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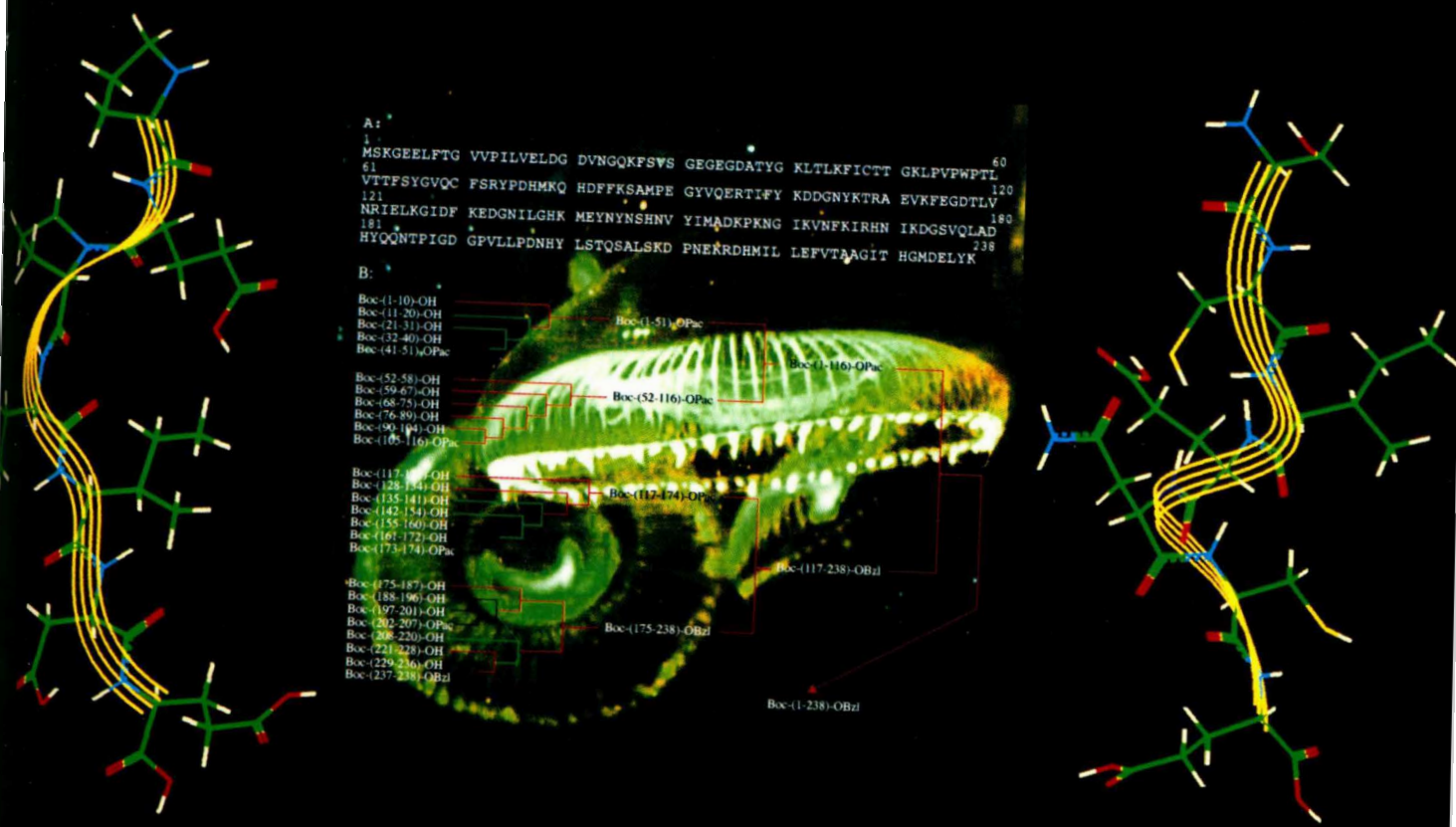
An official publication of the American Peptide Society

"Chemical Synthesis of Proteins"

Issue Editor: Shumpei Sakakibara



Editor: Charles M. Deber

Volume 51 / Number 4 / 1999

BIOPOLYMERS

ORIGINAL RESEARCH ON BIOMOLECULES

Cover Illustration: Photograph of jellyfish *Aequoria* with computer-rendered green body highlighting the scheme of total chemical synthesis of green fluorescent protein. See Sakakibara, pp. 279–29 Ribbons: Energy-minimized conformations of the sequences Pro-Glu-Pro-Thr-Ile-Asp-Glu (left) and Ser-Cys-Ile-Glu-Asn-Cys-Glu (right). The single-letter abbreviations of the residues in the tw chains are PEPTIDE SCIENCE.

Ethan Pavlo, *Editorial Production, John Wiley*

Biopolymers (Print ISSN: 0006-3525; online: ISSN 1097-0282 at Wiley Interscience, www.interscience.wiley.com) Volumes 49–52 are published 24 times, monthly except semi-monthly in April, May, 1999 and three times in January, March, July, September, and November 1999, by John Wiley & Sons, Inc., 605 Third Ave., New York, NY 10158.

 Periodicals postage paid at New York, NY, and at additional mailing offices. Biopolymers (Peptide Science) (Vol. 51, numbers 1–6) and Biopolymers (Nucleic Acids) (Vol. 52, numbers 1–4) will be sent via standard mail.

Subscription price: (Volumes 53–57, 2000): $5725.00 in US, $6025.00 in Canada or Mexico, $6280.00 outside North America. Personal rate (available only if there is an institutional subscription): $825.00 in US and Canada, $1005.00 outside North America. All subscriptions outside US will be sent by air. Subscriptions at the personal rate are available only to individuals. Payment must be made in US dollars drawn on a US bank. Claims for undelivered copies will be accepted only after the following issue has been received. Please enclose a copy of the mailing label. Missing copies will be supplied when losses have been sustained in transit and where reserve stock permits. Please allow four weeks for processing a change of addres For subscription inquiries, please call 212-850-6645 or e-mail: SUBINFO wiley.com.

Postmaster: Send address changes to *Biopolymers,* Caroline Rothaug, Director, Subscriptio Fulfillment and Distribution, Subscription Department, John Wiley & Sons, Inc., 605 Thi Avenue, New York, N.Y. 10158.

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Manuscripts for Biopolymers and *Nucleic Acid Sciences* should be submitted to the Editor, Mur Goodman, Department of Chemistry and Biochemistry, University of California, San Diego, 62 Pacific Hall, La Jolla, CA 92093. *Manuscripts for Peptide Science* should be submitted to the Edit Charles M. Deber, University of Toronto, c/o Research Institute–Structural Biology & Biochemis Research, Hospital for Sick Children, 555 University Avenue, Toronto, Ontario M5G 1X8 Cana *Information for contributors* appears in the first and last issue of each volume.

The contents of this journal are indexed in the following: *Applied Mechanics Reviews, Biol ical Abstracts, BIOSIS, Chemical Abstracts, Chemical Titles, Current Contents/Life Sciences, E neering Index, EMBASE/Excerpta Medica, Index Medicus, MEDLINE, Reference Update, Reseu Alert (ISI), Science Citation Index (ISI), and SCISEARCH Database (ISI).*

This paper meets the requirements of ANSI/NISO Z39.48-1992 (Permanence of Paper).

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PEPTIDE SCIENCE

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BIOPOLYMERS

ORIGINAL RESEARCH ON BIOMOLECULES

Publication Schedule for 1999:

Biopolymers is published according to a volume-numbering scheme and schedule that includes all sections of the journal. Full subscription: Volumes 49–52, 24 issues.

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Volume 51, Number 4 was mailed the week of December 6, 1999

Aims and Scope

Biopolymers publishes original research papers in the field of biopolymers (proteins, nucleic acids, and polysaccharides), low molecular weight molecules relevant to the study of biopolymers (including model systems, monomers, oligomers, and molecules which interact with biopolymers), and molecular dynamics and bioassemblies.

The Editors are also eager to publish Rapid Communications, which should be brief and of sufficient interest or urgency to warrant rapid publication. Such publication is not a bar to publication of a fuller account of the work at a later date.

Peptide Science and *Nucleic Acid Sciences* are also published under the aegis of *Biopolymers*. They are designed to provide a forum for large complex research projects, and to publish articles that correlate research results between the subdisciplines of the peptide sciences. Most issues of *Peptide Science* and *Nucleic Acid Sciences* will be devoted to unified themes covering the most timely subjects in their research areas.

Using the aims and scope described above, the Editors of *Biopolymers* and the Publisher strive to bring the broadest coverage of our disciplines to the scientific community. We look forward to continuing this worthy endeavor.

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Refolding of Therapeutic Proteins Produced in *Escherichia coli* as Inclusion Bodies

Abstract: *Overexpression of cloned or synthetic genes in* Escherichia coli *often results in the formation of insoluble protein inclusion bodies. Within the last decade, specific methods and strategies have been developed for preparing active recombinant proteins from these inclusion bodies. Usually, the inclusion bodies can be separated easily from other cell components by centrifugation, solubilized by denaturants such as guanidine hydrochloride (Gdn-HCl) or urea, and then renatured through a refolding process such as dilution or dialysis. Recent improvements in renaturation procedures have included the inhibition of aggregation during refolding by application of low molecular weight additives and matrix-bound renaturation. These methods have made it possible to obtain high yields of biologically active proteins by taking into account process parameters such as protein concentration, redox conditions, temperature, pH, and ionic strength.* © 1999 John Wiley & Sons, Inc. Biopoly 51: 297–307, 1999

Keywords: *refolding; inclusion body; renaturation; recombinant protein; high-level expression; therapeutic protein*

INTRODUCTION

Major advances in genetic engineering have resulted in the development of bacterial expression systems, particularly those in *Escherichia coli,* capable of producing large amounts of proteins from cloned genes.[1,2] The supply of many valuable proteins that have potential clinical or industrial use, such as hormones, cytokines, and enzymes, is often limited by their low natural availability. Initially, this approach employing *E. coli* seemed to guarantee an unlimited supply of recombinant proteins. For example, recombinant DNA technology has facilitated the efficient production of therapeutic-grade proteins such as insulin,[3] growth hormone (GH),[4] and interferon (IFN).[5] However, high-level expression of recombinant proteins in *E. coli* often results in the formation of insoluble and inactive aggregates known as inclusion bodies.[6,7] To obtain biologically active recombinant proteins from inclusion bodies, it is necessary to develop

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Biopolymers (Peptide Science), Vol. 51, 297–307 (1999)

CCC 0006-3525/99/040297-11

a simple and efficient procedure for renaturation of these proteins.[8,9]

The formation of inclusion bodies offers several advantages for the production of recombinant proteins. These proteins may be unstable in the cytoplasm of *E. coli* due to proteolysis and may be toxic to the host cell in the native conformation. Under appropriate conditions, the recombinant protein deposited in inclusion bodies amounts to about 50% or more of the total cellular protein. Because inclusion bodies have a relatively high density,[10] they can be isolated from the cellular proteins by centrifugation, and the purity of the resulting preparation may reach 90% under optimal conditions. Therefore, the production of many human therapeutic proteins as inclusion bodies is a cost-effective downstream process.[2,11,12] Recent advances in procedures for refolding inclusion body proteins have made it possible to obtain large amounts of authentic human proteins for therapeutic use. This review summarizes the improvements that have been made in the in vitro refolding of therapeutically relevant proteins containing disulfide bonds after production at high yield as inclusion bodies in *E. coli.*

HIGH-LEVEL EXPRESSION OF RECOMBINANT PROTEINS IN *E. COLI*

The expression of cloned genes in *E. coli* for the production of recombinant proteins has provided a valuable system for developing therapeutic proteins such as human insulin and human GH. Many successful *E. coli* expression systems have been described and are available from a variety of academic and commercial sources. Therefore, *E. coli* expression systems are suitable for the industrial-scale production of recombinant proteins. A number of criteria must be considered when optimizing conditions for the high-level expression of a recombinant protein. These include the stability of the mRNA,[13] the efficiency of transcription directed from a strong promoter,[14] the efficiency of protein synthesis (translation),[15] the formation of inclusion bodies, and the

Table I High-Level Expression of Recombinant Proteins for Therapeutic Use in *E. coli*[a]

Recombinant Protein	Mode of Expression	Level of Expression (% of Total Protein)	Level of Production (mg/L)	Promoter	Inclusion Body Formation	Reference
hEGF	Fusion	NE	60	*trp*	+	62
Human insulin	Fusion	20	NE	*lac*	+	3
hIFN-β	Direct	NE	20	*trp*	−	63
hIFN-γ	Direct	40	NE	*trp*	+	64
Human prourokinase	Direct	6	NE	*trp*	+	65
hGH	Direct	NE	169	*trp*	+	66
hGH	Secretion	14	$25/A_{550}$	*phoA*	−	67
hIGF-I	Fusion	20	1240	*trp*	+	68
hIGF-I	Secretion	30	8500	*phoA*	+	69
ht-PA	Direct	10	460	λP_L	+	45, 47
ht-PA	Secretion	NE	0.18	*araB*	−	70
hTIMP-1	Direct	15	NE	T7	+	51
hTIMP-2	Fusion	5	NE	T7	+	57
Human calcitonin	Fusion	NE	478	*lac*	+	71
hG-CSF derivative	Direct	15	2800	*trp*	+	72
hbFGF derivative	Direct	NE	1700	T7	−	73
hIL-2	Direct	20	700	*trp*	+	74
hIL-6	Direct	20	NE	*trp*	+	75
Human glucagon	Fusion	34.5	42	*trp*	+	76
Hirudin	Fusion	18	200	*trp*	+	77
Hirudin	Secretion	NE	1000	*trp*	−	78
Arginine deiminase	Direct	20	400	*tac*	+	25
Humanized $F(ab')_2$	Secretion	NE	2000	*phoA*	−	79
Chimeric Fab L-chain	Secretion	NE	2880	*tac*	+	80

[a] hEGF, human epidermal growth factor; hIFN, human interferon; hGH, human growth hormone; hIGF-I, human insulin-like growth factor-I; ht-PA, human tissue-type plasminogen activator; hTIMP, human tissue inhibitor of metalloproteinases; hG-CSF, human granulocyte colony-stimulating factor; hbFGF, human basic fibroblast growth factor; hIL, human interleukin. NE, not estimated.

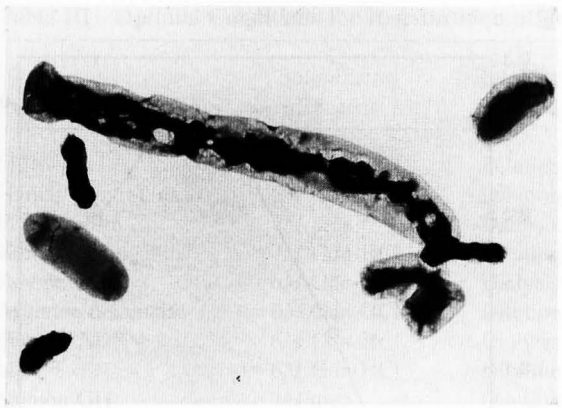


FIGURE 1 Electron micrograph of inclusion bodies containing recombinant porcine muscle adenylate kinase expressed in *E. coli.* The cells were harvested and washed with 1% NaCl and stained with 1% uranyl acetate. The dense material shown in the elongated *E. coli* is the inclusion bodies.

susceptibility of the product to proteolysis.[16,17] All of these criteria must be considered for each product individually. Representative examples of the high-level expression of recombinant proteins for therapeutic use are presented in Table I. In the majority of cases, the expressed proteins are in an insoluble form. A number of human proteins expressed in *E. coli* directly, e.g., GH, IFN-γ, interleukin-2 (IL-2), prourokinase, and tissue-type plasminogen activator (t-PA), or as fusion proteins, e.g., proinsulin, calcitonin, and insulin-like growth factor-I (IGF-I), have been shown to exist as aggregates or inclusion bodies (see Table I for references).

ISOLATION AND SOLUBILIZATION OF INCLUSION BODIES

Inclusion bodies obtained by cytosolic overexpression of a recombinant protein are large, spherical particles. Because of their refractile character, they can be observed directly in the living host cell by phase-contrast microscopy. We have shown that porcine muscle adenylate kinase is expressed in *E. coli* as inclusion bodies at high levels up to 40% of total cellular protein (Figure 1).[18] Because inclusion bodies are characterized by a relatively high specific density, they can be harvested after cell lysis by centrifugation at moderate rotor speeds.[19] To purify the inclusion bodies from their associated impurities, they can be washed with detergents such as Triton X-100, deoxycholate, or a low molar concentration of chaotroph.[2,20] However, it should be kept in mind that an excessively high concentration of urea or Gdn-HCl will lead to solubilization of the inclusion bodies themselves. Table II shows several examples of different washing solutions used for the purification of inclusion bodies.[11] On average, the purity of the inclusion body preparation may reach 90% under optimal conditions.

Next, the purified inclusion bodies must be solubilized by strong denaturants such as 6*M* Gdn-HCl or 8*M* urea. For this purpose, Gdn-HCl is usually preferable to urea for two reasons.[8] First, Gdn-HCl is a rather strong chaotroph, which may allow solubilization of extremely aggregated inclusion bodies that are resistant to solubilization by urea. Second, urea solutions may contain isocyanate, leading to carbamylation of free amino groups of the polypeptide, especially upon long-term incubation at alkaline pH values.[21] In the case of cysteine-containing proteins, the isolated inclusion bodies usually contain non-native intramolecular and intermolecular disulfide bonds,[22] which reduce the solubility of the inclusion bodies in the absence of reducing agents such as dithiothreitol (DTT), dithioerythritol, glutathione (GSH), cysteine, cystamine, or β-mercaptoethanol. Addition of these thiol reagents in combination with chaotrophs allows reduction of the disulfide bonds by thiol-disulfide

Table II Purification of Inclusion Bodies by Different Washing Solutions[a]

Recombinant Protein	Mode of Expression	Washing Solution	Reference
Human prourokinase	Direct	0.1% Triton X-100	65
ht-PA	Direct	5*M* urea, 2% Triton X-100	45
ht-PA	Direct	1% Triton X-100, 1% β-DPG	46
hM-CSF	Direct	2% Triton X-100	81
Arginine deiminase	Direct	4% Triton X-100	25
hIGF-I	Fusion	0.5% Sarcosyl	68
Bovine GH	Direct	2% deoxycholate	82
Prochymosin	Direct	0.5% Triton X-100	83
HRP	Direct	2*M* urea	84

[a] ht-PA, human tissue-type plasminogen activator; hM-CSF, human macrophage colony-stimulating factor; hIGF-I, human insulin-like growth factor-I; GH, growth hormone; HRP, horseradish peroxidase C. β-DPG, octyl-β-D-thioglucopyranoside.

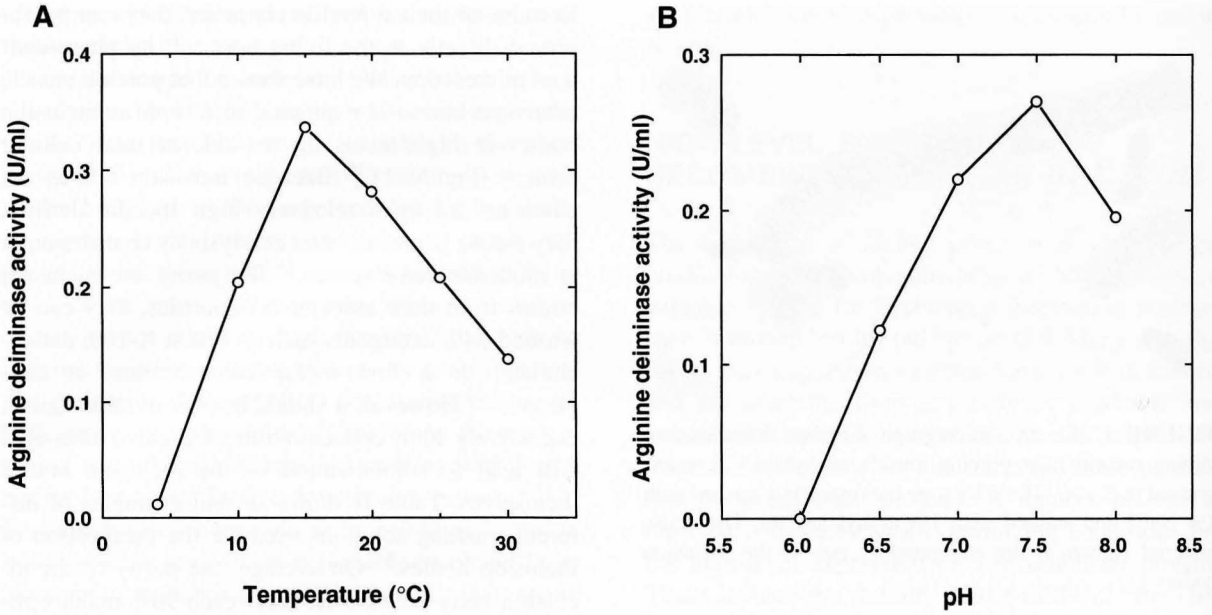


FIGURE 2 Effects of temperature and pH on renaturation of recombinant arginine deiminase (r-AD). The lyophilized inclusion bodies derived from 10 mL of cultured *E. coli* cells were solubilized in 1 mL 50 m*M* Tris HCl (pH 8.5) containing 6*M* Gdn-HCl and 10 m*M* DTT and incubated at 37°C for 1 h. The solubilized proteins were diluted rapidly with 100 mL of 10 m*M* potassium phosphate buffer, and the solutions were stirred at various temperature for 45 h at pH 7.0 (A) and at various pH values for 45 h at 15°C (B). The extent of r-AD renaturation was monitored by measuring the AD activity.

exchange.[8,23] Various experimental protocols used for the solubilization of inclusion bodies have been compared by Fischer et al.[11] If the purity of the solubilized inclusion bodies is low, purification can be achieved by reverse-phase high-performance liquid chromatography, gel filtration, or ion-exchange chromatography in the presence of a denaturant.

RENATURATION OF RECOMBINANT PROTEINS

To obtain the correctly folded proteins after solubilization of the inclusion bodies, excess denaturants and reducing thiol reagents have to be removed, and the reduced proteins transferred to oxidizing conditions. Renaturation of solubilized inclusion bodies is initiated by removal of the denaturant by either dilution or dialysis. The efficiency of renaturation depends on the competition between correct folding and aggregation.[24] To slow down the aggregation process, refolding is usually performed at low protein concentrations, within the range 10–100 μg/mL. Furthermore, the renaturation conditions must be carefully optimized with regard to external parameters such as temperature, pH, and ionic strength for each individual protein.[9,23]

Both folding and association of proteins depend strongly on temperature and pH. For example, we have shown that recombinant *Mycoplasma* arginine

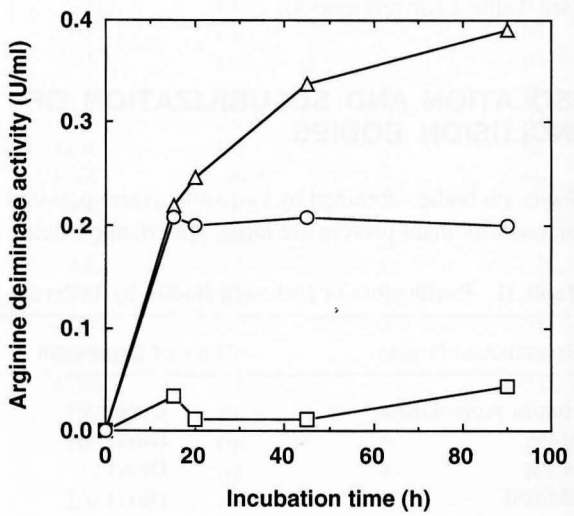


FIGURE 3 Time course of r-AD renaturation. The 6*M* Gdn-HCl-solubilized inclusion bodies containing r-AD were diluted rapidly 100-fold with 10 m*M* potassium phosphate buffer (pH 7.0) and the solutions were stirred at 4°C (□), 15°C (△), and 25°C (○) for 0–90 h. The extent of r-AD renaturation was monitored by measuring the AD activity at various time intervals.

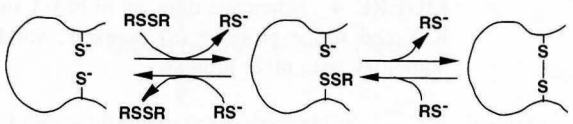
Table III Optimal Conditions for Renaturation of Proteins from Inclusion Bodies[a]

Recombinant Protein	Solubilizing Reagent	Refolding Method	pH	Temperature (°C)	Time (h)	Reference
hIFN-γ	6*M* Gdn-HCl	Dilution	7	4	Overnight	85
Human prourokinase	6*M* Gdn-HCl	Dilution	8.8	15	24	65
Prochymosin	8*M* urea	Dialysis	10.5	Room temp.	6	86
Human angiogenin	7*M* Gdn-HCl	Dilution	8.5	4	24	87
Bovine GH	6*M* Gdn-HCl	Dialysis	8.5	Room temp.	24	82
Arginine deiminase	6*M* Gdn-HCl	Dilution	7.5	15	90	25
Porcine ADK	6*M* Gdn-HCl	Dialysis	7.4	4	Overnight	18
hIGF-I	6*M* Gdn-HCl	Dilution	8	25	72	68
Salmon GH	7*M* urea	Dilution	8	4	One day	88

[a] hIFN, human interferon; GH, growth hormone; ADK, adenylate kinase; hIGF-I, human insulin-like growth factor-I.

deiminase, developed as an antitumor agent, is efficiently renatured at 15°C and at pH 7.5 by 100-fold rapid dilution of inclusion bodies solubilized with 6*M* Gdn-HCl (Figure 2).[25] The time required for complete renaturation may extend over a range of seconds to days. Upon renaturation of antibody Fab fragments from inclusion bodies, it was shown that the amount of functional antibody increased over 100 h.[26] Also, renaturation of recombinant *Mycoplasma* arginine deiminase exhibited exceedingly slow kinetics (over 90 h) even at 15°C by the rapid dilution method (Figure 3). Table III shows several of the optimal conditions for renaturation of proteins from inclusion bodies.[11]

Most secretory proteins contain disulfide bonds in their native state. If a target protein contains disulfide bonds, the renaturation buffer has to be supplemented with a redox system. Addition of a mixture of the reduced (RS^-) and oxidized (RSSR) forms of low molecular weight thiol reagents such as glutathione, cysteine, and cysteamine (molar ratios of reduced to oxidized compounds 5 : 1 to 10 : 1, respectively) usually provides the appropriate redox potential to allow formation and reshuffling of disulfides.[9,27,28] These systems increase both the rate and yield of renaturation/reoxidation by facilitating rapid reshuffling of incorrect disulfide bonds according to.[23,29]



In order to accelerate thiol–disulfide exchange, the pH of the renaturation buffer should be at the upper limit that still allows the protein to form its native structure. In order to prevent fortuitous oxidation of thiols by molecular oxygen, which is catalyzed by trace amounts of metal ions (e.g., Cu^{2+}), EDTA should be added to the buffer solutions. Reoxidation of protein disulfide bonds is performed by dilution of the reduced solubilized inclusion bodies in the "oxido-shuffling" system.[23,29] Table IV summarizes

Table IV Optimal Conditions for Renaturation and Reoxidation of Proteins from Inclusion Bodies by the Glutathion System

Recombinant Protein	Number of Disulfide Bonds	Reduced Glutathione (m*M*)	Oxidized Glutathione (m*M*)	pH	Temperature (°C)	Time (h)	Reference
Fab-fragment	5	5	0.5	8	10	150	26
ht-PA	17	0.5	0.3	8.75	15	24	45
Trancated ht-PA	9	2	0.2	8.6	20	24	32
Trancated hM-CSF	9	0.5	0.1	8.5	4	48	81
hIL-2	1	10	1	8	Room temp.	16	89
hIL-4	3	2	0.2	8	Room temp.	4	90
hIL-6	2	0.01	0.002	8.5	22	16	91
hTIMP-1	6	2	0.2	8	4	16	61
Trancated hTIMP-2	3	0.78	0.44	9.75	25	2	59

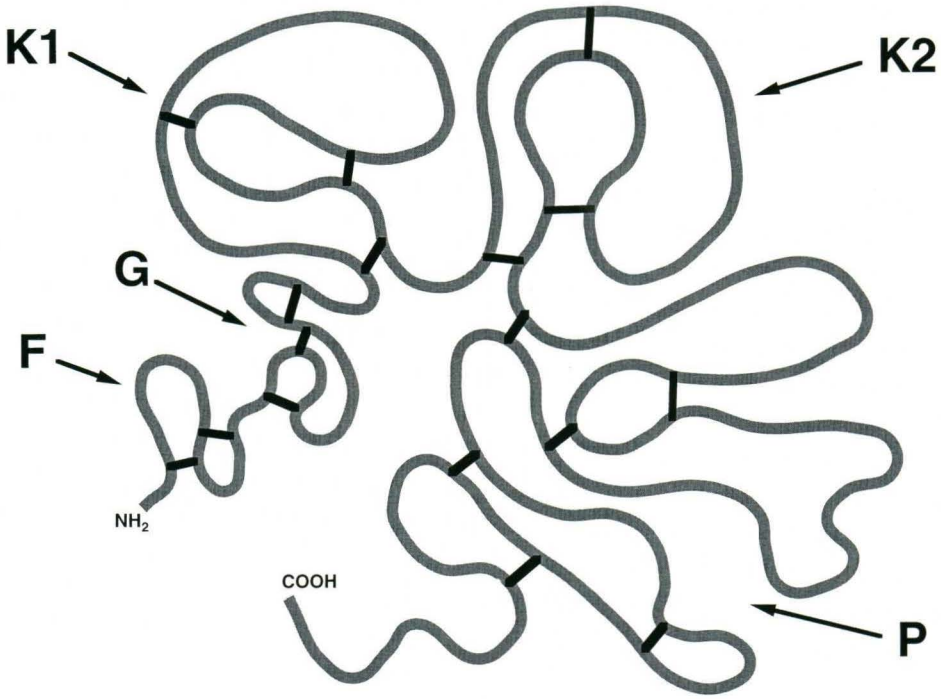


FIGURE 4 Schematic drawing of ht-PA showing the finger (F), growth factor (G), kringle (K1, K2), and serine protease (P) domains. Solid bars indicate potential disulfide bridges based on homology with other proteins.

several of the conditions for renaturation of proteins from inclusion bodies by the glutathione reoxidation system.[11]

In addition to the control of parameters such as temperature, pH, or redox conditions, the presence of low molecular weight compounds in the renaturation buffer may have a marked effect on the yield of renaturation.[8,9,30] A large series of low molecular weight additives are, in certain cases, very efficient refolding enhancers: for examples, nondenaturing concentrations of chaotrophs such as urea or Gdn-HCl are essential for the renaturation of reduced chymotrypsinogen A.[31]

The most popular additive is L-arginine.[8,9] In the case of human t-PA[29] or its truncated form,[32] the yield of renaturation is markedly increased in the presence of 0.5*M* L-arginine, whereas in its absence almost no reactivity is observed. The positive effect of L-arginine on renaturation efficiency has also been confirmed for various other proteins such as antibody Fab fragments,[26] single-chain immunotoxins,[33] and single-chain Fv fragments.[34] The mechanism by which L-arginine supports renaturation is still unknown. Although L-arginine contains a guanidino group, it does not destabilize the native folded structure as strongly as Gdn-HCl. The beneficial effect of L-arginine on protein refolding probably originates from increased solubilization of folding intermediates.[9]

In the case of bovine carbonic anhydrase B, stoichiometric amounts of polyethylene glycol (PEG) significantly enhanced the recovery of active protein by reducing aggregation.[35] Furthermore, three recombinant human proteins—deoxyribonuclease, t-PA, and IFN-γ—were refolded efficiently in the presence of PEG (MW 3350).[36] Therefore, PEG has significant potential for enhancing the recovery of active proteins from inclusion bodies.

Likewise, increased solubilization of folding intermediates can explain the positive effect of detergents on the refolding yield. Using lauryl-maltoside, CHAPS (3-[3-chloramidopropyl]dimethylammonia-1-propane sulfonate) or some other detergents during renaturation, the yield of renatured protein can be improved.[37,38] Refolding in the presence of a detergent followed by addition of cyclodextrin has been claimed to be analogous to a molecular chaperone system in terms of function.[39] To prevent aggregation during refolding, other techniques such as renaturation in reversed micelles[40] or in aqueous two-phase systems[41] have also been explored.

Another possibility for suppressing unspecific intermolecular interactions is the coupling of the denatured protein to a matrix. When denatured α-glucosidase fused to a polyarginine tag was bound to heparin-Sepharose, renaturation under conditions allowing the protein to remain bound to the matrix resulted in high

yields of active protein even at a high gel load of up to 5 mg/mL.[42] Another matrix used for this kind of renaturation is Ni^{2+}-nitrilotriacetic acid (NTA) resin, which was originally developed for efficient protein purification. After binding the denatured protein to the matrix via a His tag, the column is equilibrated with renaturation buffer, and the refolded protein can be eluted by imidazole using a pH gradient.[43]

CASE STUDIES OF THE PRODUCTION OF THERAPEUTIC PROTEINS

Example 1: t-PA

t-PA is a serine protease that has an important function in the fibrinolytic system. It catalyzes the conversion of plasminogen to plasmin in the presence of a fibrin clot. Human t-PA (ht-PA) is a glycosylated single-chain polypeptide of 527 amino acids, and contains 17 disulfide bonds.[44] A schematic drawing of ht-PA is shown in Figure 4. The molecule comprises five distinct structural domains: a finger domain, an epidermal growth factor-like domain, two kringle domains, and a C-terminal protease domain. The affinity of t-PA for fibrin, and the 100–200-fold increase in its activity in the presence of fibrin make it an attractive thrombolytic agent because it should generate plasmin locally at the fibrin surface and achieve thrombolysis without systemic activation of plasminogen.[45] Several attempts to produce ht-PA or a truncated form in *E. coli* have been reported.[29,32,45–48] Recombinant t-PA (rt-PA) accumulated as inclusion bodies in the cytoplasm. t-PA best exemplifies the challenges associated with the production of multidisulfide complex proteins in *E. coli* by refolding from inclusion bodies.

Sarmientos et al. reported that ht-PA was produced as an insoluble, aggregated form in *E. coli* (5–10% of total cellular protein), with a yield of 460 mg/L fermentation broth.[45,47] The inclusion bodies obtained after centrifugation of the sonicated extract were first washed with a solution containing 5*M* urea and 2% Triton X-100, then dissolved in 7*M* Gdn-HCl and 50 m*M* β-mercaptoethanol. The reducing agent was then removed by dialysis, and the solubilized inclusion bodies were diluted at least 50-fold into a renaturation buffer containing 2.5*M* urea, 10 m*M* lysine, and a redox coupler at appropriate concentrations (0.5 m*M* GSH and 0.3 m*M* GSSG), under carefully controlled incubation conditions (15°C, no air). After renaturation, Tween 80 was added to a final concentration of 0.01%, and the t-PA activity was measured. As a result, t-PA activity corresponding to a concentration of 2–5 μg/mL of fully active t-PA was consistently detected in the renaturation solution. Further characterization and purification of the renatured rht-PA strongly suggested that it was a fully active enzyme, very similar to natural t-PA, despite the lack of glycosylation. The purification yield of 2.8% for the overall process reflects a 20% step yield for the refolding operation, and a 56% yield for the subsequent ultrafiltration step at a refolding concentration of 2.43 mg rt-PA/L. Details of the scheme of rt-PA production from *E. coli* are shown in Figure 5.[47]

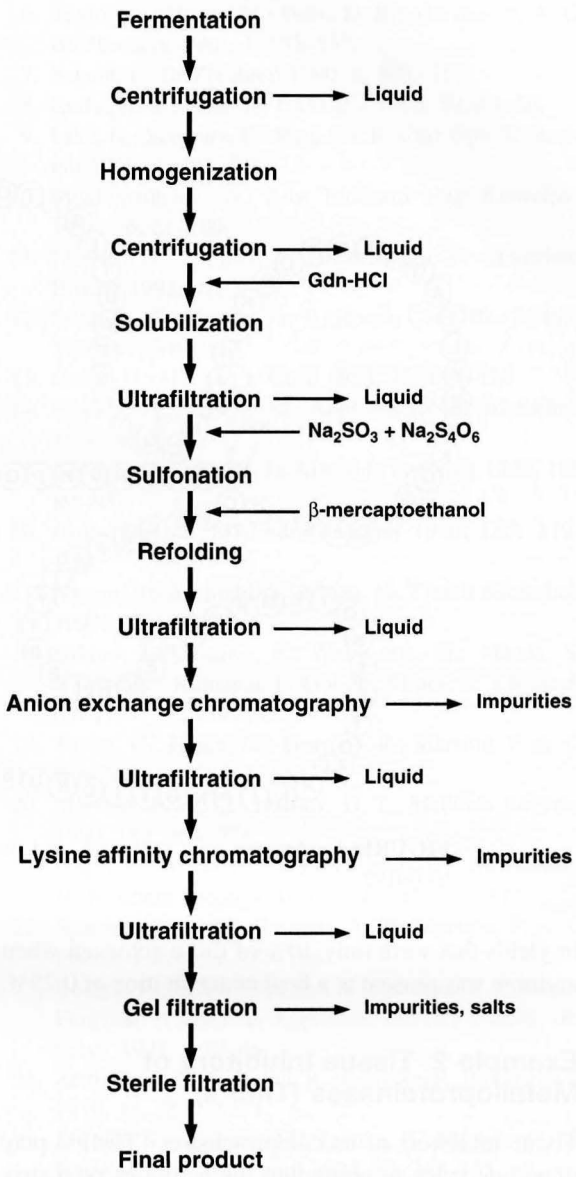


FIGURE 5 Schematic diagram for the production of renatured rt-PA from inclusion bodies expressed in *E. coli*.[10,47]

However, Grunfeld et al. reported that a 90% yield for the refolding process was achieved within an optimal concentration range of 2.6–3.7 mg rt-PA/L of reactivation mixture.[46] They also reported that absence of arginine in the reactivation mixture resulted

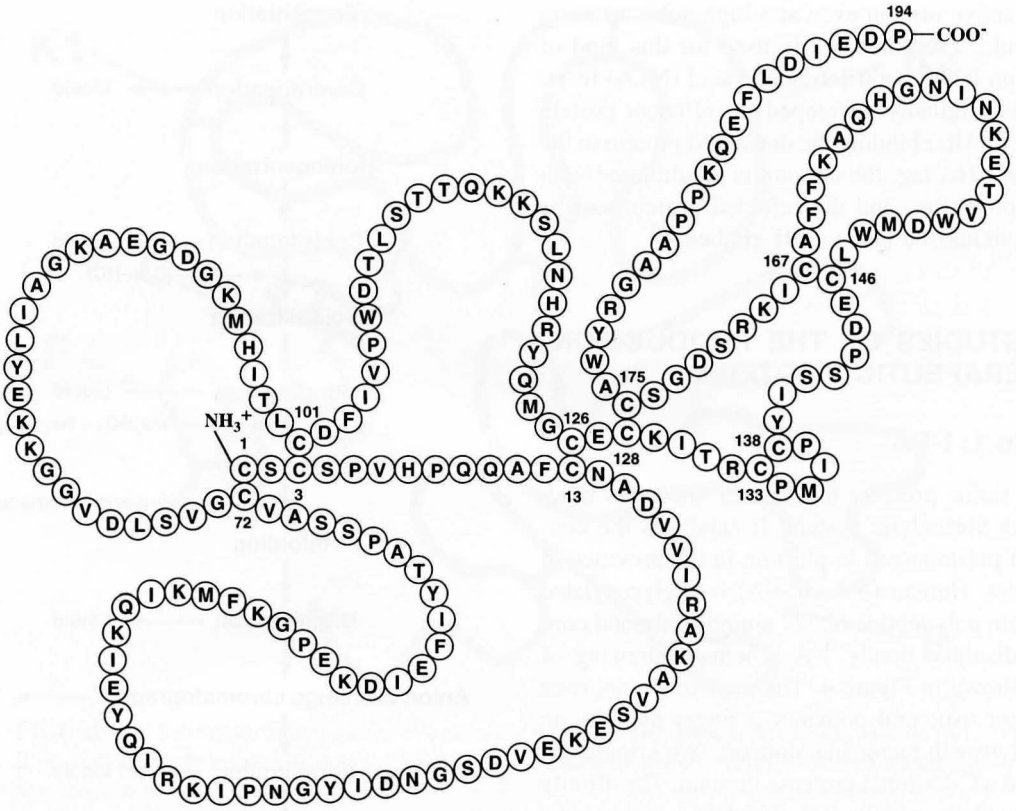


FIGURE 6 Amino acid sequence and two-dimensional representation of the structure of hTIMP-2.

in yields that were only 10% of those achieved when arginine was present at a final concentration of 0.25*M*.

Example 2: Tissue Inhibitors of Metalloproteinases (TIMPs)

Tissue inhibitors of metalloproteinases (TIMPs) play important roles in regulating the activities of matrix metalloproteinases (MMPs),[49,50] a family of enzymes responsible for the breakdown of connective tissue components. Inappropriate matrix breakdown is associated with a number of pathologies including periodontal disease, rheumatoid arthritis (RA), and tumor metastasis. The control of MMP activity is therefore an important therapeutic target, and TIMPs or low molecular weight MMP inhibitors could lead to new therapeutic strategies for the treatment of RA and cancer.[51]

Three members of the TIMP family have been identified to date: TIMP-1, TIMP-2, and TIMP-3.[52–54] The TIMP proteins show a high degree of amino acid sequence similarity including the conservation of 12 cysteinyl residues known to form 6 disulfide bonds.[55] TIMP-1 is a glycoprotein of 184 amino acids in length,[52] whereas TIMP-2 and TIMP-3 are not glycosylated.[53,54] The amino acid sequence of human TIMP-2 is shown in Figure 6. The overproduced recombinant TIMPs or the N-terminal domain of TIMPs accumulate as inclusion bodies in *E. coli,* and can be refolded into active forms.[51,56–61] Particularly, recombinant human TIMP-2 (rhTIMP-2) and rhTIMP-3 have been prepared by controlled refolding in the solid phase using an ion metal affinity column.[57,60]

Negro et al. reported that rhTIMP-2 was expressed in *E. coli* as a fusion protein with a 34-amino-acid NH_2-linked tail containing 6 histidine residues, and that the TIMP-2 fusion protein immobilized on Ni^{2+}-NTA resin was refolded at a high concentration (1 mg/mL resin) in the column.[57] The refolded rhTIMP-2 was eluted from the resin with 250 m*M* imidazole. It showed specific binding and inhibitory activity against 72-kDa gelatinase (MMP-2).

Furthermore, Negro et al. reported that rhTIMP-3 was expressed in *E. coli* as a fusion protein with a 36-amino-acid NH_2-linked tail containing 6 histidine residues, and that the rhTIMP-3 was refolded in the Ni^{2+}-NTA column by very slowly removing the denaturing and reducing agents (urea and β-mercaptoethanol).[60] The refolded rhTIMP-3 was treated with mild acid (70% formic acid) in order to remove the NH_2 tail from TIMP-3. After this treatment, the

rhTIMP-3 was loaded on the Ni^{2+}-NTA column in order to separate the cut (eluted) from the uncut (column retained) recombinant form. rhTIMP-3 from which the NH_2 tail had been removed showed inhibitory activities against both MMP-2 and MMP-9, and CD, fluorescence and second-derivative UV spectroscopic analyses supported correct refolding of rhTIMP-3. Solid-phase refolding may prove to be useful for a variety of other proteins in which correct disulfide bridging plays a critical role.

CONCLUSIONS

Although recombinant DNA technology now permits burst synthesis of heterologous proteins in *E. coli,* these proteins often accumulate as insoluble inclusion bodies, and therefore solubilization and renaturation systems are necessary in order to obtain the fully active proteins with a native conformation.

Similar to protein purification, protein refolding protocols still have to be developed on a case-by-case basis. Various procedures introduced in this review for in vitro refolding are available. Choosing the right procedure should allow renaturation of most recombinant proteins deposited in inclusion bodies, giving high yields.

Structural and functional analyses of proteins, especially those for therapeutic or industrial applications, require large amounts of recombinant proteins. The *E. coli* system for production of recombinant protein as inclusion bodies, together with a suitable renaturation procedure, provides an efficient avenue for meeting these requirements. In the near future, in vitro refolding of inclusion body proteins will become a powerful tool for commercial production of extremely complex proteins in which multidisulfide bonds play a critical role.

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