

Characterization of the active site of monkey sperm hyaluronidase

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The mammalian sperm hyaluronidase, PH-20, is active in macaque spermatozoa at neutral and acid pH. Antibodies were produced to synthesized peptides representing regions of PH-20 that may be involved in hyaluronidase activity and designated peptide 1 (amino acid sequence 142–172) and peptide 3 (amino acid sequence 277–297). Western blotting of proteins extracted from the surface of acrosome-intact spermatozoa showed that the two peptide-specific, affinity-purified IgGs label a 64 kDa band corresponding to the PH-20 molecule. Western blots of acrosome-reacted spermatozoa showed that, under reducing conditions, the two anti-peptide IgGs label the 44 kDa band only, which represents the N-terminal fragment of PH-20. Anti-peptide 3 IgG also labels the 53 kDa form of PH-20 in extracts of acrosome-reacted spermatozoa. Peptide-specific, affinity-purified Fab frag-

ments from both IgGs were shown by fluorescence microscopy and transmission electron microscopy to label the sperm plasma membrane, fused acrosomal vesicles, acrosomal matrix and inner acrosomal membrane. Fab fragments of anti-peptide 1 IgG, but not anti-peptide 3 IgG, inhibited hyaluronidase activity of PH-20 from the sperm surface and from extracts of acrosome-reacted spermatozoa at pH 7.0. Fab fragments of both anti-peptide IgGs inhibited sperm hyaluronidase activity at pH 5.0. It is concluded that the region of PH-20 encompassed by the amino acid sequence 142–172 is essential for hyaluronidase activity at neutral pH, whereas the region of amino acid sequence 277–297 may be more important at a lower pH. It is likely that these two regions are the acid/base catalyst site and the nucleophilic site, respectively, of PH-20 hyaluronidases.

Introduction

Glycosidases are a diverse group of enzymes that catalyse the hydrolysis of glycosidic bonds (Henrissat and Bairoch, 1993; Davies and Henrissat, 1995; Mark *et al.*, 1998). The hyaluronidases comprise a family of the glycosidases that has been exceedingly difficult to purify and characterize, because of their low concentration and structural instability (Kreil, 1995). The substrate for the hyaluronidases is hyaluronic acid, a ubiquitous, structurally simple molecule that is involved in numerous physiological and pathological processes (Toole, 1997). An overabundance of hyaluronic acid and increase of hyaluronidase activity are well-recognized features of tumorigenesis and are the subject of current investigation (West and Kumar, 1989; Csoka *et al.*, 1997; Lokeshwar *et al.*, 1999; Madan *et al.*, 1999).

A number of mammalian hyaluronidases have been discovered, including ubiquitous and tissue-specific forms (Csoka *et al.*, 1999). The most widely recognized tissue-specific source of hyaluronidase is the mammalian testis, identified originally as a source of 'spreading factor' (Chain

and Duthie, 1939). Hyaluronidase was identified subsequently with spermatozoa and was implicated in the mechanism of sperm–oocyte interaction (Austin, 1960). PH-20 is a glycosyl-phosphatidylinositol (GPI)-anchored sperm surface glycoprotein found on all the mammalian spermatozoa that have been examined (Myles and Primakoff, 1997). The cloning of bee venom hyaluronidase uncovered a marked cDNA sequence homology with PH-20 (Gmachl and Kreil, 1993; Lin *et al.*, 1994) and led to the recognition that PH-20 was likely to have hyaluronidase activity. There is now strong evidence that PH-20 is the only hyaluronidase in spermatozoa of guinea-pigs (Hunnicut *et al.*, 1996), rats (Hou *et al.*, 1996), macaques (Cherr *et al.*, 1996; Li *et al.*, 1997) and humans (Sabeur *et al.*, 1997).

Although the mammalian hyaluronidases are derived from different genes, it is reasonable that these enzymes share certain structural and biological characteristics with one another and with other glycosidases. The glycosidases characteristically have two acidic amino acids that function as either the acid/base catalyst or the nucleophilic site (Braithwaite *et al.*, 1997; Zverlov *et al.*, 1998). Arming *et al.* (1997) used *in vitro* mutagenesis of PH-20 to show that two acidic amino acids are critical for enzyme activity. In the present study, these putative catalytic sites of PH-20 were investigated by synthesizing peptides to the corresponding regions and producing polyclonal antibodies to the synthesized peptides.

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Materials and Methods

Reagents

The chemicals used for media preparation and experimental techniques were all purchased from Sigma Chemical Company (St Louis, MO), unless specified otherwise.

Antibodies

Recombinant cynomolgus macaque sperm PH-20 (recombinant PH-20) was generated and purified as reported by Lin *et al.* (1993) and was a gift from P. Primakoff (Department of Cell Biology and Human Anatomy, University of California, Davis) and D. Myles (Department of Molecular and Cellular Biology, University of California, Davis). Two New Zealand white rabbits were given a series of s.c. injections of 50 µg recombinant PH-20. The first injection included complete Freund's adjuvant, whereas the subsequent three injections included incomplete Freund's adjuvant. Both rabbits were exsanguinated after the fourth injection, which was approximately 2 months after the series was initiated. The serum was inactivated at 56°C for 1 h in a waterbath.

IgG was purified using ImmunoPure® IgG (protein A) purification kit (Pierce, Rockford, IL). In brief, the inactivated serum was diluted with ImmunoPure® binding buffer and applied to a pre-equilibrated protein A column. After washing with the binding buffer, the bound IgG was eluted with ImmunoPure® IgG elution buffer. After concentration with a Centricon 50 ultrafiltration tube (Millipore, Bedford, MA), the eluted IgG in elution buffer was applied to D-Salt™ cellulose desalting column (Pierce) and equilibrated with Dulbecco's PBS (DPBS) to remove salts and change the buffer to DPBS. Fab fragments were prepared from purified whole IgG as described by Cherr *et al.* (1996).

Synthetic peptides were produced and anti-peptide antibodies were developed by Zymed Laboratories (South San Francisco, CA). Two potential catalytic sites for PH-20 were identified. The region designated as peptide 1 encompasses the amino acid sequence 142–172 (AVIDWEEWRPTWARNWKPKDVYKNSIELV). A second region with characteristics of an enzyme catalytic site was designated peptide 3 and includes the amino acid sequence 277–297 (YVRNRVREAIRVSKIPDAKN). The amino acid sequence for each peptide was evaluated for probable antigenicity and the likelihood that the peptide region is located on the protein surface. The constructed peptides were coupled to the carrier protein, keyhole limpet haemocyanin. Antibody titres were evaluated by Zymed Laboratories and the rabbits were exsanguinated approximately 2 months after the initial injection. Zymed furnished the whole rabbit serum, whole IgG, specific anti-peptide IgGs and Fab fragments of the anti-peptide IgGs.

Sperm collection

All animals were housed at the California Regional Primate Research Center in compliance with the Federal

Animal Welfare Act and the National Institute of Health Guidelines for Care and Use of Laboratory Animals. Semen was collected from male cynomolgus macaques by electroejaculation (Sarason *et al.*, 1991). Each ejaculate was collected into a 15 ml conical tube and covered immediately with 5 ml Hepes-buffered Biggers, Whitten and Whittingham (BWW) medium (Irvine Scientific, Santa Ana, CA). Samples were allowed to stand at room temperature for 30 min before the upper 4 ml was removed and centrifuged for 5 min at 300 g.

Preparation of PH-20

After centrifugation, sperm pellets were resuspended in 5 ml DPBS (Life Technologies, Rockville, MD) and re-centrifuged for 5 min at 300 g. The resulting pellet was layered onto 4 ml of a suspension of 40% (v/v) Percoll in DPBS and centrifuged at 300 g for 15 min. The spermatozoa were washed two more times by centrifugation and resuspension in 10 ml DPBS before treatment as described below.

Washed acrosome-intact spermatozoa were resuspended at a concentration of 4.0×10^7 spermatozoa ml⁻¹ in DPBS containing 2 U phospholipase C–phosphatidylinositol ml⁻¹ to recover plasma membrane PH-20. Protease inhibitors (20 mmol EDTA l⁻¹, 1 mmol *p*-hydroxymercurobenzoate l⁻¹, 5 mmol *N*-ethymaleimide l⁻¹ and 1 mmol benzamidine l⁻¹) were added and the sample was rolled at a 30° angle from side to side at 28 rotations min⁻¹ at 37°C for 2 h (Li *et al.*, 1997). After the 2 h incubation, the sperm sample was microcentrifuged at 2000 g for 10 min and then passed through a 0.22 µm filter (Fisher Scientific) and stored at –80°C until needed. Before use, the phospholipase C–phosphatidylinositol-released protein extracts were pooled followed by ultracentrifugation at 100 000 g at 4°C for 1 h.

Washed spermatozoa were incubated for 2 h at 37°C in BWW medium containing 3 mg BSA ml⁻¹ to obtain endo-proteolysed PH-20 from acrosome-reacted spermatozoa. After incubation, spermatozoa were activated by addition of 1 mmol caffeine l⁻¹ and 1 mmol N₆,2'-O-dibutyryladenine 3':5'-cyclic monophosphate sodium salt l⁻¹ (dbcAMP). The spermatozoa were incubated for an additional 30 min and then washed twice with DPBS to remove the BSA. The spermatozoa were resuspended in DPBS at a concentration of 1.0×10^7 spermatozoa ml⁻¹ and were treated with 15 µmol Ca²⁺ ionophore A23187 l⁻¹ for 30 min at 37°C to induce acrosome reaction. The spermatozoa were washed and the pellet was resuspended at a concentration of 10^8 spermatozoa ml⁻¹ in DPBS containing protease inhibitors and 30 mmol *n*-ocyl *B*-D-glucopyranoside (OG) l⁻¹. OG extraction continued for 1 h at 4°C with low speed centrifugation at 300 g for 10 min, ultracentrifugation at 100 000 g for 1 h, followed by 0.22 µm filtration. The OG-extracted sperm proteins were stored at –80°C until used.

Immunoblotting

Phospholipase C–phosphatidylinositol-released sperm surface proteins were concentrated to 1 mg ml⁻¹ using

Centricon 10 ultrafiltration tubes, and then solubilized (0.3 mol Tris l⁻¹, 5% (w/v) SDS and 50% (v/v) glycerol, pH 6.8) and boiled for 3 min. The OG extract from acrosome-reacted spermatozoa was centrifuged at 300 *g* for 10 min and the supernatant was mixed 1:1 with solubilizing buffer, which included 5% (v/v) mercaptoethanol as a reducing agent.

Electrophoresis of the surface proteins of phospholipase C-phosphatidylinositol-treated acrosome-intact spermatozoa and the OG extract of acrosome-reacted spermatozoa was carried out using precast 10% (v/v) Tris-glycine gels (Fisher Scientific). Approximately 5 µg sperm protein was loaded onto each well. After electrophoresis, the gel was either silver stained (Bio-Rad, Hercules, CA) or electroblotted to nitrocellulose membranes and blocked for at least 2 h in TBS (50 mmol Tris-HCl l⁻¹, pH 7.5, 0.3 mol NaCl l⁻¹) containing 5% (w/v) nonfat dry milk and 0.1% (w/v) NaN₃. Blots were washed in TTBS (10 mmol Tris-HCl l⁻¹, pH 7.5, containing 150 mmol NaCl l⁻¹ and 0.1% (v/v) Tween 20) and incubated with each IgG for 2 h in TBS with 3% (w/v) BSA and 0.1% (w/v) NaN₃. The blots were washed thoroughly in TTBS and transferred subsequently to goat anti-rabbit IgG-alkaline phosphatase (Bio-Rad) at a concentration of 1:3000 in TBS with 3% (w/v) BSA and 0.1% (w/v) NaN₃. After washing in TTBS, immune complexes were detected using precipitating alkaline phosphatase substrate (one-step NBT-BCIP; Pierce).

Immunolocalization

Washed ejaculated spermatozoa were resuspended in 1 ml BWB-BSA (3 mg ml⁻¹) buffered with sodium bicarbonate (3 mg ml⁻¹). Each 1 ml of sperm preparation was layered onto a 3 ml column of 80% (v/v) Percoll in BWB-BSA (Tollner *et al.*, 2000) and centrifuged at 300 *g* for 15 min. Spermatozoa were washed twice by dilution in BWB-BSA (30 mg ml⁻¹) and centrifugation at 300 *g* for 10 min, were incubated overnight at 25°C, 5% CO₂, and then placed at 37°C, 5% CO₂ for 2 h before immunolocalization studies were performed.

Acrosome-intact spermatozoa were incubated for 30 min with 10 µg ml⁻¹ of affinity purified Fab fragments of each anti-peptide IgG. The spermatozoa were centrifuged at 300 *g* for 10 min and resuspended in 0.8% (w/v) paraformaldehyde in DPBS for 15 min. Fixed spermatozoa were washed three times in blocking solution (1% (w/v) BSA in DPBS with 0.2% (w/v) NaN₃), and were incubated subsequently for 1 h in a 1:10 (v/v) solution of goat anti-rabbit Fab IgG conjugated with FITC (E-Y Laboratories, Hercules, CA) in blocking solution. Labelled spermatozoa were washed thoroughly and suspended in a 1:1 solution of glycerol and 1% (w/v) paraformaldehyde in DPBS. Acrosome-reacted spermatozoa were obtained by treatment with ionophore A23187 as described earlier in the presence of Fab fragments of each anti-peptide IgG. The procedures for immunolabelling acrosome-reacted spermatozoa were the same as described earlier.

Photomicrographs were taken of representative spermatozoa using an Olympus BX-60 microscope fitted with a fluorescein filter with excitation at wavelength 480/30 nm and emission at wavelength 535/40 nm. Images were captured and processed using a Zeiss Axiocam digital camera and Axiovision software (Carl Zeiss Vision GmbH, Germany).

For fine structural observations, acrosome-intact and acrosome-reacted spermatozoa were treated as described earlier except that the secondary antibody was goat anti-rabbit Fab conjugated with either 15 nm or 10 nm gold particles (E-Y Laboratories). After incubation for 1 h in the secondary antibody, the spermatozoa were fixed again in 2.5% (w/v) glutaraldehyde in 0.2 mol cacodylate buffer l⁻¹ (pH 7.4). Spermatozoa were fixed for at least 1 h, washed twice in 0.2 mol cacodylate buffer l⁻¹ and then post-fixed in 1% (w/v) osmium tetroxide (Ted Pella Inc., Redding, CA). After exposure to osmium for 2 h, the spermatozoa were dehydrated through a graded alcohol series and embedded in epoxy resin. Sections were cut on a diamond knife, stained with lead citrate and viewed with a Phillips 400 transmission electron microscope.

Hyaluronic acid substrate gel assay

The hyaluronic acid substrate gel assay was performed using the method of Guntenhoner *et al.* (1992), as modified by Cherr *et al.* (1996). Concentrated phospholipase C-released sperm surface proteins or the OG extracts of acrosome-reacted whole spermatozoa were dissolved in SDS solubilization buffer without boiling. The samples were subjected to electrophoresis on 7.5% (w/v) SDS-polyacrylamide gels containing hyaluronic acid (Cherr *et al.*, 1996). After electrophoresis, gels were incubated for 2 h at room temperature in 50 mmol sodium acetate buffer l⁻¹ (at either pH 7.0, 5.0 or 4.0) containing 150 mmol NaCl l⁻¹ and 3% (v/v) Triton X-100 to remove SDS and restore *in situ* hyaluronidase activity. The gels were then incubated for 15 h at 37°C in 50 mmol sodium acetate buffer l⁻¹ (at either pH 7.0, 5.0 or 4.0) containing 150 mmol NaCl l⁻¹. Gels were subsequently stained with 0.5% (w/v) Alcian blue in 3% (v/v) acetic acid for 2 h, destained with 7% (v/v) acetic acid, and then counterstained with Coomassie blue (R250) for photography. Prestained protein standards (SeeBlue; Invitrogen Corp, Carlsbad, CA) were used to determine molecular masses.

Hyaluronidase activity inhibition assay

The assay for hyaluronidase activity was a modification of the method established by Frost and Stern (1997). In brief, after immobilization of biotinylated hyaluronic acid onto 96-well COVALINK-NH microtitre plates (Nunc, Placerville, NJ), the plates were equilibrated with extraction buffer (50 mmol Tris-HCl l⁻¹, pH 7.0, containing 0.15 mol NaCl l⁻¹ and 1% (v/v) Triton X-100). Each well was then filled with 100 µl ice-cold ultracentrifuged phospholipase C-phosphatidylinositol-released surface proteins or OG-extracted proteins (10⁶-10⁷ spermatozoa ml⁻¹) and incubated for 30-40 min at 37°C. Enzyme reaction was

terminated by the addition of 200 μl of 6 mol guanidine-HCl l^{-1} per well followed by three washes of 300 μl per well of PBS containing 2 mol NaCl l^{-1} , 50 mmol MgSO_4 l^{-1} and 0.05% (v/v) Tween 20. An ABC kit (Vector Laboratories, Burlingame, CA) was prepared in 10 ml PBS containing 0.1% (v/v) Tween 20, incubated for 30 min and then 100 μl was added to each well. After 30 min of incubation, followed by five washes with PBS washing buffer, 100 μl of 1 mg o-phenylenediamine ml^{-1} in citrate- Na_2HPO_4 buffer (pH 5.3) was added to each well. Plates were incubated for 10–15 min in the dark and read at 490 nm in an Emax precision microplate reader (Molecular Devices, Sunnyvale, CA). Hyaluronidase activity was determined relative to a four-parameter standard curve established using serial dilutions of a commercial bovine testicular hyaluronidase preparation (Wydase; Wyeth Laboratories Inc., Philadelphia, PA) at pH 7.0 in 50 mmol Tris-HCl l^{-1} containing 150 mmol NaCl l^{-1} . Enzyme activity was measured in turbidity reducing units ml^{-1} and standard curves were determined in triplicate for each assay. Fab fragments of each anti-peptide IgG were analysed for ability to inhibit the enzyme activity of phospholipase C-phosphatidylinositol-released PH-20 from the plasma membrane and of PH-20 extracted from whole spermatozoa to examine the effects of specific anti-peptide antibodies. Fab fragments (20 $\mu\text{g ml}^{-1}$) were combined 1:1 with the phospholipase C-phosphatidylinositol-released proteins or the OG extraction solution containing protease inhibitors, and incubated overnight at 4°C before addition to the microplate wells. Control assays containing the sperm extract and pre-immune IgG were carried out simultaneously.

The assay for hyaluronidase activity at pH 5.0 (50 mmol sodium acetate buffer l^{-1} containing 150 mmol NaCl l^{-1}) was a modification of the method established by Tung *et al.* (1994). In brief, the mixture of hyaluronic acid (final 0.8 mg ml^{-1}) and agarose (final 0.8% w/v) in distilled water was dispensed in 100 μl aliquots into each well of a 96-well microplate with a multichannel pipette and the microplate was put in the refrigerator to allow the gel to set. Each well was filled with 100 μl of a preincubated mixture of sample and Fab fragments or sample containing only protease inhibitors at pH 5.0. After 2 h pre-incubation at room temperature and 5 h incubation at 37°C, the enzyme samples were removed and each well was washed three times with 150 μl DPBS and filled with 100 μl 10% (w/v) aqueous cytochrome c. After 1 h at room temperature, the absorbance at 570 nm was measured by a microplate reader (MR600; Dynatech Laboratories Inc., Alexandria, VA).

Results

Immunoblotting of sperm proteins with anti-recombinant PH-20 IgG and anti-peptide IgGs

After electrophoresis of the surface proteins released by phospholipase C-phosphatidylinositol treatment of acrosome-intact spermatozoa, silver staining of the gel

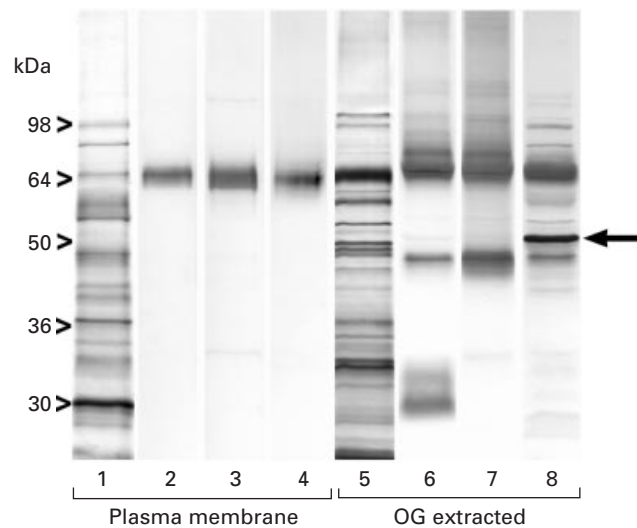


Fig. 1. Western blot analysis of phospholipase C-phosphatidylinositol-released macaque sperm surface proteins (plasma membrane) and *n*-ocyl *B*-D-glucopyranoside (OG)-extracted, whole acrosome-reacted spermatozoa. Lanes 1–4 show analysis of the phospholipase C-phosphatidylinositol-released sperm proteins. Lane 1 is a silver stained gel of the phospholipase C-phosphatidylinositol-released surface proteins. Lane 2 was probed with IgG from a polyclonal antibody to the recombinant PH-20 (r-PH-20) protein. Lanes 3 and 4 were probed with specific IgGs to the synthesized peptide 1 and peptide 3, respectively. A single 64 kDa band is recognized by all three antibodies. Lanes 5–8 show analysis of OG-extracted whole acrosome-reacted spermatozoa. The sperm proteins were reduced during solubilization. Lane 5 is a silver stained gel of the proteins that were transferred to nitrocellulose membranes in lanes 6–8. Lane 6 was probed with anti-r-PH-20 IgG, whereas lanes 7 and 8 were probed with anti-peptide 1 IgG and anti-peptide 3 IgG, respectively. After the acrosome reaction, the anti-r-PH-20 IgG labels 44 kDa and 30 kDa fragments of proteolysed PH-20 (Lane 6). The 53 kDa form of PH-20 is also recognized faintly by the anti-r-PH-20. The anti-peptide IgGs label the 44 kDa fragment clearly, but not the 30 kDa fragment that represents the carboxyl region of PH-20 (lanes 7 and 8). Anti-peptide 3 IgG labels the 53 kDa form of PH-20 (lane 8, arrow). Both of the anti-peptide IgGs also labelled the nonproteolyzed 64 kDa PH-20 protein.

revealed a number of bands (Fig. 1, lane 1). When these proteins were blotted onto nitrocellulose and probed with anti-recombinant PH-20 IgG and the anti-peptide IgGs, a single 64 kDa band was recognized by all three antibodies (Fig. 1, lanes 2–4). Electrophoresis of the OG extract of acrosome-reacted spermatozoa under reducing conditions also revealed many proteins after silver staining (Fig. 1, lane 5). On nitrocellulose membranes, the anti-recombinant PH-20 IgG recognized a 67 kDa band, as well as 44 kDa and 30 kDa bands (Fig. 1, lane 6). In contrast, the anti-peptide IgGs recognized the 67 kDa band and the 44 kDa band, but not the 30 kDa band (Fig. 1, lanes 7 and 8). The lower molecular mass forms have been attributed to endoproteolytic cleavage of the PH-20 protein into two fragments which are disulphide linked (Primakoff *et al.*, 1988). The

higher molecular mass fragment (44 kDa) is the N-terminal fragment. A 53 kDa band was recognized in the extract of acrosome-reacted spermatozoa by the anti-recombinant PH-20 IgG and the anti-peptide 3 IgG (Fig. 1, lanes 6 and 8). The anti-peptide 3 IgG had much greater affinity for this component than did the IgG to whole recombinant protein, which recognized the band only faintly. The anti-peptide 1 IgG did not label the 53 kDa band (Fig. 1, lane 7). The 53 kDa form of PH-20 is a significant component of the soluble hyaluronidase released from spermatozoa at the time of the acrosome reaction (Li *et al.*, 1997).

Immunolabelling of spermatozoa with anti-peptide IgGs

Anti-recombinant PH-20 IgG and Fab fragments of this IgG are effective in labelling acrosome-intact spermatozoa, regardless of whether spermatozoa were fixed before labelling (Overstreet *et al.*, 1995; Cherr *et al.*, 1996). In the present study, Fab fragments of anti-peptide IgGs were used for immunolocalization because these fragments resulted in more intense labelling of the sperm plasma membrane than did the whole IgGs (data not shown). In using Fab fragments rather than IgG for immunolocalization, any aggregation of surface PH-20 that is induced by incubation of spermatozoa with anti-PH-20 IgG was also avoided (Yudin *et al.*, 1998).

Fab fragments from anti-recombinant PH-20 IgG and the anti-peptide 1 IgG both labelled the entire head of acrosome-intact spermatozoa but not the sperm mid-piece or flagellum (Fig. 2a–d). Fab fragments of anti-peptide 3 IgG did not appear to label the posterior head of acrosome-intact spermatozoa and there was very faint labelling of the sperm mid-piece (Fig. 2e,f). When spermatozoa were induced to undergo the acrosome reaction in the presence of the Fab fragments from all three IgGs, the label was located over the acrosomal cap in the region of the acrosomal shroud (Fig. 3a–f). All acrosome-intact and acrosome-reacted spermatozoa appeared to have the labelling patterns shown (Figs 2 and 3, respectively).

At the ultrastructural level, Fab fragments of the anti-peptide IgGs labelled the plasma membrane over the head of the acrosome-intact spermatozoa, and there was no labelling of the sperm mid-piece or flagellum. The results with Fab fragments of anti-peptide 1 IgG demonstrate the pattern of labelling (Fig. 4a), and labelling with Fab fragments of anti-peptide 3 IgG gave similar results (not shown). The labelling pattern of acrosome-reacted spermatozoa is illustrated with Fab fragments of anti-peptide 3 IgG (Fig. 4b). Labelling with Fab fragments of anti-peptide 1 IgG gave similar results (not shown). When spermatozoa began to undergo the acrosome reaction in the presence of Fab fragments of the anti-peptide IgGs, there was immediate labelling of the externalized acrosomal matrix (Fig. 4b). The entire acrosomal shroud, including the acrosomal vesicles, composed of fused plasma membrane and outer acrosomal membrane, was labelled with immunogold particles (Fig. 4b). Recognition of PH-20 by Fab fragments of the anti-peptide IgGs appeared to be more

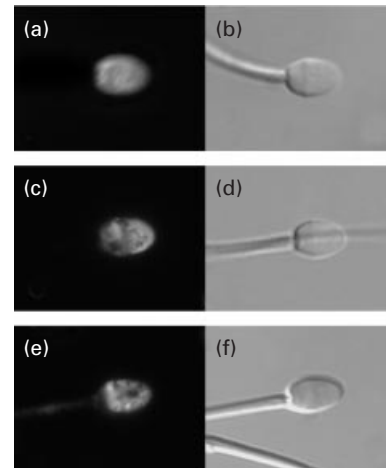


Fig. 2. Macaque spermatozoa exposed to Fab fragments of anti-PH-20 IgG before and after the acrosome reaction. (a) Localization of anti-recombinant-PH-20 Fab over the entire head of the acrosome-intact spermatozoa and (b) the corresponding phase-contrast micrograph. Localization of (c) anti-peptide 1 Fab and (e) anti-peptide 3 Fab, with the corresponding phase-contrast micrographs (d and f, respectively).

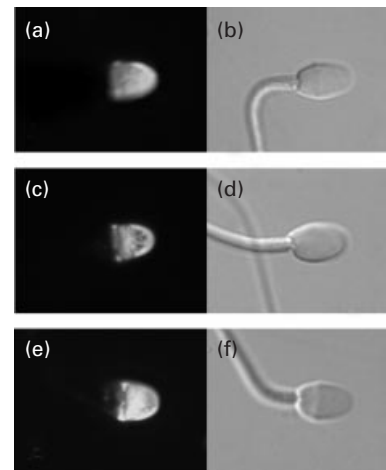


Fig. 3. Acrosome-reacted macaque spermatozoa. Immunolocalization of the Fab fragments of (a) anti-recombinant-PH-20 IgG, (c) anti-peptide 1 IgG and (e) anti-peptide 3 IgG and corresponding phase-contrast micrographs (b, d and f, respectively).

intense after the acrosome reaction, and the Fab fragments of both anti-peptide IgGs labelled the inner acrosomal membrane of acrosome-reacted spermatozoa (Fig. 4b).

Hyaluronic acid substrate gel analysis of PH-20

Hyaluronic acid substrate gel analysis of plasma membrane (phospholipase C–phosphatidylinositol-released) PH-20 revealed a 64 kDa form with highest enzyme activity at pH 7.0 (Fig. 5). A similar analysis of the (OG-extracted)

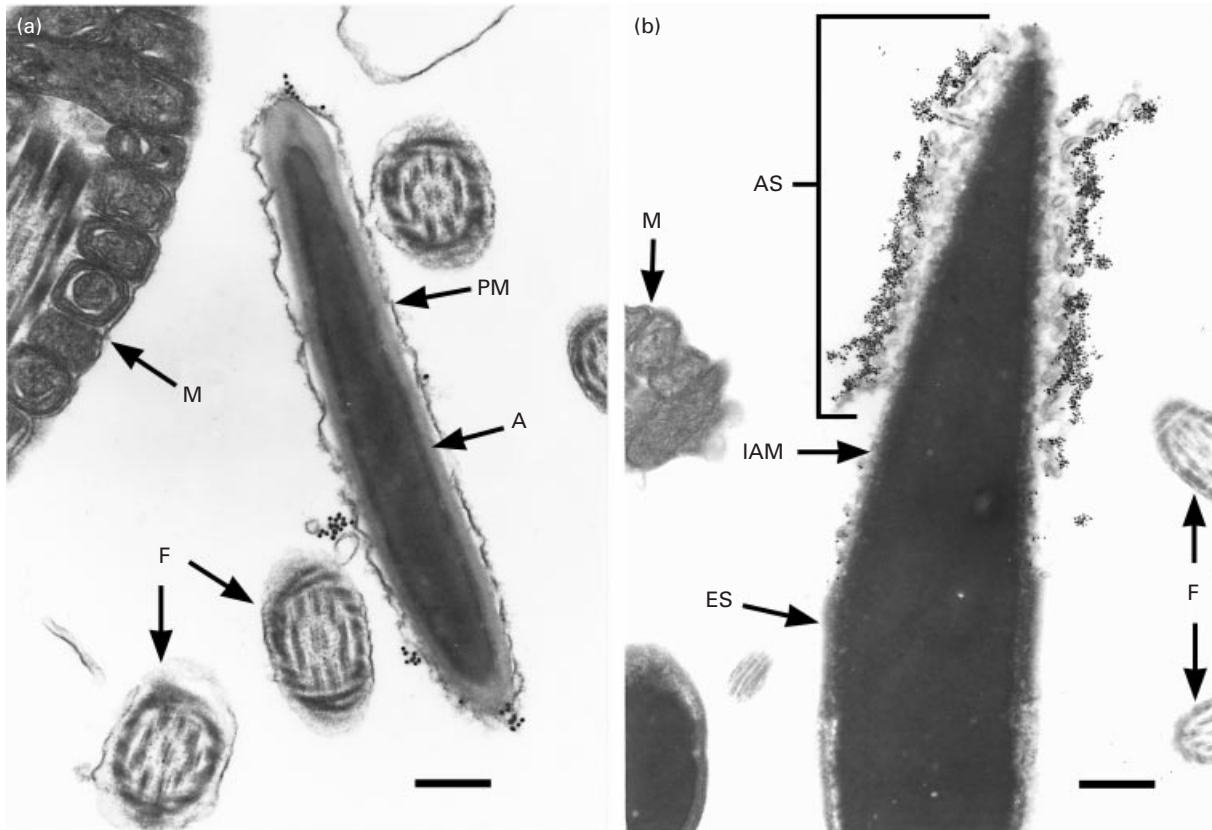
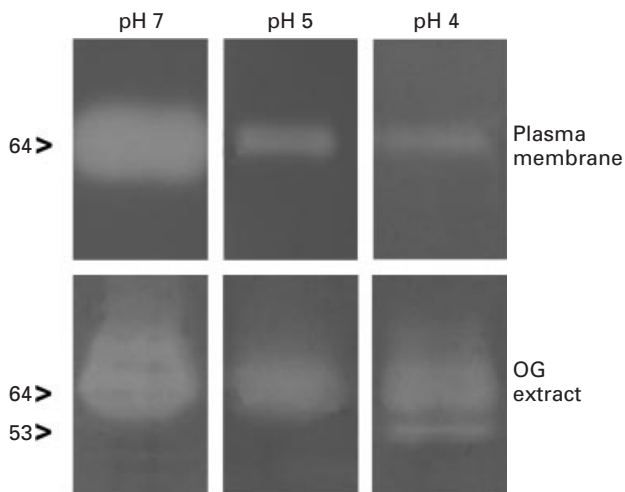


Fig. 4. (a) Immunolocalization of PH-20 on a macaque spermatozoon, using Fab fragments of anti-peptide 1 IgG. The acrosome (A) is intact and the anti-peptide 1 Fab fragments recognize a component of the plasma membrane (PM) over the entire head of the spermatozoon, whereas no gold label is found along the mid-piece (M) or flagellum (F). Scale bar represents 0.3 μm . (b) Immunolocalization of PH-20 on an acrosome-reacted macaque spermatozoon using Fab fragments of anti-peptide 3 IgG. The anti-peptide 3 Fab fragments recognize components of the entire acrosomal shroud (AS) including the acrosomal matrix and the inner acrosomal membrane (IAM). There is some label along the equatorial segment (ES) but the antibody does not recognize the plasma membrane over the mid-piece (M) and flagellum (F). Scale bar represents 0.28 μm .

endoproteolysed PH-20 from acrosome-reacted spermatozoa also revealed the 64 kDa form, as well as a 53 kDa form that was active at pH 4.0 but not at pH 5.0 or pH 7.0 (Fig. 5).



Inhibition of hyaluronidase activity by anti-peptide IgGs

Of the two anti-peptide IgGs, only Fab fragments of anti-peptide 1 IgG significantly inhibited sperm hyaluronidase activity at pH 7.0, and this inhibition was equally effective on plasma membrane PH-20 (phospholipase C-phosphatidylinositol-released) and PH-20 extracted from whole acrosome-reacted spermatozoa (OG extracted)

Fig. 5. Hyaluronic acid substrate SDS-PAGE (7.5% (w/v) gel) of plasma membrane proteins (phospholipase C-phosphatidylinositol-released) from acrosome-intact spermatozoa and *n*-ocyl *B*-D-glucopyranoside (OG) extracted proteins from whole acrosome-reacted spermatozoa. The experiment was carried out at pH 4.0, 5.0 and 7.0. The plasma membrane proteins had the highest hyaluronidase activity at pH 7.0 and had only slight activity at pH 4.0. All of the hyaluronidase activity from the plasma membrane proteins was associated with the 64 kDa form of PH-20. The OG extract had the highest hyaluronidase activity at pH 7.0 but also had significant activity at the lower pH values. The 53 kDa form of PH-20 had hyaluronidase activity at pH 4.0 only.

Table 1. Effects of anti-PH-20 antibodies at acid (pH 5.0) and neutral (pH 7.0) pH on the hyaluronidase activity of proteins released from the macaque sperm plasma membrane or of proteins from an extract of acrosome-reacted spermatozoa

Extract	Expt	pH 5.0				pH 7.0			
		Control TRU ml ⁻¹	Peptide 1 Fab (% Inhibition)	Peptide 3 Fab (% Inhibition)	r-PH-20 Fab (% Inhibition)	Control TRU ml ⁻¹	Peptide 1 Fab (% Inhibition)	Peptide 3 Fab (% Inhibition)	r-PH-20 Fab (% Inhibition)
PLC-PI (surface)	1	4.4	3.8 (14%)	3.8 (14%)	3.5 (20%)	13.7	9.3 (32%)	13.7 (0%)	5.2 (62%)
	2	5.2	4.7 (10%)	4.4 (15%)	3.7 (29%)	15.2	11.3 (26%)	14.4 (5%)	7.3 (52%)
	3	4.8	4.2 (13%)	4.0 (17%)	3.8 (21%)	10.9	7.0 (36%)	10.3 (6%)	3.6 (67%)
OG	1	2.9	2.2 (24%)	1.9 (34%)	1.5 (48%)	16.4	10.7 (35%)	15.8 (4%)	6.6 (60%)
	2	5.9	4.3 (27%)	3.6 (39%)	4.3 (27%)	11.3	8.3 (27%)	10.2 (10%)	5.9 (48%)
	3	8.1	5.9 (27%)	5.6 (31%)	5.8 (28%)	19.8	14.3 (28%)	18.5 (7%)	7.7 (61%)

Spermatozoa were treated with phospholipase C-phosphatidylinositol (PLC-PI) to extract sperm surface protein or *n*-acyl *B*-D-glucopyranoside (OG) to extract proteins from whole acrosome-reacted spermatozoa. Fab fragments of IgG from anti-recombinant-PH-20 (r-PH-20), anti-peptide 1 and anti-peptide 3 antibodies were tested. The assays for hyaluronidase activity were performed at a single concentration (20 µg ml⁻¹) of the Fab fragments. Each assay was carried out in triplicate.

TRU: enzyme activity was measured in turbidity reducing units (TRU) ml⁻¹.

% Inhibition: percentage inhibition of hyaluronidase activity compared with control values.

(Table 1). Fab fragments of anti-recombinant PH-20 IgG were more effective at inhibiting sperm hyaluronidase activity than were the Fab fragments of either anti-peptide IgG (Table 1). At pH 5.0, the two anti-peptide IgGs had similar effectiveness in inhibiting sperm hyaluronidase activity (Table 1). The inhibition appeared to be more effective on PH-20 extracted from acrosome-reacted spermatozoa than on plasma membrane PH-20 (Table 1).

Discussion

The hyaluronidases appear to have common structural features that are critically important for enzyme activity and are conserved in many species (Csoka *et al.*, 1999). In fact, the PH-20 sperm protein was recognized as a hyaluronidase because of its high degree of amino acid sequence homology with bee venom hyaluronidase (Gmachl and Kreil, 1993).

Arming *et al.* (1997) constructed a variety of recombinant human PH-20 proteins that were mutated at sites with highly conserved acidic and basic amino acids. All of the mutated proteins were shown to be deficient in hyaluronidase activity at neutral pH. In the present study, peptides 1 and 3 represent two regions of the PH-20 molecule, which include four of the five amino acid mutation sites at which mutation caused a loss of hyaluronidase activity (Arming *et al.*, 1997). Chan *et al.* (1999) found that the antibodies to PH-20, which caused infertility in female guinea-pigs, had highest titres to a region of the PH-20 molecule that is the same as the peptide 1 region in the present study.

The data obtained in the present study indicate that the peptide 1 region is essential for enzyme activity of PH-20 at neutral pH, and it is likely that it may constitute the acid/base region of the enzyme. The peptide 1 region is characterized by an abundance of aromatic amino acids flanking the catalytic residue, glutamic acid, which is a common feature of other glycosyl hydrolases (Van Roey *et al.*, 1994). Another common feature of these enzymes is the presence of basic amino acids or an organized region of basic amino acids that are used for substrate hyaluronic acid binding or docking (Withers and Aebersold, 1995). Amino acids 150–158 within peptide 1 constitute a B (×7) B region, which is commonly found in hyaluronic acid binding proteins (Yang *et al.*, 1993).

It has been reported that a natural mutation at amino acid 268 of Hyal 1, the human serum hyaluronidase, resulted in a lack of enzyme activity at acid pH in the affected individual (Triggs-Raine *et al.*, 1999). In the PH-20 molecule this site corresponds to one of the amino acids mutated by Arming *et al.* (1997), and falls within the region represented by peptide 3 in the present study. The antibody to the peptide 3 region did not affect the enzyme activity of plasma membrane PH-20 at neutral pH; however, there was evidence of inhibition of sperm hyaluronidase activity by anti-peptide 3 antibody at pH 5.0, particularly when PH-20 was extracted from acrosome-reacted spermatozoa. These data indicate that the peptide 3 region may have a role in acid-active hyaluronidase activity, a finding that is consistent with our previous reports that acid-active hyaluronidase activity is present primarily in spermatozoa that have undergone the acrosome reaction (Cherr *et al.*, 1996).

Glycosyl hydrolases commonly have two regions that appear to be involved in the catalytic activity of the enzyme (Braithwaite *et al.*, 1997; Zverlov *et al.*, 1998). The sites are centred around either glutamic acid or aspartic acid, and these amino acids act either as an acid catalyst site or as a nucleophilic site (Withers and Aebersold, 1995). It is thought that changes at either site will have a marked effect on the kinetics and the optimum pH of the enzyme reaction (Braithwaite *et al.*, 1997; Mark *et al.*, 1998). The information available on other enzymes supports the interpretation that the peptide 3 region of PH-20 could modulate the kinetics of hyaluronidase activity over the broad pH range.

The immunolocalization studies performed in the present study demonstrate that both the peptide 1 and the peptide 3 regions of PH-20 are exposed on the sperm surface, as well as in the soluble forms of the enzyme and in the forms that are bound to the inner acrosomal membrane after the acrosome reaction. The 64 kDa surface PH-20 has its highest hyaluronidase activity at neutral pH (Li *et al.*, 1997), and facilitates sperm migration through the cumulus by depolymerizing the cumulus matrix (Meyers *et al.*, 1997). The PH-20 on the inner acrosomal membrane appears to be comprised of both acid active and neutral active enzymes (Li *et al.*, 1997), and it appears to have a function in binding acrosome-reacted spermatozoa to the zona pellucida (Yudin *et al.*, 1999).

Soluble PH-20 is released by macaque spermatozoa during the acrosome reaction (Cherr *et al.*, 1996) and is composed of a 64 kDa form and a 53 kDa form; the latter is thought to be derived from the 64 kDa form on the inner acrosomal membrane (Li *et al.*, 1997). As the 53 kDa form is active only at acid pH, its biological function is not clear (Li *et al.*, 1997). It is possible that the acid active 53 kDa form could function briefly to depolymerize the cumulus matrix in the immediate vicinity of the sperm flagellum as penetration of the zona pellucida begins (Cherr *et al.*, 1996).

In the present study, it was not possible to investigate the effect of anti-peptide antibodies on the acid active 53 kDa hyaluronidase because the 53 kDa form of PH-20 is not active above pH 4.0, and the antibodies used did not bind to PH-20 proteins at pH 4.0 (data not shown). Nevertheless, a functional role for the peptide 3 site on the 53 kDa form of PH-20 is indicated by the binding of the anti-peptide 3 IgG to the 53 kDa form of PH-20 on western blots. Although the antibodies to peptide 1 did not recognize the 53 kDa form of PH-20 on western blots, this result could be due to the potential refolding of the molecule, the relatively smaller amount of this protein or a lower affinity of the anti-peptide 1 antibody for the protein.

The 53 kDa soluble form of PH-20 appears to share some characteristics with the mammalian hyaluronidases, termed Hyal 1, 2 and 3, which are also reported to have a very strict acid (< 4.5) pH requirement (Frost *et al.*, 1997; Strobl *et al.*, 1998; Flannery *et al.*, 1998). Hyal 1, 2 and 3 may promote angiogenesis, and the function of these hyaluronidases in tumour invasion and metastasis is the subject of intense investigation (Csoka *et al.*, 1997; Lepperdinger *et al.*, 1998;

Lokeshwar *et al.*, 1999; Madan *et al.*, 1999; Maingonnat *et al.*, 1999; Victor *et al.*, 1999). Progress made in understanding the actions of hyaluronidase in sperm cells may provide new insights into the mechanisms of malignant changes in other cell types.

In conclusion, the region of PH-20 represented by peptide 1 meets the criteria proposed as characteristic of other glycosidases, and antibodies to this region were shown to reduce the hyaluronidase activity of PH-20 significantly. It is concluded that the region of the PH-20 molecule encompassed by the amino acid sequence 142–172 is essential for neutral-active hyaluronidase activity and it is likely that this is the acid/base catalytic region of the enzyme. The region of the PH-20 molecule designated as peptide 3 may be critical for regulating hyaluronidase activity at lower pH and could be the nucleophilic site responsible for the enzyme kinetics.

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References

- Arming S, Strobl B, Wechselberger C and Kreil G (1997) *In vitro* mutagenesis of PH-20 hyaluronidase from human sperm *European Journal of Biochemistry* **247** 810–814
- Austin CR (1960) Capacitation and the release of hyaluronidase from the spermatozoa *Journal of Reproduction and Fertility* **3** 310–311
- Braithwaite KL, Barna T, Spurway TD *et al.* (1997) Evidence that galactanase A from *Pseudomonas fluorescens* subspecies *cellulosa* is a retaining family 53 glycosyl hydrolase in which E161 and E270 are the catalytic residues *Biochemistry* **36** 15 489–15 500
- Chain E and Duthie ES (1939) A mucolytic enzyme in testes extract *Nature* **144** 977–978
- Chan CP, Gupta S and Mark GE (1999) Identification of linear surface epitopes on the guinea pig sperm membrane protein PH-20 *Life Sciences* **64** 1989–2000
- Cherr GN, Meyers SA, Yudin AI, Vandervoort CA, Myles DG, Primakoff P and Overstreet JW (1996) The PH-20 protein in cynomolgus macaque spermatozoa: identification of two different forms exhibiting hyaluronidase activity *Developmental Biology* **175** 142–153
- Csoka AB, Scherer SW and Stern R (1999) Expression analysis of six paralogous human hyaluronidase genes clustered on chromosomes 3p21 and 7q31 *Genomics* **60** 356–361
- Csoka TB, Frost GI, Wong T and Stern R (1997) Purification and microsequencing of hyaluronidase isozymes from human urine *FEBS Letters* **417** 307–310
- Davies G and Henrissat B (1995) Structures and mechanisms of glycosyl hydrolases *Structure* **3** 853–859
- Flannery CR, Little CB, Hughes CE and Caterson B (1998) Expression and activity of articular cartilage hyaluronidases *Biochemical and Biophysical Research Communications* **251** 824–829
- Frost GI and Stern R (1997) A microtiter-based assay for hyaluronidase activity not requiring specialized reagents *Analytical Biochemistry* **251** 263–269
- Frost GI, Csoka TB, Wong T and Stern R (1997) Purification, cloning, and expression of human plasma hyaluronidase *Biochemical and Biophysical Research Communications* **236** 10–15
- Gmachi M and Kreil G (1993) Bee venom hyaluronidase is homologous to a membrane protein of mammalian sperm *Proceedings National Academy of Sciences USA* **90** 3569–3573
- Guntenhoner MW, Pogrel MA and Stern R (1992) A substrate-gel assay for hyaluronidase activity *Matrix* **12** 388–396

- Henrissat B and Bairoch A** (1993) New families in the classification of glycosyl hydrolases based on amino acid sequence similarities *Biochemical Journal* **293** 781–788
- Hou ST, Ma A, Jones R and Hall L** (1996) Molecular cloning and characterization of rat sperm surface antigen 2B1, a glycoprotein implicated in sperm–zona binding *Molecular Reproduction and Development* **45** 193–203
- Hunnicutt GR, Mahan K, Lathrop WF, Ramarao CS, Myles DG and Primakoff P** (1996) Structural relationship of sperm soluble hyaluronidase to the sperm membrane protein PH-20 *Biology of Reproduction* **54** 1343–1349
- Kreil G** (1995) Hyaluronidases – a group of neglected enzymes *Protein Science* **4** 1666–1669
- Lepperdinger G, Strobl B and Kreil G** (1998) HYAL2, a human gene expressed in many cells, encodes a lysosomal hyaluronidase with a novel type of specificity *Journal of Biological Chemistry* **273** 22 466–22 470
- Li MW, Cherr GN, Yudin AI and Overstreet JW** (1997) Biochemical characterization of the PH-20 protein on the plasma membrane and inner acrosomal membrane of cynomolgus macaque spermatozoa *Molecular Reproduction and Development* **48** 356–366
- Lin Y, Kimmel LH, Myles DG and Primakoff P** (1993) Molecular cloning of the human and monkey sperm surface protein PH-20 *Proceedings National Academy of Sciences USA* **90** 10 071–10 075
- Lin Y, Mahan K, Lathrop WF, Myles DG and Primakoff P** (1994) A hyaluronidase activity of the sperm plasma membrane protein PH-20 enables sperm to penetrate the cumulus cell layer surrounding the egg *Journal of Cell Biology* **125** 1157–1163
- Lokeshwar VB, Young MJ, Goudarzi G, Iida N, Yudin AI, Cherr GN and Selzer MG** (1999) Identification of bladder tumor-derived hyaluronidase: its similarity to HYAL1 *Cancer Research* **59** 4464–4470
- Madan AK, Yu K, Dhurandhar N, Cullinane C, Pang Y and Beech DJ** (1999) Association of hyaluronidase and breast adenocarcinoma invasiveness *Oncology Reports* **6** 607–609
- Maingonnat C, Victor R, Bertrand P, Courel MN, Maunoury R and Delpech B** (1999) Activation and inhibition of human cancer cell hyaluronidase by proteins *Analytical Biochemistry* **268** 30–34
- Mark BL, Wasney GA, Salo TJ, Khan AR, Cao Z, Robbins PW, James MN and Triggs-Raine BL** (1998) Structural and functional characterization of *Streptomyces plicatus* β -N-acetylhexosaminidase by comparative molecular modeling and site-directed mutagenesis *Journal of Biological Chemistry* **273** 19 618–19 624
- Meyers SA, Yudin AI, Cherr GN, Vandevoort CA, Myles DG, Primakoff P and Overstreet JW** (1997) Hyaluronidase activity of macaque sperm assessed by an *in vitro* cumulus penetration assay *Molecular Reproduction and Development* **46** 392–400
- Myles DG and Primakoff P** (1997) Why did the sperm cross the cumulus? To get to the oocyte. Functions of the sperm surface proteins PH-20 and fertilin in arriving at, and fusing with, the egg *Biology of Reproduction* **56** 320–327
- Overstreet JW, Lin Y, Yudin AI, Meyers SA, Primakoff P, Myles DG, Katz DF and Vandevoort CA** (1995) Location of the PH-20 protein on acrosome-intact and acrosome-reacted spermatozoa of cynomolgus macaques *Biology of Reproduction* **52** 105–114
- Primakoff P, Cowan A, Hyatt H, Tredick-Kline J and Myles DG** (1988) Purification of the guinea pig sperm PH-20 antigen and detection of a site-specific endoproteolytic activity in sperm preparations that cleaves PH-20 into two disulfide-linked fragments *Biology of Reproduction* **38** 921–934
- Sabeur K, Cherr GN, Yudin AI, Primakoff P, Li MW and Overstreet JW** (1997) The PH-20 protein in human spermatozoa *Journal of Andrology* **18** 151–158
- Sarason RL, Vandevoort CA, Mader DR and Overstreet JW** (1991) The use of nonmetal electrodes in electroejaculation of restrained but unanesthetized macaques *Journal of Medical Primatology* **20** 122–125
- Strobl B, Wechselberger C, Beier DR and Lepperdinger G** (1998) Structural organization and chromosomal localization of Hyal2, a gene encoding a lysosomal hyaluronidase *Genomics* **53** 214–219
- Tollner TL, Yudin AI, Cherr GN and Overstreet JW** (2000) Soybean trypsin inhibitor as a probe for the acrosome reaction in motile cynomolgus macaque sperm *Zygote* **8** 127–137
- Toole BP** (1997) Hyaluronan in morphogenesis *Journal of Internal Medicine* **242** 35–40
- Triggs-Raine B, Salo TJ, Zhang H, Wicklow BA and Natowicz MR** (1999) Mutations in HYAL1, a member of a tandemly distributed multigene family encoding disparate hyaluronidase activities, cause a newly described lysosomal disorder, mucopolysaccharidosis IX *Proceedings National Academy of Sciences USA* **96** 6296–6300
- Tung JS, Mark GE and Hollis GF** (1994) A microplate assay for hyaluronidase and hyaluronidase inhibitors *Analytical Biochemistry* **223** 149–152
- Van Roey P, Rao V, Plummer TH, Jr and Tarentino AL** (1994) Crystal structure of endo- β -N-acetylglucosaminidase F1, an α / β -barrel enzyme adapted for a complex substrate *Biochemistry* **33** 13 989–13 996
- Victor R, Chauzy C, Girard N, Gioanni J, d'Anjou J, Stora DN and Delpech B** (1999) Human breast-cancer metastasis formation in a nude-mouse model: studies of hyaluronidase, hyaluronan and hyaluronan-binding sites in metastatic cells *International Journal of Cancer* **82** 77–83
- West DC and Kumar S** (1989) The effect of hyaluronate and its oligosaccharides on endothelial cell proliferation and monolayer integrity *Experimental Cell Research* **183** 179–196
- Withers SG and Aebersold R** (1995) Approaches to labeling and identification of active site residues in glycosidases *Protein Science* **4** 361–372
- Yang B, Zhang L and Turley EA** (1993) Identification of two hyaluronan-binding domains in the hyaluronan receptor RHAMM *Journal of Biological Chemistry* **268** 8617–8623
- Yudin AI, Cherr GN, Vandevoort CA and Overstreet JW** (1998) Rearrangement of the PH-20 protein on the surface of macaque spermatozoa following exposure to anti-PH-20 antibodies or binding to zona pellucida *Molecular Reproduction and Development* **50** 207–220
- Yudin AI, Vandevoort CA, Li MW and Overstreet JW** (1999) PH-20 but not acrosin is involved in sperm penetration of the macaque zona pellucida *Molecular Reproduction and Development* **53** 350–362
- Zverlov V, Mahr S, Riedel K and Bronnenmeier K** (1998) Properties and gene structure of a bifunctional cellulolytic enzyme (CelA) from the extreme thermophile '*Anaerocellum thermophilum*' with separate glycosyl hydrolase family 9 and 48 catalytic domains *Microbiology* **144** 457–465

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