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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

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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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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.

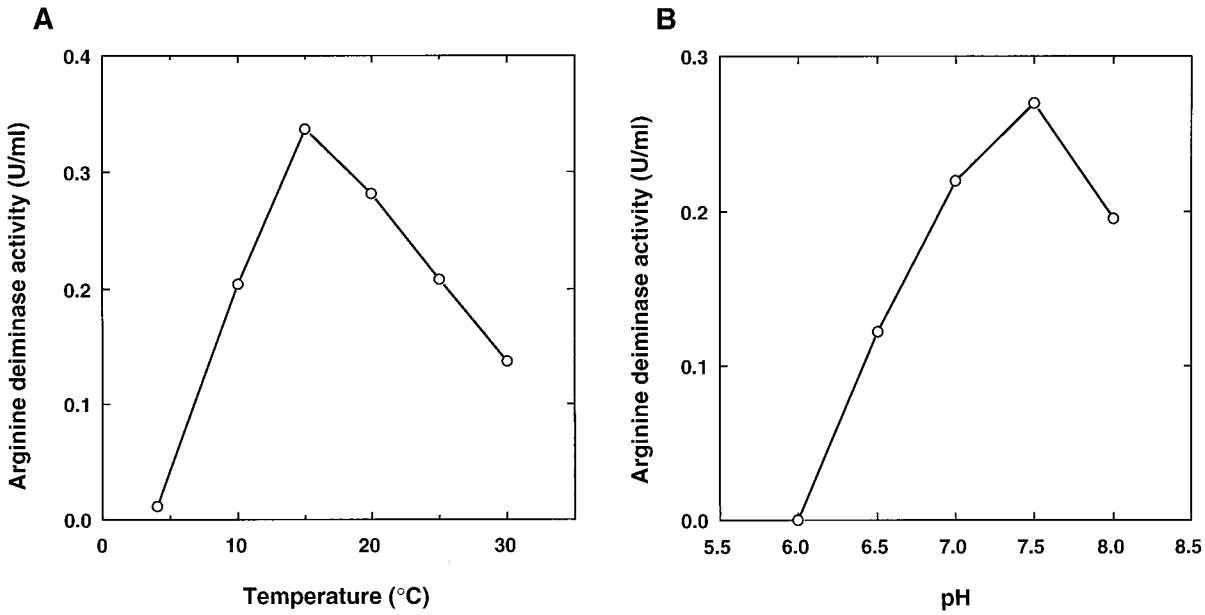


FIGURE 2 Effects of temperature and pH on renaturation of recombinant arginine deiminase (r-AD). The lyophilized inclusion bodies derived from 10 mL of cultured *E. coli* cells were solubilized in 1 mL 50 m*M* Tris HCl (pH 8.5) containing 6*M* Gdn-HCl and 10 m*M* DTT and incubated at 37°C for 1 h. The 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

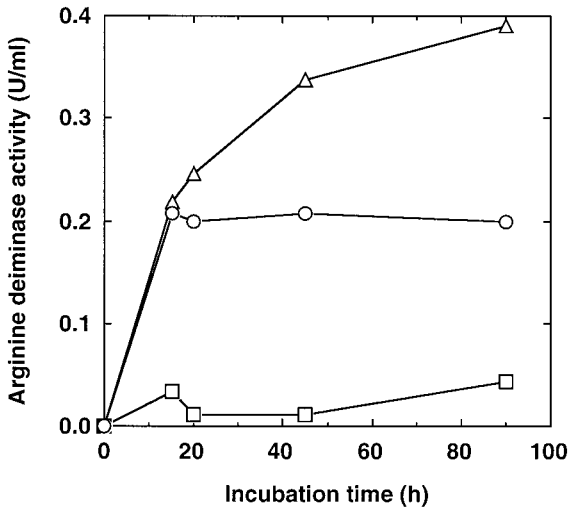


FIGURE 3 Time course of r-AD renaturation. The 6*M* Gdn-HCl-solubilized inclusion bodies containing r-AD were diluted rapidly 100-fold with 10 m*M* potassium phosphate buffer (pH 7.0) and the solutions were stirred at 4°C (□), 15°C (△), and 25°C (○) for 0–90 h. The extent of r-AD renaturation was monitored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

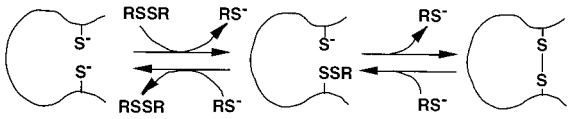
Table III Optimal Conditions for Renaturation of Proteins from Inclusion Bodies[a]

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

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