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Urea gradient size-exclusion chromatography enhanced the yield of lysozyme refolding

Zhenyu Gu[a,*], Zhiguo Su[a], Jan-Christer Janson[b]

[a]*State Key Laboratory of Biochemical Engineering, Institute of Chemical Metallurgy, Chinese Academy of Sciences, Beijing 100080, China*

[b]*Biomedical Center, Uppsala University, Uppsala, Sweden*

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Abstract

Protein refolding is still a bottleneck for large-scale production of valuable proteins expressed as inclusion bodies in *Escherichia coli*. Usually biologically active proteins cannot be obtained with high yield at a high concentration after refolding. In order to meet the challenge of protein refolding a urea gradient gel filtration-refolding system was developed in this article. A Superdex 75 column was pre-equilibrated with a linear decreased urea gradient, the denatured protein experienced the gradual decrease in urea concentration as it went through the column. The refolding of denatured lysozyme showed this method could significantly increase the activity recovery of denatured lysozyme at high protein concentration. The activity recovery of 90% was obtained from the initial protein concentration up to 17 mg/ml within 40 min.

Keywords: Gradient elution; Urea gradient; Protein refolding; Lysozyme; Proteins; Urea

1. Introduction

Genetic technique enables high level expression of extraneous proteins in *Escherichia coli*, however, high-level production of functional proteins in *E. coli* usually leads to the formation of inactive inclusion bodies. In this case the overall yield of target proteins is largely dependent on the efficiency of protein refolding process. But refolding of recombinant proteins from inclusion bodies remains a tough task for downstream engineers. Because of the exposure of inner hydrophobic core the denatured inclusion bodies are susceptible to aggregation during refolding process, which is a major reason for low refolding yield of recombinant proteins. The exact mechanism for aggregation is still under investigation. But the analysis of the kinetics of aggregation shows that the aggregation process exhibits an apparent reaction order $\geqslant 2$ [1,2], whereas the correct folding step is generally first-order reaction, which suggests the competition between refolding and aggregation might favor refolding at low protein concentrations. In typical dilution refolding, protein concentrations in the range of 10–50 μg/ml are used to get relatively high recovery [3]. But in industry scale operation, dilution refolding undoubtedly leads to the use of large refolding

*Corresponding author. +86-10-6256-1813.

E-mail address: zhenyug@yahoo.com (Z. Gu).

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reactors that produce refolded proteins at a low concentration, which makes the whole refolding process costly and time-consuming.

Many other means of inhibiting aggregation have been tried instead of lowering protein concentration simply by dilution, such as adding aggregation inhibitors into the renaturation buffer. Due to the properties of destabilising incorrectly folded proteins and preventing aggregation, arginine is one of the popular aggregation inhibitors [4]. Molecular chaperones have also been successfully used to refold proteins in different situations and the mechanism involved has been investigated thoroughly [5–7]. Refolding by chromatography is also an alternative to the dilution refolding and has been paid much attention in recent years. Those techniques include immobilization on gel matrices [8], chelating refolding [9], hydrophobic interaction chromatography refolding [10], binding to an ion-exchange matrix [11,12], and the use of gel filtration chromatography (GFC) [13–15]. Among these methods gel filtration chromatography has been a major concern as a substitute for dilution refolding because it is easy to operate and scale up. It is suggested that a reduced diffusion of proteins during gel filtration can increase the yield of active proteins and the gradual removal of denaturant in gel filtration may also inhibit the aggregation, thus enhance the correct refolding of some proteins [16,17]. In this article an improved gel filtration method was developed to refold the denatured lysozyme. A gel filtration column was pre-equilibrated with a linear decreased urea gradient (Fig. 1). After the denaturated lysozyme was applied on the top of the column where the urea concentration is the same as that in the sample, due to its large molecular mass of 14 000 it moved faster than the gradient formed by small molecules of urea. Therefore the denatured protein could pass along the gradient and refold to native conformation as urea was gradually removed. The results demonstrated that it could significantly increase the yield at high protein concentrations compared to the dilution refolding and gel filtration without urea gradient.

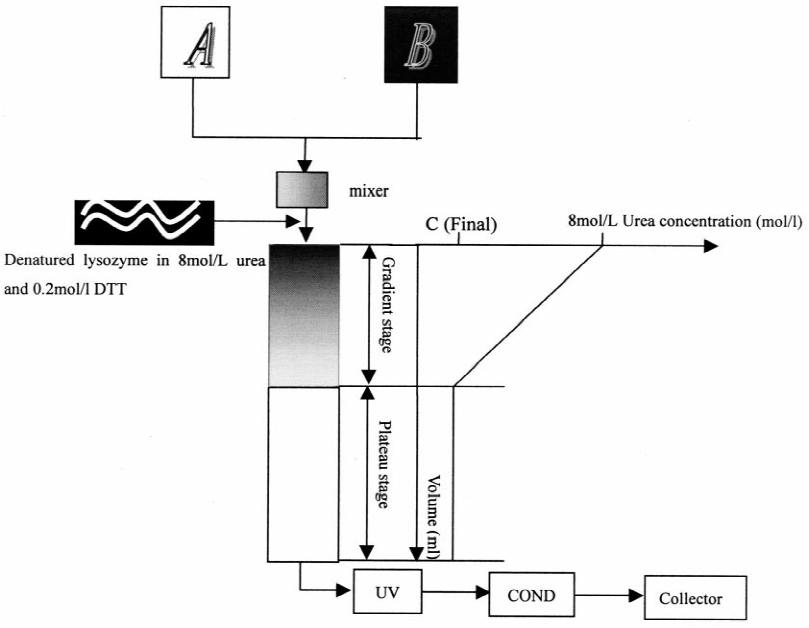

Fig. 1. Experimental system for urea gradient gel filtration refolding process. A single Superdex 75 (10/30) pre-packed column (Amersham Pharmacia) was pre-equilibrated as shown in the figure.

2. Experimental

2.1. Materials

Egg white lysozyme was purchased from Institute of Biochemical (Shanghai, China). Dithiothreitol (DTT), oxidized glutathione (GSSG) and reduced glutathione (GSH) were purchased from Sigma. Urea was purchased from Beijing Chemical Reagent. All other chemicals were analytical grade. Water used for the experimental work was ultrapure water obtained from Millipore system.

2.2. Equipment

A Superdex 75(10/30) (i.e., 300 mm×10mm I.D.) pre-packed column was connected with AKTA purifier (Amersham Pharmacia) to perform the chromatography process. A UV–Vis spectrophotometer ultrospect 2000 (Amersham Pharmacia) was used for analyzing enzyme activity and protein concentration.

2.3. Preparation of denatured proteins

Denaturation buffer: 0.1 mol/l Tris–HCl (pH 8.7) containing 8 mol/l urea and 0.2 mol/l DTT. Original denatured protein sample for refolding was prepared by incubating the native lysozyme in denaturation buffer for about 4–5 h at room temperature. For dilution refolding the original sample solution should be acidified to pH 3 by addition of 1 mol/l HCl to prevent the formation of wrong disulfide bond before refolding and DTT was removed by a Sephadex G-25 column equilibrated with the HCl-acidified Tris buffer containing 8 mol/l urea afterwards [18]. For gel filtration refolding the original sample was adjusted to the same protein concentration as that of the sample for the dilution refolding with denaturation buffer

2.4. Renaturation process

2.4.1. Pre-equilibrate gel filtration column and refolding process

Buffer A: 0.1 mol/l Tris (pH 8.7), 1 m*M* EDTA, 0.15 mol/l NaCl, the ratio of GSH to GSSG was 3 m*M*/0.3 m*M*. Buffer B: buffer A containing 8 mol/l urea. A Superdex 75 (10/30) pre-packed column was first equilibrated with the mixed buffer of various ratios of A and B followed by a gradient of various lengths to the urea concentration of 8 mol/l (100% B) (as shown in Fig. 1, dark colors represent higher urea concentrations; light colors represent lower urea concentrations). After equilibration in this manner, 200 μl denatured lysozyme of various concentrations were applied to the column and eluted with buffer B. Initial denatured lysozyme concentrations were varied from 2.6 to 30.1 mg/ml

2.4.2. Refolding by non-gradient gel filtration

A Superdex 75 (10/30) pre-packed column was equilibrated with a mixed buffer of buffer B–buffer A (25:75). The compositions of buffer A and buffer B are given in Section 2.4.1. After equilibration, 200 μl denatured lysozyme of various concentrations were applied to the column and eluted with the same equilibrating buffer. Denatured lysozyme concentrations were varied from 2.6 to 30.1 mg/ml

2.4.3. Refolding by dilution

Denatured lysozyme (200 μl) of various concentrations were rapidly diluted into the renaturation buffer, similar to the equilibrating buffer for non-gradient gel filtration refolding (as mentioned in Section 2.4.2) by a dilution factor of 40. Initial denatured lysozyme concentrations were varied from 2.6 to 30.1 mg/ml. The results obtained in both non-gradient gel filtration and dilution refolding provide the basis against which to compare the gradient gel filtration refolding process. All the refolding processes were carried out at room temperature

2.5. Protein activity assay

Lysozyme activity was determined as described by Shugar [19]. The absorbance of *M. lysodeikticus* suspension in 0.1 mol/l potassium phosphate, pH 6.2, was first adjusted to 1.3, and 50 μl of protein sample were added to 2.5 ml of the above suspension, and the initial rate of decrease in absorbance at 450 nm was measured.

2.6. Protein concentration assay

According to the Bradford method [20], Coommassie Blue G250 was used as dye reagent to measure the absorbance at 595 nm. Pure lysozyme was used to create the standard curve in stead of bovine serum albumin (BSA).

3. Results and discussions

3.1. Refolding of lysozyme by urea gradient size-exclusion chromatography (SEC)

Fig. 2 shows a chromatogram of urea gradient refolding process. The urea gradient is demonstrated by the change in conductivity curve. The buffer solution contains higher urea concentration and has lower conductivity because of the non-ionic characteristics of urea. A linear decrease of conductivity curve in the profile indicates a linear increase in urea concentration as shown in Fig. 2. By selecting a suitable gradient length, the refolded lysozyme can move exactly into the refolding buffer containing lower urea concentration at the end of the column. Therefore a true linear removal of urea was realized in this manner.

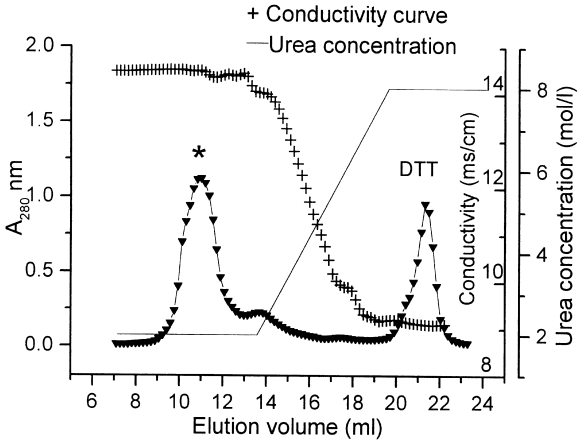

Fig. 2. Profile of urea gradient refolding process. Lysozyme loading: 200 μl at 9.7 mg/ml. Elution flow-rate: 0.3 ml/min. Buffer A: 0.1 mol/l Tris (pH 8.7), 1 m*M* EDTA, 0.15 mol/l NaCl, the ratio of GSH to GSSG was 3 m*M*/0.3 m*M*. Buffer B: buffer A containing 8 mol/l urea. Urea gradient: 6 ml from 2 mol/l (25% B) to 8 mol/l (100% B). *Represents the peak of refolded lysozyme.

3.2. Comparison of the three refolding processes

The comparison of the different renaturation processes shows the gradient gel filtration refolding has the best result with regard to activity recovery (Fig. 3). It is also demonstrated in the chromatography profile that area of the peak containing refolded lysozyme is larger in gradient gel filtration process than that in the non-gradient process (Fig. 4). The recovery decreased with the increase in the initial protein concentration in all the three refolding processes, but the urea gradient process decreased the least and the higher the initial protein concentration, the bigger the difference between the gradient refolding and the other two methods in terms of activity recovery. There is also a small peak following the first refolded lysozyme peak in the gradient refolding (Fig. 4). It is because the aggregates formed during the refolding process precipitated on the surface of the gel media or unspecific bound with the gel media and stop migrating [17]. With the urea gradient moved downwardly the precipitated aggregates can be re-solubilized by the following urea front and be eluted out from the column after the first native lysozyme peak. The higher the initial protein con-

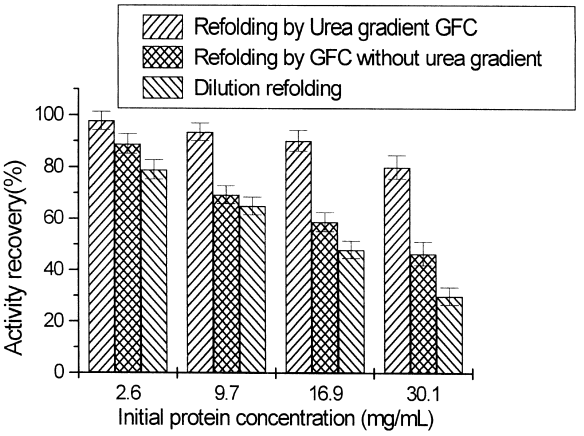

Fig. 3. Comparison of different refolding processes. For gradient refolding the conditions are the same as those in Fig. 2. For non-gradient and dilution refolding the mixed buffer of 25% B was used directly.

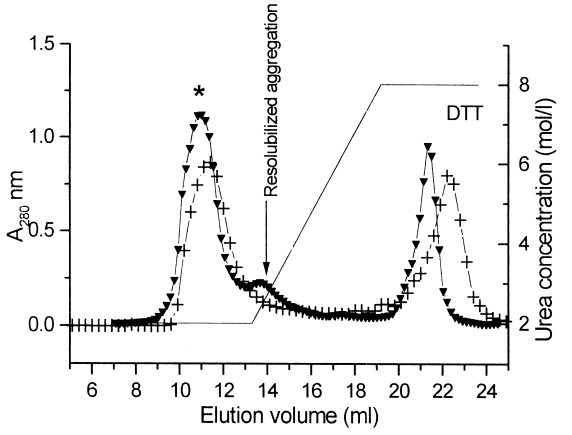

Fig. 4. Comparison of two chromatographic refolding profiles. (+) Non-gradient refolding. (▼) Urea gradient refolding. (—) Urea concentration curve. Conditions as in Fig. 2.

centration, the larger the area of the second peak (Fig. 5), which means more aggregates formed. That is the reason for the decrease in activity recovery as the initial protein concentration increased. There is only one peak of refolded lysozyme in non-gradient process (Fig. 4) because the concentration of following urea in this process was not high enough to re-solubilize the precipitation since the initial urea in sample was diluted as it went through the column. The resolubilization process provides a second chance for the precipitated protein to escape and refold [17]. The lack of resolubilization in non-gradient gel filtration refolding process is another reason for the lower activity recovery besides the quick removal of urea.

3.3. Effect of final urea concentration on the lysozyme refolding

Urea is not only a strong protein denaturant but also an effective aggregation inhibitor. It is found that urea at non-denaturing concentrations can improve the yield of correctly folded protein [21]. We found activity recovery of lysozyme decreased with the increase in the final urea concentration as the final urea concentrations were higher than 2 mol/l; however, we did not find any loss in the protein mass recovery in this case (Fig. 6). So it was quite obvious that the denatured lysozyme cannot refold to the native lysozyme completely in the presence of urea concentration higher than 2 mol/l. The protein mass recovery and activity recovery both decreased with the decrease in the urea concentration as the final urea concentration was lower than 2 mol/l (Fig. 6). It was clearly demonstrated in the elution profile that the peak area of refolded lysozyme decreased significantly at zero urea concentration compared to that at 2 mol/l urea (Fig. 7). The loss of recovery is mainly due to the loss of protein mass caused by aggregation when the protein passed quickly to the urea concentration lower than 2 mol/l. For the same

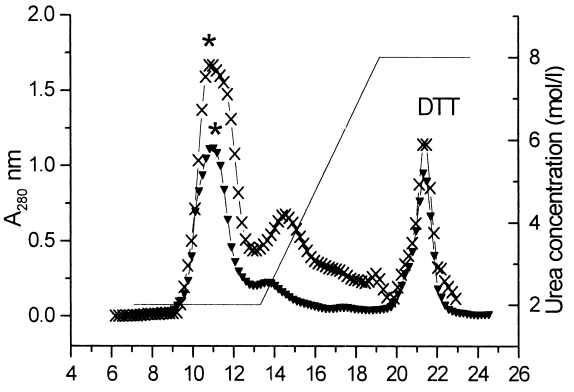

Fig. 5. Urea gradient refolding profiles at different initial protein concentrations. (▼) Initial protein concentration, 9.7 mg/ml. (×) Initial protein concentration, 16.9 mg/ml. (—) Urea concentration curve. Conditions as in Fig. 2.

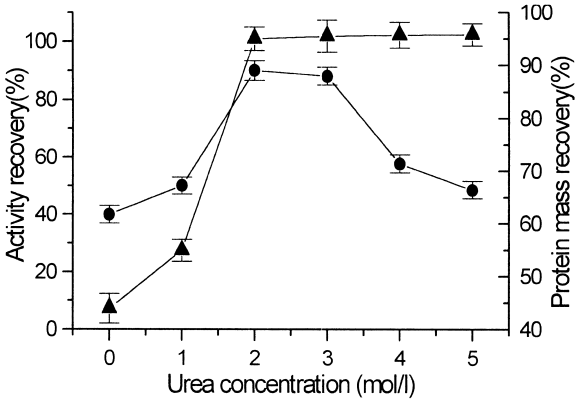

Fig. 6. Effect of final urea concentration on the activity recovery and protein mass recovery. (▲) Protein mass recovery. (●) Activity recovery. Initial protein concentration, 16.9 mg/ml. Other conditions as in Fig. 2.

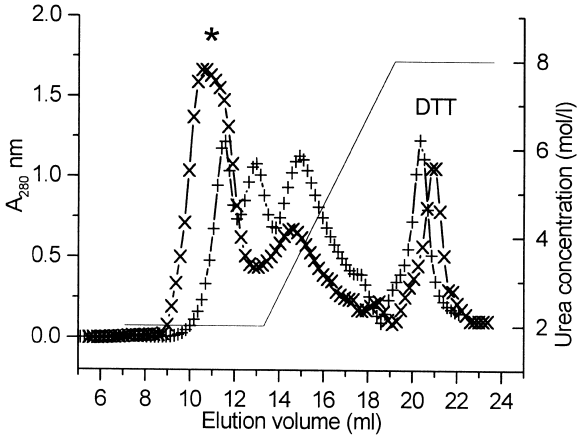

Fig. 7. Urea gradient profiles of different final urea concentrations. (×) Final urea concentration 2 mol/l. (+) Final urea concentration 0 mol/l. Other conditions as in Fig. 2. *Represents the peak of refolded lysozyme.

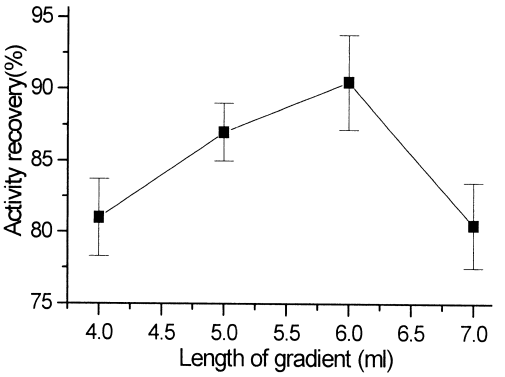

Fig. 8. Effect of gradient length on the activity recovery. Other conditions as in Fig. 2.

reason mentioned previously, the precipitated aggregates were re-dissolved and eluted out from the column followed the refolded lysozyme peak. We found two peaks containing little activity appeared at high urea concentration followed the first refolded lysozyme peak. The two peaks are the re-dissolved aggregates precipitated on the gel matrix and the area of the two peaks decreased significantly when the plateau urea concentration was 2 mol/l (Fig. 7), which means less aggregates formed at final urea concentration of 2 mol/l.

3.4. Effect of the length of urea gradient on the lysozyme refolding

The Fig. 8 shows the highest recovery was obtained at gradient length of 0.27 column volume (6 ml) for Superdex 75 (10/30). With this gradient denatured lysozyme could move exactly to the final renaturation buffer containing 2 mol/l urea at the end of column, meanwhile it provided a gradient long enough for gradual removal of urea from denatured lysozyme, thus gives the highest recovery. With a gradient length of 4 ml, although the plateau period of 2 mol/l urea was even longer to ensure denatured protein moved to the renaturation buffer at the end of the column, the sharp urea gradient made the removal of urea too quick to get high activity recovery. It is understandable that if the gradient was further sharpened to an extreme extent, namely non-gradient gel filtration, the activity recovery of non-gradient gel filtration was lower than that of gradient gel filtration as already indicated by Fig. 3. It also suggests to us that the speed of denaturant removal is closely associated with the extent of correct refolding. Denatured protein was eluted out at urea concentrations higher than 2 mol/l when the gradient length was longer than 6 ml. It has already been indicated in Fig. 6 that the highest recovery was obtained when the final urea concentration was 2 mol/l. With a shallower gradient, although the removal of urea was even gentler, the final urea concentration was too high to make the protein refold to the native conformation completely.

3.5. Effect of elution flow-rate on the lysozyme refolding

The effects of elution flow-rate on the protein refolding are shown in Fig. 9. It was clearly demonstrated that the higher the elution flow-rate, the lower the activity recovery. This phenomenon was consistent with the previous result about the effect of gradient length on the activity recovery. When the length of urea gradient was constant, the speed of urea removal increased with the increase of elution flow-rate, which resulted in lower activity recovery. In order to strike a balance between experimental time and recovery, we chose 0.3 ml/min as our

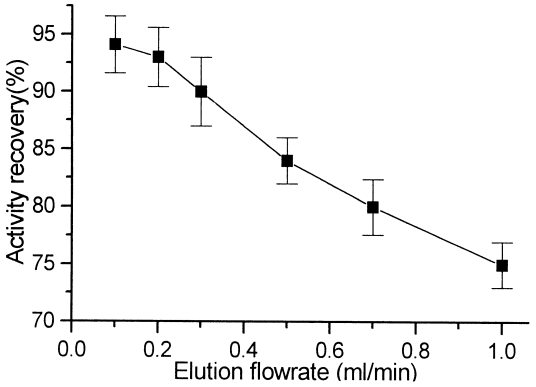

Fig. 9. Effect of elution flow-rate on the activity recovery. Other conditions as in Fig. 2.

optimal elution flow-rate. With this flow-rate, activity recovery of more than 90% was obtained within 40 min from initial protein concentration up to 17 mg/ml with a dilution factor about 40.

Table 1
Comparison of urea gradient gel filtration refolding system and size-exclusion protein refolding system (SEPROS)

	Urea gradient gel filtration refolding system	SEPROS[a]
Column volume (ml)	23.56	467.21
Lysozyme loading:		
Mass (mg)	1.9	14.5
Concentration (mg/ml)	9.7	9.6
Sample volume (ml)	0.2	1.5
Recovery:		
Mass recovery (%)	95	83
Specific recovery (%)	94.7	101
Overall activity yield (%)	90	84
Final protein concentration (mg/ml)	0.22	0.18
Efficiency (mg/ml)[b]	0.081	0.032

The comparison was made between the results obtained by the optimal conditions of both systems.

[a] SEPROS refers to a size-exclusion protein refolding system. The data are calculated from Ref. [16].

[b] The ratio of a sample volume to column volume is the refolding efficiency of the system.

4. Conclusion

The urea gradient gel filtration refolding system is a new contribution to the size-exclusion protein refolding system (SEPROS) proposed by Batas and Chaudhuri [16]. Therefore, it is important to make a comparison between the two refolding systems. The relative data obtained from both systems are summarized in Table 1. It is found that the efficiency of urea gradient gel filtration refolding is much higher than that of SEPROS, indicating the potential advantage of the present system in the protein refolding. The urea gradient gel filtration refolding system provides a gentle environment for protein renaturation. In the non-gradient gel filtration system, although the speed of urea removal may slower than dilution, the denatured protein still encounters drastic change in urea concentration, which may lead to the increase of incorrectly folded proteins. Therefore we came to the conclusion that a quick change in urea concentration may deteriorate the gradual change in protein conformation during the refolding process in the column, thus leading to the formation of incorrectly folded aggregates. On the contrary, a gradient change in urea concentration may stabilize the correctly folded intermediates of different refolding stages and direct the refolding process to further develop in a correct way until the final native conformation was reached.

It is worth noting that the gel filtration can separate proteins of different molecular mass due to the properties of a gel matrix. So with the combination of urea gradient and gel filtration process, proteins with different molecular mass may separate with each other at the same time, thus the purification and renaturation process may be realized simultaneously. Therefore, we think that the urea gradient gel filtration provides an efficient way for industrial scale protein purification and renaturation. It may also become a powerful method to analyze the association of urea concentration with the conformational change of denatured protein during refolding. Further studies should be focused on testing the efficiency of applying this newly developed process to refold other proteins, especially recombinant inclusion bodies and thoroughly investigating the mechanism involved.

Acknowledgements

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