as two modified PH20 polypeptides; a modified PH20 polypeptide and an anticancer agent, such as a chemotherapeutic compound; a modified PH20 polypeptide and a therapeutic agent (e.g. an insulin); a modified PH20 polypeptide and a plurality therapeutic and/or imaging agents, or any association thereof. Such combinations can be packaged as kits.

As used herein, a kit is a packaged combination, optionally, including instructions for use of the combination and/or other reactions and components for such use.

As used herein, "disease or disorder" refers to a pathological condition in an organism resulting from cause or condition including, but not limited to, infections, acquired conditions, genetic conditions, and characterized by identifiable symptoms.

As used herein, a hyaluronan-associated disease, disorder or condition refers to any disease or condition in which hyaluronan levels are elevated as cause, consequence or otherwise observed in the disease or condition. Hyaluronan-associated diseases and conditions are associated with elevated hyaluronan expression in a tissue or cell, increased interstitial fluid pressure, decreased vascular volume, and/or increased water content in a tissue. Hyaluronan-associated diseases, disorders or conditions can be treated by administration of a composition containing a hyaluronan degrading enzyme, such as a hyaluronidase, for example, a soluble hyaluronidase, either alone or in combination with or in addition to another treatment and/or agent. Exemplary diseases and conditions, include, but are not limited to, hyaluronan-rich cancers, for example, tumors, including solid tumors such as late-stage cancers, metastatic cancers, undifferentiated cancers, ovarian cancer, in situ carcinoma (ISC), squamous cell carcinoma (SCC), prostate cancer, pancreatic cancer, non-small cell lung cancer, breast cancer, colon cancer and other cancers. Exemplary hyaluronan-associated diseases and conditions also are diseases that are associated with elevated interstitial fluid pressure, such as diseases associated with disc pressure, and edema, for example, edema caused by organ transplant, stroke, brain trauma or other injury. Exemplary hyaluronan-associated diseases and conditions include diseases and conditions associated with elevated interstitial fluid pressure, decreased vascular volume, and/or increased water content in a tissue, including cancers, disc pressure and edema. In one example, treatment of the hyaluronan-associated condition, disease or disorder includes amelioration, reduction, or other beneficial effect on one or more of increased interstitial fluid pressure (IFP), decreased vascular volume, and increased water content in a tissue.

As used herein, "treating" a subject with a disease or condition means that the subject's symptoms are partially or totally alleviated, or remain static following treatment. Hence treatment encompasses prophylaxis, therapy and/or cure. Prophylaxis refers to prevention of a potential disease and/or a prevention of worsening of symptoms or progression of a disease. Treatment also encompasses any pharmaceutical use of a modified interferon and compositions provided herein.

As used herein, a pharmaceutically effective agent or therapeutic agent includes any bioactive agent that can exhibit a therapeutic effect to treat a disease or disorder. Exemplary therapeutic agents are described herein. Therapeutic agents include, but are not limited to, anesthetics, vasoconstrictors, dispersing agents, conventional therapeutic drugs, including small molecule drugs, including, but not limited to, bisphosphonates, and therapeutic proteins, including, but not limited to, insulin, IgG molecules, antibodies, cytokines and coagulation factors.

As used herein, "insulin" refers to a hormone, precursor or a synthetic or recombinant analog thereof that acts to increase glucose uptake and storage and/or decrease endogenous glucose production. Insulin and analogs thereof are well known to one of skill in the art, including in human and allelic and species variants thereof. Insulin is translated as a precursor polypeptide designated preproinsulin (110 amino acid for human insulin), containing a signal peptide that directs the protein to the endoplasmic reticulum (ER) wherein the signal sequence is cleaved, resulting in proinsulin. Proinsulin is processed further to release a C- or connecting chain peptide (a 31 amino acid C-chain in human insulin). The resulting insulin contains an A-chain (21 amino acid in length in human insulin; set forth in SEQ ID NO:862) and a B-chain (30 amino acid in length in human insulin; set forth in SEQ ID NO:863) which are cross-linked by disulfide bonds. A fully cross-linked human insulin contains three disulfide bridges: one between position 7 of the A-chain and position 7 of the B-chain, a second between position 20 of the A-chain and position 19 of the B-chain, and a third between positions 6 and 11 of the A-chain. Reference to an insulin includes monomeric and multimeric insulins, including hexameric insulins, as well as humanized insulins. Exemplary insulin polypeptides are those of mammalian, including human, origin. Reference to insulin includes preproinsulin, proinsulin and insulin polypeptides in single-chain or two-chain forms, truncated forms thereof that have activity, and includes allelic variants and species variants of human insulin, variants encoded by splice variants, and other variants, such as insulin analogs. An exemplary insulin is human insulin having a sequence of amino acids of the A- and B-chains of human insulin are set forth in SEQ ID NOS: 862 and 863, respectively, and variants or analogs thereof that exhibit at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity thereto to one or both of the A-chain or B-chain and that acts to increase glucose uptake and storage and/or decrease endogenous glucose production. A further exemplary insulin is porcine insulin having a sequence of amino acids for the preproinsulin as set forth in SEQ ID NO:864, whereby the A chain corresponds to amino acid residue positions 88-108 and the B-chain correspond to amino acid, and variants or analogs thereof that exhibit at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity thereto to one or both of the A-chain or B-chain and that acts to increase glucose uptake and storage and/or decrease endogenous glucose production.

As used herein, "fast-acting insulin" refers to any insulin that exhibits peak insulin levels at or about not more than four hours following subcutaneous administration to a subject. Fast-acting insulins include any insulin or any fast-acting insulin composition for acute administration to a diabetic subject in response to an actual, perceived, or anticipated hyperglycemic condition in the subject arising at the time of, or within about four hours following, administration of the fast-acting insulin (such as a prandial hyperglycemic condition resulting or anticipated to result from, consumption of a meal), whereby the fast-acting insulin is able to prevent, control or ameliorate the acute hyperglycemic condition. Fast-acting insulins include recombinant insulins and isolated insulins (also referred to as "regular" insulins) such as the insulin sold as human insulin, porcine insulins and bovine insulins, as well as rapid acting insulin analogs (also termed fast-acting insulin analogs herein) designed to be rapid acting by virtue of amino acid changes. Exemplary regular insulin preparations include, but are not limited to, human regular insulins, such as those sold under the trademarks Humulin® R, Novolin® R and Velosulin®, Insulin Human, USP and Insulin Human Injection, USP, as well as acid formulations of insulin, such as, for example, Toronto Insulin, Old Insulin, and Clear Insulin, and regular pig insulins, such as Iletin II®(porcine insulin). Regular insulins typically have an onset of action of between 30 minutes to an hour, and a peak insulin level of 2-5 hours post administration.

As used herein, rapid acting insulin analogs (also called fast-acting insulin analogs) are insulins that have a rapid onset of action. Rapid insulins typically are insulin analogs that have been engineered, such as by the introduction of one or more amino acid substitutions, to be more rapid acting than regular insulins. Rapid acting insulin analogs typically have an onset of action of 10-30 minutes post injection, with peak insulin levels observed 30-90 minutes post injection. Exemplary rapid acting insulin analogs are analogs of human insulin containing one or more amino acid changes in the A-chain and/or B-chain of human insulin set forth in SEQ ID NO:862 or 863, respectively, and that exhibit an onset of action 10-30 minutes post injection with peak insulin levels observed 30-90 minutes post injection. Exemplary rapid acting insulin analogs include, but are not limited to, for example, insulin lispro (e.g., Humalog® insulin), insulin aspart (e.g., NovoLog® insulin), and insulin glulisine (e.g., Apidra® insulin) the fast-acting insulin composition sold as VIAject® and VIAtab®(see, e.g., U.S. Pat. No. 7,279,457). The amino acid sequence of exemplary rapid acting insulin analogs have an A chain with a sequence of amino acids set forth in SEQ ID NO:862 and a B chain having a sequence of amino acids set forth in any of SEQ ID NOS:865-867. Also included are any other insulins that have an onset of action of 30 minutes or less and a peak level before 90 minutes, typically 30-90 minutes, post injection.

As used herein, a human insulin refers to an insulin that is synthetic or recombinantly produced based upon the human polypeptide, including allelic variants and analogs thereof.

As used herein, fast-acting human insulins or human fast-acting insulin compositions include any human insulin or composition of a human insulin that is fast-acting, but excludes non-human insulins, such as regular pig insulin.

As used herein, the terms "basal-acting insulins," or "basal insulins" refer to insulins administered to maintain a basal insulin level as part of an overall treatment regimen for treating a chronic condition such diabetes. Typically, a basal-acting insulin is formulated to maintain an approximately steady state insulin level by the controlled release of insulin when administered periodically (e.g., once or twice daily). Basal-acting insulins include crystalline insulins (e.g., NPH and Lente®, protamine insulin, surfen insulin), basal insulin analogs (insulin glargine, HOE 901, NovoSol Basal) and other chemical formulations of insulin (e.g., gum arabic, lecithin or oil suspensions) that retard the absorption rate of regular insulin. As used herein, the basal-acting insulins can include insulins that are typically understood as long-acting (typically reaching a relatively low peak concentration, while having a maximum duration of action over about 20-30 hours) or intermediate-acting (typically causing peak insulin concentrations at about 4-12 hours after administration).

As used herein, treatment means any manner in which the symptoms of a condition, disorder or disease or other indication, are ameliorated or otherwise beneficially altered.

As used herein, therapeutic effect means an effect resulting from treatment of a subject that alters, typically improves or ameliorates the symptoms of a disease or condition or that cures a disease or condition. A therapeutically effective amount refers to the amount of a composition, molecule or compound which results in a therapeutic effect following administration to a subject.

As used herein, the term "subject" refers to an animal, including a mammal, such as a human being.

As used herein, a patient refers to a human subject exhibiting symptoms of a disease or disorder.

As used herein, amelioration of the symptoms of a particular disease or disorder by a treatment, such as by administration of a pharmaceutical composition or other therapeutic, refers to any lessening, whether permanent or temporary, lasting or transient, of the symptoms that can be attributed to or associated with administration of the composition or therapeutic.

As used herein, prevention or prophylaxis refers to methods in which the risk of developing a disease or condition is reduced.

As used herein, a "therapeutically effective amount" or a "therapeutically effective dose" refers to the quantity of an agent, compound, material, or composition containing a compound that is at least sufficient to produce a therapeutic effect. Hence, it is the quantity necessary for preventing, curing, ameliorating, arresting or partially arresting a symptom of a disease or disorder.

As used herein, unit dose form refers to physically discrete units suitable for human and animal subjects and packaged individually as is known in the art.

As used herein, a single dosage formulation refers to a formulation containing a single dose of therapeutic agent for direct administration. Single dosage formulations generally do not contain any preservatives.

As used herein, a multi-dose formulation refers to a formulation that contains multiple doses of a therapeutic agent and that can be directly administered to provide several single doses of the therapeutic agent. The doses can be administered over the course of minutes, hours, weeks, days or months. Multidose formulations can allow dose adjustment, dose-pooling and/or dose-splitting. Because multi-dose formulations are used over time, they generally contain one or more preservatives to prevent microbial growth.

As used herein, an "article of manufacture" is a product that is made and sold. As used throughout this application, the term is intended to encompass a therapeutic agent with a soluble PH20, such as esPH20, or an esPH20 alone, contained in the same or separate articles of packaging.

As used herein, fluid refers to any composition that can flow. Fluids thus encompass compositions that are in the form of semi-solids, pastes, solutions, aqueous mixtures, gels, lotions, creams and other such compositions.

As used herein, a "control" or "standard" refers to a sample that is substantially identical to the test sample, except that it is not treated with a test parameter, or, if it is a plasma sample, it can be from a normal volunteer not affected with the condition of interest. A control also can be an internal control. For example, a control can be a sample, such as a virus, that has a known property or activity.

As used herein, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "an" agent includes one or more agents.

As used herein, the term "or" is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive.

As used herein, ranges and amounts can be expressed as "about" a particular value or range. About also includes the exact amount. Hence "about 5 bases" means "about 5 bases" and also "5 bases."

As used herein, "optional" or "optionally" means that the subsequently described event or circumstance does or does not occur, and that the description includes instances where said event or circumstance occurs and instances where it does not. For example, an optionally substituted group means that the group is unsubstituted or is substituted.

As used herein, the abbreviations for any protective groups, amino acids and other compounds, are, unless indicated otherwise, in accord with their common usage, recognized abbreviations, or the IUPAC-IUB Commission on Biochemical Nomenclature (see, (1972) *Biochem.* 11:1726).

For clarity of disclosure, and not by way of limitation, the detailed description is divided into the subsections that follow.

B. PH20 Hyaluronidase

Provided herein are modified PH20 polypeptides. PH20 (also known as sperm surface protein, sperm adhesion molecule 1 or SPAM1) is a hyaluronidase that hydrolyzes hyaluronan (also called hyaluronic acid, hyaluronate or HA) found in connective tissues such as the extracellular matrix. Hyaluronan polymers are composed of repeating disaccharide units, D-glucuronic acid (GlcA) and N-acetyl-D-glucosamine (GlcNAc), linked together via alternating β-1→4 and β-1→3 glycosidic bonds. Hyaluronan chains can reach about 25,000 disaccharide repeats or more in length, and polymers of hyaluronan can range in size from about 5,000 to 20,000,000 Da in vivo. Hyaluronan, also called hyaluronic acid or hyaluronate, is a non-sulfated glycosaminoglycan that is widely distributed throughout connective, epithelial, and neural tissues. Hyaluronan is an essential component of the extracellular matrix and a major constituent of the interstitial barrier. PH20 is an endo-β-N-acetyl-hexosaminidase that hydrolyzes the β1→4 glycosidic bond of hyaluronic acid into various oligosaccharide lengths such as tetrasaccharides and hexasaccharides. PH20 has both hydrolytic and transglycosidase activities. In addition to degrading hyaluronic acid, PH20 also can degrade chondroitin sulfates, such as C4-S and C6-S. PH20 can exhibit hyaluronidase activity at acidic pH and neutral pH.

1. Structure

PH20 cDNA has been cloned from numerous mammalian species. Exemplary PH20 precursor polypeptides include, but are not limited to, human (SEQ ID NO:6), bovine (SEQ ID NOS:15 or 17), rabbit (SEQ ID NO:23), Cynomolgus monkey (SEQ ID NO:13), guinea pig (SEQ ID NO:28), rat (SEQ ID NO:21), mouse (SEQ ID NO:19), chimpanzee (SEQ ID NO:8, SEQ ID NO:9 or SEQ ID NO:869) Rhesus monkey (SEQ ID NO:11), Fox (SEQ ID NO:30), Gibbon (SEQ ID NO:856), Marmoset (SEQ ID NO:858) or orangutan (SEQ ID NO:860) PH20 polypeptides. The mRNA transcript is typically translated to generate a precursor protein containing a 35 amino acid signal sequence at the N-terminus. Following transport to the ER, the signal peptide is removed to yield a mature PH20 polypeptide. Exemplary mature PH20 polypeptides include, but are not limited to, human (SEQ ID NO:7), bovine (SEQ ID NOS:16 or 18), rabbit (SEQ ID NO:24), Cynomolgus monkey (SEQ ID NO:14), guinea pig (SEQ ID NO:29), rat (SEQ ID NO:22), mouse (SEQ ID NO:20), chimpanzee (SEQ ID NO:10 or SEQ ID NO:870), Rhesus monkey (SEQ ID NO:12), Fox (SEQ ID NO:31), Gibbon (SEQ ID NO:857), Marmoset (SEQ ID NO:859) or orangutan (SEQ ID NO:861) PH20 polypeptides. For example, the human PH20 mRNA transcript is normally translated to generate a 509 amino acid precursor protein (SEQ ID NO:6) containing a 35 amino acid signal sequence at the N-terminus (amino acid residue positions 1-35 of SEQ ID NO:6). Thus, following transport to the ER and removal of the signal peptide, a 474 amino acid mature polypeptide with an amino acid sequence set forth in SEQ ID NO:7 is produced. Sequences of PH20 from ovine are also known (see e.g., SEQ ID NOS: 25-27).

In particular, human PH20 has the sequence of amino acids set forth in SEQ ID NO:6. The mature human PH20 lacking a signal sequence is set forth in SEQ ID NO:7. Allelic variants and other variants of PH20 are known. Other sequences of PH20 have been reported. For example, a PH20 variant is known as set forth in the precursor sequence set forth in SEQ ID NO:68 that contains an Ala at position 48 and a Trp at position 499, or the mature sequence thereof set forth in SEQ ID NO:69 containing the corresponding differences at positions 13 and 464, respectively, compared to the sequence set forth in SEQ ID NO:7 (see e.g., Gmachl et al. (1993) FEBS Lett., 336:545-548; GenBank Accession No. AAC60607). Further, a natural variant of PH20 has been identified containing a Glutamine (Gln; Q) at position 5 as compared to the precursor sequence of amino acids set forth in SEQ ID NO:6 (see e.g., SEQ ID NO:70, see also Varela et al. (2011) Nature, 469:539-542). Another natural variant contains an Alanine (Ala; A) at position 47 compared to the sequence of amino acids set forth in SEQ ID NO:6 (as set forth in SEQ ID NO: 71) and corresponding to position 12 compared to the sequence of amino acids set forth in SEQ ID NO: 3 or 7 (as set forth in SEQ ID NO:72).

The sequence and structure of PH20 polypeptides is highly conserved. Sequence identity between and among PH20 proteins from various species is about 50% to 90%. The hydrophobic N-terminal signal sequence of 35 amino acids in length is generally conserved among PH20 hyaluronidase polypeptides. PH20 hyaluronidases contain a common core hyaluronidase domain region of about 340 amino acids in length that corresponds to amino acid residues 38-374 of the precursor human PH20 sequence set forth in SEQ ID NO:6. A mature PH20 polypeptide lacking the signal sequence and containing a contiguous sequence of amino acids having a C-terminal amino acid residue corresponding to amino acid residue 464 of SEQ ID NO:6 (e.g., amino acid residues corresponding to positions 36-464 of the amino acid sequence set forth in SEQ ID NO:6) is the minimal sequence required for hyaluronidase activity (see e.g., U.S. patent application Ser. No. 10/795,095, which is issued as U.S. Pat. No. 7,767,429; see also U.S. Publication No. US20100143457).

Within the common hyaluronidase domain region, at least 57 amino acids are conserved between and among species (see e.g., Arming et al. (1997) *Eur. J. Biochem.,* 247:810-814; ten Have et al. (1998) *Reprod. Fertil. Dev.,* 10:165-72; Chowpongpang et al. (2004) *Biotechnology Letters,* 26:1247-1252). For example, PH20 hyaluronidases contain 12 conserved cysteine residues corresponding to amino acid residue 25, 189, 203, 316, 341, 346, 352, 400, 402, 408, 423 and 429 of the sequence of amino acids of a mature PH20 lacking the signal sequence such as set forth in SEQ ID NO: 3 or 7 (corresponding to amino acid residues 60, 224, 238, 351, 376, 381, 387, 435, 437, 443, 458 or 464 of full-length human PH20 set forth in SEQ ID NO:6). Cysteine residues corresponding to 25 and 316 and cysteine residues corresponding to 189 and 203 form disulfide bridges. The other cysteine residues also form disulfide bridges, are involved in posttranslational protein maturation and/or in activity modulation. For example, further four disulfide bonds are formed between the cysteine residues C376 and C387; between C381 and C435; between C437 and C443; and between C458 and C464 of the polypeptide exemplified in SEQ ID NO:6 (corresponding to positions C341 and C352; between C346 and C400; between C402 and C408; and between C423 and C429 of the mature polypeptide set forth in SEQ ID NO:3 or 7, respectively).

Amino acid residues corresponding to amino acid residue D111, E113 and E249 of the sequence of amino acids set forth in SEQ ID NO: 3 or 7 are acidic residues part of the enzyme active site and are conserved between and among PH20 species. Amino acid residues R176, R246, R252 of the sequence of amino acids set forth in SEQ ID NO: 3 or 7 are also conserved between and among species and contribute to substrate binding and/or hyaluronidase activity. Amino acid mutations D111N, E113Q, R176G, E249N and R252T result in enzymes that have no detectable enzymatic activity or residual enzymatic activity (see e.g., Arming et al. (1997) *Eur. J. Biochem.,* 247:810-814).

The results herein confirm the requirement of PH20 amino acid residues corresponding to positions 25, 111, 113, 176, 189, 203, 246, 249, 252, 316, 341, 346, 352, 400, 402, 408, 423 and 429 of the sequence of amino acids set forth in a mature PH20 lacking the signal sequence such as set forth in SEQ ID NO: 3 or 7 for hyaluronidase activity, since mutagenesis of these residues results in an enzyme that is not active (e.g., it is not expressed or is inactive when expressed, see e.g., Tables 5 and 10). The exception is that amino acid replacement corresponding to R176K and C316D resulted in mutants that generated some residual hyaluronidase activity.

Glycosylation also is required for PH20 hyaluronidase activity based on the recognition motif NxS or NxT. There are six N-linked oligosaccharides at amino acid residues corresponding to positions N47, N131, N200, N219, N333 and N358 of the sequence of amino acids set forth in SEQ ID NO: 3 or 7 (corresponding to amino acid residues N82, N166, N235, N254, N368 and N393 of human PH20 set forth in SEQ ID NO: 6). In particular, at least N-linked glycosylation sites corresponding to amino acid residues N200, N333 and N358 are required for secretion and/or activity of the enzyme (see e.g., U.S. Publication No. US20100143457). For example, a PH20 polypeptide containing amino acid mutations N200A, N333A, N358A or N333A/N393A result in inactive proteins. Single mutations of glycosylation sites N47A, N131A, N219A, N47A/N131A, N47A/N219A, N131A/N291A retain activity. The N-linked glycosylation site corresponding to amino acid residue N368 of human PH20 set forth in SEQ ID NO:6 is conserved between and among species (see e.g., Chowpongpang et al. (2004) *Biotechnology Letters,* 26:1247-1252). PH20 hyaluronidases also contains O-linked glycosylation sites. For example, human PH20 has one O-linked oligosaccharide at the amino acid residue corresponding to amino acid T440 of the sequence of amino acids set forth in SEQ ID NO:3 or 7 (corresponding to amino acid residue T475 in SEQ ID NO:6).

In addition to the catalytic sites, PH20 also contains a hyaluronan-binding site. This site is located in the Peptide 2 region, which corresponds to amino acid positions 205-235 of the precursor polypeptide set forth in SEQ ID NO:6 and positions 170-200 of the mature polypeptide set forth in SEQ ID NO:3 or 7. This region is highly conserved among hyaluronidases and is similar to the heparin binding motif. Mutation of the arginine residue at position 176 (corresponding to the mature PH20 polypeptide set forth in SEQ ID NO:3 or 7) to a glycine results in a polypeptide with only about 1% of the hyaluronidase activity of the wild type polypeptide (Arming et al., (1997) *Eur. J. Biochem.* 247: 810-814).

PH20 polypeptides contain a glycosyl phosphatidylinositol (GPI) anchor attached to the C-terminus of the protein that anchors the protein to the extracellular leaflet of the plasma membrane of cells. At least human, monkey, mouse and guinea pig PH20 are strongly attached to the plasma membrane via the GPI anchor, which can be released by treating with phosphatidylinositol-specific phospholipase C (PI-PLC; see e.g., Lin et al. (1994) *Journal of Cell Biology,* 125:1157-1163; Lin et al. (1993) *Proc. Natl. Acad. Sci.,* 90:10071-10075). Other PH20 enzymes, such as bovine PH20, are loosely attached to the plasma membrane and are not anchored via a phospholipase sensitive anchor. As discussed below, soluble active forms that, when expressed, are not attached to the membrane but are secreted can be generated by removal of all of a portion of the GPI anchor attachment signal site (see also U.S. Pat. No. 7,767,429; U.S. Publication No. US20100143457). These include, for example, soluble PH20 polypeptides set forth in any of SEQ ID NOS: 3 or 32-66, or precursor forms thereof containing a signal sequence.

GPI-anchored proteins, for example human PH20, are translated with a cleavable N-terminal signal peptide that directs the protein to the endoplasmic reticulum (ER). At the C-terminus of these proteins is another signal sequence that directs addition of a preformed GPI-anchor to the polypeptide within the lumen of the ER. Addition of the GPI anchor occurs following cleavage of the C-terminal portion at a specific amino acid position, called the ω-site (typically located approximately 20-30 amino acids from the C-terminus). Although there appears to be no consensus sequence to identify the location of the ω-site, GPI anchored proteins contain a C-terminal GPI-anchor attachment signal sequence or domain that typically contains a predominantly hydrophobic region of 8-20 amino acids, preceded by a hydrophilic spacer region of 8-12 amino acids immediately downstream of the ω-site. This hydrophilic spacer region often is rich in charged amino acids and proline (White et al. (2000) *J. Cell Sci.* 113 (Pt. 4): 721-727). There is generally a region of approximately 11 amino acids before the ω-1 position that is characterized by a low amount of predicted secondary structure, a region around the cleavage site (ω-site), from ω-1 to ω+2 that is characterized by the presence of small side chain residues, the spacer region between positions ω+3 and ω+9, and a hydrophobic tail from ω+10 to the C-terminal end (Pierleoni et al., (2008) *BMC Bioinformatics* 9:392).

Although there is no GPI-anchor attachment signal consensus sequence, various in silico methods and algorithms have been developed that can be used to identify such sequences in polypeptides (see, e.g., Udenfriend et al. (1995) *Methods Enzymol.* 250:571-582; Eisenhaber et al. (1999) *J. Mol. Chem.* 292:741-758; Kronegg and Buloz, (1999), "Detection/prediction of GPI cleavage site (GPI-anchor) in a protein (DGPI)," 129.194.185.165/dgpi/; Fankhauser et al. (2005) *Bioinformatics* 21:1846-1852; Omaetxebarria et al. (2007) *Proteomics* 7:1951-1960; Pierleoni et al. (2008) *BMC Bioinformatics* 9:392), including those that are readily available on bioinformatic websites, such as the ExPASy Proteomics tools site (expasy.ch/tools/). Thus, one of skill in the art can determine whether a PH20 polypeptide likely contains a GPI-anchor attachment signal sequence, and, therefore, whether the PH20 polypeptide is a GPI-anchored protein.

The covalent attachment of a GPI-anchor to the C-terminus of human PH20 and, therefore, the membrane-bound nature of PH20, has been confirmed using phosphatidylinositol-specific phospholipase C (PI-PLC) hydrolysis studies (see e.g., Lin et al, (1994) *J. Biol. Chem.* 125:1157-1163). Phosphatidylinositol-specific phospholipase C (PI-PLC) and D (PI-PLD) hydrolyze the GPI anchor, releasing the PH20 polypeptide from the cell membrane. The prior art literature reports that a ω-site cleavage site of human PH20 is identified between Ser-490 and Ala-491 and for monkey PH20 is identified between Ser491 and Thr492 (Lin et al. (1993) *Proc. Natl. Acad. Sci*, (1993) 90:10071-10075). Thus, the literature reports that a GPI-anchor attachment signal sequence of human PH20 is located at amino acid positions 491-509 of the precursor polypeptide set forth in SEQ ID NO:6, and the ω-site is amino acid position 490. Thus, in this modeling of human PH20, amino acids 491-509 are cleaved following transport to the ER and a GPI anchor is covalently attached to the serine residue at position 490.

2. Function

PH20 is normally expressed in sperm from a single testis-specific gene. PH20 is a sperm-associated protein involved in fertilization. PH20 is normally localized on the sperm surface, and in the lysosome-derived acrosome, where it is bound to the inner acrosomal membrane. PH20 is multifunctional and exhibits hyaluronidase activity, hyaluronan (HA)-mediated cell-signaling activity, and acts as a sperm receptor for the zona pellucida surrounding the oocyte when present on acrosome reacted (AR) sperm. For example, PH20 is naturally involved in sperm-egg adhesion and aids penetration by sperm of the layer of cumulus cells by digesting hyaluronic acid. In addition to being a hyaluronidase, PH20 also appears to be a receptor for HA-induced cell signaling, and a receptor for the zona pellucida surrounding the oocyte. Due to the role of PH20 in fertilization, PH20 can be used as an antigen for immunocontraception.

PH20 is a neutral active hyaluronidase, although it can exhibit acid-active activity in some cases. The hyaluronidase activity of PH20 is exhibited by the plasma membrane- and inner acrosomal membrane-associated PH20. The plasma membrane PH20 exhibits hyaluronidase activity only at neutral pH, while the inner acrosomal membrane-associated PH20 exhibits acid-active enzyme activity. The structural basis for these differences is due to the presence of two catalytic sites in PH20. A first catalytic site is designated the Peptide 1 region, corresponding to amino acid residues 142-172 of SEQ ID NO:6, which is involved in enzyme activity of PH20 at neutral pH. A second catalytic site is designated the peptide 3 region, corresponding to amino acid residues 277-297 of SEQ ID NO:6, which is involved in enzyme activity at lower pH. A change in the structure of the inner acrosomal membrane-associated PH20 occurs after the acrosome reaction, whereby PH20 is endoproteolytically cleaved but held together by disulfide bonds. The result of the endoproteolysis is that the peptide 3 region is activated and can thus effect neutral and acid-activity to PH20 (see e.g., Cherr et al. (2001) *Matrix Biology,* 20:515-525. Also, after the acrosome reaction, lower molecular weight forms are generated by release from the inner acrosomal membrane (e.g., a 53 kDa soluble form of PH20 is generated in monkey). The lower molecular weight form(s) also is acid active.

The hyaluronidase activity of PH20 accounts for the spreading activity observed in animal testes extracts that have been used clinically for decades to increase the dispersion and absorption of drugs (see e.g., Bookbinder et al. (2006) *J Controlled Release,* 114:230-241). For example, pharmaceutical preparations containing hyaluronidase were developed as fractionated extracts from bovine testes for therapeutic use as spreading agents and in other applications (Schwartzman (1951) *J. Pediat.,* 39:491-502). Original bovine testicular extract preparations included, for example, extracts sold under the trademarks Wydase®, Hylase®, "Dessau," Neopermease®, Alidase® and Hyazyme®. It is now known that the spreading activity of testicular extract preparations are due to PH20 hyaluronidase activity. For example, in 2001 a sperm hyaluronidase in bull was identified as the hyaluronidase PH20 (Lalancette et al. (2001) *Biol. Reprod.,* 65:628-36). By catalyzing the hydrolysis of hyaluronic acid, PH20 hyaluronidase lowers the viscosity of hyaluronic acid, thereby increasing tissue permeability. Hence, soluble forms of PH20 are used as a spreading or dispersing agent in conjunction with other agents, drug and proteins to enhance their dispersion and delivery, and to improve the pharmacokinetic and pharmacodynamic profile of the coadministered agent, drug or protein (see e.g., U.S. Pat. No. 7,767,429; Bookbinder et al. (2006) *J Controlled Release,* 114:230-241).

3. Soluble PH20 Polypeptides

PH20 can exist in membrane-bound or membrane-associated form, or can be secreted into the media when expressed from cells, and thereby can exist in soluble form. Soluble PH20 can be detected and discriminated from insoluble, membrane-bound PH20 using methods well known in the art, including, but not limited to, those using a Triton® X-114 assay. In this assay, soluble PH20 hyaluronidases partition into the aqueous phase of a Triton®

X-114 solution warmed to 37° C. (Bordier et al., (1981) *J. Biol. Chem.,* 256:1604-7) while membrane-anchored PH20 hyaluronidases partition into the detergent rich phase. Thus, in addition to using algorithms to assess whether a PH20 polypeptide is naturally GPI-anchored and hence membrane-bound, solubility experiments also can be performed.

Soluble PH20 enzymes include hyaluronidases that contain a GPI-anchor attachment signal sequence, but that are loosely attached to the membrane such that they do not contain a phospholipase sensitive anchor. For example, soluble PH20 polypeptides include ovine or bovine PH20. Various forms of such soluble PH20 hyaluronidases have been prepared and approved for therapeutic use in subjects, including humans. For example, animal-derived hyaluronidase preparations include Vitrase® (ISTA Pharmaceuticals), a purified ovine testicular hyaluronidase, and Amphadase® (Amphastar Pharmaceuticals), a bovine testicular hyaluronidase. Soluble PH20 enzymes also include truncated forms of non-human or human membrane-associated PH20 hyaluronidases that lack one or more amino acid residues of a glycosylphosphatidylinositol (GPI) anchor attachment signal sequence and that retain hyaluronidase activity (see e.g., U.S. Pat. No. 7,767,429; U.S. Publication No. US20100143457). Thus, instead of having a GPI-anchor covalently attached to the C-terminus of the protein in the ER and being anchored to the extracellular leaflet of the plasma membrane, these polypeptides are secreted when expressed from cells and are soluble. In instances where the soluble hyaluronan degrading enzyme retains a portion of the GPI anchor attachment signal sequence, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid residues in the GPI-anchor attachment signal sequence can be retained, provided the polypeptide is soluble (i.e., secreted when expressed from cells) and active.

Exemplary soluble hyaluronidases that are C-terminally truncated and lack all or a portion of the GPI anchor attachment signal sequence include, but are not limited to, PH20 polypeptides of primate origin, such as, for example, human and chimpanzee PH20 polypeptides. For example, soluble PH20 polypeptides can be made by C-terminal truncation of a polypeptide set forth in SEQ ID NOS:7, 10, 12, 14, 69, 72, 857, 859, 861 or 870 or variants thereof that exhibit at least 80%, 85%, 90%, 95% or more sequence identity to any of SEQ ID NO: 7, 10, 12, 14, 69, 72, 857, 859, 861 or 870, wherein the resulting polypeptide is active, soluble and lacks all or a portion of amino acid residues from the GPI-anchor attachment signal sequence.

Exemplary soluble PH20 polypeptides are C-terminal truncated human PH20 polypeptides that are mature (lacking a signal sequence), soluble and exhibit neutral activity, and that contain a contiguous sequence of amino acids set forth in SEQ ID NO:6 or SEQ ID NO:7 that minimally has a C-terminal truncated amino acid residue at or after amino acid residue 464 of the sequence of amino acids set forth in SEQ ID NO:6. For example, soluble PH20 polypeptides include C-terminal truncated polypeptides that minimally contain a contiguous sequence of amino acids 36-464 of SEQ ID NO:6, or includes a sequence of amino acids that has at least 85%, for example at least 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% sequence identity to a contiguous sequence of amino acids that has a C-terminal amino acid residue after amino acid 464 of SEQ ID NO:6 and retains hyaluronidase activity. Exemplary C-terminally truncated human PH20 polypeptides are mature polypeptides (lacking a signal sequence) that include a contiguous sequence of amino acids set forth in SEQ ID NO:6 with a C-terminal residue after 464 such as after amino acid position 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499 or 500 of the sequence of amino acids set forth in SEQ ID NO:6, or a variant thereof that exhibits at least 85% sequence identity, such as at least 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% sequence identity thereto and retains hyaluronidase activity. For example, exemplary C-terminal PH20 polypeptides have a sequence of amino acids 36 to 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499 or 500 of the sequence of amino acids set forth in SEQ ID NO:6, or a variant thereof that exhibits at least 85% sequence identity, such as at least 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% sequence identity thereto and retains hyaluronidase activity. Soluble PH20 polypeptides include any that has the sequence of amino acids set forth in SEQ ID NOS: 3 or 32-66 or a sequence of amino acids that exhibits at least 85% sequence identity, such as at least 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% sequence identity to the sequence of amino acids set forth in any of SEQ ID NOS: 3 or 32-66.

In particular, a soluble human PH20 polypeptide is a polypeptide that is truncated after amino acid 482 of the sequence set forth in SEQ ID NO:6. Such a polypeptide can be generated from a nucleic acid molecule containing a signal sequence and encoding amino acids 36-482, for example, as set forth in SEQ ID NO: 1 (containing an IgG kappa signal sequence) or SEQ ID NO:67 (containing the native signal sequence). Post translational processing removes the signal sequence, leaving a 447 amino acid soluble recombinant human PH20 (SEQ ID NO:3). A product produced upon expression of a vector set forth in SEQ ID NO:4 or 5, and containing a nucleic acid molecule set forth in SEQ ID NO:67, results in a secreted product, designated rHuPH20, in the culture medium that exhibits heterogeneity at the C-terminus such that the product includes a mixture of species that can include any one or more of SEQ ID NOS: 3 and 44-48 in various abundance. Typically, rHuPH20 is produced in cells that facilitate correct N-glycosylation to retain activity, such as mammalian cells, for example CHO cells (e.g., DG44 CHO cells). Hylenex® (Halozyme) is a human recombinant hyaluronidase produced by genetically engineered Chinese Hamster Ovary (CHO) cells containing nucleic acid encoding a truncated human PH20 polypeptide (designated rHuPH20).

C. Modified PH20 Polypeptides

Provided herein are modified or variant PH20 polypeptides. The modified PH20 polypeptides provided herein exhibit altered activities or properties compared to a wild-type, native or reference PH20 polypeptide. Included among the modified PH20 polypeptides provided herein are PH20 polypeptide that are active mutants, whereby the polypeptides exhibit at least 40% of the hyaluronidase activity of the corresponding PH20 polypeptide not containing the amino acid modification (e.g., amino acid replacement). In particular, provided herein are PH20 polypeptides that exhibit hyaluronidase activity and that exhibit increased stability compared to the PH20 not containing the amino acid modification. Also provided are modified PH20 polypeptides that are inactive, and that can be used, for example, as antigens in contraception vaccines.

The modifications can be a single amino acid modification, such as single amino acid replacements (substitutions), insertions or deletions, or multiple amino acid modifications, such as multiple amino acid replacements, insertions or deletions. Exemplary modifications are amino acid replacements, including single or multiple amino acid replacements. The amino acid replacement can be a conservative substitution, such as set forth in Table 2, or a non-conservative substitution, such as any described herein. Modified PH20 polypeptides provided herein can contain at least or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more modified positions compared to the PH20 polypeptide not containing the modification.

The modifications described herein can be in any PH20 polypeptide, including, including precursor, mature, or C-terminal truncated forms, so long as the modified form exhibits hyaluronidase activity. For example, the PH20 polypeptides contain modifications compared to a wildtype, native or reference PH20 polypeptide set forth in any of SEQ ID NOS: 2, 3, 6-66, 68-72, 856-861, 869 or 870, or in a polypeptide that has a sequence of amino acids that is at least 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identical to any of SEQ ID NOS: 3, 6-66, 68-72, 856-861, 869 or 870. For example, the modifications are made in a human PH20 polypeptide having the sequence of amino acids including or set forth in SEQ ID NO: 7, SEQ ID NO:69 or SEQ ID NO:72; a bovine PH20 polypeptide having a sequence of amino acids including or set forth in SEQ ID NOS:16 or 18; a rabbit PH20 polypeptide having a sequence of amino acids including or set forth in SEQ ID NO:24; a Cynomolgus monkey PH20 polypeptide having a sequence of amino acids including or set forth in SEQ ID NO:14; a guinea pig PH20 polypeptide having a sequence of amino acids including or set forth in SEQ ID NO:29; a rat PH20 polypeptide having a sequence of amino acids including or set forth in SEQ ID NO:22; a mouse PH20 polypeptide having a sequence of amino acids including or set forth in SEQ ID NO:20; a chimpanzee PH20 polypeptide having a sequence of amino acids including or set forth in SEQ ID NO:10 or 870; a Rhesus monkey PH20 polypeptide having a sequence of amino acids including or set forth in SEQ ID NO:12; a Fox PH20 polypeptide having a sequence of amino acids including or set forth in SEQ ID NO:31; a Gibbon PH20 polypeptide having a sequence of amino acids including or set forth in SEQ ID NO:857; a Marmoset PH20 polypeptide having a sequence of amino acids including or set forth in SEQ ID NO: 859; an Orangutan PH20 polypeptide having a sequence of amino acids including or set forth in SEQ ID NO:861; or a sheep PH20 polypeptide having a sequence of amino acids including or set forth in any of SEQ ID NOS: 25-27; or in sequence variants or truncated variants that exhibit at least 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 7, 10, 12, 14, 16, 18, 20, 22, 24-27, 29, 31, 69, 72, 857, 859, 861 or 870.

In particular, provided herein are PH20 polypeptides that contain modifications compared to a PH20 polypeptide set forth in SEQ ID NO: 3, 7, 32-66, 69 or 72, or a polypeptide that has a sequence of amino acids that is at least 68%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identical to any of SEQ ID NOS: 3, 7, 32-66, 69 or 72. For example, the modifications provided herein also can be made in a PH20 polypeptide set forth as SEQ ID NO: 10, 12, 14, 24, 857, 859, 861 or 870.

In particular, provided herein are modified soluble PH20 polypeptides that are PH20 polypeptides containing a modification provided herein, and that when expressed from cells are secreted into the media as a soluble protein. For example, the modifications are made in a soluble PH20 polypeptide that is C-terminally truncated within or near the C-terminus portion containing the GPI-anchor signal sequence of a PH20 polypeptide that contains a GPI-anchor signal sequence. The C-terminal truncation can be a truncation or deletion of 8 contiguous amino acids at the C-terminus, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more amino acids at the C-terminus, so long as the resulting C-terminally truncated polypeptide exhibits hyaluronidase activity and is secreted from cells (e.g., into the media) when expressed. In some examples, the modifications provided herein are made in a soluble PH20 polypeptide that is a C-terminally truncated polypeptide of SEQ ID NO:7, 10, 12, 14, 69, 72, 857, 859, 861 or 870 or a variant thereof that exhibits at least 70%, 75%, 80%, 85%, 86%), 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 7, 10, 12, 14, 69, 72, 857, 859, 861 or 870. In particular, the modifications provided herein are made in a soluble or C-terminally truncated human PH20 polypeptide having the sequence of amino acids set forth in SEQ ID NOS: 3 or 32-66 or a sequence of amino acids that exhibits at least 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% sequence identity to the sequence of amino acids set forth in any of SEQ ID NOS: 3 or 32-66. For example, modified PH20 polypeptides provided herein contain amino acid replacements or substitutions, additions or deletions, truncations or combinations thereof with reference to the PH20 polypeptide set forth in SEQ ID NO:3.

Modifications also can be made in the corresponding precursor form containing a signal peptide of any of SEQ ID NOS: 3, 7, 10, 12, 14, 16, 18, 20, 22, 24-27, 29,31,32-66, 69, 72, 857, 859, 861 or 870. For example, modifications provided herein can be made in a precursor form set forth in any of SEQ ID NOS: 2, 6, 8, 9, 11, 13, 15, 17, 19, 21, 23, 28, 30, 856, 858, 860 or 869 or in a variant thereof that exhibits at least 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 2, 6, 8, 9, 11, 13, 15, 17, 19, 21, 23, 28, 30, 856, 858, 860 or 869.

In examples of modified PH20 polypeptides provided herein, the modified PH20 polypeptide does not contain the sequence of amino acids set forth in any of SEQ ID NOS: 3-66, 68-72, 856-861, 869 or 870. Typically, the modified PH20 polypeptide is a human PH20 polypeptide, and does not contain the sequence of amino acids set forth in any of SEQ ID NOS: 8-31, 856-861, 869 or 870.

Generally, any modification, such as amino acid replacement, deletion or substitution, can be made in a PH20 polypeptide, with the proviso that the modification is not an amino acid replacement where the only modification is a single amino acid replacement that is V12A, N47A, D111N, E113Q, N131A, R176G, N200A, N219A, E249Q, R252T, N333A or N358A. Also, where the modified PH20 polypeptide contains only two amino acid replacements, the amino acid replacements are not P13A/L464W, N47A/N131A, N47A/N219A, N131A/N219A or N333A/N358A. In a further example, where the modified PH20 polypeptide contains only three amino acid replacements, the amino acid replacements are not N47A/N131A/N219A. Exemplary modifications provided herein are described in detail below.

For purposes herein, reference to positions and amino acids for modification herein, including amino acid replacement or replacements, are with reference to the PH20 polypeptide set forth in SEQ ID NO:3. It is within the level of one of skill in the art to make any of the modifications provided herein in another PH20 polypeptide by identifying the corresponding amino acid residue in another PH20 polypeptide, such as any set forth in SEQ ID NOS: 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24-27, 28, 29, 30, 31, 32-66, 68-72, 856, 857, 858, 859, 860, 861, 869 or 870 or a variant thereof that exhibits at least 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24-27, 28, 29, 30, 31, 32-66, 68-72, 856, 857, 858, 859, 860, 861, 869 or 870. Corresponding positions in another PH20 polypeptide can be identified by alignment of the PH20 polypeptide with the reference to the PH20 polypeptide set forth in SEQ ID NO:3. For example, FIG. **2** (A-L) depicts alignment of exemplary PH20 polypeptides with SEQ ID NO:3, and identification of exemplary corresponding positions. Also, since SEQ ID NOS: 3, 7, 32-66, 69 and 72 are all forms of a mature human PH20 with a different C-terminal amino acid residue, the numbering of amino acid residues in any of SEQ ID NOS: 7, 32-66, 69 and 72 is the same as SEQ ID NO:3, and hence the corresponding residues of each are identical to that set forth in SEQ ID NO:3 (see e.g., FIG. **1**). Further, SEQ ID NOS set forth in any of SEQ ID NOS: 2, 6, 70 or 71 are precursor forms thereof that differ by only the presence of a signal sequence. For purposes of modification (e.g., amino acid replacement), the corresponding amino acid residue can be any amino acid residue, and need not be identical to the residue set forth in SEQ ID NO:3. Typically, the corresponding amino acid residue identified by alignment with residues in SEQ ID NO:3 is an amino acid residue that is identical to SEQ ID NO:3, or is a conservative or semi-conservative amino acid residue thereto (see e.g., FIGS. **2**A-**2**L). It is also understood that the exemplary replacements provided herein can be made at the corresponding residue in a PH20 polypeptide, so long as the replacement is different than exists in the unmodified form of the PH20 polypeptide. Based on this description and the description elsewhere herein, it is within the level of one of skill in the art to generate a modified PH20 polypeptide containing any one or more of the described mutation, and test each for a property or activity as described herein.

Modifications in a PH20 polypeptide also can be made to a PH20 polypeptide that also contains other modifications, including modifications of the primary sequence and modifications not in the primary sequence of the polypeptide. For example, modifications described herein can be in a PH20 polypeptide that is a fusion polypeptide or chimeric polypeptide. The modified PH20 polypeptides provided herein also include polypeptides that are conjugated to a polymer, such as a PEG reagent.

Also provided herein are nucleic acid molecules that encode any of the modified PH20 polypeptides provided herein. In particular examples, the nucleic acid sequence can be codon optimized, for example, to increase expression levels of the encoded sequence. The particular codon usage is dependent on the host organism in which the modified polypeptide is expressed. One of skill in the art is familiar with optimal codons for expression in mammalian or human cells, bacteria or yeast, including for example *E. coli* or *Saccharomyces cerevisiae*. For example, codon usage information is available from the Codon Usage Database available at kazusa.or.jp.codon (see Richmond (2000) Genome Biology, 1:reports241 for a description of the database). See also, Forsburg (1994) Yeast, 10:1045-1047; Brown et al. (1991) Nucleic Acids Research, 19:4298; Sharp et al. (1988) Nucleic Acids Res., 12:8207-8211; Sharp et al. (1991) Yeast, 657-78). In some examples, the encoding nucleic acid molecules also can be modified to contain a heterologous signal sequence to alter (e.g., increased) expression and secretion of the polypeptide. Exemplary of a heterologous signal sequence is a nucleic acid encoding the IgG kappa signal sequence (set forth in SEQ ID NO:868).

The modified polypeptides and encoding nucleic acid molecules provided herein can be produced by standard recombinant DNA techniques known to one of skill in the art. Any method known in the art to effect mutation of any one or more amino acids in a target protein can be employed. Methods include standard site-directed or random mutagenesis of encoding nucleic acid molecules, or solid phase polypeptide synthesis methods. For example, nucleic acid molecules encoding a PH20 polypeptide can be subjected to mutagenesis, such as random mutagenesis of the encoding nucleic acid, error-prone PCR, site-directed mutagenesis, overlap PCR, gene shuffling, or other recombinant methods. The nucleic acid encoding the polypeptides can then be introduced into a host cell to be expressed heterologously. Hence, also provided herein are nucleic acid molecules encoding any of the modified polypeptides provided herein. In some examples, the modified PH20 polypeptides are produced synthetically, such as using solid phase or solutions phase peptide synthesis.

In the subsections below, exemplary modified PH20 polypeptide exhibiting altered properties and activities, and encoding nucleic acid molecules, provided herein are described.

1. Active Mutants

Provided herein are modified PH20 polypeptides that contain one or more amino acid replacements in a PH20 polypeptide and that exhibit hyaluronidase activity. The modified PH20 polypeptides can exhibit 40% to 5000% of the hyaluronidase activity of a wildtype or reference PH20 polypeptide, such as the polypeptide set forth in SEQ ID NOS: 3 or 7. For example, modified PH20 polypeptides provided herein exhibit at least 40% of the hyaluronidase activity, such as at least 50%, 60%, 70%, 80%, 90%, 100%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, 1000%), 2000%, 3000% or more of the hyaluronidase activity of a wildtype or reference PH20 polypeptide, such as the corresponding polypeptide not containing the amino acid modification (e.g., amino acid replacement), for example, a polypeptide set forth in SEQ ID NO:3 or 7. For example, exemplary positions that can be modified, for example by amino acid replacement or substitution, include, but are not limited to, any of positions corresponding to position 1, 2, 3, 4, 5, 6, 8, 9, 10, 11, 12, 13, 14, 15, 20, 22, 23, 24, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 54, 58, 59, 60, 61, 63, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 77, 79, 81, 82, 83, 84, 85, 86, 87, 89, 90, 91, 92, 93, 94, 96, 97, 98, 99, 102, 103, 104, 105, 106, 107, 108, 110, 114, 117, 118, 119, 120, 122, 124, 125, 127, 128, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 186, 192, 193, 195, 196, 197, 198, 200, 202, 204, 205, 206, 208, 209, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 224, 226, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 242, 245, 247, 248, 251, 253, 255, 256, 257, 258, 259, 260, 261, 263, 264, 265, 266, 267, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 297, 298, 300, 301, 302, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 320, 321, 323, 324, 325, 326, 327, 328, 331, 334, 335, 338, 339, 342, 343, 347, 348, 349, 351, 353, 356, 357, 358, 359, 360, 361, 367, 368, 369, 371, 373, 374, 375, 376, 377, 378, 379, 380, 381, 383, 385, 387, 388, 389, 391, 392, 393, 394, 395, 396, 397, 398, 399, 401, 403, 404, 405, 406, 407, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 425, 426, 427, 428, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446 or 447 with reference to amino acid positions set forth in SEQ ID NO:3. Typically, the amino acid residue that is modified (e.g., replaced with another amino acid) at the position corresponding to any of the above positions in a PH20 polypeptide is an identical residue, a conservative residue or a semi-conservative amino acid residue to the amino acid residue set forth in SEQ ID NO:3.

To retain hyaluronidase activity, modifications typically are not made at those positions that are less tolerant to change or required for hyaluronidase activity. For example, generally modifications are not made at a position corresponding to position 7, 16, 17, 18, 19, 21, 25, 53, 55, 56, 57, 62, 64, 76, 78, 80, 88, 95, 100, 101, 109, 111, 112, 113, 115, 116, 121, 123, 126, 129, 185, 187, 188, 189, 190, 191, 194, 199, 201, 203, 207, 210, 223, 225, 227, 228, 229, 241, 243, 244, 246, 249, 250, 252, 254, 262, 268, 295, 296, 299, 303, 319, 322, 329, 330, 332, 333, 336, 337, 340, 341, 344, 345, 346, 350, 352, 354, 355, 362, 363, 364, 365, 366, 370, 372, 382, 384, 386, 390, 400, 402, 408, 423, 424, 429, 430, with reference to amino acid positions set forth in SEQ ID NO:3. Also, in examples where modifications are made at any of positions 2, 3, 4, 5, 6, 8, 9, 10, 11, 12, 13, 14, 15, 20, 22, 23, 27, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 54, 58, 59, 60, 61, 63, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 77, 79, 81, 82, 83, 84, 85, 86, 87, 89, 90, 91, 92, 94, 96, 98, 99, 102, 103, 104, 105, 106, 107, 108, 110, 114, 117, 118, 119, 122, 124, 125, 127, 128, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 143, 144, 145, 149, 150, 152, 153, 154, 155, 156, 157, 158, 159, 161, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 186, 192, 193, 195, 197, 198, 200, 202, 204, 206, 208, 209, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 224, 226, 230, 231, 232, 233, 234, 235, 236, 238, 239, 240, 242, 245, 247, 248, 251, 253, 255, 256, 257, 258, 260, 261, 263, 264, 265, 266, 267, 269, 270, 271, 272, 273, 274, 275, 276, 278, 279, 280, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 297, 298, 300, 301, 302, 304, 305, 306, 307, 308, 310, 311, 312, 313, 314, 315, 316, 317, 318, 320, 321, 323, 324, 325, 326, 327, 331, 334, 335, 338, 339, 342, 343, 347, 348, 349, 351, 353, 356, 357, 358, 359, 360, 361, 367, 368, 369, 371, 373, 374, 375, 376, 377, 378, 379, 380, 381, 383, 385, 387, 388, 389, 391, 392, 393, 394, 395, 396, 397, 398, 399, 401, 403, 404, 405, 406, 410, 411, 412, 413, 414, 415, 416, 417, 419, 420, 422, 425, 426, 427, 428, 431, 432, 434, 437, 438, 439, 440, 441, 442, 443, 444, or 447 with reference to amino acid positions set forth in SEQ ID NO:3, the modification(s) is/are not the corresponding amino acid replacement(s) set forth in Table 5 or 10 herein, which are amino acid replacements that result in an inactive polypeptide. For example, if the modification is a modification at a position corresponding to position 2 with reference to SEQ ID NO:3, the modification is not replacement to a histidine (H), lysine (K), tryptophan (W) or tyrosine (Y).

Exemplary amino acid replacements at any of the above corresponding positions are set forth in Table 3. Reference to the corresponding amino acid position in Table 3 is with reference to positions set forth in SEQ ID NO:3. It is understood that the replacements can be made in the corresponding position in another PH20 polypeptide by alignment therewith with the sequence set forth in SEQ ID NO:3 (see e.g., FIGS. **1** and **2**), whereby the corresponding position is the aligned position. In particular examples, the amino acid replacement(s) can be at the corresponding position in a PH20 polypeptide as set forth in any of SEQ ID NOS: 2, 3, 6-66, 68-72, 856-861, 869 or 870 or a variant thereof having at least 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 86%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity thereto, so long as the resulting modified PH20 polypeptide exhibits at least 40% of the hyaluronidase activity of the corresponding PH20 polypeptide not containing the amino acid replacement. In particular, the replacements) can be in a corresponding position in a human PH20 polypeptide, for example, any set forth in any of SEQ ID NOS: 3, 7, 32-66, 69 or 72, or a variant thereof that exhibits at least 90%, 91%, 92%, 93%, 94%, 95%), 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 3, 7, 32-66, 69 or 72. In one example, any one or more of the replacements are in SEQ ID NO:3, so long as the resulting modified PH20 polypeptide exhibits at least 40% of the hyaluronidase activity of the PH20 polypeptide set forth in SEQ ID NO:3.

TABLE 3

Active Mutants					
Corresponding Position	Replacement	Corresponding Position	Replacement	Corresponding Position	Replacement
1	A C E F G H K N P Q R S T V W	2	A C G I L P Q S T V	3	E H L Y
4	A I S T V	5	H	6	A H K L N Q R
7	M	8	I L M P	9	K L Q R S V
10	D E G H N Q R S W	11	D G H K S	12	A E I K L N R S T
13	H S T Y	14	D I M V	15	A M V

TABLE 3-continued

Active Mutants					
Corresponding Position	Replacement	Corresponding Position	Replacement	Corresponding Position	Replacement
20	S	22	H M T Y	23	D
24	A E G H I K L M N R T V Y	26	A E G H I K M P Q R S T V W Y	27	A D E F H I K L P Q R S T W
28	A D E F I L M N P R S T V W	29	A E G H I K L M P R S T V W	30	A F G H K L M P Q R S T V W
31	A C G H I K L P R S T V W Y	32	A C F G H K L M N Q R S T V W Y	33	G M P Q R S T W
34	A E H K Q R W	35	F H L Q T V Y	36	A D G H K L N R T
37	F I K M P R W V	38	Y	39	A L N Q R T Y
40	L W	41	A C D E G H N T V W	42	A
43	N T	44	E	45	I K
46	A C E F H L M N R S T V Y	47	A D F G H K M Q R S T W Y	48	F G H I K M N Q R S V Y
49	I K R S V	50	A C D E H L M Q R S V Y	51	A N R S
52	N P Q R S T	54	A F N Q S V	58	C G H I K L N P Q R S W Y
59	Q N	60	K	61	F I M V
63	A H I K L M N R S T V W	65	R	66	H R
67	F L R V Y	68	E G H K L P Q R S T	69	A C E F G I L M P R T W Y
70	A C F G H K L N P R S T V Y	71	A D G H L M N Q R S	72	A D E H K L M Q R S Y
73	A C D G H K L M Q R S T W	74	A C E F G H K L M N P R S V W	75	A C F H L M N Q R S T Y
77	H K				
79	L T V	81	P	82	A E G H I L M N Q R S T V
83	F G H K L N Q R S T V	84	D E F G H I L M N P Q R T W Y	85	V
86	A D E F G H I K L M N P R S T V W	87	A C E G H I L M P Q R S T V Y	89	C K M P R W
90	A E G H I K L N Q R S T W	91	A Q R	92	C H L M T V
93	D E F G H I L M N P Q R S T V	94	A C D E F H L M N Q R S T	96	D L V
97	A C D E F G I L N P Q R S W Y	98	A C D E H I L M Q R S V W	99	A R S
102	A C E G H K L M N Q R S T W				
103	N	104	A C G I K M R S T	105	A C G H I P Q R S T W V
106	V				

TABLE 3-continued

Active Mutants					
Corresponding Position	Replacement	Corresponding Position	Replacement	Corresponding Position	Replacement
107	F I L	108	G	110	V
114	A G H M S	117	D	118	H K L M N Q V
119	F P Q Y	120	D F G H I L N P R S T V W Y	122	M
124	H L R	125	A H R S	127	A E G H L M N Q R S T V W
128	A C G I K L Q R S W	130	I R	131	C E F G H I L M Q R S T V Y
132	A C E F H I K L N Q S T V Y	133	I	134	L T V
135	A C D F G H K L N Q R S W Y	136	A C D F H I M N Q R S T W	137	A C I T A C H I L M N R S W Y
139	A C D E F G H K L M R S T V	140	A C D F G H I K L M R V W Y	141	A D E F G H L M Q R S T V W Y
142	C D E G H I K LM N P Q R S T	143	C E G I K L N V	144	R T W
145	A C D E G H L M N P R	146	A C E G H I K N P Q R S T V Y	147	A C D F G I L M P Q R S V W Y
148	C F G H I K L Q R S T V W Y	149	C G K L M Q R S T V	150	A C D E F G I L N P R S W Y
151	A C G H K L M N Q R S T V W Y	152	A C F I M R T V W Y	153	I L S
154	I R T V	155	A C D F G H K L M R S T V W	156	A C D G I L M Q R S T V W
157	W	158	A F G H L Q S	159	A D E G H L M N Q R S V
160	C F G H I K L M N Q R S W V Y	161	A C D E R S V	162	A D E G H L M P Q R S V W Y
163	A E G K L Q R S T V W	164	L M V W	165	A C D F N R S V W Y
166	A C E F G H L N Q R T W Y	167	A D G H K M N P R S T Y	168	H
169	L R V	170	A Q N R V	171	I V
172	A C	173	Q N R	174	A G H K M N Q R S T V W Y
175	E H T V Y	176	K L	177	V
178	G K M R				
179	A C E G I K L M N P R S T V	180	F G I K M	181	K M Q
182	L	183	E L	184	W
186	Y				
192	S T	193	F G Q R S Y	195	A G H I L N Q R S T W V
196	E G L N R S T W Y	197	A D E F G H K L M Q R S T W	198	A D E H L N Q R S T W Y
200	D T	202	M	204	P W

TABLE 3-continued

Active Mutants					
Corresponding Position	Replacement	Corresponding Position	Replacement	Corresponding Position	Replacement
205	L R S T V W Y	206	H I K L M Q R S T	208	A C K L M Q R S T V
209	A E F G L N R S T	211	L W		
212	N S T	213	A E G H K L M N Q R V W Y		
214	Q	215	A D E G H K L M Q R T V W Y	217	M
218	F M V	219	A C D E H I K L M R S T W	220	A D H I L M S T V
221	A C I M Q T V	222	D F G I K L N R S V	224	I
226	W				
230	I	231	T	232	S
233	A F G K L R Y	234	L M	235	A E G H K T
236	A G H K R S	237	A C E F H L N Q R S T W	238	D E H K Q R S T
239	N				
240	K A M P Q R S V	242	F	245	H
247	I L M	248	A H W Y	251	L M Y
253	I	255	A G N Q R S	256	A H L V
257	A C G I K L M N Q R T V	258	G H N R S	259	E G I K L N P Q R S T V W Y
260	A D E G H L M Q R S Y	261	A F K M N Q R T V W	263	A H K M R T V
264	A H	265	I	266	Y
267	M T	269	A C D S	270	M N S T
271	F G L M S V	272	D M R S T	273	H T Y
274	A F S				
275	L V	276	C D E G H I L M R S Y	277	A C D E G H K M N Q R S T Y
278	A E F G H I K N R S T V Y	279	A H Q R T	280	G Q
282	D G M Q	283	E P R S T	284	A E G H L M N Q S T Y
285	A F G H M N Q Y	286	R S W	287	I N T
288	L W	289	K S	290	I M
291	C Q R S V	292	A C F G H K N P R V W	293	A C D F G K L M P Q S V Y
294	M				
297	A	298	G I	300	R
301	A V	302	I W	303	D V
304	G I	305	D E N	306	D E S

TABLE 3-continued

Active Mutants					
Corresponding Position	Replacement	Corresponding Position	Replacement	Corresponding Position	Replacement
307	G K N Q S T V W Y	308	D G H K N P R T	309	D E G H K L M N Q R S T V W
310	A F G Q R S V Y	311	G H K Q S T	312	G K L N T
313	A E G H K L P R S T V Y	314	A D H I N Q R S T Y	315	A E G H K L M R T Y
316	D	317	A D H I K M N Q R S T W	318	D F G H I K M N Q R S T
320	E G H I K L M N R S W V Y	321	A D H K R S T Y	323	F I L
324	A D H M N R S	325	A D E G H K M N Q S V W	326	C K L V Y
327	M	328	A C G H I K L Q R S T V W Y	331	C E V
334	P T	335	S	338	Q
339	M	342	A	343	T V
347	A E G L M R S	348	D G S	349	A E K M N R T
351	A C I Q S	353	T V	356	A D H S
357	A C K S T	358	C G L T	359	D E H K M T V
360	T				
361	H	367	A C G K R S	368	A E G H K L M R S T V H R S
371	E F G H I K L M R S V	373	A E F K L M R S V	374	A H I M N P R S T V W Y
375	A G I K L M N R S T	376	A D E L M Q R S T V Y	377	D E H K P R S T
378	K N R	379	G H R S T	380	I L P T V W Y
381	E H K N Q R S V	383	A E H I K L M N S T V	385	A G H N Q R S T V
387	S	388	F H I M R T V W Y	389	A G H K L M P Q R S T Y
391	C	392	A F G K L M Q R S T V W Y	393	A D F H K L M N R S T
394	L W	395	A G H K R T W	396	A D H L Q R S T
397	R	398	L		
399	A C E K M N Q R S T V W	401	A E G Q N	403	F
404	A P T	405	A F G K M P Q R S W Y	406	A C E F G I N Q S T V Y
407	A D E F G H L M N P Q R V W	409	A D E G H I P Q R S T V	410	D K M N P Q R S T V Y
411	A H N P R S T V	412	D G H I L N Q P R S V W Y	413	A E H K N Q R S T
414	I K L M	415	G S W V Y	416	F G H I K L N Q R T V Y

TABLE 3-continued

Active Mutants					
Corresponding Position	Replacement	Corresponding Position	Replacement	Corresponding Position	Replacement
417	I	418	A E F G I L M N P Q R S V Y	419	E F G H I K L N R S W Y
420	I P	421	A E G H I K L M N Q R S T Y	422	I T
425	G I K M N R S Y	426	E G K N P Q S Y	427	H I K Q S T
428	L M P T	431	A E G H I K L N Q R S V W Y	432	E G H N S V
433	A C D E G H I K L P R S T V W	434	F G I M V	435	A C E G H R S T V Y
436	C D E G H I K L M Q R S T W Y	437	A D G H I K L M Q R S Y	438	A C D E G L N P Q R S T V W
439	A C F G H K L P Q S T V W	440	A D E F G H I L M P R S V Y	441	A D F G H K L N Q S T V Y
442	C G H K L P Q R T V W Y	443	A E F G H L M N Q R S T W	444	D E F G H I K M N R V W Y
445	A G H L M N P Q R S T V W Y	446	A C D E G H I K L M Q R T V W	447	D E F G I L M N P Q R T V W

In particular examples, provided herein is a modified PH20 polypeptide containing an amino acid replacement or replacements at a position or positions corresponding to 1, 6, 8, 9, 10, 11, 12, 14, 15, 20, 22, 24, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 46, 47, 48, 49, 50, 52, 58, 59, 63, 67, 68, 69, 70, 71, 72, 73, 74, 75, 79, 82, 83, 84, 86, 87, 89, 90, 92, 93, 94, 97, 102, 104, 107, 114, 118, 120, 127, 128, 130, 131, 132, 135, 138, 139, 140, 141, 142, 143, 144, 146, 147, 148, 149, 150, 151, 152, 155, 156, 158, 160, 162, 163, 164, 165, 166, 167, 169, 170, 172, 173, 174, 175, 178, 179, 193, 195, 196, 198, 204, 205, 206, 209, 212, 213, 215, 219, 220, 221, 222, 232, 233, 234, 235, 236, 237, 238, 240, 247, 248, 249, 257, 258, 259, 260, 261, 263, 267, 269, 271, 272, 273, 274, 276, 277, 278, 279, 282, 283, 285, 287, 289, 291, 292, 293, 298, 305, 307, 308, 309, 310, 313, 314, 315, 317, 318, 320, 321, 324, 325, 326, 328, 335, 347, 349, 351, 353, 356, 359, 367, 368, 369, 371, 373, 374, 375, 376, 377, 380, 381, 383, 385, 389, 392, 393, 395, 396, 399, 401, 404, 405, 406, 407, 409, 410, 412, 416, 418, 419, 421, 425, 427, 428, 431, 433, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446 or 447 with reference to amino acid positions set forth in SEQ ID NO:3. For example, the amino acid positions can be replacements at positions corresponding to replacement of Leucine (L) at position 1 (L1), P6, V8, I9, P10, N11, V12, F14, L15, A20, S22, F24, L26, G27, K28, F29, D30, E31, P32, L33, D34, M35, S36, L37, F38, S39, F40, I41, I46, N47, A48, T49, G50, G52, V58, D59, Y63, I67, D68, S69, I70, T71, G72, V73, T74, V75, I79, K82, I83, S84, G86, D87, L89, D90, A92, K93, K94, T97, V102, N104, M107, E114, T118, A120, D127, V128, K130, N131, R132, E135, Q138, Q139, Q140, N141, V142, Q143, L144, L146, T147, E148, A149, T150, E151, K152, Q155, E156, E158, A160, K162, D163, F164, L165, V166, E167, I169, K170, G172, K173, L174, L175, N178, H179, H193, K195, K196, G198, F204, N205, V206, K209, D212, D213, S215, N219, E220, S221, T222, T232, Q233, Q234, S235, P236, V237, A238, T240, V247, R248, E249, P257, D258, A259, K260, S261, L263, A267, T269, 1271, V272, F273, T274, Q276, V277, L278, K279, S282, Q283, E285, V287, T289, G291, E292, T293, A298, G305, L307, S308, I309, M310, M313, K314, S315, L317, L318, D320, N321, E324, T325, I326, N328, T335, Q347, Q349, V351, I353, N356, S359, P367, D368, N369, A371, Q373, L374, E375, K376, G377, F380, T381, R383, K385, E389, E392, Q393, S395, E396, Y399, S401, S404, T405, L406, S407, K409, E410, A412, D416, D418, A419, D421, A425, G427, A428, D431, F433, P436, P437, M438, E439, T440, E441, E442, P443, Q444, I445, F446 or Y447 with reference to amino acid positions set forth in SEQ ID NO:3.

Exemplary amino acid replacements in the modified PH20 polypeptides provided herein include, but are not limited, replacement with: histidine (H) at a position corresponding to position 1; A at a position corresponding to position 1; E at a position corresponding to position 1; G at a position corresponding to position 1; K at a position corresponding to position 1; Q at a position corresponding to position 1; R at a position corresponding to position 1; A at a position corresponding to position 6; M at a position corresponding to position 8; Q at a position corresponding to position 9; G at a position corresponding to position 10; H at a position corresponding to position 10; S at a position corresponding to position 11; E at a position corresponding to position 12; I at a position corresponding to position 12; K at a position corresponding to position 12; T at a position corresponding to position 12; V at a position corresponding to position 14; V at a position corresponding to position 15; M at a position corresponding to position 15; S at a position corresponding to position 20; T at a position corresponding to position 22; E at a position corresponding to position 24; H at a position corresponding to position 24; R at a position corresponding to position 24; A at a position corresponding to position 26; E at a position corresponding to position 26;

K at a position corresponding to position 26; M at a position corresponding to position 26; Q at a position corresponding to position 26; R at a position corresponding to position 26; D at a position corresponding to position 27; K at a position corresponding to position 27; R at a position corresponding to position 27; R at a position corresponding to position 28; E at a position corresponding to position 29; I at a position corresponding to position 29; K at a position corresponding to position 29; L at a position corresponding to position 29; M at a position corresponding to position 29; P at a position corresponding to position 29; R at a position corresponding to position 29; S at a position corresponding to position 29; T at a position corresponding to position 29; V at a position corresponding to position 29; G at a position corresponding to position 30; H at a position corresponding to position 30; K at a position corresponding to position 30; L at a position corresponding to position 30; M at a position corresponding to position 30; R at a position corresponding to position 30; S at a position corresponding to position 30; A at a position corresponding to position 31; C at a position corresponding to position 31; G at a position corresponding to position 31; H at a position corresponding to position 31; I at a position corresponding to position 31; K at a position corresponding to position 31; L at a position corresponding to position 31; P at a position corresponding to position 31; R at a position corresponding to position 31; S at a position corresponding to position 31; T at a position corresponding to position 31; V at a position corresponding to position 31; W at a position corresponding to position 31; C at a position corresponding to position 32; F at a position corresponding to position 32; G at a position corresponding to position 32; H at a position corresponding to position 32; W at a position corresponding to position 33; G at a position corresponding to position 33; W at a position corresponding to position 34; Q at a position corresponding to position 35; V at a position corresponding to position 35; H at a position corresponding to position 36; N at a position corresponding to position 36; F at a position corresponding to position 37; M at a position corresponding to position 37; Y at a position corresponding to position 38; A at a position corresponding to position 39; L at a position corresponding to position 39; N at a position corresponding to position 39; T at a position corresponding to position 39; L at a position corresponding to position 40; T at a position corresponding to position 41; L at a position corresponding to position 46; R at a position corresponding to position 46; D at a position corresponding to position 47; F at a position corresponding to position 47; T at a position corresponding to position 47; W at a position corresponding to position 47, with F at a position corresponding to position 48; H at a position corresponding to position 48; K at a position corresponding to position 48; N at a position corresponding to position 48; R at a position corresponding to position 49; D at a position corresponding to position 50; S at a position corresponding to position 50; M at a position corresponding to position 50; N at a position corresponding to position 52; Q at a position corresponding to position 52; R at a position corresponding to position 52; S at a position corresponding to position 52; T at a position corresponding to position 52; C at a position corresponding to position 58; K at a position corresponding to position 58; L at a position corresponding to position 58; P at a position corresponding to position 58; Q at a position corresponding to position 58; R at a position corresponding to position 58; H at a position corresponding to position 58; N at a position corresponding to position 58; Y at a position corresponding to position 58; N at a position corresponding to position 59; K at a position corresponding to position 63; L at a position corresponding to position 63;

M at a position corresponding to position 63; R at a position corresponding to position 63; W at a position corresponding to position 63; V at a position corresponding to position 67; H at a position corresponding to position 68; P at a position corresponding to position 68; Q at a position corresponding to position 68; A at a position corresponding to position 69; C at a position corresponding to position 69; E at a position corresponding to position 69; F at a position corresponding to position 69; G at a position corresponding to position 69; I at a position corresponding to position 69; L at a position corresponding to position 69; M at a position corresponding to position 69; P at a position corresponding to position 69; R at a position corresponding to position 69; T at a position corresponding to position 69; W at a position corresponding to position 69; Y at a position corresponding to position 69; A at a position corresponding to position 70; C at a position corresponding to position 70; F at a position corresponding to position 70; G at a position corresponding to position 70; H at a position corresponding to position 70; K at a position corresponding to position 70; L at a position corresponding to position 70; N at a position corresponding to position 70; P at a position corresponding to position 70; R at a position corresponding to position 70; S at a position corresponding to position 70; T at a position corresponding to position 70; V at a position corresponding to position 70; Y at a position corresponding to position 70; G at a position corresponding to position 71; N at a position corresponding to position 71; R at a position corresponding to position 71; S at a position corresponding to position 71; K at a position corresponding to position 72; M at a position corresponding to position 72; Q at a position corresponding to position 72; A at a position corresponding to position 73; H at a position corresponding to position 73; K at a position corresponding to position 73; L at a position corresponding to position 73; Q at a position corresponding to position 73; R at a position corresponding to position 73; T at a position corresponding to position 73; W at a position corresponding to position 73; A at a position corresponding to position 74; C at a position corresponding to position 74; E at a position corresponding to position 74; F at a position corresponding to position 74; G at a position corresponding to position 74; H at a position corresponding to position 74; K at a position corresponding to position 74; L at a position corresponding to position 74; M at a position corresponding to position 74; N at a position corresponding to position 74; P at a position corresponding to position 74; R at a position corresponding to position 74; S at a position corresponding to position 74; V at a position corresponding to position 74; W at a position corresponding to position 74; F at a position corresponding to position 75; L at a position corresponding to position 75; M at position corresponding to position 75; R at a position corresponding to position 75; T at a position corresponding to position 75; L at a position corresponding to position 79; L at a position corresponding to position 82; N at a position corresponding to position 82; V at a position corresponding to position 83; Q at a position corresponding to position 83; S at a position corresponding to position 83; G at a position corresponding to position 83; E at a position corresponding to position 84; F at a position corresponding to position 84; G at a position corresponding to position 84; N at a position corresponding to position 84; R at a position corresponding to position 84; A at a position corresponding to position 86; H at a position corresponding to position 86; K at a position corresponding to position 86; N at a position corresponding to position 86; S at a position corresponding to position 86; T at a position corresponding to position 86; W at a position corresponding to position 86; C at a position corresponding to position 87; G at a position corresponding to position 87; L at a position corresponding to position 87; M at a position corresponding to position 87; R at a position corresponding to position 87; S at a position corresponding to position 87; T at a position corresponding to position 87; V at a position corresponding to position 87; Y at a position corresponding to position 87; C at a position corresponding to position 89; A at a position corresponding to position 90; E at a position corresponding to position 90; H at a position corresponding to position 90; K at a position corresponding to position 90; N at a position corresponding to position 90; R at a position corresponding to position 90; C at a position corresponding to position 92; L at a position corresponding to position 92; I at a position corresponding to position 93; L at a position corresponding to position 93; Q at a position corresponding to position 93; R at a position corresponding to position 93; S at a position corresponding to position 93; T at a position corresponding to position 93; D at a position corresponding to position 94; Q at a position corresponding to position 94; R at a position corresponding to position 94; A at a position corresponding to position 97; C at an amino acid residue corresponding to position 97; D at a position corresponding to position 97; E at a position corresponding to position 97; G at a position corresponding to position 97; L at a position corresponding to position 97; S at a position corresponding to position 97; S at a position corresponding to position 102; T at a position corresponding to position 102; R at a position corresponding to position 104; L at a position corresponding to position 107; A at a position corresponding to position 114; Q at a position corresponding to position 118; H at a position corresponding to position 120; F at a position corresponding to position 120; I at a position corresponding to position 120; S at a position corresponding to position 120; V at a position corresponding to position 120; Y at a position corresponding to position 120; E at a position corresponding to position 127; H at a position corresponding to position 127; N at a position corresponding to position 127; Q at a position corresponding to position 127; R at a position corresponding to position 127; I at a position corresponding to position 128; R at a position corresponding to position 130; G at a position corresponding to position 131; I at a position corresponding to position 131; M at a position corresponding to position 131; Q at a position corresponding to position 131; R at a position corresponding to position 131; V at a position corresponding to position 131; N at a position corresponding to position 132; L at a position corresponding to position 132; D at a position corresponding to position 135; G at a position corresponding to position 135; R at a position corresponding to position 135, with L at a position corresponding to position 138; T at a position corresponding to position 139; K at a position corresponding to position 140; H at a position corresponding to position 141; R at a position corresponding to position 141; S at a position corresponding to position 141; W at a position corresponding to position 141; Y at a position corresponding to position 141; D at a position corresponding to position 142; G at a position corresponding to position 142; K at a position corresponding to position 142; N at a position corresponding to position 142; P at a position corresponding to position 142; Q at a position corresponding to position 142; R at a position corresponding to position 142; S at a position corresponding to position 142; T at a position corresponding to position 142; G at a position corresponding to position 143; K at a position corresponding to position 143; R at a position corresponding to position 144; T at a position corresponding to position 144; P at a position corresponding to position 146; R at a position corresponding to position 146; A at a position corresponding to position 147; F at a position corresponding to position 147; L at a position corresponding to position 147; R at a position corresponding to position 147; S at a position corresponding to position 147; V at a position corresponding to position 147; H at a position corresponding to position 148; K at a position corresponding to position 148; Q at a position corresponding to position 148; T at a position corresponding to position 149; V at a position corresponding to position 149; A at a position corresponding to position 150; D at a position corresponding to position 150; G at a position corresponding to position 150; N at a position corresponding to position 150; S at a position corresponding to position 150; W at a position corresponding to position 150; Y at a position corresponding to position 150; A at a position corresponding to position 151; H at a position corresponding to position 151; K at a position corresponding to position 151; L at a position corresponding to position 151; M at a position corresponding to position 151; Q at a position corresponding to position 151; R at a position corresponding to position 151; S at a position corresponding to position 151; T at a position corresponding to position 151; V at a position corresponding to position 151; W at a position corresponding to position 151; Y at a position corresponding to position 151; R at a position corresponding to position 152; T at a position corresponding to position 152; W at a position corresponding to position 152; D at a position corresponding to position 155; G at a position corresponding to position 155; K at a position corresponding to position 155; R at a position corresponding to position 155; D at a position corresponding to position 156; Q at a position corresponding to position 158; S at a position corresponding to position 158; S at a position corresponding to position 160; E at a position corresponding to position 162; A at a position corresponding to position 163; E at a position corresponding to position 163; K at a position corresponding to position 163; Q at a position corresponding to position 163; R at a position corresponding to position 163; S at a position corresponding to position 163; M at a position corresponding to position 164; V at a position corresponding to position 164; D at a position corresponding to position 165; F at a position corresponding to position 165; N at a position corresponding to position 165; S at a position corresponding to position 165; V at a position corresponding to position 165; A at a position corresponding to position 166; E at a position corresponding to position 166; F at a position corresponding to position 166; H at a position corresponding to position 166; L at a position corresponding to position 166; Q at a position corresponding to position 166; R at a position corresponding to position 166; T at a position corresponding to position 166; W at a position corresponding to position 166; Y at a position corresponding to position 166; D at a position corresponding to position 167; L at a position corresponding to position 169; R at a position corresponding to position 170; A at a position corresponding to position 172; R at a position corresponding to position 173; G at a position corresponding to position 174; K at a position corresponding to position 174; N at a position corresponding to position 174; R at a position corresponding to position 174; T at a position corresponding to position 174; T at a position corresponding to position 175; K at a position corresponding to position 178; R at a position corresponding to position 178; K at a position corresponding to position 179; Q at a position corresponding to position 193; T at a position corresponding to position 195; N at a position corresponding to position 195; with E at a position corresponding to position 196; R at a position corresponding to position 196; with D at a position corresponding to position 198; P at a position corresponding to position 204; A at a position corresponding to position 205; E at a position corresponding to position 205; L at a position corresponding to position 205; T at a position corresponding to position 205; I at a position corresponding to position 206; K at a position corresponding to position 206; L at a position corresponding to position 206; R at a position corresponding to position 206; R at a position corresponding to position 209; N at a position corresponding to position 212; S at a position corresponding to position 212; A at a position corresponding to position 213; M at a position corresponding to position 213; N at a position corresponding to position 213; H at a position corresponding to position 215; M at a position corresponding to position 215; A at a position corresponding to position 219; I at a position corresponding to position 219; K at a position corresponding to position 219; S at a position corresponding to position 219; H at a position corresponding to position 220; I at a position corresponding to position 220; L at a position corresponding to position 220; V at a position corresponding to position 220; Q at a position corresponding to position 221; G at a position corresponding to position 222; F at a position corresponding to position 232; G at a position corresponding to position 233; K at a position corresponding to position 233; R at a position corresponding to position 233; M at a position corresponding to position 234; A at a position corresponding to position 235; R at a position corresponding to position 236; C at a position corresponding to position 237; E at a position corresponding to position 237; H at a position corresponding to position 237; Q at a position corresponding to position 237; T at a position corresponding to position 237; E at a position corresponding to position 238; H at a position corresponding to amino acid position 238; S at a position corresponding to position 238; A at a position corresponding to position 240; Q at a position corresponding to position 240; I at a position corresponding to position 247; A at a position corresponding to position 248; V at a position corresponding to position 249; G at a position corresponding to position 257; T at a position corresponding to position 257; R at a position corresponding to position 257; N at a position corresponding to position 258; S at a position corresponding to position 258; P at a position corresponding to position 259; M at a position corresponding to position 260; Y at a position corresponding to position 260; A at a position corresponding to position 261; K at a position corresponding to position 261; N at a position corresponding to position 261; K at a position corresponding to position 263; R at a position corresponding to position 263; T at a position corresponding to position 267; A at a position corresponding to position 269; L at a position corresponding to position 271; M at a position corresponding to position 271; D at a position corresponding to position 272; T at a position corresponding to position 272; H at a position corresponding to position 273; Y at a position corresponding to position 273; F at a position corresponding to position 274; D at a position corresponding to position 276; H at a position corresponding to position 276; M at a position corresponding to position 276; R at a position corresponding to position 276; S at a position corresponding to position 276; Y at a position corresponding to position 276; A at a position corresponding to position 277; E at a position corresponding to position 277; H at a position corresponding to position 277; K at a position corresponding to position 277; M at a position corresponding to position 277; N at a position corresponding to position 277; Q at a position corresponding to position 277; R at a position corresponding to position 277; S at a position corresponding to position 277; T at a position corresponding to position 277; E at a position corresponding to position 278; F at a position corresponding to position 278; G at a position corresponding to position 278; H at a position corresponding to position 278; K at a position corresponding to position 278; N at a position corresponding to position 278; R at a position corresponding to position 278; S at a position corresponding to position 278; T at a position corresponding to position 278; Y at a position corresponding to position 278; H at a position corresponding to position 279; M at a position corresponding to position 282; S at a position corresponding to position 283; H at a position corresponding to position 285; T at a position corresponding to position 287; S at a position corresponding to position 289; S at a position corresponding to position 291; V at a position corresponding to position 291; C at a position corresponding to position 292; F at a position corresponding to position 292; H at a position corresponding to position 292; K at a position corresponding to position 292; R at a position corresponding to position 292; V at a position corresponding to position 292; A at a position corresponding to position 293; C at a position corresponding to position 293; D at a position corresponding to position 293; F at a position corresponding to position 293; K at a position corresponding to position 293; M at a position corresponding to position 293; P at a position corresponding to position 293; Q at a position corresponding to position 293; V at a position corresponding to position 293; Y at a position corresponding to position 293; G at a position corresponding to position 298; E at a position corresponding to position 305; G at a position corresponding to position 307; D at a position corresponding to position 308; G at a position corresponding to position 308; K at a position corresponding to position 308; N at a position corresponding to position 308; R at a position corresponding to position 308; E at a position corresponding to position 309; G at a position corresponding to position 309; H at a position corresponding to position 309; L at a position corresponding to position 309; M at a position corresponding to position 309; N at a position corresponding to position 309; Q at a position corresponding to position 309; R at a position corresponding to position 309; S at a position corresponding to position 309; T at a position corresponding to position 309; V at a position corresponding to position 309; A at a position corresponding to position 310; G at a position corresponding to position 310; Q at a position corresponding to position 310; S at a position corresponding to position 310; A at a position corresponding to position 313; G at a position corresponding to position 313; H at a position corresponding to position 313; K at a position corresponding to position 313; P at a position corresponding to position 313; R at a position corresponding to position 313; T at a position corresponding to position 313; Y at a position corresponding to position 313; with S at a position corresponding to position 314; Y at a position corresponding to position 314; A at a position corresponding to position 315; H at a position corresponding to position 315; Y at a position corresponding to position 315; A at a position corresponding to position 317; I at a position corresponding to position 317; K at a position corresponding to position 317; N at a position corresponding to position 317; Q at a position corresponding to position 317; R at a position corresponding to position 317; S at a position corresponding to position 317; T at a position corresponding to position 317; W at a position corresponding to position 317; D at a position corresponding to position 318; H at a position corresponding to position

318; K at a position corresponding to position 318; M at a position corresponding to position 318; R at a position corresponding to position 318; H at a position corresponding to position 320; K at a position corresponding to position 320; R at a position corresponding to position 320; R at a position corresponding to position 321; S at a position corresponding to position 321; N at a position corresponding to position 324; R at a position corresponding to position 324; A at a position corresponding to position 325; D at a position corresponding to position 325; E at a position corresponding to position 325; G at a position corresponding to position 325; H at a position corresponding to position 325; K at a position corresponding to position 325; M at a position corresponding to position 325; N at a position corresponding to position 325; Q at a position corresponding to position 325; S at a position corresponding to position 325; V at a position corresponding to position 325; L at a position corresponding to position 326; V at a position corresponding to position 326; C at a position corresponding to position 328; G at a position corresponding to position 328; I at a position corresponding to position 328; K at a position corresponding to position 328; L at a position corresponding to position 328; S at a position corresponding to position 328; Y at a position corresponding to position 328; S at a position corresponding to position 335; A at a position corresponding to position 347; G at a position corresponding to position 347; S at a position corresponding to position 347; M at a position corresponding to position 349; R at a position corresponding to position 349; S at a position corresponding to position 351; V at a position corresponding to position 353; with H at a position corresponding to position 356; S at a position corresponding to position 356; E at a position corresponding to position 359; H at a position corresponding to position 359; T at a position corresponding to position 359; A at a position corresponding to position 367; G at a position corresponding to position 367; K at a position corresponding to position 367; S at a position corresponding to position 367; A at a position corresponding to position 368; E at a position corresponding to position 368; K at a position corresponding to position 368; L at a position corresponding to amino acid position 368; M at a position corresponding to amino acid position 368; R at a position corresponding to position 368; T at a position corresponding to amino acid position 368; H at a position corresponding to position 369; R at a position corresponding to position 369; F at a position corresponding to position 371; H at a position corresponding to position 371; K at a position corresponding to position 371; L at a position corresponding to position 371; R at a position corresponding to position 371; S at a position corresponding to position 371; M at a position corresponding to position 373; H at a position corresponding to position 374; P at a position corresponding to position 374; A at a position corresponding to position 375; G at a position corresponding to position 375; K at a position corresponding to position 375; R at a position corresponding to position 375; D at a position corresponding to position 376; E at a position corresponding to position 376; Q at a position corresponding to position 376; R at a position corresponding to position 376; T at a position corresponding to position 376; V at a position corresponding to position 376; Y at a position corresponding to position 376; D at a position corresponding to position 377; E at a position corresponding to position 377; H at a position corresponding to position 377; K at a position corresponding to position 377; P at a position corresponding to position 377; R at a position corresponding to position 377; S at a position corresponding to position 377; T at a position corresponding to position 377; W at a position corresponding to position 380; Y at a position corresponding to position 380; S at a position corresponding to position 381; I at a position corresponding to position 383; K at a position corresponding to position 383; L at a position corresponding to position 383; S at a position corresponding to position 383; A at a position corresponding to position 385; Q at a position corresponding to position 385; V at a position corresponding to position 385; A at a position corresponding to position 389; G at a position corresponding to position 389; L at a position corresponding to position 389; K at a position corresponding to position 389; Q at a position corresponding to position 389; S at a position corresponding to position 389; A at a position corresponding to position 392; F at a position corresponding to position 392; M at a position corresponding to position 392; Q at a position corresponding to position 392; R at a position corresponding to position 392; V at a position corresponding to position 392; F at a position corresponding to position 393; M at a position corresponding to position 393; A at a position corresponding to position 395; H at a position corresponding to position 395; R at a position corresponding to position 395; A at a position corresponding to position 396; H at a position corresponding to position 396; Q at a position corresponding to position 396; S at a position corresponding to position 396; K at a position corresponding to position 399; M at a position corresponding to position 399; T at a position corresponding to position 399; V at a position corresponding to position 399; W at a position corresponding to position 399; A at a position corresponding to position 401; E at a position corresponding to position 401; A at a position corresponding to position 404; G at a position corresponding to position 405; F at a position corresponding to position 406; N at a position corresponding to position 406; A at a position corresponding to position 407; D at a position corresponding to position 407; E at a position corresponding to position 407; F at a position corresponding to position 407; H at a position corresponding to position 407; Q at a position corresponding to position 407; P at a position corresponding to position 407; A at a position corresponding to position 409; Q at a position corresponding to position 409; T at a position corresponding to position 410; Q at a position corresponding to position 412; R at a position corresponding to position 412; V at a position corresponding to position 412; L at a position corresponding to position 416; E at a position corresponding to position 418; L at a position corresponding to position 418; P at a position corresponding to position 418; R at a position corresponding to position 418; V at a position corresponding to position 418; F at a position corresponding to position 419; H at a position corresponding to position 419; I at a position corresponding to position 419; K at a position corresponding to position 419; R at a position corresponding to position 419; S at a position corresponding to position 419; Y at a position corresponding to position 419; A at a position corresponding to position 421; H at a position corresponding to position 421; K at a position corresponding to position 421; N at a position corresponding to position 421; Q at a position corresponding to position 421; R at a position corresponding to position 421; S at a position corresponding to position 421; G at a position corresponding to position 425; K at a position corresponding to position 425; Q at a position corresponding to position 427; T at a position corresponding to position 427; L at a position corresponding to position 428; A at a position corresponding to position 431; G at a position corresponding to position 431; E at a position corresponding to position

431; H at a position corresponding to position 431; K at a position corresponding to position 431; L at a position corresponding to position 431; N at a position corresponding to position 431; Q at a position corresponding to position 431; R at a position corresponding to position 431; S at a position corresponding to position 431; V at a position corresponding to position 431; A at a position corresponding to position 433; H at a position corresponding to position 433; I at a position corresponding to position 433; K at a position corresponding to position 433; L at a position corresponding to position 433; R at a position corresponding to position 433; T at a position corresponding to position 433; V at a position corresponding to position 433; W at a position corresponding to position 433; K at a position corresponding to position 436; I at a position corresponding to position 437; M at a position corresponding to position 437; A at a position corresponding to position 438; D at a position corresponding to position 438; E at a position corresponding to position 438; L at a position corresponding to position 438; N at a position corresponding to position 438; T at a position corresponding to position 438; A at a position corresponding to position 439; C at a position corresponding to position 439; K at a position corresponding to position 439; P at a position corresponding to position 439; Q at a position corresponding to position 439; T at a position corresponding to position 439; V at a position corresponding to position 439; D at a position corresponding to position 440; H at a position corresponding to position 440; M at a position corresponding to position 440; P at a position corresponding to position 440; R at a position corresponding to position 440; S at a position corresponding to position 440; A at a position corresponding to position 441; F at a position corresponding to position 441; C at a position corresponding to position 442; G at a position corresponding to position 442; R at a position corresponding to position 442; A at a position corresponding to position 443; E at a position corresponding to position 443; F at a position corresponding to position 443; G at a position corresponding to position 443; M at a position corresponding to position 443; N at a position corresponding to position 443; E at a position corresponding to position 444; H at a position corresponding to position 444; V at a position corresponding to position 444; H at a position corresponding to position 445; M at a position corresponding to position 445; N at a position corresponding to position 445; P at a position corresponding to position 445; Q at a position corresponding to position 445; S at a position corresponding to position 445; T at a position corresponding to position 445; V at a position corresponding to position 445; W at a position corresponding to position 445; A at a position corresponding to position 446; M at a position corresponding to position 446; W at a position corresponding to position 446; D at a position corresponding to position 447; E at a position corresponding to position 447; G at a position corresponding to position 447; I at a position corresponding to position 447; N at a position corresponding to position 447; P at a position corresponding to position 447; Q at a position corresponding to position 447; T at a position corresponding to position 447, and/or replacement with V at a position corresponding to position 447, each with reference to amino acid positions set forth in SEQ ID NO:3.

Exemplary of such modified PH20 polypeptides are any having the sequence of amino acids set forth in any of SEQ ID NOS: 74-855, or having a sequence of amino acids that exhibits at least 68%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS:74-855 and contains the amino acid replacement and exhibits hyaluronidase activity.

Any of the above modified PH20 polypeptides provided herein can exhibit altered, such as improved or increased, properties or activities compared to the corresponding PH20 polypeptide not containing the amino acid modification (e.g., amino acid replacement). For example, the altered activities or properties can be an increased catalytic activity and/or an increased stability under denaturing conditions.

a. Increased Activity

Provided herein are modified or variant PH20 polypeptides that contain one or more amino acid replacements in a PH20 polypeptide and that exhibit increased hyaluronidase activity compared to the corresponding PH20 polypeptide not containing the amino acid replacement(s), for example, the PH20 polypeptide set forth in any of SEQ ID NOS: 2, 3, 6-66, 68-72, 856-861, 869 or 870 or a variant thereof having at least 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 86%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity thereto. In particular, the modified or variant PH20 polypeptides provided herein exhibit increased hyaluronidase activity compared to the corresponding PH20 polypeptide not containing the amino acid replacement, for example, the PH20 polypeptide set forth in any of SEQ ID NOS: 3, 7, 32-66, 69 or 72 and in particular the PH20 polypeptide set forth in SEQ ID NO:3.

The modified PH20 polypeptide can exhibit hyaluronidase activity that is at least or about at least or 120%, 130%, 135%, 140%, 145%, 150%, 160%, 170%, 180%, 200%, 250%, 300%, 350%, 400%, 500%, 1500%, 2000%, 3000%, 4000%, 5000% of the hyaluronidase activity of the corresponding PH20 polypeptide not containing the amino acid replacement(s), for example the PH20 polypeptide set forth in any of any of SEQ ID NOS: 2, 3, 6-66, 68-72, 856-861, 869 or 870 or a variant thereof, under the same conditions. For example, the hyaluronidase activity is increased at least or about at least 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 11-fold, 12-fold, 13-fold, 14-fold, 15-fold, 16-fold, 17-fold, 18-fold, 19-fold, 20-fold, 25-fold, 30-fold, 40-fold, 50-fold, 60-fold, 70-fold, 80-fold, 90-fold, 100-fold, 200-fold, 300-fold, 400-fold or more.

In particular examples, the modified PH20 polypeptides contain an amino acid replacement at one or more amino acid positions identified as being associated with increased hyaluronidase activity. As described herein, such positions have been identified using mutagenesis and selection or screening methods to identify those positions that result in increased hyaluronidase activity. The PH20 polypeptide also can contain other modifications, such as other amino acid replacements, that alone are not associated with increased activity so long as the resulting modified PH20 polypeptide exhibits increased hyaluronidase activity compared to the PH20 not containing the amino acid modification(s), such as amino acid replacement(s). The modified PH20 polypeptide provided herein can contain 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, or more amino acid replacements. Additional modifications, such as insertions or deletions, also can be included. The amino acid replacement can be in a PH20 polypeptide as set forth in any of SEQ ID NOS: 2, 3, 6-66, 68-72, 856-861, 869 or 870 or a variant thereof having at least 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 86%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity thereto. For example, the replacement(s) can be in a human PH20 polypeptide, for example, any set forth in any of SEQ ID NOS: 3, 7, 32-66, 69 or 72 or a variant thereof.

For example, the modified PH20 polypeptides provided herein contain an amino acid replacement (substitution) at one or more amino acid positions corresponding to positions 1, 12, 15, 24, 26, 27, 29, 30, 31, 32, 33, 37, 39, 46, 48, 52, 58, 63, 67, 68, 69, 70, 71, 72, 73, 74, 75, 84, 86, 87, 92, 93, 94, 97, 118, 120, 127, 131, 135, 141, 142, 147, 148, 150, 151, 152, 155, 156, 163, 164, 165, 166, 169, 170, 174, 198, 206, 209, 212, 213, 215, 219, 233, 234, 236, 238, 247, 257, 259, 260, 261, 263, 269, 271, 272, 276, 277, 278, 282, 291, 293, 305, 308, 309, 310, 313, 315, 317, 318, 320, 324, 325, 326, 328, 347, 353, 359, 371, 377, 380, 389, 392, 395, 399, 405, 407, 409, 410, 418, 419, 421, 425, 431, 433, 436, 437, 438, 439, 440, 441, 442, 443, 445, 446 or 447 with reference to amino acid positions set forth in SEQ ID NO:3. For example, the amino acid positions can be replacements at positions corresponding to replacement of Leucine (L) at position 1 (L1), V12, L15, F24, L26, G27, F29, D30, E31, P32, L33, L37, S39, I46, A48, G52, V58, Y63, I67, D68, S69, I70, T71, G72, V73, T74, V75, S84, G86, D87, A92, K93, K94, T97, T118, A120, D127, N131, E135, N141, V142, T147, E148, T150, E151, K152, Q155, E156, D163, F164, L165, V166, I169, K170, L174, G198, V206, K209, D212, D213, S215, N219, Q233, Q234, P236, A238, V247, P257, A259, K260, S261, L263, T269, I271, V272, Q276, V277, L278, S282, G291, T293, G305, S308, I309, M310, M313, S315, L317, L318, D320, E324, T325, I326, N328, Q347, I353, S359, A371, G377, F380, E389, E392, S395, Y399, T405, S407, K409, E410, D418, A419, D421, A425, D431, F433, P436, P437, M438, E439, T440, E441, E442, P443, I445, F446 or Y447 with reference to amino acid positions set forth in SEQ ID NO:3. Exemplary of such modified PH20 polypeptides are polypeptides that exhibit at least 1.5-fold or more the activity of the corresponding PH20 polypeptide not containing the amino acid replacement.

Exemplary of amino acid replacements in the modified PH20 polypeptides provided herein include, but are not limited, replacement: with histidine (H) at a position corresponding to position 1; Q at a position corresponding to position 1; E at a position corresponding to position 12; T at a position corresponding to position 12; V at a position corresponding to position 15; E at a position corresponding to position 24; H at a position corresponding to position 24; E at a position corresponding to position 26; K at a position corresponding to position 26; K at a position corresponding to position 27; R at a position corresponding to position 27; E at a position corresponding to position 29; I at a position corresponding to position 29; L at a position corresponding to position 29; M at a position corresponding to position 29; P at a position corresponding to position 29; S at a position corresponding to position 29; V at a position corresponding to position 29; G at a position corresponding to position 30; H at a position corresponding to position 30; K at a position corresponding to position 30; M at a position corresponding to position 30; R at a position corresponding to position 30; S at a position corresponding to position 30; A at a position corresponding to position 31; C at a position corresponding to position 31; H at a position corresponding to position 31; I at a position corresponding to position 31; K at a position corresponding to position 31; L at a position corresponding to position 31; P at a position corresponding to position 31; R at a position corresponding to position 31; S at a position corresponding to position 31; T at a position corresponding to position 31; V at a position corresponding to position 31; F at a position corresponding to position 32; G at a position corresponding to position 32; H at a position corresponding to position 32; W at a position corresponding to position 33; F at a position corresponding to position 37; N at a position corresponding to position 39; T at a position corresponding to position 39; R at a position corresponding to position 46; F at a position corresponding to position 48; H at a position corresponding to position 48; N at a position corresponding to position 48; Q at a position corresponding to position 52; K at a position corresponding to position 58; Q at a position corresponding to position 58; W at a position corresponding to position 63; V at a position corresponding to position 67; H at a position corresponding to position 68; Q at a position corresponding to position 68; A at a position corresponding to position 69; C at a position corresponding to position 69; F at a position corresponding to position 69; G at a position corresponding to position 69; I at a position corresponding to position 69; L at a position corresponding to position 69; M at a position corresponding to position 69; P at a position corresponding to position 69; R at a position corresponding to position 69; W at a position corresponding to position 69; Y at a position corresponding to position 69; A at a position corresponding to position 70; C at a position corresponding to position 70; F at a position corresponding to position 70; G at a position corresponding to position 70; H at a position corresponding to position 70; K at a position corresponding to position 70; L at a position corresponding to position 70; N at a position corresponding to position 70; P at a position corresponding to position 70; R at a position corresponding to position 70; S at a position corresponding to position 70; T at a position corresponding to position 70; V at a position corresponding to position 70; R at a position corresponding to position 71; S at a position corresponding to position 71; M at a position corresponding to position 72; Q at a position corresponding to position 72; H at a position corresponding to position 73; L at a position corresponding to position 73; W at a position corresponding to position 73; A at a position corresponding to position 74; C at a position corresponding to position 74; G at a position corresponding to position 74; N at a position corresponding to position 74; P at a position corresponding to position 74; R at a position corresponding to position 74; S at a position corresponding to position 74; V at a position corresponding to position 74; W at a position corresponding to position 74; F at a position corresponding to position 75; L at a position corresponding to position 75; R at a position corresponding to position 75; T at a position corresponding to position 75; G at a position corresponding to position 84; R at a position corresponding to position 84; A at a position corresponding to position 86; C at a position corresponding to position 87; T at a position corresponding to position 87; Y at a position corresponding to position 87; C at a position corresponding to position 92; I at a position corresponding to position 93; L at a position corresponding to position 93; R at a position corresponding to position 93; T at a position corresponding to position 93; R at a position corresponding to position 94; G at a position corresponding to position 97; Q at a position corresponding to position 118; F at a position corresponding to position 120; V at a position corresponding to position 120; Y at a position corresponding to position 120; H at a position corresponding to position 127; N at a position corresponding to position 127; G at a position corresponding to position 131; R at a position corresponding to position 131; V at a position corresponding to position 131; D at a position corresponding to position 135; G at a position corresponding to position 135; R at a position corresponding to position 135, with H at a position corresponding to position 141; Y at a position corresponding to position 141; R at a position corresponding to position 142; R at a position corresponding to position 147; V at a position corresponding to position 147; K at a position corresponding to position 148; G at a position corresponding to position 150; K at a position corresponding to position 151; L at a position corresponding to position 151; M at a position corresponding to position 151; Q at a position corresponding to position 151; R at a position corresponding to position 151; R at a position corresponding to position 152; G at a position corresponding to position 155; K at a position corresponding to position 155; D at a position corresponding to position 156; A at a position corresponding to position 163; E at a position corresponding to position 163; K at a position corresponding to position 163; R at a position corresponding to position 163; M at a position corresponding to position 164; D at a position corresponding to position 165; N at a position corresponding to position 165; A at a position corresponding to position 166; F at a position corresponding to position 166; H at a position corresponding to position 166; L at a position corresponding to position 166; Q at a position corresponding to position 166; R at a position corresponding to position 166; T at a position corresponding to position 166; Y at a position corresponding to position 166; L at a position corresponding to position 169; R at a position corresponding to position 170; K at a position corresponding to position 174; D at a position corresponding to position 198; K at a position corresponding to position 206; L at a position corresponding to position 206; N at a position corresponding to position 212; M at a position corresponding to position 213; N at a position corresponding to position 213; M at a position corresponding to position 215; S at a position corresponding to position 219; K at a position corresponding to position 233; R at a position corresponding to position 233; M at a position corresponding to position 234; R at a position corresponding to position 236; E at a position corresponding to position 237; S at a position corresponding to position 238; I at a position corresponding to position 247; T at a position corresponding to position 257; P at a position corresponding to position 259; Y at a position corresponding to position 260; K at a position corresponding to position 261; N at a position corresponding to position 261; K at a position corresponding to position 263; R at a position corresponding to position 263; A at a position corresponding to position 269; L at a position corresponding to position 271; M at a position corresponding to position 271; T at a position corresponding to position 272; D at a position corresponding to position 276; S at a position corresponding to position 276; Y at a position corresponding to position 276; K at a position corresponding to position 277; R at a position corresponding to position 277; T at a position corresponding to position 277; H at a position corresponding to position 278; K at a position corresponding to position 278; N at a position corresponding to position 278; R at a position corresponding to position 278; S at a position corresponding to position 278; T at a position corresponding to position 278; Y at a position corresponding to position 278; M at a position corresponding to position 282; V at a position corresponding to position 291; A at a position corresponding to position 293; C at a position corresponding to position 293; F at a position corresponding to position 293; M at a position corresponding to position 293; P at a position corresponding to position 293; Q at a position corresponding to position 293; V at a position corresponding to position 293; E at a position corresponding to position 305; G at a position corresponding to position 308; N at a position corresponding to position 308; E at a position corresponding to position 309; L at a position corresponding to position 309; N at a position corresponding to position 309; Q at a position corresponding to position 309; R at a position corresponding to position 309; T at a position corresponding to position 309; A at a position corresponding to position 310; G at a position corresponding to position 310; K at a position corresponding to position 313; R at a position corresponding to position 313; H at a position corresponding to position 315; I at a position corresponding to position 317; K at a position corresponding to position 317; R at a position corresponding to position 317; M at a position corresponding to position 318; H at a position corresponding to position 320; K at a position corresponding to position 320; R at a position corresponding to position 320; R at a position corresponding to position 324; A at a position corresponding to position 325; D at a position corresponding to position 325; E at a position corresponding to position 325; G at a position corresponding to position 325; H at a position corresponding to position 325; K at a position corresponding to position 325; M at a position corresponding to position 325; N at a position corresponding to position 325; Q at a position corresponding to position 325; S at a position corresponding to position 325; V at a position corresponding to position 326; I at a position corresponding to position 328; K at a position corresponding to position 328; L at a position corresponding to position 328; S at a position corresponding to position 328; Y at a position corresponding to position 328; G at a position corresponding to position 347; S at a position corresponding to position 347; V at a position corresponding to position 353; with T at a position corresponding to position 359; R at a position corresponding to position 371; P at a position corresponding to position 377; T at a position corresponding to position 377; W at a position corresponding to position 380; Y at a position corresponding to position 380; K at a position corresponding to position 389; M at a position corresponding to position 392; R at a position corresponding to position 395; M at a position corresponding to position 399; T at a position corresponding to position 399; W at a position corresponding to position 399; G at a position corresponding to position 405; D at a position corresponding to position 407; Q at a position corresponding to position 407; A at a position corresponding to position 409; Q at a position corresponding to position 409; T at a position corresponding to position 410; P at a position corresponding to position 418; F at a position corresponding to position 419; I at a position corresponding to position 419; K at a position corresponding to position 419; R at a position corresponding to position 419; S at a position corresponding to position 419; H at a position corresponding to position 421; K at a position corresponding to position 421; N at a position corresponding to position 421; Q at a position corresponding to position 421; R at a position corresponding to position 421; S at a position corresponding to position 421; K at a position corresponding to position 425; A at a position corresponding to position 431; H at a position corresponding to position 431; K at a position corresponding to position 431; Q at a position corresponding to position 431; R at a position corresponding to position 431; S at a position corresponding to position 431; V at a position corresponding to position 431; L at a position corresponding to position 433; R at a position corresponding to position 433; T at a position corresponding to position 433; V at a position corresponding to position 433; K at a position corresponding to position 436; I at a position corresponding to position 437; M at a position corresponding to position

437; T at a position corresponding to position 438; V at a position corresponding to position 439; H at a position corresponding to position 440; R at a position corresponding to position 440; F at a position corresponding to position 441; R at a position corresponding to position 442; A at a position corresponding to position 443; M at a position corresponding to position 443; M at a position corresponding to position 445; P at a position corresponding to position 445; A at a position corresponding to position 446; D at a position corresponding to position 447; N at a position corresponding to position 447; and/or with Q at a position corresponding to position 447, each with reference to amino acid positions set forth in SEQ ID NO:3. The modified PH20 polypeptides can contain any one or more of the recited amino acid substitutions, in any combination, with or without additional modifications, so long at the PH20 polypeptide exhibits hyaluronidase activity, such as increased hyaluronidase activity compared to the PH20 polypeptide not containing the modification(s), for example, at least 1.5-fold increased hyaluronidase activity.

In some examples, the modified PH20 polypeptides provided herein contain one or more amino acid replacement(s) at a position(s) corresponding to position(s) 24, 29, 31, 48, 58, 69, 70, 75, 84, 97, 165, 166, 271, 278, 317, 320, 325, and/or 326 with reference to positions set forth in SEQ ID NO:3. For example, exemplary amino acid replacements include, but are not limited to, replacement with: E at a position corresponding to position 24; E at a position corresponding to position 29; V at a position corresponding to position 31; N at a position corresponding to position 48; K at a position corresponding to position 58; Q at a position corresponding to position 58; A at a position corresponding to position 69; F at a position corresponding to position 69; G at a position corresponding to position 69; P at a position corresponding to position 69; R at a position corresponding to position 69; A at a position corresponding to position 70; F at a position corresponding to position 70; G at a position corresponding to position 70; H at a position corresponding to position 70; H at a position corresponding to position 70; N at a position corresponding to position 70; R at a position corresponding to position 70; T at a position corresponding to position 70; V at a position corresponding to position 70; L at a position corresponding to position 75; T at a position corresponding to position 75; G at a position corresponding to position 84; G at a position corresponding to position 97; D at a position corresponding to position 165; L at a position corresponding to position 166; R at a position corresponding to position 166; T at a position corresponding to position 166; L at a position corresponding to position 271; H at a position corresponding to position 278; R at a position corresponding to position 278; K at a position corresponding to position 317; K at a position corresponding to position 320; E at a position corresponding to position 325, with G at a position corresponding to position 325; K at a position corresponding to position 325; N at a position corresponding to position 325; Q at a position corresponding to position 325; V at a position corresponding to position 326; each with reference to amino acid positions set forth in SEQ ID NO:3. The modified PH20 polypeptides can contain any one or more of the recited amino acid substitutions, in any combination, with or without additional modifications, so long at the PH20 polypeptide exhibits hyaluronidase activity, such as increased hyaluronidase activity compared PH20 polypeptide not containing the modification(s), for example, at least 2.0-fold increased hyaluronidase activity.

Exemplary modified PH20 polypeptides that exhibit increased activity compared to the unmodified PH20 polypeptide (e.g., set forth in SEQ ID NO:3) are any having the sequence of amino acids set forth in any of SEQ ID NOS: 73, 78, 86, 89, 91, 95, 96, 99, 100, 105, 106, 108, 109, 111, 112, 113, 115, 117, 118, 119, 120, 123-126, 128-136, 139-141, 149, 154, 155, 159, 164, 165, 167, 173, 178, 181, 191-193, 195-197, 199-205, 207-221, 225, 226, 228, 229, 231, 233, 237-239, 242, 247-254, 256, 257, 267, 269, 270, 277, 283, 293, 295, 296, 298, 300, 303, 308, 316, 318, 321, 322, 324, 325, 330, 334, 335, 338-340, 344, 348, 355, 367, 369, 371, 377, 384-388, 394, 398, 399, 401, 406-408, 410, 412, 414, 416, 419, 421-426, 428, 430, 431, 435, 448, 455, 456, 459, 462, 463, 465, 469, 478-480, 482, 484, 490, 493, 497, 501, 503, 505, 506-508, 510-512, 514, 518, 522, 523, 527, 531, 533, 537-543, 545, 551, 558, 559, 561, 563-566, 569, 572, 574, 576, 579, 581-583, 585, 587, 588, 594, 596, 602, 605, 606, 609, 613, 618-620, 624-634, 637, 640-644, 647, 648, 652, 657, 675, 695, 698, 699, 700, 712, 717, 725, 731, 732, 734, 738, 742, 746, 748-750, 757, 760, 762-765, 768-773, 775, 779, 782, 783, 786-789, 794-797, 799-801, 807, 814, 816, 819, 822, 825, 826, 830, 836, 838, 844, 847, 851, 853 or having a sequence of amino acids that exhibits at least 68%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 73, 78, 86, 89, 91, 95, 96, 99, 100, 105, 106, 108, 109, 111, 112, 113, 115, 117, 118, 119, 120, 123-126, 128-136, 139-141, 149, 154, 155, 159, 164, 165, 167, 173, 178, 181, 191-193, 195-197, 199-205, 207-221, 225, 226, 228, 229, 231, 233, 237-239, 242, 247-254, 256, 257, 267, 269, 270, 277, 283, 293, 295, 296, 298, 300, 303, 308, 316, 318, 321, 322, 324, 325, 330, 334, 335, 338-340, 344, 348, 355, 367, 369, 371, 377, 384-388, 394, 398, 399, 401, 406-408, 410, 412, 414, 416, 419, 421-426, 428, 430, 431, 435, 448, 455, 456, 459, 462, 463, 465, 469, 478-480, 482, 484, 490, 493, 497, 501, 503, 505, 506-508, 510-512, 514, 518, 522, 523, 527, 531, 533, 537-543, 545, 551, 558, 559, 561, 563-566, 569, 572, 574, 576, 579, 581-583, 585, 587, 588, 594, 596, 602, 605, 606, 609, 613, 618-620, 624-634, 637, 640-644, 647, 648, 652, 657, 675, 695, 698, 699, 700, 712, 717, 725, 731, 732, 734, 738, 742, 746, 748-750, 757, 760, 762-765, 768-773, 775, 779, 782, 783, 786-789, 794-797, 799-801, 807, 814, 816, 819, 822, 825, 826, 830, 836, 838, 844, 847, 851, 853 and contains the amino acid replacement and exhibits increased hyaluronidase activity compared to the corresponding unmodified polypeptide.

b. Increased Stability

Provided herein are PH20 polypeptides that exhibit increased stability. In particular, the PH20 polypeptides exhibit increased stability in vivo and/or in vitro. For example, the PH20 polypeptides can exhibit increased stability under various storage conditions. The modified PH20 polypeptides provided herein that exhibit increased stability display, among other parameters, increased resistance to denaturation conditions, including but not limited to, denaturation conditions caused by temperature (e.g., elevated temperature such as heat), agitation, no or low salt, and/or presence of excipients. Exemplary excipients include, but are not limited to, antiadherents, binders, coatings, fillers and diluents, flavors, colors, lubricants, glidants, preservatives, sorbents or sweeteners. For example, various excipients, such as preservatives, can act as protein denaturing agents. Modified PH20 polypeptides provided herein that exhibit increased protein stability exhibit reduced aggregation, reduced precipitation and/or increased activity when exposed to a denaturation condition compared to the corresponding PH20 not containing the amino acid replacement. For example, modified PH20 polypeptides provided herein exhibit at least or at least about or 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140%, 150%, 200%, 250%, 300%, 350%, 400%), 450%, 500% or more increased activity when exposed to a denaturation condition compared to the corresponding PH20 polypeptide not containing the amino acid replacement when exposed to the same denaturation condition.

The PH20 polypeptides provided herein that exhibit increased stability are modified or variant PH20 polypeptides that contain an amino acid replacement (substitution), deletion or insertion or other modification. Typically, the PH20 polypeptides provided herein that exhibit increased stability contain one or more amino acid replacements in a PH20 polypeptide compared to the corresponding PH20 polypeptide not containing the amino acid replacement(s), for example, the PH20 polypeptide set forth in any of SEQ ID NOS: 2, 3, 6-66, 68-72, 856-861, 869 or 870 or a variant thereof having at least 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 86%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity thereto. In particular, the modified or variant PH20 polypeptides provided herein exhibit increased stability compared to the corresponding PH20 polypeptide not containing the amino acid replacement, for example, the PH20 polypeptide set forth in any of SEQ ID NOS: 3, 7, 32-66, 69 or 72 and in particular the PH20 polypeptide set forth in SEQ ID NO:3.

In particular examples, the modified PH20 polypeptides contain an amino acid replacement at one or more amino acid positions identified as being associated with increased stability. As described herein, such positions can be identified using mutagenesis and selection or screening methods to identify those positions that result in stability (e.g., increased activity) of the polypeptide compared to the corresponding PH20 not containing the modification upon exposure to one or more denaturation conditions. The PH20 polypeptide also can contain other modifications, such as other amino acid replacements, that alone are not associated with conferring stability, so long as the resulting modified PH20 polypeptide exhibits increased stability under one or more denaturation conditions compared to the PH20 not containing the amino acid modification(s), such as amino acid replacement(s), and exhibits hyaluronidase activity. The modified PH20 polypeptide provided herein can contain 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, or more amino acid replacements. Additional modifications, such as insertions or deletions, also can be included. The amino acid replacement can be in a PH20 polypeptide as set forth in any of SEQ ID NOS: 2, 3, 6-66, 68-72, 856-861, 869 or 870 or a variant thereof having at least 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 86%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity thereto. For example, the replacements can be in a human PH20 polypeptide, for example, any set forth in any of SEQ ID NOS: 3, 7, 32-66, 69 or 72 or a variant thereof.

Exemplary of modified PH20 polypeptides provided herein are PH20 polypeptides that exhibit increased stability upon exposure to phenol compounds, high temperature (heat), and/or lack of NaCl.

i. Phenophiles

Provided herein are modified PH20 polypeptides that exhibit increased stability in the presence of phenolic compounds. Multidose formulations must contain antimicrobial preservatives to protect them from microbial contamination. For parenteral drug products, including insulin and other therapeutic agents, the most common preservatives are phenolic compounds, such as phenol, metacresol (m-cresol), benzyl alcohol, and parabens including methylparaben and propylparaben. The preservatives typically must be present at sufficient concentrations to satisfy regulatory rules. For example, regulatory requirements assert that the antimicrobial efficacy of the formulation must satisfy the preservative efficacy test (PET) requirements of the target markets. Currently different regulatory agencies have different pharmacopeial criteria for antimicrobial effectiveness for pharmaceutical products designed for multiple dosing. The PET requirements of the United States Pharmacopoeia (USP) and the European Pharmacopoeia (EP) differ considerably, imposing additional constraints in developing multidose formulations. Table 4 shows the criteria for injectable drugs to meet USP and EP criteria. Typically, formulations that meet EP (EPA or EPB) anti-microbial requirements contain more preservative than those formulated only to meet USP anti-microbial requirements.

TABLE 4

USP and EP requirement for antimicrobial effectiveness testing

			Europe	
Requirement	Time point	United States USP	EPB (Minimum)	EPA (Preferred)
Bacterial Log Reduction*	6 h			2
	24 h		1	3
	7 d	1.0	3	No recovery
	14 d	3.0	No increase	No recovery
	28 d	No increase	No increase	No recovery
Fungal Log Reduction*	7 d	No increase		2
	14 d	No increase	1	No increase
	28 d	No increase	No increase	No increase

*Log_{10} unit reduction from initial measured inoculum; No increase: not more than 0.5 log_{10} unit increase than previously measured value.

Anti-microbial preservatives can interact with proteins resulting in aggregations and negative effects on stability. Thus, although a necessary component, preservatives pose a significant problem in the development of stable, multidose formulations of proteins because they typically induce aggregation of the protein in aqueous solution. In particular, increasing or high amounts of preservatives can negatively impact the stability of a protein, including effects on physical stability (aggregation or precipitation) that can impact protein activity. For example, to meet the EP preservative efficacy requirements, relatively high amounts of phenolic compounds, such as phenol or m-cresol, can be required, which can influence stability of the protein formulation. For example, preservatives such as phenol, m-cresol, and benzyl alcohol have been shown to induce aggregation of human growth hormone (Maa and Hsu (1996) *Int. J. Pharm.* 140:155-168), recombinant interleukin-1 receptor (Remmele (1998) *Pharm. Res.* 15:200-208), human insulin-like growth factor I (Fransson (1997) *Pharm. Res.* 14:606-612), rhIFN-γ (Lam (1997) *Pharm. Res.* 14:725-729) and cytochrome c (Singh et al. (2011) *J. Pharm Sci.,* 100:1679-89). The destabilizing effect that preservatives have on proteins in solution has been a limiting factor in the development of multidose formulations, and to date, most protein therapeutics have been formulated for single use only.

PH20 hyaluronidase, such as rHuPH20, rapidly loses activity in the presence of preservatives, likely due to unfolding of the protein and subsequent aggregate formation. For example, as shown in the Examples herein, preservatives reduce PH20 enzymatic activity, particularly at elevated temperatures (see also U.S. Provisional Appl. No. 61/520,962; and U.S. application Ser. Nos. 13/507,263 and 13/507,262). For example, following incubation with 0.4% m-cresol for 4 hours, PH20 (e.g., rHuPH20) retains only about 10% of its activity (see e.g., Example 5). When incubated in the presence of 0.1% phenol and 0.15% or 0.315% m-cresol for 6 days at 37° C., PH20 (e.g., rHuPH20) retains about 0% to 15% activity, depending on the presence of other excipients or amounts of other excipients in the formulation (see e.g., Examples 9 and 10). For example, the presence of a higher concentration of salt generally increases the stability of PH20. In particular, the melting temperature of PH20, such as rHuPH20, is reduced significantly when phenolic preservatives, such as m-Cresol, are added to the formulation. For example, the unfolding temperature of rHuPH20 is reduced from 44° C. to 24° C. The lower PH20 unfolding temperatures leads to increased PH20 aggregation, especially at elevated temperatures, and reduced enzyme activity. The destabilizing effect is likely due to the hydrophobic nature of the phenolic preservatives. The hydrophobicity of the phenolic compounds can lead to interaction with rHuPH20 through nonspecific binding to the protein, ultimately perturbing the structural integrity of rHuPH20. This translates to a significant loss of rHuPH20 enzymatic activity in the presence of preservatives.

The modified PH20 polypeptides provided herein that exhibit increased stability in the presence of phenolic preservatives exhibit more than 15% enzymatic activity in the presence of at least one phenolic preservative for at least 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 24 hours, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 3 weeks, 4 weeks or more compared to the enzymatic activity of the modified PH20 polypeptide in the absence of preservative for the same time period and under the same conditions (except for the presence of preservative). In some examples, the modified PH20 polypeptides provided herein exhibit at least 16%, 17%, 18%, 19%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more enzymatic activity in the presence of a phenolic preservative compared to in the absence of preservative. For example, the phenolic preservative compound can be phenol, metacresol (m-cresol), benzyl alcohol, and/or parabens including methylparaben or propylparaben.

In particular examples, the increased stability in the presence of preservative is exhibited under temperature conditions of between or about between 0° C. to 40° C., such as between or about between 2° C. to 6° C., 24° C. to 32° C. or 35° C. to 40° C., and generally at or about at 4° C. or 5° C., 30° C. or 37° C. It is understood that since high temperature also can have a destabilizing effect on PH20 activity (see below), the percentage of enzymatic activity of a modified PH20 polypeptide provided herein in the presence of preservative is greater at lower temperatures than at higher temperatures.

Generally, the modified PH20 polypeptides provided herein exhibit increased stability, and the noted enzymatic activities, in the presence of an anti-microbial effective amount of preservative that kills or inhibits the propagation of microbial organisms in a sample of the composition. For example, the modified PH20 polypeptides provided herein exhibit increased stability in the presence of an anti-microbial effective amount of preservative that kills or inhibits the propagation of microbial organisms such that at least a 1.0 log 10 unit reduction in bacterial organisms occurs at 7 days following inoculation. In some examples, the modified PH20 polypeptides provided herein exhibit increased stability in the presence of an anti-microbial effective amount of preservative that kills or inhibits the propagation of microbial organisms such that, when tested in an antimicrobial preservative effectiveness test (APET), following inoculation of the composition with a microbial inoculum there is at least a 1.0 log 10 unit reduction in bacterial organisms at 7 days following inoculation, at least a 3.0 log 10 unit reduction of bacterial organisms at 14 days following inoculation, at least no further increase in bacterial organisms after 28 days following inoculation, and at least no increase in fungal organisms after 7 days following inoculation. In other examples, the modified PH20 polypeptides provided herein exhibit increased stability in the presence of an anti-microbial effective amount of preservative that kills or inhibits the propagation of microbial organisms such that, when tested in an antimicrobial preservative effectiveness test (APET), following inoculation of the composition with a microbial inoculum there is at least a 1.0 log 10 unit reduction of bacterial organisms at 24 hours following inoculation, at least a 3.0 log 10 unit reduction of bacterial organisms at 7 days following inoculation, no further increase in bacterial organisms after 28 days following inoculation, at least a 1.0 log 10 unit reduction of fungal organisms at 14 days following inoculation, and at least no further increase in fungal organisms after 28 days following inoculation. In yet another example, the modified PH20 polypeptides provided herein exhibit increased stability in the presence of an anti-microbial effective amount of the preservative that kills or inhibits the propagation of microbial organisms such that, when tested in an antimicrobial preservative effectiveness test (APET), following inoculation of the composition with a microbial inoculum there is at least a 2.0 log 10 unit reduction of bacterial organisms at 6 hours following inoculation, at least a 3.0 log 10 unit reduction of bacterial organisms at 24 hours following inoculation, no recovery of bacterial organisms after 28 days following inoculation of the composition with the microbial inoculum, at least a 2.0 log 10 unit reduction of fungal organisms at 7 days following inoculation, and at least no further increase in fungal organisms after 28 days following inoculation.

For example, the modified PH20 polypeptides provided herein exhibit increased stability, and above recited enzymatic activity, in the presence of a total amount of one or more phenolic preservative agents as a percentage (%) of mass concentration (w/v) that is or is between 0.05% to 0.6%, 0.1% to 0.4%, 0.1% to 0.3%, 0.15% to 0.325%, 0.15% to 0.25%, 0.1% to 0.2%, 0.2% to 0.3% or 0.3% to 0.4% inclusive.

Generally, modified PH20 polypeptides provided herein exhibit increased stability in the presence of m-cresol and/or phenol. For example, modified PH20 polypeptides provided herein exhibit increased stability in the presence of m-cresol in an amount as a % of mass concentration (w/v) in a formulation containing the modified PH20 polypeptide of between or about between 0.05% to 0.6%, 0.1% to 0.4%, 0.1% to 0.3%, 0.15% to 0.325%, 0.15% to 0.25%, 0.1% to 0.2%, 0.2% to 0.3% or 0.3% to 0.4%. In other examples, modified PH20 polypeptides provided herein exhibit increased stability in the presence of phenol in an amount at a % of mass concentration (w/v) in a formulation containing the modified PH20 polypeptide of between or about between 0.05% to 0.6%, 0.1% to 0.4%, 0.1% to 0.3%, 0.15% to

0.325%, 0.15% to 0.25%, 0.1% to 0.2%, 0.2% to 0.3% or 0.3% to 0.4% m-cresol. In further examples, modified PH20 polypeptides provided herein exhibit increased stability in the presence of phenol and m-cresol in an amount as a % of mass concentration (w/v) in a formulation containing the modified PH20 polypeptide of between or about between 0.05% to 0.25% phenol and between or about between 0.05% to 0.3% m-cresol, between or about between 0.10% to 0.2% phenol and between or about between 0.6% to 0.18% m-cresol, between or about between 0.1% to 0.15% phenol and 0.8% t 0.15% m-cresol, between or about between 0.10% to 0.15% phenol and between or about between 0.06% to 0.09% m-cresol, or between or about between 0.12% to 0.18% phenol and between or about between 0.14% to 0.22% m-cresol.

In examples herein, modified PH20 polypeptides exhibit more than 15%, such as at least 16%, 17%, 18%, 19%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more enzymatic activity in the presence of at least about between or between 0.3% to 0.4%, inclusive, m-cresol and/or phenol for at least 4 hours at 37° C. compared to the enzymatic activity of the modified PH20 polypeptide in the absence of the preservative for the same time period and under the same conditions (except for the presence of preservative). For example, modified PH20 polypeptides exhibit more than 15%, such as at least 16%, 17%, 18%, 19%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more enzymatic activity in the presence of about or 0.4% m-cresol for at least 4 hours at 37° C. compared to the enzymatic activity of the modified PH20 polypeptide in the absence of the preservative for the same time period and under the same conditions (except for the presence of preservative). Modified PH20 polypeptides provided herein also exhibit more than 15%, such as at least 16%, 17%, 18%, 19%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more enzymatic activity in the presence of at least about between or between 0.2% to 0.4%, inclusive, m-cresol and/or phenol for at least 1 day, 2 days, 3 days, 4 days, 5 days or 6 days at 37° C. compared to the enzymatic activity of the modified PH20 polypeptide in the absence of preservative for the same time period and under the same conditions (except for the presence of preservative). For example, modified PH20 polypeptides provided herein exhibit more than 15%, such as at least 16%, 17%, 18%, 19%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more enzymatic activity in the presence of about or 0.10% phenol and about or 0.15% m-cresol for at least 1 day, 2 days, 3 days, 4 days, 5 days or 6 days at 37° C. compared to the enzymatic activity of the modified PH20 polypeptide in the absence of preservative for the same time period and under the same conditions (except for the presence of preservative). In other examples, modified PH20 polypeptides provided herein exhibit more than 15%, such as at least 16%, 17%, 18%, 19%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more enzymatic activity in the presence of about or 0.315% m-cresol for at least 1 day, 2 days, 3 days, 4 days, 5 days or 6 days, generally for at least 6 days, at 37° C. compared to the enzymatic activity of the modified PH20 polypeptide in the absence of preservative for the same time period and under the same conditions (except for the presence of preservative).

For example, such modified PH20 polypeptides provided herein that exhibit increased stability to phenol compounds contain an amino acid replacement (substitution) at one or

more amino acid positions corresponding to positions 10, 12, 20, 22, 26, 34, 36, 46, 50, 52, 58, 68, 70, 74, 82, 83, 84, 86, 97, 127, 131, 138, 142, 143, 144, 166, 169, 174, 193, 195, 196, 204, 205, 206, 213, 219, 234, 237, 238, 240, 249, 261, 267, 277, 279, 291, 309, 310, 314, 315, 317, 318, 347, 367, 375, 376, 399, 401, 407, 416, 419, 421, 431, 433, 439, 440, 443 or 445 with reference to amino acid positions set forth in SEQ ID NO:3. For example, the amino acid positions can be replacements at one or more positions corresponding to replacement of (P) at position 10 (P10), V12, A20, S22, L26, D34, S36, I46, G50, G52, V58, D68, I70, T74, K82, I83, S84, Q86, T97, D127, N131, Q138, V142, Q143, L144, V166, I169, L174, H193, K195, K196, F204, N205, V206, D213, N219, Q234, V237, A238, T240, E249, S261, A267, V277, K279, G291, I309, M310, K314, S315, L317, Q347, P367, E375, K376, Y399, S401, S407, D416, A419, D421, D431, F433, E439, T440, P443 or I445 with reference to amino acid positions set forth in SEQ ID NO:3.

Exemplary of amino acid replacements in the modified PH20 polypeptides provided herein include, but are not limited to, replacement with: glycine (G) at a position corresponding to position 10; K at a position corresponding to position 12; S at a position corresponding to position 20; T at a position corresponding to position 22; M at a position corresponding to position 26; W at a position corresponding to position 34; N at a position corresponding to position 36; L at a position corresponding to position 46; M at a position corresponding to position 50; T at a position corresponding to position 52; S at a position corresponding to position 52; C at a position corresponding to position 58; K at a position corresponding to position 58; R at a position corresponding to position 58; N at a position corresponding to position 58; Y at a position corresponding to position 58; P at a position corresponding to position 58; H at a position corresponding to position 58; P at a position corresponding to position 68; V at a position corresponding to position 70; E at a position corresponding to position 74; L at a position corresponding to position 82; N at a position corresponding to position 82; V at a position corresponding to position 83; Q at a position corresponding to position 83; S at a position corresponding to position 83; G at a position corresponding to position 83; N at a position corresponding to position 84; A at a position corresponding to position 86; K at a position corresponding to position 86; E at a position corresponding to position 97; L at a position corresponding to position 97; R at a position corresponding to position 127; R at a position corresponding to position 131; L at a position corresponding to position 138; K at a position corresponding to position 142; N at a position corresponding to position 142; P at a position corresponding to position 142; S at a position corresponding to position 142; T at a position corresponding to position 142; G at a position corresponding to position 143; K at a position corresponding to position 143; T at a position corresponding to position 144; Q at a position corresponding to position 166; T at a position corresponding to position 166; L at a position corresponding to position 169; G at a position corresponding to position 174; N at a position corresponding to position 174; Q at a position corresponding to position 193; T at a position corresponding to position 195; N at a position corresponding to position 195; E at a position corresponding to position 196; R at a position corresponding to position 196; P at a position corresponding to position 204; A at a position corresponding to position 205; E at a position corresponding to position 205; I at a position corresponding to position 206; A at a position corresponding to position 213; I at a position corresponding to position 219; M at a position corresponding to position

234; T at a position corresponding to position 237; H at a position corresponding to position 238; Q at a position corresponding to position 240; V at a position corresponding to position 249; A at a position corresponding to position 261; K at a position corresponding to position 261; T at a position corresponding to position 267; K at a position corresponding to position 277; H at a position corresponding to position 279; V at a position corresponding to position 279; V at a position corresponding to position 291; E at a position corresponding to position 309; Q at a position corresponding to position 310; Y at a position corresponding to position 314; Y at a position corresponding to position 315; N at a position corresponding to position 317; W at a position corresponding to position 317; D at a position corresponding to position 318; G at a position corresponding to position 347; A at a position corresponding to position 367; R at a position corresponding to position 375; R at a position corresponding to position 376; V at a position corresponding to position 399; E at a position corresponding to position 401; A at a position corresponding to position 407; L at a position corresponding to position 416; K at a position corresponding to position 419; H at a position corresponding to position 421; E at a position corresponding to position 431; T at a position corresponding to position 433; V at a position corresponding to position 433; C at a position corresponding to position 439; P at a position corresponding to position 440; G at a position corresponding to position 443; N at a position corresponding to position 445, each with reference to amino acid residue positions set forth in SEQ ID NO:3.

The amino acid replacements) can be in a PH20 polypeptide as set forth in any of SEQ ID NOS: 2, 3, 6-66, 68-72, 856-861, 869 or 870 or a variant thereof having at least 75%, 80%, 81%, 82%, 83%), 84%, 85%, 86%, 86%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity thereto. For example, the replacement(s) can be in a human PH20 polypeptide, for example, any set forth in any of SEQ ID NOS: 3, 7, 32-66, 69 or 72 or a variant thereof.

Exemplary modified PH20 polypeptides that exhibit increased stability to phenol compounds compared to the unmodified PH20 polypeptide (e.g., set forth in SEQ ID NO:3) are any having the sequence of amino acids set forth in any of SEQ ID NOS: 83, 88, 93, 94, 101, 144, 148, 158, 171, 176, 175, 177, 178, 180, 182, 183, 184, 185, 194, 221, 240, 259, 260, 261, 262, 263, 264, 268, 270, 272, 307, 309, 327, 334, 341, 351, 352, 353, 356, 357, 358, 359, 361, 424, 426, 430, 434, 436, 443, 444, 445, 446, 447, 449, 450, 451, 454, 461, 467, 480, 487, 489, 492, 495, 504, 505, 509, 527, 544, 576, 589, 600, 603, 607, 612, 614, 647, 658, 683, 687, 733, 736, 741, 754, 763, 768, 781, 796, 797, 809, 818, 829 or 837 or having a sequence of amino acids that exhibits at least 68%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 83, 88, 93, 94, 101, 144, 148, 158, 171, 176, 175, 177, 178, 180, 182, 183, 184, 185, 194, 221, 240, 259, 260, 261, 262, 263, 264, 268, 270, 272, 307, 309, 327, 334, 341, 351, 352, 353, 356, 357, 358, 359, 361, 424, 426, 430, 434, 436, 443, 444, 445, 446, 447, 449, 450, 451, 454, 461, 467, 480, 487, 489, 492, 495, 504, 505, 509, 527, 544, 576, 589, 600, 603, 607, 612, 614, 647, 658, 683, 687, 733, 736, 741, 754, 763, 768, 781, 796, 797, 809, 818, 829 or 837 and contains the amino acid replacement, exhibits hyaluronidase activity and exhibits increased stability in the presence phenol compounds compared to the corresponding unmodified polypeptide.

In particular, provided herein is a modified PH20 polypeptide that contains an amino acid replacement with P at a position corresponding to amino acid residue 204 with reference to SEQ ID NO:3. Typically, the modified PH20 polypeptide is a human polypeptide. For example, provided herein is a modified PH20 polypeptide that contains an amino acid replacement F204P in a sequence of amino acids set forth in any of SEQ ID NOS: 3, 7, 69, 72 or 32-66, or a sequence of amino acids that exhibits at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS:3, 7, 69, 72 or 32-66 so long as the modified polypeptide contains the amino acid replacement corresponding to F204P. In other cases, the modified PH20 polypeptide is a non-human polypeptide. For example, provided herein is a modified PH20 polypeptide that contains an amino acid replacement F204P in a sequence of amino acids set forth in SEQ ID NO: 10, 12, 14, 857, 859, 861 or 870 or a sequence that exhibits at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 10, 12, 14, 857, 859, 861 or 870 so long as the modified polypeptide contains the amino acid replacement corresponding to F204P. In a further example, provided herein is a modified PH20 polypeptide that contains an amino acid replacement F205P in a sequence of amino acids set forth in SEQ ID NO:24 or Y204P in SEQ ID NO:31, or a sequence that exhibits at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO:24 or 31. Exemplary of such a modified PH20 polypeptide is a polypeptide having the sequence of amino acids set forth in SEQ ID NO:449, or having a sequence of amino acids that exhibits at least 68%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO:449 and contains the amino acid replacement F204P, exhibits increased hyaluronidase activity and exhibits increased stability to phenol compounds compared to the corresponding unmodified polypeptide (e.g., SEQ ID NO:3). In any of the above examples, the modified PH20 polypeptide that contains an amino acid replacement with P at a position corresponding to amino acid residue 204 with reference to SEQ ID NO:3 does not have the sequence of amino acids set forth in SEQ ID NO:15-22, 28 or 29.

In another example, provided herein is a modified PH20 polypeptide that contains an amino acid replacement at a position corresponding to amino acid residue 58 with reference to SEQ ID NO:3. Exemplary of amino acid replacements are replacement with lysine (K) or with arginine (R) at a position corresponding to amino acid residue 58 with reference to SEQ ID NO:3. Typically, the modified PH20 polypeptide is a human polypeptide. For example, provided herein is a modified PH20 polypeptide that contains an amino acid replacement V58K or V58R in a sequence of amino acids set forth in any of SEQ ID NOS: 3, 7, 69, 72 or 32-66, or a sequence of amino acids that exhibits at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS:3, 7, 69, 72 or 32-66. In other cases, the modified PH20 polypeptide is a non-human polypeptide. For example, provided herein is a modified PH20 polypeptide that contains an amino acid replacement V58K or V58R in a sequence of amino acids set forth in SEQ ID NO: 10, 12, 14, 20, 22, 24, 29, 857, 859, 861 or 870 or a sequence that exhibits at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 10, 12, 14, 20, 22, 24, 29, 857, 859, 861 or 870. En a further example, provided herein is a modified PH20 polypeptide that contains an amino acid replacement A58R in a sequence of amino acids set forth in SEQ ID NO:16 or 31, or a sequence that exhibits at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO:16 or 31. Exemplary of such a modified PH20 polypeptide is a polypeptide having the sequence of amino acids set forth in SEQ ID NO:182, or having a sequence of amino acids that exhibits at least 68%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO:182, which contains the amino acid replacement V58R and exhibits increased hyaluronidase activity and exhibits increased stability in the presence of phenol compounds compared to the corresponding unmodified polypeptide (e.g., SEQ ID NO:3).

ii. Thermophiles

At elevated temperatures, PH20 hyaluronidases can lose activity. Provided herein are modified PH20 polypeptides that exhibit increased stability at elevated temperatures of between or about between 30° C. to 45° C., inclusive, such as between or about between 35° C. to 42° C., in particular at or about 37° C. For example, provided herein are modified PH20 polypeptides that are stable at elevated temperatures greater than 32° C. such as 35° C. to 45° C., 37° C. to 42° C. and in particular at or about 37° C. for at least 3 hours, 4 hours, 5 hours, 6 hours, 12 hours, 1 day, 2 days, 3 days, 4 days, at least 5 days, at least 6 days or at least 7 days. Modified PH20 polypeptides that exhibit stability at elevated temperatures can be used in applications where temperatures are elevated, can fluctuate or can increase. This can occur, for example, in methods of administration utilizing pumps or other continuous infusion devices.

In particular, modified PH20 polypeptides provided herein that exhibit stability at elevated temperatures exhibit increased hyaluronidase activity at elevated temperature compared to the corresponding PH20 polypeptide not containing the modification, e.g., amino acid replacement. The PH20 polypeptides can exhibit increased hyaluronidase activity upon incubation at elevated temperatures greater than 32° C. such as 35° C. to 45° C. or 37° C. to 42° C., in particular at or about 37° C. for at least 4 hours, 5 hours, 6 hours, 12 hours, 1 day, 2 days, 3 hours, 4 hours, 5 hours, 6 hours, 12 hours, 1 day, 2 days, 3 days, 4 days, at least 5 days, at least 6 days or at least 7 days compared to the corresponding PH20 polypeptide not containing the modification incubated under the same conditions. For example, the hyaluronidase activity can be increased at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500% or more compared to the corresponding PH20 polypeptide not containing the modification incubated under the same conditions. For example, the hyaluronidase activity can be increased at least 1.1-fold, 1.2-fold, 1.3-fold, 1.4-fold, 1.5-fold, 1.6-fold, 1.7-fold, 1.8-fold, 1.9-fold, 2-fold, 3-fold, 4-fold, 5-fold or more compared to the corresponding PH20 polypeptide not containing the modification incubated under the same conditions.

In other examples, modified PH20 polypeptides provided herein that exhibit stability at elevated temperatures retain hyaluronidase activity at elevated temperatures compared to the activity of the modified PH20 polypeptide incubated at non-elevated temperatures under the same conditions (except for the differences in temperature). For example, modified PH20 polypeptides exhibit greater than or about 50%, such as greater than or at least 55%, 60%, 65%, 70%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% of the activity at elevated temperatures greater than 32° C. such as 35° C. to 45° C. or 37° C. to 42° C., in particular at or about 37° C. compared to the activity of the PH20 at non-elevated temperatures of between or about between 2° C. to 8° C. In some examples, modified PH20 polypeptides provided herein that exhibit stability at elevated temperatures exhibit increased activity at elevated temperatures compared to the activity of the modified PH20 polypeptide incubated at non-elevated temperatures under the same conditions (except for the difference in temperature). For example, modified PH20 polypeptides exhibit greater than or about 10% increased activity, such as greater than or at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500% or more of activity at elevated temperatures greater than 32° C. such as 35° C. to 45° C. or 37° C. to 42° C., in particular at or about 37° C. compared to the activity of the PH20 at non-elevated temperatures of between or about between 2° C. to 8° C. For example, modified PH20 polypeptides exhibit greater than or at least about 1.1-fold the hyaluronidase activity, such as greater than or at least 1.2-fold, 1.3-fold, 1.4-fold, 1.5-fold, 1.6-fold, 1.7-fold, 1.8-fold, 1.9-fold, 2-fold, 3-fold, 4-fold, 5-fold or more of activity at elevated temperatures greater than 32° C. such as 35° C. to 45° C. or 37° C. to 42° C., in particular at or about 37° C. compared to the activity of the PH20 at non-elevated temperatures of between or about between 2° C. to 8° C.

For example, such modified PH20 polypeptides provided herein that exhibit increased stability at elevated temperatures contain an amino acid replacement (substitution) at one or more amino acid positions corresponding to positions 1, 11, 12, 14, 20, 26, 29, 34, 50, 58, 70, 82, 83, 84, 86, 87, 140, 142, 143, 147, 152, 166, 167, 172, 174, 178, 193, 195, 206, 212, 213, 219, 233, 237, 240, 267, 277, 291, 292, 309, 313, 314, 317, 318, 347, 367, 368, 371, 374, 389, 392, 395, 396, 406, 419, 421, 439 or 443 with reference to amino acid positions set forth in SEQ ID NO:3. For example, the amino acid positions can be replacements at one or more positions corresponding to replacement of (L) at position 1 (L1), N11, V12, F14, A20, L26, F29, D34, G50, V58, I70, K82, I83, S84, Q86, D87, Q140, V142, Q143, T147, K152, V166, E167, G172, L174, N178, H193, K195, V206, D212, D213, N219, Q233, V237, T240, A267, V277, G291, E292, I309, M313, K314, L317, L318, Q347, P367, D368, A371, L374, E389, E392, S395, E396, L406, A419, D421, E439 or P443, with reference to amino acid positions set forth in SEQ ID NO:3. The resulting modified PH20 polypeptide exhibits increased stability at elevated temperatures greater than 32° C. such as 35° C. to 45° C., 37° C. to 42° C. and in particular at or about 37° C. for at least 3 hours, 4 hours, 5 hours, 6 hours, 12 hours, 1 day, 2 days, 3 days, 4 days, at least 5 days, at least 6 days, at least 7 days or more.

Exemplary amino acid replacements in the modified PH20 polypeptides provided herein include, but are not limited, replacement with: R at a position corresponding to position 1; S at a position corresponding to position 11; I at a position corresponding to position 12; V at a position corresponding to position 14; S at a position corresponding to position 20; M at a position corresponding to position 26; with R at a position corresponding to position 29; W at a position corresponding to position 34; M at a position corresponding to position 50; K at a position corresponding to position 58; Q at a position corresponding to position 58; Q at a position corresponding to position 58; V at a position corresponding to position 70; L at a position corresponding to position 82; Q at a position corresponding to position 83; R at a position corresponding to position 84; A at a position corresponding to position 86; S at a position corresponding to position 87; K at a position corresponding to position 140; S at a position corresponding to position 142; T at a position corresponding to position 142; K at a position corresponding to position 143; S at a position corresponding to position 147; T at a position corresponding to position 152; T at a position corresponding to position 166; D at a position corresponding to position 167; A at a position corresponding to position 172; G at a position corresponding to position 174; N at a position corresponding to position 174; R at a position corresponding to position 178; Q at a position corresponding to position 193; T at a position corresponding to position 195; I at a position corresponding to position 206; S at a position corresponding to position 212; A at a position corresponding to position 213; I at a position corresponding to position 219; G at a position corresponding to position 233; T at a position corresponding to position 237; A at a position corresponding to position 240; Q at a position corresponding to position 240; T at a position corresponding to position 267; E at a position corresponding to position 277; S at a position corresponding to position 291; H at a position corresponding to position 292; V at a position corresponding to position 292; S at a position corresponding to position 309; H at a position corresponding to position 313; S at a position corresponding to position 314; I at a position corresponding to position 317; T at a position corresponding to position 317; W at a position corresponding to position 317; R at a position corresponding to position 318; G at a position corresponding to position 347; A at a position corresponding to position 367; R at a position corresponding to position 368; S at a position corresponding to position 371; P at a position corresponding to position 374; A at a position corresponding to position 389; V at a position corresponding to position 392; A at a position corresponding to position 395; H at a position corresponding to position 396; N at a position corresponding to position 406; H at a position corresponding to position 419; K at a position corresponding to position 419; R at a position corresponding to position 421; S at a position corresponding to position 421; A at a position corresponding to position 439; C at a position corresponding to position 439; or G at a position corresponding to position 443, each with reference to amino acid residue positions set forth in SEQ ID NO:3.

The amino acid replacements) can be in a PH20 polypeptide as set forth in any of SEQ ID NOS: 2, 3, 6-66, 68-72, 856-861, 869 or 870 or a variant thereof having at least 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 86%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity thereto. For example, the replacement(s) can be in a human PH20 polypeptide, for example, any set forth in any of SEQ ID NOS: 3, 7, 32-66, 69 or 72 or a variant thereof.

Exemplary modified PH20 polypeptides that exhibit increased stability to phenol compounds compared to the unmodified PH20 polypeptide (e.g., set forth in SEQ ID NO:3) are any having the sequence of amino acids set forth in any of SEQ ID NOS: 79, 85, 87, 90, 93, 101, 114, 144, 171, 178, 181, 221, 259, 262, 269, 270, 282, 343, 356, 357, 359, 368, 395, 426, 429, 432, 434, 436, 441, 443, 444, 454, 460, 461, 467, 477, 487, 491, 492, 509, 525, 550, 554, 557, 584, 593, 599, 605, 611, 612, 617, 647, 658, 667, 676, 679, 709, 720, 723, 727, 740, 761, 763, 772, 773, 808, 809, or 829 or having a sequence of amino acids that exhibits at least 68%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 79, 85, 87, 90, 93, 101, 114, 144, 171, 178, 181, 221, 259, 262, 269, 270, 282, 343, 356, 357, 359, 368, 395, 426, 429, 432, 434, 436, 441, 443, 444, 454, 460, 461, 467, 477, 487, 491, 492, 509, 525, 550, 554, 557, 584, 593, 599, 605, 611, 612, 617, 647, 658, 667, 676, 679, 709, 720, 723, 727, 740, 761, 763, 772, 773, 808, 809, or 829 and contains the amino acid replacement, exhibits hyaluronidase activity and exhibits increased stability to elevated temperatures compared to the corresponding unmodified polypeptide.

iii. Absence of Salt

PH20 denatures in the presence of low salt or no salt. Thus, PH20 requires a high salt concentration of between or about between 140 mM to 200 mM to maintain stability. Other therapeutic agents, for example insulin, exhibit decreased solubility and increased crystallization/aggregation in the presence of high salt. Thus, the high salt requirements of PH20 can affect the solubility and/or activity of co-formulated therapeutic agents, while the presence of low salt can decrease the activity of PH20. This can create problems for generating PH20 co-formulations.

Provided herein are modified PH20 polypeptides that exhibit increased stability in the presence of low concentrations of salt (e.g. NaCl) less than 100 mM, for example, less than 90 mM, 80 mM, 70 mM, 60 mM, 50 mM, 40 mM, 30 mM, 25 mM, 20 mM, 15 mM, 10 mM, 5 mM or less. Generally, the modified PH20 polypeptides provided herein exhibit stability in the presence of low concentrations of salt, for example, low concentrations of NaCl of between or about between 10 mM NaCl and 100 mM NaCl, such as between or about between 15 mM to 80 mM NaCl. The modified PH20 polypeptides provided herein that exhibit stability at low concentrations of salt, such as low concentrations of NaCl (i.e., less than 100 mM or less), exhibit increased hyaluronidase activity compared to the corresponding PH20 not containing the modification(s) (e.g., amino acid replacements). For example, modified PH20 polypeptides exhibit greater than or about 10% increased activity, such as greater than or at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500% or more of activity at low concentrations of salt, such as low concentrations of NaCl (i.e., less than 100 mM), compared to the activity of the corresponding PH20 not containing the amino acid modification(s) (e.g., amino acid replacement(s) under the same conditions). For example, modified PH20 polypeptides exhibit greater than or at least about 1.1-fold the hyaluronidase activity, such as greater than or at least 1.2-fold, 1.3-fold, 1.4-fold, 1.5-fold, 1.6-fold, 1.7-fold, 1.8-fold, 1.9-fold, 2-fold, 3-fold, 4-fold, 5-fold or more of activity at low concentrations of NaCl less than 100 mM compared to the activity of the corresponding PH20 not containing the amino acid modification(s) (e.g., amino acid replacement(s) under the same conditions.

2. Inactive Mutants

Provided herein are modified PH20 polypeptides that contain one or more amino acid replacements in a PH20 polypeptide and that are inactive, whereby the polypeptides do not exhibit hyaluronidase activity or exhibit low or diminished hyaluronidase activity. The modified PH20 polypeptides provided herein that are inactive generally exhibit less than 20%, such as less than 10%, of the hyaluronidase activity of a wildtype or reference PH20 polypeptide, such as the polypeptide set forth in SEQ ID NO: 3 or 7. For example, modified PH20 polypeptides provided herein that are inactive exhibit less than 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, 0.1%, 0.05% or less of the hyaluronidase activity of a wildtype or reference PH20 polypeptide, such as the corresponding polypeptide not containing the amino acid modification (e.g., amino acid replacement), for example, a polypeptide set forth in SEQ ID NO:3 or 7.

For example, provided herein are PH20 polypeptides that are inactive and that are modified, for example by amino acid replacement or substitution, compared to a wildtype or reference PH20 polypeptide. For example, a modified PH20 polypeptide provided herein that is inactive contains one or more amino acid replacements at position(s) corresponding to position 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 25, 27, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 94, 95, 96, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 143, 144, 145, 149, 150, 152, 153, 154, 155, 156, 157, 158, 159, 161, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 197, 198, 199, 200, 201, 202, 203, 204, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 278, 279, 280, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 331, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 408, 410, 411, 412, 413, 414, 415, 416, 417, 419, 420, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 434, 437, 438, 439, 440, 441, 442, 443, 444, or 447 with reference to amino acid positions set forth in SEQ ID NO:3, so long as the resulting modified PH20 polypeptide is inactive and exhibits less than 20%, and generally less than 10%, of the hyaluronidase activity of the corresponding PH20 polypeptide not containing the amino acid replacement. Typically, the amino acid residue that is modified (e.g., replaced) at the position corresponding to any of the above positions in a PH20 polypeptide is an identical residue, a conservative residue or a semi-conservative amino acid residue to the amino acid residue set forth in SEQ ID NO:3.

Exemplary amino acid replacements at any of the above corresponding positions are set forth in Table 5. Reference to corresponding position in Table 5 is with reference to positions set forth in SEQ ID NO:3. It is understood that the replacements can be made in the corresponding position in another PH20 polypeptide by alignment therewith with the sequence set forth in SEQ ID NO:3 (see e.g., FIGS. **1** and **2**), whereby the corresponding position is the aligned position. The amino acid replacement(s) can be at the corresponding position in a PH20 polypeptide as set forth in any of SEQ ID NOS: 2, 3, 6-66, 68-72, 856-861, 869 or 870 or a variant thereof having at least 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 86%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity thereto, so long as the resulting modified PH20 polypeptide is inactive. For example, the replacement(s) can be in a corresponding position in a human PH20 polypeptide, for example, any set forth in any of SEQ ID NOS: 3, 7, 32-66, 69 or 72, or a variant thereof. In particular, any one or more of the replacements are in SEQ ID NO:3, so long as the resulting modified PH20 polypeptide is inactive and exhibits less than 20%, and generally less than 10%, of the hyaluronidase activity of the PH20 polypeptide set forth in SEQ ID NO:3

TABLE 5

Inactive Mutants

Corresponding Position	Replacement	Corresponding Position	Replacement	Corresponding Position	Replacement
2	H K W Y	3	A G K P T V	4	D E F G L P W Y
5	D G I L M N P Q R T V W Y	6	E F T V Y	7	C D F G H I K L Q R S T W Y
8	D E G H N R S W	9	C D E G N P	10	F I L M Y
11	A C F I L P T W Y	12	G H W	13	E G I L M V
14	A E G H K N P Q W	15	E F G K N P Q R S Y	16	A C D E F G H K M P R S T Y
17	D E G H I L N P Q R S T V W Y	18	C D F G H I L M P Q S T V Y	19	A C F G H I L M P Q R S V W Y
20	D E F H K L N P R T V Y	21	A C D E G H I L M R S T V W	22	C E G K P
23	A F L M N P R S T V	25	D E F G H I K L N P R S T V Y	27	C
33	C D H N V Y	34	I L N S T V	35	A D G P R S
36	C F V W Y	37	C E G N S	38	E G K L N Q R T W

TABLE 5-continued

Inactive Mutants					
Corresponding Position	Replacement	Corresponding Position	Replacement	Corresponding Position	Replacement
39	C D F W	40	A D E G K N R S T V	41	Q
42	D E H I K L M P Q R S T V	43	A E F G I K L Q R V	44	A C F G H I L N Q R S T W Y
45	A D F G P W	46	P W	47	V
48	P	49	C D G H P	50	V
51	C F I M P T W Y	52	C E F W Y	53	A C D E G H L N P Q R S T W Y
54	D E G P R Y	55	A D G H N P Q R T V Y	56	A C E G H I K L P R S T V W
57	A D F G I M P Q R V W	58	A	59	A E I L M P R T V W Y
60	A D F G H I L N P Q S T V Y	61	A E F G H N P Q R T W Y	62	A C D F I K L M P Q R S T V Y
63	C G P	64	A C D E F G H I K L P Q R S T V W	65	A C D G H I K N R S T V W Y
66	A C D E G I K L N P S T V	67	D E G P R T W	68	A C G I L P V Y
69	N T	70	Q	71	P
72	C F H I P V W	73	P	75	D G P
76	A C F G I K L P Q R S T V W	77	D E L P Q R T V	78	A D I M P T Y
79	A D F G H K N P S W Y	80	A D E F G I K L M N R S T V Y	81	A C E G H L N P S V W Y
82	Y E K	84	Y	85	A C D E F G H N Q S T
86	C P	87	P	88	A C E F G I K L M P R S T V Y
89	A D E G Q S T W Y	90	C G	91	D E F G H I L T
92	E F H K P Q R W Y	94	G P	95	A C E F G H K L M P Q S V W Y
96	S V H P R S T W	98	P	99	C E G I N P V W
100	C E F G N P R S T W Y	101	A C F H I K L M N Q R S T	102	P
103	A E F G H I L Q R T V W Y	104	F P W	105	C M N
106	A C D F H L M N P S W Y	107	A C H K P Q S V W	108	D E F K L M P Q T V Y
109	C D E L M R T W	110	F K L M P W	111	H I Q
112	C E G H L N P S	113	R V	114	I L P T V
115	A C D F G H I K L M R S V Y	116	A C D E G H I L N P Q S V W	117	D G I K N Q R S V W
118	C D E G P R W Y	119	A K I L N P R	121	A C E F G H K L M P W Y
122	A C E F I K Q R S T V	123	A C D E H L M P Q R S T V Y	124	C D E F N

TABLE 5-continued

Inactive Mutants					
Corresponding Position	Replacement	Corresponding Position	Replacement	Corresponding Position	Replacement
125	C D G L N W	126	F H I L N P Y	127	K
128	E P	129	A C D E G H L P Q S T V W	130	C D G H L N S T W Y
131	P	132	P	133	D E F G H L M N P R T V W
134	A C D F G H K P Q R S W	135	P	136	P
137	F G H N P R W Y	138	V	139	P
143	C H P R S T	144	A E F I K P Q S V Y	145	T W
149	E	149	P	150	V
152	L	153	E F M P R T V	154	D E G P S W Y
155	P Y	156	P	157	A C D E G H I K L M P Q R S T V
158	D K P R Y	159	W Y	161	W
163	C P	164	A C D E G H N P Q R	165	C H P T
166	D	167	V	168	A C D E F G K L P R S V W Y
169	A D F G H K N P Q S T Y	170	C D E G M P W Y	171	C D H M N R S W Y
172	D E I L P Q T V W Y	173	D E G H I L M P S V W Y	174	P
175	C D G K P R S	176	A C E F G H I P Q S T V W	177	A C D F G H L M Q R S T VW
178	E I L V W	180	A C E P R S	181	A C D E F H I K L R S
182	A C D E H N P Q R S T V Y	183	C D E G I K N P Q R S V	184	A C D E F G H K L M P R S V
185	A D E F G I K P R S T V W Y	186	A D G H I K L N P Q R S V W	187	A F G H I L M N Q R S T V W Y
188	A C F G H L M N P Q R S T V W	189	A E G H K L M N P R S T V W Y	190	C E F G H K L N Q R S T V W
191	A E F G K L M P Q R S T V W Y	192	C F G K L M N P Q R V W Y	193	A D K L M P V
194	A C I L P S T V	195	S	197	C
198	V W	199	E G H I K L P R S W	200	A F G H K L M P Q R S W Y
201	A F L M N P R S T V W	202	A E F G H K N P Q R V W Y	203	A D E G H L M N Q R S T V
204	A C E G H I K Q R S T	206	C D F G P Y	207	A F G M P Q R S T V W
208	D G P W	209	C P	210	A C D E G K M N P S T V W Y
211	C F G H I K M P R S T V W	212	A G H I K L M P V W	213	P S

TABLE 5-continued

Inactive Mutants					
Corresponding Position	Replacement	Corresponding Position	Replacement	Corresponding Position	Replacement
214	A C D E G H K N P R S T Y	215	C P	216	D E G H I K L M N P Q R T V
217	A C G H P Q S T V W	218	A I K L P S V	219	P
220	G K N P R W	221	D E H K P R	222	P Y
223	C D E G H K L P Q R S T V W Y	224	A D E F G M P Q R S T W Y	225	A D E G H K P Q R T V W
226	A C D E F G L N Q R S T V W Y	227	A F G H I K L M P Q R T V W Y	228	A E F G H L M N P R S T W
229	E F G K L P Q T V W	230	A E G H K M N P R S T V WY	231	A C D F G H I K L P Q R S V
232	C G H K L N P Q V Y	233	D I P S T	234	A D E G H N P S T V W
235	F L M R W Y	236	C I L N Q T Y	238	F G L P V W Y
239	C F G H I L P R S T V W Y	240	E F G N W Y	241	A C D E G I P R S T V W
242	A C D G I L M P R S T V W	243	C D F G H L M P Q R S W Y	244	A D G I V Y
245	A C F L P Q R S T V	246	A C D E G H I K L M P S T V W	247	A C F H N P Q R S T W Y
248	C D E G I M P T	249	A G H I K M Q S Y	250	C F G H K L M N P Q R S T V W
251	D F G H K P S T W	252	A D E F G H I K L N P S T Y	253	A D E G H L M N Q R S W
254	C D E G I K L P Q R T V W Y	255	C D L P V W	256	C D E G [
257	D	258	L P V W	260	C P
261	P	262	A D E G H I K Q R S T V W Y	263	E F P Q W
264	D E F G L M R T V W Y	265	A D F G H K L M N Q R S	266	A C G H M P Q R S T V W
267	D G H I K N R S W	268	A C F G H K L N P Q S T V W	269	E K L M N P Q R
270	A C E F G H I P Y	271	A D E H K T W	272	H L N P W
273	A C D G I L P Q S V W	274	C E G H N Q W Y	275	A F G I K L M Q T V W
276	F P W	278	M P	279	A C F G L W Y
280	D I M N R S T V W	281	A D G H I K N P Q R S V W	282	F L V W Y
283	A C D F W	284	C D F W	284	C I P
285	K P R T V	286	A C D F H K M P T Y	287	A C D E G K L N P Q R S
288	D E F G H I K P R T	289	A C E G H L P Q R S Y	290	D Q Y
291	A C D E F M N T W Y	292	I L T	293	E N
294	A E G H K L N P Q R S T W	295	C G H I L N P T V Y	296	C F G I K M Q R S T V W Y

TABLE 5-continued

Inactive Mutants					
Corres-ponding Posi-tion	Replacement	Corres-ponding Posi-tion	Replacement	Corres-ponding Posi-tion	Replacement
297	C E H L N P Q R S T Y	298	C E L M N P Q S T W Y	299	A C D F G H L M P Q T
300	A C D E F L M N P Q S T V W	301	E G H K M N P Q R S W Y	302	C D E F G H L M P R S T Y
303	A C D E F G K L M R W Y	304	A C D G I M N P Q S T V Y	305	L P Q R S T V Y
306	A C H I L V W Y	307	C I P	308	C F L M V W Y
310	C E F K L	311	C E F I L P V W	312	C E M V W
313	C	314	C L W	315	C I V
316	E G I K L M P R S T V W Y	317	G P	318	C P W
319	C E F G H I K M P Q R S V W Y	320	C P V	321	E M P
322	C D E G I L N P R S T V W	323	A C E G H K N R S T V	324	C F P V W Y
325	C R E G H N W	327	A E F G H N Q R S T V W Y	329	C F G H I K L N Q R S T V W Y
330	A C D E G I L M N P R S V W	331	A C D E F H K Q R S T W Y	332	A C D E F G H K L N P R S T Y
333	G H I K P R S T W Y	334	A C D E G M N R S	335	F G H I K L P V W Y
336	A E F G K N P R S T V W Y	337	C F G I K L M R T W	338	C D E F G H I K L P R T V
339	D E F G H L N P S T V W Y	340	A C D E F G H K P R S T V W	341	A E G H K L M N Q R S T V Y
342	D E F H K L M P Q R T Y	343	C D F I P W	344	F G H L M N P Q R S T W Y
345	A C E H K N Q R T V Y	346	A D F G I K L M P R S T V W	347	C F I P T V W
348	C H I L P Q R T V W Y	349	D F G P V W Y	350	A D E F H K L M N P R S T V Y
351	C D E F H N R W Y	352	A D E F G K M P Q R S T V W Y	353	C F G H K L M Q R S W
354	C D E G H I K L M P Q S V W Y	355	D F G H L M N P Q R S T V W Y	356	C G K L P R T V W
357	D E F G L M Q R	358	E H I K P Q R W	359	A F G L P W
360	A C E F G I K L M P Q R V	361	A C E G M N P Q R S V W	362	A C E G H K L M N P R S T V W
363	A C D E F G H I P Q R S T V W	364	A C D E F G K L M P R S T V Y	365	A C D E G M N P Q R S T W Y
366	A C E F G K M P Q R T W	367	E F I L M Q V	368	C P W
369	C E F I K L P Q V W	370	A D E G H K L N P Q R S V Y	371	P
372	A D E F G H K L N P R S T V W	373	C P W	374	D E
375	C F P V Y	376	I P W	377	C I L V

TABLE 5-continued

Inactive Mutants					
Corresponding Position	Replacement	Corresponding Position	Replacement	Corresponding Position	Replacement
378	D E F I L M Q T W Y	379	A C E F I L M W	380	C D E G Q R S
381	G L P W Y	382	E G H K L M N P Q R S T W Y	383	G P
384	C F M Q S T	385	C L M P W Y	386	A C F G H I L M N Q R S T V Y
387	C E F G H I L M N V W Y	388	C G P Q	389	F V
390	A C E F G H L N P R S T V W Y	391	A D G H K N P Q R S T V W Y	392	C P
393	C P	394	A D E G I K N P Q R S T V	395	C;, [
396	C F G I P Y	397	A C E F G I L M P Q T V	398	A C E G H I L N P R S T V W Y
399	D P	400	A D E F G I L MP Q R S T V Y	401	C F H K R W Y
402	A D E F L M P Q R S T V W Y	403	A C E G H K L M N P Q R T	404	C D F G H L M N R V W Y
405	C I V	406	P R	408	A E F G I K L P R S T V W Y
410	W	411	D E F G	412	E H
413	H I K LP	414	A D E G H K R S T	415	C D E P
416	C S	417	A D E F G H K M P Q R	419	D P
420	A D F G H K L N R S T W Y	422	C D G H L M N Q R S Y	423	A D E F G H L M P Q R S T V W
424	A C E G H N Q R S W Y	425	E L P W Y	426	C F M R
427	A C F L P V W Y	428	A C D E G H N R S Y	429	A D K L N P S T V W Y
430	A D E L M N S T V	431	P	432	C F I K L M P Y
434	H K P Q R W	437	T	438	Y
439	N R	440	Q	441	R
442	M N S	443	D		

3. Additional Modifications and Conjugates

The modified PH20 polypeptides include those that contain chemical or posttranslational modifications. In some examples, modified PH20 polypeptides provided herein do not contain chemical or posttranslational modifications. Chemical and post-translational modifications include, but are not limited to, PEGylation, sialation, albumination, glycosylation, farnysylation, carboxylation, hydroxylation, phosphorylation, and other polypeptide modifications known in the art.

Also, in addition to any one or more amino acid modifications, such as amino acid replacements, provided herein, modified PH20 polypeptides provided herein can be conjugated or fused to any moiety using any method known in the art, including chemical and recombinant methods, provided the resulting polypeptide retains hyaluronidase activity. For example, in addition to any one or more amino acid modifications, such as amino acid replacements, provided herein, modified PH20 polypeptides provided herein also can contain other modifications that are or are not in the primary sequence of the polypeptide, including, but not limited to, modification with a carbohydrate moiety, a polyethylene glycol (PEG) moiety, a sialic acid moiety, an Fc domain from immunoglobulin G, or any other domain or moiety. For example, such additional modifications can be made to increase the stability or serum half-life of the protein.

In some instances, the domain or other moiety is a targeted agent, including any agent that targets the conjugate to one or more cell types by selectively binding to a cell surface receptor or other cell surface moiety. For example, the domain or other moiety is a targeted agent that targets the conjugate to tumor cells. In such examples, a modified PH20 polypeptide, such as any provided herein, is linked directly or indirectly to a targeted agent. Such targeting agents include, but are not limited to, growth factors, cytokines, chemokines, antibodies, and hormones, or allelic variants, muteins, or fragments thereof so long as the targeting agent is internalized by a cell surface receptor. Exemplary, non-limiting, additional modifications are described below.

a. Decreased Immunogenicity

The modified PH20 polypeptides provided herein can be made to have decreased immunogenicity. Decreased immunogenicity can be effected by sequence changes that eliminate antigenic epitopes from the polypeptide or by altering post-translational modifications. One of skill in the art is familiar with methods of identifying antigenic epitopes in a polypeptide (see e.g., Liang et al. (2009) *BMC Bioinformatics,* 10:302; Yang et al. (2009) *Rev. Med. Virol.,* 19:77-96). In some examples, one or more amino acids can be modified in order to remove or alter an antigenic epitope.

In another example, altering the glycosylation of a protein also can effect immunogenicity. For example, altering the glycosylation of the peptide is contemplated, so long as the polypeptides minimally contain at least N-acetylglucosamine at amino acid residues corresponding to amino acid residues set forth as N200, N333 and N358 of SEQ ID NO:3 or 7.

For example, the PH20 polypeptides can be modified such that they lack fucose, particularly bifucosylation. In particular, the PH20 polypeptides provided herein are not bifucosylated. This can be achieved by expressing and producing the PH20 polypeptide in host cells that do not effect bifucosylation. Fucose is a deoxyhexose that is present in a wide variety of organisms, including mammals, insects and plants. Fucosylated glycans are synthesized by fucosyltransferases; see, e.g., Ma et al., *Glycobiology,* 16(12):158R-184R, (2006); Nakayama et al., *J. Biol. Chem.,* 276:16100-16106 (2001); and Sturla et al., *Glycobiology,* 15(10):924-935 (2005). In humans, fucose frequently exists as a terminal modification to glycan structures, and the presence of fucose α1,6-linked to N-acetylglucosamine has been shown to be important in glycoprotein processing and recognition. In insects, N-glycan core structures exhibit bifucosylation with α1,6- and α1,3-linkages. Insect cell core fucosylation with α1,3-linkages generates a carbohydrate epitope that is immunogenic in humans (see, e.g., US Publication No. 20070067855). For example, PH20 polypeptides provided herein can be generated in host cells that are incapable of bifucosylating the polypeptide. Thus, while insect cells or other cells that bifucosylate can be used for expression of the polypeptides, typically mammalian cells, such as CHO cells, are used.

In some examples, defucosylated, or fucose-deficient PH20 polypeptides can be generated in insect cells with modified glycosylation pathways, through the use of baculovirus expression vectors containing eukaryotic oligosaccharide processing genes, thereby creating "mammalianized" insect cell expression systems (see, e.g., U.S. Pat. No. 6,461,863). Alternatively, antigenicity can be eliminated by expression of PH20 polypeptides in insect cells lacking α1,3-fucosylatransferase (FT3) (see, e.g., US Publication No. 20070067855). In other examples, defucosylated or fucose-deficient PH20 polypeptides can be generated, for example, in cell lines that produce defucosylated proteins, including Led 3 CHO cells deficient in protein fucosylation (Ripka et al. *Arch. Biochem. Biophys.* 249:533-545 (1986); U.S. Pat. Pub. No. 2003/0157108; and WO 2004/056312), and knockout cell lines, such as alpha-1,6-fucosyltransferase gene, FUT8, knockout CHO cells (Yamane-Ohnuki et al. *Biotech. Bioeng.* 87:614 (2004)).

b. Conjugation to Polymers

In some examples, the modified PH20 polypeptides provided herein are conjugated to polymers. Exemplary polymers that can be conjugated to the PH20 polypeptides, include natural and synthetic homopolymers, such as polyols (i.e., poly-OH), polyamines (i.e., poly-NH_2) and polycarboxylic acids (i.e., poly-COOH), and further heteropolymers, i.e., polymers containing one or more different coupling groups, e.g., hydroxyl groups and amine groups. Examples of suitable polymeric molecules include polymeric molecules selected from among polyalkylene oxides (PAO), such as polyalkylene glycols (PAG), including polyethylene glycols (PEG), methoxypolyethylene glycols (mPEG) and polypropylene glycols, PEG-glycidyl ethers (Epox-PEG), PEG-oxycarbonylimidazole (CDI-PEG), branched polyethylene glycols (PEGs), polyvinyl alcohol (PVA), polycarboxylates, polyvinylpyrrolidone, poly-D,L-amino acids, polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, dextrans including carboxymethyl-dextrans, heparin, homologous albumin, celluloses, including methylcellulose, carboxymethylcellulose, ethylcellulose, hydroxyethylcellulose, carboxyethylcellulose and hydroxypropylcellulose, hydrolysates of chitosan, starches such as hydroxyethyl-starches and hydroxypropyl-starches, glycogen, agaroses and derivatives thereof, guar gum, pullulan, inulin, xanthan gum, carrageenan, pectin, alginic acid hydrolysates and bio-polymers.

Typically, the polymers are polyalkylene oxides (PAO), such as polyethylene oxides, such as PEG, typically mPEG, which have few reactive groups capable of cross-linking. Typically, the polymers are non-toxic polymeric molecules such as (methoxy)polyethylene glycol (mPEG) which can be covalently conjugated to the PH20 polypeptides (e.g., to attachment groups on the protein surface) using a relatively simple chemistry.

Suitable polymeric molecules for attachment to the PH20 polypeptides include, but are not limited to, polyethylene glycol (PEG) and PEG derivatives such as methoxy-polyethylene glycols (mPEG), PEG-glycidyl ethers (Epox-PEG), PEG-oxycarbonylimidazole

(CDI-PEG), branched PEGs, and polyethylene oxide (PEO) (see e.g., Roberts et al., *Advanced Drug Delivery Review* 2002, 54:459-476; Harris and Zalipsky (eds.) "Poly (ethylene glycol), Chemistry and Biological Applications" ACS Symposium Series 680, 1997; Mehvar et al., *J. Pharm. Pharmaceut. Sci.,* 3(1):125-136, 2000; Harris and Chess (2003) *Nat Rev Drug Discov.* 2(3):214-21; and Tsubery, *J Biol. Chem* 279(37):38118-24, 2004). The polymeric molecule can be of a molecular weight typically ranging from about 3 kDa to about 60 kDa. In some embodiments the polymeric molecule that is conjugated to a PH20 polypeptide provided herein has a molecular weight of 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 or more than 60 kDa.

Various methods of modifying polypeptides by covalently attaching (conjugating) a PEG or PEG derivative (i.e., "PEGylation") are known in the art (see e.g., U.S. 2006/0104968; U.S. Pat. No. 5,672,662; U.S. Pat. No. 6,737,505; and U.S. 2004/0235734). Techniques for PEGylation include, but are not limited to, specialized linkers and coupling chemistries (see e.g., Roberts, *Adv. Drug Deliv. Rev.* 54:459-476, 2002), attachment of multiple PEG moieties to a single conjugation site (such as via use of branched

PEGs; see e.g., Guiotto et al., *Bioorg. Med. Chem. Lett.* 12:177-180, 2002), site-specific PEGylation and/or mono-PEGylation (see e.g., Chapman et al., *Nature Biotech.* 17:780-783, 1999), and site-directed enzymatic PEGylation (see e.g., Sato, *Adv. Drug Deliv. Rev.*, 54:487-504, 2002) (see, also, for example, Lu and Felix (1994) *Int. J. Peptide Protein Res.* 43:127-138; Lu and Felix (1993) *Peptide Res.* 6:140-6, 1993; Felix et al. (1995) *Int. J. Peptide Res.* 46:253-64; Benhar et al. (1994) *J. Biol. Chem.* 269:13398-404; Brumeanu et al. (1995) *J Immunol.* 154:3088-95; see also, Caliceti et al. (2003) *Adv. DrugDeliv. Rev.* 55(10): 1261-77 and Molineux (2003) *Pharmacotherapy* 23 (8 Pt 2):3S-8S). Methods and techniques described in the art can produce proteins having 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more than 10 PEG or PEG derivatives attached to a single protein molecule (see e.g., U.S. 2006/0104968).

Numerous reagents for PEGylation have been described in the art. Such reagents include, but are not limited to, N-hydroxysuccinimidyl (NHS) activated PEG, succinimidyl mPEG, mPEG2-N-hydroxysuccinimide, mPEG succinimidyl alpha-methylbutanoate, mPEG succinimidyl propionate, mPEG succinimidyl butanoate, mPEG carboxymethyl 3-hydroxybutanoic acid succinimidyl ester, homobifunctional PEG-succinimidyl propionate, homobifunctional PEG propionaldehyde, homobifunctional PEG butyraldehyde, PEG maleimide, PEG hydrazide, p-nitrophenyl-carbonate PEG, mPEG-benzotriazole carbonate, propionaldehyde PEG, mPEG butryaldehyde, branched mPEG2 butyraldehyde, mPEG acetyl, mPEG piperidone, mPEG methylketone, mPEG "linkerless" maleimide, mPEG vinyl sulfone, mPEG thiol, mPEG orthopyridylthioester, mPEG orthopyridyl disulfide, Fmoc-PEG-NHS, Boc-PEG-NHS, vinylsulfone PEG-NHS, acrylate PEG-NHS, fluorescein PEG-NHS, and biotin PEG-NHS (see e.g., Monfardini et al., *Bioconjugate Chem.* 6:62-69, 1995; Veronese et al., *J. Bioactive Compatible Polymers* 12:197-207, 1997; U.S. Pat. No. 5,672,662; U.S. Pat. No. 5,932,462; U.S. Pat. No. 6,495,659; U.S. Pat. No. 6,737,505; U.S. Pat. No. 4,002,531; U.S. Pat. No. 4,179,337; U.S. Pat. No. 5,122,614; U.S. Pat. No. 5,324,844; U.S. Pat. No. 5,446,090; U.S. Pat. No. 5,612,460; U.S. Pat. No. 5,643,575; U.S. Pat. No. 5,766,581; U.S. Pat. No. 5,795,569; U.S. Pat. No. 5,808,096; U.S. Pat. No. 5,900,461; U.S. Pat. No. 5,919,455; U.S. Pat. No. 5,985,263; U.S. Pat. No. 5,990,237; U.S. Pat. No. 6,113,906; U.S. Pat. No. 6,214,966; U.S. Pat. No. 6,258,351; U.S. Pat. No. 6,340,742; U.S. Pat. No. 6,413,507; U.S. Pat. No. 6,420,339; U.S. Pat. No. 6,437,025; U.S. Pat. No. 6,448,369; U.S. Pat. No. 6,461,802; U.S. Pat. No. 6,828,401; U.S. Pat. No. 6,858,736; U.S. 2001/0021763; U.S. 2001/0044526; U.S. 2001/0046481; U.S. 2002/0052430; U.S. 2002/0072573; U.S. 2002/0156047; U.S. 2003/0114647; U.S. 2003/0143596; U.S. 2003/0158333; U.S. 2003/0220447; U.S. 2004/0013637; US 2004/0235734; U.S. 2005/0114037; U.S. 2005/0171328; U.S. 2005/0209416; EP 1064951; EP 0822199; WO 01076640; WO 0002017; WO 0249673; WO 9428024; WO 0187925; and WO 2005000360).

D. Methods for Identifying Modified Hyaluronan-Degrading Enzymes with Altered Properties or Activities

Provided herein are methods for identifying a modified or variant hyaluronan-degrading enzyme, such as a modified hyaluronidase or modified PH20 polypeptide, that exhibits an altered activity or property compared to an unmodified hyaluronan-degrading enzyme. In particular, the methods provided herein can be used to screen for one or more modified hyaluronan-degrading enzymes, such as one or more modified hyaluronidase or PH20 polypeptide, that exhibits increased activity and/or increased stability in the presence of a denaturation agent or condition. For example, the methods can be used to identify a modified or variant hyaluronan-degrading enzyme, such as a modified or variant hyaluronidase or modified or variant PH20 polypeptide, that exhibits increased stability by virtue of increased resistance to denaturation conditions, including but not limited to, denaturation conditions caused by temperature (e.g., elevated temperature such as heat), agitation, no or low salt, presence of an excipient and/or a denaturing agent. Exemplary denaturing agents or excipients include, but are not limited to, antiadherents, binders, coatings, fillers and diluents, flavors, colors, lubricants, glidants, preservatives, sorbents or sweeteners. For example, various excipients, such as preservatives, can act as protein denaturing agents. In the method, the activity also can be compared to an unmodified hyaluronan-degrading enzyme under the same denaturation condition, and a modified hyaluronan-degrading enzyme identified or selected that exhibits greater activity than the corresponding unmodified hyaluronan-degrading enzyme.

In the method, one or more modified hyaluronan-degrading enzymes are provided. In some examples, a library of modified molecules is prepared. Methods of mutagenesis and generation of libraries or collections of variant molecules is described herein and is known to one of skill in the art using standard recombinant DNA techniques. In one example, the enzymes that are tested can be pooled and screened, whereby the method permits selection of only those enzymes that exhibit a desired activity. In another example, the tested enzymes can be physically separated and screened individually, such as by formatting in arrays, such as addressable arrays.

In one aspect of the method, the modified hyaluronan-degrading enzymes are tested or screened for hyaluronidase activity in the presence and absence of one or more denaturation conditions or denaturing agent. After testing under both sets of conditions, the activities are assessed in order to identify modified hyaluronan-degrading enzymes that exhibit activity in the presence of the denaturation condition. The desired level or amount of activity selected as a cut-off in the methods can be empirically determined by the user, and is dependent on factors such as the particular hyaluronan-degrading enzyme, the desired application or use of the hyaluronan-degrading enzyme, the particular denaturation condition or denaturing agent and other similar factors. Typically, a modified hyaluronan-degrading enzyme is identified that exhibits at least 5% or 10% of the activity in the presence of a denaturing agent or condition compared to in its absence, and generally at least 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or more, for example at least 40% of the activity.

Additionally or alternatively, the activity of the modified hyaluronan-degrading enzyme in the presence of one or more denaturation conditions or denaturing agents is compared to the activity of the corresponding unmodified hyaluronan-degrading enzyme in the presence of the same denaturation agent(s) or condition(s). In such examples, it is understood that the activity of the modified and unmodified enzyme are tested under the same conditions (e.g., time, temperature, composition), except for the difference in the particular enzyme tested (unmodified versus modified). A modified hyaluronan-degrading enzyme is identified that exhibits greater activity, such as at least 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 250%, 300%, 400%, 500% or more of the activity of the unmodified hyaluronan-degrading enzyme.

The method can be performed a plurality of times, whereby the steps of the method are repeated 1, 2, 3, 4, or 5 times. The method provided herein also is iterative. In one example, after the method is performed, any identified modified hyaluronan-degrading enzyme can be modified or further modified to increase or optimize the activity.

A description of the steps of the method and components of the method are provided in the subsections that follow.

1. Hyaluronan-Degrading Enzymes and Libraries of Modified Hyaluronan-Degrading Enzymes

In the methods herein, one or more modified hyaluronan-degrading enzymes, such as a hyaluronidase or a PH20 polypeptide, are tested for a desired activity or property, such as increased stability (e.g., increased resistance to a denaturation condition). The modified hyaluronan-degrading enzyme can be modified compared to an unmodified hyaluronan-degrading enzyme, such as any hyaluronan-degrading enzyme known in the art. Hyaluronan-degrading enzymes are a family of enzymes that degrade hyaluronic acid, which is an essential component of the extracellular matrix and a major constituent of the interstitial barrier. Hyaluronan-degrading enzymes act to degrade hyaluronan by cleaving hyaluronan polymers, which are composed of repeating disaccharides units: D-glucuronic acid (GlcA) and N-acetyl-D-glucosamine (GlcNAc), linked together via alternating β1→4 and β-1→3 glycosidic bonds. By catalyzing the hydrolysis of hyaluronic acid, a major constituent of the interstitial barrier, hyaluronan-degrading enzymes lower the viscosity of hyaluronic acid, thereby increasing tissue permeability. Accordingly, hyaluronan-degrading enzymes for the uses and methods provided herein include any enzyme having the ability to catalyze the cleavage of a hyaluronan disaccharide chain or polymer. In some examples, the hyaluronan-degrading enzyme cleaves the β-1→4 glycosidic bond in the hyaluronan chain or polymer. In other examples, the hyaluronan-degrading enzyme catalyzes the cleavage of the β-1→3 glycosidic bond in the hyaluronan chain or polymer.

Hyaluronan-degrading enzymes include enzymes that are membrane-bound or that are soluble forms that are secreted from cells. Thus, where hyaluronan-degrading enzymes include a glycosylphosphatidylinositol (GPI) anchor signal sequence and/or are otherwise membrane-anchored or insoluble, such hyaluronan-degrading enzymes can be provided in soluble form by C-terminal truncation or deletion of all or a portion of the GPI anchor signal sequence to render the enzyme secreted and soluble. Thus, hyaluronan-degrading enzymes include C-terminally truncated variants, e.g., truncated to remove all or a portion of a GPI anchor signal sequence. Examples of such soluble hyaluronidases are soluble PH20 hyaluronides, such as any set forth in U.S. Pat. No. 7,767,429; U.S. Publication Nos. US 2004/0268425 and US 2010/0143457.

Exemplary hyaluronan-degrading enzymes are non-human animal or human hyaluronidases, bacterial hyaluronidases, hyaluronidases from leeches or chondroitinases that exhibit hyaluronan-degrading activity, including soluble or truncated forms thereof that are active. Exemplary non-human animal hyaluronidases are any set forth in any of SEQ ID NOS: 8-31, 856-861, 869, 870, 871-886, or mature, C-terminally truncated variants that are soluble and active, or active forms thereof. Exemplary human hyaluronidases are any set forth in any of SEQ ID NOS: 2, 3, 6, 7, 32-66, 68-72 or 887-890, or mature, C-terminally truncated variants that are soluble and active, or active forms thereof, and in particular any of SEQ ID NOS: 3, 7, 32-66, 69 or 72. Exemplary bacterial hyaluronidases are any set forth in any of SEQ ID NOS: 891-919 or mature, C-terminally truncated variants that are soluble and active, or active forms thereof. Exemplary hyaluronidases from leeches are set forth in SEQ ID NO:920 or 921, or mature, C-terminally truncated variants that are soluble and active, or active forms thereof. Exemplary chondroitinases that have hyaluronan-degrading enzyme activity are set forth in SEQ ID NO:922-924, or mature, C-terminally truncated variants that are soluble and active, or active forms thereof.

For example, one or more modified PH20 polypeptides are tested for a desired activity or property, such as increased stability (e.g., increased resistance to a denaturation condition). The modified PH20 polypeptide can be modified compared to an unmodified PH20 polypeptide, such as any known PH20 polypeptide native, wildtype or reference polypeptide. For example, the modified PH20 polypeptide is modified compared to a full-length, soluble or active form of a PH20 polypeptide, such as any set forth in any of SEQ ID NOS: 3, 7, 32-66, 69 or 72, or a polypeptide that exhibits at least 85%, such as at least 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 3, 7, 32-66, 69 or 72. In particular examples of the method herein, the starting or unmodified PH20 polypeptide has the sequence of amino acids set forth in SEQ ID NO:3.

Libraries or collections of modified hyaluronan-degrading enzymes can be screened. Hyaluronan-degrading enzymes can be modified by any process known to one of skill in the art that can alter the structure of a protein. Examples of modifications include replacement, addition, and deletion of one or more amino acids of the protein to form libraries or collections of modified hyaluronan-degrading enzymes. It is within the level of one of skill in the art to generate modified or variant proteins for use in the methods herein. Methods of mutagenesis are well known in the art and include, for example, site-directed mutagenesis such as for example QuikChange (Stratagene) or saturation mutagenesis. Mutagenesis methods include, but are not limited to, site-mediated mutagenesis, PCR mutagenesis, cassette mutagenesis, site-directed mutagenesis, random point mutagenesis, mutagenesis using uracil containing templates, oligonucleotide-directed mutagenesis, phosphorothioate-modified DNA mutagenesis, mutagenesis using gapped duplex DNA, point mismatch repair, mutagenesis using repair-deficient host strains, restriction-selection and restriction-purification, deletion mutagenesis, mutagenesis by total gene synthesis, double-strand break repair, and many others known to persons of skill. In the methods herein, mutagenesis can be effected across the full length of a protein or within a region of a protein. The mutations can be made rationally or randomly.

In some examples, the methods provided herein are performed such that the identity of each mutant protein is known a priori before the protein is tested. For example, the methods provided herein can be conducive to mutagenesis and screening or testing methods that are addressable. This can permit the ease of comparisons between the activities of tested proteins without the need for sequencing of identified proteins. For example, site-directed mutagenesis methods can be used to individually generate mutant proteins. Mutagenesis can be performed by the replacement of single amino acid residues at specific target positions one-by-one, such that each individual mutant generated is the single product of each single mutagenesis reaction. Mutant DNA molecules can be designed, generated by mutagenesis and cloned individually, such as in addressable arrays, such that they are physically separated from each other and each one is the single product of an independent mutagenesis reaction. The amino acids selected to replace the target positions on the particular protein being optimized can be either all of the remaining 19 amino acids, or a more restricted group containing only selected amino acids. In some methods provided herein, each amino acid that is replaced is independently replaced by 19 of the remaining amino acids or by less than 19 of the remaining amino acids, such as 10, 11, 12, 13, 14, 15, 16, 17 or 18 of the remaining amino acids.

2. Screening or Testing for a Desired Activity or Property

The hyaluronidase activity or other activity of a composition containing a modified hyaluronan-degrading enzyme is screened or tested under conditions that expose the hyaluronan-degrading enzyme to a denaturation condition or a denaturing agent (presence of denaturation condition or denaturing agent). The denaturing condition or denaturing agent need not be a condition or agent that is completely deadly to the enzyme, but generally is any condition or agent that destabilizes enzyme activity over time. For example, the denaturation condition can be a condition caused by temperature (e.g., elevated temperature such as greater than or about or 30° C., for example, 30° C. to 42° C. such as or about 37° C.), agitation, no or low salt (e.g., NaCl), and/or caused by the presence of a denaturing agent, such as the presence of excipients (e.g., presence of preservatives).

For purposes of selecting or identifying a modified hyaluronan-degrading enzyme that exhibits stability or increased stability under the denaturation condition, activity can be compared to activity of the modified hyaluronan-degrading enzyme in the absence of the denaturation condition and/or activity of the corresponding unmodified hyaluronan-degrading enzyme in the presence of the denaturation condition. For example, the modified hyaluronan-degrading enzyme also can be screened or tested under the same conditions, except not including a denaturing condition or denaturing agent (absence of denaturation condition or denaturing agent). If desired, the activity of the corresponding unmodified hyaluronan-degrading enzyme (e.g., the hyaluronan-degrading enzyme not containing the amino acid replacements)) can also be tested under the same conditions that expose the hyaluronan-degrading enzyme to the same denaturation condition or a denaturing agent.

For example, each member of a library or collection of modified hyaluronan-degrading enzymes is incubated under or exposed to one or more denaturation conditions. The incubation or exposure can occur in vivo or in vitro. Typically, the assay is performed in vitro. The same modified enzyme also is exposed or incubated to a reference or control condition that does not contain the denaturation condition. The activities under both conditions are compared in order to identify modified hyaluronan-degrading enzymes that exhibit stability upon exposure to a denaturation condition or conditions. Further, in screening or identifying the activity of the enzyme under the two different sets of conditions, generally the only conditions that are varied in the assay relate to the presence or absence of one or more denaturation conditions. The other conditions of the assay, including but not limited to, time, temperature and/or other incubation conditions, can be the same for both sets of conditions.

For example, exposure can be achieved by incubation of a modified hyaluronan-degrading enzyme in an assay buffer or composition that has been modified or adjusted to contain a denaturing agent such as an excipient or low or no salt. Exemplary denaturing agents or excipients include, but are not limited to, antiadherents, binders, coatings, fillers and diluents, flavors, colors, lubricants, glidants, preservatives, sorbents or sweeteners. The choice of buffer that is used can be empirically determined by one skilled in the art depending on the particular parameter or parameters being modified. Exemplary assay buffers are Good's buffers (see e.g., Good et al. (1966) Biochemistry, 5:467-477). Examples of such buffers include, but are not limited to ACES, ADA, BES, Bicine, BIS-TRIS, CAPS, HEPES, MES, MOPS, PIPES, TRIS or Trizma® buffers. Further, the amount or concentration of the excipient or salt can be empirically determined by one of skill in the art depending on the choice of excipient or salt and the desired level or activity of the modified hyaluronan-degrading enzyme.

In one example, the assay buffer or composition is modified by inclusion of an amount of a denaturing agent or denaturing excipient that is a preservative, for example; a phenolic preservative. The phenolic preservative can be phenol, metacresol (m-cresol), benzyl alcohol, and parabens including methylparaben and propylparaben. In particular, the phenolic preservative is phenol and/or m-cresol. The total amount of one or more phenolic preservative agents as a percentage (%) of mass concentration (w/v) can be between 0.05% to 0.6%, 0.1% to 0.4%, 0.1% to 0.3%, 0.15% to 0.325%, 0.15% to 0.25%, 0.1% to 0.2%, 0.2% to 0.3% or 0.3% to 0.4% inclusive. In such an example, the activity of the modified hyaluronan-degrading enzyme is tested or assessed in the presence of such a total amount (e.g., between or about between 0.05% to 0.6%) of one or more preservatives, for example, one or more phenolic preservatives. In some examples, the modified hyaluronan-degrading enzyme also can be tested or assessed under a control or reference condition in which the assay buffer or composition is not modified to contain a preservative. In certain instances, as a control, the activity of modified hyaluronan-degrading enzymes also can be compared to the corresponding unmodified hyaluronan-degrading enzyme not containing the modification(s) under conditions that contain a preservative agent and/or under conditions that do not contain a preservative agent.

In another example, the assay buffer is modified by the presence of a denaturation condition that is low or no salt. As discussed elsewhere herein, hyaluronan-degrading enzymes, such as PH20, generally require salt (e.g., NaCl, Lys-Lys or $MgCl_2$) for activity. Hence, the absence of salt or low salt is denaturing to the enzyme. In one example, the assay buffer is modified by inclusion of an amount of salt that is less than 100 mM, for example, less than 90 mM, 80 mM, 70 mM, 60 mM, 50 mM, 40 mM, 30 mM, 25 mM, 20 mM, 15 mM, 10 mM, 5 mM or less. In such an example, the activity of the modified hyaluronan-degrading enzyme is tested in the absence of salt or in the presence of salt that is less than 100 mM. In some examples, the modified hyaluronan-degrading enzyme also can be tested or assessed under a control or reference condition in which the assay buffer contains a higher salt concentration, generally between or about between 140 mM to 200 mM. In certain instances, as a control, the activity of modified hyaluronan-degrading enzymes also can be compared to the corresponding unmodified hyaluronan-degrading enzyme not containing the modification(s) under conditions that contain low or no salt, such as less than 100 mM and/or under conditions that contain salt in an amount that is between or about between 140 mM to 200 mM.

Exposure of a hyaluronan-degrading enzyme to a denaturation condition also can be achieved by incubation of a modified hyaluronan-degrading enzyme under conditions that are known to be denaturing, such as under conditions of elevated temperature such as a temperature greater than or about or 30° C. (e.g., 30° C. to 42° C. such as or about 37° C.) or agitation. For example, the activity of the modified hyaluronan-degrading enzyme is tested at elevated temperatures greater than or about or 30° C. to 42° C. In some examples, the modified hyaluronan-degrading enzyme also can be tested or assessed under a control or reference condition where the temperatures is less than 30° C., such as between or about between 0° C. to 25° C., for example, 0° C. to 5° C. or 18° C. to 25° C. In certain instances, as a control, the activity of modified hyaluronan-degrading enzymes also can be compared to the corresponding unmodified hyaluronan-degrading enzyme not containing the modification(s) under elevated temperatures greater than or about or 30° C. to 42° C. and/or temperatures is less than 30° C., such as between or about between 0° C. to 25° C., for example, 0° C. to 5° C. or 18° C. to 25° C.

The modified hyaluronan-degrading enzyme can be exposed to one or more than one of the conditions. The exposure to one condition can occur simultaneously, subsequently, intermittently or periodically to exposure to one or more other conditions.

In one example, in the method herein, the modified hyaluronan-degrading enzyme is incubated or exposed to the denaturation condition or denaturing agent prior to performing an assay for hyaluronidase activity. For example, the modified hyaluronan-degrading enzyme is incubated in the presence of a denaturing agent or exposed to one or more denaturation conditions or control conditions, such as one or more of the denaturation conditions or control conditions as described above. The incubation or exposure can be for any desired length of time, and can be empirically determined by one of skill in the art. For example, the modified hyaluronan-degrading enzyme can be incubated or exposed to one or more denaturation conditions, denaturing agents or control conditions for or about for 1 minute to 1 month, such as 1 minute to 3 weeks, 1 minute to 2 weeks, 1 minute to 1 week, 1 minute to 24 hours, 1 minute to 12 hours, such as 30 minutes to 6 hours or 1 hour to 4 hours, and generally at least or about at least 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours or 12 hours. After the time of incubation or exposure, the sample or composition containing the modified hyaluronan-degrading enzyme (or control unmodified enzyme) is assessed for hyaluronidase assay. In another example, the modified hyaluronan-degrading enzyme is exposed or incubated under one or more denaturation conditions and is simultaneously or concurrently assessed for hyaluronidase activity. In any examples where a modified hyaluronan-degrading enzyme is assessed, it is understood that an unmodified hyaluronan-degrading enzyme not containing the modifications(s) also can be assessed under similar assay conditions for comparison.

Assays to assess hyaluronidase activity are well known in the art. Examples of such assays are described in Section G. In one example, hyaluronidase activity can be assessed in a microturbidity assay, wherein the amount of undegraded HA is measured by the addition of a reagent that precipitates HA (e.g., Cetylpyridinium chloride (CPC) or acidified serum) after the enzyme is allowed to react with HA. In another example, hyaluronidase activity can be assessed using a microtiter assay in which residual biotinylated hyaluronic acid is measured following incubation with hyaluronidase (see e.g., Frost and Stern (997) *Anal. Biochem.* 251:263-269, U.S. Pat. Publication No. 20050260186). The resulting activities under each of the tested conditions is determined and compared.

3. Selection or Identification

In the method, after screening modified hyaluronan-degrading enzymes under one or more denaturation conditions, the hyaluronidase activities of the tested enzymes are compared. The method is practiced in order to identify a modified hyaluronan-degrading enzyme that is more resistant to denaturation by a condition or a denaturing agent, whereby the activity of the enzyme is indicative of the stability of the enzyme as a measure of its resistance to denaturation. It is understood that some reduction of enzyme activity, as a result of denaturation, can be tolerated in various applications, and thus the method can be practiced to select for a modified hyaluronan-degrading enzymes that exhibits a requisite activity upon exposure to a denaturation condition to permit its use or application (e.g., therapeutic activity). For example, a modified enzyme can be selected that loses activity more slowly than the corresponding unmodified or reference hyaluronan-degrading enzyme, but whose retained activity is sufficient for a particular application or purpose.

In examples of the methods herein, the activity of the modified hyaluronan degrading enzyme is assessed upon exposure to a first denaturation condition and also assessed upon exposure to a second condition that is a control or non-denaturation condition, and the resulting hyaluronidase activities compared. For comparison, in some examples, the activity can be represented as a ratio of activity or a percentage of activity under a denaturation condition compared to under a control or non-denaturation condition. For example, where the parameter that differs between the first and second condition is the presence of preservative (e.g., phenolic preservative), activity can be represented as a ratio of activity or percentage of activity observed in the presence of preservative (e.g., phenolic preservative) versus activity in the absence of preservative (e.g., phenolic preservative). In another example, where the parameter that differs between the first and second condition is temperature, activity can be represented as a ratio of activity or percentage of activity observed in the presence of elevated temperature (e.g., 30° C. to 42° C.) compared to activity in the presence of a lower temperature such as 0° C. to 25° C., for example 0° C. to 5° C. or 18° C. to 25° C.

A modified hyaluronan-degrading enzyme is selected or identified that retains or exhibits any desired activity in the presence of the denaturation condition compared to in its absence. The particular cut-off of activity for selection of enzymes herein is dependent on the particular user and/or practice of the method and can be empirically determined depending on factors such as the particular denaturation condition or denaturing agent, the particular modified hyaluronan-degrading enzyme, the desired application of the identified or selected hyaluronan-degrading enzyme and other similar factors. Generally, a selected or identified modified hyaluronan-degrading enzyme exhibits stability if any detectable activity is measured or assessed upon exposure or incubation with a denaturation condition or denaturing agent. For example, a selected or identified modified hyaluronan-degrading enzyme exhibits stability, or resistance to a denaturation condition or denaturing agent, if it exhibits at least 5% or 10% of the activity of the same enzyme in the absence of the denaturation condition or denaturing agent, and generally if the modified hyaluronan-degrading enzyme exhibits an activity that is at least 15% of the initial hyaluronidase activity prior to incubation in the presence of the denaturation condition. For example, a modified hyaluronan-degrading enzyme is selected or identified that exhibits at least (or at least about) 16%, 17%, 18%, 19%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, 110%, 120%, 130%, 140%, 150%, 200%, 300%, 400%, 500% or more of the initial hyaluronidase activity of the modified hyaluronan-degrading enzyme tested under a control or non-denaturation condition.

In other examples of the methods herein, the activity of the modified hyaluronan degrading enzyme is assessed upon exposure to a denaturation condition and the activity of the unmodified or reference hyaluronan-degrading enzyme also is assessed upon exposure to the same denaturation conditions. In such examples, the activities are compared when the enzymes are exposed to the same conditions. For comparison, the activity under a denaturation condition can be represented as a ratio of activity or a percentage of activity of a modified hyaluronan-degrading enzyme compared to an unmodified or reference hyaluronan-degrading enzyme. In such examples, a modified hyaluronan-degrading enzyme is selected that exhibits greater activity under a denaturation condition than the unmodified or reference hyaluronan-degrading enzyme. Thus, the modified hyaluronan-degrading enzyme is one that is more resistant to the condition. For example, where the denaturation condition is the presence of preservative (e.g., phenolic preservative), the activity observed in the presence of preservative (e.g., phenolic preservative) can be represented as a ratio of activity or percentage of activity of the modified hyaluronan-degrading enzyme compared to the unmodified or reference hyaluronan-degrading enzyme. In another example, where the denaturation condition is high temperature, activity observed in the presence of elevated temperature (e.g., 30° C. to 42° C.) can be represented as a ratio of activity or percentage of activity of the modified hyaluronan-degrading enzyme compared to the unmodified or reference hyaluronan-degrading enzyme.

In such examples, a modified hyaluronan-degrading enzyme, such as a modified PH20, is identified or selected that exhibits a ratio of activity that is greater than or at least 1.1, such that the enzyme exhibits greater activity than the unmodified or reference hyaluronan-degrading enzyme under the denaturation condition. For example, the ratio is at least or at least about 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 or greater. A modified hyaluronan-degrading enzyme (e.g., a modified PH20) can be selected if its activity is at least 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 250%, 300%, 400%, 500% or more of the activity of the unmodified or reference hyaluronan-degrading enzyme when tested under the same conditions. Thus, modified hyaluronan-degrading enzymes are identified that exhibit greater or improved stability compared to the unmodified hyaluronan-degrading enzyme or a reference hyaluronan-degrading enzyme as manifested by increased resistance to a denaturation condition or denaturing agent.

4. Iterative Methods

The method provided herein also is iterative. In one example, after the method is performed, any modified hyaluronan-degrading enzymes identified as exhibiting stability, such as increased stability, under a denaturation condition can be modified or further modified to increase or optimize the stability. A secondary library can be created by introducing additional modifications in a first identified modified hyaluronan-degrading enzyme. For example, modifications that were identified as conferring stability, such as increasing stability, can be combined to generate a combinatorial library. The secondary library can be tested using the assays and methods described herein.

In another example of an iterative aspect of the method, modified hyaluronan-degrading enzymes that are identified as not exhibiting stability such as increased stability (e.g., such that they are not active or do not have increased activity under the a denaturation condition), can be further modified and retested for stability under a denaturation condition. The further modifications can be targeted near particular regions (e.g., particular amino acid residues) associated with activity and/or stability of the molecule. For example, residues that are associated with activity and/or stability of the molecule generally are critical residues that are involved in the structural folding or other activities of the molecule. Hence, such residues are required for activity, generally under any condition. Critical residues can be identified because, when mutated, a normal activity of the protein is ablated or reduced. For example, critical residues can be identified that, when mutated in a hyaluronan-degrading enzyme, exhibit reduced or ablated hyaluronidase activity under a normal or control assay condition. A further library of modified proteins can be generated with amino acid mutations targeted at or near to the identified critical amino acid residues, such as adjacent to the identified critical amino acid residues. In some examples, the mutations can be amino acid replacement to any other of up to 19 other amino acid residues. The secondary library can be tested using the assays and methods described herein.

E. Production of Modified PH20 Polypeptides and Encoding Nucleic Acid Molecules

Polypeptides of a modified PH20 polypeptide set forth herein can be obtained by methods well known in the art for protein purification and recombinant protein expression. Polypeptides also can be synthesized chemically. Modified or variant, including truncated, forms can be engineered from a wildtype polypeptide using standard recombinant DNA methods. For example, modified PH20 polypeptides can be engineered from a wildtype polypeptide, such as by site-directed mutagenesis.

1. Isolation or Preparation of Nucleic Acids Encoding PH20 Polypeptides

Polypeptides can be cloned or isolated using any available methods known in the art for cloning and isolating nucleic acid molecules. Such methods include PCR amplification of nucleic acids and screening of libraries, including nucleic acid hybridization screening, antibody-based screening and activity-based screening.

For example, when the polypeptides are produced by recombinant means, any method known to those of skill in the art for identification of nucleic acids that encode desired genes can be used. Any method available in the art can be used to obtain a full length or partial (i.e., encompassing the entire coding region) cDNA or genomic DNA clone encoding a PH20, such as from a cell or tissue source.

Methods for amplification of nucleic acids can be used to isolate nucleic acid molecules encoding a desired polypeptide, including for example, polymerase chain reaction (PCR) methods. Examples of such methods include use of a Perkin-Elmer Cetus thermal cycler and Taq polymerase (Gene Amp). A nucleic acid containing material can be used as a starting material from which a desired polypeptide-encoding nucleic acid molecule can be isolated. For example, DNA and mRNA preparations, cell extracts, tissue extracts, fluid samples (e.g., blood, serum, saliva), samples from healthy and/or diseased subjects can be used in amplification methods. The source can be from any eukaryotic species including, but not limited to, vertebrate, mammalian, human, porcine, bovine, feline, avian, equine, canine, and other primate sources. Nucleic acid libraries also can be used as a source of starting material. Primers can be designed to amplify a desired polypeptide. For example, primers can be designed based on expressed sequences from which a desired polypeptide is generated. Primers can be designed based on back-translation of a polypeptide amino acid sequence. If desired, degenerate primers can be used for amplification. Oligonucleotide primers that hybridize to sequences at the 3' and 5' termini of the desired sequence can be uses as primers to amplify by PCR sequences from a nucleic acid sample. Primers can be used to amplify the entire full-length PH20, or a truncated sequence thereof, such as a nucleic acid encoding any of the soluble PH20 polypeptides provided herein. Nucleic acid molecules generated by amplification can be sequenced and confirmed to encode a desired polypeptide.

Additional nucleotide sequences can be joined to a polypeptide-encoding nucleic acid molecule, including linker sequences containing restriction endonuclease sites for the purpose of cloning the synthetic gene into a vector, for example, a protein expression vector or a vector designed for the amplification of the core protein coding DNA sequences. Furthermore, additional nucleotide sequences specifying functional DNA elements can be operatively linked to a polypeptide-encoding nucleic acid molecule. Examples of such sequences include, but are not limited to, promoter sequences designed to facilitate intracellular protein expression, and secretion sequences, for example heterologous signal sequences, designed to facilitate protein secretion. Such sequences are known to those of skill in the art. For example, exemplary heterologous signal sequences include, but are not limited to, human and mouse kappa IgG heterologous signal sequences set forth in SEQ ID NO: 868. Additional nucleotide residue sequences such as sequences of bases specifying protein binding regions also can be linked to enzyme-encoding nucleic acid molecules. Such regions include, but are not limited to, sequences of residues that facilitate or encode proteins that facilitate uptake of an enzyme into specific target cells, or otherwise alter pharmacokinetics of a product of a synthetic gene.

In addition, tags or other moieties can be added, for example, to aid in detection or affinity purification of the polypeptide. For example, additional nucleotide residue sequences such as sequences of bases specifying an epitope tag or other detectable marker also can be linked to enzyme-encoding nucleic acid molecules. Examples of such sequences include nucleic acid sequences encoding a His tag or Flag Tag.

The identified and isolated nucleic acids can then be inserted into an appropriate cloning vector. A large number of vector-host systems known in the art can be used. Possible vectors include, but are not limited to, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, or plasmids such as pCMV4, pBR322 or pUC plasmid derivatives or the Bluescript vector (Stratagene, La Jolla, Calif.). Other expression vectors include the HZ24 expression vector exemplified herein (see e.g., SEQ ID NOS:4 and 5). The insertion into a cloning vector can, for example, be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. Insertion can be effected using TOPO cloning vectors (Invitrogen, Carlsbad, Calif.).

If the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules can be enzymatically modified. Alternatively, any site desired can be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers can contain specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. In an alternative method, the cleaved vector and protein gene can be modified by homopolymeric tailing.

Recombinant molecules can be introduced into host cells via, for example, transformation, transfection, infection, electroporation and sonoporation, so that many copies of the gene sequence are generated. In specific embodiments, transformation of host cells with recombinant DNA molecules that incorporate the isolated protein gene, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the gene can be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA.

In addition to recombinant production, modified PH20 polypeptides provided herein can be produced by direct peptide synthesis using solid-phase techniques (see e.g., Stewart et al. (1969) *Solid-Phase Peptide Synthesis*, WH Freeman Co., San Francisco; Merrifield J (963) *J Am Chem Soc,* 85:2149-2154). In vitro protein synthesis can be performed using manual techniques or by automation. Automated synthesis can be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer, Foster City Calif.) in accordance with the instructions provided by the manufacturer. Various fragments of a polypeptide can be chemically synthesized separately and combined using chemical methods.

2. Generation of Mutant of Modified Nucleic Acid and Encoding Polypeptides

The modifications provided herein can be made by standard recombinant DNA techniques such as are routine to one of skill in the art. Any method known in the art to effect mutation of any one or more amino acids in a target protein can be employed. Methods include standard site-directed mutagenesis (using e.g., a kit, such as QuikChange available from Stratagene) of encoding nucleic acid molecules, or by solid phase polypeptide synthesis methods.

3. Vectors and Cells

For recombinant expression of one or more of the desired proteins, such as any modified PH20 polypeptide described herein, the nucleic acid containing all or a portion of the nucleotide sequence encoding the protein can be inserted into an appropriate expression vector, i.e., a vector that contains the necessary elements for the transcription and translation of the inserted protein coding sequence. The necessary transcriptional and translational signals also can be supplied by the native promoter for enzyme genes, and/or their flanking regions.

Also provided are vectors that contain a nucleic acid encoding the enzyme. Cells containing the vectors also are provided. The cells include eukaryotic and prokaryotic cells, and the vectors are any suitable for use therein. Generally, the cell is a cell that is capable of effecting glyosylation of the encoded protein.

Prokaryotic and eukaryotic cells containing the vectors are provided. Such cells include bacterial cells, yeast cells, fungal cells, Archea, plant cells, insect cells and animal cells. The cells are used to produce a protein thereof by growing the above-described cells under conditions whereby the encoded protein is expressed by the cell, and recovering the expressed protein. For purposes herein, for example, the enzyme can be secreted into the medium.

A host cell strain can be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed protein in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation and acylation. Post-translational processing can impact the folding and/or function of the polypeptide. Different host cells, such as, but not limited to, CHO (DG44, DXB11, CHO-K1), HeLa, MCDK, 293 and WI38 have specific cellular machinery and characteristic mechanisms for such post-translational activities and can be chosen to ensure the correct modification and processing of the introduced protein. Generally, the choice of cell is one that is capable of introducing N-linked glycosylation into the expressed polypeptide. Hence, eukaryotic cells containing the vectors are provided. Exemplary eukaryotic cells are mammalian Chinese Hamster Ovary (CHO) cells. For example, CHO cells deficient in dihydrofolate reductase (e.g., DG44 cells) are used to produce polypeptides provided herein. Note that bacterial expression of an PH20 polypeptide provided herein will not result in a catalytically active polypeptide, but when combined with proper glycosylation machinery, the PH20 can be artificially glycosylated.

Provided are vectors that contain a sequence of nucleotides that encodes the modified PH20 polypeptide, coupled to the native or heterologous signal sequence, as well as multiple copies thereof. The vectors can be selected for expression of the enzyme protein in the cell or such that the enzyme protein is expressed as a secreted protein.

A variety of host-vector systems can be used to express the protein encoding sequence. These include but are not limited to mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus and other viruses); insect cell systems infected with virus (e.g., baculovirus); microorganisms such as yeast containing yeast vectors; or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system used, any one of a number of suitable transcription and translation elements can be used.

Any methods known to those of skill in the art for the insertion of DNA fragments into a vector can be used to construct expression vectors containing a chimeric gene containing appropriate transcriptional/translational control signals and protein coding sequences. These methods can include in vitro recombinant DNA and synthetic techniques and in vivo recombinants (genetic recombination). Expression of nucleic acid sequences encoding protein, or domains, derivatives, fragments or homologs thereof, can be regulated by a second nucleic acid sequence so that the genes or fragments thereof are expressed in a host transformed with the recombinant DNA molecule(s). For example, expression of the proteins can be controlled by any promoter/enhancer known in the art. In a specific embodiment, the promoter is not native to the genes for a desired protein. Promoters which can be used include, but are not limited to, the SV40 early promoter (Bernoist and Chambon, *Nature* 290:304-310 (1981)), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al. *Cell* 22:787-797 (1980)), the herpes thymidine kinase promoter (Wagner et al., *Proc. Natl. Acad. Sci. USA* 78:1441-1445 (1981)), the regulatory sequences of the metallothionein gene (Brinster et al., *Nature* 296:39-42 (1982)); prokaryotic expression vector promoters, such as the β-lactamase promoter (Jay et al., (1981) *Proc. Natl. Acad. Sci. USA* 75:5543) or the tac promoter (DeBoer et al., *Proc. Natl. Acad. Sci. USA* 80:21-25 (1983); see also Gilbert and Villa-Komaroff, "Useful Proteins from Recombinant Bacteria," Scientific American 242:74-94 (1980)); plant expression vector promoters, such as the nopaline synthetase promoter (Herrera-Estrella et al., *Nature* 305:209-213 (1984)) or the cauliflower mosaic virus 35S RNA promoter (Gardner et al., *Nucleic Acids Res.* 9:2871 (1981)), and the promoter of the photosynthetic enzyme ribulose bisphosphate carboxylase (Herrera-Estrella et al., *Nature* 310:115-120 (1984)); promoter elements from yeast and other fungi such as the Gal4 promoter, the alcohol dehydrogenase promoter, the phosphoglycerol kinase promoter, the alkaline phosphatase promoter, and the following animal transcriptional control regions that exhibit tissue specificity and have been used in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., *Cell* 55:639-646 (1984); Ornitz et al., *Cold Spring Harbor Symp. Quant. Biol.* 50:399-409 (1986); MacDonald, *Hepatology* 7:425-515 (1987)); insulin gene control region which is active in pancreatic beta cells (Hanahan et al., *Nature* 375:115-122 (1985)), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., *Cell* 55:647-658 (1984); Adams et al., *Nature* 575:533-538 (1985); Alexander et al., *Mol. Cell. Biol* 7:1436-1444 (1987)), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., *Cell* 45:485-495 (1986)), albumin gene control region which is active in liver (Pinkert et al., *Genes and Devel.* 7:268-276 (1987)), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., *Mol. Cell. Biol.* 5:1639-1648 (1985); Hammer et al., *Science* 255:53-58 1987)), alpha-1 antitrypsin gene control region which is active in liver (Kelsey et al., *Genes and Devel.* 7:161-171 (1987)), beta globin gene control region which is active in myeloid cells (Magram et al., *Nature* 575:338-340 (1985); Kollias et al., *Cell* 46:89-94 (1986)), myelin basic protein gene control region which is active in oligodendrocyte cells of the brain (Readhead et al., *Cell* 45:703-712 (1987)), myosin light chain-2 gene control region which is active in skeletal muscle (Shani, *Nature* 574:283-286 (1985)), and gonadotrophic releasing hormone gene control region which is active in gonadotrophs of the hypothalamus (Mason et al., *Science* 234:1372-1378 (1986)).

In a specific embodiment, a vector is used that contains a promoter operably linked to nucleic acids encoding a desired protein, or a domain, fragment, derivative or homolog thereof, one or more origins of replication, and optionally, one or more selectable markers (e.g., an antibiotic resistance gene). Depending on the expression system, specific initiation signals also are required for efficient translation of a PH20 sequence. These signals include the ATG initiation codon and adjacent sequences. In cases where the initiation codon and upstream sequences of PH20 or soluble forms thereof are inserted into the appropriate expression vector, no additional translational control signals are needed. In cases where only a coding sequence, or a portion thereof, is inserted, exogenous transcriptional control signals including the ATG initiation codon must be provided. Furthermore, the initiation codon must be in the correct reading frame to ensure transcription of the entire insert. Exogenous transcriptional elements and initiation codons can be of various origins, both natural and synthetic. The efficiency of expression can be enhanced by the inclusion of enhancers appropriate to the cell system in use (Scharf et al. (1994) *Results Probl Cell Differ* 20:125-62; Bittner et al. (1987) *Methods in Enzymol,* 153:516-544).

Exemplary plasmid vectors for transformation of *E. coli* cells include, for example, the pQE expression vectors (available from Qiagen, Valencia, Calif.; see also literature published by Qiagen describing the system). pQE vectors have a phage T5 promoter (recognized by *E. coli* RNA polymerase) and a double lac operator repression module to provide tightly regulated, high-level expression of recombinant proteins in *E. coli*, a synthetic ribosomal binding site (RBS II) for efficient translation, a 6×His tag coding sequence, to and T1 transcriptional terminators, ColE1 origin of replication, and a beta-lactamase gene for conferring ampicillin resistance. The pQE vectors enable placement of a 6×His tag at either the N- or C-terminus of the recombinant protein. Such plasmids include pQE 32, pQE 30, and pQE 31 which provide multiple cloning sites for all three reading frames and provide for the expression of N-terminally 6×His-tagged proteins. Other exemplary plasmid vectors for transformation of *E. coli* cells, include, for example, the pET expression vectors (see, U.S. Pat. No. 4,952,496; available from Novagen, Madison, Wis.; see, also literature published by Novagen describing the system). Such plasmids include pET 11a, which contains the T7lac promoter, T7 terminator, the inducible *E. coli* lac operator, and the lac repressor gene; pET 12a-c, which contains the T7 promoter, T7 terminator, and the *E. coli* ompT secretion signal; and pET 15b and pET19b (Novagen, Madison, Wis.), which contain a His-Tag™ leader sequence for use in purification with a H is column and a thrombin cleavage site that permits cleavage following purification over the column, the T7-lac promoter region and the T7 terminator.

Typically, vectors can be plasmids, viral vectors, or others known in the art, used for expression of the modified PH20 polypeptide in vivo or in vitro. For example, the modified PH20 polypeptide is expressed in mammalian cells, including, for example, Chinese Hamster

Ovary (CHO) cells. An exemplary vector for mammalian cell expression is the HZ24 expression vector. The HZ24 expression vector was derived from the pCI vector backbone

(Promega). It contains DNA encoding the Beta-lactamase resistance gene (AmpR), an F1 origin of replication, a Cytomegalovirus immediate-early enhancer/promoter region (CMV), and an SV40 late polyadenylation signal (SV40). The expression vector also has an internal ribosome entry site (ERES) from the ECMV virus (Clontech) and the mouse dihydrofolate reductase (DHFR) gene.

Viral vectors, such as adenovirus, retrovirus or vaccinia virus vectors, can be employed. In some examples, the vector is a defective or attenuated retroviral or other viral vector (see U.S. Pat. No. 4,980,286). For example, a retroviral vector can be used (see Miller et al., Meth. Enzymol. 217: 581-599 (1993)). These retroviral vectors have been modified to delete retroviral sequences that are not necessary for packaging of the viral genome and integration into host cell DNA.

In some examples, viruses armed with a nucleic acid encoding a modified PH20 polypeptide can facilitate their replication and spread within a target tissue for example. The target tissue can be a cancerous tissue whereby the virus is capable of selective replication within the tumor. The virus can also be a non-lytic virus wherein the virus selectively replicates under a tissue specific promoter. As the viruses replicate, the coexpression of the PH20 polypeptide with viral genes will facilitate the spread of the virus in vivo.

4. Expression

Modified PH20 polypeptides can be produced by any method known to those of skill in the art including in vivo and in vitro methods. Desired proteins can be expressed in any organism suitable to produce the required amounts and forms of the proteins, such as for example, those needed for administration and treatment. Expression hosts include prokaryotic and eukaryotic organisms such as *E. coli*, yeast, plants, insect cells, mammalian cells, including human cell lines and transgenic animals. Expression hosts can differ in their protein production levels as well as the types of post-translational modifications that are present on the expressed proteins. The choice of expression host can be made based on these and other factors, such as regulatory and safety considerations, production costs and the need and methods for purification.

Many expression vectors are available and known to those of skill in the art and can be used for expression of proteins. The choice of expression vector will be influenced by the choice of host expression system. In general, expression vectors can include transcriptional promoters and optionally enhancers, translational signals, and transcriptional and translational termination signals. Expression vectors that are used for stable transformation typically have a selectable marker which allows selection and maintenance of the transformed cells. In some cases, an origin of replication can be used to amplify the copy number of the vector.

Modified PH20 polypeptides also can be utilized or expressed as protein fusions. For example, an enzyme fusion can be generated to add additional functionality to an enzyme. Examples of enzyme fusion proteins include, but are not limited to, fusions of a signal sequence, a tag such as for localization, e.g., a 6×His or His_6 tag or a myc tag, or a tag for purification, for example, a GST fusion, and a sequence for directing protein secretion and/or membrane association.

For long-term, high-yield production of recombinant proteins, stable expression is desired. For example, cell lines that stably express a modified PH20 polypeptide can be transformed using expression vectors that contain viral origins of replication or endogenous expression elements and a selectable marker gene. Following the introduction of the vector, cells can be allowed to grow for 1-2 days in an enriched medium before they are switched to selective media. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth and recovery of cells that successfully express the introduced sequences. Resistant cells of stably transformed cells can be proliferated using tissue culture techniques appropriate to the cell types.

Any number of selection systems can be used to recover transformed cell lines. These include, but are not limited to, the herpes simplex virus thymidine kinase (Wigler, M et al. (1977) Cell, 11:223-32) and adenine phosphoribosyltransferase (Lowy, I et al. (1980) Cell, 22:817-23) genes, which can be employed in TK− or APRT− cells, respectively. Also, antimetabolite, antibiotic or herbicide resistance can be used as the basis for selection. For example, DHFR, which confers resistance to methotrexate (Wigler, M et al. (1980) Proc. Natl. Acad. Sci, 77:3567-70); npt, which confers resistance to the aminoglycosides neomycin and G-418 (Colbere-Garapin, F et al. (1981) J. Mol. Biol., 150:1-14); and als or pat, which confer resistance to chlorsulfuron and phosphinotricin acetyltransferase, respectively, can be used. Additional selectable genes have been described, for example, trpB, which allows cells to utilize indole in place of typtophan or hisD, which allows cells to utilize histinol in place of histidine (Hartman S C and R C Mulligan (1988) Proc. Natl. Acad. Sci, 85:8047-51). Visible markers, such as but not limited to, anthocyanins, beta glucuronidase and its substrate, GUS, and luciferase and its substrate luciferin, also can be used to identify transformants and also to quantify the amount of transient or stable protein expression attributable to a particular vector system (Rhodes C A et al. (1995) Methods Mol. Biol. 55:121-131).

The presence and expression of PH20 polypeptides can be monitored. For example, detection of a functional polypeptide can be determined by testing the conditioned media for hyaluronidase enzyme activity under appropriate conditions. Exemplary assays to assess the solubility and activity of expressed proteins are provided herein.

a. Prokaryotic Cells

Prokaryotes, especially *E. coli*, provide a system for producing large amounts of proteins. Transformation of *E. coli* is a simple and rapid technique well known to those of skill in the art. Expression vectors for *E. coli* can contain inducible promoters. Such promoters are useful for inducing high levels of protein expression and for expressing proteins that exhibit some toxicity to the host cells. Examples of inducible promoters include the lac promoter, the trp promoter, the hybrid tac promoter, the T7 and SP6 RNA promoters and the temperature regulated λPL promoter.

Proteins, such as any provided herein, can be expressed in the cytoplasmic environment of *E. coli*. The cytoplasm is a reducing environment, and for some molecules, this can result in the formation of insoluble inclusion bodies. Reducing agents such as dithiothreotol and β-mercaptoethanol and denaturants, such as guanidine-HCl and urea can be used to resolubilize the proteins. An alternative approach effects protein expression in the periplasmic space of bacteria which provides an oxidizing environment and chaperonin-like and disulfide isomerases, which can aid in the production of soluble protein. Typically, a leader sequence is fused to the protein to be expressed which directs the protein to the periplasm. The leader is then removed by signal peptidases inside the periplasm. Examples of periplasmic-targeting leader sequences include the pelB leader from the pectate lyase gene and the leader derived from the alkaline phosphatase gene. In some cases, periplasmic expression allows leakage of the expressed protein into the culture medium. The secretion of proteins allows quick and simple purification from the culture supernatant. Proteins that are not secreted can be obtained from the periplasm by osmotic lysis. Similar to cytoplasmic expression, in some cases proteins can become insoluble and denaturants and reducing agents can be used to facilitate solubilization and refolding. Temperature of induction and growth 1 also can influence expression levels and solubility, typically temperatures between 25° C. and 37° C. are used. Typically, bacteria produce aglycosylated proteins. Thus, if proteins require glycosylation for function, glycosylation can be added in vitro after purification from host cells.

b. Yeast Cells

Yeasts such as *Saccharomyces cerevisae*, Schizosaccharomyces pombe, Yarrowia lipolytica, Kluyveromyces lactis and *Pichia pastoris* are well known yeast expression hosts that can be used for production of proteins, such as any described herein. Yeast can be transformed with episomal replicating vectors or by stable chromosomal integration by homologous recombination. Typically, inducible promoters are used to regulate gene expression. Examples of such promoters include GAL1, GAL7 and GAL5 and metallothionein promoters, such as CUP1, AOX1 or other *Pichia* or other yeast promoters. Expression vectors often include a selectable marker such as LEU2, TRP1, HIS3 and URA3 for selection and maintenance of the transformed DNA. Proteins expressed in yeast are often soluble. Co-expression with chaperonins such as Bip and protein disulfide isomerase can improve expression levels and solubility. Additionally, proteins expressed in yeast can be directed for secretion using secretion signal peptide fusions such as the yeast mating type alpha-factor secretion signal from *Saccharomyces cerevisae* and fusions with yeast cell surface proteins such as the Aga2p mating adhesion receptor or the *Arxula adeninivorans* glucoamylase. A protease cleavage site such as for the Kex-2 protease, can be engineered to remove the fused sequences from the expressed polypeptides as they exit the secretion pathway. Yeast also is capable of glycosylation at Asn-X-Ser/Thr motifs.

c. Insects and Insect Cells

Insect cells, particularly using baculovirus expression, are useful for expressing polypeptides such as PH20 polypeptides. Insect cells express high levels of protein and are capable of most of the post-translational modifications used by higher eukaryotes. Baculoviruses have a restrictive host range which improves the safety and reduces regulatory concerns of eukaryotic expression. Typical expression vectors use a promoter for high level expression such as the polyhedrin promoter of baculovirus. Commonly used baculovirus systems include a baculovirus, such as the *Autographa californica* nuclear polyhedrosis virus (AcNPV) or the *bombyx mori* nuclear polyhedrosis virus (BmNPV), and an insect cell line, such as Sf9 derived from *Spodoptera frugiperda, Pseudaletia unipuncta* (A7S) and *Danaus plexippus* (DpN1). For high-level expression, the nucleotide sequence of the molecule to be expressed is fused immediately downstream of the polyhedrin initiation codon of the virus. Mammalian secretion signals are accurately processed in insect cells and can be used to secrete the expressed protein into the culture medium. In addition, the cell lines *Pseudaletia unipuncta* (A7S) and *Danaus plexippus* (DpN1) produce proteins with glycosylation patterns similar to mammalian cell systems. Exemplary insect cells are those that have been altered to reduce immunogenicity, including those with “mammalianized” baculovirus expression vectors and those lacking the enzyme FT3.

An alternative expression system in insect cells employs stably transformed cells. Cell lines such as the Schnieder 2 (S2) and Kc cells (*Drosophila melanogaster*) and C7 cells (*Aedes albopictus*) can be used for expression. The *Drosophila* metallothionein promoter can be used to induce high levels of expression in the presence of heavy metal induction with cadmium or copper. Expression vectors are typically maintained by the use of selectable markers such as neomycin and hygromycin.

d. Mammalian Expression

Mammalian expression systems can be used to express proteins including PH20 polypeptides. Expression constructs can be transferred to mammalian cells by viral infection such as by adenovirus or by direct DNA transfer such as liposomes, calcium phosphate, DEAE-dextran and by physical means such as electroporation and microinjection. Expression vectors for mammalian cells typically include an mRNA cap site, a TATA box, a translational initiation sequence (Kozak consensus sequence) and polyadenylation elements. IRES elements also can be added to permit bicistronic expression with another gene, such as a selectable marker. Such vectors often include transcriptional promoter-enhancers for high-level expression, for example the SV40 promoter-enhancer, the human cytomegalovirus (CMV) promoter and the long terminal repeat of Rous sarcoma virus (RSV). These promoter-enhancers are active in many cell types. Tissue and cell-type promoters and enhancer regions also can be used for expression. Exemplary promoter/enhancer regions include, but are not limited to, those from genes such as elastase I, insulin, immunoglobulin, mouse mammary tumor virus, albumin, alpha fetoprotein, alpha 1 antitrypsin, beta globin, myelin basic protein, myosin light chain 2, and gonadotropic releasing hormone gene control. Selectable markers can be used to select for and maintain cells with the expression construct. Examples of selectable marker genes include, but are not limited to, hygromycin B phosphotransferase, adenosine deaminase, xanthine-guanine phosphoribosyl transferase, aminoglycoside phosphotransferase, dihydrofolate reductase (DHFR) and thymidine kinase. For example, expression can be performed in the presence of methotrexate to select for only those cells expressing the DHFR gene. Fusion with cell surface signaling molecules such as TCR-ζ and Fc_ϵRI-γ can direct expression of the proteins in an active state on the cell surface.

Many cell lines are available for mammalian expression including mouse, rat human, monkey, chicken and hamster cells. Exemplary cell lines include but are not limited to CHO, Balb/3T3, HeLa, MT2, mouse NS0 (nonsecreting) and other myeloma cell lines, hybridoma and heterohybridoma cell lines, lymphocytes, fibroblasts, Sp2/0, COS, NIH3T3, HEK293, 293S, 2B8, and HKB cells. Cell lines also are available adapted to serum-free media which facilitates purification of secreted proteins from the cell culture media. Examples include CHO—S cells (Invitrogen, Carlsbad, Calif., cat #11619-012) and the serum free EBNA-1 cell line (Pham et al., (2003) *Biotechnol. Bioeng.* 54:332-42.). Cell lines also are available that are adapted to grow in special mediums optimized for maximal expression. For example, DG44 CHO cells are adapted to grow in suspension culture in a chemically defined, animal product-free medium.

e. Plants

Transgenic plant cells and plants can be used to express proteins such as any described herein. Expression constructs are typically transferred to plants using direct DNA transfer such as microprojectile bombardment and PEG-mediated transfer into protoplasts, and with *agrobacterium*-mediated transformation. Expression vectors can include promoter and enhancer sequences, transcriptional termination elements and translational control elements. Expression vectors and transformation techniques are usually divided between dicot hosts, such as *Arabidopsis* and tobacco, and monocot hosts, such as corn and rice. Examples of plant promoters used for expression include the cauliflower mosaic virus promoter, the nopaline syntase promoter, the ribose bisphosphate carboxylase promoter and the ubiquitin and UBQ3 promoters. Selectable markers such as hygromycin, phosphomannose isomerase and neomycin phosphotransferase are often used to facilitate selection and maintenance of transformed cells. Transformed plant cells can be maintained in culture as cells, aggregates (callus tissue) or regenerated into whole plants. Transgenic plant cells also can include algae engineered to produce hyaluronidase polypeptides. Because plants have different glycosylation patterns than mammalian cells, this can influence the choice of protein produced in these hosts.

5. Purification

Host cells transformed with a nucleic acid sequence encoding a modified PH20 polypeptide can be cultured under conditions suitable for the expression and recovery of the encoded protein from cell culture. The protein produced by a recombinant cell is generally secreted, but may be contained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing nucleic acid encoding PH20 can be designed with signal sequences that facilitate direct secretion of PH20 through prokaryotic or eukaryotic cell membranes.

Thus, methods for purification of polypeptides from host cells will depend on the chosen host cells and expression systems. For secreted molecules, proteins are generally purified from the culture media after removing the cells. For intracellular expression, cells can be lysed and the proteins purified from the extract. When transgenic organisms such as transgenic plants and animals are used for expression, tissues or organs can be used as starting material to make a lysed cell extract. Additionally, transgenic animal production can include the production of polypeptides in milk or eggs, which can be collected, and if necessary, the proteins can be extracted and further purified using standard methods in the art.

Proteins, such as modified PH20 polypeptides, can be purified using standard protein purification techniques known in the art including but not limited to, SDS-PAGE, size fractionation and size exclusion chromatography, ammonium sulfate precipitation and ionic exchange chromatography, such as anion exchange chromatography. Affinity purification techniques also can be utilized to improve the efficiency and purity of the preparations. For example, antibodies, receptors and other molecules that bind PH20 hyaluronidase enzymes can be used in affinity purification. For example, soluble PH20 can be purified from conditioned media.

Expression constructs also can be engineered to add an affinity tag to a protein such as a myc epitope, GST fusion or His_6 and affinity purified with myc antibody, glutathione resin or Ni-resin, respectively. Such tags can be joined to the nucleotide sequence encoding a soluble PH20 as described elsewhere herein, which can facilitate purification of soluble proteins. For example, a modified PH20 polypeptide can be expressed as a recombinant protein with one or more additional polypeptide domains added to facilitate protein purification. Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp., Seattle Wash.). The inclusion of a cleavable linker sequence such as Factor XA or enterokinase (Invitrogen, San Diego, Calif.) between the purification domain and the expressed PH20 polypeptide is useful to facilitate purification. One such expression vector provides for expression of a fusion protein containing a PH20 polypeptide in and an enterokinase cleavage site. The histidine residues facilitate purification on IMIAC (immobilized metal ion affinity chromatography), while the enterokinase cleavage site provides a means for purifying the polypeptide from the fusion protein.

Purity can be assessed by any method known in the art including gel electrophoresis, orthogonal HPLC methods, staining and spectrophotometry techniques. The expressed and purified protein can be analyzed using any assay or method known to one of skill in the art, for example, any described in Section G. These include assays based on the physical and/or functional properties of the protein, including, but not limited to, analysis by gel electrophoresis, immunoassay and assays of hyaluronidase activity.

Depending on the expression system and host cells used, the resulting polypeptide can be heterogeneous due to peptidases present in the culture medium upon production and purification. For example, culture of soluble PH20 in CHO cells can result in a mixture of heterogeneous polypeptides.

6. Modification of Polypeptides by PEGylation

Polyethylene glycol (PEG) has been widely used in biomaterials, biotechnology and medicine primarily because PEG is a biocompatible, nontoxic, water-soluble polymer that is typically nonimmunogenic (Zhao and Harris, *ACS Symposium Series* 680: 458-72, 1997). In the area of drug delivery, PEG derivatives have been widely used in covalent attachment (i.e., "PEGylation") to proteins to reduce immunogenicity, proteolysis and kidney clearance and to enhance solubility (Zalipsky, Adv. Drug Del. Rev. 16:157-82, 1995). Similarly, PEG has been attached to low molecular weight, relatively hydrophobic drugs to enhance solubility, reduce toxicity and alter biodistribution. Typically, PEGylated drugs are injected as solutions.

A closely related application is synthesis of crosslinked degradable PEG networks or formulations for use in drug delivery since much of the same chemistry used in design of degradable, soluble drug carriers can also be used in design of degradable gels (Sawhney et al, *Macromolecules* 26:581-87, 1993). It also is known that intermacromolecular complexes can be formed by mixing solutions of two complementary polymers. Such complexes are generally stabilized by electrostatic interactions (polyanion-polycation) and/or hydrogen bonds (polyacid-polybase) between the polymers involved, and/or by hydrophobic interactions between the polymers in an aqueous surrounding (Krupers et al., *Eur. Polym J.* 32:785-790, 1996). For example, mixing solutions of polyacrylic acid (PAAc) and polyethylene oxide (PEO) under the proper conditions results in the formation of complexes based mostly on hydrogen bonding. Dissociation of these complexes at physiologic conditions has been used for delivery of free drugs (i.e., non-PEGylated). In addition, complexes of complementary polymers have been formed from both homopolymers and copolymers.

Numerous reagents for PEGylation have been described in the art. Such reagents include, but are not limited to, reaction of the polypeptide with N-hydroxysuccinimidyl (NHS) activated PEG, succinimidyl mPEG, mPEG2-N-hydroxysuccinimide, mPEG succinimidyl alpha-methylbutanoate, mPEG succinimidyl propionate, mPEG succinimidyl butanoate, mPEG carboxymethyl 3-hydroxybutanoic acid succinimidyl ester, homobifunctional PEG-succinimidyl propionate, homobifunctional PEG propionaldehyde, homobifunctional PEG butyraldehyde, PEG maleimide, PEG hydrazide, p-nitrophenyl-carbonate PEG, mPEG-benzotriazole carbonate, propionaldehyde PEG, mPEG butryaldehyde, branched $mPEG_2$ butyraldehyde, mPEG acetyl, mPEG piperidone, mPEG methylketone, mPEG "linkerless" maleimide, mPEG vinyl sulfone, mPEG thiol, mPEG orthopyridylthioester, mPEG orthopyridyl disulfide, Fmoc-PEG-NHS, Boc-PEG-NHS, vinylsulfone PEG-NHS, acrylate PEG-NHS, fluorescein PEG-NHS, and biotin PEG-NHS (see e.g., Monfardini et al., *Bioconjugate Chem.* 6:62-69, 1995; Veronese et al., *J. Bioactive Compatible Polymers* 12:197-207, 1997; U.S. Pat. No. 5,672,662; U.S. Pat. No. 5,932,462; U.S. Pat. No. 6,495,659; U.S. Pat. No. 6,737,505; U.S. Pat. No. 4,002,531; U.S. Pat. No. 4,179,337; U.S. Pat. No. 5,122,614; U.S. Pat. No. 5,324,844; U.S. Pat. No. 5,446,090; U.S. Pat. No. 5,612,460; U.S. Pat. No. 5,643,575; U.S. Pat. No. 5,766,581; U.S. Pat. No. 5,795,569; U.S. Pat. No. 5,808,096; U.S. Pat. No. 5,900,461; U.S. Pat. No. 5,919,455; U.S. Pat. No. 5,985,263; U.S. Pat. No. 5,990,237; U.S. Pat. No. 6,113,906; U.S. Pat. No. 6,214,966; U.S. Pat. No. 6,258,351; U.S. Pat. No. 6,340,742; U.S. Pat. No. 6,413,507; U.S. Pat. No. 6,420,339; U.S. Pat. No. 6,437,025; U.S. Pat. No. 6,448,369; U.S. Pat. No. 6,461,802; U.S. Pat. No. 6,828,401; U.S. Pat. No. 6,858,736; U.S. 2001/0021763; U.S. 2001/0044526; U.S. 2001/0046481; U.S. 2002/0052430; U.S. 2002/0072573; U.S. 2002/0156047; U.S. 2003/0114647; U.S. 2003/0143596; U.S. 2003/0158333; U.S. 2003/0220447; U.S. 2004/0013637; US 2004/0235734; WO05000360; U.S. 2005/0114037; U.S. 2005/0171328; U.S. 2005/0209416; EP 1064951; EP 0822199; WO 01076640; WO 0002017; WO 0249673; WO 9428024; and WO 0187925).

In one example, the polyethylene glycol has a molecular weight ranging from about 3 kD to about 50 kD, and typically from about 5 kD to about 30 kD. Covalent attachment of the PEG to the drug (known as "PEGylation") can be accomplished by known chemical synthesis techniques. For example, the PEGylation of protein can be accomplished by reacting NHS-activated PEG with the protein under suitable reaction conditions.

While numerous reactions have been described for PEGylation, those that are most generally applicable confer directionality, utilize mild reaction conditions, and do not necessitate extensive downstream processing to remove toxic catalysts or bi-products. For instance, monomethoxy PEG (mPEG) has only one reactive terminal hydroxyl, and thus its use limits some of the heterogeneity of the resulting PEG-protein product mixture. Activation of the hydroxyl group at the end of the polymer opposite to the terminal methoxy group is generally necessary to accomplish efficient protein PEGylation, with the aim being to make the derivatised PEG more susceptible to nucleophilic attack. The attacking nucleophile is usually the epsilon-amino group of a lysyl residue, but other amines also can react (e.g., the N-terminal alpha-amine or the ring amines of histidine) if local conditions are favorable. A more directed attachment is possible in proteins containing a single lysine or cysteine. The latter residue can be targeted by PEG-maleimide for thiol-specific modification. Alternatively, PEG hydrazide can be reacted with a periodate oxidized hyaluronan-degrading enzyme and reduced in the presence of $NaCNBH_3$. More specifically, PEGylated CMP sugars can be reacted with a hyaluronan-degrading enzyme in the presence of appropriate glycosyl-transferases. One technique is the "PEGylation" technique where a number of polymeric molecules are coupled to the polypeptide in question. When using this technique, the immune system has difficulties in recognizing the epitopes on the polypeptide's surface responsible for the formation of antibodies, thereby reducing the immune response. For polypeptides introduced directly into the circulatory system of the human body to give a particular physiological effect (i.e., pharmaceuticals) the typical potential immune response is an IgG and/or IgM response, while polypeptides which are inhaled through the respiratory system (i.e., industrial polypeptide) potentially can cause an IgE response (i.e., allergic response). One of the theories explaining the reduced immune response is that the polymeric molecule(s) shield(s) epitope(s) on the surface of the polypeptide responsible for the immune response leading to antibody formation. Another theory or at least a partial factor is that the heavier the conjugate is, the more reduced the resulting immune response is.

Typically, to make the PEGylated PH20 polypeptide provided herein, PEG moieties are conjugated, via covalent attachment, to the polypeptides. Techniques for PEGylation include, but are not limited to, specialized linkers and coupling chemistries (see e.g., Roberts, *Adv. Drug Deliv. Rev.* 54:459-476, 2002), attachment of multiple PEG moieties to a single conjugation site (such as via use of branched

PEGs; see e.g., Guiotto et al., *Bioorg. Med. Chem. Lett.* 12:177-180, 2002), site-specific PEGylation and/or mono-PEGylation (see e.g., Chapman et al., *Nature Biotech.* 17:780-783, 1999), and site-directed enzymatic PEGylation (see e.g., Sato, *Adv. Drug Deliv. Rev.*, 54:487-504, 2002). Methods and techniques described in the art can produce proteins having 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more than 10 PEG or PEG derivatives attached to a single protein molecule (see e.g., U.S. 2006/0104968).

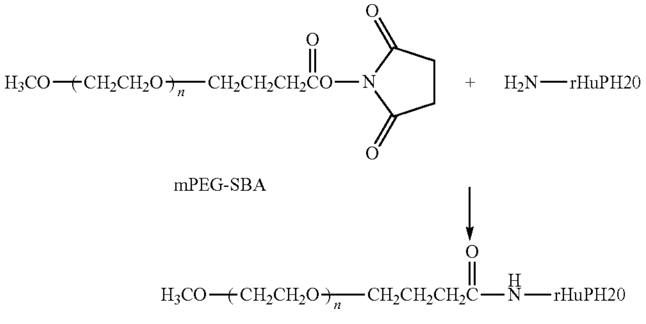
As an exemplary illustrative method for making a PEGylated PH20 polypeptide, PEG aldehydes, succinimides and carbonates have each been applied to conjugate PEG moieties, typically succinimidyl PEGs, to rHuPH20. For example, rHuPH20 has been conjugated with exemplary succinimidyl monoPEG (mPEG) reagents including mPEG-Succinimidyl Propionates (mPEG-SPA), mPEG-Succinimidyl Butanoates (mPEG-SBA), and (for attaching "branched" PEGs) mPEG2-N-Hydroxylsuccinimide. These PEGylated succinimidyl esters contain different length carbon backbones between the PEG group and the activated cross-linker, and either a single or branched PEG group. These differences can be used, for example, to provide for different reaction kinetics and to potentially restrict sites available for PEG attachment to rHuPH20 during the conjugation process.

Succinimidyl PEGs (as above) containing either linear or branched PEGs can be conjugated to PH20. PEGs can used to generate PH20s reproducibly containing molecules having, on the average, between about three to six or three to six PEG molecules per hyaluronidase. Such PEGylated rHuPH20 compositions can be readily purified to yield compositions having specific activities of approximately 25,000 or 30,000 Unit/mg protein hyaluronidase activity, and being substantially free of non-PEGylated PH20 (less than 5% non-PEGylated).

Using various PEG reagents, exemplary versions of a PEGylated PH20 polypeptide can be prepared, for example, using mPEG-SBA (30 kD), mPEG-SMB (30 kD), and branched versions based on mPEG2-NHS (40 kD) and mPEG2-NHS (60 kD). PEGylated versions of PH20 can be generated using NHS chemistries, as well as carbonates, and aldehydes, using each of the following reagents: mPEG2-NHS-40K branched, mPEG-NHS-10K branched, mPEG-NHS-20K branched, mPEG2-NHS-60K branched; mPEG-SBA-5K, mPEG-SBA-20K, mPEG-SBA-30K; mPEG-SMB-20K, mPEG-SMB-30K; mPEG-butyrldehyde; mPEG-SPA-20K, mPEG-SPA-30K; and PEG-NHS-5K-biotin. PEGylated PH20 also can be prepared using PEG reagents available from Dowpharma, a division of Dow Chemical Corporation; including PH20 polypeptides PEGylated with Dowpharma's p-nitrophenyl-carbonate PEG (30 kDa) and with propionaldehyde PEG (30 kDa).

In one example, the PEGylation includes conjugation of mPEG-SBA, for example, mPEG-SBA-30K (having a molecular weight of about 30 kDa) or another succinimidyl ester of a PEG butanoic acid derivative, to a PH20 polypeptide. Succinimidyl esters of PEG butanoic acid derivatives, such as mPEG-SBA-30K readily couple to amino groups of proteins. For example, covalent conjugation of m-PEG-SBA-30K and rHuPH20 (which is approximately 60 KDa in size) provides stable amide bonds between rHuPH20 and mPEG, as shown in Scheme 1, below.

Scheme 1



PEGylated rHuPH20

Typically, the mPEG-SBA-30K or other PEG is added to the PH20 polypeptide at a PEG:polypeptide molar ratio of 10:1 in a suitable buffer, e.g., 130 mM NaCl/10 mM HEPES at pH 6.8 or 70 mM phosphate buffer, pH 7, followed by sterilization, e.g., sterile filtration, and continued conjugation, for example, with stirring, overnight at 4° C. in a cold room. In one example, the conjugated PEG-PH20 is concentrated and buffer-exchanged.

Other methods of coupling succinimidyl esters of PEG butanoic acid derivatives, such as mPEG-SBA-30K are known in the art (see e.g., U.S. Pat. No. 5,672,662; U.S. Pat. No. 6,737,505; and U.S. 2004/0235734). For example, a polypeptide, such as a PH20 polypeptide, can be coupled to an NHS activated PEG derivative by reaction in a borate buffer (0.1 M, pH 8.0) for one hour at 4° C. The resulting PEGylated protein can be purified by ultrafiltration. Another method reacts polypeptide with mPEG-SBA in deionized water to which triethylamine is added to raise the pH to 7.2-9. The resulting mixture is stirred at room temperature for several hours to complete the PEGylation.

Methods for PEGylation of PH20 polypeptides, including, for example, animal-derived hyaluronidases and bacterial hyaluronan-degrading enzymes, are known to one of skill in the art. See, for example, European Patent No. EP 0400472, which describes the PEGylation of bovine testes hyaluorindase and chondroitin ABC lyase. Also, U.S. Publication No. 2006014968 describes PEGylation of a human hyaluronidase derived from human PH20. For example, the PEGylated hyaluronan-degrading enzyme generally contains at least 3 PEG moieties per molecule. In some examples, the PH20 polypeptide contains three to six PEG molecules. In other examples, the enzyme can have a PEG to protein molar ratio between 5:1 and 9:1, for example, 7:1.

F. Pharmaceutical Compositions and Formulations, Dosages and Administration

Pharmaceutical compositions of any of the modified PH20 polypeptides are provided herein for administration. Pharmaceutically acceptable compositions are prepared in view of approvals for a regulatory agency or other agency prepared in accordance with generally recognized pharmacopeia for use in animals and in humans. Typically, the compounds are formulated into pharmaceutical compositions using techniques and procedures well known in the art (see e.g., Ansel *Introduction to Pharmaceutical Dosage Forms*, Fourth Edition, 1985, 126).

In particular, provided herein are pharmaceutical compositions that are stable as a liquid formulation for prolonged periods of time for at least 1 month at temperatures from or from about 2° C. to 8° C., inclusive or for at least 3 days at a temperature from or from about 30° C. to 42° C., inclusive. Pharmaceutical compositions, in particular liquid formulations, can be limited by the stability of the active agent, which can be susceptible to effects of storage conditions (time or length of storage, temperature and/or agitation) and/or, formulation components contained in the composition. Hence, the stable pharmaceutical compositions generally contain a modified PH20 polypeptide as described in Section C.1.b that exhibits increased stability manifested as an increased resistance to one or more protein denaturation conditions. Such protein denaturation conditions can include, but are not limited to, elevated temperature greater than or equal to or about 30° C., agitation, low or no salt, and presence of excipients. The increased stability is characterized by improved storage time, decreased fragmentation, and/or decreased aggregate formation, while still retaining the activity of the active agent(s), e.g., the PH20 hyaluronidase. Such formulations can be provided as "ready-to use" liquid formulations without further reconstitution and/or without any requirement for further dilution. In some examples, the formulations also can be prepared in a lyophilized or concentrated form.

Pharmaceutical compositions containing a modified PH20 polypeptide can be co-administered with another therapeutic agent. In such examples, the modified PH20 polypeptides can be formulated separately as a pharmaceutical composition and administered prior to, simultaneously with, intermittently with, or subsequent to a second composition containing an active therapeutic agent. In other examples, modified PH20 polypeptides can be co-formulated with pharmaceutical formulations of other therapeutic agents.

In particular, provided herein are co-formulations containing a modified PH20 polypeptide as described herein and a therapeutic agent that is a chemotherapeutic agent, an analgesic agent, an anti-inflammatory agent, an antimicrobial agent, an amoebicidal agent, a trichomonacidal agent, an anti-parkinson agent, an anti-malarial agent, an anticonvulsant agent, an anti-depressant agent, and antiarthritics agent, an anti-fungal agent, an antihypertensive agent, an antipyretic agent, an anti-parasite agent, an antihistamine agent, an alpha-adrenergic agonist agent, an alpha blocker agent, an anesthetic agent, a bronchial dilator agent, a biocide agent, a bactericide agent, a bacteriostat agent, a beta adrenergic blocker agent, a calcium channel blocker agent, a cardiovascular drug agent, a contraceptive agent, a decongestant agent, a diuretic agent, a depressant agent, a diagnostic agent, a electrolyte agent, a hypnotic agent, a hormone agent, a hyperglycemic agent, a muscle relaxant agent, a muscle contractant agent, an ophthalmic agent, a parasympathomimetic agent, a psychic energizer agent, a sedative agent, a sympathomimetic agent, a tranquilizer agent, an urinary agent, a vaginal agent, a viricide agent, a vitamin agent, a non-steroidal anti-inflammatory agent, an angiotensin converting enzyme inhibitor agent, a polypeptide, a protein, a nucleic acid, a drug, an organic molecule or a sleep inducer. For example, modified PH20 polypeptides provided herein can be co-formulated with an antibody such as a monoclonal antibody, an Immune Globulin, an antibiotic, a bisphosphonate, a cytokine, a chemotherapeutic agent, a coagulation factor or an insulin. Exemplary therapeutic agents that can be co-formulated with a modified PH20 polypeptide are described in described in Section H. In particular, provided herein are co-formulations containing a modified PH20 polypeptide and an insulin, such as a fast-acting insulin, for example, a regular insulin or a fast-acting (rapid-acting) insulin analog. The co-formulations provided herein include stable co-formulations, whereby the active agents, i.e., the modified PH20 polypeptide and the therapeutic agent, exhibit increased stability and retain activity for prolonged periods as described herein.

Formulations containing PH20 provided herein, including separate formulations thereof and co-formulations, are stable for prolonged periods of time, including at varied temperatures and under varied storage or use conditions such as agitation. For example, the formulations provided herein are stable and retain activity of active agent(s) (e.g., PH20 hyaluronidase) at "refrigerator" conditions, for example, at 2° C. to 8° C., such as at or about 4° C., for at least at least 2 months, 3 months, 4 months, 5 months, 6 months, 7 months, at least 8 months, at least 9 months, at least 10 months, at least 11 months, at least 12 months, 13 months, 14 months, 15 months, 16 months, 17 months, 18 months, 19 months, 20 months, 21 months, 22 months, 23 months, 24 months, 25 months, 26 months, 27 months, 28 months, 29 months or 30 months or more. In another example, the formulations provided herein are stable and retain activity of active agent(s) (e.g., PH20 hyaluronidase) at room temperature for example at 18° C. to 32° C., generally 20° C. to 32° C., such as 28° C. to 32° C., for at least 2 weeks to 1 year, for example, at least 3 weeks, 4 weeks, 2 months, 3 months, 4 months, 5 months, 6 months, at least 7 months, at least 8 months, at least 9 months, or at least 1 year or more. In a further example, the formulations provided herein are stable and retain activity of active agent(s) (e.g., PH20 hyaluronidase) at elevated temperatures of about or greater than 30° C., generally from or from about 30° C. to 42° C., such as 32° C. to 37° C. or 35° C. to 37° C. or about or 37° C. for at least 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, 30 days, 35 days, 40 days, 45 days, 50 days, 60 days or more.

Compositions can take the form of solutions, suspensions, emulsions, tablets, pills, capsules, powders, and sustained release formulations. A composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and other such agents. Topical formulations also are contemplated. The formulation should suit the mode of administration.

1. Formulations—Liquids, Injectables, Emulsions

The formulation generally is made to suit the route of administration. Parenteral administration, generally characterized by injection or infusion, either subcutaneously, intramuscularly, intravenously or intradermally is contemplated herein. Preparations for parenteral administration include sterile solutions ready for injection, sterile dry soluble products, such as lyophilized powders, ready to be combined with a solvent just prior to use, including hypodermic tablets, sterile suspensions ready for injection, sterile dry insoluble products ready to be combined with a vehicle just prior to use and sterile emulsions. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. For example, the compositions containing a modified PH20 polypeptide, formulated separately or co-formulated with another therapeutic agent, can be provided as a pharmaceutical preparation in liquid form as a solution, syrup or suspension. In liquid form, the pharmaceutical preparations can be provided as a concentrated preparation to be diluted to a therapeutically effective concentration before use. Generally, the preparations are provided in a dosage form that does not require dilution for use. In another example, pharmaceutical preparations can be presented in lyophilized form for reconstitution with water or other suitable vehicle before use.

Injectables are designed for local and systemic administration. For purposes herein, local administration is desired for direct administration to the affected interstitium. The solutions can be either aqueous or nonaqueous. If administered intravenously, suitable carriers include physiological saline or phosphate buffered saline (PBS), and solutions containing thickening and solubilizing agents, such as glucose, polyethylene glycol, and polypropylene glycol and mixtures thereof.

The concentration of the pharmaceutically active compound is adjusted so that an injection or infusion provides an effective amount to produce the desired pharmacological effect. The exact dose depends on the age, weight and condition of the patient or animal as is known in the art. The unit-dose parenteral preparations can be packaged in, for example, an ampoule, a cartridge, a vial or a syringe with a needle. The volume of liquid solution or reconstituted powder preparation, containing the pharmaceutically active compound, is a function of the disease to be treated and the particular article of manufacture chosen for package. All preparations for parenteral administration must be sterile, as is known and practiced in the art. The percentage of active compound contained in such parenteral compositions is highly dependent on the specific nature thereof, as well as the activity of the compound and the needs of the subject.

Pharmaceutical compositions can include carriers or other excipients. For example, pharmaceutical compositions provided herein can contain any one or more of a diluents(s), adjuvant(s), antiadherent(s), binder(s), coating(s), fillers), flavors), color(s), lubricant(s), glidant(s), preservative(s), detergent(s), sorbent(s) or sweeteners) and a combination thereof or vehicle with which a modified PH20 polypeptide is administered. For example, pharmaceutically acceptable carriers or excipients used in parenteral preparations include aqueous vehicles, nonaqueous vehicles, antimicrobial agents, isotonic agents, buffers, antioxidants, local anesthetics, suspending and dispersing agents, emulsifying agents, sequestering or chelating agents and other pharmaceutically acceptable substances. Formulations, including liquid preparations, can be prepared by conventional means with pharmaceutically acceptable additives or excipients.

Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E. W. Martin. Such compositions will contain a therapeutically effective amount of the compound, generally in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. Such pharmaceutical carriers can be sterile liquids, such as water or oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, and sesame oil. Water is a typical carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions also can be employed as liquid carriers, particularly for injectable solutions. Examples of aqueous vehicles include Sodium Chloride Injection, Ringers Injection, Isotonic Dextrose Injection, Sterile Water Injection, Dextrose and Lactated Ringers Injection. Nonaqueous parenteral vehicles include fixed oils of vegetable origin, cottonseed oil, corn oil, sesame oil and peanut oil. Suspending and dispersing agents include, but are not limited to, sorbitol syrup, cellulose derivatives or hydrogenated edible fats, sodium carboxymethylcellulose, hydroxypropyl methylcellulose and polyvinylpyrrolidone. Emulsifying agents include, but are not limited to, lecithin or acacia. Detergents include, but are not limited to, Polysorbate 80 (TWEEN 80). Non-aqueous vehicles include, but are not limited to, almond oil, oily esters, or fractionated vegetable oils. Antimicrobial agents or preservatives include, but are not limited to, methyl or propyl-p-hydroxybenzoates or sorbic acid, m-cresol, phenol. A diluent includes, but is not limited to, lactose, sucrose, dicalcium phosphate, or carboxymethylcellulose. A lubricant includes, but is not limited to, magnesium stearate, calcium stearate or talc. A binder includes, but is not limited to, starch, natural gums, such as gum acacia, gelatin, glucose, molasses, polyvinylpyrrolidine, celluloses and derivatives thereof, povidone, crospovidones and other such binders known to those of skill in the art. Isotonic agents include, but are not limited to, sodium chloride and dextrose. Buffers include, but are not limited to, phosphate and citrate. Antioxidants include sodium bisulfate. Local anesthetics include procaine hydrochloride. A sequestering or chelating agent of metal ions includes EDTA. Other suitable pharmaceutical excipients include, but are not limited to, starch, glucose, lactose, dextrose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, saline, water, and ethanol. Pharmaceutical carriers also include ethyl alcohol, polyethylene glycol and propylene glycol for water miscible vehicles and sodium hydroxide, hydrochloric acid, citric acid or lactic acid for pH adjustment. A composition, if desired, also can contain minor amounts of non-toxic auxiliary substances such as wetting or emulsifying agents, or pH buffering agents, for example, acetate, sodium citrate, cyclodextrin derivatives, sorbitan monolaurate, triethanolamine sodium acetate, triethanolamine oleate, stabilizers, solubility enhancers, and other such agents such as for example, sodium acetate, sodium phosphate, sorbitan monolaurate, triethanolamine oleate and cyclodextrins.

In particular, antimicrobial agents (e.g., preservatives) in bacteriostatic or fungistatic concentrations (e.g., an antimicrobial effective amount) can be added to parenteral preparations packaged in multiple-dose containers, which include phenols or cresols, mercurials, benzyl alcohol, chlorobutanol, methyl and propyl p-hydroxybenzoic acid esters, thimerosal, benzalkonium chloride and benzethonium chloride.

The volume of the formulations, including the separately formulated or co-formulated PH20-containing formulations provided herein, can be any volume suitable for the container in which it is provided. In some examples, the formulations are provided in a vial, syringe, pen, reservoir for a pump or a closed loop system, or any other suitable container. For example, the formulations provided herein are between or about between 0.1 mL to 500 mL, such as 0.1 mL to 100 mL, 1 mL to 100 mL, 0.1 mL to 50 mL, such as at least or about at least or about or 0.1 mL, 1 mL, 2 mL, 3 mL, 4 mL, 5 mL, 10 mL, 15 mL, 20 mL, 30 mL, 40 mL, 50 mL or more.

a. Lyophilized Powders

Of interest herein are lyophilized powders, which can be reconstituted for administration as solutions, emulsions and other mixtures. They may also be reconstituted and formulated as solids or gels.

The sterile, lyophilized powder is prepared by dissolving a compound of enzyme in a buffer solution. The buffer solution may contain an excipient which improves the stability or other pharmacological component of the powder or reconstituted solution, prepared from the powder. Subsequent sterile filtration of the solution followed by lyophilization under standard conditions known to those of skill in the art provides the desired formulation. A liquid formulation as described herein above can be prepared. The resulting mixture is sterile filtered or treated to remove particulates and to insure sterility, and apportioned into vials for lyophilization. For example, the lyophilized powder can be prepared by dissolving an excipient, such as dextrose, sorbitol, fructose, corn syrup, xylitol, glycerin, glucose, sucrose or other suitable agent, in a suitable buffer, such as citrate, sodium or potassium phosphate or other such buffer known to those of skill in the art. Then, a selected enzyme is added to the resulting mixture, and stirred until it dissolves.

Each vial is made to contain a single dosage or multiple dosages of the compound. The lyophilized powder can be stored under appropriate conditions, such as at about 4° C. to room temperature. Reconstitution of this lyophilized powder with an appropriate buffer solution provides a formulation for use in parenteral administration,

b. Exemplary Formulations

Single dose formulations of PH20 are known in the art. For example, Hylenex® recombinant (hyaluronidase human injection) contains, per mL, 8.5 mg NaCl (145 mM), 1.4 mg dibasic sodium phosphate (9.9 mM), 1.0 mg human albumin, 0.9 mg edetate disodium (2.4 mM), 0.3 mg $CaCl_2$ (2.7 mM) and NaOH to adjust the pH to 7.4. Other formulations of human soluble hyaluronidase, such as the rHuPH20 formulations described in U.S. Pat. Pub. No. US2011/0053247, include 130 mM NaCl, 10 mM Hepes, pH 7.0; or 10 mM histidine, 130 mM NaCl, pH 6.0. Any of the modified PH20 polypeptides provided herein can be similarly formulated.

In addition to a therapeutically effective amount of a modified PH20 polypeptide and/or other therapeutic agent, exemplary pharmaceutical compositions provided herein, including separately formulated- and co-formulated-PH20 containing formulations, can contain a concentration of NaCl and are prepared at a requisite pH to maintain the stability of the active agent(s) (e.g., PH20 hyaluronidase and/or other co-formulated therapeutic agent). For multidose formulations and other formulations stored for a prolonged time, the compositions generally also contain one or more preservatives. Further stabilizing agents and other excipients also can be included. Exemplary components are described below.

i. Salt (e.g. NaCl)

In examples herein, the pharmaceutical compositions provided herein contain a concentration of salt, such as sodium chloride (NaCl), to maintain the stability of the active agent(s) (e.g., PH20 hyaluronidase). Salt, such as NaCl, is generally required to retain PH20 stability and activity. Low salt concentrations of generally less than 120 mM can have deleterious effects on PH20 activity over time and depending on temperature conditions. Hence, the absence of salt (e.g. NaCl) or a low concentration of salt (e.g. NaCl) can result in instability of the protein. In some examples herein, however, modified PH20 polypeptides that exhibit increased stability in the absence of low or no salt, such as low or no NaCl (see e.g., Section C.1.b.iii), are not susceptible to denaturation. Also, the presence of salt (e.g. NaCl) can have differing effects on other therapeutic agents. For example, the solubility of insulin and insulin analogs tends to increase with lower salt concentration (e.g., <140 mM) and high salt concentrations can result in crystallization/aggregation of insulin, especially at lower temperatures (see e.g., U.S. Provisional Appl. No. 61/520,962; U.S. application Ser. Nos. 13/507,263 and 13/507,262; and International PCT Application No. PCT/US2012/042816). Thus, pharmaceutical compositions provided herein are prepared in accordance with the requirements of the active agent(s). It is within the level of one of skill in the art to assess the stability of the active agent(s) in the formulation and under various storage conditions (see e.g., Section G). In particular examples herein, the pharmaceutical compositions, including the separately formulated or co-formulated PH20-containing formulations provided herein, contain NaCl at a concentration of between or about between 10 mM to 200 mM, such as 10 mM to 50 mM, 50 mM to 200 mM, 50 mM to 120 mM, 50 mM to 100 mM, 50 mM to 90 mM, 120 mM to 160 mM, 130 mM to 150 mM, 80 mM to 140 mM, 80 mM to 120 mM, 80 mM to 100 mM, 80 mM to 160 mM, 100 mM to 140 mM, 120 mM to 120 mM or 140 mM to 180 mM.

ii. pH and Buffer

In examples herein, the pharmaceutical compositions provided herein are prepared at a pH to maintain the stability of the active agent(s) (e.g., PH20 hyaluronidase). For example, the pharmaceutical compositions provided herein are prepared at a pH of between or about between 6.5 to 7.8 such as between or about between 6.5 to 7.2, 7.0 to 7.8, 7.0 to 7.6 or 7.2 to 7.4. Reference to pH herein is based on measurement of pH at room temperature. It is understood that the pH can change during storage over time, but typically will remain between or between about pH 6.5 to or to about 7.8. For example, the pH can vary by ±0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.2, 1.3, 1.4, 1.5 or more. Exemplary co-formulations provided herein have a pH of or of about 7.0±0.2, 7.1±0.2, 7.2±0.2, 7.3±0.2, 7.4±0.2, 7.5±0.2 or 7.6±0.2 when prepared. If necessary, pH can be adjusted using acidifying agents to lower the pH or alkalizing agents to increase the pH. Exemplary acidifying agents include, but are not limited to, acetic acid, citric acid, sulfuric acid, hydrochloric acid, monobasic sodium phosphate solution, and phosphoric acid. Exemplary alkalizing agents include, but are not limited to, dibasic sodium phosphate solution, sodium carbonate, or sodium hydroxide.

The compositions are generally prepared using a buffering agent that maintains the pH range. Any buffer can be used in formulations provided herein so long as it does not adversely affect the stability of the active agent(s) (e.g., PH20 hyaluronidase), and supports the requisite pH range required. Examples of particularly suitable buffers include Tris, succinate, acetate, phosphate buffers, citrate, aconitate, malate and carbonate. Those of skill in the art, however, will recognize that formulations provided herein are not limited to a particular buffer, so long as the buffer provides an acceptable degree of pH stability, or "buffer capacity" in the range indicated. Generally, a buffer has an adequate buffer capacity within about 1 pH unit of its pK (Lachman et al. In: The Theory and Practice of Industrial Pharmacy 3rd Edn. (Lachman, L., Lieberman, H A. and Kanig, J. L., Eds.), Lea and Febiger, Philadelphia, p. 458-460, 1986). Buffer suitability can be estimated based on published pK tabulations or can be determined empirically by methods well known in the art. The pH of the solution can be adjusted to the desired endpoint within the range as described above, for example, using any acceptable acid or base.

Buffers that can be included in the co-formulations provided herein include, but are not limited to, Tris (Tromethamine), histidine, phosphate buffers, such as dibasic sodium phosphate, and citrate buffers. Such buffering agents can be present in the co-formulations at concentrations between or about between 1 mM to 100 mM, such as 10 mM to 50 mM or 20 mM to 40 mM, such as at or about 30 mM. For example, such buffering agents can be present in the co-formulations in a concentration of or about 1 mM, 2 mM, 3 mM, 4 mM, 5 mM, 6 mM, 7 mM, 8 mM, 9 mM, 10 mM, 11 mM, 12 mM, 13 mM, 14 mM, 15 mM, 16 mM, 17 mM, 18 mM, 19 mM, 20 mM, 25 mM, 30 mM, 35 mM, 40 mM, 45 mM, 50 mM, 55 mM, 60 mM, 65 mM, 70 mM, 75 mM, or more.

iii. Preservative(s)

In examples herein, multi-dose formulations or formulations stored for prolonged periods contain an anti-microbially effective amount of preservative or mixture of preservatives in an amount to have a bacteriostatic or fungistatic effect. In particular examples, the preservatives are present in a sufficient concentration to provide the anti-microbial requirements of, for example, the United States Pharmacopoeia (USP) and the European Pharmacopoeia (EP), including the EP anti-microbial requirements (EPA) and the preferred EP anti-microbial requirements (EPB) (see Table 4). Since the presence of preservatives, and in particular phenolic preservatives, can have deleterious effects on the stability of PH20, such formulations typically contain a modified PH20 polypeptide that exhibits increased stability in the presence of preservatives, such as any described in Section C.1.b.i herein. Generally, the amount maintains the stability of the active agent(s) (e.g., PH20 hyaluronidase).

An anti-microbial effective amount of preservative is an amount that exhibits anti-microbial activity by killing or inhibiting the propagation of microbial organisms in a sample of the composition as assessed in an antimicrobial preservative effectiveness test (APET). One of skill in the art is familiar with the antimicrobial preservative effectiveness test and standards to be meet under the USP and EPA or EPB in order to meet minimum requirements. In general, the antimicrobial preservative effectiveness test involves challenging a composition with prescribed inoculums of suitable microorganisms, i.e., bacteria, yeast and fungi, storing the inoculated preparation at a prescribed temperature, withdrawing samples at specified intervals of time and counting the organisms in the sample (see, Sutton and Porter, (2002) *PDA Journal of Pharmaceutical Science and Technology* 56(4):300-311; The United States Pharmacopeial Convention, Inc., (effective Jan. 1, 2002), *The United States Pharmacopeia* 25*th* *Revision*, Rockville, Md., Chapter <51> Antimicrobial Effectiveness Testing; and European Pharmacopoeia, Chapter 5.1.3, Efficacy of Antimicrobial Preservation). The microorganisms used in the challenge generally include three strains of bacteria, namely *E. coli* (ATCC No. 8739), *Pseudomonas aeruginosa* (ATCC No. 9027) and *Staphylococcus aureus* (ATCC No. 6538), yeast (*Candida albicans* ATCC No. 10231) and fungus (*Aspergillus niger* ATCC No. 16404), all of which are added such that the inoculated composition contains 10^5 or 10^6 colony forming units (cfu) of microorganism per mL of composition. The preservative properties of the composition are deemed adequate if, under the conditions of the test, there is a significant fall or no increase, as specified in Table 3 in the number of microorganisms in the inoculated composition after the times and at the temperatures prescribed. The criteria for evaluation are given in terms of the log reduction in the number of viable microorganism as compared to the initial sample or the previous time point.

Non-limiting examples of preservatives that can be included in the co-formulations provided herein include, but are not limited to, phenol, meta-cresol (m-cresol), methylparaben, benzyl alcohol, thimerosal, benzalkonium chloride, 4-chloro-1-butanol, chlorhexidine dihydrochloride, chlorhexidine digluconate, L-phenylalanine, EDTA, bronopol (2-bromo-2-nitropropane-1,3-diol), phenylmercuric acetate, glycerol (glycerin), imidurea, chlorhexidine, sodium dehydroacetate, ortho-cresol (o-cresol), para-cresol (p-cresol), chlorocresol, cetrimide, benzethonium chloride, ethylparaben, propylparaben or butylparaben and any combination thereof. For example, formulations provided herein can contain a single preservative. In other examples, the formulations contain at least two different preservatives or at least three different preservatives. For example, formulations provided herein can contain two preservatives such as L-phenylalanine and m-cresol, L-phenylalanine and methylparaben, L-phenylalanine and phenol, m-cresol and methylparaben, phenol and methylparaben, m-cresol and phenol or other similar combinations. In one example, the preservative in the formulation contains at least one phenolic preservative. For example, the formulation contains phenol, m-cresol or phenol and m-cresol.

In the formulations provided herein, the total amount of the one or more preservative agents as a percentage (%) of mass concentration (w/v) in the formulation can be, for example, between from or between about from 0.1% to 0.4%, such as 0.1% to 0.3%, 0.15% to 0.325%, 0.15% to 0.25%, 0.1% to 0.2%, 0.2% to 0.3%, or 0.3% to 0.4%. Generally, the formulations contain less than 0.4% (w/v) preservative. For example, the co-formulations provided herein contain at least or about at least 0.1%, 0.12%, 0.125%, 0.13%, 0.14%, 0.15%, 0.16%0.17%, 0.175%, 0.18%, 0.19%, 0.2%, 0.25%, 0.3%, 0.325%, 0.35% but less than 0.4% total preservative.

In some examples, the formulations provided herein contain between or between about 0.1% to 0.25% phenol and between or about between 0.05% to 0.2% m-cresol, such as between or about between 0.10% to 0.2% phenol and between or about between 0.06% to 0.18% m-cresol, or between or about between 0.1% to 0.15% phenol and between or about between 0.08% to 0.15% m-cresol. For example, formulations provided herein contain or contain about 0.1% phenol and 0.075% m-cresol; 0.1% phenol and 0.15% m-cresol; 0.125% phenol and 0.075% m-cresol; 0.13% phenol and 0.075% m-cresol; 0.13% phenol and 0.08% m-cresol; 0.15% phenol and 0.175% m-cresol; or 0.17% phenol and 0.13% m-cresol.

iv. Stabilizers

In examples herein, the pharmaceutical compositions provided herein optionally can contain one or more other stabilizing agent to maintain the stability of the active agent(s) (e.g., PH20 hyaluronidase). Included among the types of stabilizers that can be contained in the formulations provided herein are amino acids, amino acid derivatives, amines, sugars, polyols, salts and buffers, surfactants, and other agents. The formulations provided herein contain at least one stabilizer. For example, the formulations provided herein contain at least one, two, three, four, five, six or more stabilizers. Hence, any one or more of an amino acids, amino acid derivatives, amines, sugars, polyols, salts and buffers, surfactants, and other agents can be included in the formulations herein. Generally, the formulations herein contain at least contain a surfactant and an appropriate buffer. Optionally, the formulations provided herein can contain other additional stabilizers. Other components include, for example, one or more tonicity modifiers, one or more anti-oxidation agents, or other stabilizer.

Exemplary amino acid stabilizers, amino acid derivatives or amines include, but are not limited to, L-Arginine, Glutamine, Glycine, Lysine, Methionine, Proline, Lys-Lys, Gly-Gly, Trimethylamine oxide (TMAO) or betaine. Exemplary sugars and polyols include, but are not limited to, glycerol, sorbitol, mannitol, inositol, sucrose or trehalose. Exemplary salts and buffers include, but are not limited to, magnesium chloride, sodium sulfate, Tris such as Tris (100 mM), or sodium Benzoate. Exemplary surfactants include, but are not limited to, poloxamer 188 (e.g., Pluronic® F68), polysorbate 80 (PS80), polysorbate 20 (PS20). Other stabilizers include, but are not limited to, hyaluronic acid (HA), human serum albumin (HSA), phenyl butyric acid, taurocholic acid, polyvinylpyrolidone (PVP) or zinc.

In particular examples herein, the formulations contain one or more detergents, such as surfactants, to maintain the stability of the active agent(s) (e.g., PH20 hyaluronidase). For example, surfactants can inhibit aggregation of PH20 and minimize absorptive loss. The surfactants generally are non-ionic surfactants. Surfactants that can be included in the formulations herein include, but are not limited to, partial and fatty acid esters and ethers of polyhydric alcohols such as of glycerol, or sorbitol, poloxamers and polysorbates. For example, exemplary surfactants in the -formulations herein include any one or more of poloxamer 188 (PLURONICS® such as PLURONIC® F68), TETRONICS®, polysorbate 20, polysorbate 80, PEG 400, PEG 3000, Tween® (e.g., Tween® 20 or Tween® 80), Triton® X-100, SPAN®, MYRJ®, BRIJ®, CREMOPHOR®, polypropylene glycols or polyethylene glycols. In some examples, the formulations herein contain poloxamer 188, polysorbate 20, polysorbate 80, generally poloxamer 188 (pluronic F68). The formulations provided herein generally contain at least one surfactant, such as 1, 2 or 3 surfactants.

In the formulations provided herein, the total amount of the one or more surfactants as a percentage (%) of mass concentration (w/v) in the formulation can be, for example, between from or between about from 0.005% to 1.0%, such as between from or between about from 0.01% to 0.5%, such as 0.01% to 0.1% or 0.01% to 0.02%. Generally, the formulations contain at least 0.01% surfactant and contain less than 1.0%, such as less than 0.5% or less than 0.1% surfactant. For example, the formulations provided herein can contain at or about 0.001%, 0.005%, 0.01%, 0.015%, 0.02%, 0.025%, 0.03%, 0.035%, 0.04%, 0.045%, 0.05%, 0.055%, 0.06%, 0.065%, 0.07%, 0.08%, or 0.09% surfactant. In particular examples, the formulations provided herein contain or contain about 0.01% to or to about 0.05% surfactant.

Tonicity modifiers can be included in the formulation provided herein to produce a solution with the desired osmolality. The formulations provided herein have an osmolality of between or about between 245 mOsm/kg to 305 mOsm/kg. For example, the osmolality is or is about 245 mOsm/kg, 250 mOsm/kg, 255 mOsm/kg, 260 mOsm/kg, 265 mOsm/kg, 270 mOsm/kg, 275 mOsm/kg, 280 mOsm/kg, 285 mOsm/kg, 290 mOsm/kg, 295 mOsm/kg, 300 mOsm/kg or 305 mOsm/kg. In some examples, the formulations have an osmolality of or of about 275 mOsm/kg. Tonicity modifiers include, but are not limited to, glycerin, NaCl, amino acids, polyalcohols, trehalose, and other salts and/or sugars. The particular amount can be empirically determined in order to retain enzyme activity, and/or tonicity.

In other instances, glycerin (glycerol) is included in the formulations. For example, formulations provided herein typically contain less than 60 mM glycerin, such as less than 55 mM, less than 50 mM, less than 45 mM, less than 40 mM, less than 35 mM, less than 30 mM, less than 25 mM, less than 20 mM, less than 15 mM, 10 mM or less. The amount of glycerin typically depends on the amount of NaCl present: the more NaCl present in the formulation, the less glycerin is required to achieve the desired osmolality or osmolarity. Thus, for example, in formulations containing higher NaCl concentrations, little or no glycerin need be included in the formulation. In contrast, in formulations containing slightly lower NaCl concentrations, glycerin can be included. For example, formulations provided herein can contain glycerin at a concentration of 40 mM to 60 mM, such as less than 50 mM, such as 20 mM to 50 mM, for example at or about 50 mM.

The formulations provided herein also can contain antioxidants to reduce or prevent oxidation, in particular oxidation of the PH20 polypeptide. For example, oxidation can be effected by high concentrations of surfactant or hyaluronan oligomers. Exemplary antioxidants include, but are not limited to, cysteine, tryptophan and methionine. In particular examples, the antioxidant is methionine. The formulations provided herein can include an antioxidant at a concentration from between or from about between 5 mM to or to about 50 mM, such as 5 mM to 40 mM, 5 mM to 20 mM or 10 mM to 20 mM. For example, methionine can be provided in the formulations herein at a concentration from between or from about between 5 mM to or to about 50 mM, such as 5 mM to 40 mM, 5 mM to 20 mM or 10 mM to 20 mM. For example, an antioxidant, for example methionine, can be included at a concentration that is or is about 5 mM, 10 mM, 11 mM, 12 mM, 13 mM, 14 mM, 15 mM, 16 mM, 17 mM, 18 mM, 19 mM, 20 mM, 21 mM, 22 mM, 23 mM, 24 mM, 25 mM, 26 mM, 27 mM, 28 mM, 29 mM, 30 mM, 35 mM, 40 mM, 45 mM or 50 mM. In some examples, the formulations contain 10 mM to 20 mM methionine, such as or about 10 mM or 20 mM methionine.

The formulations provided herein also can contain an amino acid stabilizer, which contributes to the stability of the preparation. The stabilizer can be a non-polar or basic amino acid. Exemplary non-polar and basic amino acids include, but are not limited to, alanine, histidine, arginine, lysine, ornithine, isoleucine, valine, methionine, glycine and proline. For example, the amino acid stabilizer is glycine or proline, typically glycine. The stabilizer can be a single amino acid or it can be a combination of 2 or more such amino acids. The amino acid stabilizers can be natural amino acids, amino acid analogues, modified amino acids or amino acid equivalents. Generally, the amino acid is an L-amino acid. For example, when proline is used as the stabilizer, it is generally L-proline. It is also possible to use amino acid equivalents, for example, proline analogues. The concentration of amino acid stabilizer, for example glycine, included in the formulation ranges from 0.1 M to 1 M amino acid, typically 0.1 M to 0.75 M, generally 0.2 M to 0.5 M, for example, at least at or about 0.1 M, 0.15 M, 0.2 M, 0.25 M, 0.3 M, 0.35 M, 0.4 M, 0.45 M, 0.5 M, 0.6 M, 0.7 M, 0.75

M or more amino acid. The amino acid, for example glycine, can be used in a form of a pharmaceutically acceptable salt, such as hydrochloride, hydrobromide, sulfate, acetate, etc. The purity of the amino acid, for example glycine, should be at least 98%, at least 99%, or at least 99.5% or more.

In examples herein, if necessary, hyaluronidase inhibitors are included in a formulation to stabilize PH20, in particular to reduce the effects of otherwise destabilizing agents and conditions, such as, for example, low salt, high pH, the presence of preservatives and elevated temperatures, present in the formulation. Such a component generally is not required for pharmaceutical compositions containing a modified PH20 polypeptide as provided herein that exhibits increased stability under such conditions. When provided, the hyaluronidase inhibitor is provided at least at its equilibrium concentration. One of skill in the art is familiar with various classes of hyaluronidase inhibitors (see e.g., Girish et al. (2009) *Current Medicinal Chemistry,* 16:2261-2288, and references cited therein). One of skill in the art knows or can determine by standard methods in the art the equilibrium concentration of a hyaluronidase inhibitor in a reaction or stable composition herein.

An exemplary hyaluronidase inhibitor for use in the compositions herein is hyaluronan (HA). Hyaluronic acid (HA, also known as hyaluronan and hyaluronate) is the natural substrate for PH20. HA is a non-sulfated glycosaminoglycan that is widely distributed throughout connective, epithelial, and neural tissues. It is a polymer of up to 25,000 disaccharide units, themselves composed of D-glucuronic acid and D-N-acetylglucosamine. The molecular weight of HA ranges from about 5 kDa to 200,000 kDa. Any size HA can be used in the compositions as a stabilizer. In some examples, the HA is a disaccharide, composed of D-glucuronic acid and D-N-acetylglucosamine. In other examples, the HA is an oligosaccharide, such as a tetrasaccharide, containing 2 repeating disaccharide units, or alternatively, the HA used in the co-formulations provided herein can contain multiple repeating disaccharide units, such as 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30 or more disaccharide units. In another example, the HA used in the formulations provided herein has a molecular weight that is from or from about 5 kDa to or to about 5,000 kDa; from or from about 5 kDa to or to about 1,000 kDa; from or from about 5 kDa to or to about 500 kDa; or from or from about 5 kDa to or to about 200 kDa. Exemplary HA oligosaccharides for use in the formulations herein have a molecular weight of or of about 6.4 kDa, 74.0 kDa, or 234.4 kDa. The formulations can contain 1 mg/mL to 20 mg/mL HA, 8 mg/mL to 12 mg/mL, such as at least or about 1 mg/mL, 2 mg/mL, 3 mg/mL, 4 mg/mL, 5 mg/mL, 6 mg/mL, 7 mg/mL, 8 mg/mL, 9 mg/mL, 10 mg/mL, 11 mg/mL, 12 mg/mL, 13 mg/mL, 14 mg/mL, 15 mg/mL, 16 mg/mL, 17 mg/mL, 18 mg/mL, 19 mg/mL or 20 mg/mL or more HA. In some examples, the molar ratio of HA to PH20 is or is about 100,000:1, 95,000:1, 90,000:1, 85,000:1, 80,000:1, 75,000:1, 70,000:1, 65,000:1, 60,000:1, 55,000:1, 50,000:1, 45,000:1, 40,000:1, 35,000:1, 30,000:1, 25,000:1, 20,000:1, 15,000:1, 10,000:1, 5,000:1, 1,000:1, 900:1, 800:1, 700:1, 600:1, 500:1, 400:1, 300:1, 200:1, or 100:1 or less.

In some examples, a nicotinic compound is used as a stabilizing agent. Nicotinic compounds include, but are not limited to, nicotinamide, nicotinic acid, niacin, niacinamide, vitamin B3 and/or salts thereof and/or any combination thereof. In particular applications, the stabilizing agent can include a nicotinic compound an amino acid or amino acids (see e.g., International Publication No. WO2010149772). For example, the amino acid can be arginine, glutamic acid and/or salts thereof or combinations thereof.

2. Compositions for Other Routes of Administration

Depending upon the condition treated other routes of administration, such as topical application, transdermal patches, oral and rectal administration are also contemplated herein.

For example, pharmaceutical dosage forms for rectal administration are rectal suppositories, capsules and tablets for systemic effect. Rectal suppositories include solid bodies for insertion into the rectum which melt or soften at body temperature releasing one or more pharmacologically or therapeutically active ingredients. Pharmaceutically acceptable substances utilized in rectal suppositories are bases or vehicles and agents to raise the melting point. Examples of bases include cocoa butter (theobroma oil), glycerin-gelatin, carbowax (polyoxyethylene glycol) and appropriate mixtures of mono-, di- and triglycerides of fatty acids. Combinations of the various bases may be used. Agents to raise the melting point of suppositories include spermaceti and wax. Rectal suppositories may be prepared either by the compressed method or by molding. The typical weight of a rectal suppository is about 2 to 3 gm. Tablets and capsules for rectal administration are manufactured using the same pharmaceutically acceptable substance and by the same methods as for formulations for oral administration. Formulations suitable for rectal administration can be provided as unit dose suppositories. These can be prepared by admixing the active compound with one or more conventional solid carriers, for example, cocoa butter, and then shaping the resulting mixture.

For oral administration, pharmaceutical compositions can take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinized maize starch, polyvinyl pyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate). The tablets can be coated by methods well-known in the art.

Formulations suitable for buccal (sublingual) administration include, for example, lozenges containing the active compound in a flavored base, usually sucrose and acacia or tragacanth; and pastilles containing the compound in an inert base such as gelatin and glycerin or sucrose and acacia.

Topical mixtures are prepared as described for the local and systemic administration. The resulting mixtures can be solutions, suspensions, emulsion or the like and are formulated as creams, gels, ointments, emulsions, solutions, elixirs, lotions, suspensions, tinctures, pastes, foams, aerosols, irrigations, sprays, suppositories, bandages, dermal patches or any other formulations suitable for topical administration.

The compounds or pharmaceutically acceptable derivatives thereof may be formulated as aerosols for topical application, such as by inhalation (see, e.g., U.S. Pat. Nos. 4,044,126, 4,414,209, and 4,364,923, which describe aerosols for delivery of a steroid useful for treatment of inflammatory diseases, particularly asthma). These formulations, for administration to the respiratory tract, can be in the form of an aerosol or solution for a nebulizer, or as a microfine powder for insufflation, alone or in combination with an inert carrier such as lactose. In such a case, the particles of the formulation will typically have diameters of less than 50 microns, or less than 10 microns.

The compounds can be formulated for local or topical application, such as for topical application to the skin and mucous membranes, such as in the eye, in the form of gels, creams, and lotions and for application to the eye or for intracisternal or intraspinal application. Topical administration is contemplated for transdermal delivery and also for administration to the eyes or mucosa, or for inhalation therapies. Nasal solutions of the active compound alone or in combination with other pharmaceutically acceptable excipients also can be administered.

Formulations suitable for transdermal administration are provided. They can be provided in any suitable format, such as discrete patches adapted to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. Such patches contain the active compound in an optionally buffered aqueous solution of, for example, 0.1 to 0.2 M concentration with respect to the active compound. Formulations suitable for transdermal administration also can be delivered by iontophoresis (see, e.g., Tyle, P, *Pharmaceutical Research* 3(6):318-326 (1986)) and typically take the form of an optionally buffered aqueous solution of the active compound.

Pharmaceutical compositions also can be administered by controlled release formulations and/or delivery devices (see e.g., in U.S. Pat. Nos. 3,536,809; 3,598,123; 3,630,200; 3,845,770; 3,916,899; 4,008,719; 4,769,027; 5,059,595; 5,073,543; 5,120,548; 5,591,767; 5,639,476; 5,674,533 and 5,733,566).

3. Dosages and Administration

The modified PH20 polypeptides provided herein can be formulated as pharmaceutical compositions for single dosage or multiple dosage administration. The PH20 polypeptide is included in an amount sufficient to exert a therapeutically useful effect in the absence of undesirable side effects on the patient treated. The therapeutically effective concentration can be determined empirically by testing the polypeptides in known in vitro and in vivo systems such as by using the assays provided herein or known in the art (see e.g., Taliani et al. (1996) *Anal. Biochem.,* 240:60-67; Filocamo et al. (1997) *J Virology,* 71:1417-1427; Sudo et al. (1996) *Antiviral Res.* 32:9-18; Bouffard et al. (1995) *Virology,* 209:52-59; Bianchi et al. (1996) *Anal. Biochem.,* 237: 239-244; Hamatake et al. (1996) *Intervirology* 39:249-258; Steinkuhler et al. (1998) *Biochem.,* 37:8899-8905; D'Souza et al. (1995) *J. Gen. Virol,* 76:1729-1736; Takeshita et al. (1997) *Anal. Biochem.,* 247:242-246; see also e.g., Shimizu et al. (1994) *J. Virol.* 68:8406-8408; Mizutani et al. (1996) *J. Virol.* 70:7219-7223; Mizutani et al. (1996) *Biochem. Biophys. Res. Commun.,* 227:822-826; Lu et al. (1996) *Proc. Natl. Acad. Sci* (*USA*), 93:1412-1417; Hahm et al., (1996) *Virology,* 226:318-326; Ito et al. (1996) *J. Gen. Virol.,* 77:1043-1054; Mizutani et al. (1995) *Biochem. Biophys. Res. Commun.,* 212:906-911; Cho et al. (1997) *J. Virol. Meth.* 65:201-207 and then extrapolated therefrom for dosages for humans.

The amount of a modified PH20 to be administered for the treatment of a disease or condition can be determined by standard clinical techniques. In addition, in vitro assays and animal models can be employed to help identify optimal dosage ranges. The precise dosage, which can be determined empirically, can depend on the particular enzyme, the route of administration, the type of disease to be treated and the seriousness of the disease.

Hence, it is understood that the precise dosage and duration of treatment is a function of the disease being treated and can be determined empirically using known testing protocols or by extrapolation from in vivo or in vitro test data. It is to be noted that concentrations and dosage values also can vary with the severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or use of compositions and combinations containing them. The compositions can be administered hourly, daily, weekly, monthly, yearly or once. Generally, dosage regimens are chosen to limit toxicity. It should be noted that the attending physician would know how to and when to terminate, interrupt or adjust therapy to lower dosage due to toxicity, or bone marrow, liver or kidney or other tissue dysfunctions. Conversely, the attending physician would also know how to and when to adjust treatment to higher levels if the clinical response is not adequate (precluding toxic side effects).

Typically, a therapeutically effective dose of a modified PH20 enzyme is at or about 10 Unit (U) to 500,000 Units, 100 Units to 100,000 Units, 500 Units to 50,000 Units, 1000 Units to 10,000 Units, 5000 Units to 7500 Units, 5000 Units to 50,000 Units, or 1,000 Units to 10,000 Units, generally 1,000 to 50,000 Units, in a stabilized solution or suspension or a lyophilized form. For example, a PH20 polypeptide, can be administered at a dose of at least or about at least or 10 U, 20 U, 30 U, 40 U, 50 U, 100 U, 150 U, 200 U, 250 U, 300 U, 350 U, 400 U, 450 U, 500 U, 600 U, 700 U, 800 U, 900 U, 1000 U, 2,000 U, 3,000 U, 4,000 Units, 5,000 U or more. The formulations can be provided in unit-dose forms such as, but not limited to, ampoules, syringes and individually packaged tablets or capsules.

The PH20 enzyme can be administered alone, or with other pharmacologically effective agent(s) or therapeutic agent(s), in a total volume of 0.1-100 mL, 1-50 mL, 10-50 mL, 10-30 mL, 1-20 mL, or 1-10 mL, typically 10-50 mL. Typically, volumes of injections or infusions of a PH20-containing composition are at least or at least about 0.01 mL, 0.05 mL, 0.1 mL, 0.2 mL, 0.3 mL, 0.4 mL, 0.5 mL, 1 mL, 2 mL, 3 mL, 4 mL, 5 mL, 6 mL, 7 mL, 8 mL, 9 mL, 10 mL, 20 mL, 30 mL, 40 mL, 50 mL or more. The formulations provided herein contain a modified PH20 polypeptide in an amount between or about between 30 Units/mL to 3000 U/mL, 300 U/mL to 2000 U/mL or 600 U/mL to 2000 U/mL or 600 U/mL to 1000 U/mL, such as at least or about at least 30 U/mL, 35 U/mL, 40 U/mL, 50 U/mL, 100 U/mL, 200 U/mL, 300 U/mL, 400 U/mL, 500 U/mL, 600 U/mL, 700 U/mL, 800 U/mL, 900 U/mL, 1000 U/mL, 2000 U/mL or 3000 U/mL. For example, the formulations provided herein contain a PH20 that is in an amount that is at least 100 U/mL to 1000 U/mL, for example at least or about at least or about or 600 U/mL.

The PH20 polypeptide can be provided as a solution in an amount that is at least or about or is 100 U/mL, 150 U/mL, 200 U/mL, 300 U/mL, 400 U/mL, 500 U/mL, 600 U/mL, 800 U/mL or 1000 U/mL, or can be provided in a more concentrated form, for example in an amount that is at least or about or is 2000 U/mL, 3000 Units/mL, 4000 U/mL, 5000 U/mL, 8000 U/mL, 10,000 U/mL or 20,000 U/mL for use directly or for dilution to the effective concentration prior to use. The PH20 polypeptide compositions can be provided as a liquid or lyophilized formulation.

When the PH20 is co-formulated with a therapeutic agent, dosages can be provided as a ratio of the amount of a PH20 polypeptide to the amount of therapeutic agent administered. For example, a PH20 polypeptide can be administered at 1 hyaluronidase U/therapeutic agent U (1:1) to 50:1 or more, for example, at or about 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, 15:1, 20:1, 25:1, 30:1, 35:1, 40:1, 45:1, 50:1 or more.

The formulations provided herein, including co-formulations and/or stable formulations, can be prepared for single dose administration, multiple dose administration or continuous infusion administrations. Implantation of a slow-release or sustained-release system, such that a constant level of dosage is maintained (see e.g., U.S. Pat. No. 3,710,795), is also contemplated herein.

For example, formulations of pharmaceutically therapeutically active compounds and derivatives thereof are provided for administration to humans and animals in unit dosage forms or multiple dosage forms. For example, compounds can be formulated as tablets, capsules, pills, powders, granules, sterile parenteral solutions or suspensions, oral solutions or suspensions, or oil-water emulsions containing suitable quantities of the compounds or pharmaceutically acceptable derivatives thereof. Each unit dose contains a predetermined quantity of therapeutically active compound(s) sufficient to produce the desired therapeutic effect, in association with the required pharmaceutical carrier, vehicle or diluent. Examples of unit dose forms include ampoules and syringes and individually packaged tablets or capsules. Unit dose forms can be administered in fractions or multiples thereof. A multiple dose form is a plurality of identical unit dosage forms packaged in a single container to be administered in segregated unit dose forms. Examples of multiple dose forms include vials, bottles of tablets or capsules or bottles of pints or gallons. Hence, multiple dose form is a multiple of unit doses that are not segregated in packaging. Generally, dosage forms or compositions containing active ingredient in the range of 0.005% to 100% with the balance made up from non-toxic carrier can be prepared.

Compositions provided herein typically are formulated for administration by subcutaneous route, although other routes of administration are contemplated, such as any route known to those of skill in the art including intramuscular, intraperitoneal, intravenous, intradermal, intralesional, intraperitoneal injection, epidural, vaginal, rectal, local, otic, transdermal administration or any route of administration. Formulations suited for such routes are known to one of skill in the art. Administration can be local, topical or systemic depending upon the locus of treatment. Local administration to an area in need of treatment can be achieved by, for example, but not limited to, local infusion during surgery, topical application, e.g., in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant. Compositions also can be administered with other biologically active agents, either sequentially, intermittently or in the same composition.

The most suitable route in any given case depends on a variety of factors, such as the nature of the disease, the tolerance of the subject to a particular administration route, the severity of the disease, and the particular composition that is used. Typically, the compositions provided herein are administered parenterally. In some examples, modified PH20 polypeptide compositions are administered so that they reach the interstitium of skin or tissues, thereby degrading the interstitial space for subsequent delivery of a therapeutic agent. Thus, in some examples, direct administration under the skin, such as by subcutaneous administration methods, is contemplated. Thus, in one example, local administration can be achieved by injection, such as from a syringe or other article of manufacture containing an injection device such as a needle. In another example, local administration can be achieved by infusion, which can be facilitated by the use of a pump or other similar device. Other modes of administration also are contemplated. For example, modified PH20 polypeptides, included conjugated forms with increased half-life such as PEGylated forms thereof, can be administered intravenously. Pharmaceutical compositions can be formulated in dosage forms appropriate for each route of administration.

Administration methods can be employed to decrease the exposure of selected modified PH20 polypeptides to degradative processes, such as proteolytic degradation and immunological intervention via antigenic and immunogenic responses. Examples of such methods include local administration at the site of treatment. PEGylation of therapeutics increases resistance to proteolysis, increases plasma half-life, and decreases antigenicity and immunogenicity. Examples of PEGylation methodologies are known in the art (see for example, Lu and Felix, Int. J. Peptide Protein Res., 43: 127-138, 1994; Lu and Felix, Peptide Res., 6: 140-6, 1993; Felix et al., Int. J. Peptide Res., 46: 253-64, 1995; Benhar et al., J. Biol. Chem., 269: 13398-404, 1994; Brumeanu et al., J. Immunol., 154: 3088-95, 1995; see also, Caliceti et al. (2003) Adv. Drug Deliv. Rev. 55(10): 1261-77 and Molineux (2003) Pharmacotherapy 23 (8 Pt 2):3S-8S). PEGylation also can be used in the delivery of nucleic acid molecules in vivo. For example, PEGylation of adenovirus can increase stability and gene transfer (see, e.g., Cheng et al. (2003) Pharm. Res. 20(9): 1444-51).

Various other delivery systems are known and can be used to administer selected PH20 polypeptides, such as but not limited to, encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the compound, receptor mediated endocytosis, and delivery of nucleic acid molecules encoding selected PH20 polypeptides such as retrovirus delivery systems.

Hence, in certain embodiments, liposomes and/or nanoparticles also can be employed with administration of soluble PH20 polypeptides. Liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs)). MLVs generally have diameters of from 25 nm to 4 μm. Sonication of MLVs results in the formation of small unilamellar vesicles (SUVs) with diameters in the range of 200 to 500 angstroms containing an aqueous solution in the core.

Phospholipids can form a variety of structures other than liposomes when dispersed in water, depending on the molar ratio of lipid to water. At low ratios of lipid to water, liposomes form. Physical characteristics of liposomes depend on the pH, ionic strength and the presence of divalent cations. Liposomes can show low permeability to ionic and polar substances, but at elevated temperatures undergo a phase transition which markedly alters their permeability. The phase transition involves a change from a closely packed, ordered structure, known as the gel state, to a loosely packed, less-ordered structure, known as the fluid state. This occurs at a characteristic phase-transition temperature and results in an increase in permeability to ions, sugars and drugs.

Liposomes interact with cells via different mechanisms: endocytosis by phagocytic cells of the reticuloendothelial system such as macrophages and neutrophils; adsorption to the cell surface, either by nonspecific weak hydrophobic or electrostatic forces, or by specific interactions with cell-surface components; fusion with the plasma cell membrane by insertion of the lipid bilayer of the liposome into the plasma membrane, with simultaneous release of liposomal contents into the cytoplasm; and by transfer of liposomal lipids to cellular or subcellular membranes, or vice versa, without any association of the liposome contents. Varying the liposome formulation can alter which mechanism is operative, although more than one can operate at the same time. Nanocapsules can generally entrap compounds in a stable and reproducible way. To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1 μm) should be designed using polymers able to be degraded in vivo. Biodegradable polyalkyl-cyanoacrylate nanoparticles that meet these requirements are contemplated for use herein, and such particles can be easily made.

4. Exemplary PH20-Insulin Co-Formulation

Provided herein are stable co-formulations of a fast acting insulin, such as a rapid acting (fast-acting) insulin analog, and a modified PH20 polypeptide. Any of the modified PH20 polypeptides provided herein can be included in a co-formulation with insulin, such as any of the co-formulations described in U.S. application Ser. No. 13/507,263 or 13/507,262 or in International PCT Application Serial No. PCT/US2012/042816.

In particular, the modified PH20 polypeptide is a modified PH20 polypeptide that exhibits increased stability under denaturation conditions, such as any set forth in Sections C.1.b. In particular, the PH20 polypeptide is a modified PH20 polypeptide that exhibits increased stability to one or more phenolic preservatives, such as any set forth in Section C.1.b.i. For example, the PH20 polypeptide is a modified PH20 polypeptide that contains an amino acid replacement with P at a position corresponding to position 204 with reference to amino acid positions set forth in SEQ ID NO:3, such as F204P with reference to any of SEQ ID NOS: 3, 7 or 32-66. In other examples, the PH20 polypeptide is a modified PH20 polypeptide that contains an amino acid replacement with R at a position corresponding to position 58 with reference to amino acid positions set forth in SEQ ID NO:3, such as V58R with reference to any of SEQ ID NOS: 3, 7 or 32-66.

The fast acting insulin can be a regular insulin or a rapid acting (fast-acting) insulin analog. Insulin is a polypeptide that when processed is composed of 51 amino acids containing an A- and B-chain. Generally, insulin contains an A-chain of about 21 amino acids and a B-chain of about 30 amino acids. The A- and B-chains are linked by disulfide bridges. Exemplary regular insulins include, for example, a human insulin (with an A chain having a sequence of amino acids set forth in SEQ ID NO:862 and a B chain having a sequence of amino acids set forth in SEQ ID NO: 863) or a porcine insulin (with an A chain having a sequence of amino acids set forth as amino acid residue positions 88-108 of SEQ ID NO: 864 and a B chain having a sequence of amino acids set forth as amino acid residue positions 25-54 of SEQ ID NO:864). Exemplary fast-acting insulin analogs are insulin variants that contain one or more amino acid modifications compared to a human insulin set forth in SEQ ID NO: 862 and 863 (A and B chains). For example, exemplary insulin analogs are known to one of skill in the art, and include, but are not limited to, glulisine having an A-chain set forth in SEQ ID NO: 862 and a B-chain that is a variant of SEQ ID NO: 863 (B-chain; LysB3, GluB29), HMR-1 153 having an A-chain set forth in SEQ ID NO:862 and a B-chain that is a variant of SEQ ID NO:863 (B-chain; LysB3, IleB28), insulin aspart having an A-chain set forth in SEQ ID NO:862 and a B-chain that is a variant of SEQ ID NO:863 (B-chain; AspB28), and insulin lispro having an A-chain set forth in SEQ ID NO:862 and a B-chain that is a variant of SEQ ID NO:863 (B-chain; LysB28, ProB29). In every instance above, the nomenclature of the analogs is based on a description of the amino acid substitution at specific positions on the A or B chain of insulin, numbered from the N-terminus of the chain, in which the remainder of the sequence is that of natural human insulin. Exemplary of such analog forms, are set forth in SEQ ID NOS:862 (A-chain) and having a B-chain set forth in any of SEQ ID NOS: 865-867.

The co-formulations are stable as a liquid formulation for prolonged periods of time for at least 1 month at temperatures from or from about 2° C. to 8° C., inclusive, or for at least 3 days at a temperature from or from about 30° C. to 42° C., inclusive. For example, the co-formulations are stable and retain activity of the PH20 hyaluronidase and insulin at "refrigerator" conditions, for example, at 2° C. to 8° C., such as at or about 4° C., for at least at least 2 months, 3 months, 4 months, 5 months, 6 months, or 7 months, at least 8 months, at least 9 months, at least 10 months, at least 11 months, at least 12 months, 13 months, 14 months, 15 months, 16 months, 17 months, 18 months, 19 months, 20 months, 21 months, 22 months, 23 months, 24 months, 25 months, 26 months, 27 months, 28 months, 29 months or 30 months or more. In another example, the formulations provided herein are stable and retain activity of the PH20 hyaluronidase and insulin at room temperature for example at 18° C. to 32° C., generally 20° C. to 32° C., such as 28° C. to 32° C., for at least 2 weeks to 1 year, for example, at least 3 weeks, 4 weeks, 2 months, 3 months, 4 months, 5 months, 6 months, at least 7 months, at least 8 months, at least 9 months, or at least 1 year or more. In a further example, the formulations provided herein are stable and retain activity of the PH20 hyaluronidase and insulin at elevated temperatures of about or greater than 30° C., generally from or from about 30° C. to 42° C., such as 32° C. to 37° C. or 35° C. to 37° C. or about or 37° C. for at least 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, 30 days, 35 days, 40 days, 45 days, 50 days, 60 days or more.

Assays to assess stability of active agents are well-known to one of skill in the art. Section G provides exemplary assays to assess stability of PH20 hyaluronidase. The stability of insulin can be assessed using similar methods well-known to one of skill in the art. For example, insulin stability and solubility can be assessed by visual assessment (e.g., including changes in color, clarity, presence of aggregates or clumping and material adhesion, or frosting), acid clarification, optical microscopy, reversed phase high performance liquid chromatography (RP-HPLC), in vitro or in vivo bioassays and denaturing and non-denaturing size exclusion chromatography (SEC). In vitro or in vivo bioassays for insulin activity include, but are not limited to, a competitive binding assay using cells expressing insulin receptors (e.g., human placental cell membranes) and a radiolabeled insulin (see e.g., Weiss et al., (2001) *J. Biol. Chem.* 276:40018-40024; Duttaroy et al., (2005) *Diabetes* 54:251-258); insulin-stimulated glucose uptake (Louveau et al., (2004) *J Endocrin.* 181:271-280, Duttaroy et al., (2005) *Diabetes* 54:251-258); assays to assess glucose production in the presence of insulin (Wang et al., (2000) *J. Biochem.,* 275:14717-14721, Duttaroy et al., (2005) *Diabetes* 54:251-258); and studies using diabetic and/or healthy animal models (Atkinson et al., (1999) *Nature Med.* 5:601-604;

Nagoya-Shibata-Yasuda (NSY) mice, Zucker diabetic fatty (ZDF) rats and Gato-Katazaki (GK) rats (Cefalu (2006) *ILAR Journal* 47:186-198).

Examples of such formulations contain 100 U/mL to 1000 U/mL of a modified PH20 polypeptide, and in particular at or about or at least 600 U/mL; 10 U/mL to 1000 U/mL of a fast-acting insulin, and in particular at or at least or about 100 U/mL; NaCl at a concentration of between or about between 80-140 mM; a pH of between or about between 7.0 to 7.8; a buffering agent that maintains the pH range of between or about between 7.0 to 7.8; 0.1% to 0.4% preservative as a mass concentration (w/v). Optionally, a further stabilizing agent can be included. For example, the co-formulations provided herein contain 1 mM to 100 mM of a buffering agent. For example, the co-formulations provided herein contain 0.005% to 0.5% surfactant. Exemplary co-formulations provided herein also can contain less than 60 mM glycerin (glycerol) and 2 mM to or to about 50 mM of an antioxidant.

The following stable formulations are exemplary only and provide a platform from which minor adjustments can be made. It is understood that very small changes in the concentrations of the various excipients and other components (e.g., ±15% of the stated concentrations), or small changes in pH, can be made while retaining some if not all of the insulin solubility and stability and PH20 stability. Further changes also can be made by adding or removing excipients. For example, the type of stabilizing surfactant can be changed.

For example, the exemplary co-formulations herein contain 100 U/mL to 1000 U/mL of a modified PH20 polypeptide, and in particular at least or about at least or about 600 U/mL of a modified PH20 polypeptide; 10 U/mL to 1000 U/mL of a fast-acting insulin, and in particular at least or about at least or about 100 U/mL of a fast-acting insulin; from or from about 10 mM to or to about 50 mM Tris (e.g., from or from about 20 mM to 40 mM Tris, such as or as about 20 mM, 25 mM, 30 mM, 35 mM or 40 mM Tris); from or from about 80 mM to or to about 160 mM NaCl (e.g., at or about 80 mM, 90 mM, 100 mM, 110 mM 120 mM, 130 mM, 140 mM, 150 mM or 160 mM NaCl); from or from about 2 mM to or to about 50 mM methionine (e.g., at or about 5 mM, 10 mM, 20 mM, 30 mM, 40 mM or 50 mM methionine); from or from about 0 mM to or to about 50 mM glycerin (e.g., at or about 5 mM, 10 mM, 20 mM, 30 mM, 40 mM or 50 mM glycerin); from or from about 0.005% to or to about 0.5% poloxamer 188, such as 0.01% to 0.05% (e.g., at or about 0.01%, 0.02%, 0.03%, 0.04% or 0.05% poloxamer 188); from or from about 0.05% to or to about 0.25% phenol (e.g., at or about 0.1%, 0.12%, 0.125%, 0.13%, 0.14%, 0.15%, 0.16% or 0.17% phenol); and from or from about 0.05% to or to about 0.4% m-cresol (e.g., at or about 0.075%, 0.08%, 0.09%, 0.1%, 0.12%, 0.13%, 0.14%, 0.15%, 0.16% or 0.17% m-cresol). The formulations are prepared with a pH from or from about 7.0 to or to about 7.6 (e.g., at or about pH 7.0, 7.1, 7.2, 7.3, 7.4, 7.5 or 7.6). In further examples, zinc is included at a concentration of or about 0.017 mg/100 U, 0.018 mg/100 U, 0.02 mg/100 U, 0.022 mg/100 U or 0.024 mg/100 U insulin.

In particular examples, the fast acting insulin is insulin aspart, insulin lispro or insulin glulisine. Exemplary co-formulations provided herein that contain a modified PH20 polypeptide and insulin lispro are those that contain from or about 25 mM to or to about 35 mM Tris (e.g., at or about 30 mM Tris); from or from about 70 mM to or to about 100 mM NaCl (e.g., at or about 80 mM or 100 mM NaCl); from or from about 10 mM to or to about 30 mM methionine (e.g., at or about 10 mM or 20 mM methionine); from or from about 40 mM to or to about 60 mM glycerin (e.g., at or about 50 mM glycerin); from or from about 0.005% to or to about 0.05% poloxamer 188 (e.g., at or about 0.01% poloxamer 188); from or from about 0.017 mg zinc/100 U insulin to or to about 0.024 mg zinc/100 U insulin (e.g., 0.017 mg zinc/100 U insulin, 0.018 mg/100 U, 0.02 mg/100 U, 0.022 mg/100 U or 0.024 mg zinc/100 U insulin); from or from about 0.08% to or to about 0.17% phenol (e.g., 0.1%, 0.125% or 0.13% phenol); and from or from about 0.07% to or to about 0.17% m-cresol (e.g., 0.075%, 0.08%, 0.13% or 0.15% m-cresol). For example, the co-formulations can contain at or about 0.1%) phenol and 0.015% m-cresol; at or about 0.125% phenol and 0.075% m-cresol; at or about 0.13% phenol and 0.075%) m-cresol; at or about 0.13% phenol and 0.08% tri-cresol; or at or about 0.17% phenol and 0.13% m-cresol. Such formulations of insulin lispro and a modified PH20 polypeptide are prepared with a pH of or about 7.0 to or to about 7.5 (typically a pH of or about pH 7.2).

Exemplary co-formulations provided herein that contain a modified PH20 polypeptide and insulin aspart are those that contain from or from about 25 mM to or to about 35 mM Tris (e.g., at or about 30 mM Tris); from or from about 70 mM to or to about 120 mM NaCl (e.g., at or about 80 mM or 100 mM NaCl); from or from about 2 mM to or to about 30 mM methionine, such as 2 mM to 10 mM or 5 mM to 30 mM methionine (e.g., at or about 5 mM, 10 mM or 20 mM methionine); from or from about 0.005% to or to about 0.05% poloxamer 188 (e.g., at or about 0.01% poloxamer 188); from or from about 0.08% to or to about 0.17% phenol (e.g., 0.1%, 0.125% or 0.13% phenol); and from or from about 0.07% to or to about 0.17% m-cresol (e.g., 0.075%, 0.08%, 0.13% or 0.15% m-cresol). For example, the co-formulations can contain at or about 0.1% phenol and 0.015% m-cresol; at or about 0.125% phenol and 0.075% m-cresol; at or about 0.13% phenol and 0.075% m-cresol; at or about 0.13% phenol and 0.08% m-cresol; or at or about 0.17% phenol and 0.13% m-cresol. Such formulations of insulin aspart and a modified PH20 polypeptide are prepared with a pH of or about 7.0 to or to about 7.6 (typically a pH of or about pH 7.4 or 7.3).

Further exemplary formulations provided herein that contain a modified PH20 polypeptide and insulin aspart are those that do not contain phenol. Such exemplary formulations contain from or from about 25 mM to or to about 35 mM Tris (e.g., at or about 30 mM Tris); from or from about 70 mM to or to about 120 mM NaCl (e.g., at or about 80 mM or 100 mM NaCl); from or from about 2 mM to or to about 30 mM methionine, such as 2 mM to 10 mM or 5 mM to 30 mM methionine (e.g., at or about 5 mM, 10 mM or 20 mM methionine); from or from about 0.005% to or to about 0.05% poloxamer 188 (e.g., at or about 0.01% poloxamer 188); and from or from about 0.07% to or to about 0.4% m-cresol, such as from or from about 0.2% to 0.4% m-cresol (e.g., 0.3%, 0.315%, 0.35%, 0.4% m-cresol). Such formulations of insulin aspart and a modified PH20 polypeptide are prepared with a pH of or about 7.0 to or to about 7.6 (typically a pH of or about pH 7.4 or 7.3).

Exemplary co-formulations provided herein that contain a modified PH20 polypeptide and insulin glulisine are those that contain from or from about 25 mM to or to about 35 mM Tris (e.g., at or about 30 mM Tris); from or from about 100 mM to or to about 150 mM NaCl (e.g., at or about 100 mM or 140 mM NaCl); from or from about 10 mM to or to about 30 mM methionine (e.g., at or about 10 mM or 20 mM methionine); from or from about 40 mM to or to about 60 mM glycerin (e.g., at or about 50 mM glycerin); from or from about 0.005% to or to about 0.05% poloxamer 188 (e.g., at or about 0.01% poloxamer 188); from or from about 0.08% to or to about 0.17% phenol (e.g., 0.1%, 0.125% or 0.13% phenol); and from or from about 0.07% to or to about 0.17% m-cresol (e.g., 0.075%, 0.08%, 0.13% or 0.15% m-cresol). For example, the co-formulations can contain at or about 0.1% phenol and 0.015% m-cresol; at or about 0.125% phenol and 0.075% m-cresol; at or about 0.13% phenol and 0.075% m-cresol; at or about 0.13% phenol and 0.08% m-cresol; or at or about 0.17% phenol and 0.13% m-cresol. Such formulations of insulin glulisine and a modified PH20 polypeptide are prepared with a pH of or about 7.0 to or to about 7.6 (typically a pH of or about pH 7.4).

5. Packaging, Articles of Manufacture and Kits

Pharmaceutical compounds of modified PH20 polypeptides, or nucleic acids encoding such polypeptides, or derivatives or variants thereof can be packaged as articles of manufacture containing packaging material, a pharmaceutical composition which is effective for treating a disease or disorder, and a label that indicates that the pharmaceutical composition or therapeutic molecule is to be used for treating the disease or disorder. Combinations of a selected modified PH20 polypeptide, or a derivative or variant thereof and an therapeutic agent also can be packaged in an article of manufacture.

The articles of manufacture provided herein contain packaging materials. Packaging materials for use in packaging pharmaceutical products are well known to those of skill in the art. See, for example, U.S. Pat. Nos. 5,323,907, 5,052,558 and 5,033,252, each of which is incorporated herein in its entirety. Examples of pharmaceutical packaging materials include, but are not limited to, blister packs, bottles, tubes, inhalers, pumps, bags, vials, containers, syringes, bottles, and any packaging material suitable for a selected formulation and intended mode of administration and treatment. The articles of manufacture can include a needle or other injection device so as to facilitate administration (e.g., sub-epidermal administration) for local injection purposes. A wide array of formulations of the compounds and compositions provided herein are contemplated including a modified PH20 polypeptide and a therapeutic agent, such as a fast-acting insulin, known to treat a particular disease or disorder. The choice of package depends on the PH20 and/or therapeutic agent, and whether such compositions will be packaged together or separately. In one example, the PH20 can be packaged as a mixture with the therapeutic agent. In another example, the components can be packaged as separate compositions

Modified PH20 polypeptides, therapeutic agents and/or articles of manufacture thereof also can be provided as kits. Kits can include a pharmaceutical composition described herein and an item for administration provided as an article of manufacture. For example a PH20 polypeptide can be supplied with a device for administration, such as a syringe, an inhaler, a dosage cup, a dropper, or an applicator. The compositions can be contained in the item for administration or can be provided separately to be added later. The kit can, optionally, include instructions for application including dosages, dosing regimens and instructions for modes of administration. Kits also can include a pharmaceutical composition described herein and an item for diagnosis. For example, such kits can include an item for measuring the concentration, amount or activity of the selected protease in a subject.

G. Methods of Assessing PH20 Activity and Stability

Assays can be used to assess the stability and activity of the PH20 polypeptides provided herein. The assays can be used to assess the hyaluronidase activity of the PH20 polypeptide under particular conditions, temperature, and/or over time. Such assays can be used, for example, to determine the stability of the PH20 polypeptide under specific denaturation conditions, including, but not limited to, elevated temperatures greater than or about or 30° C. (e.g., 30° C. to 42° C. such as or about 37° C.), agitation, presence of excipients (e.g., preservative), or low or no NaCl (salt). For example, stability under specific conditions can be monitored by assessing activity, solubility, and stability (e.g., formation of aggregates, etc.) in the absence of exposure to the denaturation condition and then at various time points thereafter in the presence of the condition. Hence, stability can be assessed over time. Stability also can be assessed by comparing any one or more of activity, solubility or aggregation in the presence of one or more denaturation conditions compared to a native, wildtype or reference PH20 polypeptide. The assays also can be used make minor adjustments to the formulations provided herein while retaining the stability of both active agents.

1. Hyaluronidase Activity

The activity of a modified PH20 polypeptide can be assessed using methods well known in the art. For example, the USP XXII assay for hyaluronidase determines activity indirectly by measuring the amount of undegraded hyaluronic acid, or hyaluronan, (HA) substrate remaining after the enzyme is allowed to react with the HA for 30 min at 37° C. (USP XXII-NF XVII (1990) 644-645 United States Pharmacopeia Convention, Inc, Rockville, Md.). A Hyaluronidase Reference Standard (USP) or National Formulary (NF) Standard Hyaluronidase solution can be used in an assay to ascertain the activity, in units, of any hyaluronidase. In one example, activity is measured using a microturbidity assay. This is based on the formation of an insoluble precipitate when hyaluronic acid binds with a reagent that precipitates it, such as acidified serum or cetylpyridinium chloride (CPC). The activity is measured by incubating hyaluronidase with sodium hyaluronate (hyaluronic acid) for a set period of time (e.g., 10 minutes) and then precipitating the undigested sodium hyaluronate with the addition of acidified serum or CPC. The turbidity of the resulting sample is measured at 640 nm after an additional development period. The decrease in turbidity resulting from hyaluronidase activity on the sodium hyaluronate substrate is a measure of hyaluronidase enzymatic activity.

In another example, hyaluronidase activity is measured using a microtiter assay in which residual biotinylated hyaluronic acid is measured following incubation with hyaluronidase (see e.g., Frost and Stern (1997) *Anal. Biochem.* 251:263-269, U.S. Pat. Publication No. 20050260186). The free carboxyl groups on the glucuronic acid residues of hyaluronic acid are biotinylated, and the biotinylated hyaluronic acid substrate is covalently coupled to a microtiter plate. Following incubation with hyaluronidase, the residual biotinylated hyaluronic acid substrate is detected using an avidin-peroxidase reaction, and compared to that obtained following reaction with hyaluronidase standards of known activity.

Other assays to measure hyaluronidase activity also are known in the art and can be used in the methods herein (see e.g., Delpech et al., (1995) *Anal. Biochem.* 229:35-41; Takahashi et al., (2003) *Anal. Biochem.* 322:257-263).

Many hyaluronidase assays have been based upon the measurement of the generation of new reducing N-acetylamino groups (Bonner and Cantey, Clin. Chim. Acta 13:746-752, 1966), or loss of viscosity (De Salegui et al., Arch. Biochem. Biophys. 121:548-554, 1967) or turbidity (Dorfman and Ott, J. Biol. Chem. 172:367, 1948). With purified substrates all of these methods suffice for determination of the presence or absence of endoglycosidase activity.

Substantially purified glycosaminoglycan substrates can also be used in a Gel Shift Assay. Glycosaminoglycans are mixed with recombinant PH20, such as a soluble PH20, to test for endoglycosidase activity that results in a shift in substrate mobility within the gel. Examples of such substrates include, but are not limited to, chondroitin-4 and 6 sulfate, dermatan sulfate, heparan-sulfate, which can be obtained from Sigma Chemical. Human umbilical cord Hyaluronan can be obtained from ICN. For example, each test substrate can be diluted to at or about 0.1 mg/mL in a buffer range from pH 3.5-7.5. In such an exemplary assay, at or about 10 μl samples of purified soluble PH20 or conditioned media from PH20 expressing cells can be mixed with at or about 90 μl of test substrate in desired buffer and incubated for 3 hours at 37° C. Following incubation, samples are neutralized with sample buffer (Tris EDTA pH 8.0, Bromophenol Blue and glycerol) followed by electrophoresis. Glycosaminoglycans can be detected using any method known in the art, for example, glycosaminoglycans can be detected by staining the gels using 0.5% Alcian Blue in 3% Glacial Acetic Acid overnight followed by destaining in 7% Glacial Acetic Acid. Degradation is determined by comparison of substrate mobility in the presence and absence of enzyme.

Hyaluronidase activity can also be detected by substrate gel zymography (Guentenhoner et al. (1992) *Matrix* 12:388-396). In this assay, a sample is applied to an SDS-PAGE gel containing hyaluronic acid and the proteins in the sample separated by electrophoresis. The gel is then incubated in an enzyme assay buffer and subsequently stained to detect the hyaluronic acid in the gel. Hyaluronidase activity is visualized as a cleared zone in the substrate gel.

The ability of a PH20 polypeptide, including a modified PH20 polypeptide provided herein, to act as a spreading or diffusing agent also can be assessed. For example, trypan blue dye can be injected subcutaneously with or without a PH20 polypeptide into the lateral skin on each side of nude mice. The dye area is then measured, such as with a microcaliper, to determine the ability of the PH20 polypeptide to act as a spreading agent (U.S. Pat. Pub. No. 20060104968).

The functional activity of a PH20 polypeptide can be compared and/or normalized to a reference standard using any of these assays. This can be done to determine what a functionally equivalent amount of a PH20 polypeptide is. For example, the ability of a PH20 polypeptide to act as a spreading or diffusing agent can be assessed by injecting it into the lateral skin of mice with trypan blue, and the amount required to achieve the same amount of diffusion as, for example, 100 units of a Hyaluronidase Reference Standard, can be determined. The amount of PH20 polypeptide required is, therefore, functionally equivalent to 100 hyaluronidase units.

2. Solubility

The solubility of a PH20 polypeptide can be determined by any method known to one of the skill in the art. One method for determining solubility is detergent partitioning. For example, a soluble PH20 polypeptide can be distinguished, for example, by its partitioning into the aqueous phase of a Triton® X-114 solution at 37° C. (Bordier et al., (1981) *J. Biol. Chem.,* 256:1604-1607). Membrane-anchored polypeptides, such as lipid-anchored hyaluronidases, including GPI-anchored hyaluronidases, will partition into the detergent-rich phase, but will partition into the detergent-poor or aqueous phase following treatment with Phospholipase C. Phospholipase C is an enzyme that cleaves the phospho-glycerol bond found in GPI-anchored proteins. Treatment with PLC will cause release of GPI-linked proteins from the outer cell membrane.

3. Purity, Crystallization or Aggregation

The stability of a PH20 polypeptide provided herein also can be assessed using other methods and assays known in the art. In addition to assessing stability based on hyaluronidase activity, stability can be assessed by visual inspection, percent recovery, protein purity and apparent melting temperature.

For example, protein purity can be measured by reversed phase high performance liquid chromatography (RP-HPLC). Protein purity, as determined by RP-HPLC, is the percent of the main PH20 protein peak present, as compared to all of the protein species present. Thus, RP-HPLC, and similar methods known to one of skill in the art, can assess degradation of the enzyme. Protein purity can be assessed over time. Protein purity also can be assessed in the presence of one or more denaturation conditions and in varying amounts thereof. Percent recovery also can be determined as the relative percentage of the polypeptide under various conditions (denaturation conditions, time of storage, mode of storage such as vessel or container, or other similar parameters that can be altered) as compared to a reference sample. PH20 polypeptide stability also can be determined by measuring the oxidation of the hyaluronidase by RP-HPLC. Percent oxidation is a measure of sum of the peak areas of the major (ox-1) and minor (ox-2) peaks.

In one example, the melting temperature of a PH20 polypeptide, such as a modified PH20 polypeptide, can be determined by measuring the hydrodynamic radius of particles by dynamic light scattering under various conditions (e.g., denaturation conditions or other storage conditions). An increase in particle size and a decrease in the melting temperature indicates denaturation and subsequent aggregation of the hyaluronidase.

Other methods known to one of skill in the art that can be used to determine the stability of the hyaluronidase in the co-formulations provided herein, include polyacrylamide gel electrophoresis (PAGE), immunoblotting, nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry, circular dichroism (CD) and dye-based fluorescence assays.

4. Pharmacodynamics/Pharmacokinetics

The effect of administration of a PH20 polypeptide, such as a modified PH20 polypeptide, alone or in combination with another therapeutic agent, on the pharmacokinetic and pharmacodynamic properties of any administered agent also can be assessed in vivo using animal models and/or human subjects, such as in the setting of a clinical trial. Pharmacokinetic or pharmacodynamic studies can be performed using animal models or can be performed during studies with patients administered with a PH20 polypeptide or modified PH20 polypeptide.

Animal models include, but are not limited to, mice, rats, rabbits, dogs, guinea pigs and non-human primate models, such as cynomolgus monkeys or rhesus macaques. In some instances, pharmacokinetic or pharmacodynamic studies are performed using healthy animals. In other examples, the studies are performed using animal models of a disease for which therapy with hyaluronan is considered, such as animal models of any hyaluronan-associated disease or disorder, for example a tumor model.

The pharmacokinetic properties of a PH20 polypeptide, such as a modified PH20 polypeptide, can be assessed by measuring such parameters as the maximum (peak) concentration (C_{max}), the peak time (i.e., when maximum concentration occurs; T_{max}), the minimum concentration (i.e., the minimum concentration between doses; C_{min}), the elimination half-life ($T_{1/2}$) and area under the curve (i.e., the area under the curve generated by plotting time versus concentration; AUC), following administration. The absolute bioavailability of the hyaluronidase can be determined by comparing the area under the curve of hyaluronidase following subcutaneous delivery (AUC_{sc}) with the AUC of hyaluronidase following intravenous delivery (AUC_{iv}). Absolute bioavailability (F), can be calculated using the formula: $F=([AUC]_{sc}\times dose_{sc})/([AUC]_{iv}\times dose_{iv})$. A range of doses and different dosing frequency of dosing can be administered in the pharmacokinetic studies to assess the effect of increasing or decreasing concentrations enzyme, such as modified PH20 polypeptide, in the dose.

H. Methods of Treatment and Combination Therapy

Provided herein are methods and uses of any of the modified PH20 polypeptides provided herein that exhibit hyaluronidase activity based on its ability to degrade glycosaminoglycan(s) such as hyaluronan. Due to such activity, the modified PH20 polypeptides can be used as a spreading factor to increase the delivery and/or bioavailability of subcutaneously administered therapeutic agents. Delivery of any therapeutic agent, including but not limited to, peptides, proteins, small molecule drugs, nucleic acids, or viruses can be facilitated or enhanced by co-administration with a modified PH20 polypeptide provided herein. For example, modified PH20 polypeptides can be used to increase the delivery of therapeutic agents such as antibodies (e.g., monoclonal antibodies), cytokines, Immune Globulin, an Insulin, or coagulation factors, to a desired locus, such as by increasing penetration of chemotherapeutic agents into solid tumors. The modified PH20 polypeptides also can be used to treat a hyaluronan-disease or disorder that is characterized by an excess or accumulation of hyaluronan. For example, modified PH20 polypeptides provided herein can be used to for treating a tumor; for treating glycosaminoglycan accumulation in the brain; for treating a cardiovascular disorder; for treating an ophthalmic disorder; for treating pulmonary disease; for treating cellulite; and/or for treating a proliferative disorder.

Other methods and uses of a modified PH20 polypeptide include any that are known to one of skill in the art. For example, various forms of PH20 hyaluronidases have been prepared and approved for therapeutic use in humans. For example, animal-derived hyaluronidase preparations include Vitrase® (ISTA Pharmaceuticals), a purified ovine testicular hyaluronidase, and Amphadase® (Amphastar Pharmaceuticals), a bovine testicular hyaluronidase. Hylenex® (Halozyme Therapeutics) is a human recombinant hyaluronidase produced by genetically engineered Chinese Hamster Ovary (CHO) cells containing nucleic acid encoding for soluble rHuPH20 (see e.g., U.S. Pat. No. 7,767,429). Approved therapeutic uses for hyaluronidases include use as an adjuvant to increase the absorption and dispersion of other therapeutic agents for hypodermoclysis (subcutaneous fluid administration), and as an adjunct in subcutaneous urography for improving resorption of radiopaque agents. In addition to these indications, hyaluronidases can be used as a therapeutic or cosmetic agent for the treatment of additional diseases and conditions. For example, hyaluronidase is commonly used, for example, for peribulbar block in local anesthesia prior ophthalmic surgery. The presence of the enzyme prevents the need for additional blocks and reduces the time to the onset of akinesia (loss of eye movement). Peribulbar and sub-Tenon's block are the most common applications of hyaluronidase for ophthalmic procedures. Hyaluronidase also can promote akinesia in cosmetic surgery, such as blepharoplasties and face lifts. It is understood that soluble PH20 hyaluronidases provided herein, including esPH20 hyaluronidases, can be used in any method of treatment or combination therapy for which a PH20 hyaluronidase is used (see e.g., U.S. Publication Nos. US20040268425; US20050260186; US20060104968; and U.S. application Ser. No. 12/381,844, published as U.S. Publication No. US20100074885; Ser. No. 12/386,249, published as U.S. Publication No. US20090311237; Ser. No. 12/387,225, published as U.S. Publication No. US20090304665; and Ser. No. 12/386,222, published as U.S. Publication No. US2010003238, each incorporated by reference in their entirely).

Exemplary, non-limiting, methods and uses are described in the following subsections.

1. Methods of Delivering Therapeutic Agents

As noted above, hyaluronidase is a spreading or diffusing substance that modifies the permeability of connective tissue through the hydrolysis of hyaluronic acid, a polysaccharide found in the intercellular ground substance of connective tissue, and of certain specialized tissues, such as the umbilical cord and vitreous humor. When no spreading factor is present, materials injected subcutaneously, such as drugs, proteins, peptides and nucleic acid, spread very slowly. Co-injection with hyaluronidase, however, can cause rapid spreading. The rate of diffusion is proportional to the amount of enzyme, and the extent of diffusion is proportional to the volume of solution.

Modified PH20 polypeptides provided herein can be used to promote or enhance the delivery agents and molecules to any of a variety of mammalian tissues in vivo. It can be used to facilitate the diffusion and, therefore, promote the delivery, of small molecule pharmacologic agents as well as larger molecule pharmacologic agents, such as proteins, nucleic acids and ribonucleic acids, and macromolecular compositions than can contain a combination of components including, but not limited to, nucleic acids, proteins, carbohydrates, lipids, lipid-based molecules and drugs (see e.g., U.S. Publication Nos. US20040268425; US20050260186; and US20060104968). Modified PH20 polypeptides can be co-administered and/or co-formulated with a therapeutic agent to improve the bioavailability as well as pharmacokinetic (PK) and/or pharmacodynamic (PD) characteristics of co-formulated or co-administered agents. PK/PD parameters that can be improved by using soluble PH20, such as esPH20, include such measures as C_{max} (the maximal concentration of agent achieved following absorption in, e.g., the bloodstream), T_{max} (the time required to achieve maximal concentration), $T_{1/2}$ (the time required for the concentration to fall by half), C_{min} (the minimal concentration of agent following metabolism and excretion), AUC (area under the curve of concentration versus time, a measure of the overall amount of bioavailability), concentrations in various tissues of interest (including, e.g., the rate of achieving desired concentrations, the overall levels, and the duration of maintaining desired levels), and E_{max} (the maximal effect achieved).

The methods of treatment provided herein include combination therapies with a therapeutic agent for the treatment of a disease or disorder for which the therapeutic agent threats. Any therapeutic agent that ameliorates and or otherwise lessens the severity of a disease or condition can be combined with a modified PH20 polypeptide provided herein in order to increase the bioavailability of such therapeutic agent. In particular, modified PH20 polypeptides provided herein can be used in each and all of the combinations described in applications see e.g., U.S. Publication Nos. US20040268425; US20050260186; US20060104968 and U.S. application Ser. No. 12/381,844, published as U.S. Publication No. US20100074885; Ser. No. 12/386,249, published as U.S. Publication No. US20090311237; Ser. No. 12/387,225, published as U.S. Publication No. US20090304665; and Ser. No. 12/386,222, published as U.S. Publication No. US2010003238 in place of the disclosed hyaluronidase or hyaluronidase degrading enzyme.

Modified PH20 polypeptides can be administered prior to, subsequent to, intermittently with or simultaneously with the therapeutic agent preparation. Generally, the modified PH20 polypeptide is administered prior to or simultaneously with administration of the therapeutic agent preparation to permit the PH20 to degrade the hyaluronic acid in the interstitial space. The PH20 can be administered at a site different from the site of administration of the therapeutic molecule or the soluble PH20 can be administered at a site the same as the site of administration of the therapeutic molecule.

Examples of pharmaceutical, therapeutic and cosmetic agents and molecules that can be administered with hyaluronidase include, but are not limited to, a chemotherapeutic or anticancer agent, an analgesic agent, an antibiotic agent, an anti-inflammatory agent, an antimicrobial agent, an amoebicidal agent, a trichomonacidal agent, an anti-parkinson agent, an anti-malarial agent, an anticonvulsant agent, an anti-depressant agent, an anti-arthritic agent, an anti-fungal agent, an antihypertensive agent, an antipyretic agent, an anti-parasitic agent, an antihistamine agent, an alpha-adrenergic agonist agent, an alpha blocker agent, an anesthetic agent, a bronchial dilator agent, a biocide agent, a bactericide agent, a bacteriostatic agent, a beta adrenergic blocker agent, a calcium channel blocker agent, a cardiovascular drug agent, a contraceptive agent, a cosmetic or esthetic agent, a decongestant agent, a diuretic agent, a depressant agent, a diagnostic agent, an electrolyte agent, a hypnotic agent, a hormone agent, a hyperglycemic agent, a muscle relaxant agent, a muscle contractant agent, an ophthalmic agent, a parasympathomimetic agent, a psychic energizer agent, a sedative agent, a sleep inducer, a sympathomimetic agent, a tranquilizer agent, a urinary agent, a vaginal agent, a viricide agent, a vitamin agent, a non-steroidal anti-inflammatory agent, or an angiotensin converting enzyme inhibitor agent, and any combination thereof. In particular, therapeutic agents include antibodies, including monoclonal antibodies, bisphosphonates, insulins, coagulation factors, cytokines and Immun Globulins.

For example, modified PH20 polypeptides provided herein can be used to increase the delivery of chemotherapeutic agents. Hyaluronidases have also been used to enhance the activity of chemotherapeutics and/or the accessibility of tumors to chemotherapeutics (Schuller et al., 1991, *Proc. Amer. Assoc. Cancer Res.* 32:173, abstract no. 1034; Czejka et al., 1990, *Pharmazie* 45:H9; Baumgartner et al. (1988) *Reg. Cancer Treat.* 1:55-58; Zanker et al. (1986) *Proc. Amer. Assoc. Cancer Res.* 27:390). Combination chemotherapy with hyaluronidase is effective in the treatment of a variety of cancers including urinary bladder cancer (Horn et al.; 1985, *J. Surg. Oncol.* 28:304-307), squamous cell carcinoma (Kohno et al., 94, *J. Cancer Res. Oncol.* 120: 293-297), breast cancer (Beckenlehner et al., 1992, *J. Cancer Res. Oncol.* 118:591-596), and gastrointestinal cancer (Scheithauer et al., 1988, *Anticancer Res.* 8:391-396). In this example, the modified PH20 hyaluronidase enhances penetration of chemotherapeutic or other anti-cancer agents into solid tumors, thereby treating the disease.

Compositions containing soluble PH20 can be injected intratumorally with anti-cancer agents or intravenously for disseminated cancers or hard to reach tumors. The anticancer agent can be a chemotherapeutic, an antibody, a peptide, or a gene therapy vector, virus or DNA. Additionally, hyaluronidase can be used to recruit tumor cells into the cycling pool for sensitization in previously chemorefractory tumors that have acquired multiple drug resistance (St Croix et al., (1998) *Cancer Lett* September 131(1): 35-44).

Exemplary anti-cancer agents that can be administered after, coincident with or before administration of a soluble PH20, such as an esPH20, include, but are not limited to Acivicins; Aclarubicins; Acodazoles; Acronines; Adozelesins; Aldesleukins; Alemtuzumabs; Alitretinoins (9-Cis-Retinoic Acids); Allopurinols; Altretamines; Alvocidibs; Ambazones; Ambomycins; Ametantrones; Amifostines; Aminoglutethimides; Amsacrines; Anastrozoles; Anaxirones; Ancitabines; Anthramycins; Apaziquones; Argimesnas; Arsenic Trioxides; Asparaginases; Asperlins; Atrimustines; Azacitidines; Azetepas; Azotomycins; Banoxantrones; Batabulins; Batimastats; BCG Live; Benaxibines; Bendamustines; Benzodepas; Bexarotenes; Bevacizumab; Bicalutamides; Bietaserpines; Biricodars; Bisantrenes; Bisantrenes; Bisnafide Dimesylates; Bizelesins; Bleomycins; Bortezomibs; Brequinars; Bropirimines; Budotitanes; Busulfans; Cactinomycins; Calusterones; Canertinibs; Capecitabines; Caracemides; Carbetimers; Carboplatins; Carboquones; Carmofurs; Carmustines with Polifeprosans; Carmustines; Carubicins; Carzelesins; Cedefingols; Celecoxibs; Cemadotins; Chlorambucils; Cioteronels; Ciplactin; Cirolemycins; Cisplatins; Cladribines; Clanfenurs; Clofarabines; Crisnatols; Cyclophosphamides; Cytarabine liposomals; Cytarabines; Dacarbazines; Dactinomycins; Darbepoetin Alfas; Daunorubicin liposomals; Daunorubicins/Daunomycins; Daunorubicins; Decitabines; Denileukin Diftitoxes; Dexniguldipines; Dexonas; Dexrazoxanes; Dezaguanines; Diaziquones; Dibrospidiums; Dienogests; Dinalins; Disermolides; Docetaxels; Dofequidars; Doxifluridines; Doxorubicin liposomals; Doxorubicin HCL; Doxorubicin HCL liposome injection; Doxorubicins; Droloxifenes; Dromostanolone Propionates; Duazomycins; Ecomustines; Edatrexates; Edotecarins; Eflornithines; Elacridars; Elinafides; Elliott's B Solutions; Elsamitrucins; Emitefurs; Enloplatins; Enpromates; Enzastaurins; Epipropidines; Epirubicins; Epoetin alfas; Eptaloprosts; Erbulozoles; Esorubicins; Estramustines; Etanidazoles; Etoglucids; Etoposide phosphates; Etoposide VP-16s; Etoposides; Etoprines; Exemestanes; Exisulinds; Fadrozoles; Fazarabines; Fenretinides; Filgrastims; Floxuridines; Fludarabines; Fluorouracils; 5-fluorouracils; Fluoxymesterones; Fluorocitabines; Fosquidones; Fostriecins; Fostriecins; Fotretamines; Fulvestrants; Galarubicins; Galocitabines; Gemcitabines; Gemtuzumabs/Ozogamicins; Geroquinols; Gimatecans; Gimeracils; Gloxazones; Glufosfamides; Goserelin acetates; Hydroxyureas; Ibritumomabs/Tiuxetans; Idarubicins; Ifosfamides; Ilmofosines; Ilomastats; Imatinib mesylates; Imexons; Improsulfans; Indisulams; Inproquones; Interferon alfa-2 as; Interferon alfa-2bs; Interferon Alfas; Interferon Betas; Interferon Gammas; Interferons; Interleukin-2s and other Lnterleukins (including recombinant Interleukins); Intoplicines; Iobenguanes [131-I]; Iproplatins; Irinotecans; Irsogladines; Ixabepilones; Ketotrexates; L-Alanosines; Lanreotides; Lapatinibs; Ledoxantrones; Letrozoles; Leucovorins; Leuprolides; Leuprorelins (Leuprolides); Levamisoles; Lexacalcitols; Liarozoles; Lobaplatins; Lometrexols; Lomustines/CCNUs; Lomustines; Lonafarnibs; Losoxantrones; Lurtotecans; Mafosfamides; Mannosulfans; Marimastats; Masoprocols; Maytansines; Mechlorethamines; Mechlorethamines/Nitrogen mustards; Megestrol acetates; Megestrols; Melengestrols; Melphalans; Melphalan L-PAMs; Menogarils; Mepitiostanes; Mercaptopurines; 6-Mercaptopurine; Mesnas; Metesinds; Methotrexates; Methoxsalens; Metomidates; Metoprines; Meturedepas; Miboplatins; Miproxifenes; Misonidazoles; Mitindomides; Mitocarcins; Mitocromins; Mitoflaxones; Mitogillins; Mitoguazones; Mitomalcins; Mitomycin Cs; Mitomycins; Mitonafides; Mitoquidones; Mitospers; Mitotanes; Mitoxantrones; Mitozolomides; Mivobulins; Mizoribines; Mofarotenes; Mopidamols; Mubritinibs; Mycophenolic Acids; Nandrolone Phenpropionates; Nedaplatins; Nelarabines; Nemorubicins; Nitracrines; Nocodazoles; Nofetumomabs; Nogalamycins; Nolatrexeds; Nortopixantrones; Octreotides; Oprelvekins; Ormaplatins; Ortataxels; Oteracils; Oxaliplatins; Oxisurans; Oxophenarsines; Paclitaxels; Pamidronates; Patupilones; Pegademases; Pegaspargases; Pegfilgrastims; Peldesines; Peliomycins; Pelitrexols; Pemetrexeds; Pentamustines; Pentostatins; Peplomycins; Perfosfamides; Perifosines; Picoplatins; Pinafides; Pipobromans; Piposulfans; Pirfenidones; Piroxantrones; Pixantrones; Plevitrexeds; Plicamycin Mithramycins; Plicamycins; Plomestanes; Plomestanes; Porfimer sodiums; Porfimers; Porfiromycins; Prednimustines; Procarbazines; Propamidines; Prospidiums; Pumitepas; Puromycins; Pyrazofurins; Quinacrines; Ranimustines; Rasburicases; Riboprines; Ritrosulfans; Rituximabs; Rogletimides; Roquinimexs; Rufocromomycins; Sabarubicins; Safingols; Sargramostims; Satraplatins; Sebriplatins; Semustines; Simtrazenes; Sizofirans; Sobuzoxanes; Sorafenibs; Sparfosates; Sparfosic Acids; Sparsomycins; Spirogermaniums; Spiromustines; Spiroplatins; Spiroplatins; Squalamines; Streptonigrins; Streptovarycins; Streptozocins; Sufosfamides; Sulofenurs; Sunitinib Malate; 6-TG; Tacedinalines; Talcs; Talisomycins; Tallimustines; Tamoxifens; Tariquidars; Tauromustines; Tecogalans; Tegafurs; Teloxantrones; Temoporfins; Temozolomides; Teniposides/VM-26s; Teniposides; Teroxirones; Testolactones; Thiamiprines; Thioguanines; Thiotepas; Tiamiprines; Tiazofurins; Tilomisoles; Tilorones; Timcodars; Timonacics; Tirapazamines; Topixantrones; Topotecans; Toremifenes; Tositumomabs; Trabectedins (Ecteinascidin 743); Trastuzumabs; Trestolones; Tretinoins/ATRA; Triciribines; Trilostanes; Trimetrexates; Triplatin Tetranitrates; Triptorelins; Trofosfamides; Tubulozoles; Ubenimexs; Uracil Mustards; Uredepas; Valrubicins; Valspodars; Vapreotides; Verteporfins; Vinblastines; Vincristines; Vindesines; Vinepidines; Vinflunines; Vinformides; Vinglycinates; Vinleucinols; Vinleurosines; Vinorelbines; Vinrosidines; Vintriptols; Vinzolidines; Vorozoles; Xanthomycin A's (Guamecyclines); Zeniplatins; Zilascorbs [2-H]; Zinostatins; Zoledronate; Zorubicins; and Zosuquidars, for example:

Aldesleukins (e.g., PROLEUKIN®); Alemtuzumabs (e.g., CAMPATH®); Alitretinoins (e.g., PANRETIN®); Allopurinols (e.g., ZYLOPRIM®); Altretamines (e.g., HEXALEN®); Amifostines (e.g., ETHYOL®); Anastrozoles (e.g., ARIMIDEX®); Arsenic Trioxides (e.g., TRISENOX®); Asparaginases (e.g., ELSPAR®); BCG Live (e.g., TICE®BCG); Bexarotenes (e.g., TARGRETIN®); Bevacizumab (AVASTDM®); Bleomycins (e.g., BLENOXANE®); Busulfan intravenous (e.g., BUSULFEX®); Busulfan orals (e.g., MYLERAN™); Calusterones (e.g., METHOSARB®); Capecitabines (e.g., XELODA®); Carboplatins (e.g., PARAPLATIN®); Carmustines (e.g., BCNU®, BiCNU®); Carmustines with Polifeprosans (e.g., GLIADEL® Wafer); Celecoxibs (e.g., CELEBREX®); Chlorambucils (e.g., LEUKERAN®); Cisplatins (e.g., PLATINOL®); Cladribines (e.g., LEU STATIN®, 2-CdA®); Cyclophosphamides (e.g., CYTOXAN®, NEOSAR®); Cytarabines (e.g., CYTOSAR-U®); Cytarabine liposomals (e.g., DepoCyt®); Dacarbazines (e.g., DTIC-Dometi): Dactinomycins (e.g., COSMEGEN®); Darbepoetin Alfas (e.g., ARANESP®); Daunorubicin liposomals (e.g. DAUNOXOME®); Daunorubicins/Daunomycins (e.g., CERUBIDINE®); Denileukin Diftitoxes (e.g., ONTAK®); Dexrazoxanes (e.g., ZINECARD®); Docetaxels (e.g., TAXOTERE®); Doxorubicins (e.g., ADRIAMYCIN®, RUBEX®); Doxorubicin liposomals, including Doxorubicin HCL liposome injections (e.g., DOXIL®); Dromostanolone propionates (e.g., DROMOSTANOLONE® and MASTERONE® Injection); Elliott's B Solutions (e.g., Elliott's B Solution®); Epirubicins (e.g., ELLENCE®); Epoetin alfas (e.g., EPOGEN®); Estramustines (e.g., EMCYT®); Etoposide phosphates (e.g., ETOPOPHOS®); Etoposide VP-16s (e.g., VEPESID®); Exemestanes (e.g., AROMASIN®); Filgrastims (e.g., NEUPOGEN®); Floxuridines (e.g., FUDR®); Fludarabines (e.g., FLUDARA®); Fluorouracils incl. 5-FUs (e.g., ADRUCIL®); Fulvestrants (e.g., FASLODEX®); Gemcitabines (e.g., GEMZAR®); Gemtuzumabs/Ozogamicins (e.g., MYLOTARG®); Goserelin acetates (e.g., ZOLADEX®); Hydroxyureas (e.g., HYDREA®); Ibritumomabs/Tiuxetans (e.g., ZEVALIN®); Idarubicins (e.g., IDAMYCIN®); Ifosfamides (e.g., IFEX®); Imatinib mesylates (e.g., GLEEVEC®); Interferon alfa-2 as (e.g., ROFERON-A®); Interferon alfa-2bs (e.g., INTRON A®); Irinotecans (e.g., CAMPTOSAR®); Letrozoles (e.g., FEMARA®); Leucovorins (e.g., WELLCOVORIN®, LEUCOVORIN®); Levamisoles (e.g., ERGAMISOL®); Lomustines/CCNUs (e.g., CeeNU®); Mechlorethamines/Nitrogen mustards (e.g., MUSTARGEN®); Megestrol acetates (e.g., MEGACE®); Melphalans/L-PAMs (e.g., ALKERAN®); Mercaptopurine incl. 6-MPs (e.g., PURINETHOL®); Mesnas (e.g., MESNEX®); Methotrexates; Methoxsalens (e.g., UVADEX®); Mitomycin Cs (e.g., MUTAMYCIN®, MITOZYTREX®); Mitotanes (e.g., LYSODREN®); Mitoxantrones (e.g., NOVANTRONE®); Nandrolone Phenpropionates (e.g., DURABOLIN-50®); Nofetumomabs (e.g., VERLUMA®); Oprelvekins (e.g., NEUMEGA®); Oxaliplatins (e.g., ELOXATIN®); Paclitaxels (e.g., PAXENE®, TAXOL®); Pamidronates (e.g., AREDIA®); Pegademases (e.g., ADAGEN®); Pegaspargases (e.g., ONCASPAR®); Pegfilgrastims (e.g., NEULASTA®); Pentostatins (e.g., NIPENT®); Pipobromans (e.g., VERCYTE®); Plicamycin/Mithramycins (e.g., MITHRACIN®); Porfimer sodiums (e.g., PHOTOFRTN®); Procarbazines (e.g., MATULANE®); Quinacrines (e.g., ATABRTNE®); Rasburicases (e.g., ELITEK®); Rituximabs (e.g., RITUXAN®); Sargramostims (e.g., PROKINE®); Streptozocins (e.g., ZANOSAR®); Sunitinib Malates (e.g., SUTENT®); Talcs (e.g., SCLEROSOL®); Tamoxifens (e.g., NOLVADEX®); Temozolomides (e.g., TEMODAR®); Teniposides/VM-26s (e.g., VUMON®); Testolactones (e.g., TESLAC®); Thioguanines incl. 6-TG; Thiotepas (e.g., THIOPLEX®); Topotecans (e.g., HYCAMTEN®); Toremifenes (e.g., FARESTON®); Tositumomabs (e.g., BEXXAR®); Trastuzumabs (e.g., HERCEPTIN®); Tretinoins/ATRA (e.g., VESANOID®); Uracil Mustards; Valrubicins (e.g., VALSTAR®); Vinblastines (e.g., VELBAN®); Vincristines (e.g., ONCOVIN®); Vinorelbines (e.g., NAVELBINE®); and Zoledronates (e.g., ZOMETA®).

For example, exemplary antibiotic agents include, but are not limited to, Aminoglycosides; Amphenicols; Ansamycins; Carbacephems; Carbapenems; Cephalosporins or Cephems; Cephamycins; Clavams; Cyclic lipopeptides; Diaminopyrimidines; Ketolides; Lincosamides; Macrolides; Monobactams; Nitrofurans; Oxacephems; Oxazolidinones; Penems, thienamycins and miscellaneous beta-lactams; Penicillins; Polypeptides antibiotics; Quinolones; Sulfonamides; Sulfones; Tetracyclines; and other antibiotics (such as Clofoctols, Fusidic acids, Hexedines, Methenamines, Nitrofurantoins Nitroxolines, Ritipenems, Taurolidines, Xibomols).

Also included among exemplary therapeutic agents are coagulation factors or other blood modifiers such as antihemophilic factors, anti-inhibitor coagulant complexes, antithrombin III, coagulation Factor V, coagulation Factor VIII, coagulation Factor IX, plasma protein fractions, von Willebrand factors; antiplatelet agents (including, for example, abciximabs, anagrelides, cilostazols, clopidogrel bisulfates, dipyridamoles, epoprostenols, eptifibatides, tirofibans; colony stimulating factors (CSFs) (including, for example, Granulocyte CSFs and Granulocyte Macrophage CSFs); erythropoiesis stimulators (including, for example, erythropoietins such as darbepoetin alfas) and epoetin alfas; hemostatics and albumins (including, for example, aprotinins, combinations of antihemophilic factors and plasma, Desmopressin Acetates, and albumins); immune globulins, as well as hepatitis B immune globulins; thrombin inhibitors (including for example direct thrombin inhibitors and lepirudin), and drotrecogin alfas; anticoagulants (including, for example, dalteparins, enoxaparins and other heparins, and warfarins).

Exemplary antibodies or other therapeutic agents include, but are not limited to, Cetuximab (IMC-C225; Erbitux®); Trastuzumab (Herceptin®); Rituximab (Rituxan®; MabThera®); Bevacizumab (Avastin®); Alemtuzumab (Campath®; Campath-1H®; Mabcampath®); Panitumumab (ABX-EGF; Vectibix®); Ranibizumab (Lucentis®); Ibritumomab; Ibritumomab tiuxetan (Zevalin®); Tositumomab; Iodine I 131 Tositumomab (BEXXAR®); Catumaxomab (Removab®); Gemtuzumab; Gemtuzumab ozogamicin (Mylotarg®); Abatacept (CTLA4-Ig; Orencia®); Belatacept (L104EA29YIg; LEA29Y; LEA); Ipilimumab (MDX-010; MDX-101); Tremelimumab (ticilimumab; CP-675,206); PRS-010 (see e.g., US20090042785); PRS-050 (US7585940; US20090305982); Aflibercept (VEGF Trap, AVE005; Holash et al., (2002) *PNAS* 99:11393-11398); Volociximab (M200); F200 (Chimeric (human/murine) IgG4 Fab fragment of Volociximab (M200)); MORAb-009 Mouse/human chimeric IgG1 (US20050054048); Soluble fusion protein: Anti-mesothelin Fv linked to atruncated *Pseudomonas* exotoxin A (SS1P (CAT-5001); US20070189962); Cixutumumab (DVIC-A12); Nimotuzumab (h-R3) (Spicer (2005) *Curr Opin Mol Ther* 7:182-191); Zalutumumab (HuMax-EGFR; Lammerts van Bueren et al. (2008) *PNAS* 105:6109-14); Necitumumab IMC-11F8 (Li et al. (2008) *Structure* 16:216-227); Sym004 (Pedersen et al. 2010 *Cancer Res* 70:588-597); and mAb-425.

In particular, therapeutic agents include, but are not limited to, immunoglobulins, Interferon beta, Interferon alpha-2 as, Interferon alpha-1s, Interferon alpha-n3s, Interferon beta-1, Interferon beta-1as, Interferon gamma-1bs, Peg-interferon alpha-2 and Peginterferon alpha-2bs, insulin, a bisphosphate (e.g., Pamidronates or Zoledronates), Docetaxels, Doxorubicins, Doxorubicin liposomals and bevacizumabs.

Other exemplary therapeutic agents that can be combined by co-administration and/or co-formulation with a modified PH20 polypeptide provided herein, include, but are not limited to, Adalimumabs, Agalsidase Betas, Alefacepts, Ampicillins, Anakinras, Antipoliomyelitic Vaccines, Anti-Thymocytes, Azithromycins, Becaplermins, Caspofungins, Cefazolins, Cefepimes, Cefotetans, Ceftazidimes, Ceftriaxones, Cetuximabs, Cilastatins, Clavulanic Acids, Clindamycins, Darbepoetin Alfas, Daclizumabs, Diphtheria, Diphtheria antitoxins, Diphtheria Toxoids, Efalizumabs, Epinephrines, Erythropoietin Alphas, Etanercepts, Filgrastims, Fluconazoles, Follicle-Stimulating Hormones, Follitropin Alphas, Follitropin Betas, Fosphenyloins, Gadodiamides, Gadopentetates, Gatifloxacins, Glatiramers, GM-CSF's, Goserelins, Goserelin acetates, Granisetrons, *Haemophilus Influenza* B's, Haloperidols, Hepatitis vaccines, Hepatitis A Vaccines, Hepatitis B Vaccines, Ibritumomab Tiuxetans, Ibritumomabs, Tiuxetans, Immunoglobulins, *Hemophilus influenza* vaccines, Influenza Virus Vaccines, Infliximabs, Insulins, Insulin Glargines, Interferons, Interferon alphas, Interferon Betas, Interferon Gammas, Interferon alpha-2a's, Interferon alpha-2b's, Interferon alpha-1's, Interferon alpha-n3's, Interferon Betas, Interferon Beta-1a's, Interferon Gammas, Interferon alpha-consensus, Iodixanols, Iohexyls, Iopamidols, Ioversols, Ketorolacs, Laronidases, Levofloxacins, Lidocaines, Linezolids, Lorazepams, Measles Vaccines, Measles virus, Mumps viruses, Measles-Mumps-Rubella Virus Vaccines, Rubella vaccines, Medroxyprogesterones, Meropenems, Methylprednisolones, Midazolams, Morphines, Octreotides, Omalizumabs, Ondansetrons, Palivizumabs, Pantoprazoles, Pegaspargases, Pegfilgrastims, Peg-Interferon Alfa-2a's, Peg-Interferon Alfa-2b's, Pegvisomants, Pertussis vaccines, Piperacillins, Pneumococcal Vaccines and Pneumococcal Conjugate Vaccines, Promethazines, Reteplases, Somatropins, Sulbactams, Sumatriptans, Tazobactams, Tenecteplases, Tetanus Purified Toxoids, Ticarcillins, Tositumomabs, Triamcinolones, Triamcinolone Acetonides, Triamcinolone hexacetonides, Vancomycins, Varicella Zoster immunoglobulins, Varicella vaccines, other vaccines, Alemtuzumabs, Alitretinoins, Allopurinols, Altretamines, Amifostines, Anastrozoles, Arsenics, Arsenic Trioxides, Asparaginases, *Bacillus* Calmette-Guerin (BCG) vaccines, BCG Live, Bexarotenes, Bleomycins, Busulfans, Busulfan intravenous, Busulfan orals, Calusterones, Capecitabines, Carboplatins, Carmustines, Carmustines with Polifeprosans, Celecoxibs, Chlorambucils, Cisplatins, Cladribines, Cyclophosphamides, Cytarabines, Cytarabine liposomals, Dacarbazines, Dactinomycins, Daunorubicin liposomals, Daunorubicins, Daunomycins, Denileukin Diftitoxes, Dexrazoxanes, Docetaxels, Doxorubicins, Doxorubicin liposomals, Dromostanolone propionates, Elliott's B Solutions, Epirubicins, Epoetin alfas, Estramustines, Etoposides, Etoposide phosphates, Etoposide VP-16s, Exemestanes, Floxuridines, Fludarabines, Fluorouracils, 5-Fluorouracils, Fulvestrants, Gemcitabines, Gemtuzumabs, Ozogamicins, Gemtuzumab ozogamicins, Hydroxyureas, Idarubicins, Ifosfamides, Imatinib mesylates, Irinotecans, Letrozoles, Leucovorins, Levamisoles, Lomustines, CCNUs, Mechlorethamines, Nitrogen mustards, Megestrols, Megestrol acetates, Melphalans, L-PAMs, Mercaptopurines, 6-Mercaptopurines, Mesnas, Methotrexates, Methoxsalens, Mitomycins, Mitomycin C's, Mitotanes, Mitoxantrones, Nandrolones, Nandrolone Phenpropionates, Nofetumomabs, Oprelvekins, Oxaliplatins, Paclitaxels, Pamidronates, Pegademases, Pentostatins, Pipobromans, Plicamycins, Mithramycins, Porfimers, Porfimer sodiums, Procarbazines, Quinacrines, Rasburicases, Rituximabs, Sargramostims, Streptozocins, Talcs, Tamoxifens, Temozolomides, Teniposides, Testolactones, Thioguanines, 6-Thioguanines, Triethylenethiophosphoramides (Thiotepas), Topotecans, Toremifenes, Trastuzumabs, Tretinoins, Uracil Mustards, Valrubicins, Vinblastines, Vincristines, Vinorelbines, Zoledronates, Acivicins, Aclarubicins, Acodazoles, Acronines, Adozelesins, Aldesleukins, Retinoic Acids, Alitretinoins, 9-Cis-Retinoic Acids, Alvocidibs, Ambazones, Ambomycins, Ametantrones, Aminoglutethimides, Amsacrines, Anaxirones, Ancitabines, Anthramycins, Apaziquones, Argimesnas, Asperlins, Atrimustines, Azacitidines, Azetepas, Azotomycins, Banoxantrones, Batabulins, Batimastats, Benaxibines, Bendamustines, Benzodepas, Bicalutamides, Bietaserpines, Biricodars, Bisantrenes, Bisnafide Dimesylates, Bizelesins, Bortezomibs, Brequinars, Bropirimines, Budotitanes, Cactinomycins, Canertinibs, Caracemides, Carbetimers, Carboquones, Carmofurs, Carubicins, Carzelesins, Cedefingols, Cemadotins, Chlorambucils, Cioteronels, Cirolemycins, Clanfenurs, Clofarabines, Crisnatols, Decitabines, Dexniguldipines, Dexormaplatins, Dezaguanines, Diaziquones, Dibrospidiums, Dienogests, Dinalins, Disermolides, Dofequidars, Doxifluridines, Droloxifenes, Duazomycins, Ecomustines, Edatrexates, Edotecarins, Eflomithines, Elacridars, Elinafides, Elsamitrucins, Emitefurs, Enloplatins, Enpromates, Enzastaurins, Epipropidines, Eptaloprosts, Erbulozoles, Esorubicins, Etanidazoles, Etoglucids, Etoprines, Exisulinds, Fadrozoles, Fazarabines, Fenretinides, Fluoxymesterones, Fluorocitabines, Fosquidones, Fostriecins, Fotretamines, Galarubicins, Galocitabines, Geroquinols, Gimatecans, Gimeracils, Gloxazones, Glufosfamides, Ilmofosines, Ilomastats, Imexons, Improsulfans, Indisulams, Inproquones, Interleukins, Interleukin-2s, recombinant Interleukins, Intoplicines, Iobenguanes, Iproplatins, Irsogladines, Ixabepilones, Ketotrexates, L-Alanosines, Lanreotides, Lapatinibs, Ledoxantrones, Leuprolides, Leuprorelins, Lexacalcitols, Liarozoles, Lobaplatins, Lometrexols, Lonafarnibs, Losoxantrones, Lurtotecans, Mafosfamides, Mannosulfans, Marimastats, Masoprocols, Maytansines, Mechlorethamines, Melengestrols, Melphalans, Menogarils, Mepitiostanes, Metesinds, Metomidates, Metoprines, Meturedepas, Miboplatins, Miproxifenes, Misonidazoles, Mitindomides, Mitocarcins, Mitocromins, Mitoflaxones, Mitogillins, Mitoguazones, Mitomalcins, Mitonafides, Mitoquidones, Mitospers, Mitozolomides, Mivobulins, Mizoribines, Mofarotenes, Mopidamols, Mubritinibs, Mycophenolic Acids, Nedaplatins, Neizarabines, Nemorubicins, Nitracrines, Nocodazoles, Nogalamycins, Nolatrexeds, Nortopixantrones, Ormaplatins, Ortataxels, Oteracils, Oxisurans, Oxophenarsines, Patupilones, Peldesines, Peliomycins, Pelitrexols, Pemetrexeds, Pentamustines, Peplomycins, Perfosfamides, Perifosines, Picoplatins, Pinafides, Piposulfans, Pirfenidones, Piroxantrones, Pixantrones, Plevitrexeds, Plomestanes, Porfiromycins, Prednimustines, Propamidines, Prospidiums, Pumitepas, Puromycins, Pyrazofurins, Ranimustines, Riboprines, Ritrosulfans, Rogletimides, Roquinimexs, Rufocromomycins, Sabarubicins, Safingols, Satraplatins, Sebriplatins, Semustines, Simtrazenes, Sizofirans, Sobuzoxanes, Sorafenibs, Sparfosates, Sparfosic Acids, Sparsomycins, Spirogermaniums, Spiromustines, Spiroplatins, Squalamines, Streptonigrins, Streptovarycins, Sufosfamides, Sulofenurs, Tacedinalines, Talisomycins, Tallimustines, Tariquidars, Tauromustines, Tecogalans, Tegafurs, Teloxantrones, Temoporfins, Teroxirones, Thiamiprines, Tiamiprines, Tiazofurins, Tilomisoles, Tilorones, Timcodars, Timonacics, Tirapazamines, Topixantrones, Trabectedins, Ecteinascidin 743, Trestolones, Triciribines, Trilostanes, Trimetrexates, Triplatin Tetranitrates, Triptorelins, Trofosfamides, Tubulozoles, Ubenimexs, Uredepas, Valspodars, Vapreotides, Verteporfins, Vinblastines, Vindesines, Vinepidines, Vinflunines, Vinformides, Vinglycinates, Vinleucinols, Vinleurosines, Vinrosidines, Vintriptols, Vinzolidines, Vorozoles, Xanthomycin A's, Guamecyclines, Zeniplatins, Zilascorbs [2-H], Zinostatins, Zorubicins, Zosuquidars, Acetazolamides, Acyclovirs, Adipiodones, Alatrofloxacins, Alfentanils, Allergenic extracts, Alpha 1-proteinase inhibitors, Alprostadils, Amikacins, Amino acids, Aminocaproic acids, Aminophyllines, Amitriptylines, Amobarbitals, Amrinones, Analgesics, Anti-poliomyelitic vaccines, Anti-rabic serums, Anti-tetanus immunoglobulins, tetanus vaccines, Antithrombin IIIs, Antivenom serums, Argatrobans, Arginines, Ascorbic acids, Atenolols, Atracuriums, Atropines, Aurothioglucoses, Azathioprines, Aztreonams, Bacitracins, Baclofens, Basiliximabs, Benzoic acids, Benztropines, Betamethasones, Biotins, Bivalirudins, Botulism antitoxins, Bretyliums, Bumetanides, Bupivacaines, Buprenorphines, Butorphanols, Calcitonins, Calcitriols, Calciums, Capreomycins, Carboprosts, Carnitines, Cefamandoles, Cefoperazones, Cefotaximes, Cefoxitins, Ceftizoximes, Cefuroximes, Chloramphenicols, Chloroprocaines, Chloroquines, Chlorothiazides, Chlorpromazines, Chondroitinsulfuric acids, Choriogonadotropin alfas, Chromiums, Cidofovirs, Cimetidines, Ciprofloxacins, Cisatracuriums, Clonidines, Codeines, Colchicines, Colistins, Collagens, Corticorelin ovine triflutates, Corticotrophins, Cosyntropins, Cyanocobalamins, Cyclosporines, Cysteines, Dacliximabs, Dalfopristins, Dalteparins, Danaparoids, Dantrolenes, Deferoxamines, Desmopressins, Dexamethasones, Dexmedetomidines, Dexpanthenols, Dextrans, Iron dextrans, Diatrizoic acids, Diazepams, Diazoxides, Dicyclomines, Digibinds, Digoxins, Dihydroergotamines, Diltiazems, Diphenhydramines, Dipyridamoles, Dobutamines, Dopamines, Doxacuriums, Doxaprams, Doxercalciferols, Doxycyclines, Droperidols, Dyphyllines, Edetic acids, Edrophoniums, Enalaprilats, Ephedrines, Epoprostenols, Ergocalciferols, Ergonovines, Ertapenems, Erythromycins, Esmolols, Estradiols, Estrogenics, Ethacrynic acids, Ethanolamines, Ethanols, Ethiodized oils, Etidronic acids, Etomidates, Factor VIIIs, Famotidines, Fenoldopams, Fentanyls, Flumazenils, Fluoresceins, Fluphenazines, Folic acids, Fomepizoles, Fomivirsens, Fondaparinuxs, Foscarnets, Fosphenyloins, Furosemides, Gadoteridols, Gadoversetamides, Ganciclovirs, Gentamicins, Glucagons, Glucoses, Glycines, Glycopyrrolates, Gonadorelins, Gonadotropin chorionics, *Haemophilus* B polysaccharides, Hemins, Herbals, Histamines, Hydralazines, Hydrocortisones, Hydromorphones, Hydroxocobalamins, Hydroxyzines, Hyoscyamines, Ibutilides, Imiglucerases, Indigo carmines, Indomethacins, Iodides, Iopromides, Iothalamic acids, Ioxaglic acids, Ioxilans, Isoniazids, Isoproterenols, Japanese encephalitis vaccines, Kanamycins, Ketamines, Labetalols, Lepirudins, Levobupivacaines, Levothyroxines, Lincomycins, Liothyronines, Luteinizing hormones, Lyme disease vaccines, Mangafodipirs, Manthtols, Meningococcal polysaccharide vaccines, Meperidines, Mepivacaines, Mesoridazines, Metaraminols, Methadones, Methocarbamols, Methohexitals, Methyldopates, Methylergonovines, Metoclopramides, Metoprolols, Metronidazoles, Minocyclines, Mivacuriums, Morrhuic acids, Moxifloxacins, Muromonab-CD3s, Mycophenolate mofetils, Nafcillins, Nalbuphines, Nalmefenes, Naloxones, Neostigmines, Niacinamides, Nicardipines, Nitroglycerins, Nitroprussides, Norepinephrines, Orphenadrines, Oxacillins, Oxymorphones, Oxytetracyclines, Oxytocins, Pancuroniums, Panthenols, Pantothenic acids, Papaverines, Peginterferon-alpha (e.g., interferon alpha 2a or 2b), Penicillin Gs, Pentamidines, Pentazocines, Pentobarbitals, Perflutrens, Perphenazines, Phenobarbitals, Phentolamines, Phenylephrines, Phenyloins, Physostigmines, Phytonadiones, Polymyxin bs, Pralidoximes, Prilocalnes, Procainamides, Procaines, Prochlorperazines, Progesterones, Propranolols, Pyridostigmine hydroxides, Pyridoxines, Quinidines, Quinupristins, Rabies immunoglobulins, Rabies vaccines, Ranitidines, Remifentanils, Riboflavins, Rifampins, Ropivacaines, Samariums, Scopolamines, Seleniums, Sermorelins, Sincalides, Somatrems, Spectinomycins, Streptokinases, Streptomycins, Succinylcholines, Sufentanils, Sulfamethoxazoles, Tacrolimuses, Terbutalines, Teriparatides, Testosterones, Tetanus antitoxins, Tetracaines, Tetradecyl sulfates, Theophyllines, Thiamines, Thiethylperazines, Thiopentals, Thyroid stimulating hormones, Tinzaparins, Tirofibans, Tobramycins, Tolazolines, Tolbutamides, Torsemides, Tranexamic acids, Treprostinils, Trifluoperazines, Trimethobenzamides, Trimethoprims, Tromethamines, Tuberculins, Typhoid vaccines, Urofollitropins, Urokinases, Valproic acids, Vasopressins, Vecuroniums, Verapamils, Voriconazoles, Warfarins, Yellow fever vaccines, Zidovudines, Zincs, Ziprasidone hydrochlorides, Aclacinomycins, Actinomycins, Adriamycins, Azaserines, 6-Azauridines, Carzinophilins, Chromomycins, Denopterins, 6-Diazo-5-Oxo-L-Norleucines, Enocitabines, Floxuridines, Olivomycins, Pirarubicins, Piritrexims, Pteropterins, Tegafurs, Tubercidins, Alteplases, Arcitumomabs, bevacizumabs, Botulinum Toxin Type A's, Botulinum Toxin Type B's, Capromab Pendetides, Daclizumabs, Dornase alfas, Drotrecogin alfas, Imciromab Pentetates, and Iodine-131's.

Delivery of Insulin

Methods provided herein include methods of co-administering a modified PH20 polypeptide and an insulin to increase subcutaneous delivery of the insulin, such as a fast-acting insulin (see e.g., U.S. Pat. No. 7,767,429; U.S. Pat. No. 7,846,431; U.S. Publication No. US20090304665; and U.S. application Ser. Nos. 13/507,263; 13/507,262 and 13/507,261). Such methods include methods of direct administration, and pump and continuous infusion methods, including open and closed pump systems. For example, exemplary insulins that can be administered with a modified PH20 hyaluronidase provided herein are fast-acting insulins or insulin analogs. For example, a co-administered insulin includes a regular insulin, insulin aspart, insulin lispro, insulin glulisine or other similar analog variants. Exemplary insulins are insulins that contain an A chain set forth in SEQ ID NO:862 and a B chain set forth in SEQ ID NO:863 or variants that contain one or more amino acid modifications compared to a human insulin set forth in SEQ ID NO: 862 and 863 (A and B chains). For example, exemplary insulin analogs are known to one of skill in the art, and include, but are not limited to, those set forth in SEQ ID NOS:862 (A-chain) and having a B-chain set forth in any of SEQ ID NOS: 865-867.

The co-formulations can be administered subcutaneously to treat any condition that is amenable to treatment with insulin. Therapeutic uses include, but are not limited to, treatment for type 1 diabetes mellitus, type 2 diabetes mellitus, gestational diabetes, and for glycemic control in critically ill patients. For example, the co-formulations of a fast acting insulin and hyaluronan degrading enzyme can be administered subcutaneously in discrete doses, such as via a syringe or insulin pen, prior to a meal as prandial insulin therapy in subjects with diabetes to achieve glycemic control. The co-formulations also can be administered subcutaneously or intraperitoneally using an insulin pump or in the context of a closed loop system to continuously control blood glucose levels throughout the day and night and/or to control post-prandial glycemic excursions. It is within the skill of a treating physician to identify such diseases or conditions.

For any disease or condition, including all those exemplified above, for which a fast-acting insulin is indicated or has been used and for which other agents and treatments are available, the co-formulations can be used in combination therewith. Depending on the disease or condition to be treated, exemplary combinations include, but are not limited to, combinations with anti-diabetic drugs, including, but not limited to, sulfonylureas, biguanides, meglitinides, thiazolidinediones, alpha-glucosidase inhibitors, peptide analogs, including glucagon-like peptide (GLP) analogs and, gastric inhibitory peptide (GIP) analogs and DPP-4 inhibitors. In another example, the co-formulations of a fast acting insulin and modified PH20 polypeptide described herein can be administered in combination with, prior to, intermittently with, or subsequent to, one or more other insulins, including fast-acting insulin, and basal-acting insulins.

2. Methods of Hyaluronan-Associated Diseases and Conditions (e.g., Tumors)

In particular, PH20 hyaluronidase can be used to treat hyaluronan-associated diseases or conditions. Typically, hyaluronan-associated diseases and conditions are associated with elevated hyaluronan expression in a tissue, cell, or body fluid (e.g., tumor tissue or tumor-associated tissue, blood, or interstitial space) compared to a control, e.g., another tissue, cell or body fluid. The elevated hyaluronan expression can be elevated compared to a normal tissue, cell or body fluid, for example, a tissue, cell or body fluid that is analogous to the sample being tested, but isolated from a different subject, such as a subject that is normal (i.e., does not have a disease or condition, or does not have the type of disease or condition that the subject being tested has), for example, a subject that does not have a hyaluronan-associated disease or condition. The elevated hyaluronan expression can be elevated compared to an analogous tissue from another subject that has a similar disease or condition, but whose disease is not as severe and/or is not hyaluronan-associated or expresses relatively less hyaluronan and thus is hyaluronan-associated to a lesser degree. For example, the subject being tested can be a subject with a hyaluronan-associated cancer, where the HA amounts in the tissue, cell or fluid are relatively elevated compared to a subject having a less severe cancer, such as an early stage, differentiated or other type of cancer. In another example, the cell, tissue or fluid contains elevated levels of hyaluronan compared to a control sample, such as a fluid, tissue, extract (e.g., cellular or nuclear extract), nucleic acid or peptide preparation, cell line, biopsy, standard or other sample, with a known amount or relative amount of HA, such as a sample, for example a tumor cell line, known to express relatively low levels of HA, such as exemplary tumor cell lines described herein that express low levels of HA, for example, the HCT 116 cell line, the HT29 cell line, the NCI H460 cell line, the DU145 cell line, the Capan-1 cell line, and tumors from tumor models generated using such cell lines.

Hyaluronan-associated diseases and conditions include those associated with high interstitial fluid pressure, such as disc pressure, proliferative disorders, such as cancer and benign prostatic hyperplasia, and edema. Edema can result from or be manifested in, for example, organ transplant, stroke or brain trauma. Proliferative disorders include, but are not limited to, cancer, smooth muscle cell proliferation, systemic sclerosis, cirrhosis of the liver, adult respiratory distress syndrome, idiopathic cardiomyopathy, lupus erythematosus, retinopathy, e.g., diabetic retinopathy or other retinopathies, cardiac hyperplasia, reproductive system associated disorders, such as benign prostatic hyperplasia (BPH) and ovarian cysts, pulmonary fibrosis, endometriosis, fibromatosis, hamartomas, lymphangiomatosis, sarcoidosis, desmoid tumors. Cancers include solid and lymphatic/blood tumors and metastatic disease, and undifferentiated tumors. The tumors amenable to treatment typically exhibit cellular and/or stromal expression of a hyaluronan, compared to a non-cancerous tissue of the same tissue type or compared to a non-metastatic tumor of the same tumor-type. Cancers include any one or more of ovarian cancer, in situ carcinoma (ISC), squamous cell carcinoma (SCC), prostate cancer, pancreatic cancer, other gastric cancers, non-small cell lung cancer, breast cancer, brain cancer and colon cancer.

Modified PH20 polypeptides provided herein, such as PEGylated forms thereof, can be used to treat tumors. Thus, in addition to its indirect anticancer effects, hyaluronidases also have direct anticarcinogenic effects. Hyaluronidase prevents growth of tumors transplanted into mice (De Maeyer et al., 1992, *Int. J. Cancer* 51:657-660) and inhibits tumor formation upon exposure to carcinogens (Pawlowski et al., 1979, *Int. J. Cancer* 23:105-109; Haberman et al., 1981, Proceedings of the 17th Annual Meeting of the American Society of Clinical Oncology, Washington, D.C., 22:105, abstract no. 415). PH20 hyaluronidase has been shown to treat various tumors (see e.g., U.S. Publication No. US2010/0003238 and U.S. application Ser. No. 13/135,817, published as U.S. Publication No. US20120020951).

The hyaluronan-rich cancer can be a cancer in which the cancer cells produce HALOs, cancers that have elevated expression of hyaluronan (as determined by immunostaining, e.g., histological staining of sections from the tumor), cancers that have elevated HAS2 (Hyaluronan synthase 2), cancers that do not produce hyaluronidase (HYAL1) in vitro. Hyaluronan-rich cancers can be identified by any method for assessing hyaluronan expression, and other known methods for assaying protein/mRNA expression.

Several hyaluronan-rich cancers have been identified. In some cases, hyaluronan expression correlates with poor prognosis, for example, decreased survival rate and/or recurrence-free survival rate, metastases, angiogenesis, cancer cell invasion into other tissues/areas, and other indicators of poor prognosis. Such correlation has been observed, for example, in hyaluronan-rich tumors including ovarian cancer, SCC, ISC, prostate cancer, lung cancer, including non-small-cell lung cancer (NSCLC), breast cancer, colon cancer and pancreatic cancer (see, for example, Anttila et al., *Cancer Research,* 60:150-155 (2000); Karvinen et al., *British Journal of Dermatology,* 148:86-94 (2003); Lipponen et al., *Eur. Journal of Cancer,* 849-856 (2001); Pirinen et al., *Int. J. Cancer:* 95:12-17 (2001); Auvinen et al., *American Journal of Pathology,* 156(2):529-536 (2000); Ropponen et al., *Cancer Research,* 58:342-347 (1998)). Thus, hyaluronan-rich cancers can be treated by administration of a hyaluronidase, such as a soluble PH20, to treat one or more symptoms of the cancer. Hyaluronan-rich tumors include, but are not limited to those of the prostate, breast, colon, ovarian, stomach, head and neck and other tumors and cancers.

Other hyaluronan-associated diseases or conditions that are associated with excess glycosaminoglycans and that can be treated with a modified PH20 polypeptide provided herein include, but are not limited to, cardiovascular disease (e.g., following ischemia reperfusion; in arteriosclerosis); vitrectomy and ophthalmic disorders and conditions (e.g., in methods to liquefy the vitreous humor of the eye; reduce postoperative pressure; other ocular surgical procedures such as glaucoma, vitreous and retina surgery and in corneal transplantation); in hypodermoclysis (i.e., infusion of fluids and electrolytes into the hypodermis of the skin); cosmetic applications (e.g., in the treatment of cellulite, "pigskin" edema or "orange peel" edema); organ transplantation (e.g., associated with interstitial edemas in connection with grafting of an organ); pulmonary disease.

3. Other Uses

In further examples of its therapeutic use, modified PH20 polypeptides provided herein, can be used for such purposes as an antidote to local necrosis from paravenous injection of necrotic substances such as vinca alkaloids (Few et al. (1987) *Amer. J. Matern. Child Nurs.* 12, 23-26), treatment of ganglion cysts (Paul et al. (1997) *J Hand Surg.* 22 (2): 219-21) and treatment of tissue necrosis due to venous insufficiency (Elder et al. (1980) *Lancet* 648-649). Modified PH20 polypeptides also can be used to treat ganglion cysts (also known as a wrist cyst, Bible cyst, or dorsal tendon cyst), which are the most common soft tissue mass of the hand and are fluid filled sacs that can be felt below the skin.

Modified PH20 polypeptides can be used in the treatment of spinal cord injury by degrading chondroitin sulfate proteoglycans (CSPGs). Following spinal cord injury, glial scars containing CSPGs are produced by astrocytes. CSPGs play a crucial role in the inhibition of axon growth. In addition, the expression of CSPG has been shown to increase following injury of the central nervous system (CNS). Soluble PH20 also can be utilized for the treatment of herniated disks in a process known as chemonucleolysis. Chondroitinase ABC, an enzyme cleaving similar substrates as hyaluronidase, can induce the reduction of intradiscal pressure in the lumbar spine. There are three types of disk injuries. A protruded disk is one that is intact but bulging. In an extruded disk, the fibrous wrapper has torn and the NP has oozed out, but is still connected to the disk. In a sequestered disk, a fragment of the NP has broken loose from the disk and is free in the spinal canal. Chemonucleolysis is typically effective on protruded and extruded disks, but not on sequestered disk injuries.

4. Contraception

Modified PH20 polypeptides provided herein can be used as vaccines in contraceptive applications. PH20 is present in the male reproductive tract, and is expressed in both the testis and epididymis and is present in sperm. PH20 plays a role in fertilization by facilitating entry of the sperm through the cumulus layer surrounding the unfertilized egg. PH20 also is able to bind to hyaluronic acid (HA) on the zona pellucida during early phases of fertilization. This binding also initiates intracellular signaling that aids in the acrosome reaction. Immunization with PH20 has been show to be an effective contraceptive in male guinea pigs (Primakoff et al. (1988) *Nature* 335:543-546, Tung et al. (1997) *Biol. Reprod.* 56:1133-1141). It also has been shown to be an effective contraceptive in female guinea pigs due to the generation of anti-PH20 antibodies that prevent sperm and egg binding. In examples herein, the modified PH20 polypeptides can be inactive enzymes, such as any described in Sections C.2. The polypeptides can be administered directly or can be administered as a recombinant virus to deliver the antigen.

I. Examples

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

Example 1

Generation of Recombinant Human PH20 Hyaluronidase (rHuPH20)

A. Generation of a Soluble rHuPH20-Expressing Cell Line

A recombinant human PH20 hyaluronidase designated rHuPH20 was generated as described in published U.S. Publication No. US20110053247. Briefly, the pCI-PH20-IRES-DHFR-SV40pa (HZ24) plasmid (set forth in SEQ ID NO:5) was used to transfect Chinese Hamster Ovary (CHO cells) (see e.g., U.S. Pat. Nos. 7,767,429 and 7,781,607 and U.S. Publication No. 2006-0104968). The HZ24 plasmid vector for expression of soluble rHuPH20 contains a pCI vector backbone (Promega), DNA encoding amino acids 1-482 of human PH20 hyaluronidase (SEQ ID NO:2), an internal ribosomal entry site (IRES) from the ECMV virus (Clontech), and the mouse dihydrofolate reductase (DHFR) gene. The pCI vector backbone also includes DNA encoding the Beta-Iactamase resistance gene (AmpR), an f1 origin of replication, a Cytomegalovirus immediate-early enhancer/promoter region (CMV), a chimeric intron, and an SV40 late polyadenylation signal (SV40). The DNA encoding the soluble rHuPH20 construct contains an NheI site and a Kozak consensus sequence prior to the DNA encoding the methionine at amino acid position 1 of the native 35 amino acid signal sequence of human PH20, and a stop codon following the DNA encoding the tyrosine corresponding to amino acid position 482 of the human PH20 hyaluronidase set forth in SEQ ID NO:2, followed by a BamHI restriction site.

Non-transfected DG44 CHO cells growing in GIBCO Modified CD-CHO media for DHFR(−) cells, supplemented with 4 mM Glutamine and 18 mL/L Plurionic F68/L (Gibco), were seeded at 0.5×10^6 cells/mL in a shaker flask in preparation for transfection. Cells were grown at 37° C. in 5% CO_2 in a humidified incubator, shaking at 120 rpm. Exponentially growing non-transfected DG44 CHO cells were tested for viability prior to transfection.

Sixty million viable cells of the non-transfected DG44 CHO cell culture were pelleted and resuspended to a density of 2×10^7 cells in 0.7 mL of 2× transfection buffer (2×HeBS: 40 mM Hepes, pH 7.0, 274 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 12 mM dextrose). To each aliquot of resuspended cells, 0.09 mL (250 μg) of the linear HZ24 plasmid (linearized by overnight digestion with Cla I (New England Biolabs) was added, and the cell/DNA solutions were transferred into 0.4 cm gap BTX (Gentronics) electroporation cuvettes at room temperature. A negative control electroporation was performed with no plasmid DNA mixed with the cells. The cell/plasmid mixes were electroporated with a capacitor discharge of 330 V and 960 μF or at 350 V and 960 μF.

The cells were removed from the cuvettes after electroporation and transferred into 5 mL of Modified CD-CHO media for DHFR(−) cells, supplemented with 4 mM Glutamine and 18 mL/L Plurionic F68/L (Gibco), and allowed to grow in a well of a 6-well tissue culture plate without selection for 2 days at 37° C. in 5% CO_2 in a humidified incubator.

Two days post-electroporation, 0.5 mL of tissue culture media was removed from each well and tested for the presence of hyaluronidase activity, using the microturbidity assay described in Example 8. The results are set forth in Table 6.

TABLE 6

Initial Hyaluronidase Activity of HZ24 Transfected DG44 CHO cells at 40 hours post-transfection

	Dilution	Activity (Units/mL)
Transfection 1 330 V	1 to 10	0.25
Transfection 2 350 V	1 to 10	0.52
Negative Control	1 to 10	0.015

Cells from Transfection 2 (350V) were collected from the tissue culture well, counted and diluted to 1×10^4 to 2×10^4 viable cells per mL. A 0.1 mL aliquot of the cell suspension was transferred to each well of five, 96 well round bottom tissue culture plates. One hundred microliters of CD-CHO media (GIBCO) containing 4 mM GlutaMAX™-1 supplement (GIBCO™, Invitrogen Corporation) and without hypoxanthine and thymidine supplements were added to the wells containing cells (final volume 0.2 mL). Ten clones were identified from the 5 plates grown without methotrexate (Table 7).

TABLE 7

Hyaluronidase activity of identified clones

Plate/Well ID	Relative Hyaluronidase
1C3	261
2C2	261
3D3	261
3E5	243
3C6	174
2G8	103
1B9	304
2D9	273
4D10	302

Six HZ24 clones were expanded in culture and transferred into shaker flasks as single cell suspensions. Clones 3D3, 3E5, 2G8, 2D9, 1E11, and 4D10 were plated into 96-well round bottom tissue culture plates using a two-dimensional infinite dilution strategy in which cells were diluted 1:2 down the plate, and 1:3 across the plate, starting at 5000 cells in the top left hand well. Diluted clones were grown in a background of 500 non-transfected DG44 CHO cells per well, to provide necessary growth factors for the initial days in culture. Ten plates were made per subclone, with 5 plates containing 50 nM methotrexate and 5 plates without methotrexate.

Clone 3D3 produced 24 visual subclones (13 from the no methotrexate treatment, and 11 from the 50 nM methotrexate treatment). Significant hyaluronidase activity was measured in the supernatants from 8 of the 24 subclones (>50 Units/mL), and these 8 subclones were expanded into T-25 tissue culture flasks. Clones isolated from the methotrexate treatment protocol were expanded in the presence of 50 nM methotrexate. Clone 3D35M was further expanded in 500 nM methotrexate giving rise to clones producing hyaluronidase activity in excess of 1,000 Units/mL in shaker flasks (clone 3D35M; or Gen1 3D35M). A master cell bank (MCB) of the 3D35M cells was then prepared.

B. Production Gen2 Cells Containing Soluble human PH20 (rHuPH20)

The Gen1 3D35M cell line described in Example 1. A was adapted to higher methotrexate levels to produce generation 2 (Gen2) clones. 3D35M cells were seeded from established methotrexate-containing cultures into CD CHO medium containing 4 mM GlutaMAX-1™ and 1.0 μM methotrexate. The cells were adapted to a higher methotrexate level by growing and passaging them 9 times over a period of 46 days in a 37° C., 7% CO_2 humidified incubator. The amplified population of cells was cloned out by limiting dilution in 96-well tissue culture plates containing medium with 2.0 μM methotrexate. After approximately 4 weeks, clones were identified and clone 3E10B was selected for expansion. 3E10B cells were grown in CD CHO medium containing 4 mM GlutaMAX-1™ and 2.0 μM methotrexate for 20 passages. A master cell bank (MCB) of the 3E10B cell line was created and frozen and used for subsequent studies.

Amplification of the cell line continued by culturing 3E10B cells in CD CHO medium containing 4 mM GlutaMAX-1™ and 4.0 μM methotrexate. After the 12*th* passage, cells were frozen in vials as a research cell bank (RCB). One vial of the RCB was thawed and cultured in medium containing 8.0 μM methotrexate. After 5 days, the methotrexate concentration in the medium was increased to 16.0 μM, then 20.0 μM 18 days later. Cells from the 8*th* passage in medium containing 20.0 μM methotrexate were cloned out by limiting dilution in 96-well tissue culture plates containing CD CHO medium containing 4 mM GlutaMAX-1™ and 20.0 μM methotrexate. Clones were identified 5-6 weeks later and clone 2B2 was selected for expansion in medium containing 20.0 μM methotrexate. After the 11*th* passage, 2B2 cells were frozen in vials as a research cell bank (RCB).

The resultant 2B2 cells are dihydrofolate reductase deficient (dhfr−) DG44 CHO cells that express soluble recombinant human PH20 (rHuPH20). The soluble PH20 is present in 2B2 cells at a copy number of approximately 206 copies/cell. Southern blot analysis of Spe I-, Xba I- and BamH I/Hind III-digested genomic 2B2 cell DNA using a rHuPH20-specific probe revealed the following restriction digest profile: one major hybridizing band of 7.7 kb and four minor hybridizing bands (~13.9, ~6.6, ~5.7 and ~4.6 kb) with DNA digested with Spe I; one major hybridizing band of ~5.0 kb and two minor hybridizing bands (~13.9 and ~6.5 kb) with DNA digested with Xba I; and one single hybridizing band of ~1.4 kb observed using 2B2 DNA digested with BamH I/Hind III.

C. Production of Gen2 Soluble rHuPH20 in 300 L Bioreactor Cell Culture

A vial of HZ24-2B2 was thawed and expanded from shaker flasks through 36 L spinner flasks in CD-CHO media (Invitrogen, Carlsbad, Calif.) supplemented with 20 μM methotrexate and GlutaMAX-1™ (Invitrogen). Briefly, the vial of cells was thawed in a 37° C. water bath, medium was added and the cells were centrifuged. The cells were resuspended in a 125 mL shake flask with 20 mL of fresh medium and placed in a 37° C., 7% CO_2 incubator. The cells were expanded up to 40 mL in the 125 mL shake flask. When the cell density reached greater than 1.5×10^6 cells/mL, the culture was expanded into a 125 mL spinner flask in a 100 mL culture volume. The flask was incubated at 37° C., 7% CO_2. When the cell density reached greater than 1.5×10^6 cells/mL, the culture was expanded into a 250 mL spinner flask in 200 mL culture volume, and the flask was incubated at 37° C., 7% CO_2. When the cell density reached greater than 1.5×10^6 cells/mL, the culture was expanded into a 1 L spinner flask in 800 mL culture volume and incubated at 37° C., 7% CO_2. When the cell density reached greater than 1.5×10^6 cells/mL the culture was expanded into a 6 L spinner flask in 5000 mL culture volume and incubated at 37° C., 7% CO_2. When the cell density reached greater than 1.5×10^6 cells/mL the culture was expanded into a 36 L spinner flask in 32 L culture volume and incubated at 37° C., 7% CO_2.

A 400 L reactor was sterilized and 230 mL of CD-CHO media were added. Before use, the reactor was checked for contamination. Approximately 30 L cells were transferred from the 36 L spinner flasks to the 400 L bioreactor (Braun) at an inoculation density of 4.0×10^5 viable cells per mL and a total volume of 260 L. Parameters were: temperature setpoint, 37° C.; Impeller Speed 40-55 RPM; Vessel Pressure: 3 psi; Air Sparge 0.5-1.5 L/Min.; Air Overlay: 3 L/min. The reactor was sampled daily for cell counts, pH verification, media analysis, protein production and retention. Also, during the run nutrient feeds were added. At 120 hrs (day 5), 10.4 L of Feed #1 Medium (4×CD-CHO+33 g/L Glucose+160 mL/L Glutamax-1™+83 mL/L Yeastolate+33 mg/L rHuInsulin) was added. At 168 hours (day 7), 10.8 L of Feed #2 (2×CD-CHO+33 g/L Glucose+80 mL/L Glutamax-1™+167 mL/L Yeastolate+0.92 g/L Sodium Butyrate) was added, and culture temperature was changed to 36.5° C. At 216 hours (day 9), 10.8 L of Feed #3 (1×CD-CHO+50 g/L Glucose+50 mL/L Glutamax-1™+250 mL/L Yeastolate+1.80 g/L Sodium Butyrate) was added, and culture temperature was changed to 36° C. At 264 hours (day 11), 10.8 L of Feed #4 (1×CD-CHO+33 g/L Glucose+33 mL/L Glutamax-1™+250 mL/L Yeastolate+0.92 g/L Sodium Butyrate) was added, and culture temperature was changed to 35.5° C. The addition of the feed media was observed to dramatically enhance the production of soluble rHuPH20 in the final stages of production. The reactor was harvested at 14 or 15 days or when the viability of the cells dropped below 40%. The process resulted in a final productivity of 17,000 Units per mL with a maximal cell density of 12 million cells/mL. At harvest, the culture was sampled for mycoplasma, bioburden, endotoxin and virus in vitro and in vivo, by Transmission Electron Microscopy (TEM) and enzyme activity.

The culture was pumped by a peristaltic pump through four Millistak filtration system modules (Millipore) in parallel, each containing a layer of diatomaceous earth graded to 4-8 μm and a layer of diatomaceous earth graded to 1.4-1.1 μm, followed by a cellulose membrane, then through a second single Millistak filtration system (Millipore) containing a layer of diatomaceous earth graded to 0.4-0.11 μm and a layer of diatomaceous earth graded to <0.1 μm, followed by a cellulose membrane, and then through a 0.22 μm final filter into a sterile single use flexible bag with a 350 L capacity. The harvested cell culture fluid was supplemented with 10 mM EDTA and 10 mM Tris to a pH of 7.5. The culture was concentrated 10× with a tangential flow filtration (TFF) apparatus using four Sartoslice TFF 30 kDa molecular weight cut-off (MWCO) polyether sulfone (PES) filter (Sartorious), followed by a 10× buffer exchange with 10 mM Tris, 20 mM Na_2SO_4, pH 7.5 into a 0.22 μm final filter into a 50 L sterile storage bag.

The concentrated, diafiltered harvest was inactivated for virus. Prior to viral inactivation, a solution of 10% Triton® X-100, 3% tri (n-butyl) phosphate (TNBP) was prepared. The concentrated, diafiltered harvest was exposed to 1%

Triton® X-100, 0.3% TNBP for 1 hour in a 36 L glass reaction vessel immediately prior to purification on the Q column.

D. Purification of Gen2 Soluble rHuPH20

A Q Sepharose (Pharmacia) ion exchange column (9 L resin, H=29 cm, D=20 cm) was prepared. Wash samples were collected for a determination of pH, conductivity and endotoxin (LAL assay). The column was equilibrated with 5 column volumes of 10 mM Tris, 20 mM Na_2SO_4, pH 7.5. Following viral inactivation, the concentrated, diafiltered harvest was loaded onto the Q column at a flow rate of 100 cm/hr. The column was washed with 5 column volumes of 10 mM Tris, 20 mM Na_2SO_4, pH 7.5 and 10 mM Hepes, 50 mM NaCl, pH7.0. The protein was eluted with 10 mM Hepes, 400 mM NaCl, pH 7.0 into a 0.22 μm final filter into sterile bag. The eluate sample was tested for bioburden, protein concentration and hyaluronidase activity. A_{280} absorbance readings were taken at the beginning and end of the exchange.

Phenyl-Sepharose (Pharmacia) hydrophobic interaction chromatography was next performed. A Phenyl-Sepharose (PS) column (19-21 L resin, H=29 cm, D=30 cm) was prepared. The wash was collected and sampled for pH, conductivity and endotoxin (LAL assay). The column was equilibrated with 5 column volumes of 5 mM potassium phosphate, 0.5 M ammonium sulfate, 0.1 mM $CaCl_2$, pH 7.0. The protein eluate from the Q sepharose column was supplemented with 2M ammonium sulfate, 1 M potassium phosphate and 1 M $CaCl_2$ stock solutions to yield final concentrations of 5 mM, 0.5 M and 0.1 mM, respectively. The protein was loaded onto the PS column at a flow rate of 100 cm/hr and the column flow thru collected. The column was washed with 5 mM potassium phosphate, 0.5 M ammonium sulfate and 0.1 mM $CaCl_2$ pH 7.0 at 100 cm/hr and the wash was added to the collected flow thru. Combined with the column wash, the flow through was passed through a 0.22 μm final filter into a sterile bag. The flow through was sampled for bioburden, protein concentration and enzyme activity.

An aminophenyl boronate column (Prometics) was prepared. The wash was collected and sampled for pH, conductivity and endotoxin (LAL assay). The column was equilibrated with 5 column volumes of 5 mM potassium phosphate, 0.5 M ammonium sulfate. The PS flow through containing purified protein was loaded onto the aminophenyl boronate column at a flow rate of 100 cm/hr. The column was washed with 5 mM potassium phosphate, 0.5 M ammonium sulfate, pH 7.0. The column was washed with 20 mM bicine, 0.5 M ammonium sulfate, pH 9.0. The column was washed with 20 mM bicine, 100 mM sodium chloride, pH 9.0. The protein was eluted with 50 mM Hepes, 100 mM NaCl, pH 6.9 and passed through a sterile filter into a sterile bag. The eluted sample was tested for bioburden, protein concentration and enzyme activity.

The hydroxyapatite (HAP) column (Biorad) was prepared. The wash was collected and tested for pH, conductivity and endotoxin (LAL assay). The column was equilibrated with 5 mM potassium phosphate, 100 mM NaCl, 0.1 mM $CaCl_2$, pH 7.0. The aminophenyl boronate purified protein was supplemented to final concentrations of 5 mM potassium phosphate and 0.1 mM $CaCl_2$ and loaded onto the HAP column at a flow rate of 100 cm/hr. The column was washed with 5 mM potassium phosphate, pH 7, 100 mM NaCl, 0.1 mM $CaCl_2$. The column was next washed with 10 mM potassium phosphate, pH 7, 100 mM NaCl, 0.1 mM $CaCl_2$. The protein was eluted with 70 mM potassium phosphate, pH 7.0 and passed through a 0.22 μm sterile filter into a sterile bag. The eluted sample was tested for bioburden, protein concentration and enzyme activity.

The HAP purified protein was then passed through a virus removal filter. The sterilized Viosart filter (Sartorius) was first prepared by washing with 2 L of 70 mM potassium phosphate, pH 7.0. Before use, the filtered buffer was sampled for pH and conductivity. The HAP purified protein was pumped via a peristaltic pump through the 20 nM virus removal filter. The filtered protein in 70 mM potassium phosphate, pH 7.0 was passed through a 0.22 μm final filter into a sterile bag. The filtered sample was tested for protein concentration, enzyme activity, oligosaccharide, monosaccharide and sialic acid profiling. The sample also was tested for process related impurities.

The protein in the filtrate was then concentrated to 10 mg/mL using a 10 kDa molecular weight cut off (MWCO) Sartocon Slice tangential flow filtration (TFF) system (Sartorius). The filter was first prepared by washing with 10 mM histidine, 130 mM NaCl, pH 6.0 and the permeate was sampled for pH and conductivity. Following concentration, the concentrated protein was sampled and tested for protein concentration and enzyme activity. A 6× buffer exchange was performed on the concentrated protein into the final buffer: 10 mM histidine, 130 mM NaCl, pH 6.0. Following buffer exchange, the concentrated protein was passed though a 0.22 μm filter into a 20 L sterile storage bag. The protein was sampled and tested for protein concentration, enzyme activity, free sulfydryl groups, oligosaccharide profiling and osmolality. Lot number WRS2 was used as a standard in the assays described below, the results showed that the test description for appearance was clear and colorless; the pH was 7.4; the endotoxin level was <0.01 EU/mL; the osmolality was 308 mOsm/Kg; the density was 1.005 g/mL; the rHuPH20 content was 1.3 ppm; and the hyaluronidase activity was 145 USP U/mL.

The sterile filtered bulk protein was then asceptically dispensed at 20 mL into 30 mL sterile Teflon vials (Nalgene). The vials were then flash frozen and stored at −20±5° C.

Example 2

Generation of PH20 Mutant Library

A. Cloning and Mutagenesis

In this example, a human hyaluronidase PH20 library was created by cloning DNA encoding human PH20 into a plasmid followed by transfection and protein expression.

The library was created by mutagenesis of a PH20 template that is a codon optimized version of PH20 with an Ig Kappa leader sequence. Specifically, for generating the library of variants, the HZ24-PH20(OHO)-IRES-SEAP expression vector (set forth in SEQ ID NO:4) was used as a template, which contains the sequence of nucleotides encoding PH20 set forth in SEQ ID NO:1, which encodes a precursor PH20 set forth in SEQ ID NO:2 or a mature PH20 set forth in SEQ ID NO:3 lacking residues 1-22 corresponding to the IgK signal sequence. The backbone of the vector was derived from the original HZ24 vector containing the DHFR selection marker (see Example 1 and SEQ ID NO:5) with the addition of an IgK leader sequence and codon optimization. The expression vector also was modified to contain the gene for secreted alkaline phosphatase (SEAP). Hence, in addition to sequence encoding PH20, the HZ24-PH20(OHO)-IRES-SEAP expression vector also contains an internal ribosome entry site (EMCV IRES) that is linked to the coding sequence for the gene for secreted alkaline phosphatase (SEAP), and a single CMV promoter that drives expression of PH20 and SEAP in the construct. It also contains a gene for ampilcillin resistance. With reference to the sequence of nucleotides set forth in SEQ ID NO:4, the sequence of nucleotides encoding PH20 corresponds to nucleotides 1058-2464 (including the IgK leader sequence), the sequence of nucleotides encoding SEAP corresponds to nucleotides 2970-4529, and the ampicillin resistance gene corresponds to nucleotides 5778-6635.

The first library was made to generate encoded variant proteins wherein each of residues 23-469 of SEQ ID NO:2 (corresponding to residues 1-447 of SEQ ID NO:3 or residues 36-482 of SEQ ID NO:6) was changed to one of about 15 amino acid residues, such that each member contained a single amino change. The resulting library contained 6753 variant members, each containing a single amino acid mutation compared to residues 23-469 of SEQ ID NO:2 (corresponding to residues 1-447 of SEQ ID NO:3 or residues 36-482 of SEQ ID NO:6). Glycerol stocks of the resulting library were prepared and stored at −80° C. The amino acid replacements (mut) in each member are listed in Table 8 below, and correspond to amino acid replacements with reference to the sequence of amino acids of PH20 set forth in SEQ ID NO:3 (and SEQ ID NOS: 7 or 32-66, which are the mature sequence of PH20 or other C-terminally truncated fragments thereof). The corresponding mutated codons (cod) of each PH20 variant in the library are also listed in Table 8, and correspond to nucleotide residue changes in the corresponding encoding nucleotide for PH20 set forth as 1058-2464 of SEQ ID NO:4. Each member was expressed and screened for hyaluoridase activity as described below.

TABLE 8

PH20 Variants

mut	cod	mut	cod	mut	cod	mut	cod	mut	cod	mut	cod
L001A	GCG	Y066S	AGT	R132N	AAT	G198T	ACT	V265G	GGT	I331K	AAG
L001C	TGT	Y066T	ACG	R132P	CCT	G198V	GTT	V265H	CAT	I331L	CTG
L001D	GAT	Y066V	GTG	R132Q	CAG	G198W	TGG	V265I	ATT	I331Q	CAG
L001E	GAG	I067C	TGT	R132S	AGT	G198Y	TAT	V265K	AAG	I331R	CGT
L001F	TTT	I067D	GAT	R132T	ACT	Y199A	GCG	V265L	CTG	I331S	AGT
L001G	GGT	I067E	GAG	R132V	GTG	Y199C	TGT	V265M	ATG	I331T	ACT
L001H	CAT	I067F	TTT	R132Y	TAT	Y199E	GAG	V265N	AAT	I331W	TGG
L001K	AAG	I067G	GGG	S133A	GCT	Y199G	GGG	V265P	CCT	I331Y	TAT
L001N	AAT	I067H	CAT	S133D	GAT	Y199H	CAT	V265Q	CAG	I332A	GCT
L001P	CCG	I067L	TTG	S133E	GAG	Y199I	ATT	V265R	AGG	I332C	TGT
L001Q	CAG	I067N	AAT	S133F	TTT	Y199K	AAG	V265S	TCT	I332D	GAT
L001R	CGG	I067P	CCG	S133G	GGG	Y199L	CTT	V265W	TGG	I332E	GAG
L001S	TCT	I067Q	CAG	S133H	CAT	Y199N	AAT	V265Y	TAT	I332F	TTT
L001T	ACG	I067R	CGG	S133I	ATT	Y199P	CCT	F266A	GCG	I332G	GGT
L001V	GTG	I067T	ACG	S133L	CTG	Y199Q	CAG	F266C	TGT	I332H	CAT
L001W	TGG	I067V	GTT	S133M	ATG	Y199R	AGG	F266D	GAT	I332K	AAG
N002A	GCT	I067W	TGG	S133N	AAT	Y199S	TCG	F266G	GGG	I332L	CTG
N002C	TGT	I067Y	TAT	S133P	CCT	Y199T	ACG	F266H	CAT	I332N	AAT
N002F	TTT	D068A	GCT	S133R	CGG	Y199W	TGG	F266L	CTT	I332P	CCT
N002G	GGG	D068C	TGT	S133T	ACT	N200A	GCT	F266M	CCG	I332R	AGG
N002H	CAT	D068E	GAG	S133V	GTT	N200D	GAT	F266P	ATG	I332S	AGT
N002I	ATT	D068G	GGG	S133W	TGG	N200F	CAG	F266Q	CAG	I332T	ACT
N002K	AAG	D068H	CAC	I134A	GCT	N200G	GGT	F266R	CGG	I332Y	TAT
N002L	TTG	D068I	ATT	I134C	TGT	N200H	CAT	F266S	TCG	N333A	GCT
N002P	CCG	D068K	AAG	I134D	GAT	N200K	AAG	F266T	ACG	N333E	GAG
N002Q	CAG	D068L	TTG	I134F	TTT	N200L	CTG	F266V	GTG	N333G	GGT
N002S	AGT	D068P	CCT	I134G	GGG	N200M	ATG	F266W	TGG	N333H	CAT
N002T	ACG	D068Q	CAG	I134H	CAT	N200P	CCT	F266Y	TAT	N333I	ATT
N002V	GTT	D068R	CGG	I134K	AAG	N200Q	CAG	A267D	GAT	N333K	AAG
N002W	TGG	D068S	TCG	I134L	TTG	N200R	AGG	A267E	GAG	N333L	CTG
N002Y	TAT	D068T	ACT	I134P	CCT	N200S	TCT	A267G	GGT	N333M	ATG
F003A	GCT	D068V	GTG	I134Q	CAG	N200T	ACT	A267H	CAT	N333P	CCT
F003E	GAG	D068Y	TAT	I134R	CGT	N200V	GTG	A267I	ATT	N333R	CGG
F003G	GGG	S069A	GCT	I134S	TCG	N200W	TGG	A267K	AAG	N333S	AGT
F003H	CAT	S069C	TGT	I134T	ACT	N200Y	TAT	A267L	CTT	N333T	ACT
F003I	ATT	S069E	GAG	I134V	GTG	G201A	GCG	A267M	ATG	N333V	GTT
F003K	AAG	S069F	TTT	I134W	TGG	G201E	GAG	A267N	AAT	N333W	TGG
F003L	TTG	S069G	GGG	E135A	GCT	G201F	TTT	A267P	CCG	N333Y	TAT
F003M	ATG	S069I	ATT	E135C	TGT	G201H	CAT	A267R	AGG	V334A	GCT
F003N	AAT	S069L	CTT	E135D	GAT	G201K	AAG	A267S	TCT	V334C	TGT
F003P	CCT	S069M	ATG	E135F	TTT	G201L	CTT	A267T	GTG	V334D	GAT
F003R	CGT	S069N	AAT	E135G	GGG	G201M	ATG	A267V	ACT	V334E	GAG
F003S	TCG	S069P	CCT	E135H	CAT	G201N	AAT	A267W	TGG	V334G	GGG
F003T	ACT	S069R	CGT	E135K	AAG	G201P	CCT	Y268A	GCT	V334H	CAT
F003V	GTG	S069T	ACG	E135L	TTG	G201Q	CAG	Y268C	TGT	V334L	TTG
F003Y	TAT	S069V	GTT	E135N	AAT	G201R	CGT	Y268F	TTT	V334M	ATG
R004A	GCG	S069W	TGG	E135P	CCT	G201S	TCG	Y268G	GGG	V334N	AAT
R004D	GAT	S069Y	TAT	E135Q	CAG	G201T	ACG	Y268H	CAT	V334P	CCT
R004E	GAG	I070A	GCT	E135R	CGG	G201V	GTG	Y268K	AAG	V334Q	CAG
R004F	TTT	I070C	TGT	E135S	TCT	G201W	TGG	Y268L	CTT	V334R	AGG
R004G	GGG	I070F	TTT	E135W	TGG	S202A	GCG	Y268N	AAT	V334S	TCT
R004I	ATT	I070G	GGG	E135Y	TAT	S202E	GAG	Y268P	CCT	V334T	ACT
R004L	TTG	I070H	CAT	L136A	GCT	S202F	TTT	Y268Q	CAG	V334Y	TAT
R004M	ATG	I070K	AAG	L136C	TGT	S202G	GGT	Y268R	CGT	T335A	GCT
R004N	AAT	I070L	TTG	L136D	GAT	S202H	CAT	Y268S	TCG	T335C	TGT

TABLE 8-continued

PH20 Variants											
mut	cod	mut	cod	mut	cod	mut	cod	mut	cod	mut	cod
R004P	CCT	I070N	AAT	L136F	TTT	S202K	AAG	Y268T	ACT	T335F	TTT
R004S	TCT	I070P	CCG	L136G	GGT	S202M	ATG	Y268V	GTG	T335G	GGT
R004T	ACG	I070Q	CAG	L136H	CAT	S202N	AAT	Y268W	TGG	T335H	CAT
R004V	GTG	I070R	CGT	L136I	ATT	S202P	CCT	T269A	GCT	T335I	ATT
R004W	TGG	I070S	TCT	L136M	ATG	S202Q	CAG	T269C	TGT	T335K	AAG
R004Y	TAT	I070T	ACT	L136N	AAT	S202R	CGT	T269D	GAT	T335L	TTG
A005D	GAT	I070V	GTT	L136P	CCT	S202T	ACG	T269E	GAG	T335N	AAT
A005G	GGG	I070Y	TAT	L136Q	CAG	S202V	GTT	T269G	GGT	T335P	CCT
A005H	CAT	T071A	GCT	L136R	CGT	S202W	TGG	T269K	AAG	T335Q	CAG
A005I	ATT	T071C	TGT	L136S	TCG	S202Y	TAT	T269L	CTG	T335S	TCT
A005L	CTT	T071D	GAT	L136T	ACT	C203A	GCG	T269M	ATG	T335V	GTG
A005M	ATG	T071E	GAG	L136W	TGG	C203D	GAT	T269N	AAT	T335W	TGG
A005N	AAT	T071G	GGG	V137A	GCT	C203E	GAG	T269P	CCG	T335Y	TAT
A005P	CCG	T071H	CAT	V137C	TGT	C203G	GGG	T269Q	CAG	L336A	GCT
A005Q	CAG	T071L	TTG	V137E	GAG	C203H	CAT	T269R	AGG	L336E	GAG
A005R	AGG	T071M	ATG	V137F	TTT	C203L	CTT	T269S	TCG	L336F	TTT
A005S	TCG	T071N	AAT	V137G	GGG	C203M	ATG	T269V	GTG	L336G	GGG
A005T	ACG	T071P	CCT	V137H	CAT	C203N	AAT	T269Y	TAT	L336H	CAT
A005V	GTG	T071Q	CAG	V137I	ATT	C203P	CCG	R270A	GCT	L336K	AAG
A005W	TGG	T071R	CGG	V137L	TTG	C203Q	CAG	R270C	TGT	L336M	ATG
A005Y	TAT	T071S	TCG	V137N	AAT	C203R	AGG	R270D	GAT	L336N	AAT
P006A	GCG	T071V	GTG	V137P	CCT	C203S	AGT	R270E	GAG	L336P	CCT
P006D	GAT	T071Y	TAT	V137Q	CAG	C203T	ACT	R270F	TTT	L336R	AGG
P006E	GAG	G072A	GCT	V137R	CGT	C203V	GTG	R270G	GGG	L336S	TCT
P006F	TTT	G072C	TGT	V137S	TCT	C203W	TGG	R270H	CAT	L336T	ACT
P006G	GGG	G072D	GAT	V137T	ACT	F204A	GCG	R270I	ATT	L336V	GTG
P006H	CAT	G072E	GAG	V137W	TGG	F204C	TGT	R270M	ATG	L336W	TGG
P006K	AAG	G072F	TTT	V137Y	TAT	F204E	GAG	R270N	AAT	L336Y	TAT
P006L	CTT	G072H	CAT	Q138A	GCT	F204G	GGG	R270P	CCT	A337C	TGT
P006N	AAT	G072I	ATT	Q138C	TGT	F204H	CAT	R270Q	CAG	A337F	TTT
P006Q	CAG	G072K	AAG	Q138E	GAG	F204I	ATT	R270S	TCG	A337G	GGG
P006R	AGG	G072L	TTG	Q138F	TTT	F204K	AAG	R270T	ACT	A337H	CAT
P006S	AGT	G072M	ATG	Q138G	GGG	F204L	CTT	R270V	GTG	A337I	ATT
P006T	ACG	G072P	CCT	Q138H	CAT	F204M	ATG	R270Y	TAT	A337K	AAG
P006V	GTG	G072Q	CAG	Q138I	ATT	F204P	CCT	I271A	GCT	A337L	TTG
P006W	TGG	G072R	CGG	Q138L	TTG	F204Q	CAG	I271D	GAT	A337M	ATG
P006Y	TAT	G072S	TCT	Q138M	ATG	F204R	AGG	I271E	GAG	A337N	AAT
P007A	GCT	G072T	ACT	Q138N	AAT	F204S	AGT	I271F	TTT	A337P	CCT
P007C	TGT	G072V	GTG	Q138R	CGT	F204T	ACT	I271G	GGG	A337R	CGG
P007D	GAT	G072W	TGG	Q138S	AGT	F204V	GTG	I271H	CAT	A337S	TCT
P007F	TTT	G072Y	TAT	Q138V	GTT	F204W	TGG	I271K	AAG	A337T	ACT
P007G	GGT	V073A	GCG	Q138W	TGG	N205A	GCG	I271L	CTT	A337V	GTT
P007H	CAT	V073C	TGT	Q138Y	TAT	N205D	GAT	I271M	ATG	A337W	TGG
P007I	ATT	V073D	GAT	Q139A	GCT	N205E	GAG	I271P	CCT	A338C	TGT
P007K	AAG	V073F	TTT	Q139C	TGT	N205F	TTT	I271R	AGG	A338D	GAT
P007L	TTG	V073G	GGG	Q139D	GAT	N205G	GGG	I271S	AGT	A338E	GAG
P007M	ATG	V073H	CAT	Q139E	GAG	N205K	AAG	I271T	ACT	A338F	TTT
P007Q	CAG	V073K	AAG	Q139F	TTT	N205L	CTG	I271V	GTT	A338G	GGG
P007R	CGG	V073L	CTT	Q139G	GGG	N205M	ATG	I271W	TGG	A338H	CAT
P007S	AGT	V073M	ATG	Q139H	CAT	N205P	CCT	V272A	GCT	A338I	ATT
P007T	ACT	V073P	CCG	Q139K	AAG	N205R	AGG	V272C	TGT	A338K	AAG
P007V	GTG	V073Q	CAG	Q139L	CTG	N205S	TCG	V272D	GAT	A338L	CTT
P007W	TGG	V073R	TGG	Q139M	ATG	N205T	ACG	V272E	GAG	A338P	CCT
P007Y	TAT	V073S	TCG	Q139P	CCT	N205V	GTG	V272G	GGG	A338Q	CAG
V008A	GCT	V073T	ACG	Q139R	CGT	N205W	TGG	V272H	CAT	A338R	CGT
V008D	GAT	V073W	CGG	Q139S	TCT	N205Y	TAT	V272K	AAG	A338S	TCG
V008E	GAG	T074A	GCT	Q139T	ACT	V206C	TGT	V272L	TTG	A338T	ACT
V008G	GGT	T074C	TGT	Q139V	GTG	V206D	GAT	V272M	ATG	A338V	GTG
V008H	CAT	T074E	GAG	Q140A	GCT	V206F	TTT	V272N	AAT	K339D	GAT
V008I	ATT	T074F	TTT	Q140C	TGT	V206G	GGG	V272P	CCT	K339E	GAG
V008L	TTG	T074G	GGT	Q140D	GAT	V206H	CAT	V272R	AGG	K339F	TTT
V008M	ATG	T074H	CAT	Q140F	TTT	V206I	ATT	V272S	TCG	K339G	GGG
V008N	AAT	T074K	AAG	Q140G	GGG	V206K	AAG	V272T	ACT	K339H	CAT
V008P	CCT	T074L	TTG	Q140H	CAT	V206L	CTT	V272W	TGG	K339L	CTG
V008Q	CAG	T074M	ATG	Q140I	ATT	V206M	ATG	F273A	GCT	K339M	ATG
V008R	CGG	T074N	AAT	Q140K	AAG	V206P	CCG	F273C	TGT	K339N	AAT
V008S	TCT	T074P	CCG	Q140L	TTG	V206Q	CAG	F273D	GAT	K339P	CCT
V008T	ACT	T074R	CGG	Q140M	ATG	V206R	CGG	F273G	GGG	K339R	CGG
V008W	TGG	T074S	TCG	Q140R	CGG	V206S	TCT	F273H	CAT	K339S	AGT
I009A	GCT	T074V	GTG	Q140S	AGT	V206T	ACG	F273I	ATT	K339T	ACT
I009C	TGT	T074W	TGG	Q140V	GTG	V206Y	TAT	F273L	CTG	K339V	GTT
I009D	GAT	V075A	GCG	Q140W	TGG	E207A	GCT	F273P	CCT	K339W	TGG
I009E	GAG	V075C	TGT	Q140Y	TAT	E207F	TTT	F273Q	CAG	K339Y	TAT
I009G	GGG	V075D	GAT	N141A	GCT	E207G	GGG	F273R	CGG	M340A	GCT
I009H	CAT	V075F	TTT	N141D	GAT	E207H	CAT	F273S	TCG	M340C	TGT
I009K	AAG	V075G	GGG	N141E	GAG	E207I	ATT	F273T	ACG	M340D	GAT

TABLE 8-continued

PH20 Variants											
mut	cod	mut	cod	mut	cod	mut	cod	mut	cod	mut	cod
I009L	CTT	V075H	CAT	N141F	TTT	E207K	AAG	F273V	GTT	M340E	GAG
I009N	AAT	V075L	CTT	N141G	GGT	E207L	TTG	F273W	TGG	M340F	TTT
I009P	CCT	V075M	ATG	N141H	CAT	E207M	ATG	F273Y	TAT	M340G	GGG
I009Q	CAG	V075N	AAT	N141L	TTG	E207P	CCG	T274A	GCG	M340H	CAT
I009R	CGG	V075P	CCG	N141M	ATG	E207Q	CAG	T274C	TGT	M340K	AAG
I009S	AGT	V075Q	CAG	N141P	CCT	E207R	AGG	T274E	GAG	M340L	CTG
I009T	ACG	V075R	CGT	N141Q	CAG	E207S	TCT	T274F	ATG	M340P	CCT
I009V	GTT	V075S	TCT	N141R	CGT	E207T	ACG	T274G	GGG	M340R	CGG
P010D	GAT	V075T	ACT	N141S	TCT	E207V	GTT	T274H	CAT	M340S	TCG
P010E	GAG	V075W	TGG	N141T	ACT	E207W	TGG	T274L	CTG	M340T	ACT
P010F	TTT	V075Y	TAT	N141V	GTT	I208A	GCT	T274N	AAT	M340V	GTG
P010G	GGT	N076A	GCT	N141W	TGG	I208C	TGT	T274P	CCT	M340W	TGG
P010H	CAT	N076C	TGT	N141Y	TAT	I208D	GAT	T274Q	CAG	C341A	GCT
P010I	ATT	N076D	GAT	V142C	TGT	I208E	GAG	T274R	CGT	C341E	GAG
P010L	CTT	N076F	TTT	V142D	GAT	I208G	GGG	T274S	AGT	C341G	GGG
P010M	ATG	N076G	GGG	V142E	GAG	I208K	AAG	T274V	GTT	C341H	CAT
P010N	AAT	N076I	ATT	V142G	GGG	I208L	TTG	T274W	TGG	C341K	AAG
P010Q	CAG	N076K	AAG	V142H	CAT	I208M	ATG	T274Y	TAT	C341L	TTG
P010R	CGG	N076L	CTG	V142I	ATT	I208P	CCG	D275A	GCT	C341M	ATG
P010S	TCG	N076P	CCT	V142K	AAG	I208Q	CAG	D275C	TGT	C341N	AAT
P010T	ACT	N076Q	CAG	V142L	TTG	I208R	CGT	D275E	GAG	C341Q	CAG
P010W	TGG	N076R	CGT	V142M	ATG	I208S	AGT	D275F	TTT	C341R	AGG
P010Y	TAT	N076S	AGT	V142N	AAT	I208T	ACG	D275G	GGG	C341S	TCT
N011A	GCG	N076T	ACT	V142P	CCT	I208V	GTG	D275I	ATT	C341T	ACT
N011C	TGT	N076V	GTT	V142Q	CAG	I208W	TGG	D275K	AAG	C341V	GTT
N011D	GAT	N076W	TGG	V142R	CGG	K209A	GCG	D275L	CTT	C341W	TGG
N011E	GAG	G077D	GAT	V142S	AGT	K209C	TGT	D275M	ATG	C341Y	TAT
N011F	TTT	G077E	GAG	V142T	ACT	K209D	GAT	D275Q	CAG	S342A	GCT
N011G	GGG	G077F	TTT	Q143C	TGT	K209E	GAG	D275R	CGT	S342D	GAT
N011H	CAT	G077H	CAT	Q143E	GAG	K209F	TTT	D275S	TCG	S342E	GAG
N011I	ATT	G077K	AAG	Q143F	TTT	K209G	GGT	D275T	ACT	S342F	TTT
N011K	AAG	G077L	TTG	Q143G	GGG	K209L	CTG	D275V	GTG	S342G	GGG
N011L	CTG	G077M	ATG	Q143H	CAT	K209N	AAT	D275W	TGG	S342H	CAT
N011P	CCG	G077N	AAT	Q143I	ATT	K209P	CCG	Q276C	TGT	S342I	ATT
N011S	TCG	G077P	CCG	Q143K	AAG	K209R	CGG	Q276D	GAT	S342K	AAG
N011T	ACG	G077Q	CAG	Q143L	TTG	K209S	AGT	Q276E	GAG	S342L	TTG
N011W	TGG	G077R	CGT	Q143M	ATG	K209T	ACT	Q276F	TTT	S342M	ATG
N011Y	TAT	G077S	TCG	Q143N	AAT	K209V	GTT	Q276G	GGG	S342P	CCT
V012A	GCT	G077T	ACG	Q143P	CCT	K209W	TGG	Q276H	CAT	S342Q	CAG
V012D	GAT	G077V	GTG	Q143R	CGG	K209Y	TAT	Q276I	ATT	S342R	CGG
V012E	GAG	G077Y	TAT	Q143S	TCG	R210A	GCG	Q276L	CTT	S342T	ACT
V012G	GGG	G078A	GCG	Q143T	ACT	R210C	TGT	Q276M	ATG	S342Y	TAT
V012H	CAT	G078C	TGT	Q143V	GTG	R210D	GAT	Q276P	CCT	Q343C	TGT
V012I	ATT	G078D	GAT	Q143Y	TAT	R210E	GAG	Q276R	CGT	Q343D	GAT
V012K	AAG	G078H	CAT	L144A	GCT	R210G	GGT	Q276S	AGT	Q343E	GAG
V012L	CTT	G078I	ATT	L144E	GAG	R210K	AAG	Q276V	GTT	Q343F	TTT
V012M	ATG	G078K	AAG	L144F	TTT	R210L	CTG	Q276W	TGG	Q343G	GGG
V012N	AAT	G078L	TTG	L144G	GGG	R210M	ATG	Q276Y	TAT	Q343I	ATT
V012P	CCG	G078M	ATG	L144I	ATT	R210N	AAT	V277A	GCT	Q343L	CTT
V012R	AGG	G078P	CCG	L144K	AAG	R210P	CCT	V277C	TGT	Q343M	ATG
V012S	TCG	G078Q	CAG	L144N	AAT	R210S	TCG	V277D	GAT	Q343P	CCT
V012T	ACT	G078R	AGG	L144P	CCT	R210T	ACT	V277E	GAG	Q343R	AGG
V012W	TGG	G078S	TCG	L144Q	CAG	R210V	GTG	V277G	GGG	Q343S	AGT
P013A	GCT	G078T	ACT	L144R	CGT	R210W	TGG	V277H	CAT	Q343T	ACT
P013E	GAG	G078V	GTG	L144S	TCT	R210Y	TAT	V277K	AAG	Q343V	GTG
P013F	TTT	G078Y	TAT	L144T	ACT	N211A	GCG	V277L	TTG	Q343W	TGG
P013G	GGG	I079A	GCT	L144V	GTT	N211C	TGT	V277M	ATG	Q343Y	TAT
P013H	CAT	I079D	GAT	L144W	TGG	N211F	TTT	V277N	AAT	V344E	GAG
P013I	ATT	I079F	TTT	L144Y	TAT	N211G	GGG	V277Q	CAG	V344F	TTT
P013L	CTT	I079G	GGG	S145A	GCT	N211H	CAT	V277R	AGG	V344G	GGG
P013M	ATG	I079H	CAT	S145C	TGT	N211I	ATT	V277S	TCT	V344H	CAT
P013Q	CAG	I079K	AAG	S145D	GAT	N211K	AAG	V277T	ACT	V344I	ATT
P013R	CGT	I079L	TTG	S145E	GAG	N211L	CTG	V277Y	TAT	V344L	CTG
P013S	TCG	I079N	AAT	S145F	TTT	N211M	ATG	L278A	GCT	V344M	ATG
P013T	ACT	I079P	CCG	S145G	GGG	N211P	CCT	L278E	GAG	V344N	AAT
P013V	GTG	I079R	CGT	S145H	CAT	N211R	CGG	L278F	TTT	V344P	CCT
P013W	TGG	I079S	AGT	S145L	TTG	N211S	AGT	L278G	GGG	V344Q	CAG
P013Y	TAT	I079T	ACT	S145M	ATG	N211T	ACT	L278H	CAT	V344R	CGT
F014A	GCG	I079V	GTT	S145N	AAT	N211V	GTT	L278I	ATT	V344S	TCG
F014D	GAT	I079W	TGG	S145P	CCT	N211W	TGG	L278K	AAG	V344T	ACT
F014E	GAG	I079Y	TAT	S145R	CGT	D212A	GCT	L278M	TTT	V344W	TGG
F014G	GGT	P080A	GCG	S145T	ACT	D212E	GAG	L278N	AAT	V344Y	TAT
F014H	CAT	P080D	GAT	S145V	GTT	D212G	GGG	L278P	CCG	L345A	GCT
F014I	ATT	P080E	GAG	S145W	TGG	D212H	CAT	L278R	CGT	L345C	TGT
F014K	AAG	P080F	TTT	L146A	GCT	D212I	ATT	L278S	TCT	L345D	GAT
F014M	ATG	P080G	GGG	L146C	TGT	D212K	AAG	L278T	ACT	L345E	GAG

TABLE 8-continued

PH20 Variants											
mut	cod	mut	cod	mut	cod	mut	cod	mut	cod	mut	cod
F014N	AAT	P080I	ATT	L146E	GAG	D212L	CTG	L278V	GTT	L345G	GGG
F014P	CCT	P080K	AAG	L146G	GGG	D212M	ATG	L278Y	TAT	L345H	CAT
F014Q	CAG	P080L	CTT	L146H	CAT	D212N	AAT	K279A	GCG	L345K	AAG
F014R	CGG	P080M	ATG	L146I	ATT	D212P	CCT	K279C	TGT	L345N	AAT
F014T	ACT	P080N	AAT	L146K	AAG	D212Q	CAG	K279D	GAT	L345P	CCT
F014V	GTG	P080R	AGG	L146N	AAT	D212S	TCG	K279F	TTT	L345Q	CAG
F014W	TGG	P080S	TCT	L146P	CCT	D212T	ACT	K279G	GGG	L345R	CGT
L015A	GCG	P080T	ACG	L146Q	CAG	D212V	GTG	K279H	CAT	L345T	ACT
L015E	GAG	P080V	GTG	L146R	CGG	D212W	TGG	K279L	CTG	L345V	GTT
L015F	TTT	P080Y	TAT	L146S	TCG	D213A	GCT	K279P	CCT	L345W	TGG
L015G	GGG	Q081A	GCT	L146T	ACT	D213E	GAG	K279Q	CAG	L345Y	TAT
L015K	AAG	Q081C	TGT	L146V	GTT	D213G	GGG	K279R	AGG	C346A	GCT
L015M	ATG	Q081E	GAG	L146Y	TAT	D213H	CAT	K279S	TCT	C346D	GAT
L015N	AAT	Q081F	TTT	T147A	GCT	D213K	AAG	K279T	ACG	C346F	TTT
L015P	CCG	Q081G	GGG	T147C	TGT	D213L	CTG	K279V	GTG	C346G	GGG
L015Q	CAG	Q081H	CAT	T147D	GAT	D213M	ATG	K279W	TGG	C346I	ATT
L015R	CGG	Q081L	CTG	T147F	TTT	D213N	AAT	K279Y	TAT	C346K	AAG
L015S	TCG	Q081M	ATG	T147G	GGT	D213P	CCT	F280D	GAT	C346L	CTT
L015T	ACT	Q081N	AAT	T147I	ATT	D213Q	CAG	F280E	GAG	C346M	ATG
L015V	GTT	Q081P	CCG	T147L	CTT	D213R	CGT	F280G	GGG	C346P	CCT
L015W	TGG	Q081R	AGG	T147M	ATG	D213S	TCG	F280H	CAT	C346Q	CAG
L015Y	TAT	Q081S	TCT	T147P	CCT	D213V	GTG	F280I	ATT	C346R	CGG
W016A	GCG	Q081V	GTT	T147Q	CAG	D213W	TGG	F280L	TTG	C346S	TCT
W016C	TGT	Q081W	TGG	T147R	CGT	D213Y	TAT	F280M	ATG	C346T	ACT
W016D	GAT	Q081Y	TAT	T147S	AGT	L214A	GCG	F280N	AAT	C346V	GTG
W016E	GAG	K082A	GCT	T147V	GTT	L214C	TGT	F280P	CCT	C346W	TGG
W016F	TTT	K082E	GAG	T147W	TGG	L214D	GAT	F280Q	CAG	Q347A	GCT
W016G	GGT	K082G	GGT	T147Y	TAT	L214E	GAG	F280R	CGT	Q347C	TGT
W016H	CAT	K082H	CAT	E148C	TGT	L214G	GGG	F280S	TCG	Q347E	GAG
W016K	AAG	K082I	ATT	E148F	TTT	L214H	CAT	F280T	ACT	Q347F	TTT
W016L	CTT	K082L	CTT	E148G	GGG	L214K	AAG	F280V	GTG	Q347G	GGT
W016M	ATG	K082M	ATG	E148H	CAT	L214N	AAT	F280W	TGG	Q347I	ATT
W016P	CCT	K082N	AAT	E148I	ATT	L214P	CCG	L281A	GCG	Q347L	TTG
W016R	CGT	K082P	CCT	E148K	AAG	L214Q	CAG	L281D	GAT	Q347M	ATG
W016S	TCG	K082Q	CAG	E148L	CTG	L214R	CGG	L281F	TTT	Q347P	CCT
W016T	ACT	K082R	CGT	E148P	CCT	L214S	TCG	L281G	GGT	Q347R	AGG
W016Y	TAT	K082S	AGT	E148Q	CAG	L214T	ACG	L281H	CAT	Q347S	TCT
A017D	GAT	K082T	ACT	E148R	CGG	L214V	GTG	L281I	ATT	Q347T	ACT
A017E	GAG	K082V	GTG	E148S	TCT	L214Y	TAT	L281K	AAG	Q347V	GTG
A017G	GGG	K082W	TGG	E148T	ACT	S215A	GCT	L281N	AAT	Q347W	TGG
A017H	CAT	K082Y	TAT	E148V	GTG	S215C	TGT	L281P	CCG	Q347Y	TAT
A017I	ATT	I083E	GAG	E148W	TGG	S215D	GAT	L281Q	CAG	E348C	TGT
A017L	CTT	I083F	TTT	E148Y	TAT	S215E	GAG	L281R	CGG	E348D	GAT
A017N	AAT	I083G	GGT	A149C	TGT	S215G	GGG	L281S	AGT	E348G	GGT
A017P	CCG	I083H	CAT	A149E	GAG	S215H	CAT	L281V	GTT	E348H	CAT
A017Q	CAG	I083K	AAG	A149F	TTT	S215K	AAG	L281W	TGG	E348I	ATT
A017R	AGG	I083L	CTG	A149G	GGT	S215L	TTG	L281Y	TAT	E348L	TTG
A017S	TCG	I083N	AAT	A149K	AAG	S215M	ATG	S282A	GCG	E348M	ATG
A017T	ACG	I083P	CCT	A149L	TTG	S215P	CCG	S282C	TGT	E348P	CCT
A017V	GTG	I083Q	CAA	A149M	ATG	S215Q	CAG	S282D	GAT	E348Q	CAG
A017W	TGG	I083R	CGT	A149P	CCT	S215R	CGG	S282E	GAG	E348R	CGG
A017Y	TAT	I083S	TCG	A149Q	CAG	S215T	ACT	S282F	TTT	E348S	TCT
W018C	TGT	I083T	ACT	A149R	CGG	S215V	GTG	S282G	GGT	E348T	ACT
W018D	GAT	I083V	GTT	A149S	TCT	S215W	TGG	S282L	CTT	E348V	GTT
W018F	TTT	I083Y	TAT	A149T	ACT	W216D	GAT	S282M	ATG	E348W	TGG
W018G	GGG	S084D	GAT	A149V	GTT	W216E	GAG	S282P	CCT	E348Y	TAT
W018H	CAT	S084E	GAG	A149W	TGG	W216G	GGT	S282Q	CAG	Q349A	GCT
W018I	ATT	S084F	TTT	A149Y	TAT	W216H	CAT	S282R	CGT	Q349D	GAT
W018L	CTG	S084G	GGT	T150A	GCT	W216I	ATT	S282T	ACT	Q349E	GAG
W018M	ATG	S084H	CAT	T150C	TGT	W216K	AAG	S282V	GTT	Q349F	TTT
W018P	CCG	S084I	ATT	T150D	GAT	W216L	CTG	S282W	TGG	Q349G	GGT
W018Q	CAG	S084L	CTT	T150E	GAG	W216M	ATG	S282Y	TAT	Q349H	CAT
W018R	CGG	S084M	ATG	T150F	TTT	W216N	AAT	Q283A	GCG	Q349K	AAG
W018S	AGT	S084N	AAT	T150G	GGG	W216P	CCT	Q283C	TGT	Q349L	CTG
W018T	ACG	S084P	CCT	T150I	ATT	W216Q	CAG	Q283D	GAT	Q349M	ATG
W018V	GTG	S084Q	CAG	T150L	TTG	W216R	CGG	Q283E	GAG	Q349N	AAT
W018Y	TAT	S084R	CGG	T150N	AAT	W216T	ACG	Q283F	TTT	Q349P	CCT
N019A	GCG	S084T	ACT	T150P	CCT	W216V	GTG	Q283G	GGG	Q349R	CGT
N019C	TGT	S084W	TGG	T150R	AGG	W216Y	TAT	Q283H	CAT	Q349S	TCG
N019F	TTT	S084Y	TAT	T150S	TCT	L217A	GCG	Q283L	CTT	Q349T	ACT
N019G	GGG	L085A	GCT	T150V	GTG	L217C	TGT	Q283N	AAT	Q349V	GTG
N019H	CAT	L085C	TGT	T150W	TGG	L217E	GAG	Q283P	CCG	Q349W	TGG
N019I	ATT	L085D	GAT	T150Y	TAT	L217G	GGT	Q283R	CGT	Q349Y	TAT
N019L	CTG	L085E	GAG	E151A	GCT	L217H	CAT	Q283S	TCT	G350A	GCT
N019M	ATG	L085F	TTT	E151C	TGT	L217I	ATT	Q283T	ACT	G350D	GAT
N019P	CCG	L085G	GGG	E151G	GGT	L217M	ATG	Q283W	TGG	G350E	GAG

TABLE 8-continued

PH20 Variants											
mut	cod	mut	cod	mut	cod	mut	cod	mut	cod	mut	cod
N019Q	CAG	L085H	CAT	E151H	CAT	L217P	CCG	Q283Y	TAT	G350F	TTT
N019R	CGT	L085K	AAG	E151K	AAG	L217Q	CAG	D284A	GCT	G350H	CAT
N019S	TCG	L085N	AAT	E151L	TTG	L217R	AGG	D284C	TGT	G350K	AAG
N019V	GTT	L085P	CCT	E151M	ATG	L217S	TCT	D284E	GAG	G350L	CTG
N019W	TGG	L085Q	CAG	E151N	AAT	L217T	ACG	D284G	GGT	G350M	ATG
N019Y	TAT	L085R	CGT	E151Q	CAG	L217V	GTG	D284H	CAT	G350N	AAT
A020D	GAT	L085S	TCG	E151R	AGG	L217W	TGG	D284I	ATT	G350P	CCT
A020E	GAG	L085T	ACT	E151S	TCG	L217Y	TAT	D284L	TTG	G350R	CGT
A020F	TTT	L085V	GTT	E151T	ACT	W218A	GCT	D284M	ATG	G350S	TCT
A020G	GGG	Q086A	GCT	E151V	GTT	W218D	GAT	D284N	AAT	G350T	ACT
A020H	CAT	Q086C	TGT	E151W	TGG	W218F	TTT	D284P	CCG	G350V	GTG
A020K	AAG	Q086D	GAT	E151Y	TAT	W218G	GGT	D284Q	CAG	G350Y	TAT
A020L	CTG	Q086E	GAG	K152A	GCT	W218H	CAT	D284S	TCT	V351A	GCT
A020N	AAT	Q086F	TTT	K152C	TGT	W218I	ATT	D284T	ACG	V351C	TGT
A020P	CCG	Q086G	GGT	K152F	TTT	W218K	AAG	D284V	GTT	V351D	GAT
A020Q	CAG	Q086H	CAT	K152G	GGT	W218L	CTT	D284Y	TAT	V351E	GAG
A020R	CGT	Q086I	ATT	K152I	ATT	W218M	ATG	E285A	GCG	V351F	TTT
A020S	TCT	Q086K	AAG	K152L	TTG	W218P	CCT	E285F	TTT	V351G	GGT
A020T	ACT	Q086L	CTG	K152M	ATG	W218Q	CAG	E285G	GGG	V351H	CAT
A020V	GTT	Q086M	ATG	K152N	AAT	W218R	CGG	E285H	CAT	V351I	ATT
A020Y	TAT	Q086N	AAT	K152P	CCT	W218S	TCG	E285K	AAG	V351L	TTG
P021A	GCG	Q086P	CCT	K152R	AGG	W218T	ACT	E285M	ATG	V351N	AAT
P021C	TGT	Q086R	CGG	K152S	TCT	W218V	GTG	E285N	AAT	V351Q	CAG
P021D	GAT	Q086S	TCT	K152T	ACT	N219A	GCG	E285P	CCT	V351R	AGG
P021E	GAG	Q086T	ACT	K152V	GTG	N219C	TGT	E285Q	CAG	V351S	TCT
P021G	GGG	Q086V	GTG	K152W	TGG	N219D	GAT	E285R	CGT	V351W	TGG
P021H	CAT	Q086W	TGG	K152Y	TAT	N219E	GAG	E285S	AGT	V351Y	TAT
P021I	ATT	D087A	GCT	A153C	TGT	N219G	GGG	E285T	ACG	C352A	GCT
P021K	AAG	D087C	TGT	A153E	GAG	N219H	CAT	E285V	GTG	C352D	GAT
P021L	CTT	D087E	GAG	A153F	TTT	N219I	ATT	E285W	TGG	C352E	GAG
P021M	ATG	D087G	GGG	A153G	GGT	N219K	AAG	E285Y	TAT	C352F	TTT
P021R	CGT	D087H	CAT	A153H	CAT	N219L	CTT	L286A	GCG	C352G	GGG
P021S	TCT	D087I	ATT	A153I	ATT	N219M	ATG	L286C	TGT	C352K	AAG
P021T	ACG	D087L	CTG	A153K	AAG	N219P	CCT	L286D	GAT	C352M	ATG
P021V	GTT	D087M	ATG	A153L	CTG	N219R	CGT	L286E	GAG	C352P	CCT
P021W	TGG	D087P	CCT	A153M	ATG	N219S	TCG	L286F	TTT	C352Q	CAG
S022A	GCT	D087Q	CAG	A153P	CCT	N219T	ACT	L286G	GGT	C352R	CGT
S022C	TGT	D087R	AGG	A153Q	CAG	N219W	TGG	L286H	CAT	C352S	AGT
S022D	GAT	D087S	TCG	A153R	CGT	E220A	GCG	L286K	AAG	C352T	ACT
S022E	GAG	D087T	ACT	A153S	AGT	E220D	GAT	L286M	ATG	C352V	GTG
S022G	GGG	D087V	GTT	A153T	ACT	E220G	GGG	L286P	CCT	C352W	TGG
S022H	CAT	D087Y	TAT	A153V	GTG	E220H	CAT	L286R	AGG	C352Y	TAT
S022K	AAG	H088A	GCT	A153W	TGG	E220I	ATT	L286S	AGT	I353A	GCT
S022L	CTG	H088C	TGT	K154A	GCT	E220K	AAG	L286T	ACG	I353C	TGT
S022M	ATG	H088E	GAG	K154C	TGT	E220L	TTG	L286W	TGG	I353E	GAG
S022N	AAT	H088F	TTT	K154D	GAT	E220M	ATG	L286Y	TAT	I353F	TTT
S022P	CCG	H088G	GGG	K154E	GAG	E220N	AAT	V287A	GCT	I353G	GGG
S022R	CGG	H088I	ATT	K154G	GGT	E220P	CCG	V287C	TGT	I353H	CAT
S022T	ACT	H088K	AAG	K154H	CAT	E220R	CGG	V287D	GAT	I353K	AAG
S022V	GTG	H088L	TTG	K154I	ATT	E220S	TCT	V287E	GAG	I353L	CTT
S022Y	TAT	H088M	ATG	K154L	CTG	E220T	ACG	V287F	TTT	I353M	ATG
E023A	GCT	H088P	CCT	K154P	CCT	E220V	GTG	V287G	GGG	I353Q	CAG
E023D	GAT	H088R	CGT	K154R	CGG	E220W	TGG	V287I	ATT	I353R	CGT
E023F	TTT	H088S	AGT	K154S	AGT	S221A	GCG	V287K	AAG	I353S	TCG
E023G	GGG	H088T	ACT	K154T	ACT	S221C	TGT	V287L	CTT	I353T	ACT
E023H	CAT	H088V	GTT	K154V	GTG	S221D	GAT	V287N	AAT	I353V	GTG
E023L	CTT	H088Y	TAT	K154W	TGG	S221E	GAG	V287P	CCT	I353W	TGG
E023M	ATG	L089A	GCT	K154Y	TAT	S221G	GGG	V287Q	CAG	R354C	TGT
E023N	AAT	L089C	TGT	Q155A	GCT	S221H	CAT	V287R	CGG	R354D	GAT
E023P	CCT	L089D	GAT	Q155C	TGT	S221I	ATT	V287S	TCT	R354E	GAG
E023Q	CAG	L089E	GAG	Q155D	GAT	S221K	AAG	V287T	ACT	R354G	GGT
E023R	CGG	L089G	GGG	Q155F	TTT	S221L	TTG	Y288D	GAC	R354H	CAT
E023S	TCT	L089K	AAG	Q155G	GGG	S221M	ATG	Y288E	GAG	R354I	ATT
E023T	ACG	L089M	ATG	Q155H	CAT	S221P	CCG	Y288F	TTT	R354K	AAG
E023V	GTG	L089N	AAT	Q155K	AAG	S221Q	CAG	Y288G	GGG	R354L	CTT
E023W	TGG	L089P	CCT	Q155L	CTT	S221R	CGG	Y288H	CAT	R354M	ATG
F024A	GCG	L089Q	CAG	Q155M	ATG	S221T	ACT	Y288I	ATT	R354P	CCT
F024C	TGT	L089R	AGG	Q155P	CCT	S221V	GTG	Y288K	AAG	R354Q	CAG
F024E	GAG	L089S	TCG	Q155R	CGG	T222A	GCG	Y288L	CTG	R354S	TCT
F024G	GGG	L089T	ACT	Q155S	AGT	T222D	GAT	Y288P	CCT	R354V	GTG
F024H	CAT	L089W	TGG	Q155T	ACT	T222E	GAG	Y288Q	CAG	R354W	TGG
F024I	ATT	L089Y	TAT	Q155V	GTT	T222F	TTT	Y288R	CGT	R354Y	TAT
F024K	AAG	D090A	GCT	Q155W	TGG	T222G	GGG	Y288S	TCT	K355D	GAT
F024L	TTG	D090C	TGT	Q155Y	TAT	T222I	ATT	Y288T	ACT	K355F	TTT
F024M	ATG	D090E	GAG	E156A	GCT	T222K	AAA	Y288V	GTG	K355G	GGG
F024N	AAT	D090G	GGG	E156C	TGT	T222L	TTG	Y288W	TGG	K355H	CAT

TABLE 8-continued

PH20 Variants											
mut	cod	mut	cod	mut	cod	mut	cod	mut	cod	mut	cod
F024P	CCT	D090H	CAT	E156D	GAT	T222N	AAT	T289A	GCT	K355L	CTG
F024R	CGT	D090I	ATT	E156G	GGT	T222P	CCG	T289C	TGT	K355M	ATG
F024T	ACG	D090K	AAG	E156I	ATT	T222R	CGG	T289E	GAG	K355N	AAT
F024V	GTT	D090L	CTT	E156K	AAG	T222S	AGT	T289G	GGT	K355P	CCT
F024Y	TAT	D090N	AAT	E156L	CTG	T222V	GTT	T289H	CAT	K355Q	CAG
C025D	GAT	D090P	CCT	E156M	ATG	T222W	TGG	T289K	AAG	K355R	CGT
C025E	GAG	D090Q	CAG	E156P	CCT	T222Y	TAT	T289L	CTT	K355S	TCT
C025F	TTT	D090R	AGG	E156Q	CAG	A223C	TGT	T289M	ATG	K355T	ACT
C025G	GGG	D090S	AGT	E156R	CGG	A223D	GAT	T289N	AAT	K355V	GTG
C025H	CAT	D090T	ACT	E156S	TCT	A223E	GAG	T289P	CCT	K355W	TGG
C025I	ATT	D090W	TGG	E156T	ACT	A223G	GGG	T289Q	CAG	K355Y	TAT
C025K	AAG	K091A	GCT	E156V	GTT	A223H	CAT	T289R	AGG	N356A	GCT
C025L	TTG	K091D	GAT	E156W	TGG	A223K	AAG	T289S	TCG	N356C	TGT
C025N	AAT	K091E	GAG	F157A	GCT	A223L	CTG	T289V	GTG	N356D	GAT
C025P	CCT	K091F	TTT	F157C	TGT	A223P	CCT	T289Y	TAT	N356F	TTT
C025R	CGT	K091G	GGG	F157D	GAT	A223Q	CAG	F290A	GCT	N356G	GGG
C025S	TCT	K091H	CAT	F157E	GAG	A223R	AGG	F290C	TGT	N356H	CAT
C025T	ACT	K091I	ATT	F157G	GGT	A223S	TCT	F290D	GAT	N356K	AAG
C025V	GTG	K091L	TTG	F157H	CAT	A223T	ACG	F290G	GGG	N356L	CTG
C025Y	TAT	K091N	AAT	F157I	ATT	A223V	GTG	F290H	CAT	N356P	CCT
L026A	GCT	K091Q	CAG	F157K	AAG	A223W	TGG	F290I	ATT	N356Q	CAG
L026E	GAG	K091R	CGT	F157L	TTG	A223Y	TAT	F290K	AAG	N356R	CGG
L026G	GGT	K091S	TCT	F157M	ATG	L224A	GCT	F290L	TTG	N356S	AGT
L026H	CAT	K091T	ACT	F157P	CCT	L224D	GAT	F290M	ATG	N356T	ACT
L026I	ATT	K091Y	TAT	F157Q	CAG	L224E	GAG	F290Q	CAG	N356V	GTG
L026K	AAG	A092C	TGT	F157R	CGG	L224F	TTT	F290R	AGG	N356W	TGG
L026M	ATG	A092E	GAG	F157S	TCG	L224G	GGG	F290S	TCG	W357A	GCT
L026P	CCG	A092F	TTT	F157T	ACT	L224I	ATT	F290T	ACT	W357C	TGT
L026Q	CAG	A092G	GGT	F157V	GTG	L224M	ATG	F290V	GTT	W357D	GAT
L026R	CGG	A092H	CAT	F157W	TGG	L224P	CCG	F290Y	TAT	W357E	GAG
L026S	TCT	A092K	AAG	E158A	GCT	L224Q	CAG	G291A	GCT	W357F	TTT
L026T	ACT	A092L	CTG	E158C	TGT	L224R	AGG	G291C	TGT	W357G	GGG
L026V	GTT	A092M	ATG	E158D	GAT	L224S	AGT	G291D	GAT	W357K	AAG
L026W	TGG	A092P	CCT	E158F	TTT	L224T	ACT	G291E	GAG	W357L	TTG
L026Y	TAT	A092Q	CAG	E158G	GGG	L224V	GTT	G291F	TTT	W357M	ATG
G027A	GCT	A092R	CGT	E158H	CAT	L224W	TGG	G291H	CAT	W357P	CCT
G027C	TGT	A092T	ACT	E158K	AAG	L224Y	TAT	G291L	CTG	W357Q	CAG
G027D	GAT	A092V	GTT	E158L	CTG	Y225A	GCG	G291M	ATG	W357R	CGT
G027E	GAG	A092W	TGG	E158N	AAT	Y225D	GAT	G291N	AAT	W357S	AGT
G027F	TTT	A092Y	TAT	E158P	CCT	Y225E	GAG	G291P	CCT	W357T	ACT
G027H	CAT	K093D	GAT	E158Q	CAG	Y225G	GGT	G291Q	CAG	W357V	GTG
G027I	ATT	K093E	GAG	E158R	CGG	Y225H	CAT	G291R	CGG	N358C	TGT
G027K	AAG	K093F	TTT	E158S	TCG	Y225K	AAG	G291S	TCT	N358D	GAT
G027L	CTG	K093G	GGT	E158V	GTG	Y225L	CTG	G291T	ACT	N358E	GAG
G027P	CCT	K093H	CAT	E158Y	TAT	Y225P	CCG	G291V	GTG	N358G	GGG
G027Q	CAG	K093I	ATT	K159A	GCT	Y225Q	CAG	G291W	TGG	N358H	CAT
G027R	CGG	K093L	CTG	K159D	GAT	Y225R	AGG	G291Y	TAT	N358I	ATT
G027S	TCG	K093M	ATG	K159E	GAG	Y225S	TCT	E292A	GCT	N358K	AAG
G027T	ACT	K093N	AAT	K159F	TTT	Y225T	ACG	E292C	TGT	N358L	CTG
G027W	TGG	K093P	CCT	K159G	GGT	Y225V	GTG	E292F	TTT	N358P	CCT
K028A	GCG	K093Q	CAG	K159H	CAT	Y225W	TGG	E292G	GGT	N358Q	CAG
K028D	GAT	K093R	CGG	K159L	CTT	P226A	GCG	E292H	CAT	N358R	CGT
K028E	GAG	K093S	AGT	K159M	ATG	P226C	TGT	E292I	ATT	N358S	TCT
K028F	TTT	K093T	ACT	K159N	AAT	P226D	GAT	E292K	AAG	N358T	ACT
K028G	GGG	K093V	GTT	K159Q	CAG	P226E	GAG	E292L	TTG	N358V	GTG
K028I	ATT	K094A	GCT	K159R	CGG	P226F	TTT	E292N	AAT	N358W	TGG
K028L	TTG	K094C	TGT	K159S	TCT	P226G	GGT	E292P	CCT	S359A	GCT
K028M	ATG	K094D	GAT	K159V	GTG	P226L	CTT	E292Q	CAG	S359C	TGT
K028N	AAT	K094E	GAG	K159W	TGG	P226N	AAT	E292R	CGG	S359D	GAT
K028P	CCT	K094F	TTT	K159Y	TAT	P226Q	CAG	E292T	ACT	S359E	GAG
K028R	CGG	K094G	GGG	A160C	TGT	P226R	AGG	E292V	GTT	S359F	TTT
K028S	AGT	K094H	CAT	A160F	TTT	P226S	TCT	E292W	TGG	S359G	GGG
K028T	ACT	K094L	TTG	A160G	GGG	P226T	ACG	T293A	GCT	S359H	CAT
K028V	GTT	K094M	ATG	A160H	CAT	P226V	GTT	T293C	TGT	S359K	AAG
K028W	TGG	K094N	AAT	A160I	ATT	P226W	TGG	T293D	GAT	S359L	TTG
F029A	GCT	K094P	CCT	A160K	AAG	P226Y	TAT	T293E	GAG	S359M	ATG
F029C	TGT	K094Q	CAG	A160L	CTG	S227A	GCT	T293F	TTT	S359P	CCT
F029E	GAG	K094R	AGG	A160M	ATG	S227F	TTT	T293G	GGT	S359R	CGG
F029G	GGG	K094S	TCT	A160N	AAT	S227G	GGG	T293K	AAG	S359T	ACT
F029H	CAT	K094T	ACT	A160Q	CAG	S227H	CAT	T293L	CTT	S359V	GTT
F029I	ATT	D095A	GCT	A160R	AGG	S227I	ATT	T293M	ATG	S359W	TGG
F029K	AAG	D095C	TGT	A160S	AGT	S227K	AAG	T293N	AAT	S360A	GCT
F029L	CTT	D095E	GAG	A160V	GTG	S227L	TTG	T293P	CCT	S360C	TGT
F029M	ATG	D095F	TTT	A160W	TGG	S227M	ATG	T293Q	CAG	S360E	GAG
F029P	CCG	D095G	GGG	A160Y	TAT	S227P	CCT	T293S	TCT	S360F	TTT
F029R	CGG	D095H	CAT	G161A	GCT	S227Q	CAG	T293V	GTG	S360G	GGG

TABLE 8-continued

PH20 Variants											
mut	cod	mut	cod	mut	cod	mut	cod	mut	cod	mut	cod
F029S	TCG	D095K	AAG	G161C	TGT	S227R	CGG	T293Y	TAT	S360I	ATT
F029T	ACG	D095L	TTG	G161D	GAT	S227T	ACG	V294A	GCT	S360K	AAG
F029V	GTG	D095M	ATG	G161E	GAG	S227V	GTG	V294C	TGT	S360L	CTG
F029W	TGG	D095P	CCT	G161H	CAT	S227W	TGG	V294E	GAG	S360M	ATG
D030A	GCG	D095Q	CAG	G161I	ATT	S227Y	TAT	V294G	GGG	S360N	AAT
D030E	GAG	D095S	TCT	G161K	AAG	I228A	GCG	V294H	CAT	S360P	CCT
D030F	TTT	D095V	GTG	G161L	CTT	I228E	GAG	V294K	AAG	S360Q	CAG
D030G	GGG	D095W	TGG	G161M	ATG	I228F	TTT	V294L	TTG	S360R	AGG
D030H	CAT	D095Y	TAT	G161Q	CAG	I228G	GGG	V294M	ATG	S360T	ACT
D030K	AAG	I096A	GCT	G161R	CGT	I228H	CAT	V294N	AAT	S360V	GTT
D030L	TTG	I096C	TGT	G161S	AGT	I228K	AAG	V294P	CCT	D361A	GCT
D030M	ATG	I096D	GAT	G161T	ACT	I228L	TTG	V294Q	CAG	D361C	TGT
D030P	CCT	I096E	GAG	G161V	GTG	I228M	ATG	V294R	AGG	D361E	GAG
D030Q	CAG	I096F	TTT	G161W	TGG	I228N	AAT	V294S	AGT	D361G	GGG
D030R	CGG	I096G	GGG	K162A	GCT	I228P	CCG	V294T	ACT	D361H	CAT
D030S	TCG	I096H	CAT	K162D	GAT	I228Q	CAG	V294W	TGG	D361L	TTG
D030T	ACT	I096L	TTG	K162E	GAG	I228R	CGT	A295C	TGT	D361M	ATG
D030V	GTT	I096N	AAT	K162F	TTT	I228S	TCT	A295D	GAT	D361N	AAT
D030W	TGG	I096P	CCT	K162G	GGG	I228T	ACT	A295E	GAG	D361P	CCT
E031A	GCG	I096R	CGT	K162H	CAT	I228W	TGG	A295F	TTT	D361Q	CAG
E031C	TGT	I096S	AGT	K162L	TTG	Y229E	GAG	A295G	GGG	D361R	AGG
E031G	GGG	I096T	ACT	K162M	ATG	Y229F	TTT	A295H	CAT	D361S	TCG
E031H	CAT	I096V	GTG	K162P	CCT	Y229G	GGT	A295I	ATT	D361V	GTT
E031I	ATT	I096W	TGG	K162Q	CAG	Y229H	CAT	A295L	CTG	D361W	TGG
E031K	AAG	T097A	GCT	K162R	CGG	Y229I	ATT	A295N	AAT	D361Y	TAT
E031L	CTG	T097C	TGT	K162S	TCG	Y229K	AAG	A295P	CCT	Y362A	GCT
E031N	AAC	T097D	GAT	K162V	GTG	Y229L	TTG	A295Q	CAG	Y362C	TGT
E031P	CCG	T097E	GAG	K162W	TGG	Y229N	AAT	A295S	AGT	Y362E	GAG
E031R	CGG	T097F	TTT	K162Y	TAT	Y229P	CCT	A295T	ACT	Y362G	GGG
E031S	TCT	T097G	GGG	D163A	GCT	Y229Q	CAG	A295V	GTT	Y362H	CAT
E031T	ACG	T097I	ATT	D163C	TGT	Y229R	CGT	A295Y	TAT	Y362K	AAG
E031V	GTG	T097L	CTT	D163E	GAG	Y229S	TCG	L296A	GCT	Y362L	CTT
E031W	TGG	T097N	AAT	D163F	TTT	Y229T	ACT	L296C	TGT	Y362M	ATG
E031Y	TAT	T097P	CCT	D163G	GGG	Y229V	GTG	L296F	TTT	Y362N	AAT
P032A	GCG	T097Q	CAG	D163H	CAC	Y229W	TGG	L296G	GGT	Y362P	CCT
P032C	TGT	T097R	CGG	D163K	AAG	L230A	GCG	L296I	ATT	Y362R	CGG
P032F	TTT	T097S	TCG	D163L	CTT	L230E	GAG	L296K	AAG	Y362S	AGT
P032G	GGG	T097W	TGG	D163P	CCT	L230G	GGG	L296M	ATG	Y362T	ACT
P032H	CAT	T097Y	TAT	D163Q	CAG	L230H	CAT	L296P	CCT	Y362V	GTG
P032K	AAG	F098A	GCT	D163R	AGG	L230I	ATT	L296Q	CAG	Y362W	TGG
P032L	CTG	F098C	TGT	D163S	TCG	L230K	AAG	L296R	CGT	L363A	GCT
P032M	ATG	F098D	GAT	D163T	ACT	L230M	ATG	L296S	TCG	L363C	TGT
P032N	AAT	F098E	GAG	D163V	GTG	L230N	AAT	L296T	ACT	L363D	GAT
P032Q	CAG	F098G	GGG	D163W	TGG	L230P	CCT	L296V	GTT	L363E	GAG
P032R	CGG	F098H	CAT	F164A	GCT	L230R	CGT	L296W	TGG	L363F	TTT
P032S	TCG	F098I	ATT	F164C	TGT	L230S	AGT	L296Y	TAT	L363G	GGG
P032T	ACT	F098L	TTG	F164D	GAT	L230T	ACT	G297A	GCT	L363H	CAT
P032V	GTG	F098M	ATG	F164E	GAG	L230V	GTT	G297C	TGT	L363I	ATT
P032W	TGG	F098P	CCT	F164G	GGG	L230W	TGG	G297E	GAG	L363P	CCT
P032Y	TAT	F098Q	CAG	F164H	CAT	L230Y	TAT	G297H	CAT	L363Q	CAG
L033C	TGT	F098R	CGT	F164L	TTG	N231A	GCT	G297I	ATT	L363R	CGG
L033D	GAT	F098S	TCG	F164M	ATG	N231C	TGT	G297L	CTT	L363S	TCG
L033G	GGG	F098V	GTT	F164N	AAT	N231D	GAT	G297N	AAT	L363T	ACT
L033H	CAT	F098W	TGG	F164P	CCT	N231F	TTT	G297P	CCT	L363V	GTG
L033I	ATT	Y099A	GCT	F164Q	CAG	N231G	GGG	G297Q	CAG	L363W	TGG
L033M	ATG	Y099C	TGT	F164R	CGG	N231H	CAT	G297R	CGG	H364A	GCT
L033N	AAT	Y099E	GAG	F164S	AGT	N231I	ATT	G297S	AGT	H364C	TGT
L033P	CCG	Y099F	TTT	F164V	GTT	N231K	AAG	G297T	ACT	H364D	GAT
L033Q	CAG	Y099G	GGT	F164W	TGG	N231L	CTT	G297V	GTG	H364E	GAG
L033R	AGG	Y099I	ATT	L165A	GCT	N231P	CCT	G297W	TGG	H364F	TTT
L033S	TCG	Y099L	TTG	L165C	TGT	N231Q	CAG	G297Y	TAT	H364G	GGG
L033T	ACT	Y099N	AAT	L165D	GAT	N231R	CGT	A298C	TGT	H364K	AAG
L033V	GTT	Y099P	CCT	L165F	TTT	N231S	TCT	A298E	GAG	H364L	CTG
L033W	TGG	Y099Q	CAG	L165G	GGG	N231T	ACG	A298G	GGG	H364M	ATG
L033Y	TAT	Y099R	AGG	L165H	CAT	N231V	GTG	A298I	ATT	H364P	CCT
D034A	GCT	Y099S	TCG	L165N	AAT	T232A	GCG	A298L	TTG	H364R	CGG
D034E	GAG	Y099T	ACT	L165P	CCT	T232C	TGT	A298M	ATG	H364S	TCT
D034G	GGT	Y099V	GTT	L165Q	CAG	T232F	TTT	A298N	AAT	H364T	ACT
D034H	CAT	Y099W	TGG	L165R	CGG	T232G	GGG	A298P	CCT	H364V	GTG
D034I	ATT	M100C	TGT	L165S	TCG	T232H	CAT	A298Q	CAG	H364Y	TAT
D034K	AAG	M100E	GAG	L165T	ACT	T232K	AAG	A298R	CGT	L365A	GCT
D034L	CTT	M100F	TTT	L165V	GTG	T232L	CTT	A298S	TCG	L365C	TGT
D034N	AAT	M100G	GGT	L165W	TGG	T232M	ATG	A298T	ACT	L365D	GAT
D034P	CCT	M100K	AAG	L165Y	TAT	T232N	AAT	A298V	GTG	L365E	GAG
D034Q	CAG	M100L	CTG	V166A	GCT	T232P	CCG	A298W	TGG	L365G	GGG
D034R	CGT	M100N	AAT	V166C	TGT	T232Q	CAG	A298Y	TAT	L365I	ATT

TABLE 8-continued

PH20 Variants											
mut	cod	mut	cod	mut	cod	mut	cod	mut	cod	mut	cod
D034S	AGT	M100P	CCT	V166D	GAT	T232R	AGG	S299A	GCT	L365M	ATG
D034T	ACG	M100Q	CAG	V166E	GAG	T232S	AGT	S299C	TGT	L365N	AAT
D034V	GTT	M100R	CGG	V166F	TTT	T232V	GTG	S299D	GAT	L365P	CCT
D034W	TGG	M100S	TCT	V166G	GGT	T232Y	TAT	S299E	GAG	L365Q	CAG
M035A	GCG	M100T	ACT	V166H	CAT	Q233A	GCG	S299F	TTT	L365R	CGG
M035D	GAT	M100V	GTT	V166L	CTT	Q233C	TGT	S299G	GGG	L365S	AGT
M035F	TTT	M100W	TGG	V166N	AAT	Q233D	GAT	S299H	CAT	L365T	ACT
M035G	GGG	M100Y	TAT	V166P	CCT	Q233F	TTT	S299I	ATT	L365V	GTG
M035H	CAT	P101A	GCT	V166Q	CAG	Q233G	GGG	S299L	CTT	L365W	TGG
M035I	ATT	P101C	TGT	V166R	CGG	Q233I	ATT	S299M	ATG	L365Y	TAT
M035L	TTG	P101F	TTT	V166T	ACT	Q233K	AAG	S299P	CCT	N366A	GCT
M035N	AAT	P101G	GGG	V166W	TGG	Q233L	CTG	S299Q	CAG	N366C	TGT
M035P	CCG	P101H	CAT	V166Y	TAT	Q233P	CCG	S299R	AGG	N366E	GAG
M035Q	CAG	P101I	ATT	E167A	GCT	Q233R	AGG	S299T	ACT	N366F	TTT
M035R	CGT	P101K	AAG	E167D	GAT	Q233S	TCG	S299Y	TAT	N366G	GGG
M035S	TCT	P101L	CTT	E167F	TTT	Q233T	ACG	G300A	GCT	N366K	AAG
M035T	ACT	P101M	ATG	E167G	GGT	Q233V	GTG	G300C	TGT	N366L	TTG
M035V	GTT	P101N	AAT	E167H	CAT	Q233W	TGG	G300D	GAT	N366M	ATG
M035Y	TAT	P101Q	CAG	E167K	AAG	Q233Y	TAT	G300E	GAG	N366P	CCT
S036A	GCG	P101R	AGG	E167L	TTG	Q234A	GCT	G300F	TTT	N366Q	CAG
S036C	TGT	P101S	TCT	E167M	ATG	Q234C	TGT	G300L	CTT	N366R	AGG
S036D	GAT	P101T	ACT	E167N	AAT	Q234D	GAT	G300M	ATG	N366S	TCT
S036F	TTT	P101Y	TAT	E167P	CCT	Q234E	GAG	G300N	AAT	N366T	ACT
S036G	GGT	V102A	GCT	E167R	AGG	Q234G	GGT	G300P	CCT	N366V	GTT
S036H	CAT	V102C	TGT	E167S	TCG	Q234H	CAT	G300Q	CAG	N366W	TGG
S036K	AAG	V102E	GAG	E167T	ACT	Q234L	CTT	G300R	AGG	P367A	GCT
S036L	TTG	V102G	GGT	E167V	GTT	Q234M	ATG	G300S	TCG	P367C	TGT
S036N	AAT	V102H	CAT	E167Y	TAT	Q234N	AAT	G300T	ACT	P367E	GAG
S036P	CCG	V102K	AAG	T168A	GCT	Q234P	CCG	G300V	GTT	P367F	TTT
S036R	CGG	V102L	TTG	T168C	TGT	Q234R	CGG	G300W	TGG	P367G	GGT
S036T	ACG	V102M	ATG	T168D	GAT	Q234S	AGT	I301A	GCT	P367H	CAT
S036V	GTT	V102N	AAT	T168E	GAG	Q234T	ACT	I301E	GAG	P367I	ATT
S036W	TGG	V102P	CCT	T168F	TTT	Q234V	GTG	I301G	GGG	P367K	AAG
S036Y	TAT	V102Q	CAG	T168G	GGG	Q234W	TGG	I301H	CAT	P367L	CTG
L037A	GCG	V102R	AGG	T168H	CAT	S235A	GCG	I301K	AAG	P367M	ATG
L037C	TGT	V102S	TCT	T168K	AAG	S235E	GAG	I301L	CTG	P367Q	CAG
L037E	GAG	V102T	ACT	T168L	CTG	S235F	TTT	I301M	ATG	P367R	CGT
L037F	TTT	V102W	TGG	T168P	CCT	S235G	GGG	I301N	AAT	P367S	TCG
L037G	GGG	D103A	GCT	T168R	CGG	S235H	CAT	I301P	CCT	P367V	GTT
L037I	ATT	D103E	GAG	T168S	TCT	S235K	AAG	I301Q	CAG	P367W	TGG
L037K	AAG	D103F	TTT	T168V	GTG	S235L	CTT	I301R	CGG	D368A	GCT
L037M	ATG	D103G	GGG	T168W	TGG	S235M	ATG	I301S	AGT	D368C	TGT
L037N	AAT	D103H	CAT	T168Y	TAT	S235P	CCT	I301V	GTT	D368E	GAG
L037P	CCT	D103I	ATT	I169A	GCT	S235Q	CAG	I301W	TGG	D368G	GGT
L037R	AGG	D103L	CTT	I169D	GAT	S235R	CGG	I301Y	TAT	D368H	CAT
L037S	TCT	D103N	AAT	I169F	TTT	S235T	ACG	V302C	TGT	D368K	AAG
L037T	ACG	D103Q	CAG	I169G	GGG	S235V	GTG	V302D	GAT	D368L	CTT
L037V	GTG	D103R	AGG	I169H	CAT	S235W	TGG	V302E	GAG	D368M	ATG
L037W	TGG	D103S	TCG	I169K	AAG	S235Y	TAT	V302F	TTT	D368P	CCT
F038A	GCG	D103T	ACT	I169L	TTG	P236A	GCT	V302G	GGT	D368R	CGT
F038C	TGT	D103V	GTT	I169N	AAT	P236C	TGT	V302H	CAT	D368S	AGT
F038E	GAG	D103W	TGG	I169P	CCT	P236E	GAG	V302I	ATT	D368T	ACT
F038G	GGG	D103Y	TAT	I169Q	CAG	P236G	GGG	V302L	TTG	D368V	GTT
F038K	AAG	N104A	GCT	I169R	CGG	P236H	CAT	V302M	ATG	D368W	TGG
F038L	CTT	N104C	TGT	I169S	TCG	P236I	ATT	V302P	CCT	D368Y	TAT
F038M	ATG	N104F	TTT	I169T	ACT	P236K	AAG	V302R	AGG	N369A	GCT
F038N	AAT	N104G	GGG	I169V	GTT	P236L	CTG	V302S	TCG	N369C	TGT
F038P	CCT	N104H	CAT	I169Y	TAT	P236N	AAT	V302T	ACT	N369E	GAG
F038Q	CAG	N104I	ATT	K170A	GCT	P236Q	CAG	V302W	TGG	N369F	TTT
F038R	AGG	N104K	AAG	K170C	TGT	P236R	CGT	V302Y	TAT	N369H	CAT
F038S	TCT	N104L	CTG	K170D	GAT	P236S	AGT	I303A	GCT	N369I	ATT
F038T	ACT	N104M	ATG	K170E	GAG	P236T	ACT	I303C	TGT	N369K	AAG
F038W	TGG	N104P	CCT	K170G	GGG	P236W	TGG	I303D	GAT	N369L	CTT
F038Y	TAT	N104R	AGG	K170I	ATT	P236Y	TAT	I303E	GAG	N369P	CCT
S039A	GCG	N104S	TCT	K170L	TTG	V237A	GCG	I303F	TTT	N369Q	CAG
S039C	TGT	N104T	ACT	K170M	ATG	V237C	TGT	I303G	GGT	N369R	CGG
S039D	GAT	N104V	GTT	K170N	AAT	V237E	GAG	I303K	AAG	N369S	TCG
S039F	TTT	N104W	TGG	K170P	CCT	V237F	TTT	I303L	TTG	N369T	ACT
S039G	GGT	L105A	GCT	K170Q	CAG	V237G	GGT	I303M	ATG	N369V	GTG
S039L	TTG	L105C	TGT	K170R	CGT	V237H	CAT	I303P	CCT	N369W	TGG
S039M	ATG	L105D	GAT	K170V	GTT	V237L	TTG	I303R	CGT	F370A	GCT
S039N	AAT	L105E	GAG	K170W	TGG	V237N	AAT	I303S	AGT	F370D	GAT
S039P	CCG	L105G	GGT	K170Y	TAT	V237P	CCT	I303V	GTG	F370E	GAG
S039Q	CAG	L105H	CAT	L171A	GCT	V237Q	CAG	I303W	TGG	F370G	GGG
S039R	CGT	L105I	ATT	L171C	TGT	V237R	CGG	I303Y	TAT	F370H	CAT
S039T	ACT	L105M	ATG	L171D	GAT	V237S	TCG	W304A	GCT	F370I	ATT

TABLE 8-continued

PH20 Variants											
mut	cod	mut	cod	mut	cod	mut	cod	mut	cod	mut	cod
S039V	GTT	L105N	AAT	L171G	GGG	V237T	ACG	W304C	TGT	F370K	AAG
S039W	TGG	L105P	CCT	L171H	CAT	V237W	TGG	W304D	GAT	F370L	CTG
S039Y	TAT	L105Q	CAG	L171I	ATT	V237Y	TAT	W304G	GGT	F370N	AAT
F040A	GCG	L105R	CGG	L171M	ATG	A238D	GAT	W304I	ATT	F370P	CCT
F040D	GAT	L105S	TCT	L171N	AAT	A238E	GAG	W304L	CTG	F370Q	CAG
F040E	GAG	L105T	ACT	L171P	CCT	A238F	TTT	W304M	ATG	F370R	AGG
F040G	GGT	L105V	GTT	L171Q	CAG	A238G	GGT	W304N	AAT	F370S	TCT
F040I	ATT	L105W	TGG	L171R	CGT	A238H	CAT	W304P	CCT	F370V	GTG
F040K	AAG	G106A	GCT	L171S	AGT	A238K	AAG	W304Q	CAG	F370Y	TAT
F040L	CTG	G106C	TGT	L171V	GTG	A238L	CTT	W304R	CGG	A371C	TGT
F040N	AAT	G106D	GAT	L171W	TGG	A238P	CCG	W304S	AGT	A371E	GAG
F040Q	CAG	G106E	GAG	L171Y	TAT	A238Q	CAG	W304T	ACT	A371F	TTT
F040R	CGG	G106F	TTT	G172A	GCT	A238R	AGG	W304V	GTG	A371G	GGG
F040S	TCT	G106H	CAT	G172C	TGT	A238S	AGT	W304Y	TAT	A371H	CAT
F040T	ACT	G106I	ATT	G172D	GAT	A238T	ACG	G305C	TGT	A371I	ATT
F040V	GTT	G106L	CTG	G172E	GAG	A238V	GTG	G305D	GAT	A371K	AAG
F040W	TGG	G106M	ATG	G172I	ATT	A238W	TGG	G305E	GAG	A371L	CTT
F040Y	TAT	G106N	AAT	G172L	CTT	A238Y	TAT	G305F	TTT	A371M	ATG
I041A	GCG	G106P	CCT	G172M	ATG	A239C	TGT	G305H	CAT	A371P	CCT
I041C	TGT	G106S	AGT	G172P	CCT	A239F	TTT	G305K	AAG	A371R	CGT
I041D	GAT	G106V	GTG	G172Q	CAG	A239G	GGT	G305L	CTT	A371S	TCG
I041E	GAG	G106W	TGG	G172R	CGT	A239H	CAT	G305N	AAT	A371T	ACT
I041F	TTT	G106Y	TAT	G172S	TCT	A239I	ATT	G305P	CCT	A371V	GTG
I041G	GGG	M107A	GCT	G172T	ACT	A239K	AAG	G305Q	CAG	A371W	TGG
I041H	CAT	M107C	TGT	G172V	GTT	T240K	AAG	G305R	CGT	I372A	GCT
I041N	AAT	M107D	GAT	G172W	TGG	A239L	TTG	G305S	TCG	I372D	GAT
I041P	CCG	M107F	TTT	G172Y	TAT	A239N	AAT	G305T	ACT	I372E	GAG
I041Q	CAG	M107G	GGG	K173D	GAT	A239P	CCT	G305V	GTG	I372F	TTT
I041R	AGG	M107H	CAT	K173E	GAG	A239R	AGG	G305Y	TAT	I372G	GGT
I041S	TCT	M107I	ATT	K173G	GGG	A239S	TCT	T306A	GCT	I372H	CAT
I041T	ACG	M107K	AAG	K173H	CAT	A239T	ACT	T306C	TGT	I372K	AAG
I041V	GTT	M107L	CTT	K173I	ATT	A239V	GTT	T306D	GAT	I372L	CTG
I041W	TGG	M107P	CCT	K173L	CTT	A239W	TGG	T306E	GAG	I372N	AAT
G042A	GCT	M107Q	CAG	K173M	ATG	A239Y	TAT	T306F	TTT	I372P	CCT
G042C	TGT	M107R	CGT	K173N	AAT	T240A	GCG	T306G	GGT	I372R	CGG
G042D	GAT	M107S	TCT	K173P	CCT	T240E	GAG	T306H	CAT	I372S	TCT
G042E	GAG	M107V	GTT	K173Q	CAG	T240F	TTT	T306I	ATT	I372T	ACT
G042H	CAT	M107W	TGG	K173R	CGG	T240G	GGG	T306L	CTG	I372V	GTG
G042I	ATT	A108D	GAT	K173S	TCG	T240L	CTT	T306P	CCT	I372W	TGG
G042K	AAG	A108E	GAG	K173V	GTG	T240M	ATG	T306R	AGG	Q373A	GCT
G042L	CTG	A108F	TTT	K173W	TGG	T240N	AAT	T306S	AGT	Q373C	TGT
G042M	ATG	A108G	GGT	K173Y	TAT	T240P	CCT	T306V	GTG	Q373E	GAG
G042P	CCT	A108H	CAT	L174A	GCT	T240Q	CAG	T306W	TGG	Q373F	TTT
G042Q	CAG	A108K	AAG	L174C	TGT	T240R	CGT	T306Y	TAT	Q373G	GGT
G042R	CGG	A108L	TTG	L174G	GGG	T240S	AGT	L307C	TGT	Q373H	CAT
G042S	TCT	A108M	ATG	L174H	CAT	T240V	GTG	L307E	GAG	Q373K	AAG
G042T	ACT	A108N	AAT	L174K	AAG	T240W	TGG	L307F	TTT	Q373L	CTG
G042V	GTT	A108P	CCT	L174M	ATG	T240Y	TAT	L307G	GGG	Q373M	ATG
S043A	GCG	A108Q	CAG	L174N	AAT	L241A	GCG	L307I	ATT	Q373N	AAT
S043D	GAT	A108R	CGG	L174P	CCT	L241C	TGT	L307K	AAG	Q373P	CCT
S043E	GAG	A108S	TCT	L174Q	CAG	L241D	GAT	L307N	AAT	Q373R	CGT
S043F	TTT	A108T	ACT	L174R	CGT	L241E	GAG	L307P	CCT	Q373S	TCT
S043G	GGT	A108V	GTG	L174S	TCG	L241F	TTT	L307Q	CAG	Q373T	ACT
S043H	CAT	A108Y	TAT	L174T	ACT	L241G	GGG	L307R	AGG	Q373V	GTT
S043I	ATT	V109A	GCT	L174V	GTT	L241I	ATT	L307S	AGT	Q373W	TGG
S043K	AAG	V109C	TGT	L174W	TGG	L241K	AAG	L307T	ACT	L374A	GCT
S043L	CTT	V109D	GAT	L174Y	TAT	L241P	CCT	L307V	GTG	L374D	GAT
S043N	AAT	V109E	GAG	L175C	TGT	L241Q	CAG	L307W	TGG	L374E	GAG
S043P	CCT	V109F	TTT	L175D	GAT	L241R	CGG	L307Y	TAT	L374G	GGT
S043Q	CAG	V109G	GGG	L175E	GAG	L241S	TCT	S308C	TGT	L374H	CAT
S043R	CGG	V109H	CAT	L175F	TTT	L241T	ACG	S308D	GAT	L374I	ATT
S043T	ACT	V109L	TTG	L175G	GGG	L241V	GTT	S308F	TTT	L374M	ATG
S043V	GTG	V109M	ATG	L175H	CAT	L241W	TGG	S308G	GGT	L374N	AAT
P044A	GCT	V109P	CCT	L175K	AAG	Y242A	GCG	S308H	CAT	L374P	CCT
P044C	TGT	V109Q	CAG	L175N	AAT	Y242C	TGT	S308K	AAG	L374R	AGG
P044E	GAG	V109R	AGG	L175P	CCT	Y242D	GAT	S308L	CTG	L374S	AGT
P044F	TTT	V109T	ACT	L175R	CGT	Y242F	TTT	S308M	ATG	L374T	ACT
P044G	GGG	V109W	TGG	L175S	TCT	Y242G	GGT	S308N	AAT	L374V	GTG
P044H	CAT	V109Y	TAT	L175T	ACT	Y242I	ATT	S308P	CCT	L374W	TGG
P044I	ATT	I110A	GCT	L175V	GTG	Y242K	AAG	S308R	CGG	L374Y	TAT
P044L	CTT	I110C	TGT	L175W	TGG	Y242L	CTT	S308T	ACT	E375A	GCT
P044N	AAT	I110D	GAT	L175Y	TAT	Y242M	ATG	S308V	GTT	E375C	TGT
P044Q	CAG	I110F	TTT	R176A	GCT	Y242P	CCG	S308W	TGG	E375F	TTT
P044R	CGT	I110G	GGG	R176C	TGT	Y242R	CGG	S308Y	TAT	E375G	GGT
P044S	TCT	I110H	CAT	R176E	GAG	Y242S	TCT	I309D	GAT	E375I	ATT
P044T	ACT	I110K	AAG	R176F	TTT	Y242T	ACG	I309E	GAG	E375K	AAG

TABLE 8-continued

PH20 Variants											
mut	cod	mut	cod	mut	cod	mut	cod	mut	cod	mut	cod
P044W	TGG	I110L	CTG	R176G	GGG	Y242V	GTT	I309G	GGT	E375L	CTT
P044Y	ACG	I110M	ATG	R176H	CAT	Y242W	TGG	I309H	CAT	E375M	ATG
R045A	GCG	I110N	AAT	R176I	ATT	V243A	GCG	I309K	AAG	E375N	AAT
R045D	GAT	I110P	CCT	R176K	AAG	V243C	TGT	I309L	CTG	E375P	CCT
R045F	TTT	I110R	CGT	R176L	CTT	V243D	GAT	I309M	ATG	E375R	CGT
R045G	GGG	I110S	AGT	R176P	CCT	V243F	TTT	I309N	AAT	E375S	TCT
R045H	CAT	I110V	GTT	R176Q	CAG	V243G	GGG	I309Q	CAG	E375T	ACT
R045I	ATT	I110W	TGG	R176S	AGT	V243H	CAT	I309R	CGT	E375V	GTT
R045K	AAG	D111C	TGT	R176T	ACT	V243L	CTT	I309S	AGT	E375Y	TAT
R045M	ATG	D111E	GAG	R176V	GTG	V243M	ATG	I309T	ACT	K376A	GCT
R045P	CCT	D111G	GGT	R176W	TGG	V243P	CCT	I309V	GTG	K376D	GAT
R045Q	CAG	D111H	CAT	P177A	GCT	V243Q	CAG	I309W	TGG	K376E	GAG
R045S	TCG	D111I	ATT	P177C	TGT	V243R	AGG	I309Y	TAT	K376G	GGG
R045T	ACG	D111K	AAG	P177D	GAT	V243S	AGT	M310A	GCT	K376I	ATT
R045V	GTG	D111L	TTG	P177F	TTT	V243T	ACG	M310C	TGT	K376L	TTG
R045W	TGG	D111M	ATG	P177G	GGG	V243W	TGG	M310E	GAG	K376M	ATG
R045Y	TAT	D111P	ACT	P177H	CAT	V243Y	TAT	M310F	TTT	K376P	CCT
I046A	GCG	D111Q	CAG	P177L	CTT	R244A	GCG	M310G	GGG	K376Q	CAG
I046C	TGT	D111R	CGG	P177M	ATG	R244D	GAT	M310K	AAG	K376R	CGT
I046E	GAG	D111S	AGT	P177Q	CAG	R244G	GGG	M310L	CTG	K376S	AGT
I046F	TTT	D111T	ACT	P177R	CGG	R244H	CAT	M310N	AAT	K376T	ACT
I046H	CAT	D111V	GTT	P177S	TCT	R244I	ATT	M310P	CCT	K376V	GTG
I046L	CTT	D111W	TGG	P177T	ACT	R244K	AAG	M310Q	CAG	K376W	TGG
I046M	ATG	D111Y	TAT	P177V	GTT	R244M	ATG	M310R	CGG	K376Y	TAT
I046N	AAT	W112C	TGT	P177W	TGG	R244N	AAT	M310S	AGT	G377C	TGT
I046P	CCT	W112D	GAT	P177Y	TAT	R244P	CCT	M310V	GTG	G377D	GAT
I046R	CGT	W112E	GAG	N178A	GCT	R244Q	CAG	M310W	TGG	G377E	GAG
I046S	TCT	W112F	TTT	N178D	GAT	R244S	TCT	M310Y	TAT	G377F	TTT
I046T	ACT	W112G	GGG	N178E	GAG	R244T	ACG	R311A	GCT	G377H	CAT
I046V	GTT	W112H	CAT	N178G	GGG	R244V	GTG	R311C	TGT	G377I	ATT
I046W	TGG	W112I	ATT	N178I	ATT	R244W	TGG	R311E	GAG	G377K	AAG
I046Y	TAT	W112L	CTT	N178K	AAG	R244Y	TAT	R311F	TTT	G377L	CTT
N047A	GCT	W112N	AAT	N178L	TTG	N245A	GCG	R311G	GGT	G377M	ATG
N047D	GAT	W112P	CCT	N178M	ATG	N245C	TGT	R311H	CAT	G377P	CCT
N047F	TTT	W112Q	CAG	N178P	CCT	N245F	TTT	R311I	ATT	G377R	AGG
N047G	GGG	W112R	CGT	N178R	CGG	N245G	GGG	R311K	AAG	G377S	TCG
N047H	CAT	W112S	TCT	N178S	AGT	N245H	CAT	R311L	TTG	G377T	ACT
N047I	ATT	W112V	GTT	N178T	ACT	N245I	ATT	R311P	CCT	G377V	GTG
N047K	AAG	W112Y	TAT	N178V	GTG	N245K	AAG	R311Q	CAG	G377Y	TAT
N047L	CTT	E113A	GCT	N178W	TGG	N245L	CTG	R311S	TCT	G378D	GAT
N047M	ATG	E113C	TGT	N178Y	TAT	N245P	CCG	R311T	ACT	G378E	GAG
N047P	CCT	E113D	GAT	H179A	GCT	N245Q	CAG	R311V	GTG	G378F	TTT
N047Q	CAG	E113F	TTT	H179C	TGT	N245R	CGG	R311W	TGG	G378I	ATT
N047R	CGG	E113G	GGG	H179E	GAG	N245S	TCG	S312A	GCT	G378K	AAG
N047S	TCT	E113H	CAT	H179G	GGG	N245T	ACG	S312C	TGT	G378L	CTG
N047T	ACG	E113L	CTT	H179I	ATT	N245V	GTG	S312E	GAG	G378M	ATG
N047V	GTG	E113P	CCT	H179K	AAG	N245W	TGG	S312F	TTT	G378N	AAT
N047W	TGG	E113Q	CAG	H179L	CTG	R246A	GCG	S312G	GGG	G378Q	CAG
N047Y	TAT	E113R	CGT	H179M	ATG	R246C	TGT	S312H	CAT	G378R	AGG
A048C	TGT	E113S	TCT	H179N	AAT	R246D	GAT	S312K	AAG	G378S	TCT
A048E	GAG	E113T	ACT	H179P	CCT	R246E	GAG	S312L	CTG	G378T	ACT
A048F	TTT	E113V	GTT	H179R	AGG	R246G	GGG	S312M	ATG	G378V	GTG
A048G	GGT	E113W	TGG	H179S	AGT	R246H	CAT	S312N	AAT	G378W	TGG
A048H	CAT	E113Y	CAT	H179T	ACT	R246I	ATT	S312P	CCT	G378Y	TAT
A048I	ATT	E114A	GCT	H179V	GTG	R246K	AAG	S312Q	CAG	K379A	GCT
A048K	AAG	E114C	TGT	H179W	TGG	R246L	TTG	S312R	CGG	K379C	TGT
A048L	CTG	E114D	GAT	L180A	GCT	R246M	ATG	S312T	ACT	K379E	GAG
A048M	ATG	E114G	GGG	L180C	TGT	R246P	CCT	S312V	GTT	K379F	TTT
A048N	AAT	E114H	CAT	L180E	GAG	R246S	AGT	S312W	TGG	K379G	GGG
A048P	CCT	E114I	ATT	L180F	TTT	R246T	ACG	M313A	GCT	K379H	CAT
A048Q	CAG	E114L	CTG	L180G	GGT	R246V	GTT	M313C	TGT	K379I	ATT
A048R	CGG	E114M	ATG	L180H	CAT	R246W	TGG	M313D	GAT	K379L	CTT
A048S	TCT	E114P	CCT	L180I	ATT	V247A	GCG	M313E	GAG	K379M	ATG
A048V	GTT	E114R	CGG	L180K	AAG	V247C	TGT	M313F	TTT	K379N	AAT
A048W	TGG	E114S	TCT	L180M	ATG	V247F	TTT	M313G	GGG	K379R	CGT
A048Y	TAT	E114T	ACT	L180N	AAT	V247H	CAT	M313H	CAT	K379S	TCT
T049A	GCG	E114V	GTG	L180P	CCT	V247I	ATT	M313K	AAG	K379T	ACT
T049C	TGT	E114W	TGG	L180R	AGG	V247L	CTG	M313L	CTT	K379V	GTT
T049D	GAT	E114Y	TAT	L180S	TCG	V247M	ATG	M313P	CCT	K379W	TGG
T049F	TTT	W115A	GCT	L180T	ACT	V247N	AAT	M313R	CGT	F380A	GCT
T049G	GGG	W115C	TGT	L180W	TGG	V247P	CCT	M313S	TCG	F380C	TGT
T049H	CAT	W115D	GAT	W181A	GCT	V247Q	CAG	M313T	ACT	F380D	GAT
T049I	ATT	W115F	TTT	W181C	TGT	V247R	CGT	M313V	GTT	F380E	GAG
T049K	AAG	W115G	GGT	W181D	GAT	V247S	TCT	M313Y	TAT	F380G	GGG
T049L	TTG	W115H	CAT	W181E	GAG	V247T	ACT	K314A	GCT	F380I	ATT
T049N	AAT	W115I	ATT	W181F	TTT	V247W	TGG	K314C	TGT	F380L	CTT

TABLE 8-continued

PH20 Variants											
mut	cod	mut	cod	mut	cod	mut	cod	mut	cod	mut	cod
T049P	CCG	W115K	AAG	W181H	CAT	V247Y	TAT	K314D	GAT	F380P	CCT
T049R	AGG	W115L	CTT	W181I	ATT	R248A	GCT	K314H	CAT	F380Q	CAG
T049S	TCG	W115M	ATG	W181K	AAG	R248C	TGT	K314I	ATT	F380R	CGG
T049V	GTT	W115P	CCT	W181L	CTG	R248D	GAT	K314L	TTG	F380S	AGT
T049W	TGG	W115R	CGG	W181M	ATG	R248E	GAG	K314N	AAT	F380T	ACT
G050A	GCG	W115S	AGT	W181N	AAT	R248G	GGG	K314P	CCT	F380V	GTG
G050C	TGT	W115V	GTG	W181Q	CAG	R248H	CAT	K314Q	CAG	F380W	TGG
G050D	GAT	W115Y	TAT	W181R	CGT	R248I	ATT	K314R	CGG	F380Y	TAT
G050E	GAG	R116A	GCT	W181S	TCT	R248L	CTT	K314S	TCG	T381A	AGC
G050F	TTT	R116C	TGT	W181V	GTG	R248M	ATG	K314T	ACT	T381E	GAG
G050H	CAT	R116D	GAT	G182A	GCT	R248P	CCG	K314V	GTT	T381F	TTT
G050L	CTT	R116E	GAG	G182C	TGT	R248S	TCG	K314W	TGG	T381G	GGT
G050M	ATG	R116G	GGG	G182D	GAT	R248T	ACG	K314Y	TAT	T381H	CAT
G050P	CCT	R116H	CAT	G182E	GAG	R248V	GTG	S315A	GCT	T381K	AAG
G050Q	CAG	R116I	ATT	G182H	CAT	R248W	TGG	S315C	TGT	T381L	TTG
G050R	CGG	R116L	CTG	G182L	CTT	R248Y	TAT	S315E	GAG	T381N	AAT
G050S	AGT	R116N	AAT	G182M	ATG	E249A	GCT	S315G	GGT	T381P	CCT
G050V	GTT	R116P	CCT	G182N	AAT	E249G	GGG	S315H	CAT	T381Q	CAG
G050W	TGG	R116Q	CAG	G182P	CCT	E249H	CAT	S315I	ATT	T381R	CGT
G050Y	TAT	R116S	TCT	G182Q	CAG	E249I	ATT	S315K	AAG	T381S	AGT
Q051A	GCG	R116T	ACT	G182R	CGT	E249K	AAG	S315L	CTG	T381V	GTG
Q051C	TGT	R116V	GTG	G182S	AGT	E249L	CTG	S315M	ATG	T381W	TGG
Q051D	GAT	R116W	TGG	G182T	ACT	E249M	ATG	S315P	CCT	T381Y	TAT
Q051F	TTT	P117D	GAT	G182V	GTT	E249P	CCT	S315R	CGG	V382E	GAG
Q051H	CAT	P117E	GAG	G182Y	TAT	E249Q	CAG	S315T	ACT	V382G	GGG
Q051I	ATT	P117F	TTT	Y183A	GCT	E249R	CGG	S315V	GTT	V382H	CAT
Q051K	AAG	P117G	GGT	Y183C	TGT	E249S	TCT	S315W	TGG	V382I	ATT
Q051M	ATG	P117H	CAT	Y183D	GAT	E249T	ACT	S315Y	TAT	V382K	AAG
Q051N	AAT	P117I	ATT	Y183E	GAG	E249V	GTG	C316A	GCT	V382L	TTG
Q051P	CCT	P117K	AAG	Y183G	GGG	E249W	TGG	C316D	GAT	V382M	ATG
Q051R	CGG	P117N	AAT	Y183I	ATT	E249Y	TAT	C316E	GAG	V382N	AAT
Q051S	TCT	P117Q	CAG	Y183K	AAG	A250C	TGT	C316G	GGG	V382P	CCT
Q051T	ACG	P117R	AGG	Y183L	TTG	A250F	TTT	C316I	ATT	V382Q	CAG
Q051W	TGG	P117S	TCG	Y183N	AAT	A250G	GGT	C316K	AAG	V382R	CGG
Q051Y	TAT	P117T	ACT	Y183P	CCT	A250H	CAT	C316L	CTG	V382S	TCG
G052A	GCT	P117V	GTT	Y183Q	CAG	A250K	AAG	C316M	ATG	V382T	ACT
G052C	TGT	P117W	TGG	Y183R	CGT	A250L	CTG	C316P	CCT	V382W	TGG
G052E	GAG	P117Y	TAT	Y183S	TCT	A250M	ATG	C316R	AGG	V382Y	TAT
G052F	TTT	T118C	TGT	Y183V	GTT	A250N	AAT	C316S	TCT	R383A	GCT
G052H	CAT	T118D	GAT	Y183W	TGG	A250P	CCT	C316T	ACT	R383E	GAG
G052K	AAG	T118E	GAG	Y184A	GCT	A250Q	CAG	C316V	GTT	R383F	TTT
G052L	CTT	T118G	GGG	Y184C	TGT	A250R	AGG	C316W	TGG	R383G	GGG
G052N	AAT	T118H	CAT	Y184D	GAT	A250S	TCT	C316Y	TAT	R383H	CAT
G052P	CCT	T118K	AAG	Y184E	GAG	A250T	ACG	L317A	GCT	R383I	ATT
G052Q	CAG	T118L	CTG	Y184F	TTT	A250V	GTG	L317C	TGT	R383K	AAG
G052R	CGG	T118M	ATG	Y184G	GGT	A250W	TGG	L317D	GAT	R383L	CTG
G052S	AGT	T118N	AAT	Y184H	CAT	I251C	TGT	L317G	GGG	R383M	ATG
G052T	ACT	T118P	CCT	Y184K	AAG	I251D	GAT	L317H	CAT	R383N	AAT
G052W	TGG	T118Q	CAG	Y184L	CTT	I251F	TTT	L317I	ATT	R383P	CCT
G052Y	TAT	T118R	CGT	Y184M	ATG	I251G	GGG	L317K	AAG	R383S	TCG
V053A	GCG	T118V	GTT	Y184P	CCT	I251H	CAT	L317M	ATG	R383T	ACT
V053C	TGT	T118W	TGG	Y184R	AGG	I251K	AAG	L317N	AAT	R383V	GTG
V053D	GAT	T118Y	TAT	Y184S	TCG	I251L	CTT	L317P	CCT	R383W	TGG
V053E	GAG	W119A	GCT	Y184V	GTG	I251M	ATG	L317Q	CAG	G384A	GCT
V053G	GGG	W119D	GAT	Y184W	TGG	I251P	CCG	L317R	AGG	G384C	TGT
V053H	CAT	W119E	GAG	L185A	GCT	I251Q	CAG	L317S	TCG	G384D	GAT
V053L	CTG	W119F	TTT	L185D	GAT	I251S	AGT	L317T	ACT	G384E	GAG
V053N	AAT	W119G	GGT	L185E	GAG	I251T	ACT	L317W	TGG	G384F	TTT
V053P	CCG	W119I	ATT	L185F	TTT	I251V	GTG	L318C	TGT	G384H	CAT
V053Q	CAG	W119K	AAG	L185G	GGG	I251W	TGG	L318D	GAT	G384I	ATT
V053R	CGG	W119L	CTG	L185I	ATT	I251Y	TAT	L318F	TTT	G384K	AAG
V053S	AGT	W119N	AAT	L185K	AAG	R252A	GCT	L318G	GGG	G384L	CTT
V053T	ACT	W119P	CCT	L185N	AAT	R252D	GAT	L318H	CAT	G384M	ATG
V053W	TGG	W119Q	CAG	L185P	CCT	R252E	GAG	L318I	ATT	G384P	CCT
V053Y	TAT	W119R	CGG	L185R	CGG	R252F	TTT	L318K	AAG	G384Q	CAG
T054A	GCG	W119S	TCT	L185S	TCG	R252G	GGT	L318M	ATG	G384R	AGG
T054D	GAT	W119V	GTT	L185T	ACT	R252H	CAT	L318N	AAT	G384S	TCG
T054E	GAG	W119Y	TAT	L185V	GTG	R252I	ATT	L318P	CCT	G384T	ACT
T054F	TTT	A120C	TGT	L185W	TGG	R252K	AAG	L318Q	CAG	K385A	GCT
T054G	GGG	A120D	GAT	L185Y	TAT	R252L	CTG	L318R	CGG	K385C	TGT
T054H	CAT	A120F	TTT	F186A	GCT	R252N	AAT	L318S	AGT	K385G	GGG
T054I	ATT	A120G	GGG	F186D	GAT	R252P	CCT	L318T	ACT	K385H	CAT
T054M	ATG	A120H	CAT	F186G	GGT	R252S	TCG	L318W	TGG	K385L	CTT
T054N	AAT	A120I	ATT	F186H	CAT	R252T	ACT	L319C	TGT	K385M	ATG
T054P	CCG	A120L	CTT	F186I	ATT	R252V	GTG	L319E	GAG	K385N	AAT
T054Q	CAG	A120N	AAT	F186K	AAG	R252Y	TAT	L319F	TTT	K385P	CCG

TABLE 8-continued

PH20 Variants											
mut	cod	mut	cod	mut	cod	mut	cod	mut	cod	mut	cod
T054R	CGT	A120P	CCT	F186L	CTT	V253A	GCG	L319G	GGG	K385Q	CAG
T054S	AGT	A120R	CGT	F186N	AAT	V253D	GAT	L319H	CAT	K385R	CGT
T054V	GTT	A120S	TCT	F186P	CCT	V253E	GAG	L319I	ATT	K385S	TCT
T054Y	TAT	A120T	ACT	F186Q	CAG	V253G	GGG	L319K	AAG	K385T	ACG
I055A	GCT	A120V	GTG	F186R	AGG	V253H	CAT	L319M	ATG	K385V	GTT
I055C	TGT	A120W	TGG	F186S	TCT	V253I	ATT	L319P	CCT	K385W	TGG
I055D	GAT	A120Y	TAT	F186V	GTT	V253L	CTG	L319Q	CAG	K385Y	TAT
I055F	TTT	R121A	GCT	F186W	TGG	V253M	ATG	L319R	AGG	P386A	GCG
I055G	GGG	R121C	TGT	F186Y	TAT	V253N	AAT	L319S	TCG	P386C	TGT
I055H	CAT	R121D	GAT	P187A	GCT	V253P	CCT	L319V	GTT	P386F	TTT
I055L	CTG	R121E	GAG	P187F	TTT	V253Q	CAG	L319W	TGG	P386G	GGG
I055N	AAT	R121F	TTT	P187G	GGG	V253R	CGG	L319Y	TAT	P386H	CAT
I055P	CCT	R121G	GGT	P187H	CAT	V253S	TCG	D320C	TGT	P386I	ATT
I055Q	CAG	R121H	CAT	P187I	ATT	V253T	ACG	D320E	GAG	P386L	CTT
I055R	CGT	R121K	AAG	P187L	CTT	V253W	TGG	D320F	TTT	P386M	ATG
I055S	TCG	R121L	CTG	P187M	ATG	S254C	TGT	D320G	GGG	P386N	AAT
I055T	ACT	R121M	ATG	P187N	AAT	S254D	GAT	D320H	CAT	P386Q	CAG
I055V	GTT	R121P	CCT	P187Q	CAG	S254E	GAG	D320I	ATT	P386R	CGT
I055Y	TAT	R121S	TCG	P187R	AGG	S254G	GGG	D320K	AAG	P386S	AGT
F056A	GCG	R121T	ACT	P187S	TCG	S254I	ATT	D320L	TTG	P386T	ACG
F056C	TGT	R121V	GTT	P187T	ACT	S254K	AAG	D320M	ATG	P386V	GTT
F056E	GAG	R121W	TGG	P187V	GTT	S254L	TTG	D320N	AAT	P386Y	TAT
F056G	GGG	R121Y	TAT	P187W	TGG	S254N	AAT	D320P	CCT	T387C	TGT
F056H	CAT	N122A	GCT	P187Y	TAT	S254P	CCT	D320R	AGG	T387E	GAG
F056I	ATT	N122C	TGT	D188A	GCT	S254Q	CAG	D320S	AGT	T387F	TTT
F056K	AAG	N122E	GAG	D188C	TGT	S254R	CGG	D320V	GTG	T387G	GGG
F056L	TTG	N122F	TTT	D188F	TTT	S254T	ACT	D320W	TGG	T387H	CAT
F056N	AAT	N122I	ATT	D188G	GGG	S254V	GTG	D320Y	TAT	T387I	ATT
F056P	CCG	N122K	AAG	D188H	CAT	S254W	TGG	N321A	GCT	T387K	AAG
F056R	CGT	N122L	CTG	D188L	CTT	S254Y	TAT	N321D	GAT	T387L	CTG
F056S	TCT	N122M	ATG	D188M	ATG	K255A	GCG	N321E	GAG	T387M	ATG
F056T	ACT	N122P	CCT	D188N	AAT	K255C	TGT	N321G	GGT	T387N	AAT
F056V	GTT	N122Q	CAG	D188P	CCT	K255D	GAT	N321H	CAT	T387Q	CAG
F056W	TGG	N122R	CGG	D188Q	CAG	K255G	GGT	N321I	ATT	T387S	TCG
Y057A	GCT	N122S	TCT	D188R	AGG	K255H	CAT	N321K	AAG	T387V	GTT
Y057D	GAT	N122T	ACT	D188S	AGT	K255L	TTG	N321L	CTG	T387W	TGG
Y057E	GAG	N122V	GTT	D188T	ACT	K255N	AAT	N321M	ATG	T387Y	TAT
Y057F	TTT	N122W	TGG	D188V	GTG	K255P	CCG	N321P	CCT	L388A	GCG
Y057G	GGG	W123A	GCT	D188W	TGG	K255Q	CAG	N321R	CGG	L388C	TGT
Y057I	ATT	W123C	TGT	C189A	GCT	K255R	CGG	N321S	TCT	L388F	TTT
Y057L	TTG	W123D	GAT	C189E	GAG	K255S	TCG	N321T	ACT	L388G	GGG
Y057M	ATG	W123E	GAG	C189G	GGT	K255T	ACT	N321V	GTG	L388H	CAT
Y057P	CCG	W123G	GGG	C189H	CAT	K255V	GTT	N321Y	TAT	L388I	ATT
Y057Q	CAG	W123H	CAT	C189K	AAG	K255W	TGG	Y322C	TGT	L388M	ATG
Y057R	CGG	W123L	CTT	C189L	TTG	K255Y	TAT	Y322D	GAT	L388P	CCT
Y057S	AGT	W123M	ATG	C189M	ATG	I256A	GCT	Y322E	GAG	L388Q	CAG
Y057T	ACG	W123P	CCT	C189N	ACT	I256C	TGT	Y322F	TTT	L388R	CGT
Y057V	GTG	W123Q	CAG	C189P	CCT	I256D	GAT	Y322G	GGT	L388S	TCG
Y057W	TGG	W123R	AGG	C189R	AGG	I256E	GAG	Y322H	CAT	L388T	ACG
V058A	GCT	W123S	AGT	C189S	TCG	I256G	GGG	Y322I	ATT	L388V	GTT
V058C	TGT	W123T	ACT	C189T	ACT	I256H	CAT	Y322L	CTG	L388W	TGG
V058D	GAT	W123V	GTT	C189V	GTG	I256L	CTT	Y322N	AAT	L388Y	TAT
V058G	GGT	W123Y	TAT	C189W	TGG	I256M	ATG	Y322P	CCT	E389A	GCT
V058H	CAT	K124A	GCT	C189Y	TAT	I256N	AAT	Y322R	CGT	E389F	TTT
V058I	ATT	K124C	TGT	Y190C	TGT	I256P	CCG	Y322S	TCT	E389G	GGT
V058K	AAG	K124D	GAT	Y190E	GAG	I256Q	CAG	Y322T	ACT	E389H	CAT
V058L	CTT	K124E	GAG	Y190F	TTT	I256R	AGG	Y322V	GTG	E389I	ATT
V058N	AAT	K124F	TTT	Y190G	GGG	I256T	ACG	Y322W	TGG	E389K	AAG
V058P	CCT	K124G	GGG	Y190H	CAT	I256V	GTT	M323A	GCT	E389L	CTG
V058Q	CAG	K124H	CAT	Y190K	AAG	I256W	TGG	M323C	TGT	E389M	ATG
V058R	CGG	K124I	ATT	Y190L	CTT	P257A	GCG	M323E	GAG	E389P	CCT
V058S	TCG	K124L	CTT	Y190N	AAT	P257C	TGT	M323F	TTT	E389Q	CAG
V058W	TGG	K124N	AAT	Y190P	CCT	P257D	GAT	M323G	GGG	E389R	CGG
V058Y	TAT	K124P	CCT	Y190Q	CAG	P257G	GGG	M323H	CAT	E389S	TCG
D059A	GCT	K124R	CGG	Y190R	CGT	P257I	ATT	M323I	ATT	E389T	ACT
D059E	GAG	K124S	TCT	Y190S	TCT	P257K	AAG	M323K	AAG	E389V	GTT
D059G	GGG	K124T	ACT	Y190T	ACT	P257L	CTT	M323L	TTG	E389Y	TAT
D059H	CAT	K124V	GTG	Y190V	GTG	P257M	ATG	M323N	AAT	D390A	GCG
D059I	ATT	K124W	TGG	Y190W	TGG	P257N	AAT	M323P	CCT	D390C	TGT
D059L	CTT	P125A	GCT	N191A	GCT	P257Q	CAG	M323R	CGG	D390E	GAG
D059M	ATG	P125C	TGT	N191E	GAG	P257R	CGT	M323S	AGT	D390F	TTT
D059N	AAT	P125D	GAT	N191F	TTT	P257S	TCG	M323T	ACT	D390G	GGG
D059P	CCT	P125G	GGG	N191G	GGG	P257T	ACG	M323V	GTT	D390H	CAT
D059Q	CAG	P125H	CAT	N191K	AAG	P257V	GTG	E324A	GCT	D390L	CTT
D059R	CGT	P125I	ATT	N191L	TTG	P257W	TGG	E324C	TGT	D390N	AAT
D059T	ACG	P125L	CTT	N191M	ATG	D258A	GCG	E324D	GAT	D390P	CCG

TABLE 8-continued

PH20 Variants											
mut	cod	mut	cod	mut	cod	mut	cod	mut	cod	mut	cod
D059V	GTG	P125N	AAT	N191P	CCT	D258E	GAG	E324F	TTT	D390R	CGG
D059W	TGG	P125Q	CAG	N191Q	CAG	D258G	GGG	E324G	GGG	D390S	AGT
D059Y	TAT	P125R	CGT	N191R	CGG	D258H	CAT	E324H	CAT	D390T	ACT
R060A	GCG	P125S	TCG	N191S	TCG	D258I	ATT	E324L	TTG	D390V	GTG
R060D	GAT	P125T	ACT	N191T	ACT	D258L	CTT	E324M	ATG	D390W	TGG
R060F	TTT	P125V	GTG	N191V	GTT	D258N	AAT	E324N	AAT	D390Y	TAT
R060G	GGT	P125W	TGG	N191W	TGG	D258P	CCG	E324P	CCT	L391A	GCT
R060H	CAT	P125Y	TAT	N191Y	TAT	D258Q	CAG	E324R	CGG	L391C	TGT
R060I	ATT	K126A	GCT	H192C	TGT	D258R	CGT	E324S	AGT	L391D	GAT
R060K	AAG	K126D	GAT	H192F	TTT	D258S	AGT	E324V	GTG	L391G	GGG
R060L	CTT	K126E	GAG	H192G	GGT	D258T	ACG	E324W	TGG	L391H	CAT
R060N	AAT	K126F	TTT	H192K	AAG	D258V	GTG	E324Y	TAT	L391K	AAG
R060P	CCG	K126G	GGT	H192L	CTT	D258W	TGG	T325A	GCT	L391N	AAT
R060Q	CAG	K126H	CAT	H192M	ATG	D258Y	TAT	T325C	TGT	L391P	CCT
R060S	TCG	K126I	ATT	H192N	AAT	A259E	GAG	T325D	GAT	L391Q	CAG
R060T	ACG	K126L	CTG	H192P	CCT	A259G	GGG	T325E	GAG	L391R	CGG
R060V	GTT	K126M	ATG	H192Q	CAG	A259I	ATT	T325G	GGT	L391S	TCT
R060Y	TAT	K126N	AAT	H192R	CGT	A259K	AAG	T325H	CAT	L391T	ACT
L061A	GCT	K126P	CCT	H192S	TCG	A259L	TTG	T325I	ATT	L391V	GTG
L061E	GAG	K126Q	CAG	H192T	ACT	A259M	ATG	T325K	AAG	L391W	TGG
L061F	TTT	K126R	AGG	H192V	GTT	A259N	AAT	T325M	ATG	L391Y	TAT
L061G	GGG	K126S	TCT	H192W	TGG	A259P	CCT	T325N	AAT	E392A	GCT
L061H	CAT	K126T	ACT	H192Y	TAT	A259Q	CAG	T325Q	CAG	E392C	TGT
L061I	ATT	K126V	GTG	H193A	GCT	A259R	CGT	T325R	CGG	E392F	TTT
L061M	ATG	K126W	TGG	H193C	TGT	A259S	AGT	T325S	TCG	E392G	GGG
L061N	AAT	K126Y	TAT	H193D	GAT	A259T	ACT	T325V	GTG	E392K	AAG
L061P	CCT	D127A	GCT	H193F	TTT	A259V	GTG	T325W	TGG	E392L	CTG
L061Q	CAG	D127E	GAG	H193G	GGG	A259W	TGG	I326A	GCT	E392M	ATG
L061R	AGG	D127F	TTT	H193K	AAG	A259Y	TAT	I326C	TGT	E392P	CCT
L061T	ACT	D127G	GGT	H193L	TTG	K260A	GCG	I326D	GAT	E392Q	CAG
L061V	GTT	D127H	CAT	H193M	ATG	K260C	TGT	I326E	GAG	E392R	AGG
L061W	TGG	D127K	AAG	H193P	CCG	K260D	GAT	I326G	GGG	E392S	AGT
L061Y	TAT	D127L	CTG	H193Q	CAG	K260E	GAG	I326H	CAT	E392T	ACT
G062A	GCG	D127M	ATG	H193R	AGG	K260G	GGG	I326K	AAG	E392V	GTT
G062C	TGT	D127N	AAT	H193S	TCT	K260H	CAT	I326L	CTT	E392W	TGG
G062D	GAT	D127Q	CAG	H193T	ACG	K260L	TTG	I326N	AAT	E392Y	TAT
G062F	TTT	D127R	CGT	H193V	GTG	K260M	ATG	I326P	CCT	Q393A	GCG
G062I	ATT	D127S	AGT	H193Y	TAT	K260P	CCG	I326R	CGG	Q393C	TGT
G062K	AAG	D127T	ACT	Y194A	GCT	K260Q	CAG	I326S	TCT	Q393D	GAT
G062L	CTT	D127V	GTT	Y194C	TGT	K260R	CGG	I326V	GTG	Q393F	TTT
G062M	ATG	D127W	TGG	Y194E	GAG	K260S	TCT	I326W	TGG	Q393G	GGT
G062P	CCT	V128A	GCT	Y194F	TTT	K260V	GTT	I326Y	TAT	Q393H	CAT
G062Q	CAG	V128C	TGT	Y194G	GGG	K260W	TGG	L327A	GCT	Q393I	ATT
G062R	CGT	V128E	GAG	Y194I	ATT	K260Y	TAT	L327D	GAT	Q393K	AAG
G062S	AGT	V128F	TTT	Y194L	TTG	S261A	GCG	L327E	GAG	Q393L	TTG
G062T	ACT	V128G	GGG	Y194N	AAT	S261E	GAG	L327F	TTT	Q393M	ATG
G062V	GTG	V128H	CAT	Y194P	CCT	S261F	TTT	L327G	GGG	Q393N	AAT
G062Y	TAT	V128I	ATT	Y194Q	CAG	S261G	GGG	L327H	CAT	Q393P	CCG
Y063A	GCG	V128K	AAG	Y194R	AGG	S261I	ATT	L327M	ATG	Q393R	CGT
Y063C	TGT	V128L	CTG	Y194S	TCG	S261K	AAG	L327N	AAT	Q393S	TCG
Y063G	GGT	V128P	CCT	Y194T	ACG	S261L	CTT	L327Q	CAG	Q393T	ACG
Y063H	CAT	V128Q	CAG	Y194V	GTG	S261M	ATG	L327R	CGG	F394A	GCG
Y063I	ATT	V128R	AGG	Y194W	TGG	S261N	AAT	L327S	AGT	F394D	GAT
Y063K	AAG	V128S	TCG	K195A	GCG	S261P	CCT	L327T	ACT	F394E	GAG
Y063L	CTG	V128W	TGG	K195E	GAG	S261Q	CAG	L327V	GTG	F394G	GGG
Y063M	ATG	V128Y	TAT	K195F	TTT	S261R	CGT	L327W	TGG	F394I	ATT
Y063N	AAT	Y129A	GCT	K195G	GGT	S261T	ACT	L327Y	TAT	F394K	AAG
Y063P	CCT	Y129C	TGT	K195H	CAT	S261V	GTT	N328A	GCT	F394L	CTG
Y063R	AGG	Y129D	GAT	K195I	ATT	S261W	TGG	N328C	TGT	F394N	AAT
Y063S	TCT	Y129E	GAG	K195L	TTG	P262A	GCG	N328D	GAT	F394P	CCG
Y063T	ACG	Y129G	GGG	K195N	AAT	P262D	GAT	N328G	GGT	F394Q	CAG
Y063V	GTG	Y129H	CAT	K195Q	CAG	P262E	GAG	N328H	CAT	F394R	CGT
Y063W	TGG	Y129L	TTG	K195R	CGT	P262F	TTT	N328I	ATT	F394S	TCG
Y064A	GCT	Y129M	ATG	K195S	TCT	P262G	GGG	N328K	AAG	F394T	ACT
Y064C	TGT	Y129P	CCT	K195T	ACT	P262H	CAT	N328L	CTT	F394V	GTT
Y064D	GAT	Y129Q	CAG	K195V	GTG	P262I	ATT	N328Q	CAG	F394W	TGG
Y064E	GAG	Y129R	CGG	K195W	TGG	P262K	AAG	N328R	AGG	S395A	GCG
Y064F	TTT	Y129S	AGT	K195Y	TAT	P262Q	CAG	N328S	AGT	S395C	TGT
Y064G	GGT	Y129T	ACT	K196A	GCT	P262R	CGT	N328T	ACT	S395D	GAT
Y064H	CAT	Y129V	GTT	K196C	TGT	P262S	TCT	N328V	GTG	S395E	GAG
Y064I	ATT	Y129W	TGG	K196D	GAT	P262T	ACT	N328W	TGG	S395G	GGG
Y064K	AAG	K130C	TGT	K196E	GAG	P262V	GTG	N328Y	TAT	S395H	CAT
Y064L	CTT	K130D	GAT	K196G	GGG	P262W	TGG	P329C	TGT	S395K	AAG
Y064P	CCT	K130E	GAG	K196I	ATT	P262Y	TAT	P329F	TTT	S395L	CTT
Y064Q	CAG	K130G	GGG	K196L	TTG	L263A	GCT	P329G	GGT	S395M	ATG
Y064R	CGG	K130H	CAT	K196N	AAT	L263E	GAG	P329H	CAT	S395P	CCT

TABLE 8-continued

PH20 Variants											
mut	cod	mut	cod	mut	cod	mut	cod	mut	cod	mut	cod
Y064S	AGT	K130I	ATT	K196P	CCG	L263F	TTT	P329I	ATT	S395R	CGG
Y064T	ACT	K130L	TTG	K196R	CGT	L263G	GGG	P329K	AAG	S395T	ACG
Y064V	GTT	K130N	AAT	K196S	TCG	L263H	CAT	P329L	CTG	S395V	GTT
Y064W	TGG	K130Q	CAG	K196T	ACT	L263K	AAG	P329N	AAT	S395W	TGG
P065A	GCT	K130R	AGG	K196V	GTG	L263M	ATG	P329Q	CAG	S395Y	TAT
P065C	TGT	K130S	TCT	K196W	TGG	L263N	AAT	P329R	CGT	E396A	GCG
P065D	GAT	K130T	ACT	K196Y	TAT	L263P	CCG	P329S	AGT	E396C	TGT
P065F	TTT	K130V	GTG	P197A	GCT	L263Q	CAG	P329T	ACT	E396D	GAT
P065G	GGG	K130W	TGG	P197C	TGT	L263R	CGG	P329V	GTT	E396F	TTT
P065H	CAT	K130Y	TAT	P197D	GAT	L263S	AGT	P329W	TGG	E396G	GGG
P065I	ATT	N131C	TGT	P197E	GAG	L263T	ACT	P329Y	TAT	E396H	CAT
P065K	AAG	N131E	GAG	P197F	TTT	L263V	GTT	Y330A	GCT	E396I	ATT
P065N	AAT	N131F	TTT	P197G	GGT	L263W	TGG	Y330C	TGT	E396L	CTT
P065R	CGG	N131G	GGG	P197H	CAT	P264A	GCG	Y330D	GAT	E396P	CCG
P065S	TCG	N131H	CAT	P197K	AAG	P264D	GAT	Y330E	GAG	E396Q	CAG
P065T	ACG	N131I	ATT	P197L	TTG	P264E	GAG	Y330F	TTT	E396R	AGG
P065V	GTT	N131L	CTT	P197M	ATG	P264F	TTT	Y330G	GGT	E396S	TCT
P065W	TGG	N131M	ATG	P197Q	CAG	P264G	GGT	Y330I	ATT	E396T	ACT
P065Y	TAT	N131P	CCT	P197R	CGT	P264H	CAT	Y330L	CTG	E396V	GTG
Y066A	GCG	N131Q	CAG	P197S	AGT	P264L	CTT	Y330M	ATG	E396Y	TAT
Y066C	TGT	N131R	CGG	P197T	ACT	P264M	ATG	Y330N	AAT	K397A	GCT
Y066D	GAT	N131S	AGT	P197W	TGG	P264N	AAT	Y330P	CCT	K397C	TGT
Y066E	GAG	N131T	ACT	G198A	GCT	P264R	CGG	Y330R	AGG	K397E	GAG
Y066G	GGT	N131V	GTG	G198C	TGT	P264S	AGT	Y330S	AGT	K397F	TTT
Y066H	CAT	N131Y	TAT	G198D	GAT	P264T	ACT	Y330V	GTT	K397G	GGT
Y066I	ATT	R132A	GCT	G198E	GAG	P264V	GTT	I331V	GTG	K397I	ATT
Y066K	AAG	R132C	TGT	G198H	CAT	P264W	TGG	Y330W	TGG	K397L	TTG
Y066L	CTG	R132E	GAG	G198L	CTG	P264Y	TAT	I331A	GCT	K397M	ATG
Y066N	AAT	R132F	TTT	G198N	AAT	V265A	GCG	I331C	TGT	K397N	AAT
Y066P	CCT	R132H	CAT	G198P	CCG	V265C	TGT	I331D	GAT	K397P	CCG
Y066R	CGG	R132I	ATT	G198Q	CAG	V265D	GAT	I331E	GAG	K397Q	CAG
K397T	ACT	R132K	AAG	G198R	AGG	V265E	GAG	I331F	TTT	K397R	AGG
K397V	GTT	R132L	TTG	G198S	TCT	V265F	TTT	I331H	CAT	K397S	TCG
F398A	GCT	L406P	CCT	K415G	GGT	C423T	ACT	A432L	TTG	E441D	GAT
F398C	TGT	L406Q	CAG	K415L	CTG	C423V	GTG	A432M	ATG	E441F	TTT
F398E	GAG	L406R	CGG	K415M	ATG	C423W	TGG	A432N	AAT	E441G	GGG
F398G	GGT	L406S	AGT	K415P	CCG	I424A	GCT	A432P	CCT	E441H	CAT
F398H	CAT	L406T	ACG	K415Q	CAG	I424C	TGT	A432R	AGG	E441K	AAG
F398I	ATT	L406V	GTT	K415R	CGG	I424E	GAG	A432S	TCT	E441L	CTT
F398L	CTT	L406Y	TAT	K415S	TCT	I424G	GGG	A432V	GTG	E441N	AAT
F398N	AAT	S407A	GCG	K415T	ACT	I424H	CAT	A432Y	TAT	E441Q	CAG
F398P	CCT	S407D	GAT	K415V	GTG	I424K	AAG	F433A	GCT	E441R	CGG
F398R	AGG	S407E	GAG	K415W	TGG	I424L	CTT	F433C	TGT	E441S	AGT
F398S	TCT	S407F	TTT	K415Y	TAT	I424N	AAT	F433D	GAT	E441T	ACT
F398T	ACT	S407G	GGT	D416C	TGT	I424Q	CAG	F433E	GAG	E441V	GTG
F398V	GTT	S407H	CAT	D416F	TTT	I424R	CGG	F433G	GGG	E441Y	TAT
F398W	TGG	S407L	CTG	D416G	GGT	I424S	TCG	F433H	CAT	E442C	TGT
F398Y	TAT	S407M	ATG	D416H	CAT	I424T	ACT	F433I	ATT	E442G	GGG
Y399A	GCG	S407N	AAT	D416I	ATT	I424V	GTT	F433K	AAG	E442H	CAT
Y399C	TGT	S407P	CCT	D416K	AAG	I424W	TGG	F433L	TTG	E442K	AAG
Y399D	GAT	S407Q	CAG	D416L	CTT	I424Y	TAT	F433P	CCT	E442L	CTT
Y399E	GAG	S407R	CGG	D416N	AAT	A425C	TGT	F433R	CGG	E442M	ATG
Y399G	GGG	S407T	ACG	D416Q	CAG	A425D	GAT	F433S	AGT	E442N	AAT
Y399K	AAG	S407V	GTG	D416R	CGG	A425E	GAG	F433T	ACT	E442P	CCT
Y399M	ATG	S407W	TGG	D416S	TCT	A425G	GGT	F433V	GTG	E442Q	CAG
Y399N	AAT	C408A	GCG	D416T	ACG	A425I	ATT	F433W	TGG	E442R	CGG
Y399P	CCT	C408E	GAG	D416V	GTG	A425K	AAG	L434F	TTT	E442S	AGT
Y399Q	CAG	C408F	TTT	D416W	TGG	A425L	TTG	L434G	GGT	E442T	ACT
Y399R	CGG	C408G	GGG	D416Y	TAT	A425M	ATG	L434H	CAT	E442V	GTG
Y399S	TCG	C408I	ATT	T417A	GCT	A425N	AAT	L434I	ATT	E442W	TGG
Y399T	ACG	C408K	AAG	T417D	GAT	A425P	CCT	L434K	AAG	E442Y	TAT
Y399V	GTT	C408L	CTT	T417E	GAG	A425R	AGG	L434M	ATG	P443A	GCT
Y399W	TGG	C408N	AAT	T417F	TTT	A425S	AGT	L434N	AAT	P443D	GAT
C400A	GCG	C408P	CCT	T417G	GGG	A425V	GTG	L434P	CCT	P443E	GAG
C400D	GAT	C408R	CGT	T417H	CAT	A425W	TGG	L434Q	CAG	P443F	TTT
C400E	GAG	C408S	TCG	T417I	ATT	A425Y	TAT	L434R	CGG	P443G	GGG
C400F	TTT	C408T	ACT	T417K	AAG	D426A	GCT	L434S	AGT	P443H	CAT
C400G	GGG	C408V	GTT	T417L	TTG	D426C	TGT	L434T	ACT	P443I	ATT
C400I	ATT	C408W	TGG	T417M	ATG	D426E	GAG	L434V	GTT	P443L	CTT
C400L	CTG	C408Y	TAT	T417P	CCT	D426F	TTT	L434W	TGG	P443M	ATG
C400M	ATG	K409A	GCG	T417Q	CAG	D426G	GGG	L434Y	TAT	P443N	AAT
C400P	CCG	K409C	TGT	T417R	CGT	D426I	ATT	K435A	GCT	P443Q	CAG
C400Q	CAG	K409D	GAT	T417S	TCG	D426K	AAG	K435C	TGT	P443R	AGG
C400R	CGG	K409E	GAG	T417W	TGG	D426L	CTG	K435E	GAG	P443S	TCT
C400S	AGT	K409G	GGT	D418A	GCT	D426M	ATG	K435F	TTT	P443T	ACT
C400T	ACG	K409H	CAT	D418C	TGT	D426N	AAT	K435G	GGT	P443W	TGG

TABLE 8-continued

PH20 Variants											
mut	cod	mut	cod	mut	cod	mut	cod	mut	cod	mut	cod
C400V	GTG	K409I	ATT	D418E	GAG	D426P	CCT	K435H	CAT	Q444C	TGT
C400Y	TAT	K409L	CTG	D418F	TTT	D426Q	CAG	K435I	ATT	Q444D	GAT
S401A	GCT	K409P	CCG	D418G	GGT	D426R	CGT	K435L	CTG	Q444E	GAG
S401C	TGT	K409Q	CAG	D418I	ATT	D426S	TCG	K435P	CCT	Q444F	TTT
S401D	GAT	K409R	AGG	D418L	TTG	D426Y	TAT	K435R	AGG	Q444G	GGG
S401E	GAG	K409S	TCG	D418M	ATG	G427A	GCT	K435S	TCT	Q444H	CAT
S401F	TTT	K409T	ACG	D418N	AAT	G427C	TGT	K435T	ACT	Q444I	ATT
S401G	GGG	K409V	GTG	D418P	CCT	G427F	TTT	K435V	GTT	Q444K	AAG
S401H	CAT	K409W	TGG	D418Q	CAG	G427H	CAT	K435W	TGG	Q444L	CTG
S401K	AAG	A412Y	TAT	D418R	CGG	G427I	ATT	K435Y	TAT	Q444M	ATG
S401L	CTT	E410D	GAT	D418S	TCG	G427K	AAG	P436C	TGT	Q444N	AAT
S401N	AAT	E410G	GGG	D418V	GTG	G427L	CTG	P436D	GAT	Q444R	CGG
S401Q	CAG	E410I	ATT	D418Y	TAT	G427P	CCT	P436E	GAG	Q444V	GTT
S401R	CGT	E410K	AAG	A419D	GAT	G427Q	CAG	P436G	GGG	Q444W	TGG
S401T	ACT	E410L	CTT	A419E	GAG	G427R	CGT	P436H	CAT	Q444Y	TAT
S401W	TGG	E410M	ATG	A419F	TTT	G427S	AGT	P436I	ATT	I445A	GCT
S401Y	TAT	E410N	AAT	A419G	GGG	G427T	ACT	P436K	AAG	I445C	TGT
C402A	GCT	E410P	CCG	A419H	CAT	G427V	GTG	P436L	CTG	I445D	GAT
C402D	GAT	E410Q	CAG	A419I	ATT	G427W	TGG	P436M	ATG	I445G	GGG
C402E	GAG	E410R	CGT	A419K	AAG	G427Y	TAT	P436Q	CAG	I445H	CAT
C402F	TTT	E410S	TCG	A419L	CTT	V428A	GCT	P436R	CGG	I445K	AAG
C402G	GGG	E410T	ACG	A419N	AAT	V428C	TGT	P436S	TCT	I445L	CTT
C402L	TTG	E410V	GTG	A419P	CCT	V428D	GAT	P436T	ACT	I445M	ATG
C402M	ATG	E410W	TGG	A419R	CGG	V428E	GAG	P436W	TGG	I445N	AAT
C402P	CCT	E410Y	TAT	A419S	TCT	V428F	TTT	P436Y	TAT	I445P	CCT
C402Q	CAG	K411A	GCT	A419T	ACT	V428G	GGT	P437A	GCT	I445Q	CAG
C402R	CGG	K411D	GAT	A419W	TGG	V428H	CAT	P437D	GAT	I445R	AGG
C402S	TCT	K411E	GAG	A419Y	TAT	V428L	CTT	P437F	TTT	I445S	AGT
C402T	ACG	K411F	TTT	V420A	GCT	V428M	ATG	P437G	GGT	I445T	ACT
C402V	GTT	K411G	GGG	V420D	GAT	V428N	AAT	P437H	CAT	I445V	GTG
C402W	TGG	K411H	CAT	V420F	TTT	V428P	CCT	P437I	ATT	I445W	TGG
C402Y	TAT	K411I	ATT	V420G	GGT	V428R	CGG	P437K	AAG	I445Y	TAT
Y403A	GCT	K411L	CTG	V420H	CAT	V428S	TCG	P437L	CTG	F446A	GCT
Y403C	TGT	K411N	AAT	V420I	ATT	V428T	ACT	P437M	ATG	F446C	TGT
Y403E	GAG	K411P	CCT	V420K	AAG	V428Y	TAT	P437Q	CAG	F446D	GAT
Y403F	TTT	K411R	AGG	V420L	CTT	C429A	GCT	P437R	CGT	F446E	GAG
Y403G	GGT	K411S	TCG	V420N	AAT	C429D	GAT	P437S	TCT	F446G	GGG
Y403H	CAT	K411T	ACT	V420P	CCT	C429G	GGT	P437T	ACT	F446H	CAT
Y403K	AAG	K411V	GTT	V420R	AGG	C429I	ATT	P437W	TGG	F446I	ATT
Y403L	TTG	K411W	TGG	V420S	TCT	C429K	AAG	P437Y	TAT	F446K	AAG
Y403M	ATG	A412D	GAT	V420T	ACT	C429L	TTG	M438A	GCT	F446L	TTG
Y403N	AAT	A412E	GAG	V420W	TGG	C429M	ATG	M438C	TGT	F446M	ATG
Y403P	CCG	A412G	GGG	V420Y	TAT	C429N	AAT	M438D	GAT	F446Q	CAG
Y403Q	CAG	A412H	CAT	D421A	GCT	C429P	CCT	M438E	GAG	F446R	CGG
Y403R	CGG	A412I	ATT	D421E	GAG	C429R	CGG	M438G	GGG	F446T	ACT
Y403S	TCT	A412L	CTG	D421G	GGT	C429S	TCG	M438L	TTG	F446V	GTT
Y403T	ACG	A412N	AAT	D421H	CAT	C429T	ACT	M438N	AAT	F446W	TGG
S404A	GCT	A412P	CCT	D421I	ATT	C429V	GTT	M438P	CCT	Y447D	GAT
S404C	TGT	A412Q	CAG	D421K	AAG	C429W	TGG	M438Q	CAG	Y447E	GAG
S404D	GAT	A412R	CGG	D421L	TTG	C429Y	TAT	M438R	AGG	Y447F	TTT
S404F	TTT	A412S	AGT	D421M	ATG	I430A	GCT	M438S	TCG	Y447G	GGT
S404G	GGT	A412V	GTT	D421N	AAT	I430D	GAT	M438T	ACT	Y447I	ATT
S404H	CAT	A412W	TGG	D421Q	CAG	I430E	GAG	M438V	GTG	Y447K	AAG
S404L	CTT	D413A	GCG	D421R	CGG	I430G	GGG	M438W	TGG	Y447L	CTT
S404M	ATG	D413E	GAG	D421S	TCG	I430H	CAT	M438Y	TAT	Y447M	ATG
S404N	AAT	D413F	TTT	D421T	ACT	I430K	AAG	E439A	GCT	Y447N	AAT
S404P	CCT	D413G	GGT	D421W	TGG	I430L	TTG	E439C	TGT	Y447P	CCT
S404R	AGG	D413H	CAT	D421Y	TAT	I430M	ATG	E439F	TTT	Y447Q	CAG
S404T	ACG	D413I	ATT	V422A	GCT	I430N	AAT	E439G	GGG	Y447R	AGG
S404V	GTG	D413K	AAG	V422C	TGT	I430P	CCT	E439H	CAT	Y447T	ACT
S404W	TGG	D413L	CTG	V422D	GAT	I430R	AGG	E439K	AAG	Y447V	GTT
S404Y	TAT	D413N	AAT	V422E	GAG	I430S	TCT	E439L	CTT	Y447W	TGG
T405A	GCG	D413P	CCG	V422G	GGG	I430T	ACT	E439N	AAT		
T405C	TGT	D413Q	CAG	V422H	CAT	I430V	GTT	E439P	CCT		
T405F	TTT	D413R	CGT	V422I	ATT	I430W	TGG	E439Q	CAG		
T405G	GGG	D413S	TCG	V422L	CTG	D431A	GCT	E439R	CGG		
T405I	ATT	D413T	ACT	V422M	ATG	D431E	GAG	E439S	TCG		
T405K	AAG	D413W	TGG	V422N	AAT	D431G	GGT	E439T	ACT		
T405L	TTG	V414A	GCG	V422P	CCT	D431H	CAT	E439V	GTT		
T405M	ATG	V414D	GAT	V422Q	CAG	D431I	ATT	E439W	TGG		
T405P	CCG	V414E	GAG	V422R	CGT	D431K	AAG	T440A	GCT		
T405Q	CAG	V414F	TTT	V422S	TCG	D431L	CTT	T440D	GAT		
T405R	CGT	V414G	GGT	V422T	ACT	D431N	AAT	T440E	GAG		
T405S	TCT	V414H	CAT	V422W	TGG	D431P	CCT	T440F	TTT		
T405V	GTG	V414I	ATT	V422Y	TAT	D431Q	CAG	T440G	GGG		
T405W	TGG	V414K	AAG	C423A	GCT	D431R	CGT	T440H	CAT		

TABLE 8-continued

PH20 Variants											
mut	cod	mut	cod	mut	cod	mut	cod	mut	cod	mut	cod
T405Y	TAT	V414L	TTG	C423D	GAT	D431S	TCT	T440I	ATT		
L406A	GCT	V414M	ATG	C423E	GAG	D431V	GTT	T440L	CTT		
L406C	TGT	V414Q	CAG	C423F	TTT	D431W	TGG	T440M	ATG		
L406D	GAT	V414R	AGG	C423G	GGG	D431Y	TAT	T440P	CCT		
L406E	GAG	V414S	TCG	C423H	CAT	A432C	TGT	T440Q	CAG		
L406F	TTT	V414T	ACT	C423L	CTG	A432E	GAG	T440R	AGG		
L406G	GGT	V414Y	TAT	C423M	ATG	A432F	TTT	T440S	AGT		
L406I	ATT	K415A	GCG	C423P	CCT	A432G	GGG	T440V	GTG		
L406N	AAT	K415C	TGT	C423Q	CAG	A432H	CAT	T440Y	TAT		
		K415D	GAT	C423R	AGG	A432I	ATT	E441A	GCT		
		K415E	GAG	C423S	TCG	A432K	AAG	E441C	TGT		

2. Expression

For expression of each mutant, HZ24-PH20-IRES-SEAP plasmid DNA containing cDNA encoding one of the variant PH20 or encoding wildtype PH20 was transfected into monolayer CHO—S cells (Invitrogen, Cat. No. 11619-012) using Lipofectamine 2000 (Invitrogen, Cat. No. 11668-027) according to the protocol suggested by the manufacturer. CHO—S cells were seeded the night before transfection and grown in DMEM with 10% FBS to be 80% confluent the next day. Then, the medium of the CHO—S cells was replaced with Opti-MEM. A mixture of plasmid DNA and lipofectamine was made (0.2 μg DNA and 0.5 μL Lipofetamine). The Lipofectamine/DNA mixture was added to CHO—S cells and incubated overnight. The next day, the cells were supplemented with CD-CHO serum free medium (Invitrogen, Cat. No. 10743-029). Supernatant from transfected cells was collected at various time points after transfection, and generally 96 hours after transfection. The supernatant, containing the variant PH20 protein or wildtype PH20 having a sequence of amino acids set forth in SEQ ID NO:3, was stored at −20° C. Activities of the supernatants were screened as described in the following examples.

Example 3

Screening of Library with a Hyaluronidase Activity Assay to Identify Activity Mutants

In this example, supernatants of expressed PH20 variants generated in Example 2 were screened using a hyaluronidase activity assay to assess activity of each mutant. In addition, activity of the secreted alkaline phosphatase (SEAP) was also measured to allow for normalizing PH20 activity of the expressed mutants to the PH20 wildtype. Active and inactive mutants were identified.

1. Generation of Biotinylated HA (bHA) Substrate

A 1.2-MDa FLA (Lifecore) was biotinylated for use as a substrate in the hyaluronidase activity assay. First, 1.2 grams (g) of 1.2 MDa HA was dissovled at 4° C. in 600 mL ddH$_2$O for a week at a concentration of 2 mg/mL with stirring. Next, 645.71 mg Biotin Hydrazide was dissolved in 100 mL DMSO to a concentration of 25 mM (6.458 mg/mL, 247.8 mg in 38.37 mL DMSO). The biotin solution was warmed briefly at 37° C. until the solution was clear. Also, 368.61 mg Sulfo-NHS in 20 mL ddH$_2$O was dissolved to make a 100× solution (18.4 mg/mL Sulfo-NHS). A 30 mM (1000×) water-soluble carbodiimide EDC solution was made by dissolving 17.63 mg EDC in 3 mL ddH20 at a concentration of 5.7513 mg/mL right before the reaction was started.

To four (4) 1000-mL sterile capped bottles, the following components were added at room temperature (RT) and in the following order with stirring: 1) 200 mL of 2 mg/mL HA solution; 2) 80 mL of 0.5M MES, pH 5.0 with gentle mixing; and 3) 91.6 mL of ddH$_2$O with gentle mixing. Next, 24 mL of 25 mM Biotin-Hydrazide and 4 mL of 100× Sulfo-NHS solution were added sequentially, immediately followed by the addition of 500 μL EDC. After the addition of each component, the solution was mixed by inverting three times and stirring. After the addition of the last component, the solution was mixed by stirring overnight at 4° C. Then, Guanidine hydrochloride was added to a final concentration of 4 M by adding 38.2 g per 100 mL and was allowed to dissolve completely before adjusting the solution volume to 600 mL with ddH$_2$O.

For dialysis, 200 mL from each batch of the conjugated HA guanidine hydrochloride solution was transferred into dialysis membranes. Over the course of three days, the solution was dialyzed against ddH$_2$O with a change in ddH$_2$O at least six times. The resulting volume of about 840 mL was adjusted to a final volume of 1000 mL with ddH$_2$O. The final concentration of the biotinylated hyaluronan (bHA) was 0.4 mg/mL.

2. Hyaluronidase Activity Assay

The enzyme assay was a modification of the method described by Frost et al. (1997) (A Microtiter-Based Assay for Hyaluronidase Activity Not Requiring Specialized Reagents. Analytical Biochemistry (1997) 251:263-269) that provides a measure of PH20 hyaluronidase activity.

First, biotinylated HA (bHA) substrate was bound to plastic microtiter plates to generate assay plates. Briefly, 100 μl of b-HA at 1 mg/mL in 0.5 M carbonate buffer (pH 9.6) was dispensed into each well of a high bind microplate (Immunolon 4 HBX extra high binding; Thermo Scientific). The plate was covered with a plate sealer and stored between 2-8° C. for 24-48 hours.

Then, the assay plate was washed with 1× phosphate buffered saline (PBS) wash buffer containing 0.05% (v/v) Tween 20 (PBST). PBST was generated from 1×PBS (generated from Catalog No. P5368, Sigma (10 mM Phosphate Buffer, 2.7 mM Potassium Chloride, 137 mM Sodium Chloride, pH 7.4) by placing the contents of one packet of PBS into a 1-L graduated cylinder with 800 mL deionized water, dissolved by stirring or shaking and adding sufficient quantity of water to 1 L) by adding 500 μl Tween 20 (Catalog No. 6505; EMD Bioscience) to 900 mL of 1×PBS and adding sufficient quantity of water to 1 L. Washing was done using the BioTek ELx405 Select CW plate washer (BioTek) by washing five (5) times with 300 μl PBST wash buffer per well for each wash. At the end of each wash, the plate was tapped on a paper towel to remove excess liquid from each well. Prior to incubation with samples, 200 μl Blocking Buffer (1.0% w/v Bovine Serum Albumin (BSA) in PBS) was added to each well and the assay plate was incubated at 37° C. for approximately 1 hour prior. The Blocking buffer was generated by adding 2.5 g of BSA (Catalog No. 001-000-162; Jackson Immuno Research) to 200 mL 1×PBS, stirring, adding a sufficient quantity of 1×PBS to 250 mL and filtering through an 0.2 μM PES filter unit.

Transfected variant or wildtype PH20 supernatants generated as described in Example 1 were diluted in duplicate 1:25 in assay diluent buffer (pH 7.4 HEPES buffer; 10 mM HEPES, 50 mM NaCl, 1 mM $CaCl_2$, 1 mg/mL BSA, pH 7.4°, 0.05% Tween-20) in uncoated 4×HB high bound microplates. For the standard curve, 1:3 serial dilutions of rHuPH20 (generated as described in Example 1 with a specific activity of 145 U/mL) were made in assay diluent buffer in duplicate starting from 3 U/mL for standards as follows: 3 U/mL, 1 U/mL, 1/3 U/mL, 1/9 U/mL, 1/27 U/mL, 1/81 U/mL, and 1/243 U/mL. One hundred microliters (100 μl) of each standard and sample were transferred to the assay plates and incubated for approximately 1.5 hours at 37° C.

After the incubation, the plate was washed with PBST using the BioTek ELx405 Select CW plate washer by washing five (5) times with 300 μl PBST wash buffer per well for each wash. At the end of each wash, the plate was tapped on a paper towel to remove excess liquid from each well. Then, 100 μl of 1:5000 diluted Streptavidin-HRP (SA-HRP) was added to each well of the plate and incubated at ambient temperature for approximately 1 hour. For the dilution, a 1 mg/mL stock of Streptavidin-HRP conjugate (Catalog No. 21126; Thermo Scientific) was diluted 1:5000 into dilution buffer (1 mg/mL BSA, 0.025% Tween20, 137 mM NaCl, 20 mM Tris pH 7.5). After the incubation, the plate was washed with PBST using the BioTek ELx405 Select CW plate washer by washing five (5) times with 300 μl PBST wash buffer per well for each wash. At the end of each wash, the plate was tapped on a paper towel to remove excess liquid from each well. Then, 100 μl of TMB solution (Catalog No. 52-00-03, KPL; ambient temperature and protected from light) was added to each well for approximately five (5) minutes at room temperature or until an optimal color development was yielded. To stop the reaction, 100 μl 1.0 N Sulfuric Acid or TMB Stop solution (Catalog No. 50-85-06) were added to each well and the plates tapped to mix. Optical density was measured at 450 nm within 30 minutes of adding the stop solution. Since more PH20 in a standard or sample would lead to less bHA available to bind SA-HRP, the optical density (450 nm) value was inversely proportional to the concentration of hyaluronidase activity in each specimen.

3. SEAP Activity

Activity of secreted alkaline phosphatase (SEAP) in the cell culture supernatant also was measured using a colorimetric assay of placental alkaline phosphatase using pNPP as a phosphatase substrate (Anaspec SensoLyte pNPP SEAP kit; Catalog No. 72144, Anaspec) according to the manufacturer's instructions. The absorbance signal was measured at optical density (OD) of 405 nm.

The criteria for the high throughput (HTP) screening were that the transfected supernatant resulted in a SEAP signal of ≥0.1 and the signal for the rHuPH20 wildtype control produced a signal of ≥1 U/mL. Also, the criteria for each screen were that the standard curves had a signal to noise ratio (S/N) for the 0 U/mL standard versus the 3 u/mL standard at OD_{405} of ≥5, had less than three (3) standards with a coefficient of variation (CV) ≥10%, and at least four (4) of the standards were in the linear range.

Example 4

Selected PH20 Variants with Altered Hyaluronidase Activity

Each generated variant was screened for hyaluonidase activity as described in Example 3. The SEAP expression was used to normalize PH20 activity of each variant to the PH20 wildtype. Mutants were identified that exhibited altered hyaluronidase activity compared to wildtype.

1. Active Mutants

Active mutants were selected whereby at least one duplicate sample exhibited greater than 40% of wildtype activity when normalized to SEAP activity. The identified active mutants are set forth in Table 9. The Table sets forth the amino acid replacement compared to the sequence of amino acids of PH20 set forth in SEQ ID NO:3. The amino acid sequence of exemplary mutants also is set forth by reference to a SEQ ID NO. The Table also sets forth the average hyaluronidase activity of tested duplicates normalized by SEAP values compared to average of wildtype PH20 activities in each plate, which were also normalized by their own SEAP values. For example, a value of 0.40 indicates that the variant exhibits 40% of the hyaluronidase activity of wildtype PH20, a value of 1 indicates that the variant exhibits a similar hyaluronidase activity of wildtype and a value of 3.00 indicates that the variant exhibits 300% of the hyaluronidase activity of wildtype PH20 or 3-fold increased activity compared to wildtype.

The results in Table 9 show that over 600 tested mutants exhibit activity that is increased compared to wildtype. For example, about 536 mutants exhibit 120% or greater than 120% of the hyaluronidase activity of wildtype PH20 and about 75 of the mutants exhibit 300% or greater than 300% of the hyaluronidase activity of wildtype PH20. In particular, the results in Table 9 show that that hyaluronidase activity compared to wildtype of mutant S69A is about 22-fold; mutant S69R is about 14-fold; mutant I70A is about 27-fold; mutant I70K is about 14-fold; mutant I70R is about 14-fold; and mutant I271L is about 10-fold.

TABLE 9

ACTIVE MUTANTS

mutant	SEQ ID NO	AvgNorm Act.
L001A	74	0.95
L001C		0.89
L001E	75	0.55
L001F		0.41
L001G	76	0.62
L001H	73	1.90
L001K	77	1.39
L001N		0.87
L001P		0.92
L001Q	78	3.27
L001R	79	0.72
L001S		0.74
L001T		0.99
L001V		1.00
L001W		0.88
N002A		0.61
N002C		0.4
G291C		0.27
N002G		0.44

TABLE 9-continued

ACTIVE MUTANTS

mutant	SEQ ID NO	AvgNorm Act.
N002L		0.46
N002P		0.54
N002Q		0.84
N002S		0.78
N002T		1.05
N002V		0.65
F003E		0.42
F003H		0.68
F003L		0.59
F003Y		0.50
R004A		0.73
R004I		0.54
R004S		0.60
R004T		0.66
R004V		1.09
A005H		0.44
P006A	80	0.78
P006H		0.58
P006K		0.80
P006L		0.76
P006N		0.40
P006Q		0.89
P006R		0.56
P007M		0.57
V008I		1.17
V008L		0.53
V008M	81	0.47
V008P		0.33
I009K		0.69
I009L		1.08
I009R		0.53
I009S		0.98
I009V		0.84
P010D		0.62
P010E		0.66
P010G	83	0.55
P010H	84	0.43
P010N		0.55
P010Q		0.89
P010R		0.73
P010S		0.55
P010W		0.59
N011D		0.54
N011G		0.45
N011H		0.69
N011K		0.58
N011S	85	0.39
M310F		0.30
V012A		0.56
V012E	86	1.86
V012I	87	0.68
V012K	88	0.65
V012L		0.44
V012N		0.46
V012R		0.50
V012S		0.75
V012T	89	1.50
P013H		0.46
P013S		0.68
P013T		0.90
P013Y		0.51
F014D		0.64
F014I		0.42
F014M		0.47
F014V	90	0.46
L015A		0.65
L015M	92	0.45
L015V	91	2.20
A020S	93	0.50
S022H		0.57
S022M		0.49
S022T	94	0.48
S022Y		0.45
E023D		0.97
F024A		0.69
F024E	95	3.99
F024G		0.75
F024H	96	2.07
F024I		0.70
F024K		0.96
F024L		0.62
F024M		0.85
F024N		0.60
F024R	97	1.22
F024T		1.18
F024V		1.15
F024Y		0.90
L026A	98	1.30
L026E	99	3.22
L026G		0.81
L026H		0.97
L026I		0.51
L026K	100	1.88
L026M	101	1.43
L026P		0.55
L026Q	102	1.44
L026R	103	1.43
L026S		0.78
L026T		0.87
L026V		0.52
L026W		0.53
L026Y		0.52
G027A		0.79
G027D	104	1.22
G027E		1.18
G027F		0.61
G027H		1.11
G027I		0.41
G027K	105	2.71
G027L		0.76
G027P		0.46
G027Q		1.12
G027R	106	1.88
G027S		0.94
G027T		0.61
G027W		0.76
K028A		0.78
K028D		0.62
K028E		0.54
K028F		0.75
K028I		0.55
K028L		0.51
K028M		0.67
K028N		0.58
K028P		0.40
K028R	107	0.71
K028S		0.46
K028T		0.68
K028V		0.76
K028W		0.51
F029A		0.90
F029E	108	4.03
F029G		1.05
F029H		0.82
F029I	109	1.53
F029K	110	1.34
F029L	111	2.36
F029M	112	2.08
F029P	113	3.79
F029R	114	1.24
F029S	115	2.21
F029T	116	0.85
F029V	117	1.65
F029W		0.48
D030A		1.12
D030F		0.84

TABLE 9-continued

ACTIVE MUTANTS		
mutant	SEQ ID NO	AvgNorm Act.
D030G	118	2.02
D030H	119	1.69
D030K	120	2.63
D030L	121	1.32
D030M	122	1.85
D030P		1.19
D030Q		0.84
D030R	123	1.82
D030S	124	1.62
D030T		0.57
D030V		0.46
D030W		0.62
E031A	125	2.05
E031C	126	2.95
E031G	127	1.27
E031H	128	2.74
E031I	129	3.89
E031K	130	3.13
E031L	131	2.62
E031P	132	1.51
E031R	133	2.27
E031S	134	1.70
E031T	135	3.96
E031V	136	4.57
E031W	137	1.26
E031Y		1.13
P032A		0.92
P032C	138	0.40
P032F	139	2.71
I326C		0.39
I331C		0.27
P032G	140	1.60
P032H	141	2.08
P032K		1.04
P032L		0.82
P032M		0.67
P032N		0.70
P032Q		1.11
P032R		1.17
P032S		1.01
P032T		0.77
P032V		0.81
P032W		0.54
P032Y		1.01
L033G	143	0.57
L033M		0.69
L033P		0.87
L033Q		0.45
L033R		0.61
L033S		0.48
L033T		0.45
L033W	142	1.58
D034A		0.38
D034E		0.58
D034H		0.41
D034K		0.54
D034Q		0.59
D034R		1.17
D034W	144	0.46
M035F		0.87
M035H		0.60
M035L		0.52
M035T		0.83
M035Y		0.78
S036A		0.45
S036D		0.32
S036G		0.64
S036H	147	0.54
S036K		0.83
S036L		0.71
S036R		1.09
Q347L		0.39
V351Q		0.34
S036T		0.51

TABLE 9-continued

ACTIVE MUTANTS		
mutant	SEQ ID NO	AvgNorm Act.
L037F	149	3.33
L037I		0.62
L037K		0.43
L037M	150	1.46
L037P		0.63
L037R		0.51
L037V		0.57
F038Y	151	1.29
S039A	152	1.06
S039L	153	0.80
S039N	154	2.32
S039Q		1.10
S039R		0.56
S039T	155	1.57
S039Y		0.56
F040L	156	0.92
F040W		1.11
I041A		0.67
I041C		0.53
I041D		0.78
I041E		0.51
I041G		0.76
I041H		0.77
I041N		0.40
I041T	157	1.47
I041V		0.73
I041W		0.66
G042A		0.64
S043T		0.43
P044E		0.59
R045I		0.45
R045K		0.53
I046A		1.04
I046C		0.37
I046E		0.43
I046F		0.73
I046H		0.82
I046L	158	1.08
I046M		1.00
I046N		0.66
I046R	159	2.29
I046S		0.64
I046T		0.55
I046V		1.01
I046Y		0.76
N047A		0.48
N047D	160	0.82
N047F	161	1.32
N047G		0.82
N047H		1.16
N047K		0.67
N047M		0.77
N047Q		0.69
N047R		0.84
N047S		0.85
N047T	162	1.49
N047W	163	0.63
N047Y		0.45
A048F	164	2.51
A048G		0.83
A048H	165	1.99
A048I		0.64
A048K	166	1.28
A048M		0.76
A048N	167	4.25
A048Q		1.05
A048R		0.66
A048S		1.06
A048V		0.60
A048Y		0.81
T049I		0.42
T049K		0.85
T049R	168	1.41
T049S		0.92

TABLE 9-continued

ACTIVE MUTANTS		
mutant	SEQ ID NO	AvgNorm Act.
T049V		0.45
G050A		0.93
G050C		0.41
G050D	169	1.37
G050E		0.78
G050H		0.74
G050L		0.43
G050M	171	0.47
G050Q		0.86
G050R		0.86
G050S	170	1.24
G050V		0.3
G050Y		0.58
Q051N		0.60
Q051S		0.46
G052N	172	0.89
G052P		0.43
G052Q	173	3.71
G052R	174	0.53
G052S	175	1.32
E375I		0.36
F380V		0.39
G052T	176	0.49
T054A		0.43
T054F		0.56
T054N		0.48
T054Q		0.91
T054S		0.70
T054V		0.66
V058C	177	0.55
V058G		0.54
V058H	183	1.09
V058I		0.57
V058K	178	4.08
V058L	179	1.54
V058N	184	0.49
V058P	180	0.90
V058Q	181	4.54
V058R	182	1.92
V058S		0.83
V058W		0.65
V058Y	185	1.07
D059Q		0.40
D059N	186	1.27
R060K		0.69
L061I		0.42
L061M		0.73
L061V		0.59
Y063A		0.63
Y063H		1.07
Y063I		1.03
Y063K	187	1.36
Y063L	188	1.33
Y063M	189	1.32
Y063N		0.96
Y063R	190	1.40
Y063S		1.00
Y063T		1.07
Y063V		0.43
Y063W	191	1.53
P065R		0.57
Y066H		0.47
Y066R		0.51
I067F		1.00
I067L		0.45
I067R		0.24
I067V	192	1.80
I067Y		0.55
D068E		0.72
D068H	193	2.06
D068K		1.08
D068L		0.43
D068P	194	0.50

TABLE 9-continued

ACTIVE MUTANTS		
mutant	SEQ ID NO	AvgNorm Act.
D068Q	195	1.67
D068R		0.70
D068S		0.81
D068T		0.75
S069A	196	22.06
S069C	197	1.97
S069E	198	1.48
S069F	199	8.75
S069G	200	6.06
S069I	201	3.12
S069L	202	3.44
S069M	203	2.67
S069P	204	8.14
S069R	205	14.06
S069T	206	0.58
S069W	207	2.18
S069Y	208	2.71
I070A	209	27.00
I070C	210	2.57
I070F	211	5.69
I070G	212	6.22
I070H	213	9.09
I070K	214	14.64
I070L	215	3.05
I070N	216	6.19
I070P	217	3.03
I070R	218	13.95
I070S	219	3.63
I070T	220	5.43
I070V	221	6.34
I070Y	222	1.26
T071A		0.86
T071D		0.50
T071G	223	1.41
T071H		0.93
T071L		1.09
T071M		0.89
T071N	224	1.21
T071Q		0.68
T071R	225	2.17
T071S	226	1.54
G072A		0.45
G072D		0.60
S395W		0.4
G072E		0.69
G072H		0.46
G072K	227	1.39
G072L		0.43
G072M	228	3.11
G072Q	229	2.33
G072R		0.65
G072S		0.51
V073A	230	1.38
V073C		0.84
V073D		0.94
V073G		1.17
V073H	231	1.54
V073K	232	1.42
V073L	233	1.59
V073M		0.68
V073Q	234	0.96
V073R	235	0.72
V073S		0.86
K297R		0.34
S401Q		0.39
V073T	236	1.34
V073W	237	1.91
T074A	238	2.28
T074C	239	2.18
T074E	240	1.38
T074F	241	1.43
T074G	242	2.75
T074H	243	1.40
T074K	244	1.29

TABLE 9-continued

ACTIVE MUTANTS		
mutant	SEQ ID NO	AvgNorm Act.
T074L	245	1.43
T074M	246	0.52
T074N	247	2.12
T074P	248	2.45
T074R	249	2.22
T074S	250	1.80
T074V	251	2.27
T074W	252	2.13
V075A		0.71
V075C		0.46
V075F	253	2.00
V075H		0.62
V075L	254	5.22
V075M	255	1.16
V075N		0.81
V075Q		1.51
V075R	256	3.02
V075S		0.76
V075T	257	4.34
V075Y		0.63
G077H		0.32
I079L	258	1.44
I079T		0.79
I079V		1.01
Q081P		0.60
K082A		0.94
K082E		0.50
K082G		0.64
K082H		0.44
K082I		1.01
K082L	259	0.87
K082M		0.58
K082N	260	0.96
K082Q		0.76
K082R		0.85
K082S		0.62
K082T		0.56
K082Y		0.32
K082V		0.57
I083F		0.57
I083G	264	1.05
I083L		0.93
I083N		0.82
I083Q	262	1.07
I083R		0.45
I083S	263	0.79
I083T		0.95
I083V	261	0.99
S084D		0.98
S084E	265	0.52
S084F	266	0.72
S084G	267	8.68
S084H		0.96
S084I		0.90
S084L		0.92
S084M		0.77
S084N	268	0.89
S084P		0.57
S084Q		0.86
S084R	269	1.89
S084T		0.82
S084W		0.86
S084Y		0.30
S221I		0.35
L085V		0.42
Q086A	270	2.70
Q086D		0.88
Q086E		1.18
Q086F		0.54
Q086G		1.02
Q086H	271	1.70
Q086I		0.65

TABLE 9-continued

ACTIVE MUTANTS		
mutant	SEQ ID NO	AvgNorm Act.
Q086K	272	0.97
Q086L		0.92
Q086M		1.06
Q086N	273	1.28
Q086P		0.42
Q086R		0.93
Q086S	274	0.85
Q086T	275	0.58
Q086V		0.97
Q086W	276	1.21
D087A		1.00
D087C	277	1.77
D087E		0.86
D087G	278	1.00
D087H		0.72
D087I		0.53
D087L	279	0.55
D087M	280	0.58
D087P		0.31
D087Q		1.05
D087R	281	1.28
D087S	282	0.99
D087T	283	1.70
A412H		0.39
D413H		0.31
V414K		0.3
K415V		0.39
D087V	284	0.66
D087Y	285	2.72
L089C	286	1.46
L089R		0.34
L089K		0.45
L089M		0.63
D090A	287	1.48
D090E	288	1.15
D090G		0.41
D090H	289	1.24
D090I		1.10
D090K	290	1.36
D090L		1.15
D090N	291	1.18
D090Q		1.11
D090R	292	1.49
D090S		1.15
D090T		1.02
D090W		0.81
K091A		0.89
K091Q		0.43
K091R		0.67
A092C	293	1.97
A092H		0.22
A092L	294	1.29
A092M		0.86
A092T		0.70
A092V		1.09
K093D		0.71
K093E		0.83
K093F		0.50
K093G		0.97
K093H		0.61
K093I	295	3.25
R248H		0.4
K093L	296	1.53
K093M		0.70
K093N		0.71
K093Q	297	0.84
K093R	298	1.52
K093S	299	1.25
K093T	300	3.93
K093V		0.24
K094A		0.64
K094D	301	0.93
K094E		0.79
K094F		0.59

TABLE 9-continued

ACTIVE MUTANTS		
mutant	SEQ ID NO	AvgNorm Act.
K094H		0.72
K094L		0.52
K094M		0.66
K094N		0.99
K094Q	302	1.22
K094R	303	3.94
K094S		0.94
K094T		1.14
I096D		0.69
I096L		0.46
I096V		0.68
T097A	304	1.25
T097C	305	0.53
T097D	306	1.31
T097E	307	1.19
T097F		0.75
P257C		0.36
D426K		0.26
G427H		0.35
T097G	308	4.84
T097I		0.85
T097L	309	1.22
T097N		1.10
T097P		0.62
T097Q		1.17
T097R		0.95
T097S	310	1.21
T097W		0.53
T097Y		0.74
F098A		0.60
F098C		0.58
F098D		0.47
F098E		0.44
F098H		1.06
F098I		0.52
F098L		0.58
F098M		0.87
F098Q		0.65
P436C		0.39
F098R		0.72
F098S		0.56
F098V		0.46
F098W		0.81
Y099A		0.33
Y099R		0.53
Y099S		0.43
V102A		0.83
V102C		0.69
V102E		0.90
V102G		0.67
V102H		0.88
V102K		1.03
V102L		0.71
V102M		0.77
V102N		1.02
V102Q		1.03
V102R		0.94
V102S	311	1.41
V102T	312	1.26
V102W		0.76
D103N		0.39
N104A		0.69
N104C		0.41
N104G		0.48
N104K		0.88
N104M		0.61
N104R	313	1.25
N104S		1.03
N104T		0.71
L105A		0.54
L105G		0.51
L105I		0.94
L105P		0.84
L105Q		0.90

TABLE 9-continued

ACTIVE MUTANTS		
mutant	SEQ ID NO	AvgNorm Act.
L105R		0.65
L105S		0.61
L105T		0.51
L105W		0.34
L105V		0.99
G106V		0.43
M107F		0.91
M107I		0.67
M107L	314	1.32
A108G		0.47
I110V		0.51
E114A	315	1.44
E114G		0.73
E114H		0.75
E114M		0.44
E114S		0.69
P117D		0.56
T118H		0.47
T118K		0.53
T118L		1.09
T118M		0.53
T118N		0.67
T118Q	316	3.37
T118V		0.79
W119F		0.53
W119P		0.36
W119Y		1.08
A120D		0.76
A120F	318	2.62
A120G		1.03
A120H	317	1.11
A120I	319	1.33
A120L		1.25
A120N		0.81
A120P		0.42
A120R		0.82
A120S	320	1.21
A120T		0.62
A120V	321	1.53
A120W		0.59
A120Y	322	1.95
N122M		0.56
K124L		0.34
K124R		0.62
P125H		0.43
P125R		0.63
P125S		0.54
D127A		0.89
D127E	323	1.31
D127G		0.97
D127H	324	2.33
D127L		0.84
D127M		0.4
D127N	325	1.69
D127Q	326	1.21
D127R	327	0.51
D127S		0.77
D127T		1.11
D127V		0.56
D127W		0.44
V128A		0.53
V128C		0.68
V128G		0.49
V128I	328	1.25
V128K		1.16
V128L		0.95
V128Q		0.55
V128R		0.74
V128S		0.53
V128W		0.50
K130I		0.50
K130R	329	1.42
N131C		0.60

TABLE 9-continued

ACTIVE MUTANTS

mutant	SEQ ID NO	AvgNorm Act.
N131E		0.44
N131F		0.63
N131G	330	2.47
N131H		0.80
N131I	331	1.40
N131L		0.82
N131M	332	0.99
N131Q	333	1.24
N131R	334	2.81
N131S		0.76
N131T		1.02
N131V	335	2.08
N131Y		0.85
R132A		0.68
R132C		0.58
R132E		0.70
R132F		0.60
R132H		0.66
K279A		0.27
E285A		0.34
R132I		0.56
R132K		1.05
R132L	337	0.76
R132N	336	1.28
R132Q		0.69
R132S		0.79
R132T		0.61
R132V		0.73
R132Y		0.78
S133I		0.54
I134L		1.04
I134T		0.60
I134V		1.08
E135A		0.99
E135C		0.77
E135D	338	2.68
E135F		0.73
E442L		0.4
E135G	339	2.79
E135H		0.79
E135K		1.15
E135L		0.82
E135N		0.56
E135Q		1.59
E135R	340	2.08
E135S		1.13
E135W		0.63
E135Y		0.50
L136A		0.73
L136C		0.56
L136D		0.47
L136F		0.96
L136H		1.00
L136I		0.65
L136M		1.05
L136N		0.48
L136Q		0.61
L136R		0.74
L136S		0.80
L136T		0.72
L136W		1.11
V137A		0.48
V137I		1.01
V137T		0.51
Q138A		0.69
Q138C		0.65
Q138H		0.71
Q138I		0.54
Q138L	341	0.59
Q138M		0.68
Q138N		0.61
Q138R		0.53
Q138S		0.48
Q138W		0.41

TABLE 9-continued

ACTIVE MUTANTS

mutant	SEQ ID NO	AvgNorm Act.
Q138Y		0.60
Q139A		0.92
Q139C		0.44
Q139D		0.48
Q139E		0.94
Q139F		0.53
Q139G		0.65
Q139H		0.56
Q139K		0.73
Q139L		0.70
Q139M		0.95
Q139R		0.79
Q139S		0.81
Q139T	342	1.31
Q139V		0.77
Q140A		0.96
Q140C		0.50
Q140D		0.59
Q140F		0.66
Q140G		0.73
Q140H		0.84
Q140I		0.75
Q140K	343	0.93
Q140L		0.51
Q140M		0.80
Q140R		0.85
Q140V		0.61
Q140W		0.59
Q140Y		0.41
N141A		1.12
N141D		1.09
N141E		0.67
N141F		0.81
N141G		1.15
N141H	344	2.03
N002I		0.37
G297A		0.57
N141L		0.61
N141M		0.48
N141Q		1.16
N141R	345	1.40
N141S	346	0.72
N141T		0.45
N141V		0.50
N141W	347	0.83
N141Y	348	1.55
V142C		0.61
V142D	349	0.71
V142E		0.87
V142G	350	0.98
V142H		1.11
V142I		0.81
V142K	351	1.40
V142L		0.75
V142M		0.76
V142N	352	0.98
V142P	353	0.88
V142Q	354	1.04
V142R	355	1.53
V142S	356	0.93
V142T	357	1.19
Q143E		0.77
Q143G	358	0.62
Q143I		0.44
Q143K	359	1.30
I009Q	82	0.4
Q143L		0.56
Q143N		0.73
Q143V		0.57
L144T	361	1.02
L144W		0.79
S145A		0.58
S145C		0.44
S145D		0.48

TABLE 9-continued

ACTIVE MUTANTS

mutant	SEQ ID NO	AvgNorm Act.
S145E		0.56
S145G		0.94
S145H		0.56
S145L		0.44
S145M		0.56
S145N		0.58
S145P		1.04
S145R		0.97
L146A		0.52
L146C		0.42
G305N		0.36
M310Y		0.38
L146E		0.50
L146G		0.62
L146H		0.78
L146I		0.82
L146K		0.84
L146N		0.57
L146P	362	0.93
L146Q		0.84
L146R	363	1.47
L146S		0.71
L146T		0.74
L146V		0.84
L146Y		0.80
S312K		0.38
T147A	364	1.20
T147C		0.47
T147D		0.71
T147F	365	1.24
T147G		1.05
T147I		0.85
T147L	366	1.30
T147M		0.79
T147P		1.09
T147Q		1.29
T147R	367	2.11
T147S	368	1.27
T147V	369	2.04
T147W		0.97
T147Y		1.04
E148C		0.66
E148F		0.42
E148G		1.05
E148H	370	1.24
E148I		0.73
E148K	371	1.63
E148L		0.85
E148Q	372	1.44
E148R		0.97
E148S		1.15
E148T		0.82
E148V		0.99
E148W		0.43
E148Y		0.95
A149C		1.15
A149G		0.52
A149K		0.51
A149L		0.88
A149M		0.88
A149Q		1.15
A149R		1.02
A149S		1.08
A149T	373	1.24
A149V	374	1.34
T150A	375	1.21
T150C		0.70
T150D	376	1.24
T150E		1.05
T150F		0.71
T150G	377	2.19
T150I		0.52
T150L		0.70

TABLE 9-continued

ACTIVE MUTANTS

mutant	SEQ ID NO	AvgNorm Act.
T150N	378	0.91
T150P		0.88
T150R		0.90
T150S	379	0.92
T150W	380	1.25
T150Y	381	1.36
E151A	382	1.27
E151C		1.00
E151G		1.06
E151H	383	1.34
E151K	384	2.05
E151L	385	1.03
E151M	386	1.26
E151N		0.95
E151Q	387	2.01
D320L		0.37
E151R	388	1.61
E151S	389	1.28
E151T	390	1.21
E151V	391	1.38
E151W	392	1.31
E151Y	393	1.31
K152A		0.51
K152C		0.52
K152F		0.61
K152I		0.65
K152M		0.75
K152R	394	1.85
K152T	395	1.20
K152V		0.82
K152Y		0.67
A153I		0.93
A153L		0.51
K154R		0.86
K154T		0.83
K154V		0.46
Q155A		0.91
Q155C		0.60
Q155D	397	1.49
Q155F		0.70
Q155G	398	1.61
Q155H		1.03
Q155K	399	1.57
Q155L		0.86
Q155M		0.97
Q155R	400	1.27
Q155S		0.77
Q155T		0.76
Q155V		0.73
Q155W		0.91
E156A		0.79
E156D	401	1.95
E156G		0.49
E156I		0.51
E156L		0.43
E156M		0.87
E156Q		0.84
E156R		0.43
E156S		0.62
E156T		0.69
E156V		0.45
E156W		0.49
F157W		0.61
E158A		0.56
E158F		0.51
E158H		0.54
I326S		0.95
I331E		0.34
E158L		0.44
E158Q	402	1.25
E158S	403	0.95
K159A		0.64
K159D		0.52
K159E		0.49

TABLE 9-continued

ACTIVE MUTANTS		
mutant	SEQ ID NO	AvgNorm Act.
K159H		0.74
K159L		0.62
K159M		0.66
K159N		0.73
K159Q		0.92
K159R		0.88
K159S		0.67
K159V		0.41
A160C		0.61
A160F		0.79
A160G		0.75
A160H		0.47
A160I		0.43
A160K		0.91
A160L		0.67
M035Q		0.37
A160M		0.77
A160N		0.56
A160Q		0.65
A160R		0.89
A160S	404	1.35
A160V		0.73
A160Y		1.07
G161A		0.99
G161C		0.44
G161D		0.86
G161E		0.49
G161R		0.48
S036N	148	0.38
G161S		0.77
G161V		0.42
K162A		0.50
K162D		0.77
K162E	405	0.51
V351C		0.35
W357K		0.36
K162G		0.56
K162H		0.62
K162L		0.54
K162M		1.04
K162P		0.64
K162Q		0.58
K162R		0.52
K162S		0.47
K162V		0.52
K162W		1.01
K162Y		0.72
D163A	406	1.52
D163E	407	1.63
D163G		1.15
D163K	408	1.90
D163L		1.18
D163Q	409	1.40
D163R	410	1.80
D163S	411	1.34
D163T		1.13
D163V		0.76
F164L		1.13
F164M	412	1.66
F164V	413	1.23
S043N		0.34
F164W		0.72
L165A		0.48
L165D	414	5.79
L165F	415	1.23
L165N	416	2.19
L165R		0.59
L165S	417	1.31
L165V	418	1.22
L165W		1.14
A371G		0.38
L165Y		0.66
V166A	419	2.85
V166C		1.16

TABLE 9-continued

ACTIVE MUTANTS		
mutant	SEQ ID NO	AvgNorm Act.
V166E	420	1.28
V166F	421	1.67
V166G		1.11
V166H	422	1.74
V166L	423	4.38
V166Q	424	3.61
V166R	425	5.56
V166T	426	4.26
V166W	427	1.26
V166Y	428	2.08
E167A		0.84
E167D	429	0.69
E167G		0.60
E167H		0.89
E167K		0.91
E167M		0.87
E167N		0.83
E167P		0.58
E167R		1.02
E167S		1.17
E167T		0.59
E167Y		0.55
T168H		0.46
I169L	430	2.08
I169R		0.54
I169V		0.74
K170N		0.72
K170R	431	2.58
K170V		0.58
L171I		0.73
L171V		0.64
G172A	432	1.20
G172C		1.03
K173N		0.44
K173R	433	0.82
L174A		1.20
L174G	434	0.40
L174K	435	2.39
L174M		0.79
L174N	436	1.36
L174Q		0.99
L174R	437	1.50
L174S		0.85
L174T	438	1.12
L174V		0.62
L174W		0.78
L174Y		1.06
L175E		0.43
Q051A		0.34
L175H		0.57
L175T	439	1.43
L175V		0.94
L175Y		0.66
R176K		0.67
N178G		0.85
N178K	440	0.85
N178M		0.88
K376L		0.37
F380T		0.39
N178R	441	1.10
H179A		1.06
H179C		0.94
H179E		0.62
H179G		0.86
H179I		0.90
H179K	442	1.39
H179L		0.73
H179M		0.63
H179N		0.96
H179P		0.44
H179R		0.96
H179S		0.51
H179T		0.43
H179V		0.42

TABLE 9-continued

ACTIVE MUTANTS

mutant	SEQ ID NO	AvgNorm Act.
L180F		0.59
L180G		0.62
L180K		0.44
L180M		0.64
W181M		0.88
L061F		0.3
W181Q		0.88
G182L		0.90
Y183L		0.70
F186Y		0.59
H192S		0.49
H192T		0.50
H193G		0.68
H193Q	443	0.82
H193S		0.42
H193Y		0.58
K195A		0.51
K195G		0.45
K195H		0.45
K195I		0.50
K195L		0.45
K195N	445	0.74
K195Q		0.71
K195R		0.85
K195S		0.42
K195T	444	0.58
K195W		0.49
K196E	446	0.43
D068G		0.37
K196G		0.41
K196L		0.65
K196R	447	0.58
K196S		0.68
K196T		1.18
K196W		0.55
P197A		0.81
P197D		0.58
P197E		0.52
P197F		0.48
P197G		0.75
P197H		0.62
P197K		0.99
P197L		0.56
P197M		1.03
P197Q		0.69
P197R		0.58
P197S		0.70
P197T		0.41
G198A		0.80
G198D	448	1.99
G198E		0.49
G198H		0.84
G198L		0.48
G198N		0.80
G198Q		0.55
G198R		0.58
G198S		0.76
G198T		0.41
G198Y		0.81
N200D		0.46
S202M		0.40
F204P	449	0.63
N205A	450	1.30
N205D		0.85
N205E	451	1.94
N205F		0.52
N205G		0.79
N205K		0.76
N205M		0.58
N205P		0.75
N205R		0.54
N205S		0.80
N205T	453	0.85
N205V		0.49
N205W		0.41
V206H		0.50
V206I	454	0.94
V206K	455	1.75
V206L	456	1.57
S395T		0.39
V206M		0.43
V206R	457	1.30
V206S		0.72
G072Y		0.35
V206T		0.59
I208A		0.62
I208C		0.48
I208K		0.91
I208L		0.84
I208M		0.88
I208Q		0.77
I208R		1.14
I208S		0.62
I208T		1.01
I208V		1.07
K209A		0.53
K209E		0.46
K209G		0.44
K209N		0.50
F398L		0.35
S404T		0.37
K209R	458	0.68
K209S		0.50
K209T		0.50
D212N	459	1.52
D212S	460	0.93
D212T		0.76
D213A	461	0.85
D213E		0.79
D213G		0.81
D213H		0.75
D213K		0.82
D213L		0.56
D213M	462	1.56
D213N	463	1.53
D213Q		1.04
D213R		0.92
D213V		0.47
D213W		0.49
D213Y		0.49
L214Q		0.57
S215A		0.74
S215D		0.62
S215E		0.74
S215G		0.88
S215H	464	0.91
S215K		0.99
S215L		0.60
S215M	465	1.77
S215Q		0.79
G077K		0.32
S215R		0.71
S215T		0.80
S215V		0.69
S215W		0.52
W216Y		0.48
L217M		0.51
W218F		0.57
N219A	466	1.29
N219C		0.43
N219D		0.75
N219E		0.95
N219H		0.97
N219I	467	0.60
N219K	468	1.45
N219L		0.72
N219M		1.02

TABLE 9-continued

ACTIVE MUTANTS

mutant	SEQ ID NO	AvgNorm Act.
I083H		0.4
N219R		1.10
N219S	469	2.48
N219T		0.82
N219W		0.48
E220A		0.75
E220H	470	1.40
E220I	471	1.34
E220L	472	1.45
E220S		0.62
E220T		0.91
E220V	473	1.35
S221A		0.72
S221C		0.59
S221M		0.46
S221Q	474	1.37
S221T		0.94
S221V		1.04
T222D		0.43
T222F		0.43
T222G	475	0.49
T222K		0.75
T222L		0.64
T222N		0.80
T222R		0.75
E220D		0.39
T222I		0.4
T222S		0.63
T222V		0.79
L224I		0.61
L230I		0.87
N231T		1.10
T232F	476	0.73
T232S		0.76
Q233A		0.71
Q233F		0.53
Q233G	477	0.46
Q233K	478	1.69
Q233L		0.69
Q233R	479	1.50
Q233Y		0.50
Q234M	480	1.65
S235A	481	0.47
S235E		1.00
S235G		0.95
S235H		0.44
S235K		0.53
S235T		0.66
P236A		1.07
P236G		1.09
P236H		0.46
P236K		0.71
P236R	482	3.09
Q234L		0.40
P236S		0.91
V237A		0.90
V237E	484	1.93
V237F		0.41
A412Q	751	0.35
A413Q		0.38
V414L		0.36
D418G		0.45
V237H	485	0.75
V237L		1.12
V237N		0.67
L089W		0.26
V237Q	486	1.46
V237R		0.71
V237S		1.03
V237T	487	1.01
V237W		0.52
A238D		0.75
A238E	488	0.59

TABLE 9-continued

ACTIVE MUTANTS

mutant	SEQ ID NO	AvgNorm Act.
A238H	489	0.60
A238K		0.60
A238Q		1.02
A238R		0.49
A238S	490	2.62
A238T		0.44
T240K		1.13
T240A	491	0.48
T240M		0.48
T240P		0.56
T240Q	492	0.75
T240R		0.91
A239N		0.32
T240S		0.74
T240V		0.77
Y242F		1.08
N245H		0.50
V247I	493	2.01
V247L		0.83
V247M		0.52
R248A	494	0.43
R248W		0.52
R248Y		0.67
I251Y		0.37
I251L		0.58
I251M		0.43
V253I		0.76
K255A		0.40
K255N		0.52
K255Q		0.91
K255R		0.71
K093P		0.38
K255S		0.43
I256A		0.42
I256H		0.51
I256L		0.64
I256V		0.51
P257A		0.82
P257G	496	0.51
P257I		1.07
P257K		0.92
P257L		0.69
P257M		0.90
P257N		0.69
P257Q		0.61
P257R	498	1.38
P257T	497	2.04
P257V		0.88
D258H		0.84
D258N	499	1.44
D258R		0.45
D258S	500	1.44
D258G		0.39
D426S		0.36
G427I		0.54
A259E		0.85
A259G		0.68
A259I		0.46
A259K		0.76
A259L		0.53
A259N		0.49
A259P	501	1.54
A259Q		0.70
A259R		0.72
A259S		0.63
A259T		0.51
A259V		0.41
A259W		0.55
A259Y		0.51
K260A		0.66
K260D		0.41
K260E		0.58
K260H		0.87
K260L		0.60

TABLE 9-continued

ACTIVE MUTANTS

mutant	SEQ ID NO	AvgNorm Act.
E249V	495	
K260M	502	0.85
K260Q		0.58
K260R		0.83
K260S		0.66
K260G		0.37
K260Y	503	1.73
S261A	504	0.74
S261F		0.73
S261K	505	2.54
S261M		0.56
S261N	506	1.98
S261Q		0.76
S261R		1.19
S261T		0.66
S261V		0.48
S261W		0.44
L263A		0.76
L263K	507	2.73
L263M		0.89
L263R	508	1.63
L263T		0.49
N104I		0.35
L263V		0.75
P264A		0.43
P264H		0.60
V265I		0.58
F266Y		0.58
A267M		0.45
A267T	509	1.34
T269A	510	1.63
T269C		0.75
T269D		0.76
T269S		1.01
R270M		0.46
R270N		0.52
R270S		0.69
I271F		0.72
I271G		1.29
L105C		0.33
I271L	511	10.62
V272E		0.39
I271M	512	3.24
I271S		0.42
I271V		1.05
V272D	513	1.36
V272R		0.74
V272S		0.96
V272T	514	1.61
F273H	515	1.41
F273T		0.48
F273Y	516	0.90
T274A		0.51
T274F	517	1.28
T274S		0.62
Q276C		0.88
Q276D	518	1.69
Q276E		1.05
Q276H	519	1.20
Q276I		0.51
Q276L		0.48
W119Q		0.72
Q276M	520	1.14
Q276R	521	1.30
Q276S	522	1.63
Q276Y	523	1.94
V277A	524	0.65
V277C		0.41
V277D		0.79
V277E	525	1.02
V277G		1.18
V277H	526	1.09
V277K	527	1.51
V277M	528	0.94

TABLE 9-continued

ACTIVE MUTANTS

mutant	SEQ ID NO	AvgNorm Act.
V277N	529	1.15
V277Q	530	0.82
V277R	531	1.63
V277S	532	0.83
K124H		0.35
V277T	533	1.94
V277Y		0.66
L278A		1.13
L278E	534	1.03
L278F	535	1.26
L278G	536	1.33
L278H	537	4.50
L278I		0.93
L278K	538	1.75
D275V		0.4
L278N	539	1.74
L278R	540	5.87
L278S	541	1.67
L278T	542	1.66
L278V		0.44
L278Y	543	1.51
K279H	544	0.44
K279Q		0.84
K279R		1.10
K279T		0.86
F280G		0.47
F280Q		0.43
S282D		0.41
S282G		0.54
S282M	545	2.64
S282Q		0.41
Q283E		0.63
Q283P		1.18
Q283R		0.59
Q283S	546	1.73
Q283T		0.65
D284A		0.58
D284E		1.21
D284G		0.60
D284H		0.51
D284L		0.50
D284M		0.56
D284N		0.40
D284Q		0.95
D284S		0.99
E285F		0.47
E285G		0.52
E285H	547	1.30
E285M		0.43
E285N		0.40
E285Q		0.59
E285Y		0.99
L286S		0.46
D284T		0.39
L286R		0.53
V287I		0.51
V287T	548	0.50
Y288L		0.79
Y288W		0.49
T289K		0.75
T289S	549	0.48
F290I		0.41
F290M		1.03
G291Q		0.80
G291R		0.45
G291S	550	0.41
G291V	551	1.63
E292A		0.66
E292C	552	0.71
E292F	553	0.90
E292G		0.41
E292H	554	1.26
E442W		0.38

TABLE 9-continued

ACTIVE MUTANTS

mutant	SEQ ID NO	AvgNorm Act.
E292K	555	1.27
E292N		0.99
E292P		1.05
E292R	556	0.42
E292V	557	1.28
E292W		0.83
T293A	558	1.90
T293C	559	1.67
T293D	560	1.46
V137C		0.37
V137S		0.36
V137L		0.21
Q143C		0.28
L144R	360	0.26
K152W	396	0.37
A153S		0.34
K154I		0.38
E156C		0.35
E158G		0.37
K159G		0.38
A160W		0.39
G161V		0.42
D163W		0.38
D163F		0.39
L165C		0.27
V166N		0.47
E167F		0.31
K170A		0.40
K170Q		0.40
K173Q		0.32
L174H		0.38
R176L		0.40
P177V		0.36
L180I		0.38
W181K		0.29
Y183E		0.32
Y184W		0.39
H193R		0.33
H193F		0.38
K195V		0.36
K196N		0.39
K196Y		0.39
P197W		0.39
G198W		0.29
N200T		0.37
F204W		0.39
N205L	452	0.39
N205Y		0.4
V206Q		0.33
K209F		0.4
K209L		0.38
N211L		0.41
N211W		0.51
W218M		0.38
W218V		0.28
T293F	561	1.94
T293G		1.00
T293K	562	1.35
T293L		1.00
T293M	563	2.29
T293P	564	1.64
T293Q	565	1.83
T293S		0.89
T293V	566	2.15
T293Y	567	1.49
V294M		0.41
A298G	568	0.43
A298I		0.41
G300R		0.42
I301A		0.88
I301V		0.88
V287N		0.35
V302W		0.46
V302I		0.45
I303V		0.47
W304G		1.13
W304I		1.17
G305D		1.00
G305E	569	1.62
T306D		0.76
T306E		0.52
T306S		1.02
L307K		0.43
L307N		0.76
L307Q		0.61
L307S		0.86
L307T		1.08
L307V		0.48
L307W		0.64
L307Y		0.60
S308D	571	0.92
S308G	572	1.73
S308H		1.15
S308K	573	1.33
S308N	574	2.33
S308P		0.65
S308R	575	1.34
S308T		0.72
I309D		0.72
I309E	576	1.99
I309G	577	1.44
I303D		0.34
I309H	578	1.30
I309K		0.98
I309L	579	1.72
I309M	580	1.47
I309N	581	3.11
I309Q	582	1.64
I309R	583	2.27
I309S	584	1.16
I309T	585	2.09
I309V	586	0.60
I309W		0.88
M310A	587	1.50
M310G	588	2.73
M310Q	589	0.59
M310R		0.50
M310S	590	1.61
M310V		0.70
R311G		0.53
L307G	570	0.32
R311G		0.54
R311H		0.48
R311K		0.72
R311Q		0.43
R311S		0.84
R311T		0.52
S312G		0.49
S312N		1.26
S312T		0.75
M313A	591	1.34
M313E		0.63
M313G	592	0.56
M313H	593	1.23
M313K	594	2.85
S312L		0.38
M313L		1.05
M313P	595	1.11
M313R	596	2.30
M313S		0.88
M313T	597	0.67
M313V		0.99
M313Y	598	1.12
K314A		0.82
K314D		0.53
K314H		1.10
K314I		0.54
K314N		0.57

TABLE 9-continued

ACTIVE MUTANTS

mutant	SEQ ID NO	AvgNorm Act.
K314Q		0.62
K314R		0.95
K314S	599	0.61
K314T		0.61
K314Y	600	0.45
S315A	601	0.85
S315E		0.41
S315G		0.72
S315H	602	2.04
S315K		0.62
S315L		0.42
S315M		0.63
S315R		1.04
S315T		0.97
S315Y	603	0.50
C316D		0.41
L317A	604	1.27
L317D		0.61
L317H		1.05
L317I	605	1.76
L317K	606	5.11
L317M		1.20
L317N	607	0.73
L317Q	608	1.67
L317R	609	2.41
L317S	610	1.03
L317T	611	0.93
L317W	612	0.84
L318D	614	0.46
L318F		0.51
L318G		0.49
L318H	615	0.45
L318I		0.70
L318K	616	1.36
L318M	613	1.68
L318N		0.52
L318Q		0.71
L318R	617	1.34
L318S		0.71
L318T		0.63
D320E		0.78
D320G		0.83
D320H	618	1.75
D320I		1.00
D320K	619	6.42
D320M		0.79
D320N		0.52
D320R	620	3.19
D320S		1.19
D320W		0.40
D320V		0.35
D320Y		0.86
N321A		1.01
N321D		1.25
N321H		0.92
N321K		1.29
N321R	621	1.23
N321S	622	1.26
N321T		0.64
N321Y		0.40
M323F		0.64
M323I		0.55
M323L		0.55
E324A		0.59
E324D		1.15
E324H		0.79
E324M		0.50
E324N	623	1.01
E324R	624	2.28
E324S		0.62
T325A	625	1.87
T325D	626	1.78
T325E	627	4.03
T325G	628	4.21
T325H	629	3.45
T325K	630	4.37
T325M	631	2.11
T325N	632	4.64
T325Q	633	5.08
T325S	634	3.19
T325V	635	1.24
T325W		0.62
I326K		0.95
I326L	636	1.50
I326V	637	6.29
I326Y		0.77
L327M		0.52
N328A		0.67
N328C	638	1.25
N328G	639	0.56
N328H		0.88
N328I	642	1.85
N328K	640	2.12
N328L	641	2.01
N328Q		1.13
N328R		0.68
N328S	643	2.22
N328T		0.59
N328V		1.16
N328Y	644	1.66
I331V		0.94
N328W		0.33
V334T		0.39
V334P		0.46
T335S	645	0.47
A338Q		0.63
K339M		0.61
S342A		0.68
Q343T		0.49
Q343V		0.51
Q347A	646	0.78
Q347E		0.78
Q347G	647	2.68
Q347M		0.61
Q347R		0.55
Q347S	648	2.38
E348D		0.67
E348G		0.55
E348S		0.44
Q349A		0.47
Q349E		0.83
Q349K		0.93
Q349M	649	0.70
Q349N		0.44
M035V	146	0.37
Q349R	650	0.73
Q349T		0.49
V351A		1.14
V351S	651	0.92
I353T		0.42
I353V	652	1.61
N356A		0.41
N356D		0.79
N356H	653	0.82
N356S	654	0.46
W357A		0.80
W357C		0.67
L037W		0.36
W357S		0.41
W357T		0.62
N358C		0.66
N358G		0.41
N358T		0.58
V351I		0.36
N358L		0.38
S359D		0.45
S359E	655	1.05

TABLE 9-continued

ACTIVE MUTANTS

mutant	SEQ ID NO	AvgNorm Act.
S359H	656	0.44
S359K		0.66
S359M		0.63
S359T	657	2.11
S359V		0.65
S360T		0.50
P367A	658	0.55
P367C		0.83
P367G	659	0.47
P367K	660	0.57
P367R		0.46
P367S	661	0.52
D368A	662	1.34
D368E	663	1.28
D368G		0.49
D368H		0.96
D368K	664	1.31
D368L	665	0.64
D368M	666	0.78
D368R	667	1.31
D368S		0.93
D368T	668	0.80
D361H		0.37
D368V		0.41
N369H	669	1.33
N369R	670	0.55
N369S		0.54
A371E		1.05
A371F	671	0.52
A371H	672	1.20
A371I		0.50
A371K	673	1.76
L374W		0.34
A371L	674	0.57
A371M		0.57
A371R	675	1.51
A371S	676	1.45
A371V		0.94
Q373A		0.65
Q373E		0.81
Q373F		0.62
Q373K		0.73
Q373L		0.84
Q373M	677	1.43
Q373R		0.68
Q373S		0.87
Q373V		1.05
L374A		0.60
L374H	678	1.42
L374I		0.80
L374M		1.11
L374N		0.43
L374P	679	0.43
L374R		0.83
L374S		0.58
L374T		0.47
L374V		0.56
L374Y		0.66
E375A	680	0.42
E375G	681	0.90
E375K	682	1.49
E375L		0.46
E375M		0.54
E375N		0.81
E375R	683	0.43
E375S		0.77
E375T		1.17
K376A		0.95
K376D	684	0.78
K376E	685	0.88
K376M		0.46
K376Q	686	0.69
K376R	687	0.67
K376S		0.80
K376T	688	0.53
K376V	689	0.58
K376Y	690	0.42
G377D	691	1.35
G377E	692	0.59
G377H	693	1.49
G377K	694	1.50
G377P	695	2.30
G377R	696	1.28
G377S	697	1.80
Q051R		0.36
G377T	698	3.83
G378K		1.22
G378N		0.64
G378R		1.03
K379G		0.52
K379H		0.57
K379R		0.74
K379S		0.46
K379T		0.4
M035Q	145	0.37
F380I		0.56
F380L		0.67
F380P		0.47
F380W	699	2.15
F380Y	700	1.50
T381H		0.48
T381K		1.06
T381N		0.51
T381Q		0.84
T381R		0.87
T381S	701	0.87
T381V		0.89
R383A		0.51
R383E		0.51
R383H		0.71
R383I	702	0.71
R383K	703	1.30
R383L	704	1.31
R383M		0.61
R383N		0.77
T381E		0.35
R383S	705	0.87
R383T		0.98
R383V		1.05
K385A	706	1.12
K385G		0.62
K385H		0.50
K385N		0.41
K385Q	707	0.73
K385R		0.94
K385S		1.05
K385T		0.46
K385V	708	0.43
T387S		0.93
L388F		0.92
L388H		0.47
L388I		0.98
L388M		0.79
L388R		0.60
L388T		0.51
L388V		0.78
L388W		0.77
L388Y		1.18
E392W		0.31
E389A	709	1.14
E389G	710	0.91
E389H		1.17
E389K	712	1.91
E389L	711	0.65
E389M		0.60
E389P		0.75
E389Q	713	0.69
E389R		0.94

TABLE 9-continued

ACTIVE MUTANTS

mutant	SEQ ID NO	AvgNorm Act.
E389S	714	1.08
E389T		0.70
E389Y		0.77
L391C		0.90
E392A	715	0.58
E392F	716	0.54
E392G		1.00
E392K		0.66
E392L		0.80
E392M	717	1.54
E392Q	718	1.01
E392R	719	0.66
E392S		0.52
E392T		0.72
E392V	720	1.27
E392Y		0.92
Q393A		1.26
Q393D		0.45
Q393F	721	1.23
Q393H		1.05
Q393K		0.80
Q393L		0.91
Q393M	722	0.80
Q393N		0.72
Q393R		0.74
Q393S		1.15
Q393T		0.41
F394L		0.56
F394W		0.41
S395A	723	1.10
S395G		0.77
S395H	724	0.56
S395K		0.96
S395R	725	1.98
E396A	726	0.52
E396D		0.64
E396H	727	0.47
E396Q	728	0.73
E396R		0.61
E396S	729	0.61
E396T		0.89
E396L		0.39
Y399A		1.01
Y399C		0.46
Y399E		1.49
S407L		0.4
Y399K	730	1.94
Y399M	731	2.70
Y399N		0.52
Y399Q		1.18
Y399R		1.20
Y399S		1.01
Y399T	732	2.40
Y399V	733	1.44
Y399W	734	1.92
S401A	735	0.82
S401E	736	0.46
S401N		0.42
Y403F		0.62
S404A	737	0.63
S404P		0.64
S401G		0.38
T405F		0.36
T405A		0.56
T405G	738	2.32
T405K		0.74
T405M		0.48
T405P		0.64
T405Q		0.75
T405R		0.60
T405S		0.94
T405W		0.73
T405Y		0.44
L406A		0.70
L406C		0.98
L406E		0.73
L406F	739	1.42
L406G		1.00
L406I		0.61
L406N	740	0.76
L406Q		0.93
L406S		0.47
L406T		0.83
L406V		0.87
L406Y		0.74
S407A	741	1.16
S407D	742	1.52
S407E	743	1.38
S407F	744	1.42
S407G		0.75
S407H	745	1.34
S407M		0.74
K411H		0.33
S407N		0.72
S407P	747	0.94
S407Q	746	1.71
S407R		1.04
S407V		0.56
S407W		0.41
K409A	748	2.18
K409D		0.65
K409E		0.62
K409G		0.50
K409H		0.64
K409I		0.51
K409P		0.48
K409Q	749	3.33
K409R		0.84
K409S		0.72
I083K		0.30
K409T		0.63
K409V		0.48
A412Y		0.66
E410D		0.47
E410K		0.70
E410M		0.42
E410N		0.67
E410P		0.73
E410Q		0.85
E410R		0.61
E410S		0.81
E410T	750	1.54
E410V		0.65
E410Y		0.62
K411A		0.48
K411N		1.02
K411P		0.42
K411R		0.97
K411S		1.21
K411T		0.63
K411V		0.99
A412D		0.74
A412G		0.80
A412I		0.81
E220M		0.36
P226W		0.51
A412L		0.65
A412N		0.86
A412P		0.77
A412R	752	0.66
A412S		0.86
A412V	753	0.53
A412W		0.54
D413E		0.52
D413K		0.42
D413N		0.94
D413R		0.50
D413T		0.41

TABLE 9-continued

ACTIVE MUTANTS

mutant	SEQ ID NO	AvgNorm Act.
V414I		1.12
V414M		0.53
K415G		0.40
K415S		0.42
K415W		0.42
D416F		0.41
D416G		0.67
D416H		0.57
D416I		0.63
D416K		0.76
D416L	754	0.75
D416N		0.73
D416Q		0.83
D416R		0.46
V237C	483	0.35
D416T		0.85
D416V		0.59
D416Y		0.40
T417I		1.22
D413A		0.38
D413S		0.39
K415Y		0.39
D418A		0.92
D418E	755	1.31
D418F		0.81
L089P		0.38
D418G		0.45
D418I		0.99
D418L	756	1.28
D418M		1.09
D418N		0.91
D418P	757	2.11
D418Q		1.05
D418R	758	1.18
D418S		0.78
D418V	759	1.43
D418Y		0.97
A419E		0.45
A419F	760	2.17
A419G		0.42
A419H	761	1.21
A419I	762	1.64
A419K	763	1.88
A419L		0.56
A419N		0.53
V421I		0.39
A419R	764	1.81
A419S	765	2.65
A419W		0.69
A419Y	766	1.44
V420I		1.04
V420P		0.48
D421A	767	1.28
D421E		0.81
D421G		0.62
D421H	768	1.98
K255G		0.39
D421K	769	2.42
D421L		0.73
D421M		0.94
D421N	770	1.89
D421Q	771	1.54
D421R	772	2.21
D421S	773	2.12
K094C		0.33
D421T		0.80
D421Y		0.66
V422I		0.42
V422T		0.49
A425G	774	1.20
A425I		0.44
A425K	775	1.75
A425M		0.70
A425N		0.46
A425R		0.49
A425S		0.47
D426E		0.62
D426G		0.85
D426N		0.61
D426P		1.03
D426Q		0.42
D426Y		0.43
G427K		0.52
G427S		0.42
V428L	778	1.25
A425Y		0.39
G427T	777	0.35
G427Q	776	0.39
V428M		0.42
V428P		0.82
V428T		0.62
D431A	779	2.42
D431E	781	1.27
D431G	780	0.55
D431H	782	3.13
D431I		1.05
D431K	783	1.83
D431L	784	0.62
D431N	785	1.30
D431Q	786	2.16
D431R	787	2.20
D431S	788	1.91
D431V	789	1.52
D431W		0.56
D431Y		0.85
A432E		0.60
A432G		0.52
A432H		0.34
A432N		0.51
A432S		0.61
A432V		0.56
F433A	790	0.97
R270T		0.40
F433C		0.69
F433D		0.95
F433E		0.82
F433G		0.54
F433H	791	0.83
F433I	792	1.06
F433K	793	1.36
F433L	794	1.87
F433P		0.95
F433R	795	1.63
F433S		0.86
F433T	796	1.86
F433V	797	1.63
F433W	798	1.28
L434F		0.41
L434G		0.47
L263H		0.36
L434I		0.89
L434M		0.60
L434V		0.46
K435A		1.08
K435C		0.53
K435E		0.78
K435G		0.64
K435H		1.05
K435R		1.01
K435S		1.03
K435T		0.73
K435V		0.44
K435Y		0.50
P436D		1.19
P436E		0.74
P436G		1.19
L105H		0.36
P436H		0.72

TABLE 9-continued

ACTIVE MUTANTS		
mutant	SEQ ID NO	AvgNorm Act.
V272M		0.31
P436I		0.84
P436K	799	2.05
P436L		0.63
P436M		0.61
P436Q		0.86
P436R		1.00
P436S		0.92
P436T		0.59
P436W		0.43
P436Y		0.49
P437A		0.56
P437D		0.62
P437G		0.50
P437H		1.11
P437I	800	2.46
P437K		0.83
P437L		0.51
P437M	801	2.55
P437Q		0.96
D275L		0.24
P437R		0.85
P437S		0.57
P437Y		0.42
M438A	802	0.75
M438C		0.63
M438D	803	0.87
M438E	804	0.72
M438G		0.83
M438L	805	0.86
M438N	806	1.08
M438P		0.81
M438Q		0.85
M438R		0.99
M438S		0.83
M438T	807	3.99
M438V		0.85
P125A		0.36
M438W		0.57
E439A	808	1.20
E439C	809	0.58
E439F		1.00
E439G		1.22
E439H		0.74
E439K	810	1.20
E439L		0.88
E439P	811	1.16
Q276G		0.36
E439Q	812	1.32
E439S		1.02
E439T	813	1.15
E439V	814	1.57
E439W		0.62
T440A		1.22
T440D	815	1.03
T440E		1.00
T440F		0.85
T440G		0.86
T440H	816	3.00
T440I		1.04
T440L		0.97
T440M	817	1.08
T440P	818	0.88
T440R	819	1.77
T440S	820	1.17
T440V		1.02
T440Y		1.11
E441A	821	1.47
E441D		0.67
E441F	822	3.91
E441G		0.87
E441H		0.65
E441K		0.80
E441L		0.82
E441N		0.82
E441Q		0.81
E441S		0.79
E441T		0.66
E441V		0.54
E441Y		0.51
E442C	823	1.38
E442G	824	0.51
E442H		0.76
E442K		0.73
E442P		0.91
E442Q		0.74
D284Y		0.37
L286W		0.38
E442R	825	3.94
E442T		0.61
E442V		0.65
E442Y		0.60
P443A	826	1.63
P443E	827	1.07
P443F	828	0.70
P443G	829	1.12
P443H		1.08
P443L		1.19
P443M	830	1.99
P443N	831	1.25
P443Q		0.96
P443R		1.04
P443S		0.99
P443T		0.87
P443W		0.64
Q444M		0.37
Q444D		0.97
Q444E	832	1.19
Q444F		0.66
Q444G		0.93
Q444H	833	0.97
Q444I		0.58
Q444K		1.03
Q444N		1.01
Q444R		0.85
Q444V	834	1.12
Q444W		0.64
Q444Y		0.67
I445A		0.97
I445G		0.98
I445H	835	1.35
I445L		1.06
I445M	836	1.57
I445N	837	1.24
I445P	838	1.67
I445Q	839	1.26
I445R		1.08
I445S	840	1.21
I445T	841	1.38
I445V	842	1.25
I445W	843	0.69
I445Y		0.53
F446A	844	1.58
F446C		0.75
F446D		1.18
F446E		1.10
F446G		1.12
F446H		1.28
F446I		1.06
F446K		0.94
F446L		0.93
F446M	845	1.31
F446Q		0.72
F446R		0.89
F446T		0.89
F446V		0.91
F446W	846	1.40
Y447D	847	3.25

TABLE 9-continued

ACTIVE MUTANTS		
mutant	SEQ ID NO	AvgNorm Act.
Y447E	848	1.36
Y447F		1.41
Y447G	849	0.92
Y447I	850	1.36
Y447L		1.09
Y447M		0.90
Y447N	851	1.58
Y447P	852	1.46
Y447Q	853	2.37
Y447R		1.12
Y447T	854	1.90
Y447V	855	1.38
Y447W		1.07

2. Inactive Mutants

The other mutants that exhibited less than 20% hyaluronidase activity of wildtype PH20, in at least one of the duplicates, were rescreened to confirm that the dead mutants are inactive. To confirm the inactive mutants, the hyaluronidase activity assay described in Example 3 was modified to incorporate an overnight 37° C. substrate-sample incubation step prior to measurement of enzymatic activity. The modified assay is intended to detect PH20 activities below 0.2 U/mL.

The preparation of the bHA coated plates and blocking of the plates prior to addition of the transfected variant supernatants or wildtype PH20 was the same as described in Example 3. The assay was modified as follows. First, transfected variant supernatants or wildtype PH20 not containing a mutation generated as described in Example 2 were diluted in duplicate 1:25 in assay diluent. For the standard curve, 1:3 serial dilutions of rHuPH20 (generated as described in Example 1) were made in assay diluent in duplicate starting from 0.1 U/mL down to 0.00014 U/mL. A blank well also was included. Then, 100 μl of the diluted samples or standard were added to pre-designated wells of the bHA-coated and blocked plate and allowed to incubate at 37° C. overnight. After the incubation, the plates were washed and binding to bHA detected as described above in Example 3. Optical density was measured at 450 nm within 30 minutes of adding the stop solution.

The identified reconfirmed inactive mutants are set forth in Table 10. The Table sets forth the amino acid replacement compared to the sequence of amino acids of PH20 set forth in SEQ ID NO:3.

TABLE 10

Inactive Mutants							
N002H	R060V	R121W	C189P	P236I	V287N	L336W	G377V
N002K	R060Y	R121Y	C189R	P236L	V287P	L336Y	G378D
N002W	L061A	N122A	C189S	P236N	V287Q	A337C	G378E
N002Y	L061E	N122C	C189T	P236Q	V287R	A337F	G378F
F003A	L061F	N122E	C189V	P236T	V287S	A337G	G378I
F003G	L061G	N122F	C189W	P236Y	Y288D	A337I	G378L
F003K	L061H	N122I	C189Y	A238F	Y288E	A337K	G378M
F003P	L061N	N122K	Y190C	A238G	Y288F	A337L	G378Q
F003T	L061P	N122Q	Y190E	A238L	Y288G	A337M	G378T
F003V	L061Q	N122R	Y190F	A238P	Y288H	A337R	G378W
R004D	L061R	N122S	Y190G	A238V	Y288I	A337T	G378Y
R004E	L061T	N122T	Y190H	A238W	Y288K	A337W	K379A
R004F	L061W	N122V	Y190K	A238Y	Y288P	A338C	K379C
R004G	L061Y	W123A	Y190L	A239C	Y288R	A338D	K379E
R004L	G062A	W123C	Y190N	A239F	Y288T	A338E	K379F
R004P	G062C	W123D	Y190Q	A239G	T289A	A338F	K379I
R004W	G062D	W123E	Y190R	A239H	T289C	A338G	K379L
R004Y	G062F	W123H	Y190S	A239I	T289E	A338H	K379M
A005D	G062I	W123L	Y190T	A239L	T289G	A338I	K379W
A005G	G062K	W123M	Y190V	A239P	T289H	A338K	F380C
A005I	G062L	W123P	Y190W	A239R	T289L	A338L	F380D
A005L	G062M	W123Q	N191A	A239S	T289P	A338P	F380E
A005M	G062P	W123R	N191E	A239T	T289Q	A338R	F380G
A005N	G062Q	W123S	N191F	A239V	T289R	A338T	F380Q
A005P	G062R	W123T	N191G	A239W	T289S	A338V	F380R
A005Q	G062S	W123V	N191K	A239Y	T289Y	K339D	F380S
A005R	G062T	W123Y	N191L	T240E	F290D	K339E	T381G
A005T	G062V	K124C	N191M	T240F	F290Q	K339F	T381L
A005V	G062Y	K124D	N191P	T240G	F290Y	K339G	T381P
A005W	Y063C	K124E	N191Q	T240N	G291A	K339H	T381W
A005Y	Y063G	K124F	N191R	T240W	G291C	K339L	T381Y
P006E	Y063P	K124N	N191S	T240Y	G291D	K339N	V382E
P006F	Y064A	P125C	N191T	L241A	G291E	K339P	V382G
P006T	Y064C	P125D	N191V	L241C	G291F	K339S	V382H
P006V	Y064D	P125G	N191W	L241D	G291M	K339T	V382K
P006Y	Y064E	P125L	N191Y	L241E	G291N	K339V	V382L
P007C	Y064F	P125N	H192C	L241G	G291T	K339W	V382M
P007D	Y064G	P125W	H192F	L241I	G291W	K339Y	V382N
P007F	Y064H	K126F	H192G	L241P	G291Y	M340A	V382P
P007G	Y064I	K126H	H192K	L241R	E292I	M340C	V382Q
P007H	Y064K	K126I	H192L	L241S	E292L	M340D	V382R
P007I	Y064L	K126L	H192M	L241T	E292T	M340E	V382S
P007K	Y064P	K126N	H192N	L241V	T293E	M340F	V382T
P007L	Y064Q	K126P	H192P	L241W	T293N	M340G	V382W
P007Q	Y064R	K126Y	H192Q	Y242A	V294A	M340H	V382Y
P007R	Y064S	D127K	H192R	Y242C	V294E	M340K	R383G

TABLE 10-continued

Inactive Mutants							
P007S	Y064T	V128E	H192V	Y242D	V294G	M340P	R383P
P007T	Y064V	V128P	H192W	Y242G	V294H	M340R	G384C
P007W	Y064W	Y129A	H192Y	Y242I	V294K	M340S	G384F
P007Y	P065A	Y129C	H193A	Y242L	V294L	M340T	G384M
V008D	P065C	Y129D	H193D	Y242M	V294N	M340V	G384Q
V008E	P065D	Y129E	H193K	Y242P	V294P	M340W	G384S
V008G	P065G	Y129G	H193L	Y242R	V294Q	C341A	G384T
V008H	P065H	Y129H	H193M	Y242S	V294R	C341E	K385C
V008N	P065I	Y129L	H193P	Y242T	V294S	C341G	K385L
V008R	P065K	Y129P	H193V	Y242V	V294T	C341H	K385M
V008S	P065N	Y129Q	Y194A	Y242W	V294W	C341K	K385P
V008W	P065R	Y129S	Y194C	V243C	A295C	C341L	K385W
I009C	P065S	Y129T	Y194I	V243D	A295G	C341M	K385Y
I009D	P065T	Y129V	Y194L	V243F	A295H	C341N	P386A
I009E	P065V	Y129W	Y194P	V243G	A295I	C341Q	P386C
I009G	P065W	K130C	Y194S	V243H	A295L	C341R	P386F
I009N	P065Y	K130D	Y194T	V243L	A295N	C341S	P386G
I009P	Y066A	K130G	Y194V	V243M	A295P	C341T	P386H
P010F	Y066C	K130H	K195S	V243P	A295T	C341V	P386I
P010I	Y066D	K130L	P197C	V243Q	A295V	C341Y	P386L
P010L	Y066E	K130N	G198V	V243R	A295Y	S342D	P386M
P010M	Y066G	K130S	G198W	V243S	L296C	S342E	P386N
P010Y	Y066I	K130T	Y199E	V243W	L296F	S342F	P386Q
N011A	Y066K	K130W	Y199G	V243Y	L296G	S342H	P386R
N011C	Y066L	K130Y	Y199H	R244A	L296I	S342K	P386S
N011F	Y066N	N131P	Y199I	R244D	L296K	S342L	P386T
N011I	Y066P	R132P	Y199K	R244G	L296M	S342M	P386V
N011L	Y066S	S133D	Y199L	R244I	L296Q	S342P	P386Y
N011P	Y066T	S133E	Y199P	R244V	L296R	S342Q	T387C
N011T	Y066V	S133F	Y199R	R244Y	L296S	S342R	T387E
N011W	I067D	S133G	Y199S	N245A	L296T	S342T	T387F
N011Y	I067E	S133H	Y199W	N245C	L296V	S342Y	T387G
V012G	I067G	S133L	N200A	N245F	L296W	Q343C	T387H
V012H	I067P	S133M	N200F	N245L	L296Y	Q343D	T387I
V012W	I067R	S133N	N200G	N245P	G297C	Q343F	T387L
P013E	I067T	S133P	N200H	N245Q	G297E	Q343I	T387M
P013G	I067W	S133R	N200K	N245R	G297H	Q343P	T387N
P013I	D068A	S133T	N200L	N245S	G297L	Q343W	T387V
P013L	D068C	S133V	N200M	N245T	G297N	V344F	T387W
P013M	D068G	S133W	N200P	N245V	G297P	V344G	T387Y
P013V	D068I	I134A	N200Q	R246A	G297Q	V344H	L388C
F014A	D068L	I134C	N200R	R246C	G297R	V344L	L388G
F014E	D068P	I134D	N200S	R246D	G297S	V344M	L388P
F014G	D068V	I134F	N200W	R246E	G297T	V344N	L388Q
F014H	D068Y	I134G	N200Y	R246G	G297Y	V344P	L388S
F014K	S069N	I134H	G201A	R246H	A298C	V344Q	E389F
F014N	S069T	I134K	G201F	R246I	A298E	V344R	E389V
F014P	I070Q	I134P	G201L	R246K	A298L	V344S	D390A
F014Q	T071P	I134Q	G201M	R246L	A298M	V344T	D390C
F014W	G072C	I134R	G201N	R246M	A298N	V344W	D390E
L015E	G072F	I134S	G201P	R246P	A298P	V344Y	D390F
L015F	G072H	I134W	G201R	R246S	A298Q	L345A	D390G
L015G	G072I	E135P	G201S	R246T	A298S	L345C	D390H
L015K	G072P	L136P	G201T	R246V	A298T	L345E	D390L
L015N	G072V	V137F	G201V	R246W	A298W	L345H	D390N
L015P	G072W	V137G	G201W	V247A	A298Y	L345K	D390P
L015Q	V073P	V137H	S202A	V247C	S299A	L345N	D390R
L015R	V075D	V137N	S202E	V247F	S299C	L345Q	D390S
L015S	V075G	V137P	S202F	V247H	S299D	L345R	D390T
L015Y	V075P	V137R	S202G	V247N	S299F	L345T	D390V
W016A	N076A	V137W	S202H	V247P	S299G	L345V	D390W
W016C	N076C	V137Y	S202K	V247Q	S299H	L345Y	D390Y
W016D	N076F	Q138V	S202N	V247R	S299L	C346A	L391A
W016E	N076G	Q139P	S202P	V247S	S299M	C346D	L391D
W016F	N076I	Q143C	S202Q	V247T	S299P	C346F	L391G
W016G	N076K	Q143H	S202R	V247W	S299Q	C346G	L391H
W016H	N076L	Q143P	S202V	V247Y	S299T	C346I	L391K
W016K	N076P	Q143R	S202W	R248C	G300A	C346K	L391N
W016M	N076Q	Q143S	S202Y	R248D	G300C	C346L	L391P
W016P	N076R	Q143T	C203A	R248E	G300D	C346M	L391Q
W016R	N076S	L144A	C203D	R248G	G300E	C346P	L391R
W016S	N076T	L144E	C203E	R248I	G300F	C346R	L391S
W016T	N076V	L144F	C203G	R248M	G300L	C346S	L391T
W016Y	N076W	L144I	C203H	R248P	G300M	C346T	L391V
A017D	G077D	L144K	C203L	R248T	G300N	C346V	L391W
A017E	G077E	L144P	C203M	E249A	G300P	C346W	L391Y
A017G	G077L	L144Q	C203N	E249G	G300Q	Q347C	E392C
A017H	G077P	L144S	C203Q	E249H	G300S	Q347F	E392P

TABLE 10-continued

Inactive Mutants							
A017I	G077Q	L144V	C203R	E249I	G300T	Q347I	Q393C
A017L	G077R	L144Y	C203S	E249K	G300V	Q347P	Q393P
A017N	G077T	S145T	C203T	E249M	G300W	Q347T	F394A
A017P	G077V	S145W	C203V	E249Q	I301E	Q347V	F394D
A017Q	G078A	A149E	F204A	E249S	I301G	Q347W	F394E
A017R	G078D	A149P	F204C	E249Y	I301H	E348C	F394G
A017S	G078I	T150V	F204E	A250C	I301K	E348H	F394I
A017T	G078M	K152L	F204G	A250F	I301M	E348I	F394K
A017V	G078P	A153E	F204H	A250G	I301N	E348L	F394N
A017W	G078T	A153F	F204I	A250H	I301P	E348P	F394P
A017Y	G078Y	A153M	F204K	A250K	I301Q	E348Q	F394Q
W018C	I079A	A153P	F204Q	A250L	I301R	E348R	F394R
W018D	I079D	A153R	F204R	A250M	I301S	E348T	F394S
W018F	I079F	A153T	F204S	A250N	I301W	E348V	F394T
W018G	I079G	A153V	F204T	A250P	I301Y	E348W	F394V
W018H	I079H	K154D	V206C	A250Q	V302C	E348Y	S395C
W018I	I079K	K154E	V206D	A250R	V302D	Q349D	S395L
W018L	I079N	K154G	V206F	A250S	V302E	Q349F	S395M
W018M	I079P	K154P	V206G	A250T	V302F	Q349G	S395P
W018P	I079S	K154S	V206P	A250V	V302G	Q349P	E396C
W018Q	I079W	K154W	V206Y	A250W	V302H	Q349V	E396F
W018S	I079Y	K154Y	E207A	I251D	V302L	Q349W	E396G
W018T	P080A	Q155P	E207F	I251F	V302M	Q349Y	E396I
W018V	P080D	Q155Y	E207G	I251G	V302P	G350A	E396P
W018Y	P080E	E156P	E207M	I251H	V302R	G350D	E396Y
N019A	P080F	F157A	E207P	I251K	V302S	G350E	K397A
N019C	P080G	F157C	E207Q	I251P	V302T	G350F	K397C
N019F	P080I	F157D	E207R	I251S	V302Y	G350H	K397E
N019G	P080K	F157E	E207S	I251T	I303A	G350K	K397F
N019H	P080L	F157G	E207T	I251W	I303C	G350L	K397G
N019I	P080M	F157H	E207V	R252A	I303D	G350M	K397I
N019L	P080N	F157I	E207W	R252D	I303E	G350N	K397L
N019M	P080R	F157K	I208D	R252E	I303F	G350P	K397M
N019P	P080S	F157L	I208G	R252F	I303G	G350R	K397P
N019Q	P080T	F157M	I208P	R252G	I303K	G350S	K397Q
N019R	P080V	F157P	I208W	R252H	I303L	G350T	K397T
N019S	P080Y	F157Q	K209C	R252I	I303M	G350V	K397V
N019V	Q081A	F157R	K209P	R252K	I303R	G350Y	F398A
N019W	Q081C	F157S	R210A	R252L	I303W	V351C	F398C
N019Y	Q081E	F157T	R210C	R252N	I303Y	V351D	F398E
A020D	Q081G	F157V	R210D	R252P	W304A	V351E	F398G
A020E	Q081H	E158D	R210E	R252S	W304C	V351F	F398H
A020F	Q081L	E158K	R210G	R252T	W304D	V351H	F398I
A020H	Q081N	E158P	R210K	R252Y	W304G	V351N	F398L
A020K	Q081P	E158R	R210M	V253A	W304I	V351R	F398N
A020L	Q081S	E158Y	R210N	V253D	W304M	V351W	F398P
A020N	Q081V	K159W	R210P	V253E	W304N	V351Y	F398R
A020P	Q081W	K159Y	R210S	V253G	W304P	C352A	F398S
A020R	Q081Y	G161W	R210T	V253H	W304Q	C352D	F398T
A020T	K082W	D163C	R210V	V253L	W304S	C352E	F398V
A020V	K082Y	D163P	R210W	V253M	W304T	C352F	F398W
A020Y	I083E	F164A	R210Y	V253N	W304V	C352G	F398Y
P021A	I083K	F164C	N211C	V253Q	W304Y	C352K	Y399D
P021C	S084Y	F164D	N211F	V253R	G305L	C352M	Y399P
P021D	L085A	F164E	N211G	V253S	G305P	C352P	C400A
P021E	L085C	F164G	N211H	V253W	G305Q	C352Q	C400D
P021G	L085D	F164H	N211I	S254C	G305R	C352R	C400E
P021H	L085E	F164N	N211K	S254D	G305S	C352S	C400F
P021I	L085F	F164P	N211M	S254E	G305T	C352T	C400G
P021L	L085G	F164Q	N211P	S254G	G305V	C352V	C400I
P021M	L085H	F164R	N211R	S254I	G305Y	C352W	C400L
P021R	L085N	L165C	N211S	S254K	T306A	C352Y	C400M
P021S	L085Q	L165H	N211T	S254L	T306C	I353C	C400P
P021T	L085S	L165P	N211V	S254P	T306H	I353F	C400Q
P021V	L085T	L165T	N211W	S254Q	T306I	I353G	C400R
P021W	Q086C	V166D	D212A	S254R	T306L	I353H	C400S
S022C	Q086P	E167V	D212G	S254T	T306V	I353K	C400T
S022E	D087P	T168A	D212H	S254V	T306W	I353L	C400V
S022G	H088A	T168C	D212I	S254W	T306Y	I353M	C400Y
S022K	H088C	T168D	D212K	S254Y	L307C	I353Q	S401C
S022P	H088E	T168E	D212L	K255C		I353R	S401F
E023A	H088F	T168F	D212M	K255D	L307I	I353S	S401H
E023F	H088G	T168G	D212P	K255L	L307P	I353W	S401K
E023L	H088I	T168K	D212V	K255P	S308C	R354C	S401R
E023M	H088K	T168L	D212W	K255V	S308F	R354D	S401W
E023N	H088L	T168P	D213P	K255W	S308L	R354E	S401Y
E023P	H088M	T168R	D213S	I256C	S308M	R354G	C402A
E023R	H088P	T168S	L214A	I256D	S308V	R354H	C402D

TABLE 10-continued

Inactive Mutants							
E023S	H088R	T168V	L214C	I256E	S308W	R354I	C402E
E023T	H088S	T168W	L214D	I256G	S308Y	R354K	C402F
E023V	H088T	T168Y	L214E	I256P	M310C	R354L	C402L
C025D	H088V	I169A	L214G	P257D	M310E	R354M	C402M
C025E	H088Y	I169D	L214H	D258L	M310F	R354P	C402P
C025F	L089A	I169F	L214K	D258P	M310K	R354Q	C402Q
C025G	L089D	I169G	L214N	D258V	M310L	R354S	C402R
C025H	L089E	I169H	L214P	D258W	R311C	R354V	C402S
C025I	L089G	I169K	L214R	K260C	R311E	R354W	C402T
C025K	L089Q	I169N	L214S	K260P	R311F	R354Y	C402V
C025L	L089S	I169P	L214T	S261P	R311I	K355D	C402W
C025N	L089T	I169Q	L214Y	P262A	R311L	K355F	C402Y
C025P	L089W	I169S	S215C	P262D	R311P	K355G	Y403A
C025R	L089Y	I169T	S215P	P262E	R311V	K355H	Y403C
C025S	D090C	I169Y	W216D	P262F	R311W	K355L	Y403E
C025T	D090G	K170C	W216E	P262G	S312C	K355M	Y403G
C025V	K091D	K170D	W216G	P262H	S312E	K355N	Y403H
C025Y	K091E	K170E	W216H	P262I	S312M	K355P	Y403K
G027C	K091F	K170G	W216I	P262K	S312V	K355Q	Y403L
L033C	K091G	K170M	W216K	P262Q	S312W	K355R	Y403M
L033D	K091H	K170P	W216L	P262R	M313C	K355S	Y403N
L033H	K091I	K170W	W216M	P262S	K314C	K355T	Y403P
L033N	K091L	K170Y	W216N	P262T	K314L	K355V	Y403Q
L033V	K091N	L171C	W216P	P262V	K314W	K355W	Y403R
L033Y	K091T	L171D	W216Q	P262W	S315C	K355Y	Y403T
D034I	A092E	L171H	W216R	P262Y	S315I	N356C	S404C
D034L	A092F	L171M	W216T	L263E	S315V	N356G	S404D
D034N	A092H	L171N	W216V	L263F	C316E	N356K	S404F
D034S	A092K	L171R	L217A	L263P	C316G	N356L	S404G
D034T	A092P	L171S	L217C	L263Q	C316I	N356P	S404H
D034V	A092Q	L171W	L217G	L263W	C316K	N356R	S404L
M035A	A092R	L171Y	L217H	P264D	C316L	N356T	S404M
M035D	A092W	G172D	L217P	P264E	C316M	N356V	S404N
M035G	A092Y	G172E	L217Q	P264F	C316P	N356W	S404R
M035P	K094G	G172I	L217S	P264G	C316R	W357D	S404V
M035R	K094P	G172L	L217T	P264L	C316S	W357E	S404W
M035S	D095A	G172P	L217V	P264M	C316T	W357F	S404Y
S036C	D095C	G172Q	L217W	P264R	C316V	W357G	T405C
S036F	D095E	G172T	W218A	P264T	C316W	W357L	T405I
S036V	D095F	G172V	W218I	P264V	C316Y	W357M	T405V
S036W	D095G	G172W	W218K	P264W	L317G	W357Q	L406P
S036Y	D095H	G172Y	W218L	P264Y	L317P	W357R	L406R
L037C	D095K	K173D	W218P	V265A	L318C	N358E	C408A
L037E	D095L	K173E	W218S	V265D	L318P	N358H	C408E
L037G	D095M	K173G	W218V	V265F	L318W	N358I	C408F
L037N	D095P	K173H	N219P	V265G	L319C	N358K	C408G
L037S	D095Q	K173I	E220G	V265H	L319E	N358P	C408I
F038E	D095S	K173L	E220K	V265K	L319F	N358Q	C408K
F038G	D095V	K173M	E220N	V265L	L319G	N358R	C408L
F038K	D095W	K173P	E220P	V265M	L319H	N358W	C408P
F038L	D095Y	K173S	E220R	V265N	L319I	S359A	C408R
F038N	I096A	K173V	E220W	V265Q	L319K	S359F	C408S
F038Q	I096C	K173W	S221D	V265R	L319M	S359G	C408T
F038R	I096G	K173Y	S221E	V265S	L319P	S359L	C408V
F038T	I096H	L174P	S221H	F266A	L319Q	S359P	C408W
F038W	I096P	L175C	S221K	F266C	L319R	S359W	C408Y
S039C	I096R	L175D	S221P	F266G	L319S	S360A	E410W
S039D	I096S	L175G	S221R	F266H	L319V	S360C	K411D
S039F	I096T	L175K	T222P	F266M	L319W	S360E	K411E
S039W	I096W	L175P	T222Y	F266P	L319Y	S360F	K411F
F040A	F098P	L175R	A223C	F266Q	D320C	S360G	K411G
F040D	Y099C	L175S	A223D	F266R	D320P	S360I	A412E
F040E	Y099E	R176A	A223E	F266S	D320V	S360K	A412H
F040G	Y099G	R176C	A223G	F266T	N321E	S360L	D413H
F040K	Y099I	R176E	A223H	F266V	N321M	S360M	D413I
F040N	Y099N	R176F	A223K	F266W	N321P	S360P	D413K
F040R	Y099P	R176G	A223L	A267D	Y322C	S360Q	D413L
F040S	Y099V	R176H	A223P	A267G	Y322D	S360R	D413P
F040T	Y099W	R176I	A223Q	A267H	Y322E	S360V	V414A
F040V	M100C	R176P	A223R	A267I	Y322G	D361A	V414D
I041Q	M100E	R176Q	A223S	A267K	Y322I	D361C	V414E
G042D	M100F	R176S	A223T	A267N	Y322L	D361E	V414G
G042E	M100G	R176T	A223V	A267R	Y322N	D361G	V414H
G042H	M100N	R176V	A223W	A267S	Y322P	D361M	V414K
G042I	M100P	R176W	A223Y	A267W	Y322R	D361N	V414R
G042K	M100R	P177A	L224A	Y268A	Y322S	D361P	V414S
G042L	M100S	P177C	L224D	Y268C	Y322T	D361Q	V414T
G042M	M100T	P177D	L224E	Y268F	Y322V	D361R	K415C

TABLE 10-continued

Inactive Mutants							
G042P	M100W	P177F	L224F	Y268G	Y322W	D361S	K415D
G042Q	M100Y	P177G	L224G	Y268H	M323A	D361V	K415E
G042R	P101A	P177H	L224M	Y268K	M323C	D361W	K415P
G042S	P101C	P177L	L224P	Y268L	M323E	Y362A	D416C
G042T	P101F	P177M	L224Q	Y268N	M323G	Y362C	D416S
G042V	P101H	P177Q	L224R	Y268P	M323H	Y362E	T417A
S043A	P101I	P177R	L224S	Y268Q	M323K	Y362G	T417D
S043E	P101K	P177S	L224T	Y268S	M323N	Y362H	T417E
S043F	P101L	P177T	L224W	Y268T	M323R	Y362K	T417F
S043G	P101M	P177V	L224Y	Y268V	M323S	Y362L	T417G
S043I	P101N	P177W	Y225A	Y268W	M323T	Y362M	T417H
S043K	P101Q	N178E	Y225D	T269E	M323V	Y362N	T417K
S043L	P101R	N178I	Y225E	T269K	E324C	Y362P	T417M
S043Q	P101S	N178L	Y225G	T269L	E324F	Y362R	T417P
S043R	P101T	N178V	Y225H	T269M	E324P	Y362S	T417Q
S043V	V102P	N178W	Y225K	T269N	E324V	Y362T	T417R
P044A	D103A	N178Y	Y225P	T269P	E324W	Y362V	A419D
P044C	D103E	H179W	Y225Q	T269Q	E324Y	Y362W	A419P
P044F	D103F	L180A	Y225R	T269R	T325C	L363A	V420A
P044G	D103G	L180C	Y225T	R270A	T325R	L363C	V420D
P044H	D103H	L180E	Y225V	R270C	I326E	L363D	V420F
P044I	D103I	L180P	Y225W	R270E	I326G	L363E	V420G
P044L	D103L	L180R	P226A	R270F	I326H	L363F	V420H
P044N	D103Q	L180S	P226C	R270G	I326N	L363G	V420K
P044Q	D103R	W181A	P226D	R270H	I326W	L363H	V420L
P044R	D103T	W181C	P226E	R270I	L327A	L363I	V420N
P044S	D103V	W181D	P226F	R270P	L327E	L363P	V420R
P044T	D103W	W181E	P226G	R270Y	L327F	L363Q	V420S
P044W	D103Y	W181F	P226L	I271A	L327G	L363R	V420T
P044Y	N104F	W181H	P226N	I271D	L327H	L363S	V420W
R045A	N104P	W181I	P226Q	I271E	L327N	L363T	V420Y
R045D	N104W	W181K	P226R	I271H	L327Q	L363V	V422C
R045F	L105C	W181L	P226S	I271K	L327R	L363W	V422D
R045G	L105M	W181R	P226T	I271T	L327S	H364A	V422G
R045P	L105N	W181S	P226V	I271W	L327T	H364C	V422H
R045W	G106A	W181V	P226W	V272A	L327V	H364D	V422L
I046P	G106C	G182A	P226Y	V272H	L327W	H364E	V422M
I046W	G106D	G182C	S227A	V272L	L327Y	H364F	V422N
N047V	G106F	G182D	S227F	V272N	P329C	H364G	V422Q
A048P	G106H	G182E	S227G	V272P	P329F	H364K	V422R
T049C	G106L	G182H	S227H	V272W	P329G	H364L	V422S
T049D	G106M	G182N	S227I	F273A	P329H	H364M	V422Y
T049G	G106N	G182P	S227K	F273C	P329I	H364P	C423A
T049H	G106P	G182Q	S227L	F273D	P329K	H364R	C423D
T049P	G106S	G182R	S227M	F273G	P329L	H364S	C423E
	G106W	G182S	S227P	F273I	P329N	H364T	C423F
Q051C	G106Y	G182T	S227Q	F273L	P329Q	H364V	C423G
Q051F	M107A	G182V	S227R	F273P	P329R	H364Y	C423H
Q051I	M107C	G182Y	S227T	F273Q	P329S	L365A	C423L
Q051M	M107H	Y183C	S227V	F273S	P329T	L365C	C423M
Q051P	M107K	Y183D	S227W	F273V	P329V	L365D	C423P
Q051T	M107P	Y183E	S227Y	F273W	P329W	L365E	C423Q
Q051W	M107Q	Y183G	I228A	T274C	P329Y	L365G	C423R
Q051Y	M107S	Y183I	I228E	T274E	Y330A	L365M	C423S
G052C	M107V	Y183K	I228F	T274G	Y330C	L365N	C423T
G052E	M107W	Y183N	I228G	T274H	Y330D	L365P	C423V
G052F	A108D	Y183P	I228H	T274N	Y330E	L365Q	C423W
G052W	A108E	Y183Q	I228L	T274Q	Y330G	L365R	I424A
G052Y	A108F	Y183R	I228M	T274W	Y330I	L365S	I424C
V053A	A108K	Y183S	I228N	T274Y	Y330L	L365T	I424E
V053C	A108L	Y183V	I228P	D275A	Y330M	L365W	I424G
V053D	A108M	Y184A	I228R	D275F	Y330N	L365Y	I424H
V053E	A108P	Y184C	I228S	D275G	Y330P	N366A	I424N
V053G	A108Q	Y184D	I228T	D275I	Y330R	N366C	I424Q
V053H	A108T	Y184E	I228W	D275K	Y330S	N366E	I424R
V053L	A108V	Y184F	Y229E	D275L	Y330V	N366F	I424S
V053N	A108Y	Y184G	Y229F	D275M	Y330W	N366G	I424W
V053P	V109C	Y184H	Y229G	D275Q	I331A	N366K	I424Y
V053Q	V109D	Y184K	Y229K	D275T	I331C	N366M	A425E
V053R	V109E	Y184L	Y229L	D275V	I331D	N366P	A425L
V053S	V109L	Y184M	Y229P	D275W	I331E	N366Q	A425P
V053T	V109M	Y184P	Y229Q	Q276F	I331F	N366R	A425W
V053W	V109R	Y184R	Y229T	Q276P	I331H	N366T	A425Y
V053Y	V109T	Y184S	Y229V	Q276W	I331K	N366W	D426C
T054D	V109W	Y184V	Y229W	L278M	I331Q	P367E	D426F
T054E	I110F	L185A	L230A	L278P	I331R	P367F	D426M
T054G	I110K	L185D	L230E	K279A	I331S	P367I	D426R
T054P	I110L	L185E	L230G	K279C	I331T	P367L	G427A

TABLE 10-continued

Inactive Mutants							
T054R	I110M	L185F	L230H	K279F	I331W	P367M	G427C
T054Y	I110P	L185G	L230K	K279G	I331Y	P367Q	G427F
I055A	I110W	L185I	L230M	K279L	I332A	P367V	G427L
I055D	D111H	L185K	L230N	K279W	I332C	D368C	G427P
I055G	D111I	L185P	L230P	K279Y	I332D	D368P	
I055H	D111Q	L185R	L230R	F280D	I332E	D368W	G427V
I055N	W112C	L185S	L230S	F280I	I332F	N369C	G427W
I055P	W112E	L185T	L230T	F280L	I332G	N369E	G427Y
I055Q	W112G	L185V	L230V	F280M	I332H	N369F	V428A
I055R	W112H	L185W	L230W	F280N	I332K	N369I	V428C
I055T	W112L	L185Y	L230Y	F280R	I332L	N369K	V428D
I055V	W112N	F186A	N231A	F280S	I332N	N369L	V428E
I055Y	W112P	F186D	N231C	F280T	I332P	N369P	V428G
F056A	W112S	F186G	N231D	F280V	I332R	N369Q	V428H
F056C	E113R	F186H	N231F	F280W	I332S	N369V	V428N
F056E	E113V	F186I	N231G	L281A	I332T	N369W	V428R
F056G	E114I	F186K	N231H	L281D	I332Y	F370A	V428S
F056H	E114L	F186L	N231I	L281G	N333G	F370D	V428Y
F056I	E114P	F186N	N231K	L281H	N333H	F370E	C429A
F056K	E114T	F186P	N231L	L281I	N333I	F370G	C429D
F056L	E114V	F186Q	N231P	L281K	N333K	F370H	C429K
F056P	W115A	F186R	N231Q	L281N	N333P	F370K	C429L
F056R	W115C	F186S	N231R	L281P	N333R	F370L	C429N
F056S	W115D	F186V	N231S	L281Q	N333S	F370N	C429P
F056T	W115F	F186W	N231V	L281R	N333T	F370P	C429S
F056V	W115G	P187A	T232C	L281S	N333W	F370Q	C429T
F056W	W115H	P187F	T232G	L281V	N333Y	F370R	C429V
Y057A	W115I	P187G	T232H	L281W	V334A	F370S	C429W
Y057D	W115K	P187H	T232K	S282F	V334C	F370V	C429Y
Y057F	W115L	P187I	T232L	S282L	V334D	F370Y	I430A
Y057G	W115M	P187L	T232N	S282V	V334E	A371P	I430D
Y057I	W115R	P187M	T232P	S282W	V334G	A371W	I430E
Y057L	W115S	P187N	T232Q	S282Y	V334M	I372A	I430L
Y057M	W115V	P187Q	T232V	Q283A	V334N	I372D	I430M
Y057P	W115Y	P187R	T232Y	Q283C	V334R	I372E	I430N
Y057Q	R116A	P187S	Q233D	Q283D	V334S	I372F	I430S
Y057R	R116C	P187T	Q233I	Q283F	T335F	I372G	I430T
Y057V	R116D	P187V	Q233P	Q283W	T335G	I372H	I430V
Y057W	R116E	P187W	Q233S	D284C	T335H	I372K	D431P
V058A	R116G	P187Y	Q233T	D284I	T335I	I372L	A432C
D059A	R116H	D188A	Q234A	D284P	T335K	I372N	A432F
D059E	R116I	D188C	Q234D	E285K	T335L	I372P	A432I
D059I	R116L	D188F	Q234E	E285P	T335P	I372R	A432K
D059L	R116N	D188G	Q234G	E285R	T335V	I372S	A432L
D059M	R116P	D188H	Q234H	E285T	T335W	I372T	A432M
D059P	R116Q	D188L	Q234N	E285V	T335Y	I372V	A432P
D059R	R116S	D188M	Q234P	L286A	L336A	I372W	A432Y
D059T	R116V	D188N	Q234S	L286C	L336E	Q373C	L434H
D059V	R116W	D188P	Q234T	L286D	L336F	Q373P	L434K
D059W	P117D	D188Q	Q234V	L286F	L336G	Q373W	L434P
D059Y	P117G	D188R	Q234W	L286H	L336K	L374D	L434Q
R060A	P117I	D188S	S235F	L286K	L336N	L374E	L434R
R060D	P117K	D188T	S235L	L286M	L336P	E375C	L434W
R060F	P117N	D188V	S235M	L286P	L336R	E375F	P437T
R060G	P117Q	D188W	S235R	L286T	L336S	E375P	M438Y
R060H	P117R	C189A	S235W	L286Y	L336T	E375V	E439N
R060I	P117S	C189E	S235Y	V287A	L336V	E375Y	E439R
R060L	P117V	C189G	P236C	V287C	R121G	K376I	T440Q
R060N	P117W	C189H	W119L	V287D	R121H	K376P	E441R
R060P	T118C	C189K	W119N	V287E	R121K	K376W	E442M
R060Q	T118D	C189L	W119P	V287G	R121L	G377C	E442N
R060S	T118E	C189M	W119R	V287K	R121M	G377I	E442S
R060T	T118G	C189N	R121A	V287L	R121P	G377L	P443D
T118R	T118P	T118W	R121C	R121F	G378D	G377V	G378E
T118Y	W119I	W119A	W119K	R121E	G378F	G378I	

Example 5

Assay for Hyaluronidase Activity Under Temperature and Phenophilic Conditions

Supernatants from PH20 activity variants set forth in Table 9, as identified in Example 4, were tested for stability under thermophilic and/or phenophilic conditions. The assay to measure hyaluronidase activity under temperature and phenophile conditions using biotinylated-HA (bHA) as substrate for measuring hyaluronidase activity was modified from the original assay described in Example 3 in that it incorporated a 4-hour 37° C. incubation of samples with or without m-cresol prior to measurement of enzymatic activity. The assay was used to identify PH20 mutants with thermophilic properties (activity greater at 37° C. condition than at 4° C.) and/or with phenolphilic properties (greater activity in the presence of m-cresol than wildtype PH20).

1. Primary Screen

Prior to incubating samples with bHA, variant PH20 samples were diluted into designated wells of an uncoated 4×HB plate for pre-incubation at 37° C. for 4 hours under the following conditions: 1) pre-incubation at 37° C. with 0.4% m-cresol; and 2) pre-incubation at 37° C. without 0.4% m-cresol. For the preincubation at 37° C. with 0.4% m-cresol, a 1% m-cresol intermediate stock was prepared from 50% (v/v) m-cresol stock solution. Briefly, in a 2 mL Wheaton glass vial a 50% stock of m-cresol (Fluka, Catalog No. 65996; Spectrum, Catalog No. C2773) was made in methanol based on the density (D=1.034 g/L). The vial was sealed and stored at −20° C. with protection from light in small aliquotes. Then, the 1% intermediate stock was generated by dilution in HEPES assay buffer (10 mM HEPES, 50 mM NaCl, 1 mM $CaCl_2$, 1 mg/mL BSA, pH 7.4, 0.05% Tween-20) daily immediately prior to use in a fume hood with vortexing.

Then, duplicates of transfected variant supernatant samples set forth in Table 9, generated as described above in Example 2, were each separately subjected to a 1:2.5 dilution of 1% m-cresol in HEPES assay buffer/transfected supernatant to obtain 0.4% final concentration of m-cresol. For the preincubation at 37° C. without 0.4% m-cresol, transfected variant supernatant samples were subjected to a 1:2.5 dilution in HEPES assay buffer/transfected supernatant. In addition, for each condition, an internal killing control was also tested by spiking in 3 U/mL of rHuPH20 in pH 7.4 HEPES buffer (generated as described in Example 1) that was diluted the same as described above for the transfected samples. The plates were sealed with plate sealers and incubated at 37° C. for 4 hours.

The preparation of the bHA coated plates and blocking of the plates prior to addition of the transfected variant supernatants or wildtype PH20 was the same as described in Example 3. The assay was further modified as follows. First, samples were diluted in duplicate 1:10 in HEPES assay buffer in 4×HB plates. For each variant, the samples that were tested were 1) non-preincubated transfected variant supernatant (no incubation; 4° C.); 2) preincubated transfected variant supernatants preincubated at 37° C. for 4 hours with 0.4% m-cresol (Cresol); or 3) preincubated transfected variant supernatant preincubated at 37° C. for 4 hours without 0.4% m-cresol (no cresol; 37° C.). In addition, the spiked-in samples also were tested. A standard curve using rHuPH20 was made as described in Example 3 without m-cresol. One hundred microliters (100 μl) of each standard and sample were transferred to pre-designated wells of the bHA-coated and blocked plate and incubated for approximately 1.5 hours at 37° C. Thus, each sample of each variant was tested in quadruplicate due to the preincubation of duplicate samples of each transfected variant supernatants in the pre-incubation step and the further duplicate of each sample in the bHA assay.

After the incubation, the plates were washed and binding to bHA detected as described above in Example 3. Optical density was measured at 450 nm within 30 minutes of adding the stop solution.

The U/mL activity was calculated from the standard curve and compared. The results were depicted as the percent (%) activity remaining under each of the following parameters: ratio of activity at 1) 37° C. preincubation without m-cresol/4° C.; 2) 37° C. after preincubation with m-cresol/4° C.; and 3) 37° C. after preincubation with m-cresol/after preincubation at 37° C. without m-cresol. Initial phenophile hits for reconfirmation were identified as those that in a duplicate assay exhibited a percentage of remaining activity under condition 3) of ≥20% of the original activity at 37° C.

Initial Hits were rescreened using a 6-well plate rescreen assay. For the rescreen, plasmid DNA corresponding to the potential Hit was transformed into *E. coli* bacteria and plasmid DNA prepared and purified using MaxiPrep according to the manufacturers instructions. The DNA sequence was confirmed.

The plasmid DNA was transfected into monolayer CHO—S cells (Invitrogen, Cat. No. 11619-012) grown on 6-well plates at a density of about 50-80% confluency using Lipofectamine 2000 (Invitrogen, Cat. No. 11668-027) according to the protocol suggested by the manufacturer. Transfections were performed in duplicate. The cells were incubated at 37° C. in a CO_2 incubator for 96 hours post-transfection before collecting the supernatant for the assay. As controls, cells also were transfected with the HZ24-PH20 (OHO)-IRES-SEAP expression vector (SEQ ID NO:4) that contains a codon-optimized wildtype PH20 sequence (OHO). Mock cells also were included as controls.

Ninety-Six (96) hours post-transfections, supernatant was collected from each sample, including the OHO and mock controls, and assayed for hyaluronidase activity under various conditions as described above: 1) non-preincubated transfected variant supernatant (no incubation; 4° C.); 2) preincubated transfected variant supernatants preincubated at 37° C. for 4 hours with 0.4% m-cresol (Cresol; 37° C.); or 3) preincubated transfected variant supernatant preincubated at 37° C. for 4 hours without 0.4% m-cresol (no cresol; 37° C.). Hyaluronidase activity was determined as described above using the bHA assay.

The results were assessed as described above. Absolute hyaluronidase activity (U/mL) was generated from the standard curve. In addition, percent activity was determined as a ratio of activity at 37° C./4° C., 37° C. plus m-cresol/4° C., and 37° C. plus m-cresol/37° C. The results are set forth in Tables 11 and 12 below.

TABLE 11

Absolute Hyaluronidase Activity

Mutant	No incubation (4° C.)		37° C. no cresol (37° C.)		37° C. with m-cresol (37° C. plus m-cresol)	
L001A	2.993	2.511	3.529	3.214	0.287	0.295
L001E	2.669	2.539	2.862	3.179	0.376	0.341
L001G	0.348	0.583	0.596	0.676	0.055	0.031
L001Q	5.135	6.443	6.133	5.719	0.621	0.636
L001R	5.603	4.390	6.576	7.042	0.458	0.396
P006A	2.965	3.208	4.088	3.495	0.404	0.435
V008M	1.376	1.401	1.856	1.678	0.000	0.008
I009Q	0.447	0.381	0.469	0.476	0.031	0.030
P010G	0.747	0.564	0.820	0.688	0.123	0.114
P010H	0.473	0.485	0.624	0.548	0.000	0.000
N011S	0.862	0.962	1.313	1.263	0.094	0.064
V012E	11.019	5.519	5.312	5.528	0.753	0.934
V012I	2.804	3.844	3.610	6.566	0.106	0.090
V012K	1.691	1.963	2.479	2.243	0.330	0.321
F014V	0.144	0.165	0.222	0.242	0.003	0.000
L015M	0.902	1.073	1.026	0.901	0.017	0.017
A020S	1.494	2.205	2.822	2.620	0.413	0.397
S022T	3.035	3.788	3.375	3.273	0.684	0.748
L026M	1.482	1.226	2.027	1.704	0.224	0.178
K028R	0.944	0.845	1.043	0.925	0.112	0.095
F029R	1.195	1.511	1.848	1.839	0.140	0.140
F029S	3.019	3.615	3.566	3.521	0.250	0.283
F029T	1.451	1.712	1.839	2.065	0.220	0.212
P032C	0.370	0.419	0.476	0.534	0.006	0.040
L033G	0.566	0.700	0.686	0.627	0.001	0.026
D034W	0.340	0.321	0.499	0.471	0.076	0.069

TABLE 11-continued

Mutant	Absolute Hyaluronidase Activity					
	No incubation (4° C.)		37° C. no cresol (37° C.)		37° C. with m-cresol (37° C. plus m-cresol)	
M035V	0.887	0.639	0.721	0.652	0.116	0.023
S036H	1.109	0.752	1.178	1.135	0.117	0.026
S036N	0.797	0.933	0.893	0.859	0.171	0.260
L037M	0.574	0.404	0.455	0.353	0.049	0.032
F040L	2.603	3.941	3.515	4.148	0.277	0.361
I046L	3.027	2.959	4.011	3.342	0.513	0.557
N047D	2.222	2.359	2.573	2.639	0.032	0.021
N047W	0.404	0.415	0.423	0.456	0.000	0.017
A048N	12.398	45.971	14.252	23.873	0.797	0.902
T049R	7.893	13.334	9.685	12.102	0.563	0.649
G050D	3.287	3.148	3.084	3.020	0.242	0.264
G050M	1.763	2.333	2.780	3.244	0.250	0.393
G052N	7.217	9.809	6.939	13.978	1.109	1.083
G052T	1.542	1.224	1.795	1.433	0.381	0.463
G052S	2.152	1.999	2.120	1.963	0.498	0.566
V058C	1.428	1.312	1.321	1.301	0.212	0.210
V058K	28.000	28.000	61.016	61.016	23.586	23.586
V058R	5.719	4.688	5.542	4.822	3.134	3.149
V058N	1.200	1.175	1.550	1.525	0.200	0.175
V058Y	1.040	0.770	1.071	1.088	0.388	0.454
V058Q	11.956	15.363	18.458	45.092	1.567	2.166
V058P	3.360	2.949	2.799	5.121	0.592	0.884
V058H	3.790	5.074	7.590	9.222	0.826	1.205
D068P	0.215	0.215	0.213	0.180	0.001	0.184
S069T	1.927	2.179	2.671	2.671	0.289	0.240
I070P	1.284	1.593	1.306	1.589	0.010	0.032
I070V	1.818	2.437	3.099	3.335	0.433	0.363
V073Q	4.846	5.441	5.880	5.827	0.383	0.477
V073R	0.522	0.803	0.720	0.804	0.018	0.059
T074E	2.903	3.834	3.868	3.871	0.666	0.626
T074M	0.569	0.744	0.656	0.771	0.079	0.083
T074N	2.792	1.905	2.565	2.995	0.281	0.204
T074P	2.331	1.593	2.525	2.648	0.309	0.265
T074R	0.999	0.820	0.806	1.066	0.060	0.023
T074V	1.186	1.280	1.365	1.460	0.101	0.080
V075M	0.917	1.087	1.233	1.321	0.003	0.028
K082L	1.362	1.311	1.563	3.302	0.325	0.354
K082N	3.202	3.411	3.396	3.244	0.792	0.861
I083V	3.706	2.633	5.194	3.615	1.552	1.017
I083Q	2.376	1.946	2.665	3.674	0.720	0.510
I083S	0.841	1.054	0.880	1.005	0.235	0.268
I083G	2.276	2.443	2.418	1.866	0.545	0.601
S084E	1.470	1.484	1.834	1.683	0.115	0.115
S084F	1.179	1.212	0.982	1.103	0.025	0.000
S084N	2.255	1.888	3.268	2.476	0.597	0.547
S084R	8.534	14.779	10.230	30.016	1.117	1.494
Q086A	2.084	2.120	2.845	3.310	0.405	0.322
Q086H	1.187	1.000	1.218	1.296	0.087	0.065
Q086K	0.127	0.110	0.126	0.072	0.032	0.023
Q086S	2.528	2.082	2.539	2.149	0.173	0.241
Q086T	3.018	2.542	2.832	4.562	0.290	0.406
D087G	2.755	2.176	2.252	1.971	0.034	0.122
D087L	2.070	2.277	2.195	2.311	0.324	0.299
D087M	2.262	2.325	2.510	2.038	0.191	0.335
D087S	5.210	10.305	6.983	14.399	0.569	0.928
D087V	1.361	1.364	1.553	1.187	0.142	0.189
D090E	8.251	12.299	7.666	19.836	1.093	1.234
D090N	2.812	2.775	3.123	2.737	0.379	0.290
K093Q	2.491	2.065	2.267	1.971	0.132	0.131
K093R	2.986	2.862	3.094	2.842	0.362	0.465
K094D	2.393	2.088	2.071	2.132	0.135	0.211
K094R	1.407	1.542	1.764	1.676	0.158	0.166
T097C	0.330	0.618	0.545	0.505	0.044	0.087
T097D	0.520	0.565	0.643	0.664	0.055	0.073
T097E	1.096	1.410	1.394	1.623	0.217	0.262
T097L	0.899	1.198	1.065	1.241	0.246	0.300
N104R	2.508	2.356	2.876	2.790	0.279	0.238
A120H	2.155	2.551	2.028	2.883	0.168	0.199
D127R	0.264	0.339	0.149	0.199	0.105	0.068
V128I	3.120	3.313	3.546	3.401	0.389	0.504
N131M	15.335	20.678	27.143	15.899	0.505	0.447
N131R	8.195	8.748	7.724	8.392	1.645	1.626
N131V	1.656	1.870	2.280	1.962	0.233	0.214
R132L	3.306	3.235	3.259	2.966	0.337	0.430
Q138L	1.494	1.660	1.611	1.521	0.410	0.347
Q140K	2.829	4.065	4.996	4.464	0.546	0.559
N141R	1.290	1.320	1.334	1.527	0.058	0.035
N141S	2.201	2.708	2.900	2.966	0.135	0.164
N141W	1.475	1.568	1.927	1.643	0.100	0.105
V142D	2.552	2.186	2.914	3.193	0.128	0.067
V142G	1.357	1.796	1.597	1.621	0.211	0.219
V142K	3.532	2.381	3.867	3.681	0.571	0.575
V142N	0.432	0.567	0.672	0.589	0.103	0.087
V142P	4.624	7.213	7.722	7.021	1.074	1.081
V142Q	5.090	6.900	7.618	6.897	0.678	0.678
V142R	1.968	2.595	2.941	2.689	0.364	0.330
V142S	2.789	2.988	4.763	3.497	0.416	0.591
V142T	1.926	3.260	4.313	4.031	0.495	0.472
Q143G	3.922	4.903	5.632	4.846	0.782	0.780
Q143K	3.634	3.671	7.285	5.008	1.043	1.039
L144R	3.810	4.581	5.191	5.107	0.556	0.520
L144T	1.496	1.681	1.941	1.831	0.285	0.219
L146P	0.818	0.782	0.954	0.904	0.011	0.031
T147S	0.984	1.149	1.399	1.497	0.055	0.039
T150N	0.442	0.585	0.622	0.684	0.039	0.046
T150S	1.747	1.400	1.875	1.988	0.120	0.121
E151A	2.870	2.269	2.965	2.860	0.359	0.337
E151L	3.365	3.289	4.446	4.007	0.218	0.251
E151S	5.187	4.591	5.987	6.262	0.371	0.294
E151T	2.442	3.000	3.134	3.309	0.000	0.000
E151V	3.998	4.247	4.459	4.232	0.326	0.314
E151W	7.166	14.248	11.352	13.524	0.131	0.121
K152T	1.204	1.377	1.796	1.883	0.100	0.067
K152W	2.084	1.795	2.549	2.406	0.063	0.069
E158S	0.339	0.397	0.451	0.407	0.000	0.000
K162E	0.168	0.195	0.114	0.080	0.004	0.024
L165F	4.775	5.250	5.075	5.075	0.600	0.725
V166Q	1.883	2.507	2.937	2.958	0.392	0.324
V166T	0.993	1.315	1.821	1.800	0.231	0.235
E167D	0.811	0.910	1.109	1.480	0.111	0.056
I169L	1.812	1.796	2.540	2.196	0.335	0.341
K170R	1.578	2.054	2.536	1.995	0.209	0.201
G172A	0.413	0.581	0.692	0.777	0.052	0.056
K173R	1.654	1.551	1.766	2.083	0.173	0.156
L174G	0.184	0.087	0.210	0.230	0.026	0.031
L174N	1.616	2.276	2.494	2.872	0.331	0.543
L174T	0.552	0.566	0.689	0.820	0.090	0.050
N178K	2.931	4.375	4.891	4.513	0.258	0.362
N178R	8.160	13.820	16.287	20.033	0.665	0.790
H193Q	1.060	1.367	2.264	1.888	0.346	0.346
K195T	1.227	0.806	1.548	1.911	0.348	0.292
K195N	1.266	1.437	1.649	1.385	0.369	0.353
K196E	0.732	0.660	0.663	1.017	0.244	0.239
K196R	2.246	2.285	2.383	2.174	0.315	0.384
F204P	3.500	4.550	2.925	3.750	2.475	4.725
N205A	0.515	0.837	0.717	0.854	0.153	0.160
N205E	1.011	2.004	1.627	1.870	0.314	0.346
N205L	1.084	1.029	1.165	0.000	0.123	0.088
N205T	0.295	0.367	0.428	0.406	0.043	0.053
V206I	0.317	0.508	0.600	0.565	0.079	0.088
K209R	2.041	2.453	2.445	1.951	0.291	0.077
D212N	5.568	4.549	6.271	6.016	0.167	0.322
D212S	1.987	1.502	2.442	2.222	0.204	0.152
D213A	0.235	0.283	0.432	0.438	0.116	0.060
D213M	1.664	2.080	2.650	2.046	0.181	0.142
S215H	2.448	3.056	2.670	2.414	0.268	0.139
S215M	1.497	2.175	2.618	1.630	0.110	0.146
N219I	0.338	0.250	0.860	0.728	0.076	0.082
E220V	3.783	3.828	4.993	4.349	0.371	0.257
T222G	3.528	5.262	5.399	5.549	0.033	0.044
T232F	0.539	1.242	0.716	0.781	0.089	0.153
Q233G	0.041	0.095	0.115	0.121	0.000	0.000
Q234M	6.029	6.031	5.764	4.871	1.286	0.988
S235A	0.550	0.502	0.714	0.607	0.079	0.073
V237C	0.623	0.708	0.860	0.824	0.000	0.000
V237H	0.303	0.316	0.370	0.459	0.046	0.034
V237T	0.152	0.196	0.254	0.247	0.054	0.053
A238E	2.050	1.800	1.945	2.559	0.159	0.171

TABLE 11-continued

	Absolute Hyaluronidase Activity					
Mutant	No incubation (4° C.)		37° C. no cresol (37° C.)		37° C. with m-cresol (37° C. plus m-cresol)	
A238H	0.579	0.363	0.345	0.743	0.090	0.062
T240A	1.107	0.900	1.564	1.302	0.143	0.118
T240Q	0.333	0.510	0.542	0.617	0.080	0.085
R248A	2.274	2.499	2.575	3.115	0.027	0.075
E249V	3.001	3.894	4.284	4.325	0.655	0.712
P257G	3.981	4.452	4.985	5.022	0.039	0.034
K260M	0.719	0.960	0.839	0.935	0.072	0.068
S261A	3.253	3.117	1.872	2.686	1.264	1.451
S261K	6.089	5.421	9.860	6.297	1.583	1.437
S261N	14.149	40.257	20.219	14.303	2.115	1.917
A267T	0.052	0.095	0.102	0.106	0.036	0.041
F273H	0.340	0.436	0.417	0.519	0.025	0.031
F273Y	0.558	0.505	0.668	0.519	0.052	0.050
Q276H	2.706	1.877	2.027	1.997	0.181	0.201
Q276M	0.775	0.768	0.762	0.806	0.043	0.000
Q276R	6.080	9.717	7.383	14.593	0.807	1.281
Q276S	1.353	1.212	1.497	1.681	0.149	0.147
V277A	1.202	1.643	1.692	2.129	0.118	0.110
V277E	2.440	2.340	4.289	4.577	0.161	0.239
V277H	5.548	5.302	7.181	7.300	0.227	0.512
V277K	8.950	8.996	33.627	33.627	4.442	4.045
V277M	1.279	1.622	1.754	1.818	0.264	0.270
V277N	14.351	4.306	12.865	11.772	0.938	0.796
V277Q	5.459	5.461	6.547	6.343	0.373	0.493
V277R	18.300	12.038	17.581	20.641	2.737	2.023
V277S	14.351	10.444	9.509	15.135	0.727	0.716
V277T	8.412	7.804	8.497	11.184	0.679	0.871
L278E	4.416	2.795	3.330	2.800	0.170	0.202
L278G	7.502	7.456	9.173	7.760	0.596	0.612
K279H	0.888	1.087	1.234	1.339	0.185	0.269
V287T	0.580	0.667	0.843	0.832	0.139	0.100
T289S	0.783	1.019	0.819	1.001	0.008	0.007
G291S	0.227	0.322	0.419	0.385	0.051	0.016
G291V	3.662	3.707	4.131	5.599	0.821	0.706
E292C	1.344	1.599	1.711	1.617	0.138	0.144
E292F	6.106	4.697	8.422	6.216	0.520	0.363
E292H	2.620	3.316	4.458	3.830	0.389	0.451
E292R	2.810	2.178	3.155	2.829	0.398	0.339
E292V	0.891	1.121	1.453	1.494	0.193	0.177
T293A	1.986	3.110	2.546	1.789	0.086	0.076
A298G	0.161	0.274	0.342	0.236	0.030	0.022
L307G	0.616	0.661	0.726	0.605	0.000	0.000
S308D	0.264	0.325	0.337	0.344	0.014	0.010
S308K	0.651	0.722	0.826	0.716	0.011	0.000
S308N	3.995	4.406	6.808	6.128	0.386	0.362
I309E	3.166	2.819	3.921	3.663	0.637	0.528
I309G	6.651	5.429	6.824	6.194	0.503	0.400
I309L	0.326	0.403	0.501	0.431	0.048	0.047
I309M	2.809	2.473	3.467	3.383	0.278	0.239
I309N	4.865	5.191	5.444	5.054	0.380	0.327
I309S	10.719	28.759	18.217	158.604	0.748	1.367
I309T	3.052	2.509	2.989	3.735	0.228	0.207
I309V	1.705	1.292	1.929	1.787	0.029	0.062
M310G	4.514	6.397	7.568	7.084	0.866	0.915
M310Q	3.648	3.179	3.912	3.380	1.088	0.955
M313G	0.252	0.325	0.348	0.355	0.034	0.036
M313H	3.767	5.276	10.243	10.395	0.380	0.404
M313K	12.689	12.122	15.085	12.984	0.129	0.072
M313P	4.050	2.951	4.198	3.919	0.209	0.177
M313R	4.634	10.863	7.288	3.568	0.337	0.296
M313T	2.903	4.474	4.705	4.467	0.331	0.313
M313Y	1.063	1.262	1.276	1.300	0.096	0.089
K314S	2.848	4.450	4.042	5.879	0.391	0.533
K314Y	0.093	0.131	0.226	0.182	0.013	0.020
S315A	1.472	1.082	1.345	1.484	0.222	0.148
S315H	2.412	3.242	3.648	3.414	0.440	0.371
S315Y	0.279	0.626	0.477	0.362	0.146	0.143
L317A	3.254	2.845	4.019	3.776	0.280	0.317
L317I	1.078	1.524	2.021	1.687	0.257	0.180
L317K	12.129	9.382	11.668	12.591	0.402	0.445
L317N	2.907	3.066	3.703	3.717	0.445	0.540
L317R	8.631	15.187	20.585	15.106	0.796	0.857
L317S	11.586	29.267	10.535	25.114	1.637	1.613
L317T	1.338	1.073	1.953	1.656	0.136	0.018
L317W	0.810	1.128	1.326	1.665	0.158	0.171
L318D	1.750	1.970	1.847	1.930	0.322	0.322
L318H	1.073	0.806	1.072	1.005	0.046	0.074
L318R	2.856	3.464	4.583	4.187	0.258	0.260
N321R	3.069	4.409	5.059	4.946	0.482	0.426
N321S	0.683	0.710	0.700	0.772	0.058	0.035
E324N	4.309	2.530	4.508	3.321	0.348	0.303
T325E	1.071	1.270	1.337	1.352	0.193	0.143
N328G	0.379	0.504	0.747	0.553	0.031	0.040
N328Y	2.629	4.543	4.758	4.543	0.490	0.477
T335S	0.905	0.787	0.977	0.986	0.113	0.062
Q347A	8.316	11.961	8.432	11.508	0.918	1.266
Q347G	1.358	1.120	3.021	2.319	0.253	0.209
Q349M	1.493	1.629	1.486	1.760	0.178	0.217
Q349R	0.451	0.572	0.663	0.598	0.078	0.079
V351S	1.379	1.633	1.804	1.647	0.000	0.000
I353V	2.335	1.954	3.090	2.697	0.323	0.321
N356H	0.445	0.451	0.445	0.588	0.038	0.023
N356S	0.262	0.253	0.136	0.318	0.000	0.008
S359E	2.616	2.635	3.547	3.560	0.382	0.333
S359H	0.403	0.371	0.445	0.374	0.000	0.000
P367A	0.643	0.782	1.074	0.996	0.139	0.131
P367G	0.593	0.530	0.686	0.650	0.000	0.000
P367K	0.707	0.767	0.890	0.513	0.045	0.052
P367S	3.967	3.478	2.946	3.073	0.424	0.505
D368A	1.762	2.321	2.143	1.895	0.031	0.040
D368E	3.464	4.944	5.772	4.842	0.530	0.555
D368L	0.557	0.566	0.607	0.619	0.000	0.006
D368M	0.861	1.065	1.031	1.104	0.028	0.028
D368R	4.503	5.270	7.418	6.226	0.754	0.735
D368T	2.345	1.993	2.512	2.525	0.072	0.085
N369R	1.548	2.719	2.503	2.022	0.160	0.125
A371F	2.760	5.207	4.974	3.980	0.308	0.222
A371H	8.101	86.587	77.531	77.531	1.403	1.316
A371H	3.509	4.058	3.900	3.879	0.000	0.334
A371K	2.903	3.546	3.963	4.055	0.509	0.505
A371L	11.018	40.668	76.587	43.516	1.159	0.964
A371L	3.328	3.445	3.472	2.075	0.000	0.025
A371R	25.855	25.855	n/a	n/a	2.851	3.634
A371R	6.592	7.733	7.987	7.576	0.000	0.196
A371S	3.329	3.505	4.916	4.611	0.412	0.781
L374P	2.939	7.129	11.522	8.771	0.665	0.646
E375A	0.627	0.507	0.557	0.683	0.000	0.014
E375G	1.596	1.299	2.025	1.806	0.209	0.265
E375R	0.937	1.132	1.529	1.318	0.201	0.260
K376D	0.458	0.312	0.518	0.515	0.064	0.026
K376E	1.572	1.094	1.572	1.674	0.213	0.174
K376Q	0.727	0.940	0.910	0.846	0.116	0.102
K376R	2.086	1.351	1.704	2.690	0.539	0.279
K376T	0.847	1.001	1.026	1.135	0.153	0.064
K376V	0.834	0.861	1.036	1.021	0.033	0.026
K376Y	1.316	0.777	1.353	0.747	0.125	0.097
G377D	1.159	1.332	1.285	1.763	0.202	0.186
G377E	0.877	0.926	1.144	1.189	0.092	0.088
G377H	3.037	3.432	4.460	3.598	0.372	0.364
G377K	3.445	4.101	6.405	4.911	0.283	0.245
G377R	1.096	1.257	1.312	1.191	0.077	0.085
G377S	0.453	0.452	0.492	0.457	0.034	0.036
G377T	2.198	2.313	2.474	2.522	0.424	0.461
F380W	17.497	27.987	25.734	29.353	2.566	2.716
T381S	2.861	3.161	3.886	3.558	0.521	0.367
R383I	1.959	6.936	10.340	6.820	0.655	0.513
R383S	2.429	2.548	3.228	3.044	0.339	0.321
K385A	0.479	0.669	0.604	0.754	0.028	0.000
K385Q	1.746	2.089	2.403	2.609	0.217	0.196
K385V	1.232	1.750	1.387	1.410	0.071	0.042
E389A	6.872	10.944	21.081	24.610	0.449	0.449
E389G	0.166	0.203	0.188	0.284	0.004	0.000
E389L	1.814	2.142	2.598	2.403	0.370	0.303
E389Q	2.547	3.432	3.459	3.423	0.411	0.437
E389S	1.847	2.640	3.059	2.456	0.000	0.007
E392A	1.797	1.370	2.021	2.133	0.147	0.136
E392F	1.575	1.407	1.821	2.023	0.071	0.079
E392Q	5.826	4.653	6.583	4.364	0.693	0.729

TABLE 11-continued

Absolute Hyaluronidase Activity						
Mutant	No incubation (4° C.)		37° C. no cresol (37° C.)		37° C. with m-cresol (37° C. plus m-cresol)	
E392R	4.555	5.306	5.900	6.548	0.218	0.193
E392V	3.817	2.936	4.747	4.544	0.367	0.291
Q393F	1.754	2.186	2.455	2.222	0.260	0.226
Q393M	1.252	1.826	1.749	1.588	0.028	0.049
S395A	4.220	6.127	8.788	6.906	1.141	0.856
S395H	1.609	2.261	2.574	2.564	0.323	0.268
E396A	1.135	1.184	1.497	1.524	0.126	0.149
E396H	0.357	0.532	0.751	0.684	0.069	0.022
E396Q	1.310	1.625	1.611	1.559	0.162	0.160
E396S	3.375	5.709	5.274	6.380	0.146	0.129
Y399T	2.538	3.250	3.313	3.989	0.000	0.002
Y399V	2.738	2.697	3.028	3.129	0.484	0.557
Y399W	1.400	1.883	1.715	1.946	0.236	0.233
S401A	2.636	3.171	3.216	3.148	0.447	0.410
S401E	1.685	1.601	2.110	2.060	0.344	0.309
S404A	1.288	1.635	1.924	1.724	0.000	0.019
L406F	0.706	0.490	0.867	0.716	0.000	0.000
L406N	0.617	0.795	0.943	1.044	0.060	0.070
S407A	2.428	2.949	3.432	3.255	0.389	0.548
S407D	2.090	5.790	5.038	5.682	0.569	0.575
S407P	2.660	2.708	3.812	3.301	0.261	0.366
A412Q	2.001	2.918	2.925	2.902	0.279	0.247
A412R	4.562	5.132	6.390	6.347	0.570	0.596
A412V	2.581	3.451	3.789	3.511	0.189	0.189
D416L	0.610	0.817	0.737	1.043	0.130	0.160
D418R	4.541	4.847	5.347	5.438	0.406	0.583
A419H	10.409	20.311	25.109	38.221	2.214	2.293
A419K	12.835	10.298	24.536	208.289	2.556	3.173
D421A	5.968	5.617	6.094	16.940	0.761	0.764
D421H	48.012	48.012	160.106	32.481	16.300	28.113
D421K	5.527	5.225	6.864	5.346	0.523	0.725
D421N	9.060	8.635	10.039	8.645	1.502	1.422
D421Q	7.529	5.581	7.858	8.016	0.842	0.994
D421R	6.637	5.463	9.211	7.537	0.815	0.737
D421S	5.556	5.355	7.899	8.898	0.869	0.762
A425G	10.421	8.827	7.796	10.676	0.827	1.189
G427Q	1.008	1.252	1.342	1.230	0.031	0.106
G427T	1.330	1.380	1.664	1.643	0.080	0.065
V428L	2.138	2.769	2.930	3.029	0.053	0.030
D431E	2.810	2.220	1.972	2.112	0.519	0.438
D431H	2.154	3.185	4.017	3.028	0.294	0.301
D431K	8.123	16.953	19.563	11.575	2.272	2.339
D431L	1.211	1.215	1.564	1.448	0.164	0.170
D431N	11.819	12.063	16.358	15.131	1.601	1.399
D431Q	6.077	9.828	14.157	10.760	1.533	1.153
D431S	14.523	10.220	11.338	9.075	0.853	0.829
F433A	4.035	4.673	5.943	4.649	0.581	0.595
F433H	1.836	2.397	2.574	2.108	0.347	0.356
F433I	2.754	2.643	2.990	2.299	0.338	0.382
F433K	17.815	14.495	16.240	49.615	1.806	1.790
F433R	8.198	6.719	10.572	8.960	1.113	0.857
F433T	6.005	5.941	9.716	8.019	1.327	1.542
F433V	10.645	7.762	150.315	8.696	2.415	1.505
F433W	0.526	0.795	0.784	0.903	0.082	0.068
P437I	0.759	0.996	1.130	1.066	0.027	0.019
M438A	1.996	1.518	2.125	2.060	0.214	0.210
M438D	2.849	2.522	3.002	2.857	0.305	0.074
M438E	4.681	4.992	5.386	5.680	0.431	0.518
M438L	10.127	5.268	6.663	11.324	0.670	0.739
M438N	6.172	5.531	8.050	5.568	0.649	0.662
M438T	2.218	2.411	2.308	2.500	0.309	0.304
E439A	3.557	4.432	4.883	4.235	0.568	0.596
E439A	1.099	0.998	1.694	1.470	0.080	0.109
E439C	0.148	0.256	0.286	0.286	0.042	0.045
E439K	0.466	0.588	0.580	0.616	0.077	0.065
E439P	2.868	3.736	3.394	3.267	0.529	0.490
E439Q	1.070	0.848	1.087	1.080	0.116	0.115
E439T	1.965	1.889	2.179	2.323	0.313	0.263
T440D	4.148	4.443	4.931	3.533	0.568	0.651
T440H	2.317	1.982	3.297	2.595	0.147	0.196
T440M	3.397	3.305	2.878	2.873	0.254	0.367
T440P	3.562	3.593	3.987	3.277	0.540	0.566
T440S	2.522	2.207	2.533	2.895	0.283	0.284
E441F	1.402	1.407	1.813	1.560	0.204	0.178
E442G	2.871	3.340	3.193	3.347	0.327	0.367
P443E	0.907	0.710	0.856	0.928	0.044	0.063
P443F	1.830	2.370	2.683	2.321	0.301	0.286
P443G	4.077	2.921	9.751	4.614	0.835	0.756
Q444E	8.293	3.861	6.800	6.213	0.581	0.594
Q444H	3.823	3.936	5.746	4.710	0.486	0.513
Q444V	2.193	2.107	2.847	2.583	0.384	0.284
I445M	5.265	4.438	4.480	4.489	0.773	0.691
I445N	3.375	4.024	3.592	3.515	0.499	0.455
I445W	2.289	2.694	2.683	2.695	0.314	0.296
Y447E	2.373	2.464	2.363	2.685	0.391	0.345
Y447G	0.945	1.352	1.358	1.401	0.187	0.162
Y447P	0.991	1.383	1.379	1.490	0.190	0.183
positive control (OHO)	2.919	2.173	2.773	2.105	0.145	0.178
	3.984	4.463	4.215	4.823	0.189	0.253
	3	2.725	3	3.325	0.1	0.125
	2.501	2.883	2.370	3.158	0.452	0.522
	7.629	2.989	10.835	3.914	0.485	0.219
	5.783	5.356	2.609	3.643	0.542	0.402
	5.279	5.422	2.815	4.026	0.618	0.401
	4.775	4.385	2.845	3.327	0.718	0.540
	3.617	4.264	3.322	3.427	0.633	0.479
	5.881	4.511	5.518	4.359	0.743	0.848
	6.754	4.932	3.902	4.120	0.665	0.724
	3.911	3.494	3.911	5.179	0.726	0.841
	5.406	7.559	4.018	4.620	0.735	0.429
	4.015	3.887	3.9400	3.4080	0.3340	0.3410
	2.604	2.339	2.4430	2.3910	0.2350	0.2330
	3.736	3.473	3.6210	3.0560	0.3100	0.2770
	3.759	3.509	3.6330	3.0490	0.3600	0.3030

n/a (not available; e.g., beyond detection limit)

TABLE 12

Percent (%) Activity						
	duplicate 1			duplicate 2		
	% activity at 37° C./4° C.	% activity 37° C. + m-cresol/37° C.	% activity 37° C. + m-cresol/4° C.	% activity at 37° C./4° C.	% activity 37° C. + m-cresol/37° C.	% activity 37° C. + m-cresol/4° C.
L001A	117.908	8.13	9.59	127.997	9.179	11.75
L001E	107.231	13.14	14.09	125.207	10.727	13.43
L001G	171.264	9.23	15.80	115.952	4.586	5.32
L001Q	119.435	10.13	12.09	88.763	11.121	9.87
L001R	117.366	6.96	8.17	160.410	5.623	9.02
P006A	137.875	9.88	13.63	108.946	12.446	13.56
V008M	134.884	0.00	0.00	119.772	0.477	0.57
I009Q	104.922	6.61	6.94	124.934	6.303	7.87

TABLE 12-continued

Percent (%) Activity						
	duplicate 1			duplicate 2		
	% activity at 37° C./4° C.	% activity 37° C. + m-cresol/37° C.	% activity 37° C. + m-cresol/4° C.	% activity at 37° C./4° C.	% activity 37° C. + m-cresol/37° C.	% activity 37° C. + m-cresol/4° C.
P010G	109.772	15.00	16.47	121.986	16.570	20.21
P010H	131.924	0.00	0.00	112.990	0.000	0.00
N011S	152.320	7.16	10.90	131.289	5.067	6.65
V012E	48.208	14.18	6.83	100.163	16.896	16.92
V012I	128.745	2.94	3.78	170.812	1.371	2.34
V012K	146.600	13.31	19.52	114.264	14.311	16.35
F014V	154.167	1.35	2.08	146.667	0.000	0.00
L015M	113.747	1.66	1.88	83.970	1.887	1.58
A020S	188.889	14.64	27.64	118.821	15.153	18.00
S022T	111.203	20.27	22.54	86.404	22.854	19.75
L026M	136.775	11.05	15.11	138.989	10.446	14.52
K028R	110.487	10.74	11.86	109.467	10.270	11.24
F029R	154.644	7.58	11.72	121.707	7.613	9.27
F029S	118.119	7.01	8.28	97.400	8.037	7.83
F029T	126.740	11.96	15.16	120.619	10.266	12.38
P032C	128.649	1.26	1.62	127.446	7.491	9.55
L033G	121.201	0.15	0.18	89.571	4.147	3.71
D034W	146.765	15.23	22.35	146.729	14.650	21.50
M035V	81.285	16.09	13.08	102.034	3.528	3.60
S036H	106.222	9.93	10.55	150.931	2.291	3.46
S036N	112.045	19.15	21.46	92.069	30.268	27.87
L037M	79.268	10.77	8.54	87.376	9.065	7.92
F040L	135.036	7.88	10.64	105.252	8.703	9.16
I046L	132.507	12.79	16.95	112.944	16.667	18.82
N047D	115.797	1.24	1.44	111.869	0.796	0.89
N047W	104.703	0.00	0.00	109.880	3.728	4.10
A048N	114.954	5.59	6.43	51.931	3.778	1.96
T049R	122.704	5.81	7.13	90.760	5.363	4.87
G050D	93.824	7.85	7.36	95.934	8.742	8.39
G050M	157.686	8.99	14.18	139.048	12.115	16.85
G052N	96.148	15.98	15.37	142.502	7.748	11.04
G052T	116.407	21.23	24.71	117.075	32.310	37.83
G052S	98.513	23.49	23.14	98.199	28.833	28.31
V058C	92.507	16.05	14.85	99.162	16.141	16.01
V058K	217.914	38.66	84.24	217.914	38.655	84.24
V058R	96.905	56.55	54.80	102.858	65.305	67.17
V058N	129.167	12.90	16.67	129.787	11.475	14.89
V058Y	102.981	36.23	37.31	141.299	41.728	58.96
V058Q	154.383	8.49	13.11	293.510	4.804	14.10
V058P	83.304	21.15	17.62	173.652	17.262	29.98
V058H	200.264	10.88	21.79	181.750	13.067	23.75
D068P	99.070	0.47	0.47	83.721	102.222	85.58
S069T	138.609	10.82	15.00	122.579	8.985	11.01
I070P	101.713	0.77	0.78	99.749	2.014	2.01
I070V	170.462	13.97	23.82	136.849	10.885	14.90
V073Q	121.337	6.51	7.90	107.094	8.186	8.77
V073R	137.931	2.50	3.45	100.125	7.338	7.35
T074E	133.241	17.22	22.94	100.965	16.172	16.33
T074M	115.290	12.04	13.88	103.629	10.765	11.16
T074N	91.870	10.96	10.06	157.218	6.811	10.71
T074P	108.323	12.24	13.26	166.227	10.008	16.64
T074R	80.681	7.44	6.01	130.000	2.158	2.80
T074V	115.093	7.40	8.52	114.063	5.479	6.25
V075M	134.460	0.24	0.33	121.527	2.120	2.58
K082L	114.758	20.79	23.86	251.869	10.721	27.00
K082N	106.059	23.32	24.73	95.104	26.541	25.24
I083V	140.151	29.88	41.88	137.296	28.133	38.63
I083Q	112.163	27.02	30.30	188.798	13.881	26.21
I083S	104.637	26.70	27.94	95.351	26.667	25.43
I083G	106.239	22.54	23.95	76.381	32.208	24.60
S084E	124.762	6.27	7.82	113.410	6.833	7.75
S084F	83.291	2.55	2.12	91.007	0.000	0.00
S084N	144.922	18.27	26.47	131.144	22.092	28.97
S084R	119.873	10.92	13.09	203.099	4.977	10.11
Q086A	136.516	14.24	19.43	156.132	9.728	15.19
Q086H	102.612	7.14	7.33	129.600	5.015	6.50
Q086K	99.213	25.40	25.20	65.455	31.944	20.91
Q086S	100.435	6.81	6.84	103.218	11.215	11.58
Q086T	93.837	10.24	9.61	179.465	8.900	15.97
D087G	81.742	1.51	1.23	90.579	6.190	5.61
D087L	106.039	14.76	15.65	101.493	12.938	13.13
D087M	110.964	7.61	8.44	87.656	16.438	14.41

TABLE 12-continued

	Percent (%) Activity					
	duplicate 1			duplicate 2		
	% activity at 37° C./4° C.	% activity 37° C. + m-cresol/37° C.	% activity 37° C. + m-cresol/4° C.	% activity at 37° C./4° C.	% activity 37° C. + m-cresol/37° C.	% activity 37° C. + m-cresol/4° C.
D087S	134.031	8.15	10.92	139.728	6.445	9.01
D087V	114.107	9.14	10.43	87.023	15.922	13.86
D090E	92.910	14.26	13.25	161.281	6.221	10.03
D090N	111.060	12.14	13.48	98.631	10.596	10.45
K093Q	91.008	5.82	5.30	95.448	6.646	6.34
K093R	103.617	11.70	12.12	99.301	16.362	16.25
K094D	86.544	6.52	5.64	102.107	9.897	10.11
K094R	125.373	8.96	11.23	108.690	9.905	10.77
T097C	165.152	8.07	13.33	81.715	17.228	14.08
T097D	123.654	8.55	10.58	117.522	10.994	12.92
T097E	127.190	15.57	19.80	115.106	16.143	18.58
T097L	118.465	23.10	27.36	103.589	24.174	25.04
N104R	114.673	9.70	11.12	118.421	8.530	10.10
A120H	94.107	8.28	7.80	113.015	6.903	7.80
D127R	56.439	70.47	39.77	58.702	34.171	20.06
V128I	113.654	10.97	12.47	102.656	14.819	15.21
N131M	177.000	1.86	3.29	76.888	2.811	2.16
N131R	94.253	21.30	20.07	95.930	19.376	18.59
N131V	137.681	10.22	14.07	104.920	10.907	11.44
R132L	98.578	10.34	10.19	91.685	14.498	13.29
Q138L	107.831	25.45	27.44	91.627	22.814	20.90
Q140K	176.600	10.93	19.30	109.815	12.522	13.75
N141R	103.411	4.35	4.50	115.682	2.292	2.65
N141S	131.758	4.66	6.13	109.527	5.529	6.06
N141W	130.644	5.19	6.78	104.783	6.391	6.70
V142D	114.185	4.39	5.02	146.066	2.098	3.06
V142G	117.686	13.21	15.55	90.256	13.510	12.19
V142K	109.485	14.77	16.17	154.599	15.621	24.15
V142N	155.556	15.33	23.84	103.880	14.771	15.34
V142P	166.998	13.91	23.23	97.338	15.397	14.99
V142Q	149.666	8.90	13.32	99.957	9.830	9.83
V142R	149.441	12.38	18.50	103.622	12.272	12.72
V142S	170.778	8.73	14.92	117.035	16.900	19.78
V142T	223.936	11.48	25.70	123.650	11.709	14.48
Q143G	143.600	13.88	19.94	98.837	16.096	15.91
Q143K	200.468	14.32	28.70	136.421	20.747	28.30
L144R	136.247	10.71	14.59	111.482	10.182	11.35
L144T	129.746	14.68	19.05	108.923	11.961	13.03
L146P	116.626	1.15	1.34	115.601	3.429	3.96
T147S	142.175	3.93	5.59	130.287	2.605	3.39
T150N	140.724	6.27	8.82	116.923	6.725	7.86
T150S	107.327	6.40	6.87	142.000	6.087	8.64
E151A	103.310	12.11	12.51	126.047	11.783	14.85
E151L	132.125	4.90	6.48	121.830	6.264	7.63
E151S	115.423	6.20	7.15	136.397	4.695	6.40
E151T	128.337	0.00	0.00	110.300	0.000	0.00
E151V	111.531	7.31	8.15	99.647	7.420	7.39
E151W	158.415	1.15	1.83	94.919	0.895	0.85
K152T	149.169	5.57	8.31	136.747	3.558	4.87
K152W	122.313	2.47	3.02	134.039	2.868	3.84
E158S	133.038	0.00	0.00	102.519	0.000	0.00
K162E	67.857	3.51	2.38	41.026	30.000	12.31
L165F	106.283	11.82	12.57	96.667	14.286	13.81
V166Q	155.975	13.35	20.82	117.990	10.953	12.92
V166T	183.384	12.69	23.26	136.882	13.056	17.87
E167D	136.745	10.01	13.69	162.637	3.784	6.15
I169L	140.177	13.19	18.49	122.272	15.528	18.99
K170R	160.710	8.24	13.24	97.128	10.075	9.79
G172A	167.554	7.51	12.59	133.735	7.207	9.64
K173R	106.771	9.80	10.46	134.300	7.489	10.06
L174G	114.130	12.38	14.13	264.368	13.478	35.63
L174N	154.332	13.27	20.48	126.186	18.907	23.86
L174T	124.819	13.06	16.30	144.876	6.098	8.83
N178K	166.871	5.27	8.80	103.154	8.021	8.27
N178R	199.596	4.08	8.15	144.957	3.943	5.72
H193Q	213.585	15.28	32.64	138.113	18.326	25.31
K195T	126.161	22.48	28.36	237.097	15.280	36.23
K195N	130.253	22.38	29.15	96.381	25.487	24.57
K196E	90.574	36.80	33.33	154.091	23.500	36.21
K196R	106.100	13.22	14.02	95.142	17.663	16.81
F204P	83.571	84.62	70.71	82.418	126.000	103.85
N205A	139.223	21.34	29.71	102.031	18.735	19.12

TABLE 12-continued

	Percent (%) Activity					
	duplicate 1			duplicate 2		
	% activity at 37° C./4° C.	% activity 37° C. + m-cresol/37° C.	% activity 37° C. + m-cresol/4° C.	% activity at 37° C./4° C.	% activity 37° C. + m-cresol/37° C.	% activity 37° C. + m-cresol/4° C.
N205E	160.930	19.30	31.06	93.313	18.503	17.27
N205L	107.472	10.56	11.35	0.000	#DIV/0!	8.55
N205T	145.085	10.05	14.58	110.627	13.054	14.44
V206I	189.274	13.17	24.92	111.220	15.575	17.32
K209R	119.794	11.90	14.26	79.535	3.947	3.14
D212N	112.626	2.66	3.00	132.249	5.352	7.08
D212S	122.899	8.35	10.27	147.936	6.841	10.12
D213A	183.830	26.85	49.36	154.770	13.699	21.20
D213M	159.255	6.83	10.88	98.365	6.940	6.83
S215H	109.069	10.04	10.95	78.992	5.758	4.55
S215M	174.883	4.20	7.35	74.943	8.957	6.71
N219I	254.438	8.84	22.49	291.200	11.264	32.80
E220V	131.985	7.43	9.81	113.610	5.909	6.71
T222G	153.033	0.61	0.94	105.454	0.793	0.84
T232F	132.839	12.43	16.51	62.882	19.590	12.32
Q233G	280.488	0.00	0.00	127.368	0.000	0.00
Q234M	95.605	22.31	21.33	80.766	20.283	16.38
S235A	129.818	11.06	14.36	120.916	12.026	14.54
V237C	138.042	0.00	0.00	116.384	0.000	0.00
V237H	122.112	12.43	15.18	145.253	7.407	10.76
V237T	167.105	21.26	35.53	126.020	21.457	27.04
A238E	94.878	8.17	7.76	142.167	6.682	9.50
A238H	59.585	26.09	15.54	204.683	8.345	17.08
T240A	141.283	9.14	12.92	144.667	9.063	13.11
T240Q	162.763	14.76	24.02	120.980	13.776	16.67
R248A	113.237	1.05	1.19	124.650	2.408	3.00
E249V	142.752	15.29	21.83	111.068	16.462	18.28
P257G	125.220	0.78	0.98	112.803	0.677	0.76
K260M	116.690	8.58	10.01	97.396	7.273	7.08
S261A	57.547	67.52	38.86	86.173	54.021	46.55
S261K	161.931	16.05	26.00	116.159	22.820	26.51
S261N	142.901	10.46	14.95	35.529	13.403	4.76
A267T	196.154	35.29	69.23	111.579	38.679	43.16
F273H	122.647	6.00	7.35	119.037	5.973	7.11
F273Y	119.713	7.78	9.32	102.772	9.634	9.90
Q276H	74.908	8.93	6.69	106.393	10.065	10.71
Q276M	98.323	5.64	5.55	104.948	0.000	0.00
Q276R	121.431	10.93	13.27	150.180	8.778	13.18
Q276S	110.643	9.95	11.01	138.696	8.745	12.13
V277A	140.765	6.97	9.82	129.580	5.167	6.70
V277E	175.779	3.75	6.60	195.598	5.222	10.21
V277H	129.434	3.16	4.09	137.684	7.014	9.66
V277K	375.721	13.21	49.63	373.799	12.029	44.96
V277M	137.138	15.05	20.64	112.084	14.851	16.65
V277N	89.645	7.29	6.54	273.386	6.762	18.49
V277Q	119.930	5.70	6.83	116.151	7.772	9.03
V277R	96.071	15.57	14.96	171.465	9.801	16.81
V277S	66.260	7.65	5.07	144.916	4.731	6.86
V277T	101.010	7.99	8.07	143.311	7.788	11.16
L278E	75.408	5.11	3.85	100.179	7.214	7.23
L278G	122.274	6.50	7.94	104.077	7.887	8.21
K279H	138.964	14.99	20.83	123.183	20.090	24.75
V287T	145.345	16.49	23.97	124.738	12.019	14.99
T289S	104.598	0.98	1.02	98.234	0.699	0.69
G291S	184.581	12.17	22.47	119.565	4.156	4.97
G291V	112.807	19.87	22.42	151.039	12.609	19.05
E292C	127.307	8.07	10.27	101.126	8.905	9.01
E292F	137.930	6.17	8.52	132.340	5.840	7.73
E292H	170.153	8.73	14.85	115.501	11.775	13.60
E292R	112.278	12.61	14.16	129.890	11.983	15.56
E292V	163.075	13.28	21.66	133.274	11.847	15.79
T293A	128.197	3.38	4.33	57.524	4.248	2.44
A298G	212.422	8.77	18.63	86.131	9.322	8.03
L307G	117.857	0.00	0.00	91.528	0.000	0.00
S308D	127.652	4.15	5.30	105.846	2.907	3.08
S308K	126.882	1.33	1.69	99.169	0.000	0.00
S308N	170.413	5.67	9.66	139.083	5.907	8.22
I309E	123.847	16.25	20.12	129.940	14.414	18.73
I309G	102.601	7.37	7.56	114.091	6.458	7.37
I309L	153.681	9.58	14.72	106.948	10.905	11.66
I309M	123.425	8.02	9.90	136.797	7.065	9.66
I309N	111.901	6.98	7.81	97.361	6.470	6.30

TABLE 12-continued

	Percent (%) Activity					
	duplicate 1			duplicate 2		
	% activity at 37° C./4° C.	% activity 37° C. + m-cresol/37° C.	% activity 37° C. + m-cresol/4° C.	% activity at 37° C./4° C.	% activity 37° C. + m-cresol/37° C.	% activity 37° C. + m-cresol/4° C.
I309S	169.951	4.11	6.98	551.493	0.862	4.75
I309T	97.936	7.63	7.47	148.864	5.542	8.25
I309V	113.138	1.50	1.70	138.313	3.470	4.80
M310G	167.656	11.44	19.18	110.739	12.916	14.30
M310Q	107.237	27.81	29.82	106.323	28.254	30.04
M313G	138.095	9.77	13.49	109.231	10.141	11.08
M313H	271.914	3.71	10.09	197.024	3.886	7.66
M313K	118.882	0.86	1.02	107.111	0.555	0.59
M313P	103.654	4.98	5.16	132.802	4.516	6.00
M313R	157.272	4.62	7.27	32.845	8.296	2.72
M313T	162.074	7.04	11.40	99.844	7.007	7.00
M313Y	120.038	7.52	9.03	103.011	6.846	7.05
K314S	141.924	9.67	13.73	132.112	9.066	11.98
K314Y	243.011	5.75	13.98	138.931	10.989	15.27
S315A	91.372	16.51	15.08	137.153	9.973	13.68
S315H	151.244	12.06	18.24	105.305	10.867	11.44
S315Y	170.968	30.61	52.33	57.827	39.503	22.84
L317A	123.510	6.97	8.60	132.724	8.395	11.14
L317I	187.477	12.72	23.84	110.696	10.670	11.81
L317K	96.199	3.45	3.31	134.204	3.534	4.74
L317N	127.382	12.02	15.31	121.233	14.528	17.61
L317R	238.501	3.87	9.22	99.467	5.673	5.64
L317S	90.929	15.54	14.13	85.810	6.423	5.51
L317T	145.964	6.96	10.16	154.334	1.087	1.68
L317W	163.704	11.92	19.51	147.606	10.270	15.16
L318D	105.543	17.43	18.40	97.970	16.684	16.35
L318H	99.907	4.29	4.29	124.690	7.363	9.18
L318R	160.469	5.63	9.03	120.872	6.210	7.51
N321R	164.842	9.53	15.71	112.180	8.613	9.66
N321S	102.489	8.29	8.49	108.732	4.534	4.93
E324N	104.618	7.72	8.08	131.265	9.124	11.98
T325E	124.837	14.44	18.02	106.457	10.577	11.26
N328G	197.098	4.15	8.18	109.722	7.233	7.94
N328Y	180.981	10.30	18.64	100.000	10.500	10.50
T335S	107.956	11.57	12.49	125.286	6.288	7.88
Q347A	101.395	10.89	11.04	96.213	11.001	10.58
Q347G	222.459	8.37	18.63	207.054	9.013	18.66
Q349M	99.531	11.98	11.92	108.042	12.330	13.32
Q349R	147.007	11.76	17.29	104.545	13.211	13.81
V351S	130.819	0.00	0.00	100.857	0.000	0.00
I353V	132.334	10.45	13.83	138.025	11.902	16.43
N356H	100.000	8.54	8.54	130.377	3.912	5.10
N356S	51.908	0.00	0.00	125.692	2.516	3.16
S359E	135.589	10.77	14.60	135.104	9.354	12.64
S359H	110.422	0.00	0.00	100.809	0.000	0.00
P367A	167.030	12.94	21.62	127.366	13.153	16.75
P367G	115.683	0.00	0.00	122.642	0.000	0.00
P367K	125.884	5.06	6.36	66.884	10.136	6.78
P367S	74.263	14.39	10.69	88.355	16.433	14.52
D368A	121.623	1.45	1.76	81.646	2.111	1.72
D368E	166.628	9.18	15.30	97.937	11.462	11.23
D368L	108.977	0.00	0.00	109.364	0.969	1.06
D368M	119.744	2.72	3.25	103.662	2.536	2.63
D368R	164.735	10.16	16.74	118.140	11.805	13.95
D368T	107.122	2.87	3.07	126.693	3.366	4.26
N369R	161.693	6.39	10.34	74.366	6.182	4.60
A371F	180.217	6.19	11.16	76.436	5.578	4.26
A371H	957.055	1.81	17.32	89.541	1.697	1.52
A371H	111.143	0.00	0.00	95.589	8.610	8.23
A371K	136.514	12.84	17.53	114.354	12.454	14.24
A371L	695.108	1.51	10.52	107.003	2.215	2.37
A371L	104.327	0.00	0.00	60.232	1.205	0.73
A371R	#VALUE!	#VALUE!	11.03	#VALUE!	#VALUE!	14.06
A371R	121.162	0.00	0.00	97.970	2.587	2.53
A371S	147.672	8.38	12.38	131.555	16.938	22.28
L374P	392.038	5.77	22.63	123.033	7.365	9.06
E375A	88.836	0.00	0.00	134.714	2.050	2.76
E375G	126.880	10.32	13.10	139.030	14.673	20.40
E375R	163.180	13.15	21.45	116.431	19.727	22.97
K376D	113.100	12.36	13.97	165.064	5.049	8.33
K376E	100.000	13.55	13.55	153.016	10.394	15.90
K376Q	125.172	12.75	15.96	90.000	12.057	10.85

TABLE 12-continued

	Percent (%) Activity					
	duplicate 1			duplicate 2		
	% activity at 37° C./4° C.	% activity 37° C. + m-cresol/37° C.	% activity 37° C. + m-cresol/4° C.	% activity at 37° C./4° C.	% activity 37° C. + m-cresol/37° C.	% activity 37° C. + m-cresol/4° C.
K376R	81.687	31.63	25.84	199.112	10.372	20.65
K376T	121.133	14.91	18.06	113.387	5.639	6.39
K376V	124.221	3.19	3.96	118.583	2.547	3.02
K376Y	102.812	9.24	9.50	96.139	12.985	12.48
G377D	110.871	15.72	17.43	132.357	10.550	13.96
G377E	130.445	8.04	10.49	128.402	7.401	9.50
G377H	146.855	8.34	12.25	104.837	10.117	10.61
G377K	185.922	4.42	8.21	119.751	4.989	5.97
G377R	119.708	5.87	7.03	94.749	7.137	6.76
G377S	108.609	6.91	7.51	101.106	7.877	7.96
G377T	112.557	17.14	19.29	109.036	18.279	19.93
F380W	147.077	9.97	14.67	104.881	9.253	9.70
T381S	135.827	13.41	18.21	112.559	10.315	11.61
R383I	527.820	6.33	33.44	98.328	7.522	7.40
R383S	132.894	10.50	13.96	119.466	10.545	12.60
K385A	126.096	4.64	5.85	112.706	0.000	0.00
K385Q	137.629	9.03	12.43	124.892	7.512	9.38
K385V	112.581	5.12	5.76	80.571	2.979	2.40
E389A	306.767	2.13	6.53	224.872	1.824	4.10
E389G	113.253	2.13	2.41	139.901	0.000	0.00
E389L	143.219	14.24	20.40	112.185	12.609	14.15
E389Q	135.807	11.88	16.14	99.738	12.767	12.73
E389S	165.620	0.00	0.00	93.030	0.285	0.27
E392A	112.465	7.27	8.18	155.693	6.376	9.93
E392F	115.619	3.90	4.51	143.781	3.905	5.61
E392Q	112.993	10.53	11.89	93.789	16.705	15.67
E392R	129.528	3.69	4.79	123.407	2.947	3.64
E392V	124.365	7.73	9.61	154.768	6.404	9.91
Q393F	139.966	10.59	14.82	101.647	10.171	10.34
Q393M	139.696	1.60	2.24	86.966	3.086	2.68
S395A	208.246	12.98	27.04	112.714	12.395	13.97
S395H	159.975	12.55	20.07	113.401	10.452	11.85
E396A	131.894	8.42	11.10	128.716	9.777	12.58
E396H	210.364	9.19	19.33	128.571	3.216	4.14
E396Q	122.977	10.06	12.37	95.938	10.263	9.85
E396S	156.267	2.77	4.33	111.753	2.022	2.26
Y399T	130.536	0.00	0.00	122.738	0.050	0.06
Y399V	110.592	15.98	17.68	116.018	17.801	20.65
Y399W	122.500	13.76	16.86	103.346	11.973	12.37
S401A	122.003	13.90	16.96	99.275	13.024	12.93
S401E	125.223	16.30	20.42	128.670	15.000	19.30
S404A	149.379	0.00	0.00	105.443	1.102	1.16
L406F	122.805	0.00	0.00	146.122	0.000	0.00
L406N	152.836	6.36	9.72	131.321	6.705	8.81
S407A	141.351	11.33	16.02	110.376	16.836	18.58
S407D	241.053	11.29	27.22	98.135	10.120	9.93
S407P	143.308	6.85	9.81	121.898	11.088	13.52
A412Q	146.177	9.54	13.94	99.452	8.511	8.46
A412R	140.070	8.92	12.49	123.675	9.390	11.61
A412V	146.804	4.99	7.32	101.739	5.383	5.48
D416L	120.820	17.64	21.31	127.662	15.340	19.58
D418R	117.749	7.59	8.94	112.193	10.721	12.03
A419H	241.224	8.82	21.27	188.179	5.999	11.29
A419K	191.165	10.42	19.91	2022.616	1.523	30.81
D421A	102.111	12.49	12.75	301.584	4.510	13.60
D421H	333.471	10.18	33.95	67.652	86.552	58.55
D421K	124.190	7.62	9.46	102.316	13.562	13.88
D421N	110.806	14.96	16.58	100.116	16.449	16.47
D421Q	104.370	10.72	11.18	143.630	12.400	17.81
D421R	138.783	8.85	12.28	137.964	9.778	13.49
D421S	142.171	11.00	15.64	166.162	8.564	14.23
A425G	74.810	10.61	7.94	120.947	11.137	13.47
G427Q	133.135	2.31	3.08	98.243	8.618	8.47
G427T	125.113	4.81	6.02	119.058	3.956	4.71
V428L	137.044	1.81	2.48	109.390	0.990	1.08
D431E	70.178	26.32	18.47	95.135	20.739	19.73
D431H	186.490	7.32	13.65	95.071	9.941	9.45
D431K	240.835	11.61	27.97	68.277	20.207	13.80
D431L	129.149	10.49	13.54	119.177	11.740	13.99
D431N	138.404	9.79	13.55	125.433	9.246	11.60
D431Q	232.960	10.83	25.23	109.483	10.716	11.73
D431S	78.069	7.52	5.87	88.796	9.135	8.11

TABLE 12-continued

	Percent (%) Activity					
	duplicate 1			duplicate 2		
	% activity at 37° C./4° C.	% activity 37° C. + m-cresol/37° C.	% activity 37° C. + m-cresol/4° C.	% activity at 37° C./4° C.	% activity 37° C. + m-cresol/37° C.	% activity 37° C. + m-cresol/4° C.
F433A	147.286	9.78	14.40	99.486	12.798	12.73
F433H	140.196	13.48	18.90	87.943	16.888	14.85
F433I	108.569	11.30	12.27	86.984	16.616	14.45
F433K	91.159	11.12	10.14	342.290	3.608	12.35
F433R	128.958	10.53	13.58	133.353	9.565	12.75
F433T	161.799	13.66	22.10	134.977	19.229	25.96
F433V	1412.071	1.61	22.69	112.033	17.307	19.39
F433W	149.049	10.46	15.59	113.585	7.530	8.55
P437I	148.880	2.39	3.56	107.028	1.782	1.91
M438A	106.463	10.07	10.72	135.705	10.194	13.83
M438D	105.370	10.16	10.71	113.283	2.590	2.93
M438E	115.061	8.00	9.21	113.782	9.120	10.38
M438L	65.794	10.06	6.62	214.958	6.526	14.03
M438N	130.428	8.06	10.52	100.669	11.889	11.97
M438T	104.058	13.39	13.93	103.691	12.160	12.61
E439A	137.279	11.63	15.97	95.555	14.073	13.45
E439A	154.140	4.72	7.28	147.295	7.415	10.92
E439C	193.243	14.69	28.38	111.719	15.734	17.58
E439K	124.464	13.28	16.52	104.762	10.552	11.05
E439P	118.340	15.59	18.44	87.446	14.998	13.12
E439Q	101.589	10.67	10.84	127.358	10.648	13.56
E439T	110.891	14.36	15.93	122.975	11.322	13.92
T440D	118.877	11.52	13.69	79.518	18.426	14.65
T440H	142.296	4.46	6.34	130.928	7.553	9.89
T440M	84.722	8.83	7.48	86.929	12.774	11.10
T440P	111.931	13.54	15.16	91.205	17.272	15.75
T440S	100.436	11.17	11.22	131.174	9.810	12.87
E441F	129.315	11.25	14.55	110.874	11.410	12.65
E442G	111.216	10.24	11.39	100.210	10.965	10.99
P443E	94.377	5.14	4.85	130.704	6.789	8.87
P443F	146.612	11.22	16.45	97.932	12.322	12.07
P443G	239.171	8.56	20.48	157.960	16.385	25.88
Q444E	81.997	8.54	7.01	160.917	9.561	15.38
Q444H	150.301	8.46	12.71	119.665	10.892	13.03
Q444V	129.822	13.49	17.51	122.591	10.995	13.48
I445M	85.090	17.25	14.68	101.149	15.393	15.57
I445N	106.430	13.89	14.79	87.351	12.945	11.31
I445W	117.213	11.70	13.72	100.037	10.983	10.99
Y447E	99.579	16.55	16.48	108.969	12.849	14.00
Y447G	143.704	13.77	19.79	103.624	11.563	11.98
Y447P	139.152	13.78	19.17	107.737	12.282	13.23
positive control (OHO)	94.998	5.23	4.97	96.871	8.456	8.19
	105.798	4.48	4.74	108.066	5.246	5.67
	100.000	3.33	3.33	82.7780	3.759	4.59
	94.762	19.07	18.07	109.539	16.529	18.11
	142.024	4.48	6.36	130.947	5.595	7.33
	45.115	20.77	9.37	68.017	11.035	7.51
	53.324	21.95	11.71	74.253	9.960	7.40
	59.581	25.24	15.04	75.872	16.231	12.31
	91.844	19.05	17.50	80.371	13.977	11.23
	93.828	13.47	12.63	96.630	19.454	18.80
	57.773	17.04	9.85	83.536	17.573	14.68
	100.000	18.56	18.56	148.226	16.239	24.07
	74.325	18.29	13.60	61.119	9.286	5.68
	98.132	8.48	8.32	87.677	10.006	8.77
	93.817	9.62	9.02	102.223	9.745	9.96
	96.922	8.56	8.30	87.993	9.064	7.98
	96.648	9.91	9.58	86.891	9.938	8.63

n/a (not available; e.g., beyond detection limit)

2. Summary of Results for F204P

For mutant F204P, the results above of tested supernatant from transient transfection of CHO—S cells incubated in the presence of m-cresol in a bHA enzymatic activity assay showed that the F204P mutant protein was highly resistant to 0.4% m-cresol treatment. The results showed that the activity that remained after 4 hours incubation with 0.4% m-cresol at 37° C. was approximately equal to the activity observed when the enzyme was incubated at either 4° C. or at 37° C. in the absence of m-cresol. The positive control (WT PH20-OHO) showed a reduction in activity of 75% and 83% on the day of the assay (as assayed from two different OHO transfections). This demonstrated that the F204P phenophile was able to retain 60% to 90% or greater of its activity above the residual activity of the wildtype PH20 control enzyme.

In order to confirm the stability of F204P upon m-cresol treatment or exposure to increased temperature, a second transfection of F204P was performed in duplicate using CHO—S cells, and clarified supernatant was again tested for its stability at 4° C., at 37° C. for 4 hours with 0.4% m-cresol and at 37° C. for 4 hours without 0.4% m-cresol. The results confirmed that the F204P mutant enzyme retained a high amount of hyaluronidase activity after the 4 hour incubation in m-cresol at 37° C. The results were similar to the results seen in the first screening of the mutant, with F204P retaining anywhere from 57% to greater than 90% of its activity above the residual activity of the wildtype PH20 control enzyme after the 4 hour incubation.

A summary of the enzyme activity of F204P compared to the wildtype control is set forth in Table 13.

TABLE 13

Summary of Enzyme Activity

	Remaining Activity after 4 h incubation (37° C. + m-cre/37° C.)		Net % Increase	Remaining Activity after 4 h incubation (37° C. + m-cre/4° C.)		Net % Increase
Transfection #	F204P	WT (OHO)	in Activity Over WT (37° C.)	F204P	WT (OHO)	in Activity Over WT (4° C.)
1	73.6%	16.4%	57.2%	86.0%	25.3%	60.7%
2	122.3%	25.2%	97.1%	109.7%	16.6%	93.1%

Example 6

Large Scale Expression and Purification of PH20 Hit Variant

1. Expression and Purification

HZ24-PH20-IRES-SEAP plasmid DNA containing cDNA encoding one of the variant PH20 was transfected into monolayer CHO—S cells as generally described in Example 2. CHO—S cells were cultured in shaker flasks using CD-CHO media supplemented with GlutaMAX (8 mM). On the day of transfection, 15 flasks were prepared of approximately 300 mL volume containing the CHO—S cells at an approximate density of 1.0×10^6 cells/mL. Each 300 mL flask was transfected using 375 μg of plasmid DNA encoding the F204P mutant combined with 375 μL of Freestyle MAX transfection reagent. The transfected plasmid DNA had a sequence of nucleotides set forth in SEQ ID NO:4 containing a codon change of TTC to CCT at nucleotide positions 1733-1735, thereby encoding the F204P mutant. The transfected cells were then allowed to remain in culture for 96 hours, whereupon the cells and media were harvested and pooled. The cells were pelleted by centrifugation (4000× g, 20'), and the supernatant retained for purification of the F204P protein (approximately 4.5 liters).

The crude supernatant was concentrated 10× using a 30 kDa Tangential flow filter (TFF) system (Millipore Pellicon XL, Bimax 30, 200 mL void volume; 50 cm^2 filter surface area) until the volume was approximately 450 mL. The permeate was saved for assay to detect flow through of the F204P protein. A free-flow buffer exchange for the retentate was then performed using 4 liters of buffer (10 mM $NaPO_4$; 25 mM NaCl, pH 7.2). The volume of the retentate was reduced again to approximately 200 mL, and then the remaining permeate in the system was purged (void volume ~200 mL) and the system was flushed using approximately 50 mL of buffer to yield a final concentrated product of approximately 450 mL.

An anti-rHuPH20 affinity column was prepared by coupling antigen affinity purified Rabbit anti-rHuPH20 IgG to CNBr-activated Sepharose 4 Fast Flow (GEHealth catalog No. 17-0981-01). Briefly, 0.7 g of pre-activated Sepharose 4 powder was suspended in 1 mM HCl in a 10 mL glass column for 30 minutes to allow the powder to swell. The solution was drained from the column and washed with 15 gel volumes (about 30 mL) of cold 1 mM HCl by gravity. The column was washed with 5 gel volumes of coupling buffer (0.1M $NaHCO_3$, 0.5M NaCl at pH 8.3). Next, 5 mg of Rabbit anti-rHuPH20 IgG at >1.0 mg/mL in coupling buffer was added to the column at a protein/gel ratio of 2-3 mg/mL gel. The column was rotated head to head at 4° C. overnight. The flow-through was collected for coupling efficiency determination. The gel was washed with 2 gel volumes of coupling buffer, and then washed and resuspended in 1 M ethanolaminine pH 9.5 for 2 hours at room temperature to block unused activated sites. The gel was washed 6 times with 5 gel volumes per wash alternating coupling buffer and 0.1 NaAc, 0.5M NaCl, pH 4.5. The gel was then washed with 10 gel volumes of TBS (20 mM Tris-HCl, 0.15 M NaCl, pH 7.5). The coupling efficiency was determined (1-post-coupling protein concentration/pre-coupling protein concentration×100%). The antibody coupled gel was stored in TBS with 0.02% NaN_3 at 4° C.

The concentrated supernatant product was subsequently loaded onto a anti-rHuPH20 affinity column at an approximate rate of 5 mL/min. The elution was performed according to standard procedure using a GE™ AKTA FPLC purification system (GE Healthcare, Product No. 18-1900-26), whereby the protein was eluted via a low pH glycine wash (0.1 M glycine-HCl, pH 2.5) in 1 mL fractions. Each fraction was immediately neutralized by the addition of 100 μL of 1M Tris, pH 7.5.

The eluted protein was assayed by resolving protein bands on a 4-20% SDS-PAGE gradient Tris-glycine gel. See Blue®Plus2 Pre-stained MW standards (Life Technologies; Catalog No. LC5925) were used as molecular weight standards, and 50 ng rHuPH20 (as described in Example 1) was used as a positive control. The polyacrylamide gel was stained with Instant Blue to show total protein from each fraction. To confirm the bands on the gel are PH20, the gel was transferred to a PVDF membrane (Invitrogen), which was subjected to Western Blot using a Rabbit anti-PH20 primary antibody generated by immunizing rabbits with rHuPH20 and an HRP-Goat anti-rabbit secondary antibody (Calbiochem, Cat. No. DC03L).

Then, the flow-through from the initial loading of the affinity column was re-loaded onto the column twice due to the low capacity of the affinity column. All fractions containing the protein were then combined resulting in a total volume that was approximately 13 mL. This product was then dialyzed overnight versus four liters of buffer (10 mM $NaPO_4$, 140 mM NaCl, pH 7.2) using a Slide-A-Lyzer Dialysis Cassette G2 (20,000 MWCO) with a 15 mL capacity. The buffer was then changed and the product dialyzed against a second fresh four liters of the same buffer. The F204P protein was then concentrated using an Amicon Ultra Centrifugation column (Millipore; 10,000 MWCO) to a final volume of approximately 450 μL (10 minutes at 4000×g).

2. Characterization of Protein

The purified protein was characterized for its protein concentration, activity, and purity.

To determine the protein concentration of the purified protein, a quantification ELISA was performed as described in Example 7. Also, hyaluronidase activity was determined as described in Example 3. The protein concentration after centrifugation was estimated to be approximately 400 μg/mL. The purified protein also was resolved on a 4-20% SDS-PAGE gradient Tris-glycine gel, which was then stained with Instant Blue. The staining results demonstrated that the protein was essentially a single molecular weight protein of approximately 63 kDa, similar to the rHuPH20 control. No appreciable degradative products were detected by this method. Approximate yields of the protein at various timepoints and activity during the purification are described in Table 14.

TABLE 14

Characterization of Purification Steps

		Activity Assay		Quant ELISA Assay		
Purification Step	Volume (mL)	Activity (U/mL)	Total Activity (U)	Protein Conc. (μg/mL)	Total Protein (μg)	Specific Activity (U/μg)
Supernatant	4500	2.66	11,700	0.046	207	56.5
Conc. after TFF & Buffer Exchange	450	42	18,900	0.4	178	105.9
Pooled Fractions 5-7 after AC, Dialysis & Conc. - A280	0.45	11,741	5283	396	180	35.3

The purity of the purified protein was determined by Reverse Phase HPLC (RP-HPLC). The elution time from the reverse phase column was essentially identical as that observed with the recombinant human hyaluronidase (HUB), and provides a basis for crude estimation of the purity of the sample at approximately 80-90%.

Example 7

Quantification Using ELISA

The quantification of PH20 or variants were performed using an ELISA that captures the protein using a monoclonal anti-rHuPH20 capture antibody. Specifically, one day prior to performing the ELISA, 96-well 4HBX plates were coated with capture antibody (Protein G purified rabbit polyclonal anti-PH20 antibody generated by immunizing rabbits with rHuPH20; 1 mg/mL stock) at 1 μg/mL in 100 mM phosphate (pH 7.2) in a total volume of 100 μL per well. The plates were stored at 4° C. overnight. On the next day, the plates were washed 5× with 1×PBS at 300 μL/well with a plate washer. After each wash, the plated were patted dry on paper towels. Then, the plates were blocked with 200 μL PBS containing Tween 20 (1×PBST) per well at room temperature for 1 hour.

The standards and samples were added to the plate. For generation of the standard, a 1 mg/mL stock of rHuPH20 (Example 1) was freshly diluted to 50 μg/mL in HEPES pH 7.4 assay buffer as an intermediate stock. Then, for the standards, the 50 μg/mL stock was diluted in duplicates into 360 μL of 0.5×PBST at 300 ng/mL for the first standard (first row). For the other standard rows, 240 μL 0.5×PBST were added to each well, and 1:3 serial dilutions made. For the transfected supernatant samples, 360 μL per well was added in duplicate into the first row, and each were also serially diluted as described above into 0.5×PBST. For purified samples, 100 μL was added per well. The plates were incubated for 2 hours at room temperature. After incubation, the plates were washed 5× with 1×PBST at 300 μL/well using a plate washer. After each wash, the plates were patted dry on paper towels.

An HRP-conjugated anti-PH20 antibody was prepared for detection using an HRP conjugation kit (Pierce, Thermo-Fisher; Catalog No. 31489). 1 mg of a Protein G purified rabbit polyclonal antibody generated by immunizing rabbits with rHuPH20 was diluted in 1 mL PBS and 1 mL of 2× carbonate kit buffer. Next, 100 μL of peroxidase were added to 1 mL of the above antibody solution and incubated at room temperature for 1 hour. Then, 10 μL $NaBH_4$ stock was added in a fume hood, and the sample incubated at room temperature for 20 minutes. To quench the reaction, 20 μL of ethanolamine was added and incubated at room temperature for 15 minutes. To this, 1/25 volume 5% human serum albumin (0.1 mL syringe) was added to give a 2 mg/mL albumin stock reaction. The pH was adjusted to about 7.9 by addition of 250 μL of 1 M Tris pH 7.4. The concentration of the stock was 400 μg/mL. The stock solution was further diluted 1/10 in PBS Tween20 (0.05%) containing 0.5% human serum albumin and preservatives, and then was sterile filtered. The stock was stored at 4° C. or was frozen at −20° C.

Antibodies were detecting using the HRP-conjugated anti-PH20 antibody that was diluted 1000× into 0.5×PBST. 100 μL of the diluted antibody was added to all wells of the plate and the plate incubated for a further 2 hours at room temperature. After incubation, the plates were washed 5× with 1×PBST at 300 μL/well using a plate washer. After each wash, the plates were patted dry on paper towels. Then, 100 μL of TMB substrate were added to each well and the reaction was stopped after 5-10 minutes by adding 100 μL of stop solution per well. The plate was read at OD_{450}.

Example 8

Determination of Enzymatic Activity of PH20

Enzymatic activity of PH20 in samples such as cell cultures, purification fractions and purified solutions was determined using a turbidimetric assay, which is based on the formation of an insoluble precipitate when hyaluronic acid binds with cetylpyridinium chloride (CPC). The activity is measured by incubating PH20 with hyaluronan for a set period of time (30 minutes) and then precipitating the undigested hyaluronan with the addition of CDC. The turbidity of the resulting sample is measured at 640 nm. The decrease in turbidity resulting from enzyme activity on the hyaluronan substrate is a measure of the PH20 enzymatic activity. The method is run using a calibration curve generated with dilutions of a PH20 assay working reference standard (rHuPH20 standard generated as described in Example 1), and sample activity measurements are made relative to this calibration curve.

Dilutions of the sample and standards were prepared in Enzyme Diluent Solution (70 mM NaCl, 0.1% human serum albumin [HSA], 0.67 g/L gelatin hydrolysate in 25 mM PIPES buffer, pH 5.5). The samples were diluted to an appropriate concentration. Hyaluronic acid (HA, average MW of 20-50 kDa) from Lifecore Biomedical (Chaska, Minn.) also was prepared at 1 mg/mL in substrate solution that contains 25 mM PIPES, 70 mM NaCl at pH 5.5. Equal amounts of the above two solutions were mixed to prepare a 1 mL reaction mixture and incubated at 37° C. for 30 min. The reaction was stopped by addition of 4 mL of Cetylpyridinium Chloride Solution (CPC, 5.0 mg/mL). After brief vortexing, the turbidity of the sample mixture was read at 640 nm and the activity was determined by fitting against a standard curve. Specific activity (Units/mg) was calculated by dividing the enzyme activity (U/mL) by the protein concentration (mg/mL).

Example 9

Stability of F204P-PH20 Variant in Preservative

To confirm the screening results, an amount estimated to be about 450 U/mL of the purified F204P protein as described in Example 6 was formulated in 10 mM sodium phosphate, pH 6.5, 120 mM NaCl, 10 mM methionine, 0.01% Pluronic F-68, 0.1% phenol and 0.15% m-cresol. A test article that also contained an amount estimated to be about 450 U/mL wild type rHuPH20 (generated as described in Example 1) in the same formulation was also prepared to serve as a control. Each formulation solution was aliquotted in 0.5 mL and filled into 2 mL USP Type I borosilicate glass with a chlorobutyl rubber stopper and an aluminum seal. The vials were incubated at 5° C., 30° C. or 37° C. Samples were withdrawn from the incubator at various times and enzymatic activity was measured as described in Example 8.

The results of the enzymatic activity measurements are shown in Table 15. As can be seen, the rHuPH20 wild type control showed a rapid decrease in activity when incubated at 37° C. in the presence of phenolic preservatives. In contrast, the F204P mutant showed no significant loss in activity throughout the study. The results also show that activity of PH20 is retained after incubation for up to 4 weeks at 5° C. and 30° C. compared to the activity of the rHuPH20 wildtype control not containing the mutation. These results confirm that F204P tolerates EPB level of preservative (0.1% phenol and 0.15% m-cresol) and is stable at 37° C. for at least up to 6 days at at 5° C. and 30° C. for greater than one month.

TABLE 15

Stability of rHuPH20 wildtype and F204P mutant incubated at with preservative

	PH20 relative activity (%) at 5° C.			PH20 relative activity (%) at 30° C.			PH20 relative activity (%) at 37° C.		
ID	T0	2 w	4 w	6 d	2 w	4 w	2 d	4 d	6 d
F204P	100	—	91.8	84.1	100	96.6	105	91.1	95.9
wildtype control	100	—	81.9	66.7	61.7	60.5	48.6	29.6	15.2

Example 10

Stability of F204P-PH20 Variant in Insulin Coformulation

The PH20 variant F204P was tested for its stability in a coformulation containing an insulin analog (insulin aspart or insulin lispro).

In the tested coformulations, the insulin lispro was a commercial product (Insulin Lispro: Eli Lilly Humalog® (insulin Lispro) 100 U/mL, Lot A572364).

In the tested coformulations, the insulin aspart analog was a reprocessed aspart prepared by pooling 12 vials (10 mL each) of a commercial product (Insulin Aspart: Novo Nordisk, NovoRapid® (insulin Aspart), Lot XS60195), which was then concentrated using an Amicon Ultracel-10 K column concentrator until the final concentration was about 5 times the original concentration. The insulin analog was precipitated by addition of 1 M sodium acetate, pH 5.3 and 30 mM zinc chloride ($ZnCl_2$, EMD, Cat No. ZX0065-1) at 1/10 of the protein solution volume. The solution was placed on ice for 30 minutes followed by centrifugation at 5600 rpm for 20 minutes in an Avanti J-E Centrifuge with JS-5.3 swinging bucket rotor (Beckman Coulter). The supernatant was decanted and the pellet was resuspended and washed with 20 mM sodium acetate, 2 mM zinc chloride, pH 5.5 solution. The resuspended solution was centrifuged as described above. The washing step was repeated a total of 5 times. A final wash was performed with 20 mM sodium acetate, pH 5.5 to remove all traces of zinc chloride. The resulting protein paste was dissolved with water containing 20 mM HCl. After complete dissolution, 250 mM Tris, pH 10.7 was added to a final Tris concentration of 20 mM. The pH of the resulting solution was adjusted such that the insulin analog was formulated as described below and the protein concentration was adjusted to about 15-20 mg/mL. An insulin analog prepared in this way typically had a yield of about 90%, with a residual preservative concentration at less than 100 times the starting material.

Briefly, three (3) formulations were generated each containing 600 Units (U) of PH20-F204P or wildtype rHuPH20 (generated as described in Example 1) for a total of 6 formulations as set forth in Table 16:

TABLE 16

Summary of Insulin Formulations

		Buffer		Tonicity					Preservatives		API	
ID	pH	NaPO$_4$	Tris/ HCl	modifier NaCl	Anti-Ox Methionine	Glycerin	Metal Zn	Surfactant F68	Phenol	m-Cresol	PH20 (U/mL)	Analog (mg/mL)
F1. Humalog + F204P	7.0-7.8	13.2 mM				173.7 mM	0.242 mM			0.315%	600	3.5
F2. Humalog + wt	7.0-7.8	13.2 mM				173.7 mM	0.242 mM			0.315%	600	3.5
F3. Aspart + F204P	7.3		30 mM	100 mM	5 mM			0.010%	0.100%	0.150%	600	3.5
F4. Aspart + wt	7.3		30 mM	100 mM	5 mM			0.010%	0.100%	0.150%	600	3.5
F5. Aspart + F204P	7.3		30 mM	100 mM	5 mM			0.010%		0.315%	600	3.5
F6. Aspart + wt	7.3		30 mM	100 mM	5 mM			0.010%		0.315%	600	3.5

Each formulation solution was dispensed in 0.5 mL aliquots into 2 mL USP Type I borosilicate glass vials with a chlorobutyl rubber stopper and an aluminum seal. The vials were incubated at 5° C., 30° C. and 37° C. Samples were withdrawn from the incubator at scheduled time points for enzymatic activity measurements as described in Example 8.

The results of the enzymatic activity measurements for samples incubated at 37° C., 30° C. and 5° C. are shown in Tables 17-19, respectively. At 37° C., the enzymatic activity of samples containing wildtype rHuPH20 (F2, F4 and F6) were almost totally lost within two days of incubation. In contrast, after 6 days incubation at 37° C., formulation F3 and F5, which contains PH20-F204P, lost only about 10% and 30%, respectively. The PH20-F204P formulated in commercial Humalog (F1) lost most of its activity within 2 days at 37° C. most likely due to the lack of NaCl in the formulation.

A similar trend for enzymatic activities of ampoules incubated at 30° C. was noted between the PH20-F204P and rHuPH20. For formulations that contain an EPA preservative level, the differences between wild type and F204P were dramatic (Table 17; F1 and F5 vs. F2 and F6). When the preservative concentration was reduced to an EPB level (F3 and F4), the F204P still outperformed wildtype rHuPH20, although there was slightly higher rHuPH20 stability compared to EPA conditions. In both EPA and EPB preservative levels, PH20-F204P was able to maintain its activity up to 14 days at 30° C. when 100 mM of NaCl was included in the formulation.

TABLE 17

Enzymatic activity of rHuPH20 wild type and F204P mutant incubated at 37° C.

	PH20 activity U/mL, (% of remaining activity)				
ID	Initial Activity	2 d	4 d	6 d	2 w
F1. Humalog + F204P	583 (100%)	61 (10%)	15 (3%)	10 (2%)	—
F2. Humalog + wt	439 (100%)	4 (1%)	—	—	—
F3. Aspart + F204P	625 (100%)	613 (98%)	496 (79%)	570 (91%)	532 (85%)
F4. Aspart + wt	566 (100%)	58 (10%)	24 (4%)	4 (1%)	—
F5. Aspart + F204P	657 (100%)	484 (74%)	462 (70%)	478 (73%)	360 (55%)
F6. Aspart + wt	596 (100%)	−1 (0%)	—	—	—

TABLE 18

Enzymatic activity of rHuPH20 wild type and F204P mutant incubated at 30° C.

	PH20 activity U/mL, (% of remaining activity)			
ID	Initial Activity	6 d	2 w	4 w
F1. Humalog + F204P	583 (100%)	345 (59%)	250 (43%)	111 (19%)
F2. Humalog + wt	439 (100%)	1 (0%)	16 (4%)	−1
F3. Aspart + F204P	625 (100%)	601(96%)	650(104%)	579 (93%)
F4. Aspart + wt	566 (100%)	428 (76%)	390 (69%)	277 (49%)
F5. Aspart + F204P	657 (100%)	632 (96%)	655 (100%)	561 (85%)
F6. Aspart + wt	596 (100%)	145 (24%)	65 (11%)	9 (1.5%)

TABLE 19

Enzymatic Activity at 5° C.			
	PH20 activity (U/mL) at 5° C.		
ID	Initial Activity	2 w	4 w
F1. Humalog + F204P	583	544	565
F2. Humalog + wt	439	428	404
F3. Aspart + F204P	625	647	607
F4. Aspart + wt	566	580	496
F5. Aspart + F204P	657	695	574
F6. Aspart + wt	596	583	519

Example 11

Stability of V58R-PH20 in Insulin Coformulation

A. Stability of V58R-PH20

The PH20 variant V58R was expressed in CHO—S cells as described in Example 2 or Example 6. The transfected plasmid DNA had a sequence of nucleotides set forth in SEQ ID NO:4 containing a codon change of GTG to CGG at nucleotide positions 1295-1297, thereby encoding the V58R mutant. The V58R mutant was tested for its stability in a coformulation containing insulin aspart (insulin aspart analog prepared as described in Example 10) and under EPA or EPB preservative levels. Briefly, four (4) formulations were generated each containing 600 Units (U) of PH20-V58R or wildtype rHuPH20 (generated as described in Example 1) as set forth in Table 20. Formulations F1 and F2 represent the EPB preservative levels while formulations F3 and F4 represent the EPA preservative levels.

TABLE 20

Summary of Insulin Formulations

		Buffer		Tonicity modifier	Anti-Ox		Metal	Surfactant	Preservatives		API	
ID	pH	NaPO$_4$	Tris/HCl	NaCl	Methionine	Glycerin	Zn	F68	Phenol	m-Cresol	PH20 (U/mL)	Analog (mg/mL)
F1. Aspart + V58R	7.3		30 mM	100 mM	5 mM			0.010%	0.100%	0.150%	600	3.5
F2. Aspart + rHuPH20 wt	7.3		30 mM	100 mM	5 mM			0.010%	0.100%	0.150%	600	3.5
F3. Aspart + V58R	7.3		30 mM	100 mM	5 mM			0.010%		0.315%	600	3.5
F4. Aspart + rHuPH20 wt	7.3		30 mM	100 mM	5 mM			0.010%		0.315%	600	3.5

Each formulation solution was dispensed in 0.5 mL aliquots into 2 mL USP Type I borosilicate glass vials with a chlorobutyl rubber stopper and an aluminum seal. The vials were incubated at 30° C. and 37° C. Samples were withdrawn from the incubator at scheduled time points for enzymatic activity measurements as described in Example 8.

The results of the enzymatic activity measurements for samples incubated at 37° C. and 30° C. are shown in Table 21 and Table 22. At 37° C., the enzymatic activity of samples containing wildtype rHuPH20 (F2 and F4) were almost totally lost within two days of incubation. In contrast, after 6 days incubation at 37° C., formulations F1 (EPB) and F3 (EPA), containing V58R-PH20, lost only about 25% and 40% activity, respectively. At 30° C., the enzymatic activity of samples containing wildtype rHuPH20 also was dramatically reduced in the presence of EPA or EPB preservatives levels within one month of incubation, although there was a slightly less dramatic loss in activity in the presence of EPB preservative levels. In contrast, for V58R-PH20, there was no loss of enzymatic activity for either tested formulation up to 1 month.

TABLE 21

Enzymatic activity of rHuPH20 wild type and V58R mutant incubated at 37° C.				
	PH20 activity U/mL			
Formulation	Initial Activity	2 d	4 d	6 d
F1. Aspart + V58R	1350	1099	1094	1006
F2. Aspart + rHuPH20 wt	677	53	−3	—
F3. Aspart + V58R	1189	793	581	464
F4. Aspart + rHuPH20 wt	744	12	−9	—

TABLE 22

Enzymatic activity of rHuPH20 wild type and V58R mutant incubated at 30° C.			
	PH20 activity U/mL		
Formulation	Initial Activity	2 weeks	4 weeks
F1. Aspart + V58R	1350	1368	1208
F2. Aspart + rHuPH20 wt	677	422	256
F3. Aspart + V58R	1189	1228	1171
F4. Aspart + rHuPH20 wt	744	21	−5

B. Comparison of Stability of F204P and V58R

The PH20 variant V58R-PH20 was compared to F204P for its stability in a coformulation containing insulin aspart (insulin aspart analog prepared as described in Example 10) and under EPA or EPB preservative levels. Briefly, eight (8) formulations were generated as set forth in Table 23. Formulations F1-F4 represent the EPB preservative levels while formulations F5-F8 represent the EPA preservative levels. Formulations F3 and F4 and formulations F7 and F8 were identical and represent the wildtype control formulations formulations used for the EPB or EPA studies, respectively.

TABLE 23

Summary of Insulin Formulations

		Buffer		Tonicity					Preservatives		API	
ID	pH	$NaPO_4$	Tris/ HCl	modifier NaCl	Anti-Ox Methionine	Glycerin	Metal Zn	Surfactant F68	Phenol	m- Cresol	PH20 U/mL	Analog mg/mL
F1. Aspart + V58R	7.3		30 mM	100 mM	5 mM			0.010%	0.100%	0.150%	600	3.5
F2 Aspart + F204P	7.3		30 mM	100 mM	5 mM			0.010%	0.100%	0.150%	600	3.5
F3. Aspart + rHuPH20 wt(1)	7.3		30 mM	100 mM	5 mM			0.010%	0.100%	0.150%	600	3.5
F4. Aspart + rHuPH20 wt(2)	7.3		30 mM	100 mM	5 mM			0.010%	0.100%	0.150%	600	3.5
F5. Aspart + V58R	7.3		30 mM	100 mM	5 mM			0.010%		0.315%	600	3.5
F6 Aspart + F204P	7.3		30 mM	100 mM	5 mM			0.010%		0.315%	600	3.5
F7. Aspart + rHuPH20 wt(1)	7.3		30 mM	100 mM	5 mM			0.010%		0.315%	600	3.5
F8. Aspart + rHuPH20 wt(2)	7.3		30 mM	100 mM	5 mM			0.010%		0.315%	600	3.5

Each formulation solution was dispensed in 0.5 mL aliquots into 2 mL USP Type I borosilicate glass vials with a chlorobutyl rubber stopper and an aluminum seal. The vials were incubated at 30° C. and 37° C. Samples were withdrawn from the incubator at scheduled time points for enzymatic activity measures as described in Example 8.

The results show that the percentage hyaluronidase activity in the tested formulations after preincubation at 37° C. was slightly greater for both PH20 mutants when formulated in EPB and not EPA preservative levels. While the percent of activity remaining was greater than 80% for both tested mutants after 6 days incubation in formulations containing EPB preservative levels, it was less in the presence of EPA preservative levels. For example, the activity remaining at 6 days in EPA preservative levels was slightly less than 80% after 6 days for F204P-PH20, while it was only about 40% for V58R-PH20. Hence, the results also show that at 37° C., V58R-PH20 is somewhat less stable than the F204P-PH20, in particular in a formulation with EPA preservative levels. After incubation at 30° C. for at least a week, the F204P-PH20 and V58R-PH20 were stable and exhibited almost 100% initial activity in the presence of both EPA and EPB preservative levels. In contrast, rHuPH20 exhibited only about 40% of its initial activity after 4 weeks at 30° C. in the presence of EPB preservative levels, while it exhibited no detectable activity after 4 weeks at 30° C. in the presence of EPA preservative levels.

Example 12

Expression of F204P-PH20 Using a Lentivirus Expression Vector

A lentivirus expression vector, pLV-EF1a-PH20(F204P)-IRES-GFP-Bsd was generated containing a codon-optimized mutant hyaluronidase cDNA encoding F204P-PH20. The sequence of pLV-EF1a-PH20(F204P)-IRES-GFP-Bsd is set forth in SEQ ID NO:925. The pLV-EF1a-PH20 (F204P)—IRES-GFP-Bsd vector contains an ampicillin resistance gene (AmpR) located at nucleotides 8611-9471, an EF1a promoter at residues 1933 to 2327, an IRES at residues 4786-5370, a GFP-Bsd at residues 5394-6527 and nucleotides encoding F204P-PH20 at residues 3369-4781.

Lentivirus was produced as described in Bandaranayake et al. ((2011) Nucleic Acids Research, 39:e143). Briefly, 293T cells (ATCC) were plated at 6×10^6 cells onto 10 cm tissue culture plates. After 24 hours, 6 μg of psPAX2 (SEQ ID NO:926; Addgene plasmid No. 12260), 3 μg of PMD2.G (SEQ ID NO:927; Addgene plasmid #12259) and 9 μg lentiviral vector plasmid pLV-EF1a-PH20(F204P)-IRES-GFP-Bsd were mixed in 1.5 mL Opti-MEM (Life Technologies). 45 μL of Lipofectamine 2000 (LF2000; Life Technologies) were diluted into 1.5 mL Opti-MEM (Life Technologies). The DNA and LF2000 were mixed gently, and incubated at room temperature for 20 minutes to allow the DNA and lipid to form complexes. In the meantime, the overnight culture medium was replaced with 5.0 mL DMEM+10% FBS without antibiotics. A volume of 3.0 mL containing the DNA-LF2000 complexes were added to the 293T cells. The medium containing the DNA-LF2000 complexes was replaced with 10 mL complete medium at 12-16 hours post-transfection. The supernatant was collected at 48 hours post-transfection and the medium was transferred to a polypropylene storage tube. The virus-containing medium was spun at 1300 rpm for 5 minutes to pellet any 293T cells that were carried over during collection. The supernatant was carefully transferred to a sterile polypropylene storage tube.

CHO—S cells (Invitrogen) were grown in CHO—S media (Invitrogen) with shaking at 120 rpm at 37° C. and 5% CO_2 in vented 125-mL shake flasks (Nalgene). For transduction, CHO—S cells were added to wells of a six-well plate at 2*10^6 cells per well in 2 ml of CHO—S media containing 4 μg/mL hexadimethrine bromide at a final concentration of 4 μg/mL (Polybrene; SIGMA). Virus was added to each well at a multiplicity of infection (MOI) of 10 and the cells were incubated with shaking (120 rpm) at 37° C. and 5% CO_2 for 6 hours. The cells were then harvested and pelleted by low speed centrifugation (500×g, 5 min). The transduction medium was removed and replaced with 10 mL of fresh CHO—S medium (Invitrogen) supplemented with GlutaMax (50 mL/liter) and transferred to a T-25 flask. Three days post infection, blasticidin (Invitrogen) was added to the growth medium at a concentration of 1 μg/mL. The medium was changed regularly at 3-4 day intervals, and the cells were transferred to a T75 flask for expansion. Two weeks after the initial infection, the cells were expanded to shaker flasks and maintained in culture using medium containing 1 μg/mL blasticidin. F204P-PH20 protein secreted into the CHO—S medium was collected and purified by affinity chromatography using an anti-rHuPH20 affinity column as described in Example 6. The protein was prepared in standard API buffer (10 mM Histidine, 130 mM NaCl, pH 6.5).

Example 13

Analysis of Secondary Structure and Melting Temperature

The secondary structure and melting temperature of the PH20 variant F204P was tested and compared to wild-type rHuPH20 (generated as described in Example 1) to further assess stability of the variant. The secondary structure was tested by circular dichroism. A Jasco J-810-150S equipped with PTC-424S was employed for the CD spectral measurement and the CD spectra were collected by Spectra Manager (Version 1.5, Jasco). Procedures for instrumental set up and data collection are described in Table 24.

TABLE 24

CD Spectroscopy Operation Conditions

Parameters	Conditions
Nitrogen flow rate	25 ft^3/h
Sample temperature	30-75° C.
Sample concentration	Approx. 0.1 mg/mL
Cell pathlength	1 mm
Wavelength	220 nm
Data pitch	1° C.
Delay time	60 seconds
Temperature slope	1° C./min
Sensitivity	standard
Response	4 seconds
Band width	1 nm

1. Sample Preparation and Measurement

Two hundred (200) μL of a 0.1 mg·mL protein sample diluted in McIlvaine's buffer (McIlvaine (1921) JBC 49:183) adjusted to pH 6.5 were prepared. A series of samples of the F204P variant were also generated that varied in pH by adjustment using McIlvaine's buffer to a pH range from 5.0 to 7.5 as set forth in Table 25. In addition, samples also were generated by adjusting the NaCl concentration to 17.5 mM to 140 mM as set forth in Table 26. Samples were filtered using a 0.2 μm syringe filter prior to measurement. Similar samples were generated for rHuPH20. Then, 200 μL samples were transferred to a rectangular cuvetted having a 1 mm width and seated on Jasco J-810 spectropolarimeter. CD spectra of the samples were collected under the conditions described in Table 20. The melting temperature (T_m) was calculated using Spectra Manager (v 1.5, Jasco) from the CD spectral intensity measured at the temperature range from 30° C. to 75° C. The cuvettes were cleaned by Chromerge® cleaner (C577-12, Fisher scientific) between individual sample loading and after the run.

TABLE 25

Sample pH and concentration

Target pH	Actual pH	F204P (μL)	Buffer (μL)	F204P concentration (mg/mL)
5.0	4.92	25	175	0.1
5.5	5.38	25	175	0.1
6.0	5.99	25	175	0.1
6.5	6.49	25	175	0.1
7.0	7.00	25	175	0.1
7.5	7.5	25	175	0.1

TABLE 26

Sodium Concentration in Samples at pH 6.5

Target NaCl concentration (mM)	NaCl, 2.8M (μL)	F204P (μL)	Buffer at pH 6.5 (μL)	F204P concentration (mg/mL)
17.5	0.00	25	175	0.1
50.0	2.32	25	172.7	0.1
75.0	4.11	25	170.9	0.1
100.0	5.89	25	169.1	0.1
140.0	8.75	25	166.3	0.1

2. Results

The results show that the secondary structure of F204P is similar to rHuPH20. As a function of temperature, circular dichroism showed that a change in the absorption was measured with increasing temperatures. As a function of pH, the T_m distribution was closely comparable for both F204P and rHuPH20 and the highest T_m for each was obtained between pH 5.5 and pH 6.0. The results, however, showed that T_m of the F204P variant was approximately 9° C. higher at all tested ranges than wildtype rHuPH20. This result indicated that the F204P mutant is more stable against thermal stress conditions. As a function of salt, the results show that the F204P and wildtype rHuPH20 both exhibited an increasing T_m with higher salt concentration, showing that both have a proportional inclination toward salt concentration.

Example 14

Assessment of Enzymatic Activity in an Intradermal Trypan Blue Dispersion Assay

Spreading activity of the PH20 variant F204P was assessed using a dye dispersion in vivo assay. Briefly, purified PH20 variant F204P (prepared as described in Example 12) and wild-type rHuPH20 (prepared as described in Example 1) were both formulated in API buffer (10 mM Histidine, 130 mM NaCl, pH 6.5) at a concentration of 10,000 U/mL. The stocks were further diluted to three target concentrations of 1000, 100 and 10 U/mL by serial 1:10 dilutions in API buffer. Purified proteins (either rHuPH20 or F204P-PH20) were diluted 1:1 with 0.4% Trypan Blue (0.4% liquid solution; Catalog No. 15250, Invitrogen) to give a final concentration of 5, 50 and 500 U/mL protein, each containing 0.2% trypan blue. A vehicle control (API buffer) also was prepared. Forty-two (42) female NCr nu/nu homozygous mice were used in the study with six mice used per group as set forth in Table 27.

TABLE 27

Summary of Treatment Groups for Dye Dispersion Study					
Group	No. of Mice	Test Article	Final Dose with Trypan Blue (Units/mL)	Trypan Blue	Injection Volume (mL)
1	6	Control	0	0.2%	0.04
2	6	rHuPH20	5	0.2%	0.04
3	6	rHuPH20	50	0.2%	0.04
4	6	rHuPH20	500	0.2%	0.04
5	6	F204P-PH20	5	0.2%	0.04
6	6	F204P-PH20	50	0.2%	0.04
7	6	F204P-PH20	500	0.2%	0.04

Forty (40) μL of samples were administered by a single intradermal injection. The area of dye dispersion was measured at 2.5, 5, 10, 15 and 20 minutes post-injection and was recorded by photographic imaging by photograph of the injection site with a Nikon D90 digital camera with 60 mm prime micro-lens. A laser distance meter (Leica D3) was used to accurately position the camera at a pre-determined distance from the Trypan Blue dye area on the animal. The area of the dye was determined using Image-Pro Analyzer 7.0 (MediaCybernetics, Inc). The calculated areas were expressed as mm^2.

The results are set forth in Table 28. The results showed that the dispersion activity of the PH20 variant F204P was substantially identical to the dispersion activity of rHuPH20. The ability to increase the area of dye dispersion was dose-dependent, with both proteins having greatest activity at 500 U/mL. The results also showed that the area of dye dispersion increased with time post-intradermal injection. The areas of dye dispersion of rHuPH20 and F204P-PH20 were significantly greater than the areas of dye dispersion for the controls ($p<0.05$) at all time points when formulated at all concentrations (5, 50 and 500 U/mL) with the exception of rHuPH20 at the lowest concentration (5 U/mL). When compared to each other, rHuPH20 and F204P-PH20 showed similar dispersion effects, although there was a significant difference in dispersion between the two groups at 5 U/mL and 500 U/mL but not at 50 U/mL. In sum, the results show that both rHuPH20 and F204P-PH20 provided a statistically significant increase in the area of dye dispersion compared to the vehicle control.

TABLE 28

Trypan Blue Dispersion					
Group	Area (mm2)				
Avg. (n = 6)	2.5 min	5 min	10 min	15 min	20 min
1: Control	37.44 ± 2.81	38.16 ± 3.33	43.71 ± 2.12	45.70 ± 2.38	48.77 ± 2.14
2: rHuPH20 (5 U/mL)	36.68 ± 2.83	42.31 ± 2.57	45.41 ± 2.75	46.72 ± 3.35	49.61 ± 2.97
3: rHuPH20 (50 U/mL)	39.24 ± 1.20	44.90 ± 1.44	46.96 ± 1.70	50.08 ± 2.07	53.50 ± 1.59
4: rHuPH20 (500 U/mL)	44.72 ± 1.35	50.21 ± 1.92	57.47 ± 1.29	59.77 ± 1.25	57.17 ± 3.28
5: F204P (5 U/mL)	39.65 ± 1.53	46.09 ± 2.73	48.07 ± 1.43	52.54 ± 2.01	54.11 ± 1.01
6: F204P (50 U/mL)	38.10 ± 1.92	47.07 ± 2.12	51.48 ± 2.14	55.24 ± 1.90	58.34 ± 2.89
7: F204P (500 U/mL)	46.58 ± 1.67	54.06 ± 2.52	58.96 ± 1.85	64.37 ± 1.72	64.44 ± 2.17

Example 15

Assessment of Enzymatic Activity by Dermal Barrier Reconstitution

Activity of F204P-PH20 was assessed and compared to rHuPH20 to measure the amount of time required for the dermal barrier to reconstitute itself after intradermal hyaluronidase administration. Dermal reconstitution was evaluated by comparing the duration of the hyaluronidase spreading activity as assessed by monitoring the area of diffusion of 0.4% Trypan Blue over time. The proteins used in the study were purified PH20 variant F204P (prepared as described in Example 12) and wild-type rHuPH20 (prepared as described in Example 1) that were both formulated in API buffer (10 mM Histidine, 130 mM NaCl, pH 6.5). Vehicle (API buffer) was used as a control. Male NCr nu/nu homozygous mice were used in the study with three animals per time point for a total of fifteen mice used per group as set forth in Table 29.

TABLE 29

Summary of Treatment Groups for Dermal Barrier Reconstitution Study					
Group	No. of Mice	Time Points (h)	Test Article	Final Dose (Units/mL)	Injection Volume (mL)
1	15	0.5, 1, 4, 24, 48	Control	0	0.04
2	15	0.5, 1, 4, 24, 48	rHuPH20	100	0.04
3	15	0.5, 1, 4, 24, 48	F204P	100	0.04

All mice received two intradermal doses of vehicle control or rHuPH20 or F204P-PH20 at 100 U/mL in 0.04 mL at study time 0. The same control or test article was injected on the opposing lateral sides of each animal (right, R; left, L). Injection sites were marked with a permanent marker. Trypan Blue Stain (0.4% liquid solution; 15250, Invitrogen) was administered at a volume of 0.04 mL by intradermal injection at the same injection site at 0.5, 1, 4, 24 and 48 hours post-injection of test article or control. At 5 and 20 minutes post-injection of the Trypan Blue Stain, the area of the dye at the injection site was measured by digital imaging of the region as described in Example 14.

The results are set forth in Table 30. The results show that when the area of dye dispersion was measured at various time points after administration of the test article or control, there was a statistically significant increase in the area of dye dispersion at 30 min and 1 hour post-injection of rHuPH20 or F204P-PH20. By 4 hours post-administration of the enzymes, however, there was not a statistically significant increase in the area of dye dispersion compared to control. In addition, no statistically significant differences in the area of dye dispersion was observed between the rHuPH20 and F204P-PH20 treatment groups. Therefore, the duration of the spreading activity of rHuPH20 and F204P were similar and show that rHuPH20 and F204P-PH20 have comparable in vivo performance.

TABLE 30

Dermal Reconstitution

time Point	min post-injection	Vehicle	rHuPH20	F204P-PH20
30	5	49.96 ± 2.05	80.84 ± 8.03	80.76 ± 4.46
	20	64.42 ± 2.49	94.55 ± 7.09	95.75 ± 5.18
1 hour	5	58.01 ± 3.21	82.56 ± 6.40	77.11 ± 3.18
	20	65.19 ± 6.21	96.19 ± 6.39	91.45 ± 1.73
4 hour	5	52.10 ± 3.47	67.19 ± 2.39	67.33 ± 3.93
	20	57.69 ± 3.92	81.15 ± 4.45	82.21 ± 4.14
24 hour	5	49.87 ± 3.25	59.01 ± 2.15	54.91 ± 3.54
	20	57.15 ± 3.47	67.65 ± 2.27	62.91 ± 3.30
48 hour	5	53.64 ± 2.99	53.53 ± 4.88	55.64 ± 7.19
	20	61.57 ± 4.02	66.33 ± 4.12	63.11 ± 5.97

Example 16

In Vivo Pharmacokinetics of F204P-PH20 Compared to rHuPH20

The pharmacokinetics (PK) of rHuPH20 and F204P-PH20 were compared following intravenous tail-vein administration by measuring the plasma hyaluronidase levels over time after administration. The proteins used in the study were purified PH20 variant F204P (prepared as described in Example 12; batch concentration 1.02 mg/mL) and wild-type rHuPH20 (prepared as described in Example 1; batch concentration 0.95 mg/mL) formulated in API buffer (10 mM Histidine, 130 mM NaCl, pH 6.5). The proteins were prepared at a concentration of 0.087 mg/mL in API buffer for a dose volume of about 5 mL. An animal that was not administered with protein was used a control (pre-dose control). Forty two (42) male CD-1 mice (~20-30 grams) were used in the study with six animals per treatment group as set forth in Table 31.

TABLE 31

Pharmacokinetics of Single Intravenous Dose of rHuPH20 or F204P-PH20

Group	number of animals (No.)	Test Article	Dose (mg/kg)	Dose Volume (mL/kg)	Euthanasia
1	6 (Nos. 1-6)	no treatment	N/A	N/A	pre-dose
2	6 (Nos. 7-12)	rHuPH20	0.433	5	1 min
3	6 (Nos. 13-18)	rHuPH20	0.433	5	5 ± 1 min
4	6 (Nos. 19-24)	rHuPH20	0.433	5	10 ± 2 min
5	6 (Nos. 25-30)	F204P-PH20	0.433	5	1 min
6	6 (Nos. 21-36)	F204P-PH20	0.433	5	5 ± 1 min
7	6 (Nos. 37-42)	F204P-PH20	0.433	5	10 ± 2 min

Mice were intravenously administered 0.433 mg/kg rHuPH20 or F204P-PH20 by tail vein injection. Blood samples were obtained from animals 1 minute, 5 minutes and 10 minutes post-administration. Blood samples were obtained by terminal bleed (cardiac puncture) and collected into blood collection tubes containing the anti-coagulant EDTA for the preparation of plasma. Blood samples were centrifuged at 500 g for 10 minutes and the plasma removed and frozen at −80° C. until assessment of hyaluronidase activity using the microturbidity assay described in Example 8.

The results are set forth in Table 32. The results show that hyaluronidase activity is detected in plasma prior to treatment with the hyaluronidase. Within 1 minute post-treatment with either rHuPH20 or F204P-PH20 hyaluronidase, there is a detectably high amount of hyaluronidase activity present in the plasma, which is similar between both treatment groups. Over time, the hyaluronidase activity rapidly decreases for both treatment groups, although there is detectably hyaluronidase activity present in the plasma 10 minutes post-administration. At the 5 minute and 10 minute post-administration time points, activity in the plasma in animals treated with F204P-PH20 is greater than in animals treated with rHuPH20. This shows that F204P-PH20 exhibits somewhat greater activity for a prolonged time period, and therefore exhibits greater half-life in vivo than rHuPH20.

TABLE 32

rHuPH20 and F204P-PH20 Activity (U/mL) in Mouse Plasma K2EDTA

	Time Point (min)							
	Predose		1 minute		5 minute		10 minute	
Protein	Animal No.	U/mL	Animal No.	U/mL	Animal No.	U/mL	Animal No.	U/mL
rHuPH20	1	BQL	7	235^{a}	13	18.3	19	3.76
	2	BQL	8	13.5	14	7.70	20	3.70
	3	BQL	9	278	15	8.85	21	2.64
	4	BQL	10	328	16	10.5	22	2.70
	5	BQL	11	356	17	12.8	23	2.36
	6	BQL	12	287	18	18.0	24	2.80
F204P-PH20	1	BQL	25	249	31	48.0	37	11.5
	2	BQL	26	223	32	21.6	38	11.4
	3	BQL	27	246	33	38.4	39	10.1
	4	BQL	28	246	34	38.6	40	12.2
	5	BQL	20	0.696	35	38.2	41	10.8
	6	BQL	30	257	36	28.5	42	10.2

BQL—Below Quantifiable Limit <0.625 U/mL with minimum required dilution

[a] Hemolyzed

Since modifications will be apparent to those of skill in this art, it is intended that this invention be limited only by the scope of the appended claim.

SEQUENCE LISTING

The patent contains a lengthy "Sequence Listing" section. A copy of the "Sequence Listing" is available in electronic form from the USPTO web site (http://seqdata.uspto.gov/?pageRequest=docDetail&DocID=US09447401B2). An electronic copy of the "Sequence Listing" will also be available from the USPTO upon request and payment of the fee set forth in 37 CFR 1.19(b)(3).

The invention claimed is:

1. A modified PH20 polypeptide that exhibits increased stability in the presence of a phenolic preservative, comprising an amino acid replacement in an unmodified PH20 polypeptide, wherein:

the amino acid replacement confers the increased stability;

the amino acid replacement is replacement with proline at a position corresponding to position 204 in a PH20 polypeptide with reference to amino acid residue positions set forth in SEQ ID NO:3;

corresponding amino acid positions are identified by alignment of the modified PH20 polypeptide with the polypeptide whose sequence is set forth in SEQ ID NO:3;

the modified PH20 polypeptide has at least 85% sequence identity to the sequence of amino acids set forth in SEQ ID NO: 3 or 7; and

stability is increased compared to the unmodified polypeptide without the amino acid replacement, wherein stability is measured by resistance to denaturation in the presence of a phenolic preservative.

2. The modified PH20 polypeptide of claim **1**, wherein the unmodified PH20 polypeptide is a soluble PH20 polypeptide that consists of the sequence of amino acids set forth in any of SEQ ID NOS: 3, 7 and 32-66.

3. The modified PH20 polypeptide of claim **1**, wherein:

the unmodified PH20 polypeptide consists of the sequence of amino acids set forth in SEQ ID NO:3 or 7; and

the modified PH20 polypeptide has at least 95% sequence identity to SEQ ID NO:3.

4. The modified PH20 polypeptide of claim **1**, that has only up to 10 amino acid replacements compared to the polypeptide of SEQ ID NO: 7 or 3.

5. The modified PH20 polypeptide of claim **1**, wherein the modified PH20 polypeptide exhibits at least 15% of the hyaluronidase activity in the presence of the phenolic preservative(s) for at least 4 hours compared to the hyaluronidase activity in the absence of the phenolic preservative(s), wherein the activity is compared under the same conditions except for the presence of the phenolic preservative(s).

6. The modified PH20 polypeptide of claim **1**, wherein the modified PH20 polypeptide is stable in the presence of an anti-microbially effective amount of one or more phenolic preservatives.

7. The modified PH20 polypeptide of claim **6**, wherein the anti-microbially effective amount is a total amount of one or more phenolic preservative agents as a percentage (%) of mass concentration (w/v) that is from about 0.05% to 0.6%.

8. The modified PH20 polypeptide of claim **1**, wherein the phenolic preservative is selected from among one or more of phenol, metacresol (m-cresol), benzyl alcohol and a paraben.

9. The modified PH20 polypeptide of claim **1**, wherein the preservative is a phenolic preservative that is m-cresol, phenol, or a combination of m-cresol and phenol.

10. The modified PH20 polypeptide of claim **1**, further comprising at least one amino acid replacement at the amino acid position selected from the group consisting of 58, 10, 12, 20, 22, 26, 34, 36, 46, 50, 52, 68, 70, 74, 82, 83, 84, 86, 97, 127, 131, 138, 142, 143, 144, 166, 169, 174, 193, 195, 196, 205, 206, 213, 219, 234, 237, 238, 240, 249, 261, 267, 277, 279, 291, 309, 310, 314, 315, 317, 318, 347, 367, 375, 376, 399, 401, 407, 416, 419, 421, 431, 433, 439, 440, 443 and 445 with reference to amino acid positions set forth in SEQ ID NO:3, wherein amino acid position is identified by alignment of the modified PH20 polypeptide with SEQ ID NO:3.

11. The modified PH20 polypeptide of claim **10**, comprising at least one amino acid replacement selected from the group consisting of: G at a position corresponding to position 10; K at a position corresponding to position 12; S at a position corresponding to position 20; T at a position corresponding to position 22; M at a position corresponding to position 26; W at a position corresponding to position 34; N at a position corresponding to position 36; L at a position corresponding to position 46; M at a position corresponding to position 50; T at a position corresponding to position 52; S at a position corresponding to position 52; C at a position corresponding to position 58; K at a position corresponding to position 58; R at a position corresponding to position 58; N at a position corresponding to position 58; Y at a position corresponding to position 58; P at a position corresponding to position 58; H at a position corresponding to position 58; P at a position corresponding to position 68; V at a position corresponding to position 70; E at a position corresponding to position 74; L at a position corresponding to position 82; N at a position corresponding to position 82; V at a position corresponding to position 83; Q at a position corresponding to position 83; S at a position corresponding to position 83; G at a position corresponding to position 83; N at a position corresponding to position 84; A at a position corresponding to position 86; K at a position corresponding to position 86; E at a position corresponding to position 97; L at a position corresponding to position 97; R at a position corresponding to position 127; R at a position corresponding to position 131; L at a position corresponding to position 138; K at a position corresponding to position 142; N at a position corresponding to position 142; P at a position corresponding to position 142; S at a position corresponding to position 142; T at a position corresponding to position 142; G at a position corresponding to position 143; K at a position corresponding to position 143; T at a position corresponding to position 144; Q at a position corresponding to position 166; T at a position corresponding to position 166; L at a position corresponding to position 169; G at a position corresponding to position 174; N at a position corresponding to position 174; Q at a position corresponding to position 193; T at a position corresponding to position 195; N at a position corresponding to position 195; E at a position corresponding to position 196; R at a position corresponding to position 196; A at a position corresponding to position 205; E at a position corresponding to position 205; I at a position corresponding to position 206; A at a position corresponding to position 213; I at a position corresponding to position 219; M at a position corresponding to position 234; T at a position corresponding to position 237; H at a position corresponding to position 238; Q at a position corresponding to position 240; V at a position corresponding to position 249; A at a position corresponding to position 26t; K at a position corresponding to position 261; T at a position corresponding to position 267; K at a position corresponding to position 277; H at a position corresponding to position 279; V at a position corresponding to position 279; V at a position corresponding to position 291; E at a position corresponding to position 309; Q at a position corresponding to position 310; Y at a position corresponding to position 314; Y at a position corresponding to position 315; N at a position corresponding to position 317; W at a position corresponding to position 317; D at a position corresponding to position 318; G at a position corresponding to position 347; A at a position corresponding to position 367; R at a position corresponding to position 375; R at a position corresponding to position 376; V at a position corresponding to position 399; E at a position corresponding to position 401; A at a position corresponding to position 407; L at a position corresponding to position 416; K at a position corresponding to position 419; H at a position corresponding to position 421; E at a position corresponding to position 431; T at a position corresponding to position 433; V at a position corresponding to position 433; C at a position corresponding to position 439; P at a position corresponding to position 440; G at a position corresponding to position 443; and N at a position corresponding to position 445, with reference to amino acid sequence of SEQ ID NO:3.

12. The modified PH20 polypeptide of claim **11**, comprising at least one amino acid replacement selected from the group consisting of: T at a position corresponding to position 52, K at a position corresponding to position 58, R at a position corresponding to position 58, P at a position corresponding to position 68, V at a position corresponding to position 83, P at a position corresponding to position 204, A at a position corresponding to position 261, T at a position corresponding to position 267, K at a position corresponding to position 277 and H at a position corresponding to position 421, with reference to amino acid residue positions set forth in SEQ ID NO:3.

13. The modified PH20 polypeptide of claim **12**, comprising replacement with R at a position corresponding to position 58 in a PH20 polypeptide with reference to amino acid positions set forth in SEQ ID NO:3.

14. The modified PH20 polypeptide of claim **1** that consists of a sequence of amino acid residues having at least 98% sequence identity to SEQ ID NO:49 and comprises replacement with P at a position corresponding to position 204 in a PH20 polypeptide with reference to amino acid residue positions set forth in SEQ ID NO:49.

15. The modified PH20 polypeptide of claim **1** that is purified or isolated.

16. The modified PH20 polypeptide of claim **1** that is further modified by a modification selected from among glycosylation, sialation, albumination, farnysylation, carboxylation, hydroxylation and phosphorylation.

17. The modified PH20 polypeptide of claim **1**, wherein the modified PH20 polypeptide is glycosylated, whereby the polypeptide comprises at least an N-acetylglucosamine moiety linked to each of at least three asparagine (N) residues.

18. The modified PH20 polypeptide of claim **17**, wherein the three asparagine residues correspond to amino acid residues 200, 333 and 358 of SEQ ID NO:3.

19. The modified PH20 polypeptide of claim **1** that is modified by conjugation to a polymer.

20. The modified PH20 polypeptide of claim **19**, wherein the polymer is dextran or PEG.

21. The modified PH20 polypeptide of claim **1**, wherein the modified PH20 polypeptide is conjugated to a moiety selected from among a multimerization domain, toxin, detectable label or drug.

22. A pharmaceutical composition, comprising the modified PH20 polypeptide of claim **1**.

23. The pharmaceutical composition of claim **22**, wherein the pharmaceutical composition comprises a pharmaceutically acceptable excipient.

24. The pharmaceutical composition of claim **22**, comprising an anti-microbially effective amount of a preservative or mixture of preservatives.

25. The pharmaceutical composition of claim **24**, comprising at least one phenolic preservative.

26. The pharmaceutical composition of claim **25**, wherein the preservative(s) is(are) selected from among phenol, metacresol (m-cresol), benzyl alcohol, and a paraben.

27. The pharmaceutical composition of claim **24**, wherein the anti-microbially effective amount is a total amount of one or more preservative agents as a percentage (%) of mass concentration (w/v) that is or is between 0.05% to 0.6%.

28. The pharmaceutical composition of claim **22**, comprising a therapeutically active agent.

29. The pharmaceutical composition of claim **28**, wherein the therapeutic agent is a polypeptide, a protein, a nucleic acid, a drug, a small molecule or an organic molecule.

30. The pharmaceutical composition of claim **28**, wherein the therapeutically active agent is selected from the group consisting of a chemotherapeutic agent, an analgesic agent, an anti-inflammatory agent, an antimicrobial agent, an amoebicidal agent, a trichomonacidal agent, an anti-parkinson agent, an anti-malarial agent, an anticonvulsant agent, an anti-depressant agent, and antiarthritics agent, an antifungal agent, an antihypertensive agent, an antipyretic agent, an anti-parasite agent, an antihistamine agent, an alpha-adrenergic agonist agent, an alpha blocker agent, an anesthetic agent, a bronchial dilator agent, a biocide agent, a bactericide agent, a bacteriostat agent, a beta adrenergic blocker agent, a calcium channel blocker agent, a cardiovascular drug agent, a contraceptive agent, a decongestant agent, a diuretic agent, a depressant agent, a diagnostic agent, a electrolyte agent, a hypnotic agent, a hormone agent, a hyperglycemic agent, a muscle relaxant agent, a muscle contractant agent, an ophthalmic agent, a parasympathomimetic agent, a psychic energizer agent, a sedative agent, a sympathomimetic agent, a tranquilizer agent, an urinary agent, a vaginal agent, a viricide agent, a vitamin agent, a non-steroidal anti-inflammatory agent, an angiotensin converting enzyme inhibitor agent, and a sleep inducer.

31. The pharmaceutical composition of claim **28**, wherein the therapeutic agent is selected from the group consisting of Adalimumabs, Agalsidase Betas, Alefacepts, Ampicillins, Anakinras, Antipoliomyelitic Vaccines, Anti-Thymocytes, Azithromycins, Becaplermins, Caspofungins, Cefazolins, Cefepimes, Cefotetans, Ceftazidimes, Ceftriaxones, Cetuximabs, Cilastatins, Clavulanic Acids, Clindamycins, Darbepoetin Alfas, Daclizumabs, Diphtheria, Diphtheria antitoxins, Diphtheria Toxoids, Efalizumabs, Epinephrines, Erythropoietin Alphas, Etanercepts, Filgrastims, Fluconazoles, Follicle-Stimulating Hormones, Follitropin Alphas, Follitropin Betas, Fosphenytoins, Gadodiamides, Gadopentetates, Gatifloxacins, Glatiramers, GM-CSFs, Goserelin acetates, Granisetrons, *Haemophilus Influenza* B's, Haloperidols, Hepatitis vaccines, Hepatitis A Vaccines, Hepatiti B Vaccines, Ibritumomab Tiuxetans, Ibritumomabs, Tiuxetans, Immunoglobulins, *Hemophilus influenza* vaccines, Influenza Virus Vaccines, Infliximabs, Insulin lispro, 75% neutral protamine lispro (NPL)/25% insulin lispro, 50% neutral protamine Hagedorn (NPH)/50% regular insulin, 70% NPH/30% regular insulin; Regular insulin, NPH insulin, Ultra insulin, Ultralente insulin, and Insulin Glargines, Interferons, Interferon alpha, Interferon Betas, Interferon Gammas, Interferon alpha-2a, Interferon alpha 2-b, Interferon Alphacon, Interferon alpha-n, Interferon Betas, Interferon Beta-1 a's, Interferon Gammas, Interferon alpha-con, Iodixanols, Iohexols, Iopamidols, Ioversols, Ketorolacs, Laronidases, Levofloxacins, Lidocaines, Linezolids, Lorazepams, Measles Vaccines, Measles virus, Mumps viruses, Measles-Mumps-Rubella Virus Vaccines, Rubella vaccines, Medroxyprogesterones, Meropenems, Methylprednisolones, Midazolams, Morphines, Octreotides, Omalizumabs, Ondansetrons, Palivizumabs, Pantoprazoles, Pegaspargases, Pegfilgrastims, Peg-Interferon Alpha-2a's, Peg-Interferon Alpha-2b's, Pegvisomants, Pertussis vaccines, Piperacillins, Pneumococcal Vaccines and Pneumococcal Conjugate Vaccines, Promethazines, Reteplases, Somatropins, Sulbactams, Sumatriptans, Tazobactams, Tenecteplases, Tetanus Purified Toxoids, Ticarcillins, Tositumomabs, Triamcinolones, Triamcinolone Acetonides, Triamcinolone hexacetonides, Vancomycins, Varicella Zoster immunoglobulins, Varicella vaccines, other vaccines, Alemtuzumabs, Alitretinoins, Allopurinols, Altretamines, Amifostines, Anastrozoles, Arsenics, Arsenic Trioxides, Asparaginases, *Bacillus* Calmette-Guerin (BCG) vaccines, BCG Live, Bexarotenes, Bleomycins, Busulfans, Busulfan intravenous, Busulfan orals, Calusterones, Capecitabines, Carboplatins, Carmustines, Carmustines with Polifeprosans, Celecoxibs, Chlorambucils, Cisplatins, Cladribines, Cyclophosphamides, Cytarabines, Cytarabine liposomals, Dacarbazines, Dactinomycins, Daunorubicin liposomals, Daunorubicins, Denileukin Diftitoxes, Dexrazoxanes, Docetaxels, Doxorubicins, Doxorubicin liposomals, Dromostanolone propionates, Elliotts B Solutions, Epirubicins, Epoetin alfas, Estramustines, Etoposide phosphates, Exemestanes, Floxuridines, Fludarabines, Fluorouracils, Fulvestrants, Gemcitabines, Gemtuzumabs, Ozogamicins, Gemtuzumab ozogamicins, Hydroxyureas, Idarubicins, Ifosfamides, Imatinib mesylates, Irinotecans, Letrozoles, Leucovorins, Levamisoles, Lomustines, Mechlorethamines, Nitrogen mustards, Megestrols, Megestrol acetates, Melphalans, Mercaptopurines, Mesnas, Methotrexates, Methoxsalens, Mitomycins, Mitomycin C's, Mitotanes, Mitoxantrones, Nandrolones, Nandrolone Phenpropionates, Nofetumomabs, Oprelvekins, Oxaliplatins, Paclitaxels, Pamidronates, Pegademases, Pentostatins, Pipobromans, Plicamycins, Porfimer sodiums, Procarbazines, Quinacrines, Rasburicases, Rituximabs, Sargramostims, Streptozocins, Talcs, Tamoxifens, Temozolomides, Teniposides, Testolactones, Thioguanines, Triethylenethiophosphoramides (Thiotepas), Topotecans, Toremifenes, Trastuzumabs, Tretinoins, Uracil Mustards, Valrubicins, Vinblastines, Vincristines, Vinorelbines, Zoledronates, Acivicins, Aclarubicins, Acodazoles, Acronines, Adozelesins, Retinoic Acids, 9-Cis-Retinoic Acids, Alvocidibs, Ambazones, Ambomycins, Ametantrones, Aminoglutethimides, Amsacrines, Anaxirones, Ancitabines, Anthramycins, Apaziquones, Argimesnas, Asperlins, Atrimustines, Azacitidines, Azetepas, Azotomycins, Banoxantrones, Batabulins, Batimastats, Benaxibines, Bendamustines, Benzodepas, Bicalutamides, Bietaserpines, Biricodars, Bisantrenes, Bisnafide Dimesylates, Bizelesins, Bortezomibs, Brequinars, Bropirimines, Budotitanes, Cactinomycins, Canertinibs, Caracemides, Carbetimers, Carboquones, Carmofurs, Carubicins, Carzelesins, Cedefingols, Cemadotins, Cioteronels, Cirolemycins, Clanfenurs, Clofarabines, Crisnatols, Decitabines, Dexniguldipines, Dexormaplatins, Dezaguanines, Diaziquones, Dibrospidiums, Dienogests, Dinalins, Disermolides, Dofequidars, Doxifluridines, Droloxifenes, Duazomycins, Ecomustines, Edatrexates, Edotecarins, Eflomithines, Elacridars, Elinafides, Elsamitrucins, Emitefurs, Enloplatins, Enpromates, Enzastaurins, Epipropidines, Eptaloprosts, Erbulozoles, Esorubicins, Etanidazoles, Etoglucids, Etoprines, Exisulinds, Fadrozotes, Fazarabines, Fenretinides, Fluoxymesterones, Flurocitabines, Fosquidones, Fostriecins, Fotretamines, Galarubicins, Galocitabines, Geroquinols, Gimatecans, Gimeracils, Gloxazones, Glufosfamides, Ilmofosines, Ilomastats, Imexons, Improsulfans, Indisulams, Inproquones, Interleukins, Interleukin-2s, recombinant Interleukins, Intoplicines, Iobenguanes, Iproplatins, Irsogladines, Ixabepilones, Ketotrexates, L-Alanosines, Lanreotides, Lapatinibs, Ledoxantrones, Leuprolides, Lexacalcitols, Liarozoles, Lobaplatins, Lometrexols, Lonafamibs, Losoxantrones, Lurtotecans, Mafosfamides, Mannosulfans, Marimastats, Masoprocols, Maytansines, Melengestrols, Menogarils, Mepitiostanes, Metesinds, Metomidates, Metoprines, Meturedepas, Miboplatins, Miproxifenes, Misonidazoles, Mitindomides, Mitocarcins, Mitocromins, Mitoflaxones, Mitogillins, Mitoguazones, Mitomalcins, Mitonafides, Mitoquidones, Mitospers, Mitozolomides, Mivobulins, Mizoribines, Mofarotenes, Mopidamols, Mubritinibs, Mycophenolic Acids, Nedaplatins, Nelarabines, Nemorubicins, Nitracrines, Nocodazoles, Nogalamycins, Nolatrexeds, Nortopixantrones, Ormaplatins, Ortataxels, Oteracils, Oxisurans, Oxophenarsines, Patupilones, Peldesines, Peliomycins, Pelitrexols, Pemetrexeds, Pentamustines, Peptomycins, Perfosfamides, Perifosines, Picoplatins, Pinafides, Piposulfans, Pirfenidones, Piroxantrones, Pixantrones, Plevitrexeds, Plomestanes, Porfiromycins, Prednimustines, Propamidines, Prospidiums, Pumitepas, Puromycins, Pyrazofurins, Ranimustines, Riboprines, Ritrosulfans, Rogletimides, Roquinimexs, Sabarubicins, Safingols, Satraplatins, Sebriplatins, Semustines, Simtrazenes, Sizofirans, Sobuzoxanes, Sorafenibs, Sparfosates, Sparfosic Acids, Sparsomycins, Spirogermaniums, Spiromustines, Spiroplatins, Squalamines, Streptonigrins, Streptovarycins, Sufosfamides, Sulofenurs, Tacedinalines, Talisomycins, Tallimustines, Tariquidars, Tauromustines, Tecogalans, Tegafurs, Teloxantrones, Temoporfins, Teroxirones, Thiamiprines, Tiamiprines, Tiazofurins, Tilomisoles, Tilorones, Timcodars, Timonacics, Tirapazamines, Topixantrones, Trabectedins, Trestolones, Triciribines, Trilostanes, Trimetrexates, Triplatin Tetranitrates, Triptorelins, Trofosfamides, Tubulozotes, Ubenimexs, Uredepas, Valspodars, Vapreotides, Verteporfins, Vindesines, Vinepidines, Vinflunines, Vinformides, Vinglycinates, Vinleucinols, Vinleurosines, Vinrosidines, Vintriptols, Vinzolidines, Vorozoles, Xanthomycin A's, Guamecyclines, Zeniplatins, Zilascorbs [2-H], Zinostatins, Zorubicins, Zosuquidars, Acetazolamides, Acyclovirs, Adipiodones, Alatrofloxacins, Alfentanils, Allergenic extracts, Alpha 1-proteinase inhibitors, Alprostadils, Amikacins, Amino acids, Aminocaproic acids, Aminophyllines, Amitriptylines, Amobarbitals, Amrinones, Analgesics, Anti-poliomyelitic vaccines, Anti-rabic serums, Anti-tetanus immunoglobulins, tetanus vaccines, Antithrombin Ills, Antivenom serums, Argatrobans, Arginines, Ascorbic acids, Atenolols, Atracuriums, Atropines, Aurothioglucoses, Azathioprines, Aztreonams, Bacitracins, Baclofens, Basiliximabs, Benzoic acids, Benztropines, Betamethasones, Biotins, Bivalirudins, Botulism antitoxins, Bretyliums, Bumetanides, Bupivacaines, Buprenorphines, Butorphanols, Calcitonins, Calcitriols, Calciums, Capreomycins, Carboprosts, Carnitines, Cefamandoles, Cefoperazones, Cefotaximes, Cefoxitins, Ceftizoximes, Cefuroximes, Chloramphenicols, Chloroprocaines, Chloroquines, Chlorothiazides, Chlorpromazines, Chondroitinsulfuric acids, Choriogonadotropin alfas, Chromiums, Cidofovirs, Cimetidines, Ciprofloxacins, Cisatracuriums, Clonidines, Codeines, Colchicines, Colistins, Collagens, Corticorelin ovine triflutates, Corticotrophins, Cosyntropins, Cyanocobalamins, Cyclosporines, Cysteines, Dacliximabs, Dalfopristins, Dalteparins, Danaparoids, Dantrolenes, Deferoxamines, Desmopressins, Dexamethasones, Dexmedetomidines, Dexpanthenols, Dextrans, Iron dextrans, Diatrizoic acids, Diazepams, Diazoxides, Dicyclomines, Digibinds, Digoxins, Dihydroergotamines, Diltiazems, Diphenhydramines, Dipyridamoles, Dobutamines, Dopamines, Doxacuriums, Doxaprams, Doxercalciferols, Doxycyclines, Droperidols, Dyphyllines, Edetic acids, Edrophoniums, Enalaprilats, Ephedrines, Epoprostenols, Ergocalciferols, Ergonovines, Ertapenems, Ery~hromycins, Esmolols, Estradiols, Estrogenics, Ethacrynic acids, Ethanolamines, Ethanols, Ethiodized oils, Etidronic acids, Etomidates, Famotidines, Fenoldopams, Fentanyls, Flumazenils, Fluoresceins, Fluphenazines, Folic acids, Fomepizoles, Fomivirsens, Fondaparinuxs, Foscarnets, Fosphenytoins, Furosemides, Gadoteridols, Gadoversetamides, Ganciclovirs, Gentamicins, Glucagons, Glucoses, Glycines, Glycopyrrolates, Gonadorelins, Gonadotropin chorionics, *Haemophilus* B polysaccharides, Hemins, Herbals, Histamines, Hydralazines, Hydrocortisones, Hydromorphones, Hydroxocobalamins, Hydroxyzines, Hyoscyamines, Ibutilides, Imiglucerases, Indigo carmines, Indomethacins, Iodides, Iopromides, Iothalamic acids, Ioxaglic acids, Ioxilans, Isoniazids, Isoproterenols, Japanese encephalitis vaccines, Kanamycins, Ketamines, Labetalols, Lepirudins, Levobupivacaines, Levothyroxines, Lincomycins, Liothyronines, Luteinizing hormones, Lyme disease vaccines, Mangafodipirs, Manthtols, Meningococcal polysaccharide vaccines, Meperidines, Mepivacaines, Mesoridazines, Metaraminols, Methadones, Methocarbamols, Methohexitals, Methyldopates, Methylergonovines, Metoclopramides, Metoprolols, Metronidazoles, Minocyclines, Mivacuriums, Morrhuic acids, Moxifloxacins, Muromonab-CD3s, Mycophenolate mofetils, Nafcillins, Nalbuphines, Nalmefenes, Naloxones, Neostigmines, Niacinamides, Nicardipines, Nitroglycerins, Nitroprussides, Norepinephrines, Orphenadrines, Oxacillins, Oxymorphones, Oxytetracyclines, Oxytocins, Pancuroniums, Panthenols, Pantothenic acids, Papaverines, Peginterferon alpha 2As, Penicillin Gs, Pentamidines, Pentazocines, Pentobarbitals, Perflutrens, Perphenazines, Phenobarbitals, Phentolamines, Phenylephrines, Phenytoins, Physostigmines, Phytonadiones, Polymyxin, Pralidoximes, Prilocaines, Procainamides, Procaines, Prochlorperazines, Progesterones, Propranolols, Pyridostigmine hydroxides, Pyridoxines, Quinidines, Quinupristins, Rabies immunoglobulins, Rabies vaccines, Ranitidines, Remifentanils, Riboflavins, Rifampins, Ropivacaines, Samariums, Scopolamines, Seleniums, Sermorelins, Sincalides, Somatrems, Spectinomycins, Streptokinases, Streptomycins, Succinylcholines, Sufentanils, Sulfamethoxazoles, Tacrolimuses, Terbutalines, Teriparatides, Testosterones, Tetanus antitoxins, Tetracaines, Tetradecyl sulfates, Theophyllines, Thiamines, Thiethylperazines, Thiopentals, Thyroid stimulating hormones, Tinzaparins, Tirofibans, Tobramycins, Tolazolines, Tolbutamides, Torsemides, Tranexamic acids, Treprostinils, Trifluoperazines, Trimethobenzamides, Trimethoprims, Tromethamines, Tuberculins, Typhoid vaccines, Urofollitropins, Urokinases, Valproic acids, Vasopressins, Vecuroniums, Verapamils, Voriconazoles, Warfarins, Yellow fever vaccines, Zidovudines, Zincs, Ziprasidone hydrochlorides, Aclacinomycins, Actinomycins, Adriamycins, Azaserines, 6-Azauridines, Carzinophilins, Chromomycins, Denopterins, 6 Diazo 50xo-L-Norleucines, Enocitabines, Floxuridines, Olivomycins, Pirarubicins, Piritrexims, Pteropterins, Tegafurs, Tubercidins, Alteplases, Arcitumomabs, bevacizumabs, Botulinum Toxin Type A's, Botulinum Toxin Type B's, Capromab Pendetides, Daclizumabs, Dornase alphas, Drotrecogin alphas, Imciromab Pentetates, Iodine-131's, an antibiotic agent; an angiogenesis inhibitor; anti-cataract and anti-diabetic retinopathy substances; carbonic anhydrase inhibitors; mydriatics; photodynamic therapy agents; prostaglandin analogs; growth factor; anti-neoplastics; anti-metabolites; anti-viral; amebicides and anti-protozoals; anti-tuberculosis and anti-leprotic; antitoxins and antivenins; antihemophilic factor, anti-inhibitor coagulant complex, antithrombin III, coagulations Factor V, coagulation Factor IX, plasma protein fraction, von Willebrand factor; anti-platelet agent a colony stimulating factor (CSF); an erythropoiesis stimulator; hemostatics and albumins; Immune Globulins; thrombin inhibitors; anticoagulants; a steroidal anti-inflammatory drug selected from among alclometasones, algestones, beclomethasones, betamethasones, budesonides, clobetasols, clobetasones, clocortolones, cloprednols, corticosterones, col~isones, cortivazols, deflazacorts, desonides, desoximetasones, dexamethasones, diflorasones, diflucortolones, difluprednates, enoxolones, fluazacorts, flucloronides, flumethasones, flunisolides, fluocinolones, fluocinonides, fluocortins, fluocortolones, fluorometholones, fluperolones, fluprednidenes, fluprednisolones, flurandrenolides, fluticasones, formocortals, halcinonides, halobetasols, halometasones, halopredones, hydrocortamates, hydrocortisones, loteprednol etabonate, mazipredones, medrysones, meprednisones, methylprednisolones, mometasone furoate, paramethasones, prednicarbates, prednisolones, prednisones, prednivals, prednylidenes, rimexolones, tixocortols and triamcinolones; docosenoid, prostaglandins, prostaglandin analogs, antiprostaglandins and prostaglandin precursors; miotics, cholinergics and anti-cholinesterase; and anti-allergenics.

32. The pharmaceutical composition of claim **28**, wherein the therapeutic agent is selected from the group consisting of an antibody, an Immune Globulin, a bisphosphonate, a cytokine, a chemotherapeutic agent, a coagulation factor and an insulin.

33. The pharmaceutical composition of claim **32**, wherein the therapeutic agent is an insulin that is a fast-acting insulin.

34. The pharmaceutical composition of claim **33**, wherein the fast-acting insulin is regular insulin or is an insulin analog.

35. The pharmaceutical composition of claim **34**, wherein the fast-acting insulin is a regular insulin that is a human insulin or a pig insulin.

36. The pharmaceutical composition of claim **35**, wherein:

the fast-acting insulin comprises an A chain having the sequence of amino acids set forth in SEQ ID NO:862 and a B chain having the sequence of amino acids set forth in SEQ ID NO:863; or

the fast-acting insulin comprises an A chain with the sequence of amino acids set forth as amino acid residue positions 88-108 of SEQ ID NO:864 and a B chain with the sequence of amino acids set forth as amino acid residue positions 25-54 of SEQ ID NO:864.

37. The pharmaceutical composition of claim **34**, wherein the fast-acting insulin is an insulin analog selected from among insulin lispro, insulin aspart and insulin glulisine.

38. The pharmaceutical composition of claim **37**, wherein the insulin analog is an insulin having an A chain with the sequence of amino acids set forth in SEQ ID NO:862 and a B chain having the sequence of amino acids set forth in any of SEQ NOS:865-867.

39. The pharmaceutical composition of claim **33**, wherein:

the amount of the modified PH20 polypeptide is from about 100 U/mL to 1000 U/mL; and

the amount of the fast-acting insulin is from about 10 U/mL to 1000 U/mL.

40. The pharmaceutical composition of claim **39**, further comprising:

a pH from about 7.0 to 7.6;

NaCl at a concentration from about 0.1 mM to 200 mM; and

an anti-microbially effective amount of one or more preservatives, wherein at least one preservative is a phenolic preservative.

41. The pharmaceutical composition of claim **40**, wherein the anti-microbially effective amount is a total amount of one or more preservative agents as a percentage (%) of mass concentration (w/v) that is between 0.05% to 0.6%, inclusive.

42. The pharmaceutical composition of claim **40**, wherein the preservative(s) is(are) selected from the group consisting of phenol, metacresol (m-cresol), benzyl alcohol, and a paraben.

43. The pharmaceutical composition of claim **40**, further comprising: a surfactant in an amount as a percentage (%) of mass concentration (w/v) in the formulation composition that is at least or at least about 0.001%; a buffering agent that is a non-metal binding agent or is a metal binding agent, wherein the concentration of the buffering agent is between about 1 mM to 100 mM; glycerin in a concentration less than 60 mM; an antioxidant at a concentration between about 2 mM to 50 mM; and/or zinc at a concentration of between about 0.001 to 0.1 mg per 100 units of insulin (mg/100 U).

44. The pharmaceutical composition of claim **43**, wherein the surfactant is selected from the group consisting of a polypropylene glycol, polyethylene glycol, glycerin, sorbitol, poloxamer and polysorbate.

45. The pharmaceutical composition of claim **43**, wherein the buffering agent is selected from the group consisting of Tris, histidine, phosphate and citrate.

46. The pharmaceutical composition of claim **43**, wherein the antioxidant is selected from the group consisting of cysteine, tryptophan and methionine.

47. A closed loop system, comprising the pharmaceutical composition of claim **33**.

48. An insulin pump, comprising the pharmaceutical composition of claim **33**.

49. An insulin pen, comprising the pharmaceutical composition of claim **33**.

* * * * *