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Chromatographic refolding of recombinant human interferon gamma by an immobilized sht GroEL191-345 column

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Abstract

Minichaperone sht GroEL191-345 was covalently coupled to NHS-activated Sepharose Fast Flow gel. Refolding of recombinant human interferon gamma (rhIFN-γ) was carried out on a chromatographic column packed with immobilized minichaperone. The effects of salt concentration, urea concentration gradient, elution flow rate and protein loading on the refolding efficiency were investigated. The results indicated that immobilized sht GroEL191-345 chromatography was an effective protocol for the refolding of rhIFN-γ. When loading 100 μl denatured rhIFN-γ (17.8 mg/ml), the protein mass recovery and total activity obtained in this optimal process reached 74.25% and 6.74×10^6 IU/ml, respectively with the immobilized minichaperone column which was reused for 10 times with 25% decrease of renaturation capacity.

Keywords: Minichaperone; sht GroEL191-345; Immobilization; Recombinant human interferon gamma; Chromatographic refolding; Inclusion body

1. Introduction

The production of genetically engineered proteins from *Escherichia coli* was limited by 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]. However, protein aggregation appeared in the refolding step limited the total yield of downstream processing, and isolation of product from aggregates must be subsequently undertaken. Many attempts [2–4] have been made to improve in vitro refolding, such as optimization of the physicochemical properties of the refolding environment and developments of new refolding strategy. In recent years, refolding by chromatography methods [5] has been developed rapidly, including ion-exchange chromatography [6], chelating chromatography [7], hydrophobic interaction chromatography [8] and size-exclusion chromatography (SEC) [9], etc.

In 1996, Zahn [10] isolated the monomeric apical domain of GroEL that had residual activity (the minichaperone core) and expressed the fragment in *E. coli*. Minichaperone GroEL, containing a full-length substrate binding domain, could bind to the unfolded protein in solution, thus efficiently preventing aggregation of folding intermediates [11,12]. The sht GroEL191-345 with a His_6 tag at N-terminal (molecular weight 18 kD), one of the recombinant GroEL minichaperones, was proved to facilitate the in vitro refolding of several proteins such as rhodanese and cyclophilin A in the absence of GroES and ATP, and had some of the activities of intact GroEL in vivo [13]. Altamiranno [14] successfully employed immobilized minichaperones both in column chromatography and batchwise to refold indole 3-glycerol phosphate synthase lacking residues 1–48 in high yield with biological activity. Recently, chaperone-induced refolding of the scorpion toxin Cn5, a substrate recalcitrant to any conventional refolding procedure in significant recovery, demonstrated unambiguously the effectiveness of minichaperones both when immobilized and free in solution [15]. The smaller plasmid of mini-GroEL facilitates the genetic operation and chaperone preparation. This is valuable to simplify the refolding process of recombinant proteins and decrease the cost in commercial scale.

Interferon gamma is a product of activated T lymphocytes and natural killer (NK) cells and exhibits pleiotropic biological activities. Now the recombinant DNA techniques made it possible to

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produce human IFN-γ in large amounts. However, recombinant human interferon gamma (rhIFN-γ) was expressed as inclusion bodies in *E. coli*, and the recovery of biologically active proteins became imperative. Our previous work [16] demonstrated that the efficient in vitro refolding of rhIFN-γ was possible in the presence of free sht GroEL191-345. Furthermore 6 × His tagged mini-chaperone was immobilized on Ni-NTA resin by affinity interaction in the course of mini-chaperone purification [7], which was an integrated process of protein purification and immobilization and could simplify the operation steps. But we found that the immobilization efficiency was not high enough due to the interference of impurities. With the aim of enhancing and widening the use of the immobilized sht GroEL191-345 chromatography, here mini-chaperone was immobilized on NHS-activated Sepharose Fast Flow gel by covalent bonding interaction after the purification of protein and we applied this protocol to the refolding of rhIFN-γ and optimized the operation parameters.

2. Experimental

2.1. Plasmid and strain

The plasmid pRSET A carrying the full length of sht GroEL191-345 cDNA was a gift from Professor Fersht in Centre for Protein Engineering MRC, Cambridge. *E. coli* BL21(λDE3) [F^-, ompT, hsdSB, r_B^-, m_B^- (λ Ci857, ind1, Sam7, nin5, lacUV5-T7.AL)] pLysS was used as the cloning and expression host cells. Genetically engineered bacteria (pBV220/IFN-γDH5α), wish cell and Vesicular Stomatitis Virus (VSV) were kept by Institute of Bioengineering in Zhejiang University.

2.2. Chemicals

Guanidine hydrochloride (GdnHCl), tris(hydroxymethyl) aminomethane (Tris), ethylenediamine tetraacetic disodium salt (EDTA-Na), dithiothreitol (DTT), Triton X-100, NaH_2PO_4 and Na_2HPO_4 were purchased from Sigma (St. Louis, USA). All other chemicals were of analytical grade. Buffers were prepared with deionized water filtrated by 0.22 μm membrane (Millipore, Bedford, USA). Ni-NTA agarose was obtained from Qiagen (Hilden, Germany). NHS-activated Sepharose Fast Flow was from Amersham Biosciences (Uppsala, Sweden).

2.3. Analytical methods

Cells growth was monitored by measuring the optical density at 600 nm. SDS-PAGE with 15% running gel and 5% stacking gel was used to detect proteins, which were stained with Coomassie brilliant blue R-250 dye [17]. The protein purity was evaluated by the GD 2000 gel documentation system (Bio-rad, Hercules, USA). The total protein concentration was determined by a modified Bradford's method [18] using bovine serum albumin (BSA) as a reference. The rhIFN-γ samples were taken in triplicate for activity assay [19], and the average value was used to denote the total activity.

2.4. Production of sht GroEL191-345

E. coli strain BL21(DE3) pLysS was transformed with pRSET A plasmid and transformants were screened on Luria–Bertani broth (LB) plates containing 50 μg/ml ampicillin (Amp). Several transformant colonies were transferred to 50 ml of 2 × YT medium in 250 ml shaking flasks and cultured overnight at 37 °C with rotating speed 220 rpm, then the broth was inoculated into 500 ml shaking flasks with 150 ml fortified M9 medium, where both seed and fermentation medium were supplemented with 50 μg/ml Amp. The bacteria were cultured at 37 °C until the optical density (OD_{600}) reached 0.4–0.6, and then IPTG was added to a final concentration of 1 mM. After induction at 33 °C for 4 h, the cells were harvested by centrifugation at 5000 × *g* for 5 min at 4 °C, and then resuspended in 50 mM Tris buffer pH 8.0 (300 mM NaCl and 10 mM imidazole). Followed by ultrasonication on ice bath for 3 s × 120 to disrupt the cells, the supernatant was then recovered by centrifugation at 17,000 × *g* for 60 min at 4 °C. The clarified supernatant was directly loaded on a Ni-NTA agarose column (16 mm × 40 mm) at a flow rate 0.5 ml/min and monitored by absorbance at 280 nm. The column was washed by two column volumes (CVs) of 50 mM Tris buffer pH 8.0 (300 mM NaCl and 10 mM imidazole) and eluted with two CVs of 50 mM Tris buffer pH 8.0 (300 mM NaCl and 75 mM imidazole). Protein fractions were collected and desalted using HiPrep 26/10 desalting column (Amersham Biosciences, Uppsala, Sweden). The purification procedure was performed on ÄKTA Explorer 100 (Amersham Biosciences, Uppsala, Sweden). Finally, the minichaperone was concentrated by Amicon Ultra-4 Filter Units (NMWL 5000).

2.5. Immobilization of sht GroEL191-345

The sht GroEL191-345 was immobilized on NHS-activated Sepherose Fast Flow gel described as Altamirano [15]. Firstly minichaperone was rapidly diluted to about 15 mg/ml in the coupling buffer pH 8.3 (50 mM $NaHCO_3$ and 500 mM NaCl) and mixed with the gel end-over-end for 6 h at room temperature, followed by washing with the coupling buffer. Then nonreacted ligands were blocked by the blocking buffer pH 8.3 (2.5 M ethanolamine and 500 mM NaCl) for 4 h at room temperature. Finally, the gels were washed with five cycles of 100 mM Tris buffer pH 7.8 (500 mM NaCl) followed by 100 mM acetate buffer pH 4.0 (500 mM NaCl) to remove nonimmobilized minichaperone. The coupling density of sht GroEL191-345 was approximately 5 mg protein per ml of the swollen gel.

2.6. Solubilization of rhIFN-γ inclusion bodies

The rhIFN-γ was expressed in an insoluble form as inclusion bodies, as previously reported [20]. The cell pellets were collected by centrifugation, resuspended in 100 ml of 50 mM Tris buffer pH 8.0 (1 mM EDTA) and lysed by ultrasonication on ice bath. Cellular lysates were centrifuged at 8000 × *g* for 15 min. Then inclusion bodies were washed with 15 ml of 50 mM Tris buffer pH 8.0 (0.5% Triton X-100) and incubated at 37 °C for 2 h. Finally the pellets were solubilized in 50 ml denaturation buffer

pH 7.7 (30 mM sodium phosphate, 8 M urea, 10 mM DTT, 1 mM EDTA and 150 mM NaCl) at 37 °C for 12 h.

2.7. Batchwise refolding of rhIFN-γ by free and immobilized sht GroEL191-345

Denatured rhIFN-γ, diluted to 5 ml with the refolding buffer, was refolded for 2 h at room temperature. The sample was then slowly dialyzed at 4 °C for 12 h in 30 mM sodium phosphate buffer pH 7.7 and insoluble materials were removed by centrifugation at 8000 × *g* for 15 min.

Denatured rhIFN-γ was rapidly mixed with a suspension of immobilized sht GroEL191-345 gel to the final system volume 5 ml containing 1 ml of the swollen gel. After incubation for 2 h at room temperature end-over-end, the supernatant was collected for dialysis as above.

2.8. Chromatographic refolding of rhIFN-γ by immobilized sht GroEL191-345

Chromatographic refolding was conducted on a 5 ml immobilized sht GroEL191-345 column at room temperature. Firstly the column was equilibrated with urea, then denatured rhIFN-γ sample of 17.8 mg/ml was applied directly to the column at a flow rate of 1 ml/min and eluted with the refolding buffer containing 150 mM NaCl and 1 M urea. The fractions were pooled for dialysis after refolding. In the experiment, the rhIFN-γ refolding with an immobilized chaperone fragment column chromatography was performed on ÄKTA Explorer 100. Each experiment was carried out in triplicate, the mean values were adopted. The effects of NaCl concentration in the equilibrium buffer (150, 300 and 500 mM), pre-equilibrium mode (urea non-gradient of buffer A containing 500 mM NaCl and 1 M urea, and a linear urea gradient of buffer A containing 500 mM NaCl and 1 M urea and buffer B containing 500 mM NaCl and 8 M urea), elution flow rate (0.025, 0.05, 0.1 and 0.2 ml/min) and loading amount (50, 100, 200 and 500 μl) on the refolding were investigated in detail.

3. Results and discussion

3.1. Expression of rhIFN-γ as inclusion bodies

The rhIFN-γ expression, detected by SDS-PAGE was shown in Fig. 1. The molecular weight (M_W) of the rhIFN-γ is 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, which would otherwise interfere with subsequent SDS-PAGE analysis. 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 (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 IBs was up to 85%. Then the purified IBs were solubilized with the denaturing buffer.

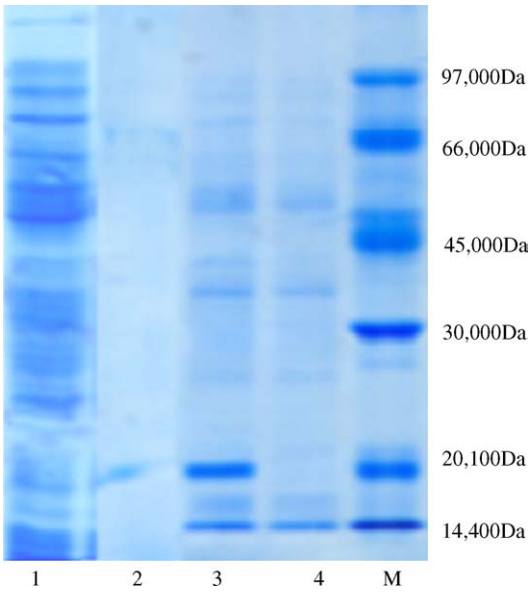

Fig. 1. SDS-PAGE of expression of rhIFN-γ as inclusion bodies in *E. coli*. Lane 1: supernatant of cell lysate after centrifugation; lane 2: supernatant of cell culture broth; lane 3: pellet of cell lysate after centrifugation; lane 4: cells not induced; M: marker.

3.2. Comparison of batchwise refolding of rhIFN-γ by free and immobilized sht GroEL191-345

A comparison of the batchwise refolding of rhIFN-γ by free and immobilized sht GroEL191-345 was given in Table 1. The refolding buffer is 30 mM sodium phosphate buffer pH 7.7 containing 1 M urea, 1 mM EDTA and 150 mM NaCl and the optimal mol ratio of sht GroEL191-345 versus rhIFN-γ in the refolding system is 2:1, according to our previous work [7]. During the refolding, the immobilization of sht GroEL191-345 on agarose prevented the co-aggregation of sht GroEL191-345 and rhIFN-γ while raised the chance of hydrophobic binding individually. As a result, the recovery of active refolded rhIFN-γ enhanced.

3.3. Chromatographic refolding of rhIFN-γ by immobilized sht GroEL191-345

As stated above, immobilized sht GroEL191-345 refolding system is the preferred mode of rhIFN-γ renaturation. We then packed a column (1 cm × 5 cm) with immobilized sht GroEL191-345 by NHS-activated Sepharose Fast Flow gel. Experiments on chromatographic refolding of rhIFN-γ by immobilized chaperone fragment were carried out and the process mechanism was discussed.

3.3.1. Effect of NaCl concentration in the equilibrium buffer

Immobilized sht GroEL191-345 chromatographic refolding of rhIFN-γ is based on the interaction between the hydropho-

Table 1
Comparison of batchwise refolding of rhIFN-γ by free and immobilized sht GroEL191-345 (protein loading per refolding system volume 150 μg/ml)

Refolding mode	Protein mass recovery (%)	Total activity (10^5) (IU/ml)	Specific activity (10^7) (IU/mg)
Dilution	14.77 ± 0.76	2.88 ± 0.15	1.95 ± 0.08
Free sht GroEL191-345 mediated	32.50 ± 1.40	6.63 ± 0.28	2.04 ± 0.12
Immobilized sht GroEL191-345 mediated	47.50 ± 1.90	9.67 ± 0.39	2.04 ± 0.09

bic surfaces of the minichaperone and denatured rhIFN-γ. Since the refolding mechanism is quite similar to hydrophobic interaction chromatography (HIC) [21], the rhIFN-γ refolding was characterized with the variation of the NaCl concentration (150, 300 and 500 mM, respectively) in the equilibrium buffer. The chromatograms obtained from different NaCl concentrations in the equilibrium buffer were shown in Fig. 2. The peaks eluting at volume of 3 ml (peak I) and 8 ml (peak II) were assigned to be unbound and refolded rhIFN-γ, respectively. Fig. 2 demonstrated that the increase of the NaCl concentration in the equilibrium buffer between 150 and 500 mM enhanced the hydrophobic adsorption between the minichaperone and denatured rhIFN-γ because the peak area of unbound rhIFN-γ decreased and that of refolded protein increased. Fractions collected between elution volume of 6.5 and 12 ml were pooled and assayed. The results shown in Fig. 3 indicated that higher NaCl concentrations in the equilibrium buffer than in the refolding buffer benefited the rhIFN-γ refolding.

3.3.2. *Pre-equilibrium with a linear urea gradient*

Gu [9] developed a size-exclusion chromatography (SEC) pre-equilibrated with a linear decreased urea gradient for lysozyme refolding. Due to its larger molecular mass, the proteins migrated faster than the gradient formed by small molecules of urea. Therefore, the denatured proteins experienced the gradual decrease in urea concentrations while they went through the column, and refolded into the native conformation. In order to enhance the refolding recovery of rhIFN-γ, we injected samples of denatured rhIFN-γ on the immobilized sht GroEL191-345 column equilibrated with a urea non-gradient,

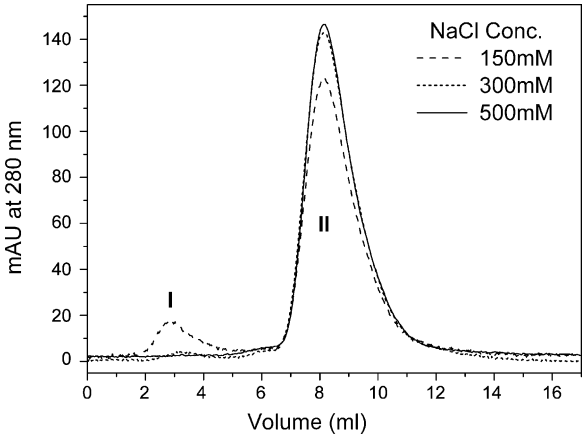

Fig. 2. Chromatograms of rhIFN-γ refolding by immobilized sht GroEL191-345 chromatography (peak I: unbound rhIFN-γ; peak II: refolded rhIFN-γ. The equilibrium buffer containing 1 M urea, elution flow rate 0.05 ml/min, protein loading 100 μl).

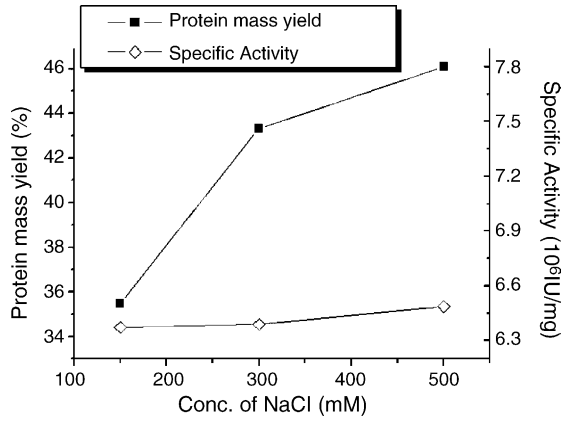

Fig. 3. Effects of NaCl concentration in the equilibrium buffer on rhIFN-γ refolding by immobilized sht GroEL191-345 chromatography.

i.e. buffer A (500 mM NaCl and 1 M urea) and with a linear urea gradient, i.e. a linear gradient of buffer A (500 mM NaCl and 1 M urea) and buffer B (500 mM NaCl and 8 M urea), respectively. The results of these experiments are shown in Fig. 4. As expected, an obvious enhancement of protein mass recovery and total activity of refolded rhIFN-γ was observed after the column was equilibrated with a linear urea gradient.

3.3.3. *Effect of elution flow rate on the refolding*

Elution flow rate influenced the retention time of rhIFN-γ in the immobilized sht GroEL191-345 column, which of course determined refolding time of rhIFN-γ. To study the effect of the flow rate on the refolding recovery, denatured rhIFN-γ was eluted at 0.025, 0.05, 0.1 and 0.2 ml/min. As shown in Fig. 5,

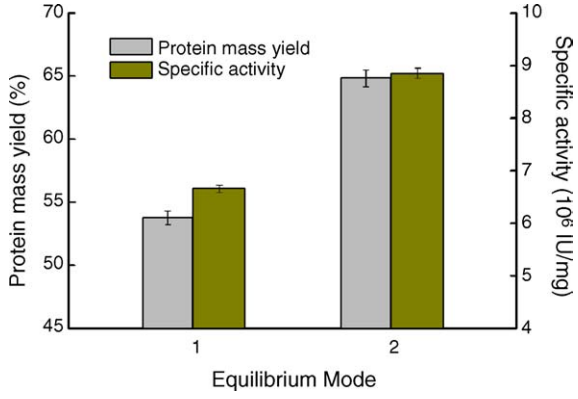

Fig. 4. Comparison of rhIFN-γ refolding by immobilized sht GroEL191-345 chromatography pre-equilibrated with urea non-gradient of buffer A (mode 1) and with a linear urea gradient of buffer A and B (mode 2) (buffer A: 500 mM NaCl and 1 M urea, buffer B: 500 mM NaCl and 8 M urea, elution flow rate 0.05 ml/min, protein loading 100 μl).

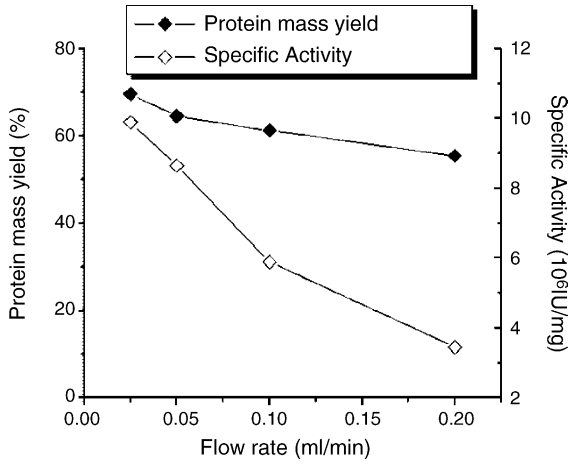

Fig. 5. Effects of flow rate on rhIFN-γ refolding by immobilized sht GroEL191-345 chromatography (pre-equilibrium with a linear urea gradient of buffer A containing 500 mM NaCl and 1 M urea and buffer B containing 500 mM NaCl and 8 M urea, protein loading 100 μl).

both protein mass recovery and total activity of refolded rhIFN-γ increased by reducing the elution flow rate. The reason is presumably that urea was removed from unfolded rhIFN-γ more gently in the lower elution flow rate, ensuring that protein experienced more favorable microenvironment for renaturation.

3.3.4. Effect of protein loading on the refolding

Different amounts of denatured rhIFN-γ (0.89–8.9 mg) were correspondingly injected on the immobilized sht GroEL191-345 column. In the kinetic competition between folding and aggregation, the folding process is a first-order reaction, while aggregation is an intermolecular reaction involving at least two polypeptide chains [3]. Thus, the key factors affecting refolding recovery of rhIFN-γ in this case were expected to be the amount of protein loading and 50, 100, 200 and 500 μl denatured rhIFN-γ (17.8 mg/ml) were investigated, which was shown in Fig. 6. As the amount of protein loading increased, a sharp decrease of protein mass recovery and total activity of refolded rHuIFN-γ occurred.

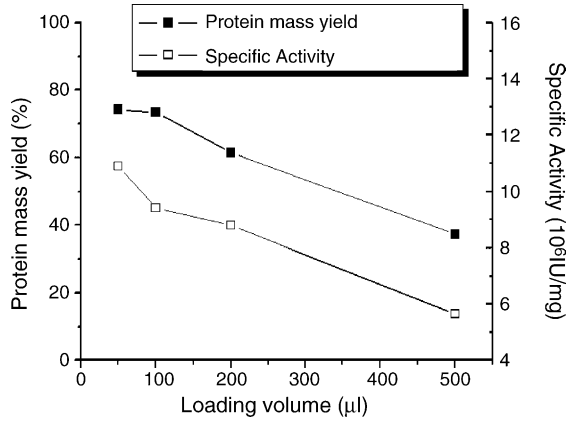

Fig. 6. Effects of protein loading on rhIFN-γ refolding by immobilized sht GroEL191-345 chromatography (pre-equilibrium with a linear urea gradient of buffer A containing 500 mM NaCl and 1 M urea and buffer B containing 500 mM NaCl and 8 M urea, elution flow rate 0.05 ml/min).

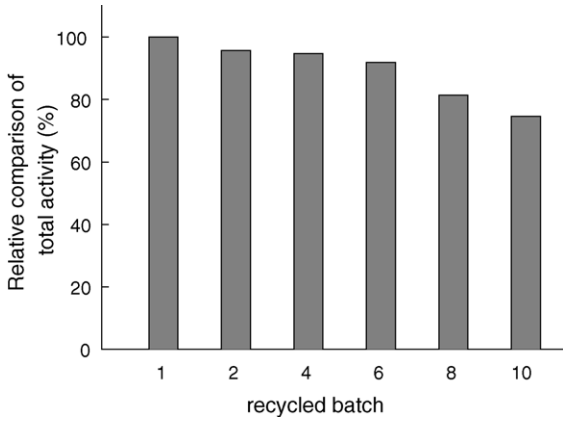

Fig. 7. Protein total activity of sequential 10 batches operation on rhIFN-γ refolding by immobilized sht GroEL191-345 (pre-equilibrium with a linear urea gradient of buffer A containing 500 mM NaCl and 1 M urea and buffer B containing 500 mM NaCl and 8 M urea, elution flow rate 0.05 ml/min, protein loading 100 μl).

Compared with the typical amounts loaded for column refolding on an ion-exchange column, the amounts of protein loaded onto the column were not high enough. The reason may be that when loading samples of denatured rhIFN-γ onto the immobilized mini-chaperone column, mini-chaperone itself possibly was partly denatured because the buffer included denaturant 8 M urea. This problem is being studied.

3.4. Reutilization of the immobilized sht GroEL191-345 chromatographic column

Guise [22] reported that by ultrafiltration recovering evident lost of GroEL was observed after continuous recycled use in the refolding assisted by free chaperone. In our batchwise refolding experiment, the affinity chromatography was employed due to the short histidine tag attached to the GroEL191-345. But compared with chromatographic refolding, the addition of protein refolding assistant of free chaperone will burden the downstream processing. Commercially the immobilized sht GroEL(191-345) column generally should be recycled to reduce the cost. Here taking the activity of refolded rhIFN-γ as 100% for the first batch refolding, the total activity decreased 25% after 10 batches reuse (see Fig. 7). It was estimated that 5–10% of minichaperone lost during each run. Hence immobilization of mini-GroEL to NHS-activated Sepharose resin not only was easy to be done, but also could be reused.

4. Conclusions

Results reported here demonstrated an effective refolding technique for rhIFN-γ recovered from *E. coli* inclusion bodies, which might be potential in the biotechnology industry. Immobilized sht GroEL191-345 chromatographic column was pre-equilibrated with a linear urea gradient, then samples were loaded onto the column in a high-salt buffer (8 M urea and 500 mM NaCl) and eluted by a low-salt buffer (1 M urea and 150 mM NaCl). Considering the practical application, the appropriate flow rate was ascertained to be 0.05 ml/min. When 100 μl

denatured rhIFN-γ (17.8 mg/ml) was loaded on 5 ml chromatographic column, i.e. about 350 μg protein loading per ml of the swollen gel, the product concentration was 0.3 mg/ml after elution. The protein mass recovery and total activity obtained in this optimal process reached 74.25% and 6.74×10^6 IU/ml, respectively with the immobilized minichaperone column which was reused for 10 times with 25% decrease of renaturation ability.

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