On-Column Refolding of Recombinant Human Interferon-γ with an Immobilized Chaperone Fragment

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On-Column Refolding of Recombinant Human Interferon-γ with an Immobilized Chaperone Fragment

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The chaperone mini-GroEL is a soluble recombinant fragment containing the 191−345 amino acid sequence of GroEL with a 6×His tag. The refolding protocol assisted with mini-GroEL was studied for the activity recovery of rhIFN-γ inclusion bodies. In a suspended system, mini-GroEL showed significant enhancement of the activity recovery of rhIFN-γ, applyed with a 1−5:1 stoichiometry of mini-GroEL to rhIFN-γ at 25 °C. Moreover, 1 M urea in the renaturation buffer had a synergistic effect on suppressing the aggregation and improving the activity recovery. Finally, a novel chromatographic column, containing 1 cm height of Sephadex G 200 at the top of column and packed with immobilized mini-GroEL to promote refolding, was devised. The total activity recovered per milligram of denatured rhIFN-γ was up to 3.93×10^6 IU with the immobilized mini-GroEL column, which was reused four times without evident loss of renaturation ability. A convenient technique with the integrated process of chaperon preparation and rhIFN-γ folding in vitro was developed.

Introduction

More and more genes of interest are being expressed in foreign cells. The expression of heterologous genes in *E. coli* is by far the most available and inexpensive means for obtaining large amounts of target proteins for research or commercial purposes (*1*). However, such proteins are often produced as inactive inclusion bodies (IBs). In general, protein refolding from IBs involves three steps: isolating the IBs, dissolving them in strong denaturants, and recovering the biological activity in vitro. Correct and efficient refolding depends on many variables, which may differ from one protein to another. Protein refolding is a limiting step for bioprocess industry, and it has been a focus for basic research and application (*2*).

Since the reports that molecular chaperone played an essential role in the correct folding of newly synthesized polypeptides in vivo, researchers attempted to develop the strategy of in vitro protein refolding that was assisted by molecular chaperone (*3*, *4*). Among the variety of molecular chaperones, the system of GroEL/GroES is mostly investigated (*5*). GroEL/GroES binds to unfolded polypeptides or the folding intermediates, preventing improper interactions of polypeptide chains that lead to aggregation (*6*). Especially, recent research indicated that the *E. coli* chaperone GroEL could promote refolding of glycerol dehydrogenase and creatine kinase in the absence of GroES and ATP (*7*, *8*). With the understanding of the molecular chaperone system, the useful fragment of GroEL was cloned and expressed in *E. coli*. The soluble recombinant fragment (called mini-GroEL) containing the 191−345 amino acid sequence of GroEL and a histidine tag had efficient chaperone activity in the refolding of cyclophilin A and rhodanese (*9*). The smaller plasmid of mini-GroEL facilitates the genetic operation and chaperone preparation. This is quite valuable to simplify the refolding process and lower the cost in commercial scale.

The human interferons (hIFNs) are a family of proteins mainly consisting of hIFN-α (leucocyte), hIFN-β (fibroblast), and hIFN-γ (immune). hIFN-γ is more potent than the other two in the effects of immunomodulatory activities and anti-proliferation (*10*). Recombinant hIFN-γ is a good solution to meet the requirement of clinical therapy in large amounts. However, rhIFN-γ was expressed as IBs in *E. coli*, and it is imperative that it be refolded in vitro to get the correct conformation. Although the protocols of rhIFN-γ refolding have been established, little research on refolding assisted with chaperone (especially mini-GroEL) has been reported. Additionally, all of these protocols required large refolding reactors and quantities of buffer (*11*−*14*).

Because the preparation of molecular chaperones is time-consuming and large quantities of chaperones are needed in facilitating protein refolding, reuse of chaperones is crucial for large-scale production. In this work, a novel protocol for rhIFN-γ refolding by reuse of mini-GroEL, which combines preparation with immobilization of mini-GroEL, was studied. On the basis of the optimum renaturation condition with mini-GroEL in suspended system, an affinity chromatographic column packed with Ni-NTA resin immobilized mini-GroEL was developed to facilitate rhIFN-γ refolding. The setup not only avoided the damage of mini-GroEL by guanidine hydrochloride but also had high refolding efficiency.

Materials and Methods

Materials. Plasmid pRSET A/GroEL(191−345) cDNA, with N-terminal inserted 17-residue histidine tag gene, was transformed into *E.coli* host cell BL21. Details about

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transforming and fermentation production of mini-GroEL has been described earlier (*15*). The Ni-NTA resin was the product of Qiagen Company (Germany). ÄKTA Explorer 100, column (XK16/20), and electrophoresis apparatus were obtained from Amersham Biosciences. Gel doc 2000 was purchased from Bio-Rad Corporation. Guanidine hydrochloride (GdnCl), dithiothreitol (DTT), and trizma base were purchased from Sigma. All other chemicals commercially available were of analytical grade.

Production of rhIFN-γ Inclusion Bodies. Genetically engineered *E.coli* pBV220/IFN-DH5 contains plasmid of hIFN-cDNA inserted thermal sensitive P_RP_L promoter. A single bacterial colony was inoculated into 20 mL of LB medium containing 100 μg/mL ampicillin and grown at 30 °C O/N at 220 rpm. Then 2 mL of seed culture was transformed to a 1 L flask with 200 mL of 5×M9+LB sterile medium (15 g of tryptone, 7.5 g of yeast extract, 5 g of NaCl, 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 1 g of NH_4Cl, 3 mg of $CaCl_2$ per liter; before use, 1 mL of 1 M $MgSO_4 \cdot 7H_2O$ and 10 mL of 20% sucrose were added, and the concentration of ampicillin was adjusted to 100 μg/mL). While optical density at 600 nm was about 0.8, the cells were induced by shifting the temperature of the culture from 30 to 42 °C, and rhIFN-γ was expressed. The culture was allowed to continue for further 6 h.

The cells were then harvested by centrifugation at 1500*g* for 10 min. The precipitation was gently suspended to a homogeneous paste with 200 mL of buffer A (0.05 M Tris-HCl, pH 8.0) and centrifuged again. The washed cells were resuspended in 10 volumes of buffer A, kept in a ice−water bath and disrupted with ultrasonic for 5 s on, 3 s off, 100 rounds under 500 W of power. Followed by centrifugation at 6000*g* for 15 min at 4 °C, the pellets of rhIFN-γ IBs were obtained. The IBs were further resuspended with buffer B (buffer A containing 1.5 M urea, 0.4% Triton-X 100, 10 mM EDTA) and incubated for 30 min at 37 °C. After centrifugation, the precipitate was purified IBs.

Preparation of Solubilized rhIFN-γ. Wet IBs of 1 g were solubilized by incubating in 10 mL of buffer C (buffer A containing 6 M GdnCl and 0.01 M DTT) for 90 min at 37 °C with rolling at 120 rpm. After centrifugation at 6000*g* for 60 min at 4 °C to remove insoluble materials and cell debris, the supernatant, containing solubilized but denatured rhIFN-γ with a concentration of 13.6 mg/mL, was aliquoted into eppendorf tubes and stored at −20 °C as stock solution.

Batchwise Refolding in Suspended System. Denatured rhIFN-γ was diluted by 60-fold into renaturation buffer D (pH 7.0, 0.05 M PBS containing 5 mM EDTA; 20 mM KCl; 6.3, 63, or 126 μM mini-GroEL; 0.5, 1, 1.5 or 2 M urea, respectivelly) by rapidly vortexing at 20 °C. The renaturation system was set in a incubator rolling at 100 rpm.

Refolding with Immobilized Mini-GroEL Column Chromatography. The mini-GroEL with 6×His affinity tag could bind to the Ni-NTA resin. After mini-GroEL expression in *E. coli*, harvesting and crashing the cells, the supernatant together with Ni-NTA resin was equilibrated for 1 h with gentle mixing. The coupling density was 2.1 mg/mL resin. Then a chromatographic column (XK16/20) was packed with 12 mL of the mini-GroEL immobilized resin. At the top of the Ni-NTA resin, 1 cm of Sephadex G 200 was packed. Refolding with this column was performed using ÄKTA Explorer 100. Prior to refolding, the column was equilibrated with buffer D. After the denatured rhIFN-γ was applied, the column was instantly eluted with buffer D, and the eluted protein peak was pooled for determination of protein concentration and biological activity.

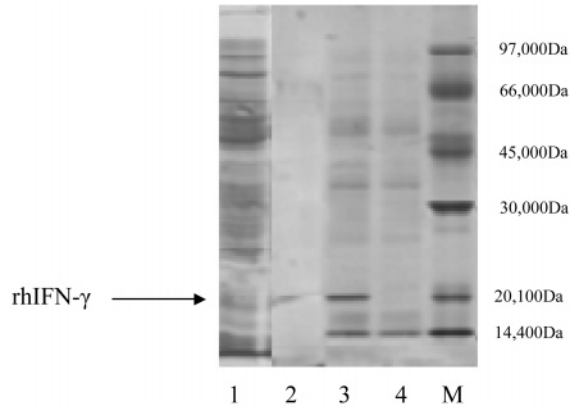

Figure 1. Expression of rhIFN-γ as inclusion bodies in *E. coli.* Lane 1: supernatant after cell disruption and centrifugation. Lane 2: supernatant after cells harvesting. Lane 3: precipitate of the supernatant in lane 1. Lane 4: cells not be induced. M: marker.

Determination of Biological Activity and Protein Concentration. After renaturation, the sample was dialyzed against buffer A at 4 °C overnight and syringed with 0.22 μm sterile membrane to remove bacteria. Antiviral activity of refolded rhIFN-γ was evaluated as described by Du (*16*). According to the standard, the assay was performed by incubating a fixed count of WISH cells with a serially diluted sample followed by challenging the cells with a defined plaque-forming units of vesicular stomatitis virus (VSV). The activity was calculated as the reciprocal of the dilution in the well of the titer plate where 50% of the WISH cell monolayer is protected from the cytopathic effect of the challenging virus. Protein concentration was determined by Coomassie Brilliant Blue assay (*17*).

SDS−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE was performed to assess rhIFN-γ expression and confirm the binding of mini-GroEL with Ni-NTA. The experiments were carried out according to Laemmli (*18*), and the results were recorded with Bio-Rad gel doc 2000.

Results and Discussion

Expression of rhIFN-γ as Inclusion Bodies. rhIFN-γ expression detected by SDS−PAGE is shown in Figure 1. The molecular weight (M_w) of the rhIFN-γ was 18 kDa, and there was no band at the position of 18 kDa (see lane 4) if the cells were not induced at 42 °C. It demonstrated that the host cells did not contain endogenous protein with M_w of 18 kDa. After induction, the target protein was not excreted into supernatant of the culture medium (See lane 2), and there was an obvious band with M_w of 18 kDa only in the pellet after cell disruption and centrifugation (see lanes 1 and 3). It could be concluded that rhIFN-γ was intracellular aggregate, i.e., expressed as inclusion bodies.

Refolding of rhIFN-γ in Suspension System. ***Effect of Temperature on Activity Recovery.*** rhIFN-γ IBs were solubilized into a high concentration of denaturant and subsequently allowed to refold into natural conformation to recover biological activity (*1*). As shown in Figure 2, the activity of refolded rhIFN-γ increased by 5- to 6-fold in the presence of mini-GroEL compared to that in the absence of mini-GroEL. It showed that mini-GroEL had a distinct effect on promoting the rhIFN-γ renaturation. When operated at 4, 25, and 37

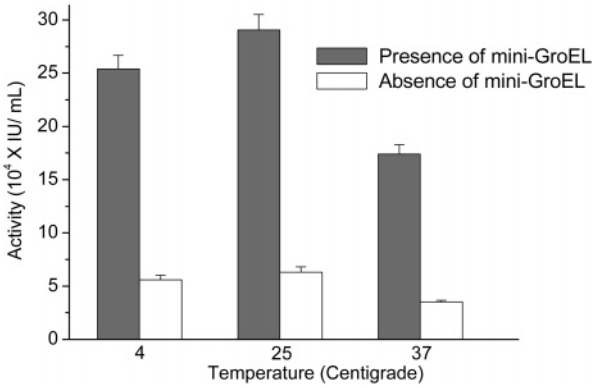

Figure 2. Effect of temperature on rhIFN-γ refolding assisted by mini-GroEL. The molar ratio of mini-GroEL to rhIFN-γ was 1:1, and the concentration of denatured rhIFN-γ was 13.6 mg/mL.

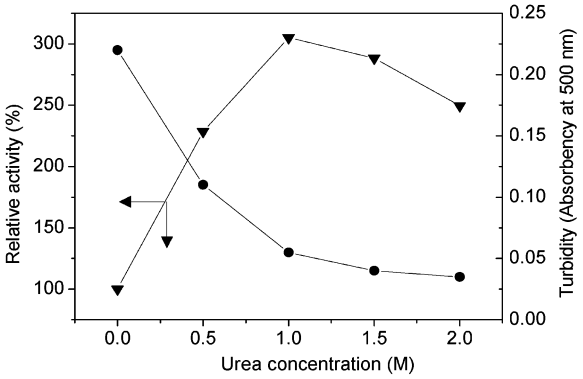

Figure 3. Effect of urea on IFN-γ refolding assisted by mini-GroEL. The molar ratio of mini-GroEL to IFN-γ was 1:1: (▼) relative activity; (●) turbidity.

Table 1. Effect of Molar Ratio of Mini-GroEL to rhIFN-γ on Refolding Efficiency

	molar ratio of mini-GroEL to rhIFN-γ			
	0.5	1	5	10
activity ($\times 10^5$ IU/mL)	3.97	7.03	8.11	5.57
total activity per mg of denatured rhIFNγ ($\times 10^6$ IU)	1.75	3.10	3.58	2.46
relative comparison (%)	56.3	100	115.4	80.0

°C in the presence of mini-GroEL, the activities of refolded rhIFN-γ were 2.54×10^5, 2.91×10^5, and 1.74×10^5 IU/mL, respectively. The formation of aggregates could be observed immediately when renaturation occurred at 37 °C, which indicated that the rate of aggregates formation was much faster, so that the mini-GroEL had no effect on aid of refolding. On the other hand, the oscillation and diffusion of molecules were too slow at 4 °C and opposed combining and releasing of mini-GroEL to rhIFN-γ. The optimum temperature was ascertained as 25 °C for refolding of rhIFN-γ in a suspension system assisted by mini-GroEL, which was in accordance with refolding cyclophilin A and rhodanese by Zahn et al. (*9*).

Effect of Urea on Activity Recovery. Denaturant concentration in the renaturation buffer is a key factor for mediating the refolding of denatured protein. As a weak chaotropic agent, urea may not only alleviate 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, optimum concentration of urea is a very efficient strategy to suppress aggregation (*2*, *19*). The synergetic effect of urea on rhIFN-γ refolding assisted by mini-GroEL was first investigated (Figure 3). After addition of 0.5, 1, 1.5, and 2 M urea to buffer D, the activities of refolded rhIFN-γ were increased by 2.3-, 3.1-, 2.9-, and 2.5-fold, respectively, compared to that with no urea present. The samples were incubated for 1 h, and the turbidity qualitatively describing the accumulation of aggregates was monitored at 500 nm by light scattering. There was a sharp decrease in the turbidity of renaturation buffer with the increase of urea from 0 to 1 M and then a slight decrease (Figure 3). So presence of urea had a dose-dependent effect on the aggregation. Golbik et al. (*20*) reported that 50% of the mini-GroEL lost renaturation activity when incubated in 2.9 M urea solution. The concentration of urea for suppressing protein aggregation in renaturation buffer commonly recommended is 2−3 M. However, the optimum urea was 1 M for activity recovery of rhIFN-γ with mini-GroEL present, which referred to the side effect of a high concentration of urea to mini-GroE. It was also implied that mini-GroEL played an important role in rhIFN-γ refolding.

Effect of Amount of Mini-GroEL on Activity Recovery. To promote protein refolding in vitro, the mini-GroEL should bind with rhIFN-γ molecules. The effect of the molar ratio of mini-GroEL to rhIFN-γ on refolding efficiency is shown in Table 1. The results denote that the molar ratio also has an evident influence on the activity recovered of rhIFN-γ when refolding is assisted with mini-GroEL, and the maximum activity and the maximum of total activity recovered per mg denatured rhIFN-γ were 8.11×10^5 IU/mL and 3.58×10^6 IU at the molar ratio of 5. Taking the activity recovered at the molar ratio of 1 as 100%, the relative activity was 56.3%, 115.4%, and 80.0% when the molar ratio was 0.5, 5, and 10, respectively. The activity recovery decreased while mini-GroEL was at high concentrations; the possible reason was that superfluous mini-GroEL molecules increased intermolecular hydrophorbic interactions of rhIFN-γ leading to nonspecific aggregation. The mini-GroEL should be reusable so that rhIFN-γ refolding in vitro with mini-GroEL is practicable in industrial production. The immobilization of mini-GroEL may be a good method.

Refolding of rhIFN-γ with Immobilized Mini-GroEL. ***Immobilization of Mini-GroEL by Ni-NTA Resin.*** The 6×His tag was introduced to genetically engineered technology so that it could be more convenient to capture the desired protein, and it allows the immobilization of mini-GroEL with a 6×His tag protein on metal-chelating surfaces such as Ni-NTA.

After Ni-NTA resin was incubated in the supernatant of disrupted cells, the purity of desired protein binding on resin could be detected. Then elution of column packed with immobilized mini-GroEL resin was performed using ÄKTA explorer 100; the elution profile is depicted in Figure 4. Large quantities of impurities were eluted with buffer I, and subsequently the peak of mini-GroEL was collected when eluted with buffer II containing imidazole. As determined by SDS−PAGE, there was only one band of mini-GroEL in the collected fractions (Figure 5). This demonstrated that high purity mini-GroEL could be obtained using Ni-NTA resin affinity attachment. In most cases, the 6×His tag does not interfere with the structure or function of the purified protein, and hence studies on refolding of rhIFN-γ with immobilized mini-GroEL chromatography can proceed.

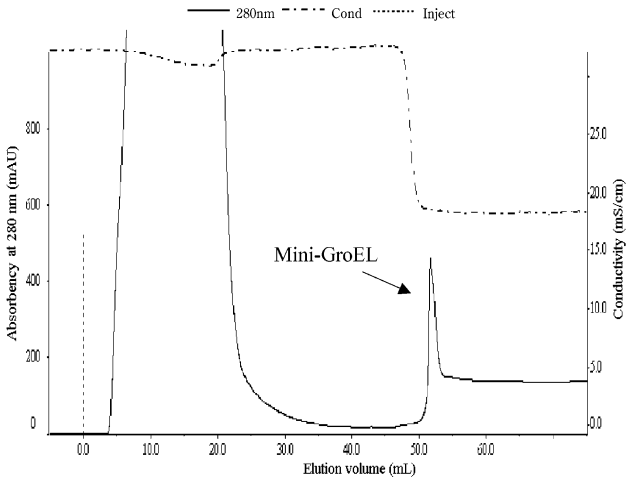

Figure 4. Elution profile for purification of mini-GroEL with Ni-NTA affinity chromatography (XK16/20 column packed with 12 mL of Ni-NTA, flow rate 0.5 mL/min). Buffer I: elution of unbound protein, pH 8.2, 0.05 M Tris-HCl, 0.3 M NaCl. Buffer II: elution desired protein, pH 8.2, 0.05 M Tris-HCl, 0.15 M NaCl, 0.25 M imidazole).

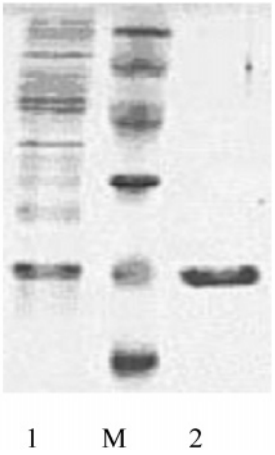

Figure 5. Purification of mini-GroEL with Ni-NTA affinity chromatography. Lane 1: the supernatant. Lane 2: elution of desired protein. M: marker, as shown in Figure 1.

Immobilized Mini-GroEL Column for Refolding. The stability of the 6×His and Ni-NTA binding is not affected by strong denaturants such as 6 M GdnCl or 8 M urea. However, the concentration of GdnCl was relatively high at the top of column when the denatured rhIFN-γ in 6 M GdnCl was loaded. This would impair the mini-GroEL renaturation activity, because it was reported that 50% of the mini-GroEL lost renaturation activity under the effect of 2.9 M urea solution (*20*). An immobilized mini-GroEL column for rhIFN-γ refolding was devised to avoid the damage and guarantee the process of refolding, as shown in Figure 6. At the front of the column, Sephadex G 200 was packed to a height of 1 cm. The exclusive molecular weight of the gel ranges between 5000 and 6×10^5 Da, so GdnCl and most of the rhIFN-γ molecules could penetrate into the gel pore. The separation between GdnCl and denatured rhIFN-γ was slowly proceeded to avoid aggregate formation, and meanwhile, GdnCl was diluted moderately. When the rhIFN-γ sample passed through the resin with immobilized mini-GroEL and was eluted by renaturation buffer, the process of refolding could be achieved with the aid of mini-GroEL. Finally, the refolded rhIFN-γ was outflowing from the column.

On-Column Refolding of rhIFN−γ with Immobilized Mini-GroEL. When loading 0.1 mL of 0.05 M PBS containing 6 M GdnCl and eluting to test the immobilized mini-GroEL column, the absorbency at 280 nm remained at almost zero. It showed that there was little negative influence of 6 M GdnCl on the immobilization.

As indicated (*21*), the apparent rate of lysozyme refolding decreased as a result of the resistance of diffusion between the resin and fluid phase after chaperones were immobilized, and more time should be needed for refolding than in suspended system. On the other hand, the steric exclusion of immobilized chaperones lowered the collision efficiency of protein with chaperone molecules. Hence the activity of refolded lysozyme increased with the decrease of elution flow rate. In the present research, the relatively low flow rate was adopted to prolong the retention time of rhIFN-γ. Figure 7 describes the elution profile of refolded rhIFN-γ at a linear flow rate of 1.25×10^{-3} m/min; the peak of elution was pooled for activity assay.

According to the retention theory of chromatography, the equation is written as

$$K = \frac{V_e - V_0}{V_t - V_0}$$

where V_t, V_0, and V_e denote the total gel volume in the column, void volume, and average retention volume of the desired protein, respectively. K is the partition coefficient of protein, and the magnitude of K shows how the desired protein interacts with the matrix. The present column for rhIFN-γ refolding contained 12 mL of Ni-NTA resin and 2 mL of Sephadex G 200, giving a void volume of 4.2 mL and 0.56 mL. The retention volume is 20.7 mL as shown in Figure 7. Accordingly, the predicted value of K is 1.72. It was confirmed that the mini-GroEL interacted strongly with rhIFN-γ molecules when they passed through the column.

From the above designed chromatography, the pooled volume, protein concentration, activity of refolded rhIFN-γ, and the total activity recovered per milligram of denatured rhIFN-γ were 6.0 mL, 0.14 mg/mL, 8.90×10^5 IU/mL, and 3.93×10^6 IU, respectively. The total activity per milligram of denatured rhIFN-γ with immobilized mini-GroEL column refolding was higher than that with free mini-GroEL refolding in a suspended system. The reason might be as follow. First, the soluble and partially folded intermediates were formed under optimum concentration of GdnCl in gel media, and it was helpful to increase the rate of rhIFN-γ refolding when samples subsequently entered the immobilized mini-GroEL column. Second, comparted by gel media pore, protein aggregation was reduced. In addition, the microdomain structure of rhIFN-γ was formed transiently when it bound to mini-GroEL, and the structure was broken under the effect of fluid flowing. However, the microdomain structure of rhIFN-γ was formed again when binding to the next mini-GroEL and then broken instantly again. During the cycle of the microdomain structure formed-broken, the correct folded structure was retained and the incorrect folded structure was reverted to refold again, driven by thermodynamic stability.

Reuse of Immobilized Mini-GroEL. The immobilized mini-GroEL was reused for four times. Taking the activity of refolded rhIFN-γ as 100% for the first batch refolding, the relative activities varied in the range of 90% and 105% for the other three batches of refolding (Figure 8), and there was no evident difference in activity

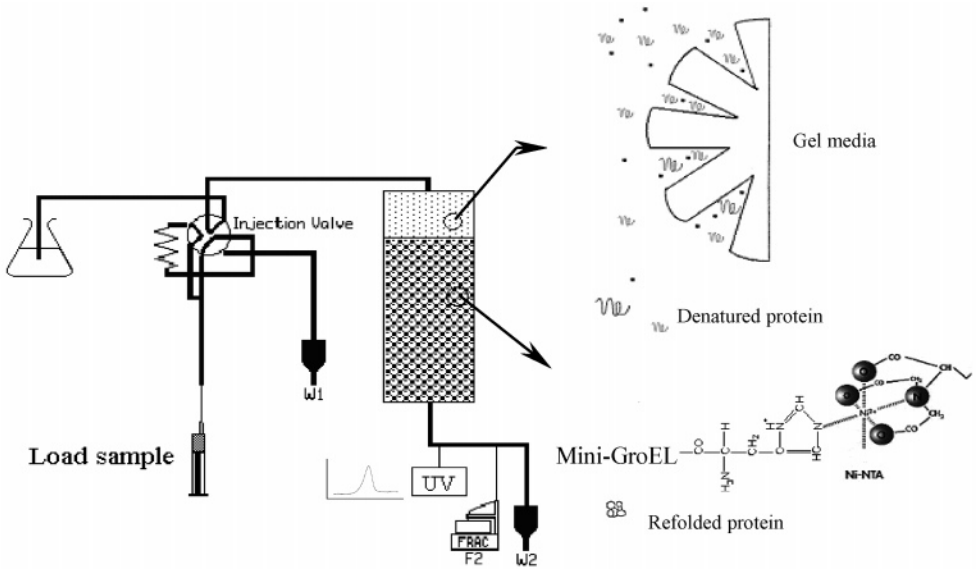

Figure 6. Schematic diagram of experimental setup and the process of refolding assisted with immobilized mini-GroEL.

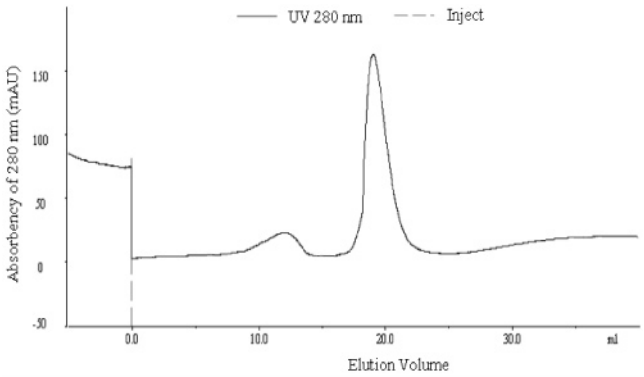

Figure 7. Elution profile of refolded rhIFN-γ with immobilized mini-GroEL chromatography. Application volume and concentration were 0.1 mL and 13.6 mg/mL; renaturation buffer containing 1 M urea.

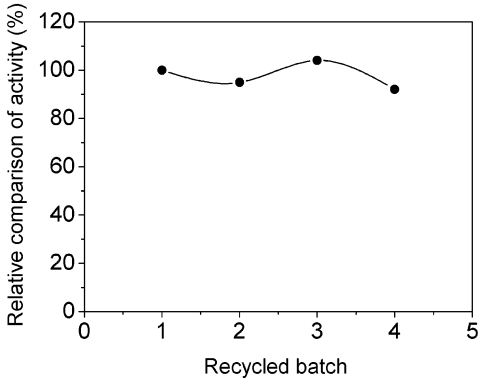

Figure 8. Reuse of immobilized mini-GroEL column for rhIFN-γ refolding

recovery for each batch. It was reported that by ultrafiltration recovery, 50% of GroEL was lost after continuous recycled use for five times (*21*). Hence not only was immobilization of mini-GroEL to Ni-NTA resin easy to do but it also allowed the mini-GroEL to be reused.

Conclusions

Although the specific mechanism of protein refolding promoted by chaperone is not entirely clear at the present, the central cavity provided by the system of GroEL/GroES was used to illuminate protein refolding in the presence of chaperone (*22*). The refolding of rhIFN-γ molecules does not necessarily require the central cavity of intact GroEL. However, a 1−5:1 stoichiometry of mini-GroEL to rhIFN-γ was indeed helpful for activity recovery, where the value of K was 1.72. The monodispersal of the mini-GroEL had the assistant effect of combining protein molecules so that they could be folded in a segregated unit, as mimicking some aspects of a "folding cage".

The recovery of bioactive proteins from IBs is a complex process, and many methods have been developed for protein refolding in vitro (*23*). To be practicable for commercial process, refolding protocols should be inexpensive, highly efficient, and easy to operate. In this paper, the correct refolding of rhIFN-γ inclusion bodies was enhanced under the synergistic effect of mini-GroEL and urea. Finally, a novel setup with an immobilized mini-GroEL column, which was easy to operate and reusable, was developed for rhIFN-γ refolding. The total activity recovered per milligram of denatured rhIFN-γ IBs was 3.93×10^6 IU. With the integrative process of chaperon preparation and protein refolding, the immobilized mini-GroEL chromatography is a successful trial for rhIFN-γ folding in vitro. According to the mechanism of "folding cage", this protocol could possibly be used to refold other recombinant proteins that would bind with mini-GroEL.

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