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Renaturation of Recombinant Human Pro-Urokinase Expressed in *Escherichia coli*

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A synthetic gene encoding human pro-urokinase (pro-UK) with E. coli-favored codon usage was cloned into plasmid pET-3d and expressed in E. coli BL21(DE3) LysS strain. The expressed products, which accumulated as inactive inclusion bodies, were denatured and renatured in vitro. A broad range of parameters such as pH, protein concentration, denaturant concentration, the use of cosolvent polyethylene glycol and presence of basic or acidic amino acid was examined. At optimal renaturation condition, pro-UK activity of more than 1000I.U. was obtained from 1 milliliter cell culture.

Plasminogen activators are serine proteases which play a role in fibrinolysis by converting the proenzyme plasminogen to plasmin. Plasmin, in turn, degrades fibrin to soluble products (1). Human pro-urokinase, one of the plasminogen activators, is initially synthesized as a single-chain molecule we refer to as pro-UK (2). Human pro-UK is a 411 amino acid glycoprotein and contains up to twelve disulfide bonds (3). The molecule contains three structural domains, an EGF-like domain (9-44aa), a kringle domain (45-133aa) and a serine-protease domain (134-411aa).

Numerous attempts to express the human pro-UK cDNA in mammalian cells (4), yeast (5) and Escherichia coli (3,6,7,8,9) have been reported. However, the secretion levels of correctly refolded pro-UK by mammalian cells and yeast cells (10) were not sufficiently high. When expressed in E. coli, recombinant pro-UK accumulated as inclusion bodies in the cytoplasm and the refolding of pro-UK from inclusion bodies was inefficient (6,8,11,12). Efforts were made to get biologically active pro-UK in E. coli (13,14,15), but the yield was still low.

The feasibility of in vitro renaturation of a protein has been established with pioneering experiments by Anfisen (16). However the renaturation of large, cysteine-rich and multi-domain proteins is often a difficult exercise. The difficulties encountered in the reactivation of pro-UK from E. coli inculsion bodies has limited further development of this thrombolytic agent.

In the present study, we describe and assess the feasibility of affecting the yield of the renaturation of bacterially expressed human pro-UK in its active conformation.

MATERIALS AND METHODS

Materials. Plasmid pET-3d and strain BL21(DE3) LysS were kindly provided by Dr. F. William Studier (Brookhaven National Laboratory, USA). Fibrinogen was purchased from Sigma. Thromin was from Tianjing Biological Reagent Factory. Reduced (GSH) and oxidized (GSSG) glutathione, arginine, lysine, glutamic acid, polyethylene glycol-6000 (PEG-6000) and protein molecular weight standards were from Shanghai Dongfong Reagent Factory.

Construction of pro-UK expression plasmid pET-3d-pro-UK. A human pro-UK encoding gene with E. coli-favored codon usage was synthesized (17) and cloned in pUC-9. The pro-UK gene has BspHI site at its 5′ end, containing the ATG start codon, and HindIII site just downstream the stop codon. After HindIII digestion, Klenow blunting and BspHI digestion, the DNA fragment encoding pro-UK was inserted into pET-3d plasmid treated with BamHI digestion, Klenow blunting and NcoI digestion. In the resulting plasmid pET-3d-pro-UK, the pro-UK gene is under the under of T7 promoter.

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Protein expression. A culture from a single colony was grown at 37°C overnight and innoculated into LB medium containing 100μg/ml ampicillin and 34μg/ml chloramphenicol using 2% of the final growth volume, then grown at 37°C until the OD600 reached 0.6. The induction of pro-UK expression was begun by addition of 0.1mM IPTG. After expressed for 3 hours, bacteria were harvested by centrifugation.

Denaturation and renaturation. Centrifuged cells from 60ml flask culture were suspended in 4ml 0.1M Na phosphate buffer, pH7.5 and disrupted by sonication. The suspension was centrifuged at 10,000rpm for 10 minutes and the collected pellet was solubilized in 8M urea, 50mM β-mercaptoethanol, 0.1M.Na phosphate buffer, pH7.5 at 4°C for 16 hours, then dialyzed against 8M urea, 1mM β-mercaptoethanol, 0.1M Na phosphate buffer, pH7.5 for 6 hours and renatured by 50-fold dilution in renaturation buffer at 4°C for 18–24 hours. After dialysis against 0.01M Na phosphate buffer, pH7.5, fibrinolytic activity was assayed on fibrin plate prepared as described by Ploug and Kieldgaard (18).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (*SDS–PAGE*). SDS-PAGE was performed according to Laemmli (19) with 4% stacking gels and 12 % separating gels. Samples were treated by heating at 95°C for 10 minutes in 50mM Tris-HC1, pH6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol, 0.02% bromphenol blue before loading on gel. Proteins were visualized by coomassie blue staining.

Protein assay. Protein concentrations were measured as described by Bradford (20) with bovine serum albumin as standard.

RESULTS

A chemically synthesized human full length pro-urokinase gene with E. coli-favored codon usage (17) was cloned into pET-3d plasmid and transformed into E. coli BL21(DE3), BL21(DE3) LysS and BL21(DE3) LysE strains. The expression level of recombinant human pro-UK was 15–18% of total cellular protein in E. coli BL21(DE3)LysS strain, better than in BL21(DE3) and BL21(DE3) LysE strains (data not shown). Pro-UK is expressed essentially in an insoluble denatured form as indicated by observation that there was nearly no urokinase activity in the bacterial lysate.

After sonication of the expression cells, the insoluble inclusion bodies were separated by centrifugation. Most products in inclusion bodies were in the single chain form, pro-UK, as shown in lane 1 of Fig. 1. The insoluble pellets were solubilized in 8M urea. Addition of β-mercaptoethanol as reduction agent was necessary for total solubilization of pro-UK because these aggregates are partially linked by sulfide bonds (9). The subsequent renaturation of pro-UK was carried out by dialysis again 8M urea, 1mM β-mercaptoethanol, 0.1M Na phosphate buffer, pH7.5 to reduce the concentration of the reducing agent and then 50-fold dilution in renaturation buffer. To promote disulfide bond formation and rearrangement, renaturation reactions contained reduced and oxidized glutathione in a ratio of 5:1. After renaturation, pro-UK activity was measured using the classical fibrin plate technique. The yield of renaturation is represented as enzyme activity (I.U.) per ml culture in order to make an easy comparison with the yield of secretively fibrinolytically active pro-UK expressed in E. coli (15).

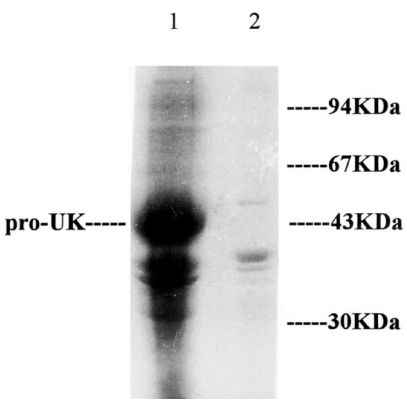

FIG. 1. SDS–PAGE analysis of cellular lysis pellets. Lane 1: lysis pellets from cells harboring expression plasmid pET-3d-pro-UK; Lane 2: lysis pellets from cells harboring pET-3d plasmid.

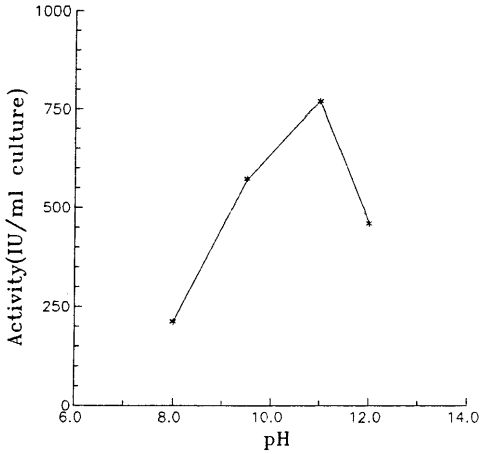

FIG. 2. Effect of pH on the yield of the renaturation process. Renaturation buffer: 2.5M urea, 50mM Tris-HC1, 5mM EDTA, 10mM NaCl, 1.25mM GSH, 0.25mM GSSG, 10mM lysine with different pH.

To determined optimal condition for obtaining maximum yield of the renaturation process, a broad range of parameter was examined. Among these were: pH, protein concentration, denaturant concentration, presence of acidic or basic amino acids and use of cosolvent polyethylene glycol.

The effect of pH on the yield of renaturation was shown in Fig. 2. Renaturation of the protein reached a maximum at pH11. The presence of free basic amino acids, lysine and arginine, had important influence on the renaturation process while acidic amino acid, glutamic acid, significantly decreased the yield of renaturation as shown in Fig. 3. The effect of lysine on renaturation process was better than that of arginine (Fig. 4). The reason why glutamic acid inhibits the renaturation process of pro-UK is still unknown.

Maintenance of a relative high urea concentration (2.5M) was very important in renaturation process as shown in Fig. 5. It can prevent the unfolded proteins from re-aggregating. At low urea concentration condition, especially 0.5M urea, there were more aggregates reformed in the renaturation process that easily observed by eye.

Cleland reported that the use of cosolvent PEG could enhance protein folding and decrease

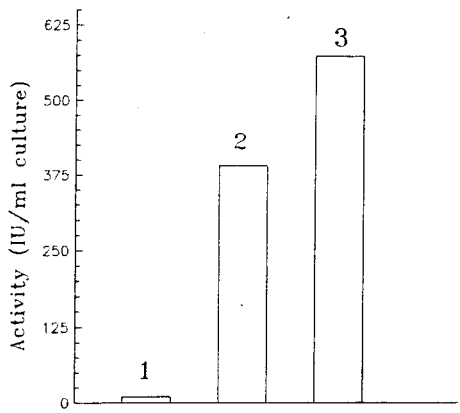

FIG. 3. Effect of basic or acidic amino acid on the yield of the renaturation process. Renaturation buffer: 2.5M urea, 50mM Tris-HC1, pH9.5, 5mM EDTA, 10mM NaCl, 1.25mM GSH, 0.25mM GSSG with 1: 10mM glutamic acid; 2: no amino acid; 3: 10mM lysine.

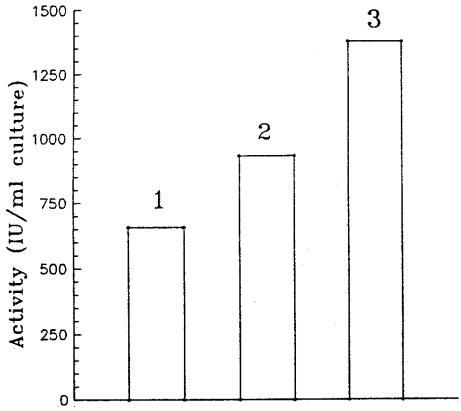

FIG. 4. Effect of basic amino acids on the yield of the renaturation process. Renaturation buffer: 2.5M urea, 50mM Tris-HC1, pH1l, 5mM EDTA, 10mM NaCl, 1.25mM GSH, 0.25mM GSSG with 1: no amino acid; 2: 10mM arginine; 3: 10mM lysine.

aggregation (21). The effect of PEG-6000 on renaturation of pro-UK was assessed as shown in Fig. 6. At concentration of 0.002g/L PEG-6000, recovery of pro-UK activity reached maximum.

The importance of the pro-UK protein concentration in the renaturation mixture is evident. Fig. 7 showed that in the presence of PEG-6000, low protein concentration greatly favored renaturation process. When protein concentration increased from 3.2mg/L to 10mg/L, the renaturation yield decreased dramatically. Even at high protein concentration, the presence of lysine still had effect on renaturation. The presence of low concentration of PEG-6000 together with lysine had greater effect on the renaturation process. At optimal renaturation condition, pro-UK activity of more than 1000I.U. was obtained from 1ml cell culture.

DISCUSSION

In this paper we describe high level expression of human pro-UK in E. coli and an efficient renaturation procedure for recovery of recombinant pro-UK activity from inclusion bodies. The expression level we achieved, obviously higher than previously reported (3,6,7,8,9), may be at-

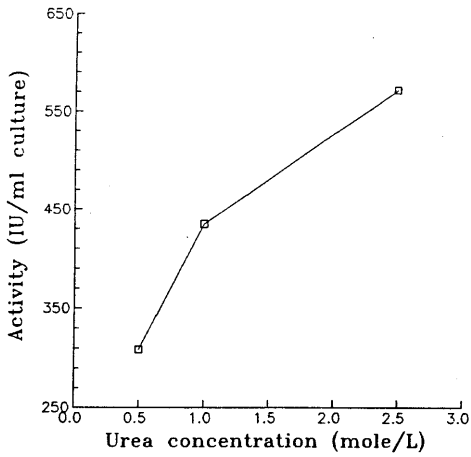

FIG. 5. Effect of urea concentration on the yield of the renaturation process. Renaturation buffer: 2.5M urea, 50mM Tris-HC1, pH9.5, 5mM EDTA, 10mM NaCl, 1.25mM GSH, 0.25mM GSSG, 10mM lysine with different urea concentration.

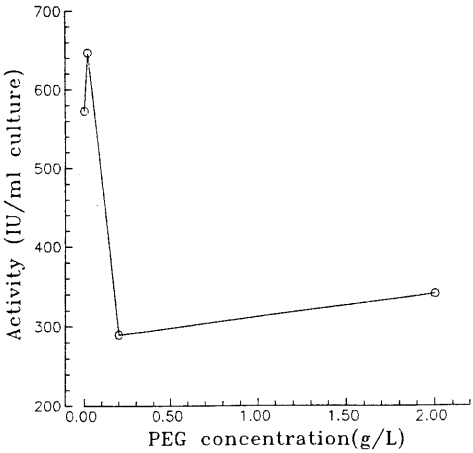

FIG. 6. Effect of PEG-6000 concentration on the yield of the renaturation process. Renaturation buffer: 2.5M urea, 50mM Tris-HCl, pH9.5, 5mM EDTA, 10mM NaCl, 1.25mM GSH, 0.25mM GSSG, 10mM lysine with different PEG-6000 concentration.

tributed to the use of a synthetic pro-UK encoding gene with E. coli-favored codon usage. Although no particularly novel method for renaturation was used in this work, our renaturation yields in optimal condition are notably higher than those previously reported by others (6,9,11,12).

During the refolding process, the protein may reform inactive protein aggregates. These aggregates significantly reduce the yield of active protein. Therefore, it is essential to choose conditions such as use of cosolvent PEG, low protein concentration, maintenance of denaturant concentration which minimize the formation of aggregates during refolding. The pH value and presence of some ligand-like molecules in renaturation process may have significant effect on the yield of renaturation.

These results suggest that each parameter of the renaturation reaction has to be carefully opti-

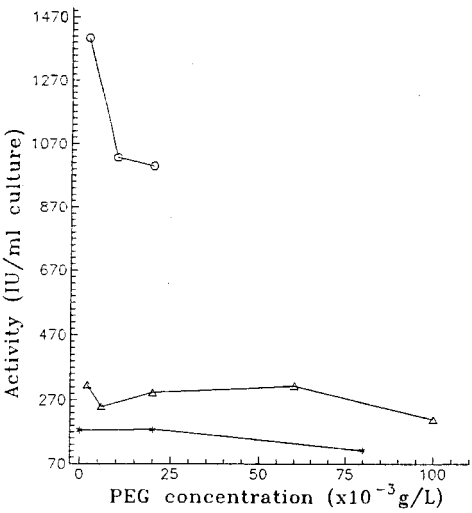

FIG. 7. Effect of protein concentration on the yield of the renaturation process. Renaturation buffer: 2.5M urea, 50mM Tris-HC1, 5mM EDTA, 10mM NaCl, 1.25mM GSH, 0.25mM GSSG with–○–: 3.2mg/L recombinant pro-UK, 10mM lysine, pH11;–△–: 10mg/L recombinant pro-UK, 10mM lysine, pH11;–*–: 12.9mg/L recombinant pro-UK, pH9.5.

mized in order to reach significant yields. Our data demonstrate the possibility of efficiently renaturing even complex molecules like pro-UK.

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