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Dual gradient ion-exchange chromatography improved refolding yield of lysozyme

Ming Li, Guifeng Zhang, Zhiguo Su*

State Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, P.O. Box 353, Beijing, 100080, China

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Abstract

Protein refolding at high concentrations always leads to aggregation, which limits commercial application. An ion-exchange chromatography process with gradient changes in urea concentration and pH was developed to refold denatured lysozyme at high concentration. After adsorption of the denatured protein onto an ion-exchange medium, elution was carried out in combination with a gentle decrease in urea concentration and elevation of pH. Protein would gradually refold along the column with high activity yield. Denatured and reduced lysozyme at 40 mg/ml was loaded into a column filled with SP Sepharose Fast Flow, resulting in 95% activity recovery and 98% mass yield within a short period of time. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Gradient elution; Protein folding; pH effects; Lysozyme; Proteins; Urea

1. Introduction

High heterologous expression levels of recombinant proteins in bacteria often lead to the formation of inactive aggregates termed ‘‘inclusion bodies’’. Protein products in inclusion body form can be advantageous over the soluble form, including relatively high product concentration, less proteolytic degradation, and reduced toxicity to the host cells. The challenge is to convert inactive and misfolded inclusion body proteins into soluble bioactive products [1].

The denaturing and reducing agents at high concentration, which solubilize the inclusion bodies, have to be removed to induce protein refolding. Several conventional methods, including dilution, dialysis, and diafiltration have been developed to remove the excess denaturants or exchange the solubilization buffer. All these methods, though investigated widely, suffer more or less from a number of limitations [2]. Among them, dilution has been used commercially. The process is simple as to dilute the denatured protein solution directly into refolding buffer. However, the concentration of denatured protein in refolding buffer has to be strictly controlled at low level to prevent aggregate formation [3]. This would inevitably increase the total processing volume, a heavy burden for the downstream steps. Furthermore, this process is time consuming and buffer consuming. Large production vessels and quantities of denaturants are required.

*Corresponding author. Fax: +86-10-6256-1822.
E-mail address: zgsu@home.icm.ac.cn (Z. Su).

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Dialysis always causes the adhesion of protein on the membrane and takes a long time [4]. Diafiltration might be a good alternative because of its faster removal of denaturants than dialysis. However, protein aggregation will cause membrane clogging and restricts its application on a large scale.

Liquid chromatography for buffer exchange to induce protein refolding has appeared in recent years. Size-exclusion chromatography (SEC) is the focus of attention. The SEC column can separate denaturants from protein to promote renaturation. The process has demonstrated its advantages in refolding of thioredoxin [5], Rets-1, RNase A, IHF [6], PDGF [7] and u-PA [8]. But the sharp decrease in denaturant concentration during feed loading stage inevitably causes aggregate formation and low refolding yield [9]. The decrease in protein solubility or large aggregate formation due to fast removal of denaturant could also bring flow obstruction within the column. The aggregation may be alleviated by adsorption of the individual denatured polypeptide chains onto the solid phase, such as ion-exchange chromatography (IEC) media [10,11], hydrophobic interaction chromatography media [12], and other adsorption solid support. Furthermore, the solid support could also be covalently connected to some functional groups before the adsorption of polypeptides, such as liposome [13,14], molecular chaperone GroEL [15], nickel chelating group [16], and antibody. The immobilized groups may enhance protein adsorption, induce refolding, stabilize the native state or increase the solubility of refolding intermediates. Though innovative, chromatographic refolding still needs further improvement so as to increase its recovery and prevent column clogging.

A way to improve chromatographic refolding is to control the denaturant concentration in the process. Correct refolding and proper associations of different domains depend strongly on the circumstance in which proteins refold. Gently removal of denaturants would enhance refolding yield. Adding denatured lysozyme and carbonic anhydrase into refolding buffer by fed-batch model could lead to increased activity recovery at high protein concentration [17]. Linear decrease in the urea concentration during diafiltration was very effective in inhibiting aggregation at relatively high protein concentration [18]. Maeda et al. [19] proved that mild removal of urea from denatured protein by means of dialysis would be useful to renature protein effectively. The study in this laboratory [20] has demonstrated that gradient decrease in urea concentration during size-exclusion chromatography could obtain high activity recovery at relatively high protein concentration. However, for IEC, Creighton [10] found that linear removal of urea in IEC had only 10% refolding yield for lysozyme. Low renaturation yield may be attributed to the total removal of urea in the refolding buffer and some lysozyme was not eluted out from the column. Investigation by Wetlaufer and Xie [21] and Hevehan and De Bernardez Clark [3] showed that the low concentration of denaturants in the refolding buffer could result in increased renaturation yield. The low concentration of urea in the IEC refolding buffer may help the lysozyme leave from the media and bring high refolding yield.

The effects of pH on refolding yield and rate, especially on the formation of disulfide bonds, have not received much attention in chromatographic refolding. Both folding and association of proteins rely on pH [22,23]. Usually, aggregation is reduced when the pH of the medium is far away from the isoelectric point of protein [24]. In general, the basic circumstances help the formation of disulfide bonds. The nearby charged residues on the oxidation potential also make a difference [25,26]. At a pH below the pK_a of the cysteine thiol, the effects of nearby charges on the oxidation should be small since both the thiol and disulfide states are neutral. At pH values above the thiol pK_a, nearby positive charges should stabilize the reduced state and it will be difficult to form the disulfide bonds, while nearby negative charges should have the opposite effect. Moreover, the different stages of refolding have an effect on the formation of disulfide bonds [27].

Considering the importance of denaturant concentration and pH in refolding, a dual-gradient ion-exchange chromatography process was developed to improve the refolding recovery at high protein concentration. The denatured hen egg-white lysozyme was bound on the ion-exchange medium, and gently eluted with refolding buffer by gradient decreasing urea concentration from 6 to 1 mol/l and increasing pH from 6.2 to 10. Protein would gradually refold through the column resulting in an increased renaturation recovery. The result demon-

strated that the new renaturation process had remarkable advantages over conventional methods.

2. Experimental

2.1. Materials and equipments

Hen egg-white lysozyme was purchased from the Institute of Biochemistry (Shanghai, China), dithiothreitol (DTT) and *M. lysodeikticus* were from Sigma. Reduced glutathione (GSH) and oxidized glutathione (GSSG) were from Beijing Chemical Reagent Co. All other reagents were of analytical grade. Ultrapure water for all the experiments was obtained from a Milli-Q system (Millipore Inc.).

An SP Sepharose High-Performance Hitrap column (5 ml) for IEC and a Superdex 75 (10/30) pre-packed column for SEC were purchased from Pharmacia Biotech. All chromatography experiments were carried out on ÄKTA Purifier Workstation (Pharmacia Biotech). A UV–Vis spectrophotometer, Ultrospect 2000 (Pharmacia Biotech), was used to analyze protein activity and concentration.

2.2. Preparation of denatured lysozyme

Native hen egg-white lysozyme was dissolved in denaturant buffer (0.05 mol/l Tris–HCl at pH 8.7 containing 8 mol/l urea and 0.1 mol/l DTT). After incubation for 4–5 h at room temperature, the protein activity was analyzed to confirm that the protein has been denatured completely. The denaturation buffer and all other buffers used in all processes should be prepared and used within 1 day.

2.3. Refolding by dilution

Denatured proteins at various concentrations were directly diluted with refolding buffer (0.1 mol/l Tris–HCl at pH 8.7 containing 2 mol/l urea, 3 mmol/l GSH, 0.3 mmol/l GSSG and 0.15 mol/l NaCl). The additives in refolding buffer should be adjusted to obtain higher activity recovery in dilution.

2.4. Refolding by IEC without gradient

An ion-exchange column (5 ml) was equilibrated with buffer A (0.05 mol/l Tris–HCl at pH 8.7 containing 2 mol/l urea, 3 mmol/l GSH and 0.3 mmol/l GSSG). A 200-μl aliquot of denatured protein at 40 mg/ml was loaded onto the column. After rinsing with buffer A for two-column volume (10 ml), the column was directly eluted with buffer B (0.1 mol/l Tris–HCl at pH 8.7 containing 2 mol/l urea, 0.3 mol/l NaCl, 3 mmol/l GSH and 0.3 mmol/l GSSG). The flow-rate was controlled at 0.4 ml/min in all processes. Fractions of elution were collected and analyzed for activity and concentration.

2.5. Refolding by gradient IEC

The same column was used and the feed loading was kept the same. For chromatographic refolding with a single gradient of urea concentration, two buffers were used. Buffer A contained 0.05 mol/l Tris–HCl (pH 8.7), 6 mol/l urea, 3 mmol/l GSH and 0.3 mmol/l GSSG. Buffer B contained 0.1 mol/l Tris–HCl (pH 8.7), 1 mol/l urea, 0.3 mol/l NaCl, 3 mmol/l GSH and 0.3 mmol/l GSSG. Buffer B also contained 0.3 mol/l NaCl to elute the bound protein from the column at the end of the gradient. After loading of the protein, the column was rinsed with buffer A. Elution was started by gradually increasing the ratio of buffer B/buffer A from 0:100 to 100:0 at 0.4 ml/min within one-column volume. For chromatographic refolding with pH gradient only, the pH in buffer A was 6.0–6.2 and in buffer B 10.0, while the concentration of urea in buffer A and buffer B was kept the same as 2 mol/l. The gradient length was also one-column volume.

For the dual-gradient (urea concentration and pH) refolding with the same column, the pH was 6.0–6.2 in buffer A and 10.0 in buffer B, coupled with 6 mol/l urea in buffer A and 1 mol/l in buffer B. After loading and rinsing with buffer A, the column was eluted by gradually increasing the ratio of buffer B/buffer A from 0 to 100% at 0.4 ml/min within one-column volume. A schematic illustration of the dual-gradient process system is shown in Fig. 1. It actually indicates the dynamic change in the environment for protein binding and moving through the column. The urea concentration had decreased from

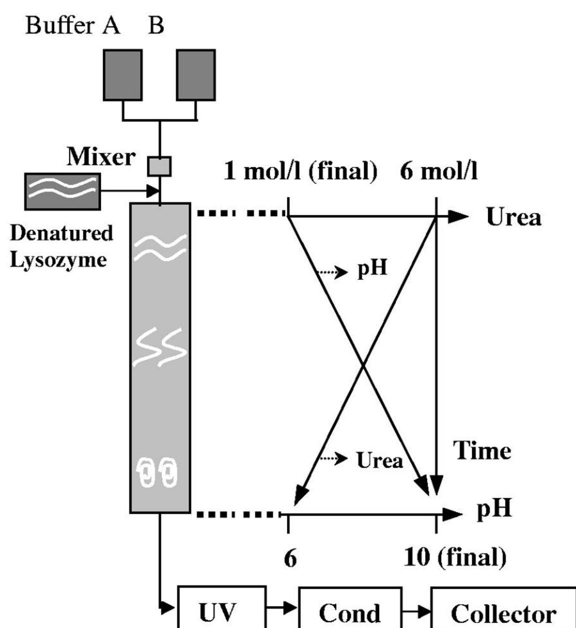

Fig. 1. Experimental system for dual gradient IEC refolding process.

6 to 1 mol/l and pH was elevated from 6.2 to 10.0 when the protein exited the IEC column.

2.6. Further purification of refolding proteins by size-exclusion chromatography

A Superdex 75 column was equilibrated with 0.1 mol/l Tris–HCl at pH 8.7 containing 0.15 mol/l NaCl and 2 mol/l urea to further purify the refolding lysozyme. The refolding lysozyme solution collected from IEC was then loaded onto the column and eluted with the same buffer at a flow-rate of 0.4 ml/min.

2.7. Protein activity assay

Lysozyme activity was determined by Jolles' method [28]. The absorbance at 450 nm of *M. Lysodeikticus* suspended in 0.06 mol/l potassium phosphate (pH 6.2) solution was adjusted to 0.7. After addition of 10 μl of protein solution to the suspension, the initial rate of decrease in absorbance at 450 nm was measured. The specific activity of native lysozyme was used as the standard native activity.

2.8. Protein concentration assay

According to Bradford [29], Coomassie Blue G250 was used as dye reagent to measure the absorbance at 595 nm, using the absorbance of pure lysozyme as the standard curve.

3. Results and discussion

3.1. Refolding of lysozyme by dilution

Experiments on the renaturation of lysozyme were first carried out by the dilution method, which was for comparative purpose. Fig. 2 shows the dynamic refolding process with time. The results indicated that addition of 0.1 mol/l NaCl would improve the yield compared to no NaCl or a higher concentration of NaCl added in the refolding buffer. GSH/GSSG redox system added showed a twofold greater yield than by air oxidation. The appropriate pH in the refolding buffer was about 8.5. After incubation for 2 h, the specific activity of refolded lysozyme at 30 μg/ml was 12 900 U/mg. The lower the protein

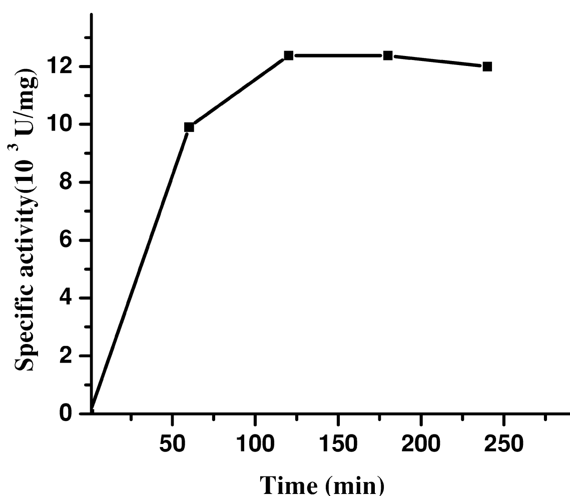

Fig. 2. Lysozyme refolding curve by dilution. Denatured protein at 3 mg/ml was directly diluted with refolding buffer (0.1 mol/l Tris–HCl at pH 8.7 containing 2 mol/l urea, 3 mmol/l GSH, 0.3 mmol/l GSSG and 0.15 mol/l NaCl) in a 100:1 ratio.

concentration in refolding solution, the higher the refolding yields. It has been known in the literature that the kinetic competition between folding and aggregating occurred in the refolding solution. The folding process is a first-order reaction, while the aggregation is an intermolecular reaction involving at least two polypeptide chains. Thus, the rate of aggregate formation increases according to some power (≥2) of the protein concentration [2]. The concentration of protein concentration should be kept at low levels during dilution, which limited its large-scale application.

3.2. Refolding by IEC

Fig. 3 shows one of the chromatographic refolding curves obtained by dual-gradient IEC. Urea shows the change in its concentration and NaCl represents the conductivity change in the buffer. The first peak at 280 nm describes the absorbance of DTT in denatured buffer, which is the reducing agent to break the disulfide bonds of native lysozyme. The second peak shows the absorbance of refolded lysozyme.

Fig. 4 describes the comparison of different processes on refolding yields. It shows the increase in refolding yields as the refolding method improved. A simple IEC process without any gradient (method 2 in Fig. 4) is much better than the conventional dilution (method 1) by onefold increase in the specific activity. Binding of the denatured lysozyme on the ion-exchange medium prevented the interaction between the polypeptide chains and decreased the aggregate formation. In situ purification also was achieved by washing the bound protein before elution. Compared with the dilution, the specific activity of protein from IEC refolding without gradient was 25 200 U/mg, 1.9-fold of that achieved by dilution. The concentration of the collected fractions was from 0.8 to 1.2 mg/ml.

IEC with denaturant gradient (method 3 in Fig. 4) improved the specific activity of the IEC process by 25%. As described in the refolding process of SEC in the Introduction section, the polypeptide chains directly loaded onto the ion-exchange chromatographic column filled with a low concentration of denaturants would contact with each other to form an aggregate. Urea gradient may minimize the rapid structure collapse. High concentrations of urea in the column kept the denatured protein in the unfolded state. After being bound by the medium, the denatured proteins were induced to refold by gradually decreasing the urea concentration during the elution stage. Intermolecular interactions leading to aggregate were reduced when the individual protein was

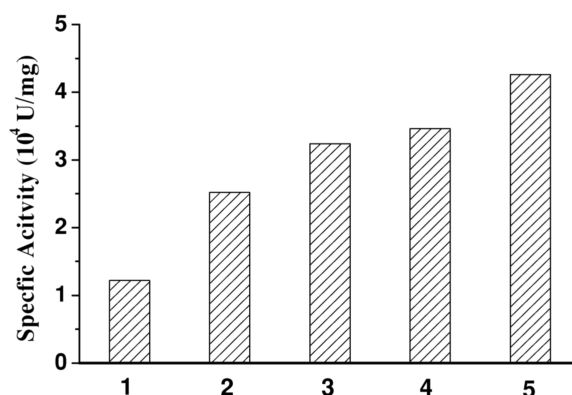

Fig. 4. Comparison of different refolding processes. 1, Dilution; 2, IEC without gradient; 3, IEC with urea gradient only; 4, IEC with pH gradient only; 5, IEC with dual (urea and pH) gradient. The denatured lysozyme loading during IEC was 200 μl at 40 mg/ml.

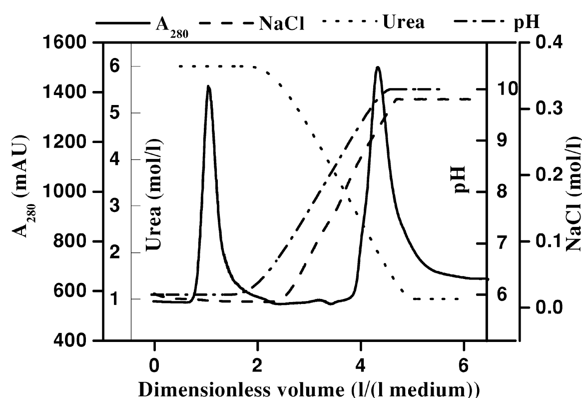

Fig. 3. Chromatographic curve on lysozyme refolding with dual-gradient IEC. Equilibrated and rinsed buffer A was 0.05 mol/l Tris–HCl at pH 6.2 containing 6 mol/l urea, 3 mmol/l GSH, and 0.3 mmol/l GSSG. Elution buffer B was 0.1 mol/l Tris–HCl at pH 10 containing 1 mol/l urea, 0.3 mol/l NaCl, 3 mmol/l GSH and 0.3 mmol/l GSSG. Flow-rate was controlled at 0.4 ml/min. Protein loading was 8 mg; gradient length was 5 ml from 100% buffer A to 100% buffer B.

isolated from each other through binding to the support.

The pH gradient (method 4 in Fig. 4) is very important. The basic thiol exchange reaction involves the ionized form of thiol and is therefore pH-dependent [30]. The optimal pH in dilution process showed the average request to oxidize all the cysteine thiols. But the need of pH to form disulfide bonds at different sites of proteins and at different refolding status would have a little difference, which might be one reason that refolding by dilution needs much longer time. The gradient of pH would pass the pH values from acidic to basic point and satisfy the need for formation of different disulfide bonds, therefore it might decrease the chance of wrong disulfide bond formation. If the pH values were too high, the chance of formation of wrong-paired disulfide bonds would increase and also have little opportunity to rearrange by the red/ox system of GSH/GSSG (Fig. 5). High pH would cause the degradation of peptide chains [21], which therefore decreases the refolding yield. It has been found that the adsorption ability of denatured protein to the medium was lower than that of native formation. The buffer with low pH (6.0–6.2) in which protein was injected into the column would enhance the adsorption of the denatured protein. High pH (10.0) helped the protein that had adsorbed on the medium to leave.

The urea gradient and the pH gradient can be integrated into one process as a dual-gradient system (method 5 in Fig. 4). Urea concentration gradient in combination with pH elevation proved effective in fulfilling the refolding at linearly decreasing denaturant concentration and the formation of disulfide bonds. Recovery of activity and mass of refolded protein were higher than in processes without gradient or with only one gradient. The specific activity of refolded protein after dual-gradient IEC process was 42 618 U/mg and 7.8 mg protein was recovered.

3.3. Effect of protein loading on the lysozyme refolding

Fig. 6 indicates that the amount of protein loading into the column affects the refolding yield. For comparison purpose, we take the activity yield (48 318 U/mg) of 2 mg loading as 100%, and others were relative values to it. When the protein loading onto the column was kept low, there were enough adsorption sites for the denatured protein to bind and refold. The unfolded polypeptide chains also had less chance to contact with each other because of their immobilization at different sites. The refolding process would complete without intermolecular interaction and a high activity yield would be obtained. As the protein loading increased, the number of protein molecules would exceed the number of binding sites on the solid support at the top of the column. Interaction between the molecules was inevitable. This would influence the correct refolding and result in aggregation. The refolding yield was decreased. From an economic point of view, we need

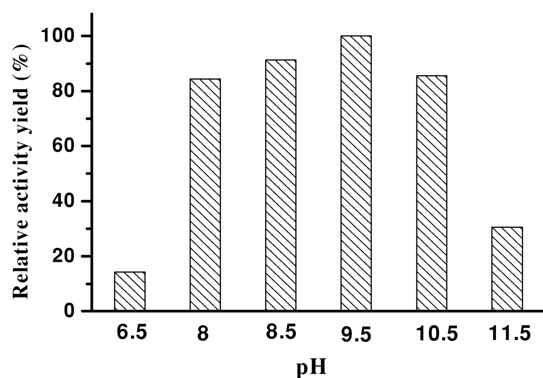

Fig. 5. Effect of pH of buffer B on the refolding of lysozyme by dual IEC. The operation condition was the same as that in Fig. 3. Lysozyme loading was 1.6 g/l medium.

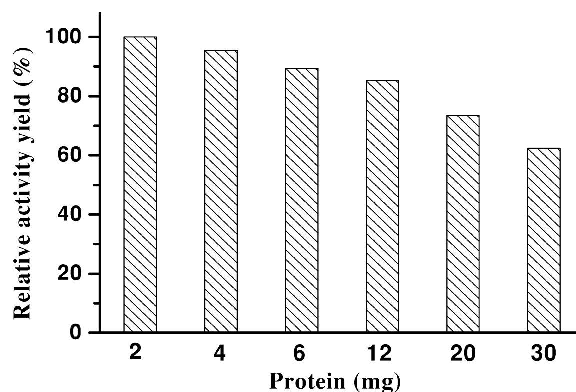

Fig. 6. Effect of amount of lysozyme loaded onto the column. The operating conditions were as in Fig. 3.

to balance between the activity yield and protein loading. Fig. 6 demonstrates the trade-off. Although aggregation was greatly inhibited, it was not completely stopped. The relative activity yield would decrease from 100 to 62% when the amount loaded on the column (5 ml) was increased from 2 to 30 mg (protein concentration from 0.4 to 7.5 g/l medium).

SEC was carried out to analyze the aggregation (Fig. 7). If the concentration of lysozyme loaded into the ion-exchange column is low (0.8 g/l media), the refolding lysozyme solution has only one peak in the SEC curve. The retention time is the same as that of native lysozyme. When high concentration of denatured lysozyme (6 g/l media) was applied onto the IEC column for refolding, two peaks are observed in the SEC curve. The retention time of the first peak was less than that of native formation and the specific activity was one-fifth of the native form, which suggests that there was formation of protein aggregates at high concentrations of protein loading.

3.4. *Effect of elution flow-rate on the refolding recovery*

Fig. 8 shows the effects of elution flow-rate on activity recovery of refolding during dual-gradient IEC, which demonstrates that there was an optimum at 0.3 ml/min. High flow-rate of elution would leave too short a time for target protein to refold completely, whereas a slow flow-rate of elution would provide the chance for protein contact as they move through the column, increasing the possibility of aggregation. Furthermore, the slow elution rate was also not preferred since it would take a long time to complete this process.

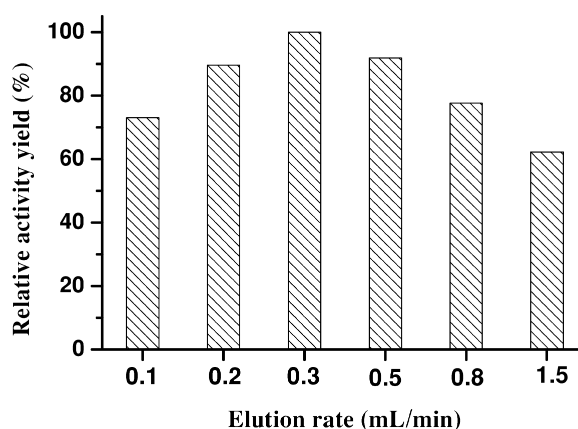

Fig. 8. Effect of elution rate on refolding of lysozyme. Other operation conditions as in Fig. 3.

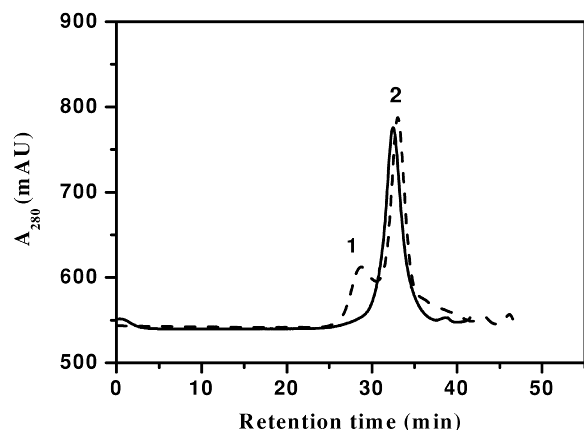

Fig. 7. Size-exclusion chromatographic curve of refolding lysozyme solution. (—) Protein solution collected when injected into the ion-exchange column with 4 mg lysozyme. (- - -) Protein solution collected when injected into the ion-exchange column with 30 mg lysozyme. Elution buffer was 0.1 mol/l Tris–HCl at pH 8.7 containing 0.15 mol/l NaCl and 2 mol/l urea. Column was Superdex 75 (10/30) and the elution rate was controlled at 0.4 ml/min. Peak 1 shows the aggregation.

4. Conclusion

Urea concentration and pH gradient are first used in the IEC process to refold denatured proteins. Compared with Creighton's method, this process needs only two kinds of buffer and results in saving on steps and time. Existence of denaturants at low concentration in the refolding buffer increased the activity yield and mass recovery. A pH gradient in this process satisfied the formation of all disulfide bonds of protein, assisting the denatured protein to adsorb on the ion-exchange medium in acidic conditions, driving the protein to refold in basic conditions and to desorb from the medium. It was also found that one column volume of gradient length could be sufficient to obtain high activity yield, which took less than 1 h to complete in the dual-gradient process, while it would take at least 2 h for the dilution refolding process to complete as described in Table 1.

Table 1
Comparison of dual gradient IEC refolding system with dilution

	Dual gradient IEC	Dilution
Protein loaded (mg)	8 (200 μl of 40 mg/ml)	
Time for complete refolding (h)	<1	2
Final protein concentration (mg/ml)	0.8–1.2	0.03
Mass recovery (%)	98	100
Specific activity (U/mg)	42 618	12 900
Activity recovery (%)	95	29
Overall activity yield (%)	93	29

The IEC process is one of the most valuable methods for protein purification because of its simple operation, good biological compatibility and high capacity. Refolding proteins by IEC had been done in the literature, and it was shown that this process was efficient. This study further enhances the technique by adding dual-gradient operation, which can be easily realized on column chromatography. The ion-exchange column could be reused at least 30–50 times in this process, which decreased the cost of materials. A regeneration procedure is necessary at inter-run to remove the residual proteins tightly bound on the medium. This new process could satisfy the request for decreasing denaturant mildly and driving disulfide bond formation at different pH values. It is possible that the dual-gradient IEC could be very useful in refolding inclusion body protein on a large scale.

Acknowledgements

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