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Lysozyme refolding at high concentration by dilution and size-exclusion chromatography

GAO Yong-gui(高永贵)[1,2], GUAN Yi-xin (关怡新)[1],
YAO Shan-jing (姚善泾)[1†], CHO Man-gi[3]

([1] *Department of Chemical and Biochemical Engineering*;
[2] *College of Life Science, Zhejiang University, Hangzhou 310027, China*)
([3] *Engineering Research Center, Dong-Seo University, 69-1Jurea-dong Sasanggu, Busan 617-716, Korea*)

[†] E-mail: yaosj@che.zju.edu.cn

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Abstract: This study of renaturation by dilution and size exclusion chromatography (SEC) addition of urea to improve yield as well as the initial and final protein concentrations showed that although urea decreased the rate of lysozyme refolding, it could suppress protein aggregation to sustain the pathway of correct refolding at high protein concentration; and that there existed an optimum urea concentration in renaturation buffer. Under the above conditions, lysozyme was successfully refolded from initial concentration of up to 40 mg/mL by dilution and 100 mg/mL by SEC, with the yield of the former being more than 40% and that of the latter being 34.8%. Especially, under the condition of 30 min interval time, i.e. $\tau > 2(t_{R2} - t_{R1})$, the efficiency was increased by 25% and the renaturation buffer could be recycled for SEC refolding in continuous operation of downstream process.

Key words: Lysozyme refolding, Downstream process, Dilution, Size exclusion chromatography, Urea
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INTRODUCTION

More and more genes of interest could be expressed in foreign cells to obtain large amounts of desired proteins. However, such proteins are often produced as inactive inclusion bodies in *Escherichia coli*. Protein refolding from inclusion bodies involves a series of operations, i.e., isolating the inclusion bodies, dissolving them in strong denaturants and recovering the biological activity by the controlled removal of the denaturant. Because protein refolding is a limiting step for bioprocess industry, it has been a focus for basic research and application (De Bernardez Clark, 2001). Protein refolding by traditional dilution method was used to develop size-exclusion chromatography protein refolding system (SEPROS) to increase the yield during the refolding of lysozyme and bovine carbonic anhydrase (Batas et al., 1996; 1999). Use of SEC, successfully achieved renaturaton of heterodimeric platelet-derived growth factor and urokinase plasminogen activator from inclusion bodies (Müller et al., 1999; Fahey et al., 2000). Researchers reported recently that yield was enhanced by urea gradient SEC; and that the aggregates could also be suppressed. However, it was not easy to operate under gradient elution with ordinary apparatus (Gu et al., 2001).

It is known that the refolding yield decreases with the increase of the concentration of denatured proteins because of the kinetic competition between the protein aggregation and protein refolding (Batas et al., 1996). However, refolding at low protein concentration often leads to the requirement of large refolding reactor and quantities of buffer; and increasing difficulty in protein recovery. How to refold proteins at high initial concentration with high recovery is valuable and challengable. Denatured lysozyme refolding has

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† Author for correspondence

been a subject of interest for many years, and recently the lysozyme is becoming popular for evaluation of new refolding strategies (Rozema et al., 1996; Katoh et al., 2000).

This paper reports studies on refolding of lysozyme at high concentration by dilution and SEC with optimum amount of urea in renaturation buffer as dilution additives.

MATERIALS AND METHODS

1. Materials

Hen egg white lysozyme, dried *Micrococcus lysodeikticus* cells, reduced glutathione (GSH), oxidized glutathione (GSSG), dithiothreitol (DTT) and Trizma base were purchased from Sigma. ÄKTA Explorer 100, superdex 75 and XK16/20 column were obtained from Phamacia Biotech. All other chemicals were analytical grade.

2. Preparation of denatured lysozyme

Native lysozyme was denatured by incubation in 0.1 mol/L Tris-HCl, pH 8.5 containing 8 mol/L urea and 0.03 mol/L DTT for 4 h at room temperature; then the denatured lysozyme was aliquoted into eppendorf tube and stored at −20 ℃ as stock solution. To remove DTT in denatured lysozyme solution, the solution was acidified to pH 3 by the addition of 1 mol/L HCl and dialyzed against 0.1 mol/L acetic acid at 4 ℃ O/N.

3. Refolding by dilution

Denatured lysozyme was diluted into renaturation buffer(0.1 mol/L Tris-HCl, pH 8.0, containing 1 mmol/L EDTA, 0.15 mmol/L NaCl, 3 mmol/L GSH, 0.3 mmol/L GSSG and a definite amount of urea) by rapidly vortexing at 20℃; after incubation at regular intervals , the activity of a mixture sample was assayed.

4. Refolding using SEC

Size-exclusion chromatography refolding was performed using a XK16/20 column packed with Superdex 75 gel media. The column was equilibrated with renaturation buffer, following sample injection, the elution fractions were analyzed for enzymatic activity.

5. Enzyme activity and protein assay

Lysozyme activity was determined by the method of F.I.P (Stellmach et al., 1992) and protein concentration was determined by Coomassie Brilliant Blue Assay (Brandford, 1976).

RESULTS AND DISCUSSION

1. Refolding by dilution

Fig.1 shows that there was no obvious difference with respect to the activity recovery of Process A and Process B. GSH/GSSG in renaturation buffer could change the redox potential of the refolding mixture to shuffle the disulfide bond of the lysozyme (Rozema et al., 1996). However, after 20-fold dilution, the concentration of DTT existing in the denatured lysozyme solution was very low (only 1.5 mmol/L) and had little effect on the formation of the lysozyme disulfide bond. Under the denatured condition with 30 mmol/L DTT, it was not necessary to remove DTT in the denatured lysozyme by dialysis or gel filtration before refolding. It is very helpful for industrial process to decrease cost and the number of steps.

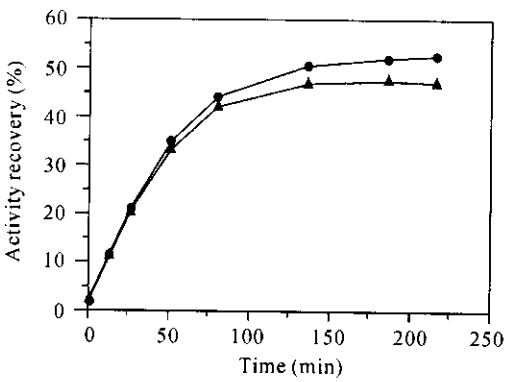

Fig.1 Influence of DTT in denatured solution on the refolding by dilution (3 mol/L urea in renaturation buffer and dilution by 20 fold, Process A: ●, directly diluted the denatured lysozyme solution; Process B: ▲, refolding by dilution after removing DTT by dialysis against 0.1 mol/L acetic acid at 4℃ O/N)

Almost full activity could be recovered at the lowest initial concentration of 1 mg/ml diluted 80 fold, whereas the yield fell to 25% at the initial concentration of 40 mg/mL(Fig.2). While the initial concentration increased, the refolding yield significantly decreased at the same dilution factor. The refolding yield increased with the increase of the dilution factor at the same initial

concentration. However, at a dilution factor of 80, the refolding yields were as low as 47% and 25% at initial concentrations of 20 mg/mL and 40 mg/mL, respectively. Moreover, while the initial concentration was low, the final protein concentration was lower after dilution; it was even more difficult to recover the active protein.

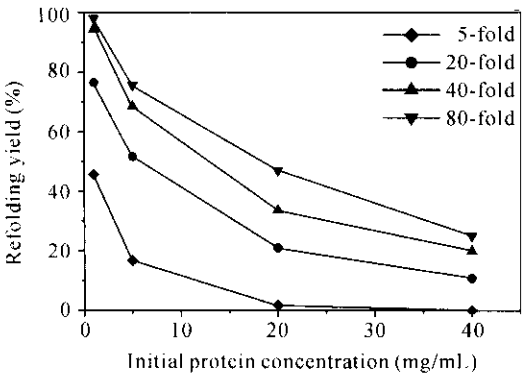

Fig.2 Effect of initial protein concentration and dilution factor on lysozyme refolding by dilution (the initial protein concentrations were 1 mg/mL, 5 mg/mL, 20 mg/mL and 40 mg/mL, respectively, renaturation buffer containing 2 mol/L urea, refolding at 20℃ O/N)

Adopting the conditions for maximum yield of reactivation (Goldberg et al., 1991), it was found that refolding at initial concentration of 40 mg/mL diluted 20-fold and 40-fold or 20 mg/mL diluted 20-fold, protein aggregates were observed. For 5-fold dilution in which the final urea was about 3.2 mol/L in the refolding mixture, no protein precipitation was observed.

Considering that urea can suppress the protein aggregation, which is the main cause for low recovery of protein activity, so we investigated the effect of urea in renaturation buffer during protein refolding at high concentration. Urea had great impact on both yield and rate of the lysozyme refolding (Fig. 3). In order to obtain high recovery, the optimum urea concentration was suggested to be increased with the increase of denatured lysozyme concentration. The initial concentration was 5 mg/mL, the refolding buffer should contain 3 mol/L urea, while the initial concentration was 40 mg/mL, the optimum concentration of urea was increased to 4 mol/L. During refolding, two types of interactions including: correct intrachain and incorrect interchain interactions occurred. The former led to refolding and the latter resulted in aggregation. Kinetic competition existed between these interactions. The rate of aggregation increased faster than that of refolding if the protein concentration was increased (Batas et al., 1996). In the present research, higher concentration of urea suppressed the protein aggregation, and decreased the rate of aggregation faster than that of refolding. This effect can cause the unfolded protein to continue to fold at high concentration, thereby, promoting the folding and improving the yield.

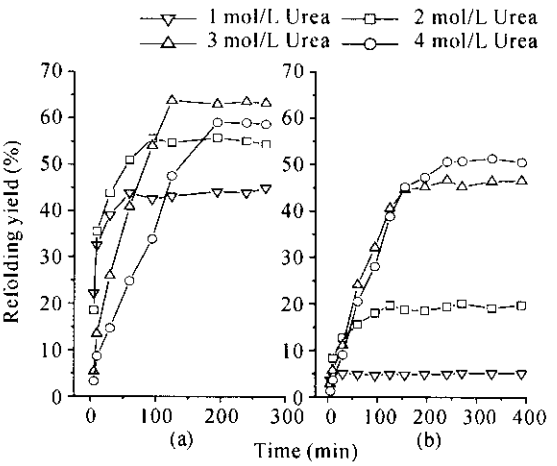

Fig.3 Effect of urea on kinetic of lysozyme refolding at low and high initial protein concentration
(a) 5 mg/mL initial protein concentration;
(b) 40 mg/mL initial protein concentration

2. Refolding Using SEC

(1) Choice of column height

Superdex 75 is a novel SEC gel media with high performance, and exclusive molecular weight ranging from 3 kDa to 70 kDa. The molecular weight of lysozyme is 14.3 kDa, DTT and other denaturants are low molecular materials (molecular weight lower than 3 kDa). In order to refold lysozyme, the key procedure is to slowly remove the denaturant from the lysozyme solution; which can be done by SEC using a XK16/20 column packed with Superdex 75 gel media. When the applied sample was 0.1 mL (20 mg/mL lysozyme) and the volumetric flow rate was 0.3 mL/min, the separation peak of lysozyme and low molecular denaturant was as shown in Fig. 4. The resolutions (Rs) were 0.9, 1.4 and 2.1 at the column height of 3.5 cm, 5.0 cm and 7.0 cm, respectively, calcu-

lated by UNICORE 3.0 operation system. According to chromatography theory, when Rs was more than 1.5, the refolded lysozyme and the denaturant were to be separated completely, hence the column d height of 7 cm was adopted to refold lysozyme.

(2) Effect of urea on lysozyme refolding

Like refolding by dilution, SEC refolding with renaturation buffer addition of various amounts of urea was tested, the elution profile is as represented in Fig.5 and the result is shown in Table 1. Molecule Stokes radius(S_r) was calculated by using the equation of the elution volume (V_e) and S_r reported by Batas et al.(1997).

$$S_r = (0.94 - 6.3/V_e) \quad (1)$$

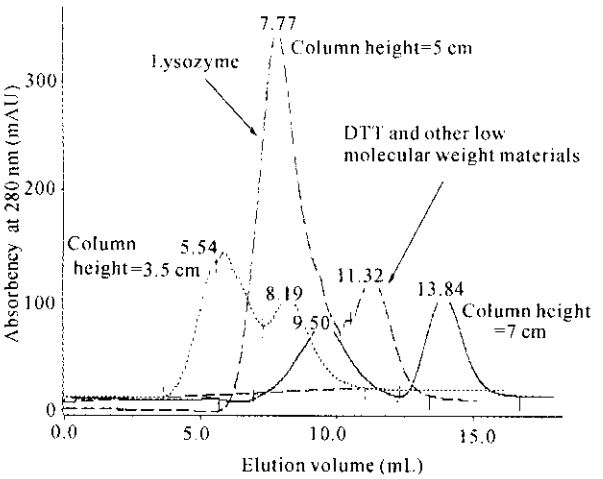

Fig.4 Elution profile for SEC refolding at different column height

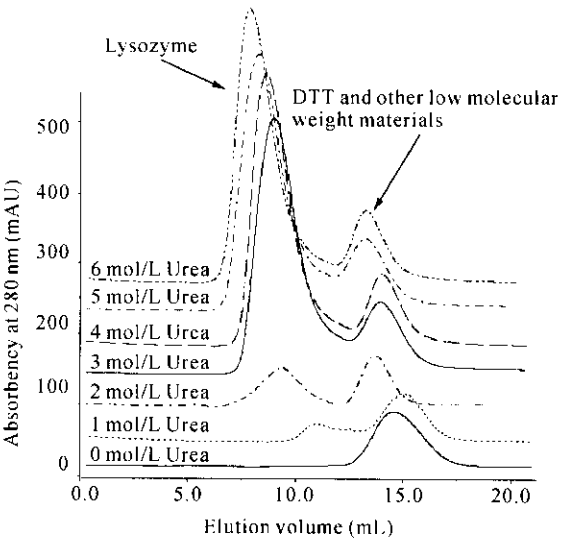

Fig.5 Elution profile for SEC refolding lysozyme with renaturation buffer containing various amount of urea (Denatured lysozyme concentration: 20 mg/mL; applied sample: 0.1 ml ; volumetric flow rate: 0.3 mL/min)

Table 1 Effect of urea on lysozyme refolding using size-exclusion chromatography

Urea (mol/L)	0	1	2	3	4	5	6
Elution volume (ml)	0	11.13	9.50	9.16	8.84	8.55	8.18
Stokes radius (nm)	/	2.67	3.61	3.96	4.40	4.92	5.89
Activity recovery (%)	0	15.8	42.3	75.6	83.1	30.2	21.7

As the concentration of urea increased from 1 mol/L to 6 mol/L, the elution volume of the refolded lysozyme decreased from 11.3 mL to 8.18 mL; while the S_r of lysozyme molecule increased from 2.67 nm to 5.89 nm. During lysozyme refolding, the protein molecule developed a more compact and native-like structure called molten globule which was easy to fold. At this stage, the protein had a smaller S_r and could move further into the pores of the gel media. The average partition coefficient of molten globule was larger than that of denatured lysozyme which contributed a lot to separate them. Because of separation by gel pores, intermolecular aggregation was reduced. It should be noted that for renaturation buffer without urea, no refolded lysozyme peak was detected; probably because of the high viscosity of the solution at high lysozyme concentration (Batas et al., 1997). The viscosity increased while the diffusive rate of protein into the gel pores decreased. The optimum urea in renaturation buffer was 4 mol/L, when the yield recovery was 83.1% (Table 1). The lysozyme refolding yield decreased when the urea concen-

tration was 6 mol/L and 5 mol/L. For urea concentration higher than 4 mol/L, the denaturation effect was too strong to inhibit protein molecule from going through the high-energy transient state, which was the free energy landscape for protein folding (Adkevich et al., 1994).

(3) Effect of protein concentration on lysozyme refolding

Under the above conditions, lysozyme refolding was investigated by SEC at initial concentrations of 20 mg/mL, 50 mg/mL and 100 mg/mL, respectively(Table 2). The yield recovery was the total active protein recovery including enzymatic activity recovery and protein recovery. Protein refolding at high initial protein concentration and with high yield is the focus of industrial process. The highest yield recovery was 83.1% at 20 mg/mL, with the initial concentration increasing, the specific activity recovery and yield recovery decreased. Although the concentration was up to 100 mg/mL, the yield recovery and the concentration recovered were still as high as 34.8% and 1.06 mg/mL.

Table 2 Lysozyme refolding using SEC at high initial concentration

Initial concentration (mg/mL)	Protein applied (mg)	Concentration recovered (mg/mL)	Specific activity recovery (%)	Yield recovery (%)
20	2	0.32	89.4	83.1
50	5	0.63	79.4	54.1
100	10	1.06	74.6	34.8

(4) SEC refolding with sample application in a continuous operation

A good protocol for protein refolding should possess high yield capacity. In addition to the scale up of column using SEC refolding, sample application in a continuous operation could also improve the yield capacity and save renaturation buffer with isogradient elution. As shown in Fig.6, if the interval time τ was under the condition $\tau > 2(t_{R2} - t_{R1})$, where t_{R2} and t_{R1} were the retention times of the first and second elution peak, there was no mutual influence for applying samples twice in continuous operation. The activity recoveries were 82.5% and 81.8%, compared with that of batch refolding using SEC (83.1%, see Table 1); there was no obvious difference. In the present experiment, τ was 30 min and $2(t_{R2} - t_{R1})$ was 29.4, hence 30 min was saved (60 min needed for batch refolding), i.e., the efficiency was increased by 25%.

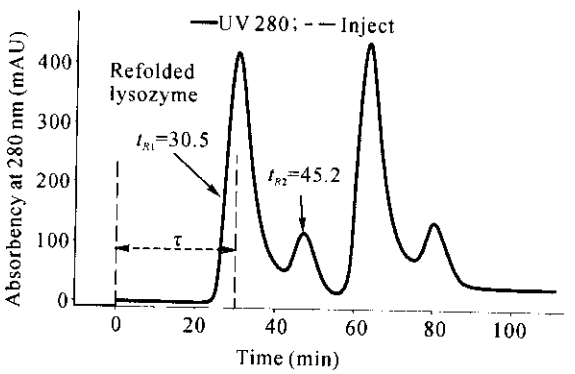

Fig. 6 Elution profile of lysozyme refolding using SEC in continuous operation

CONCLUSIONS

The results here illustrated that renaturation buffer addition of urea was a very useful strategy to improve activity recovery when refolding by dilution or SEC at high initial protein concentration. However, the optimum concentration of urea varied with the changing of initial concentration of protein. Dilution refolding is a basic method for other refolding protocols. The activity recovery by SEC refolding was increased by 10% ~ 20% more than that by dilution refolding using lysozyme as model protein. Lysozyme refolding with XK16/26 column packed Superdex 75, could not detect the peak of protein aggregate. If a high-resolution gel media was chosen, or a prepacked column was used, the protein aggregates and refolded proteins could be further separated (Zhang et al., 1992; Batas et al., 1996; Müller et al., 1999).

The recovery of bioactive proteins from inclusion bodies is a complex process. Various

protocols have now been developed for protein refolding *in vitro* . To be acceptable for commercial process, refolding protocols should yield relatively high concentration, be highly efficient and operation easily. Compared with SEPROS by Batas et al., (1996) and gradient urea SEC refolding by Gu et al. (2001), it was found that SEC with isogradient buffer containing 4 mol/L urea was slightly less effective in total activity recovery, although the final concentration and efficiency were improved (Table 3).

Table 3 Comparison of Batas', Gu' and this work

	Batas	Gu	This work
Column volume (mL)	467.2	23.6	14.0
Lysozyme application (mL × mg/mL)	1.5 × 9.6	0.2 × 9.7	0.2 × 20
Lysozyme amount (mg)	14.5	1.9	4
Total activity recovery (100)	90	84	81
Final concentration (mg/mL)	0.22	0.18	0.54
Renaturation buffer recycle	Recycled	Not recycled	Recycled
Efficiency for one batch (mg/mL)	0.032	0.081	0.286
Efficiency in continuous production (mg/h•mL)	No report	0.062	0.358

Note: The ratio of sample application to column volume is the refolding efficiency for one batch, and the ratio value per hour is the refolding efficiency which directly relates to productivity.

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