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Refolding and Purification of Recombinant Human Interferon-γ Expressed as Inclusion Bodies in *Escherichia coli* Using Size Exclusion Chromatography

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Abstract A size exclusion chromatography (SEC) process, in the presence of denaturant in the refolding buffer was developed to refold recombinant human interferon-γ (rhIFN-γ) at a high concentration. The rhIFN-γ was overexpressed in *E. coli*, resulting in the formation of inactive inclusion bodies (IBs). The IBs were first solubilized in 8 M urea as the denaturant, and then the refolding process performed by decreasing the urea concentration on the SEC column to suppress protein aggregation. The effects of the urea concentration, protein loading mode and column height during the refolding step were investigated. The combination of the buffer-exchange effect of SEC and a moderate urea concentration in the refolding buffer resulted in an efficient route for producing correctly folded rhIFN-γ, with protein recovery of 67.1% and specific activity up to 1.2×10^7 IU/mg.

Keywords: size exclusion chromatography (SEC), recombinant human interferon-γ, protein refolding, purification, inclusion bodies

INTRODUCTION

The production of genetically engineered proteins from *E. coli* was limited by the high level expression of the cloned gene product as inclusion bodies (IBs). These aggregates have no biological activity and there is a need to solubilize the inclusion bodies and refold the protein into its native structure [1]. The general strategy used to recover active protein involves three steps: inclusion body isolation and washing; solubilization of the aggregated protein; and refolding of the solubilized protein [2]. After dissolution of the IBs in a buffer containing strong chaotropic agents, such as 6 M guanidine hydrochloride or 8 M urea, reducing agents, such as dithiothreitol (DTT) or β-mercaptoethanol, were added to reduce the disulfide bonds. Finally, the denatured protein was transferred into a non-denaturing environment and refolded towards its native conformation, which was usually achieved by dilution or dialysis [3]. However, the protein aggregation problem that occurred in the refolding step limited the total downstream processing yield, so isolation of the product from the aggregates must subsequently be undertaken. Many attempts have been made to improve *in vitro* refolding, such as optimizations of the physico chemical properties of the refolding environment, the development of new refolding methods and solid-phase refolding [4-7].

In recent years, refolding using chromatography methods has rapidly developed, including ion exchange chromatography, chelating chromatography, hydrophobic interaction chromatography and size exclusion chromatography (SEC) [8-11], *etc.* Batas [12] indicated that refolding in a SEC packed column was based on an alternative buffer-exchange mechanism to remove high-concentration denaturant to promote renaturation. Small molecules, like DTT and urea, entered the pores of the SEC gel and were isolated from unfolded protein molecules, such that the refolding was initiated. Refolding by SEC has a notable advantage that could be combined with the simultaneous partial purification of proteins.

Interferon-γ (IFN-γ), also known as type II interferon, is endogenously produced by lymphoid cells in response to antigenic stimuli [13] and has always been an important clinical pharmaceutical due to its unique biological activities, such as antiviral, antitumor and immunoregulation effects [14]. Recombinant human IFN-γ (rhIFN-γ) is a good solution for meeting the large amounts required for clinical therapy. However, rhIFN-γ is expressed as IBs in *E. coli*, and the renaturation of the protein is crucial for a high production yield. In this paper, a method is described for the refolding and purification of rhIFN-γ produced as IBs in *E. coli* in order to obtain the purified rhIFN-γ with high activity.

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MATERIALS AND METHODS

Strain and Reagents

Genetically engineered bacteria (pBV220/IFN-γDH5α), the wish cell and vesicular stomatitis virus (VSV), were kept by the Institute of Bioengineering at Zhejiang University. The guanidine hydrochloride (GdnHCl), urea, ethylenediamine tetracetic acid (EDTA), dithiothreitol (DTT), Triton X-100, tris (hydroxymethyl) aminomethane (Tris) and ampicillin were purchased from Sigma. The Superdex 75 gel for SEC was from Amersham Biosciences, Sweden. Ultrapure water was used for all the experiments. All other agents were of analytical grade.

Production of rhIFN-γ Inclusion Bodies

Recombinant *E. coli* pBV220/IFN-γDH5α, carrying the hIFN-γ-cDNA plasmid, including the temperature-inducible P_RP_L promoter, was used as the expression host. The host cells were grown overnight at 30°C, with shaking at 220 rpm in 20 mL LB medium (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl at pH 7.5), containing 100 μg/mL ampicillin. The freshly grown culture was transferred into a 3.7-L fermentor (Bioengineering, Switzerland) with 2 L of sterile 5 × M9 + LB medium (30 g tryptone, 15 g yeast extract, 25.2 g $Na_2HPO_4{\cdot}12H_2O$, 6 g KH_2PO_4, 10 g NaCl, and 2 g NH_4Cl, adding 2 mL of 1 M $MgSO_4$ and 30 mL of 20% glucose after sterilization) containing 100 μg/mL ampicillin. The cells were grown at 30°C and pH 6.8 until the absorbance of the culture suspension achieved an optical density of 0.8 at 600 nm. Protein expression was induced by shifting the temperature from 30 to 42°C, and the cells then allowed to grow for a further 6 h. The cells were harvested by centrifugation at 5,000 rpm and 4°C for 10 min. The collected pellets were re-suspended in 100 mL of lysis buffer (1 mM EDTA and 50 mM phosphate buffer at pH 7.0) and crushed using an ultrasonic homogenizer (500 w for 20 min). The cell homogenate was then centrifuged at 8,000 rpm and 4°C for 15 min. The collected inclusion bodies were re-suspended in 100 mL of detergent buffer (0.5% Triton X-100, 2 M urea, 1 mM EDTA and 50 mM phosphate buffer at pH 7.0) and stirred with the magnetic agitator for 2 h. After centrifugation at 8,000 rpm for 15 min, the pellets of then obtained IBs were stored at −20°C until use.

Preparation of Denatured rhIFN-γ

A 1 g pellet of the washed IBs was solubilized into 10 mL denaturing buffer (8 M urea, 10 mM DTT, 1 mM EDTA and 50 mM phosphate buffer at pH 7.0) and stirred at room temperature for 6 h. The solution containing the denatured rhIFN-γ was then centrifuged at 10,000 rpm and 4°C for 30 min, to remove the insoluble particles. After determination of the protein concentration, the solubilized rhIFN-γ was ready for refolding by SEC.

Refolding of rhIFN-γ by Size Exclusion Chromatography

The size exclusion chromatography was carried out at room temperature using a XK16/20 column (Amersham Biosciences, Sweden) packed with Superdex 75 gel medium. The buffers were treated by microfiltration (0.45 μm) and degassed prior to chromatography. The packed column was equilibrated with 5 CV (column volume) of refolding buffer (3 M urea, 10 mM EDTA, 0.15 M NaCl and 50 mM phosphate buffer at pH 7.0). The denatured protein sample was loaded through a sample loop, and eluted with the same buffer, at a flow rate of 0.4 mL/min. The eluted factions were collected and assayed for total protein concentration, rhIFN-γ activity and SDS-PAGE analysis.

Analytical Methods

Total Protein Concentration

The total protein concentration was determined by the Coomassie Brilliant Blue assay [15], using bovine serum albumin as a reference.

Biological Activity Assay [16]

After renaturation, the sample was dialyzed overnight against 50 mM phosphate buffer at pH 7.5 and 4°C, and then syringed with a 0.22 μm sterile membrane to remove any bacteria. The assay was performed by incubating the WISH cells as a monolayer with a serially diluted sample on a 96 well plate in a CO_2 incubator at 37°C. The cells were then challenged by the vesicular stomatitis virus (VSV). The antiviral activity was calculated as the reciprocal of the dilution, where 50% of the WISH cells were protected from the cytopathic effect of the attacking virus.

Electrophoresis Analysis

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [17]. The concentrations of the running and stacking gels were 15% and 5%, respectively. The results were evaluated using a Gel Doc 2000 (Bio-Rad, USA).

RESULTS AND DISCUSSION

Expression of rhIFN-γ as Inclusion Bodies

The rhIFN-γ expression, detected by SDS-PAGE, is shown in Fig. 1. The molecular weight (M_W) of the rhIFN-γ was 18 kDa, and there was no band at the 18 kDa (see lane 4) position if the cells were not induced at 42°C, demonstrating that the host cells did not contain endogenous protein with a M_W of 18 kDa, which would otherwise interfere with subsequent SDS-PAGE analysis. After induction, the target protein was not excreted into the supernatant of the culture medium (see lane 2), and there was a clear band with M_W of 18 kDa only in the pellet after cell disruption and centrifugation (see lanes 1

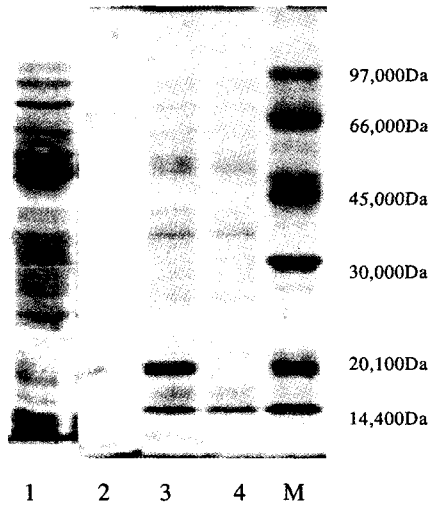

Fig. 1. SDS-PAGE of the expression of rhIFN-γ as an inclusion body in *E. coli*. Lane 1: supernatant of the cell lysate after centrifugation; Lane 2: supernatant of the cell culture broth; Lane 3: pellet of the cell lysate after centrifugation; Lane 4: none induced cells; M: marker.

and 3). It could be concluded that rhIFN-γ was an intracellular aggregate, *i.e.*, expressed as inclusion bodies (IBs). The production of the rhIFN-γ IBs was approximately 1.6 g/L culture. The rhIFN-γ IBs were easily isolated from the heterologous proteins in the cell by washing and centrifuging. After washed with the detergent buffer, the purity of the IBs was up to 85%. The purified IBs were then solubilized into denaturing buffer, and following centrifugation, the supernatant contained the denatured rhIFN-γ at a concentration of 15.6 mg/mL.

Integration of rhIFN-γ Refolding and Purification by SEC

SEC is a process that can be used for the simultaneous initiation of protein refolding, and purification from contaminants. In order to refold rhIFN-γ, the key procedure was to slowly exchange the denaturing buffer around the unfolded protein with refolding buffer, which can be achieved by SEC using a XK 16/20 column packed with Superdex 75 gel medium. A 0.1 mL sample of denatured rhIFN-γ (9.7 mg/mL) in denaturing buffer was injected onto the SEC column that had previously been equilibrated with refolding buffer, at a flow rate of 0.4 mL/min, followed by washing with refolding buffer. Small molecules such as DTT and urea were separated from the unfolded protein molecules that have a higher Stokes radius (Sr). Therefore, the denaturant concentration around the unfolded proteins decreased, and the protein refolding gradually performed under the refolding conditions. Fig. 2 shows the chromatogram of rhIFN-γ refolding by SEC, where the first peak refers to the proteins and the second peak, coupled with a high conductivity and the same retention time as the column volume, to the small molecules of DTT, *etc.* Fractions 3, 4, 5 and 6 were pooled separately, and the concentration of the refolded protein determined and the purity detected by SDS-PAGE analysis. It can be seen that only fraction 3 had a contaminant band, between 30 kDa and 45 kDa, and the rhIFN-γ in fractions 4, 5 and 6 had an electrophoresis grade purity. The results of the concentration measurement indicated that the protein mass of fraction 3 accounted for 30.1% of the total proteins in all fractions. It was expected that the purification steps after the refolding process could be simplified if the fraction 3 had been optimized further.

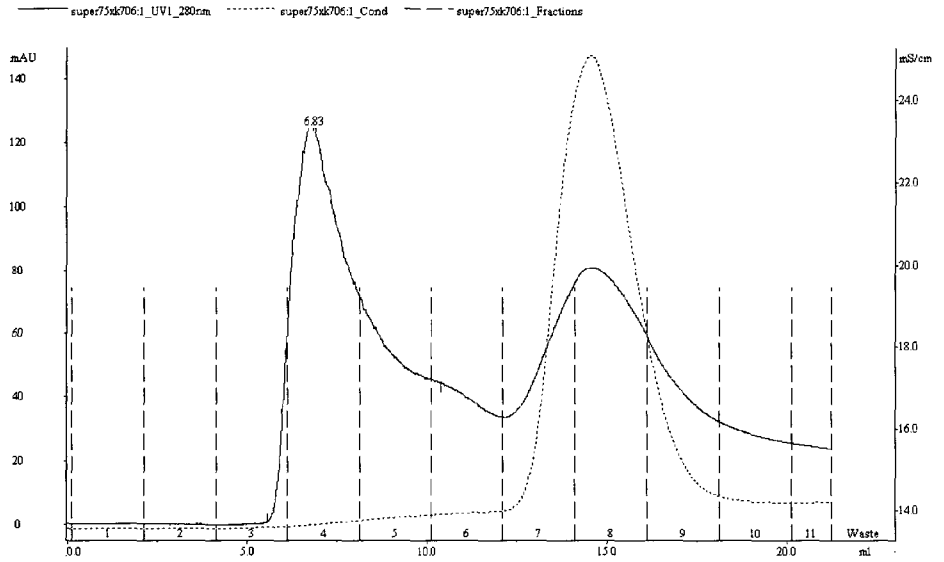

Fig. 2. Elution profile and collected elution samples of rhIFN-γ using SEC. Flow rate: 0.4 mL/min; Applied sample: 0.1 mL × 9.7 mg/mL; Column height: 7 cm; Refolding buffer: 3 M urea, 10 mM EDTA, 0.15 M NaCl and 0.05 M PBS, at pH 7.0.

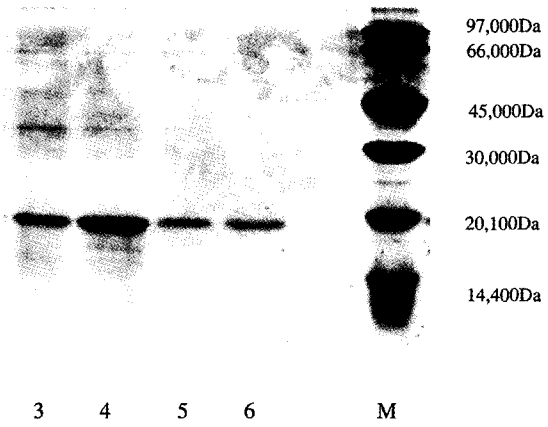

Fig. 3. Detection of eluted samples from the SEC by SDS-PAGE. Lanes 3, 4, 5 and 6 denote the collected samples shown in Fig. 2; M: marker.

Effect of Urea Concentration on rhIFN-γ Refolding

The concentration of the denaturant in the renaturation buffer was a key factor in mediating the refolding of the denatured protein [18,19]. As a weak chaotropic agent, urea may not only alleviate the intermolecular interactions of the denatured protein, which lead to aggregation, but also influence the solubility and the stability of the native, denatured intermediate states during refolding. Thus, optimizing the concentration of urea was a very efficient strategy for suppressing aggregation [20]. The refolding of rhIFN-γ was performed by SEC in the presence of urea in the renaturation buffer. The elution profiles were represented in Fig. 4, and the results shown in Fig. 5. As the concentration of urea was increased to 4 M, the area of the protein peak, *i.e.* the protein concentration, increased rapidly. Therefore, it is reasonable to postulate that urea in the refolding buffer at a non-denaturing concentration could prevent the aggregation of the unfolded proteins. However, the activity of rhIFN-γ refolded by SEC decreased sharply when the concentration of urea reached 4 M. This can be explained by urea concentrations higher than 3 M [21] having a strong denaturant effect, which was detrimental to the bioactivity of the refolded rhIFN-γ, and the optimum urea concentration in the refolding buffer has a dose-dependent effect on the aggregation. At the optimized conditions, the final protein concentration of rhIFN-γ was 0.19 mg/mL and the specific activity of 4.75×10^6 IU/mg with the urea-to-protein concentration ratio 18.

Effect of Loading Mode on rhIFN-γ Refolding

To study the influence of the loading mode on the protein refolding [22], different concentrations and volumes of denatured rhIFN-γ (mode 1: 0.1 mL × 15.6 mg/mL, mode 2: 0.2 mL × 7.8 mg/mL and mode 3: 0.5 mL × 3.12 mg/mL) were injected onto the column. Refolding buffer (3 M urea, 10 mM EDTA, 0.15 M NaCl and 50 mM phosphate buffer at pH 7.0) was used as the mobile phase. The collected fractions were pooled, and the concentration and activity of refolded rhIFN-γ determined by the methods mentioned above. Protein recovery was defined as the ratio of the eluted rhIFN-γ mass to that of the denatured protein applied. As shown in Table 1, although the protein recoveries of the three loading modes were nearly constant, obvious differences in the specific and total activities were obtained with the different loading modes. It was assumed that higher feed concentration resulted in more protein aggregates. With the lower feed concentration of 3.12 mg/mL denatured rhIFN-γ, the protein recovery was the highest, but with a lower specific activity than with mode 2. Mode 2 was used a moderate sample application, which not only gave the highest total activity, but a relatively higher recovery also.

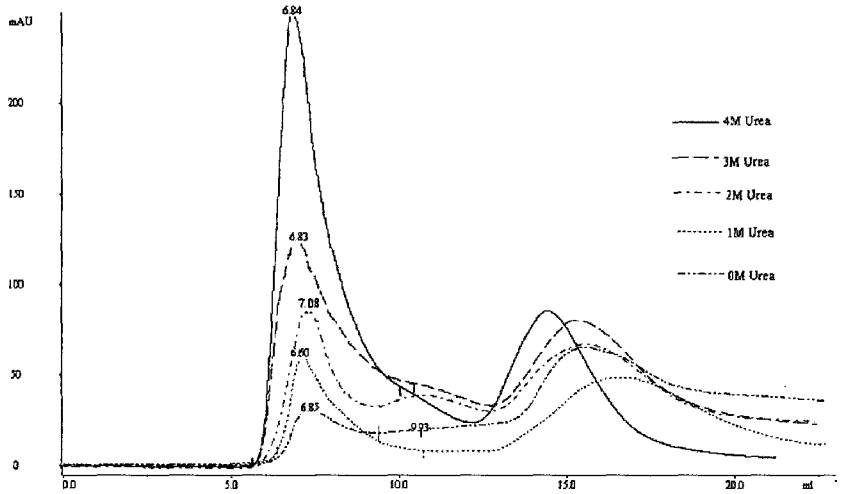

Fig. 4. Elution profiles for the SEC refolding rhIFN-γ with renaturation buffer containing different concentrations of urea. Flow rate: 0.4 mL/min; Applied sample: 0.1 mL × 9.7 mg/mL; Column height: 7 cm; Refolding buffer: urea, 10 mM EDTA, 0.15 M NaCl and 0.05 M PBS, at pH 7.0.

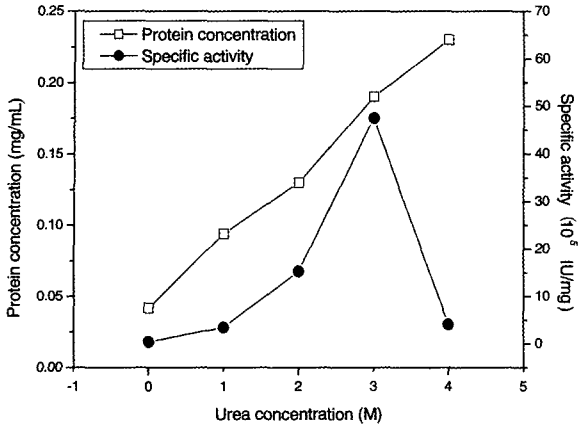

Fig. 5. Effect of urea concentration on rhIFN-γ refolding.

Table 1. Effect of protein loading mode on rhIFN-γ refolding

Application mode	1	2	3
Volume (mL) × Concentration (mg/mL)	0.1 × 15.6	0.2 × 7.8	0.5 × 3.12
Specific activity (× 10^5 IU/mg)	8.6	39.8	14.7
Protein recovery (%)	65.2	70.7	78.5
Active protein (× 10^5 IU)	8.7	43.9	18.0

Effect of Column Height on rhIFN-γ Refolding

Under the above optimized conditions, the rhIFN-γ refolding was first performed by SEC with column heights of 3.5, 5.0, and 7.0 cm, with resolutions of 0.9, 1.4 and 2.1, respectively. Basically, complete separation of the components can be assumed, using chromatography principles, with a resolution larger that 1.5. Therefore, a column height of 7.0 cm was adopted for performing the rhIFN-γ refolding. Generally, an increase in the column height benefits the separation efficiency of various intermediates, extending the refolding time for the transformation of refolding intermediates into their natural structures [23,24]. In order to determine the optimum refolding conditions, rhIFN-γ refolding using a 12-cm column was studied further under the same conditions. The results were shown in Table 2. As expected, the protein refolding was greatly improved, with the specific activity increasing to 11.9 × 10^6 IU/mg.

Process Design of Active rhIFN-γ Production

Based on the studies on fermentation of the genetically engineered bacterium pBV220/IFN-γDH5α, the purification of IBs and rhIFN-γ refolding by SEC, and the technical aspects of the production of active rhIFN-γ developed, have focused on the optimization of rhIFN-γ refolding *in vitro*. From Fig. 6, more than 1 mg quantities of correctly folded rhIFN-γ were finally obtained, with a specific activity up to 1.2 × 10^7 IU/mg.

Table 2. Effect of column height on IFN-γ refolding

Column height (cm)	7	12
Sample application (mL × mg/mL)	0.2 × 7.8	0.2 × 7.8
Protein recovery (%)	70.7	67.1
Specific activity (× 10^6 IU/mg)	4.0	11.9
Total activity (× 10^6 IU)	4.4	12.5

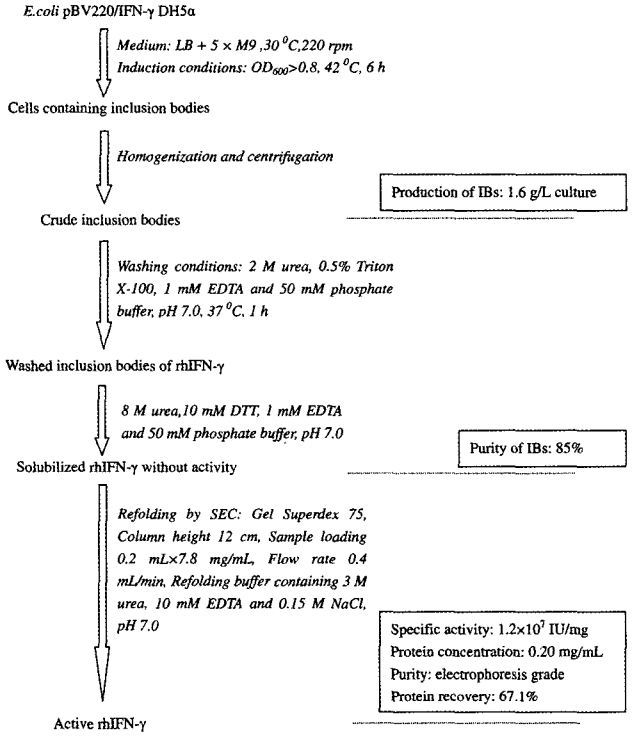

Fig. 6. Technical aspects on the production of active rhIFN-γ from genetically engineered *E. coli*.

CONCLUSION

The rhIFN-γ can successfully be simultaneously refolded and purified from inactive IBs by a process based on the SEC technique. To overcome the high insolubility of the protein, a reliable method has been developed for recovering recombinant protein, using 3 M urea in the refolding buffer, and has a dose-dependent effect on the aggregation. The recovery of bioactive proteins from IBs is a complex process, and many methods have been developed for a commercial process, and the refolding protocols should be inexpensive, highly efficient, and easy to operate. The SEC process for rhIFN-γ refolding is a valuable method, which could be used to obtain large amounts of native and active rhIFN-γ for use in clinical therapy.

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