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REFOLDING OF RECOMBINANT HUMAN INTERFERON α-2a FROM *Escherichia coli* BY UREA GRADIENT SIZE EXCLUSION CHROMATOGRAPHY

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Protein refolding is still a puzzle in the production of recombinant proteins expressed as inclusion bodies (IBs) in *Escherichia coli*. Gradient size exclusion chromatography (SEC) is a recently developed method for refolding of recombinant proteins in IBs. In this study, we used a decreasing urea gradient SEC for the refolding of recombinant human interferon α-2a (rhIFNα-2a) which was overexpressed as IBs in *E. coli*. In chromatographic process, the denatured rhIFNα-2a would pass along the 8.0–3.0 M urea gradient and refold gradually. Several operating conditions, such as final concentration of urea along the column, gradient length, the ratio of reduced to oxidized glutathione and flow rate were investigated, respectively. Under the optimum conditions, 1.2×10^8 IU/mg of specific activity and 82% mass recovery were obtained from the loaded 10 ml of 1.75 mg/ml denatured protein, and rhIFNα-2a was also purified during this process with the purity of higher than 92%. Compared with dilution method, urea gradient SEC was more efficient for the rhIFNα-2a refolding in terms of specific activity and mass recovery.

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Nowadays, a large number of recombinant proteins have been produced in recombinant strain of *Escherichia coli*. However, highly expressed recombinant proteins in *E. coli* often result in the formation of the aggregation, known as "inclusion bodies (IBs)" [1, 2]. IBs are insoluble and inactive, therefore it is necessary to solubilize them and refold the protein into its native structure. The usually employed method for the refolding of recombinant protein is dilution method. Although it is simple and easy to perform, refolding by dilution need to perform at extremely low protein concentrations (5–100 μg/ml) to minimize aggregation and misfolding which are often responsible for low refolding yield [3]. Additionally, this traditional refolding method used for commercial applications requires larger volumes and long refolding times. So, it is necessary to explore novel methods for the refolding of recombinant proteins.

Size exclusion chromatography (SEC), as one of the effective ways for protein refolding has been attracted much attention in recent years [4–9]. The principle of protein refolding by SEC is to separate the denaturants from the unfolded protein by the gel media of SEC while they pass through the column. As the denaturant concentration surrounding the unfolded protein is reduced, the protein begins to fold into the native state [10]. Additionally the protein can be partially purified during the chromatographic process which significantly reduced the processing steps and times [11]. To fully explore the advantage of SEC, Gu with co-workers developed urea gradient SEC [12], in which urea concentration around the denatured proteins was decreased linearly and gently which could significantly increase the refolding yield at a high protein concentration. The gradient chromatography has been used in refolding studies during recent years [13–17]. However, there are only a few investigations on refolding of recombinant proteins, especially disulfide bonded proteins up to the present.

Interferon α-2a (IFNα-2a) is a therapeutic protein with a wide spectrum of biological activities including virus-unspecific antiviral effect, cell growth inhibition and immunomodulatory properties [18, 19]. It consists of 165 amino acids with 4 cysteine residues involved in the formation of 2 disulfide bridges [20]. IFNα-2a has being used extensively to treat chronic hepatitis B and C and several types of cancer, i.e. hairy cell leukemia, melanoma, multiple myeloma, condylomata acuminate, AIDS-related Kaposi's sarcoma, and in combination with retinoids, IFNα-2a induces regression in advanced squamous carcinomas of the cervix [21, 22].

In this study, we used a urea gradient SEC method for the refolding of rhIFNα-2a. The gel filtration column was pre-equilibrated with urea at decreasing concentration (Fig. 1). From the top of column to the end of the gradient, urea concentration was gradually de-

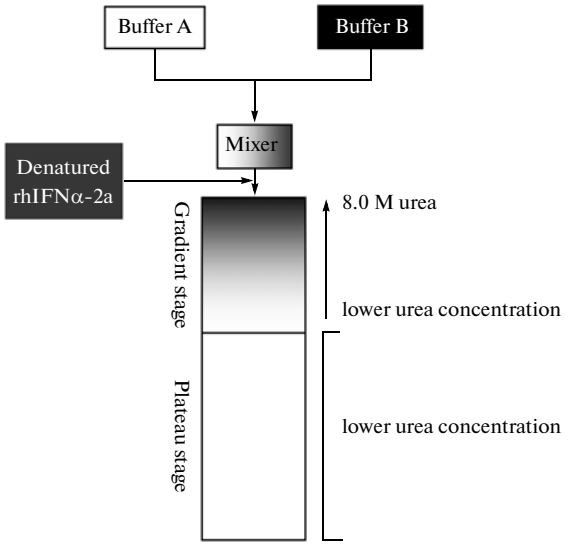

Fig. 1. Experimental system for the urea gradient SEC refolding process.

creased from 8.0 M to a lower concentration. Due to its larger molecular weight of 19.2 kDa, the rhIFNα-2a migrated faster than the gradient formed by small molecules of urea. When unfolded rhIFNα-2a passed through the column filled with a linear decreasing urea gradient, the urea concentration around the denatured rhIFNα-2a molecules would decrease gradually, and rhIFNα-2a would refold step by step. Several operating conditions which significantly influence the refolding efficiency of rhIFNα-2a were investigated, respectively. And we also compared its refolding yield with that obtained by dilution method.

Therefore, the aim of the present study was to introduce an efficient method for refolding of rhIFNα-2a which was overexpressed as IBs in *E. coli* and to examine the effect of operating conditions on this novel refolding performance and thus for exploit of gradient SEC advantages for refolding applications.

MATERIALS AND METHODS

Materials. The *E. coli* strain BL21 (DE3) and the vector pBV220-rhIFNα-2a were provided by the Protein Expression Section, Qufu Normal University (China). Human amnion WISH cells and standard interferon-α were purchased from the National Institute for Control of Pharmaceutical and Biological Products (China). Vesicular stomatitis virus (VSV) was obtained from Wuhan Institute of Virology, Chinese Academy of Sciences (China). Oxidized glutathione (GSSG), reduced glutathione (GSH), urea and tris(hydroxymethyl)aminomethane (Tris) were purchased from Sigma (USA). ÄKTA systems, chromatography media and electrophoresis apparatus were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). All other chemicals were of analytical grade.

Expression and isolation of rhIFNα-2a inclusion bodies. The *E. coli* strain BL21 (DE3) was transformed by the vector pBV220-rhIFNα-2a for the production of rhIFNα-2a. Single colonies were picked and grown overnight at 30°C in Luria Bertani (LB) medium (g/l): tryptone – 10, yeast extract – 5 and NaCl – 10, containing 100 μg/ml of ampicillin. The culture mixture was then inoculated to fresh LB medium (1 : 100 dilution) containing ampicillin and grown at 30°C until the cells OD_{600} reached to 0.6. Then, the cells were induced by shifting the culture temperature quickly to 42°C. After a further 5 h, the cells were harvested and rhIFNα-2a expression was analyzed by SDS-PAGE. The cell pellets were resuspended in 50 mM Tris-HCl buffer (pH 8.0) containing 1.0 mM EDTA at the ratio of 1 : 20 (w : v) and disrupted by ultrasonication on ice bath for 3 s × 200 W. After centrifugation at 8,000 *g* for 20 min, the IBs were washed 3 times with 50 mM Tris-HCl buffer (pH 8.0) containing 0.5% Triton X-100 and 1.0 mM EDTA.

Solubilization of the rhIFNα-2a IBs. The IBs were solubilized at the ratio of 1 : 10 (w : v) in the denaturing buffer (50 mM Tris-HCl, 8.0 M urea, 50 mM β-mercaptoethanol, 1.0 mM EDTA, pH 8.0) by incubation at 4°C for 8 h with agitation and then centrifuged at 12000 *g* for 20 min to remove any insoluble matter.

Refolding of rhIFNα-2a by urea gradient SEC. Urea gradient SEC for the refolding of rhIFNα-2a was carried out using a pre-packed Sephacryl S-200 column (100 cm × 3.5 cm). Two buffers were used in the chromatographic process. Buffer A: 50 mM Tris-HCl, pH 8.0, 1.0 mM EDTA, 15% glycerol (v/v), 0–3.3 mM GSH, 0–1.8 mM GSSG; buffer B: buffer A containing 8.0 M urea. The column was first equilibrated with the mixed buffer of various ratios of buffer A and buffer B. Then, gradually increasing buffer B up to 100%, urea gradient was formed. As shown in Fig. 1, urea concentrations in the column were varied from 8.0 M to a lower concentration. Dark colors represent higher urea concentrations, light colors represent lower urea concentrations. Then, various volumes of denatured rhIFNα-2a prepared above were loaded onto the column and eluted with buffer B. The experiments were performed at 4°C and the peak fractions containing rhIFNα-2a were collected and tested for protein activity.

To investigate the effect of operating conditions, one of them was varied while keeping other conditions unchanged. Four parameters in the refolding process, including gradient length, final urea concentration along the column, the ratio of GSH/GSSG and flow rate were investigated, respectively.

Refolding of rhIFNα-2a by dilution. In order to compare with refolding by gradient SEC, denatured rhIFNα-2a prepared above in various volume was diluted with a refolding buffer (50 mM Tris-HCl, 3.0 M

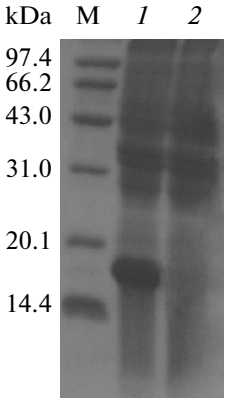

Fig. 2. SDS-PAGE analysis of the rhIFNα-2a expression in *E. coli*. M – molecular weight markers; *1* – after induction at 42°C; *2* – before induction.

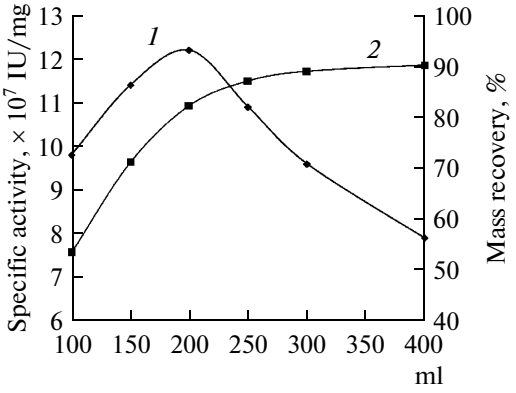

Fig. 3. Effect of urea gradient length (ml) on the rhIFNα-2a refolding. Buffer A contained 2.7 mM GSH and 0.9 mM GSSG. Urea gradient: 8.0 M urea (100% B) to 3.0 M (37.5% B) in various gradient length. The rhIFNα-2a loading: 10 ml with 1.75 mg/ml, flow rate 1.0 ml/min. *1* – specific activity; *2* – mass recovery.

urea, 1 mM EDTA, 15% glycerol (v/v), 3.0 mM GSH and 0.6 mM GSSG, pH 8.0) to a volume of 100 ml, which corresponds to the peak width of rhIFNα-2a in the urea gradient SEC. After incubation at 4°C for 24 h, the protein activity was determined.

Analytical methods. Protein concentrations were determined by Bradford's method [23]. Protein expression was analyzed by a reducing SDS-PAGE using 15% running gel and 4.5% stacking gel. The protein purity was analyzed by the GD 1000 gel documentation system (Bio-Rad, USA).

The mass recovery (MR, %) of rhIFNα-2a was defined as:

$$MR = \frac{M_{I,F}}{M_{I,IB}} = \frac{(C_F V_F P_F)}{(C_{IB} V_{IB} P_{IB})} \times 100\%,$$

where $M_{I,F}$ is the mass of rhIFNα-2a in the finally obtained rhIFNα-2a solution (mg); C_F is total protein concentration in the finally obtained rhIFNα-2a solution (mg/ml); V_F is the volume of the finally obtained rhIFNα-2a solution (ml); P_F is the purity of rhIFNα-2a in the finally obtained rhIFNα-2a solution; $M_{I,IB}$ is the mass of rhIFNα-2a in the injected solution of IBs (mg); C_{IB} is total protein concentration in the injected solution of IBs (mg/ml); V_{IB} is the volume of the injected solution of IBs (ml); P_{IB} is the purity of rhIFNα-2a in the injected solution of IBs.

Assay of rhIFNα-2a activity. The antiviral activity of rhIFNα-2a was measured by protection of human amnion WISH cells against the cytopathic action of VSV infection as described by traditional method [24]. Antiviral activity was expressed as the amount of rhIFNα-2a required for 50% protection of the death of WISH cells from VSV infection.

RESULTS AND DISCUSSION

Expression of rhIFNα-2a as IBs. The expression of rhIFNα-2a analyzed by SDS-PAGE is shown in Fig. 2. After 42°C induction an obvious band with molecular weight of 19.2 kDa was revealed in the pellet after cell disruption and centrifugation (see lane 1). The rhIFNα-2a was expressed as IBs and it was found that after 5 h of induction, the yield of the rhIFNα-2a IBs was around 2.6 g of wet weight per 1 l of culture. After washing with the detergent buffer, the purified IBs were dissolved in 8.0 M urea and the concentration of rhIFNα-2a was measured to be 1.75 mg/ml with a purity of 60%.

Effect of the length of urea gradient on rhIFNα-2a refolding. In this experiment, the column was pre-equilibrated with a linear decreased urea gradient. The denatured rhIFNα-2a was loaded onto the top of the gradient where the urea concentration was 8.0 M, which was the same as that in the loading sample. The denatured rhIFNα-2a went through the column gradually from 8.0 M urea to the final renaturation buffer containing a low concentration of urea. If the length of the gradient was too short, the change of the urea concentration was too fast to make the rhIFNα-2a refolding correctly. However, if the gradient length was too long, the denatured protein was eluted out with higher urea concentration than the optimum for protein refolding. Experiments were conducted to select an optimal length of urea gradient for the rhIFNα-2a refolding. Fig. 3 shows that the specific activity and mass recovery of rhIFNα-2a changed with the length of urea gradient. It can be seen that the mass recovery of rhIFNα-2a increased with the increase of the urea gradient length. That is because the increase of urea gradient length could make a slower change of urea concentration around the denatured rhIFNα-2a, thus reducing the chance of getting aggregations. The high-

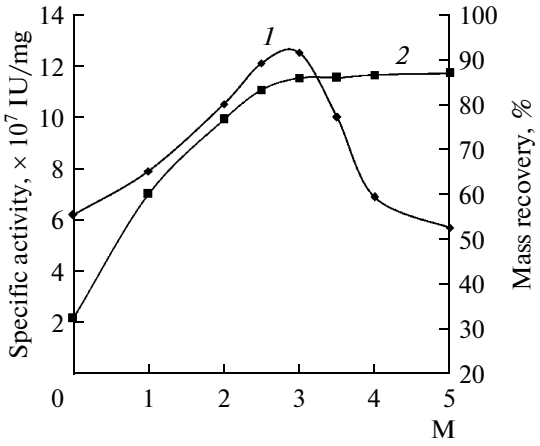

Fig. 4. Effect of final urea concentration (M) on the rhIFNα-2a refolding. The length of urea gradient was 200 ml, other conditions were the same as those indicated in Fig. 3 except for final concentration of urea. *1* – specific activity; *2* – mass recovery.

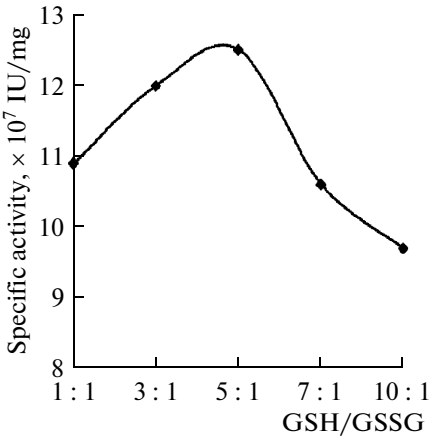

Fig. 5. Effect of the ratio of GSH/GSSG on the rhIFNα-2a refolding. The final urea concentration was 3.0 M, other conditions were the same as those indicated in Fig. 4 except for the ratio of GSH/GSSG.

est specific activity was obtained at a urea gradient with a length of 200 ml. With this gradient, denatured rhIFNα-2a could move exactly to the final renaturation buffer containing a low concentration of urea at the end of column, meanwhile this length of the urea gradient had to be long enough for gradual removal of urea from denatured protein, thus giving the highest protein specific activity. Therefore, we chose the gradient length of 200 ml for the subsequent refolding experiments.

Effect of final urea concentration on the refolding of rhIFNα-2a. As we know, urea can not only denature the protein but also prevent the protein forming aggregation. It was found that a suitable concentration of urea in refolding buffer can increase the yield of correctly refolded protein [12, 25, 26]. With the aim of finding the optimum urea concentration for rhIFNα-2a refolding, the effects of final urea concentration in urea gradient SEC was investigated (Fig. 4). It can be seen that both specific activity and mass recovery of rhIFNα-2a increased with the increase of final urea concentration before 3.0 M (37.5% B). This indicates that too low urea concentration is not enough to prevent protein molecules forming aggregation with each other. However, when the final urea concentration was higher than 3.0 M, the specific activity decreased rapidly with the increase of urea concentration, but the protein mass recovery didn't have any loss. This implies that higher than 3.0 M urea concentration is enough for preventing aggregation, but this concentration is unfavorable for denatured rhIFNα-2a refolding to their active state. The above results indicate that both protein specific activity and mass recovery reach maxima at 3.0 M urea (37.5% B) which is potent enough to suppress the aggregation of the protein and simultaneously make the denatured rhIFNα-2a refold to their activity structure. Therefore, we chose this concentration for subsequent gradient SEC refolding studies.

Effect of the ratio of GSH/GSSG on rhIFNα-2a refolding. The rhIFNα-2a molecule contains two intra-molecular disulfide bonds, and both are required for its correct conformation and consequently its bioactivity [20]. Thus, the formation of right disulfide bonds plays an important role during the course of the rhIFNα-2a refolding. It was reported that an appropriate redox pairs in refolding buffer presumably helped to reoxidize the reduced disulfide bonds of protein to oxidized state [27]. The most commonly used redox agent is glutathione in the reduced and oxidized forms (known as GSH and GSSG). Previous refolding studies showed that a suitable ratio of GSH/GSSG was able to provide a favorable refolding environment which could influence the formation of disulfide bonds and hence the rate of correct refolding [28, 29]. In this work, experiments were conducted to select a suitable ratio of GSH/GSSG for the rhIFNα-2a refolding by using urea gradient SEC. The highest specific activity of rhIFNα-2a was achieved when the ratio of GSH/GSSG was 5 : 1 (Fig. 5). Therefore, we chose the ratio of GSH/GSSG of 5 : 1 in the buffer for rhIFNα-2a refolding.

Effect of flow rate on the refolding of rhIFNα-2a. The flow rate is also a significant impact in gradient SEC. It was reported that the elution flow rate influenced the retention time of the proteins in the column, which determined refolding time of the proteins [30]. To research the effect of the elution flow rate on the rhIFNα-2a refolding, denatured rhIFNα-2a was eluted out at 0.5, 1.0, 1.5, 2.0 and 2.5 ml/min, respectively (Fig. 6). It was demonstrated that the specific activity of rhIFNα-2a decreased with the increase of

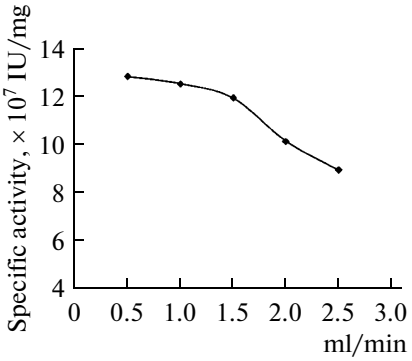

Fig. 6. Effect of flow rate (ml/min) on the rhIFNα-2a refolding. The ratio of GSH/GSSG was 5 : 1, other conditions were the same as those indicated in Fig. 5 except for the elution flow rate.

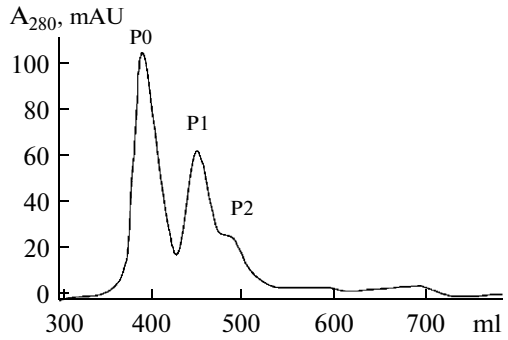

Fig. 7. Profile of rhIFNα-2a refolding by urea gradient SEC. Buffer A contained 3.0 mM GSH and 0.6 mM GSSG. Urea gradient: 200 ml from 8.0 M (100% B) to 3.0 M (37.5% B). The rhIFNα-2a loading: 10 ml with 1.75 mg/ ml, flow rate 1.5 ml/min.

elution flow rate. That occured because higher flow rate means faster removing of urea from unfolded rhIFNα-2a, which was not benefit for the refolding of rhIFNα-2a. Besides that, a slow elution flow rate could give the protein sufficient refolding time (residence time) to stay inside of the SEC column for refolding. In order to strike a balance between experimental time and protein activity, we used a flow rate of 1.5 ml/min as an optimal elution flow rate.

Comparison of rhIFNα-2a refolding by urea gradient SEC and dilution method. Dilution is the most often used method for the refolding of proteins produced by *E. coli*. In this work, a comparison between the urea gradient SEC and the dilution refolding was conducted (Table). Both of specific activity and mass recovery of rhIFNα-2a obtained by dilution method are lower in comparison with results after urea gradient SEC at the same sample loading volume. It can also be seen in Table that the protein specific activity and mass recovery decreased with the increase of the sample loading volume for the two refolding methods. It was because a large sample loading volume could increase the loading amount (loading volume × concentration) of denatured rhIFNα-2a, and thus increase the chance of intermolecular collision and the protein aggregations. However, with the increase of the sample loading volume, the protein specific activity and mass recovery obtained by gradient SEC decreased slower than that by dilution method, and the larger the loading volume, the bigger the difference between the two refolding methods. That was because gradient SEC could suppress aggregations more effectively by gradual removal of urea [15]. We can conclude that urea gradient SEC is more efficient than dilution method for rhIFNα-2a refolding.

Optimal conditions for the refolding of rhIFNα-2a by urea gradient SEC. Fig. 7 represents the chromatography profiles of rhIFNα-2a refolding at the opti-

Comparison of the rhIFNα-2a refolding by urea gradient SEC and dilution method*

Loading volume, ml	Specific activity, ×10^7 IU/mg		Mass recovery,%	
	urea gradient SEC	dilution	urea gradient SEC	dilution
5	13.4 ± 1.1	9.2 ± 1.9	88.7 ± 5.0	24.9 ± 2.6
7.5	12.9 ± 1.5	8.3 ± 2.1	85.9 ± 4.7	21.9 ± 1.8
10	12.1 ± 1.4	6.8 ± 1.6	82.3 ± 5.8	14.6 ± 2.7
12.5	9.5 ± 1.9	5.3 ± 1.3	78.1 ± 3.2	10.1 ± 1.7
15	8.7 ± 1.7	3.4 ± 1.1	74.6 ± 3.5	8.3 ± 2.5

* For urea gradient SEC, the length of urea gradient was 200 ml, the elution flow rate was 1.5 ml/min, the ratio of GSH/GSSG was 5 : 1, other conditions were the same as those demonstrated in Fig. 3. For dilution refolding, the conditions were the same as those demonstrated in the section devoted to refolding of rhIFNα-2a by dilution.

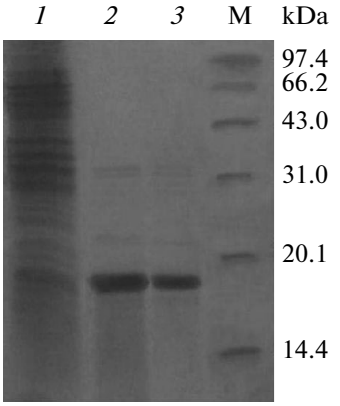

Fig. 8. SDS-PAGE analysis of the rhIFNα-2a refolding by urea gradient SEC. M – molecular weight markers; *1* – fraction from peak P0; *2* – fraction from peak P1; *3* – fraction from peak P2.

mum experimental conditions determined above by urea gradient SEC. Three peaks (P0, P1 and P2) were found during refolding process. Analysis of peak P0 by electrophoresis (Fig. 8, lane *1*) revealed compounds of large molecular weights, mainly impurities, and there was no activity of rhIFNα-2a. So, we suppose this peak may contain some impurities in the IBs. On the other hand, peaks P1 and P2 contained predominantly refolded rhIFNα-2a (Fig. 8, lanes *2* and *3*). The specific activities of the protein in the P1 and P2 peaks were similar, around 1.2×10^8 IU/mg. From Fig. 7, we can see that peak P2 is a little refolding rhIFNα-2a following peak P1. It was most likely due to a little aggregation and precipitation of the protein on the gel matrix after the denatured rhIFNα-2a passed through the column [10, 31]. With the urea gradient moved downwardly, the precipitated aggregation could be resolubilized by the following urea and be eluted out from the column following the refolding rhIFNα-2a peak (Fig. 7, peak P1). That is the reason why the higher protein mass recovery can be achieved by urea gradient SEC refolding. In addition, another remarkable advantage of this method is that the rhIFNα-2a can be purified during chromatographic process; its purity was about 92% scanned by Bio-Rad Gel Doc (Fig. 8).

The advantage of the gradient SEC refolding must relate to its gentle, smooth exchange of the buffer around the unfolded protein, from the denaturing buffer to the refolding buffer, which can increase both of its specific activity and mass recovery by preventing the formation of inactive protein and aggregations. In this paper, we have successfully refolded rhIFNα-2a overexpressed in *E. coli* by urea gradient SEC. Several operating conditions such as gradient length, final urea concentration along the column, the ratio of GSH/GSSG and flow rate have been investigated. Under optimal conditions, when 10 ml of denatured rhIFNα-2a at a concentration of 1.75 mg/ml was loaded onto the SEC column, rhIFNα-2a with specific activity of 1.2×10^8 IU/mg, purity of 92%, and mass recovery of 82% was obtained. In conclusion, urea gradient SEC is a high efficiency refolding method for rhIFNα-2a, and we hope our studies could be useful in refolding of other recombinant proteins expressed in *E. coli* as IBs.

ACKNOWLEDGMENTS

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