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Hydrophobic interaction chromatography correctly refolding proteins assisted by glycerol and urea gradients

Jing-Jing Li[a], Yong-Dong Liu[a], Fang-Wei Wang[b,c], Guang-Hui Ma[a], Zhi-Guo Su[a,*]

[a] *National Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, P.O. Box 353, Beijing 100080, China*
[b] *Dalian University of Technology, DaLian, LiaoNing 116024, China*
[c] *Second Clinical College, Dalian Media University, DaLian, LiaoNing 116027, China*

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Abstract

Chromatographic columns packed with commercially available hydrophobic interaction chromatography (HIC) media were found to be able to suppress aggregation and nevertheless had a tendency to promote the structural misfolding resulting in higher soluble protein recovery and lower specific activity than that by dilution when they were used to refold lysozyme, a model protein. Moreover, this misfolding effect was exacerbated with increasing hydrophobicity of media. A novel strategy involving the combination of glycerol, a typical osmolyte, a urea gradient and commercially available HIC media was introduced to facilitate protein refolding correctly as well as improve mass recovery by providing a gradual change of the refolding environment in the HIC column. In this process, unfolded lysozyme was bound to Poros PE HIC column at high salt concentration and was released by a urea gradient followed by elution with refolding buffer in the presence of 50 % (v/v) glycerol, resulting in 86.3% activity yield and 85% mass recovery with the refolded product of native specific activity. For the absence of glycerol, only 50.9% activity yield and 59% specific activity recovery was obtained although mass recovery was closed to that in the presence of glycerol. It was also discovered that glycerol addition during elution process was necessary for correct refolding compared to mixing of glycerol with post-column fraction. The possible mechanism for refolding with this system was proposed to be relevant to the formation of an on-pathway intermediate that could slowly reactivate.

Keywords: Refolding; Hydrophobic interaction chromatography; Glycerol; Lysozyme; Gradient

1. Introduction

In vitro refolding is a necessary and critical operation unit for processing recombinant proteins that are over-expressed and frequently form inactive inclusion bodies in *Escherichia coli*. [1]. During refolding process, aggregation that arises from assembly of kinetically trapped intermediates by intermolecular hydrophobic interaction often competitively occurs and dominates over on-pathway refolding at increasing protein concentration due to its higher reaction order than refolding resulting in the low yield of active protein of interest [2,3]. To minimize aggregate, classical dilution refolding way has to be performed at very low protein concentration, which is unfavorable for industrial process [4]. An efficient way that promotes refolding and simultaneously minimizes aggregation at high protein concentration will significantly improve the yield of target protein as well as reduce the cost of production [5].

Utilization of chromatographic columns for refolding is a good strategy because the solid media can provide inner particular environment able to separate molecules from each other efficiently during refolding, thereby minimizing the aggregation [6]. Size-exclusion chromatography (SEC)

* Corresponding author. Fax: +86 10 62561822.
E-mail address: Zgsu@home.ipe.ac.cn (Z.-G. Su).

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can promote refolding by restricting the diffusion of protein molecules of various structural forms in the refolding mixture with the removal of denaturant [7,8]. Unfolded proteins can also be immobilized onto the column by various interactions. Refolding of bound proteins to ion-exchanger through electrostatic adsorption can be achieved by elution with increased salt concentration in refolding buffer [9]. Immobilized metal ion affinity chromatography (IMAC) is able to specifically capture target proteins equipped with polyhistidine tags under denaturation condition. Bound protein can be released by refolding buffer containing particular additives, e.g. imidazole of certain concentration resulting in simultaneous purification and refolding [10]. However, commercially available hydrophobic interaction chromatography was seldom reported to successfully refold proteins although self-made PEG support was reported as a medium for HIC refolding [11].

In principle, commercially available hydrophobic interaction chromatography (HIC) media can be used to assist in vitro refolding because the hydrophobic interaction between HIC ligands and denatured proteins as well as refolding intermediates likely minimize aggregate. However, compared with SEC, IEC and IMAC that supply hydrophilic environment favorable for hydrophobic collapse of denatured proteins, commercially available HIC-assisted refolding may bring about the problem that hydrophobic ligands of HIC of relative high density, in view of the commercial purpose to improve capacity for separation, possibly alter thermodynamic free-energy that impels the refolding process of unfolded proteins and then lead to formation of incorrect structure. Moreover, partially refolded proteins may also be denatured again in HIC especially in the case of using strong hydrophobic interaction supports [12]. A strategy to utilize commercially available HIC to correctly refold protein is to gradually increase the hydrophilicity of media with additives and promote intra-molecular hydrophobic collapse of protein during refolding proceeding. Osmolytes can be considered as the ideal candidate in that they are able to strengthen the compactness of protein of inner hydrophobic moieties and prevent their exposure resulting in enhanced stability [13]. Some osmolytes such as sugars, polyols (e.g. glycerol) and certain amino acids, called folding enhancer, was able to enhance refolding and even directly acted as the solvent for protein refolding [14–16]. Thus the combination of osmolytes and HIC is anticipated to both facilitate correctly refolding and prevent aggregation.

In the present work, we developed a new strategy of protein refolding involving the combination of a commercially available HIC media, Poros PE, and a typical osmolyte, glycerol, with lysozyme as the model protein. In addition, the technique of gradient utilization, which was developed in our previous study on SEC and IEC refolding [17,18], was introduced into the elution program to provide a gradual change of the refolding environment. In this process, unfolded lysozyme was adsorbed to Poros PE HIC column at high salt concentration. A urea gradient was used to release bound protein followed by elution with refolding buffer containing glvcerol of high concentration. The effects of various HIC media and the addition of glycerol on refolding efficiency were investigated. A possible mechanism of refolding with our strategy was proposed.

2. Materials and methods

2.1. Materials

Hen egg white lysozyme, *Micrococus lysodeikticus* dried cells were obtained from Sigma. All chemical reagents were commercially available and were of analytical grade. Poros PE, a perfusion hydrophobic interaction support, was purchased from Applied Biosystems, USA. Hitrap Phenyl Sepharose HP (1 ml), Hitrap Butyl Sepharose FF (1 ml) and Hitrap Octyl Sepharose FF (1 ml), Superdex 75 HR 10/30 column (300 mm × 10 mm internal diameter (i.d.)), AKTA purifier 10, ultrospect 2000 UV–vis spectrophotometer were products of GE Healthcare, Uppsala, Sweden.

2.2. Denaturation of proteins

Ten milligrams lysozyme was dissolved in 1 ml unfolding buffer (6 M guanidine hydrochloride (Gdn-HCl), 100 mM Tris–HCl, 1 mM EDTA, 150 mM dithiothreitol (DTT), pH 8.5) and incubated for 3 h at 37 °C to confirm complete denaturation. And then denatured proteins of various concentrations were prepared by dilution of the protein described above with the same unfolding buffer.

2.3. Refolding by dilution

In order to compare with refolding by HIC, 20 μl denatured lysozyme was directly diluted into 1 ml refolding buffer (100 mM Tris–HCl, 1 mM EDTA, 3 mM GSH, 0.3 mM GSSG, pH 8.5) with or without the addition of glycerol.

2.4. Refolding bypassing through HIC column

One hundred microlitres denatured lysozyme was directly applied to different HIC columns (including Packed Poros PE column (1 ml), Hitrap Butyl Sepharose FF (1 ml), Hitrap Octyl Sepharose FF (1 ml) and Hitrap Phenyl Sepharose HP (1 ml)) respectively pre-equilibrated with refolding buffer (100 mM Tris, 1 mM EDTA, 3 mM GSH, 0.3 mM GSSG, pH 8.5) and then was eluted with the buffer at 0.2 ml/min flow rate on AKTA Purifier.

Refolding was also carried out in Packed Poros PE column (128 mm × 10 mm i.d.) with the same buffer.

2.5. Refolding by adsorption–elution way in HIC column

One hundred microlitres unfolded lysozyme of 10 mg/ml was applied to the Poros PE HIC column (128 mm × 10 mm

i.d.) equilibrated with the buffer of high salt concentration (A: 3.6 M $(NH_4)_2SO_4$, 50 mM Tris–HCl, 1 mM EDTA, 3 mM GSH, 0.3 mM GSSG, pH 8.5) followed by 2 ml gradient of A to the denaturant (B: 8 M urea, 50 mM Tris–HCl, 1 mM EDTA, 3 mM GSH, 0.3 mM GSSG, pH 8.5) and subsequent 1 column volume (CV) washing with A resulting in adsorption as well as prevention of aggregation. After refolding buffer (C: 100 mM Tris–HCl, 1 mM EDTA, 0.4 M $(NH_4)_2SO_4$, 0.01% (v/v) β-mercaptoethanol ((β-ME), pH 8.5) continuously washed the column to decrease ionic strength, 4 ml gradient of increasing urea concentration from C to B and 4 ml gradient of decreasing urea concentration from B to C in turn was then introduced into the column to release bound lysozyme followed by elution with C to promote released protein refolding. Flow rate was 0.5 ml/min. All steps were carried out on AKTA purifier.

In the other experiments, 50% (v/v) of final glycerol concentration was added into the buffer C and then the steps described above were performed. The elution process by the gradient of urea and glycerol in the column and mobile phase is modified according to reference [17], as shown in Fig. 1. As a control experiment, glycerol was immediately mixed with pooled refolded lysozyme.

2.6. Enzyme activity assay

After incubation overnight at room temperature, refolded lysozyme obtained with above methods was adjusted to lower than 50 μg/ml of protein concentration with working buffer (0.06 M sodium phosphate, pH 6.2) to increase the sensitivity of reaction. The activity was measured according to the method described by Shugar [19]. Activity yield was the percentage ratio of the activity of refolded lysozyme to that of native lysozyme of the same mass. Specific activity was represented by the activity of refolded lysozyme per milligram. Specific activity recovery was the percentage ratio of the specific activity of refolded lysozyme to that of native lysozyme.

On the other hand, pooled protein was diluted with 200 mM NaH_2PO_4 into about 50 μg/ml at different intervals and then was assayed to observe post-column refolding kinetics.

2.7. Protein concentration assay

The concentrations of proteins were determined according to Bradford method [20]. Pure native lysozyme was used as referenced protein instead of BSA. Mass recovery or soluble protein recovery of was the ratio of total mass of refolded lysozyme to that of loaded unfolded protein for HIC refolding. It was the ratio of protein mass in the supernatant upon centrifugation of refolded solution at 12,000 × *g* to remove sediment to adopted unfolded protein mass for dilution refolding.

2.8. SEC analysis

Fifty microlitres refolded lysozyme was applied to Superdex 75 HR 10/30 (300 mm × 10 mm i.d.) equilibrated with mobile phase (4 M urea, 20 mM Na_2HPO_4, pH 7.0, 0.16 M NaCl) and developed with above buffer at flow rate of 0.5 ml/min.

3. Results and discussion

3.1. Effects of hydrophobicity of HIC media on refolding and aggregation

A series of columns packed with various commercially available HIC media were attempted to refold lysozyme by passing-through way. Unfolded lysozyme was loaded to the columns pre-equilibrated with the refolding buffer followed by elution with the same buffer. As the control experiment, sample was directly 50-fold diluted into refolding buffer at

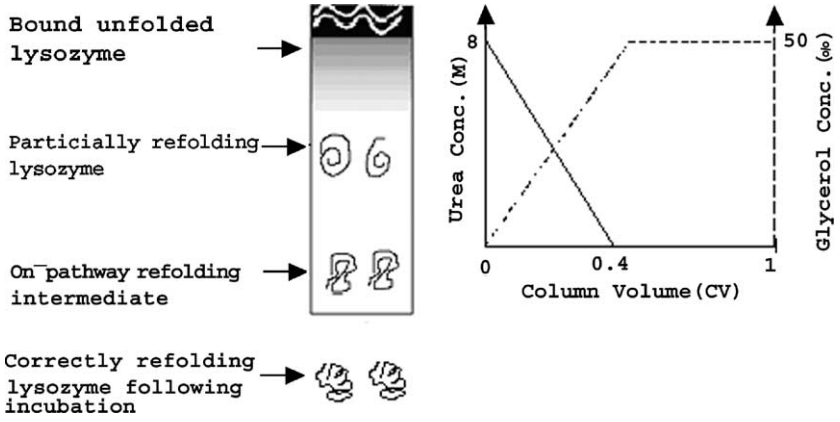

Fig. 1. A schematic diagram for HIC column refolding with gradients of with urea and glycerol. The rectangle represents the HIC column in which curves denote unfolded, partially refolding lysozyme, on-pathway refolding intermediate and correctly refolded lysozyme from the top to the bottom respectively. The gradually weak shadow indicates a gradient of decreasing urea concentration to increasing glycerol concentration. The coordinates system on the right illustrates that concentration of urea (solid line) and glycerol (dotted line) linearly alters with the volume of the column. The *X*-coordinate represents the corresponding position in the column with the volume of zero as the top of the column and 1 CV as the bottom. Conc., concentration.

Table 1
Effects of various HIC media on refolding efficiency by passing through way

Refolding ways	Activity yield (%)	Soluble protein recovery (%)	Specific activity recovery (%)
Dilution	26.8	32.6	82.2
Poros PE column	52	69.7	74.7
Butyl FF column	55.7	79.5	70
Octyl FF column	59.3	81.2	69.8
Phenyl HP column	55.9	89.2	62.7

For the dilution refolding, 20 μl denatured lysozyme at the protein concentration of 10 mg/ml was directly diluted into 1 ml refolding buffer (100 mM Tris–HCl, 1 mM EDTA, 3 mM GSH, 0.3 mM GSSG, pH 8.5). For HIC-assisted refolding, 100 μl denatured lysozyme of 10 mg/ml was directly applied to different HIC columns (including Packed Poros PE column (1 ml), Hitrap Butyl Sepharose FF (1 ml), Hitrap Octyl Sepharose FF (1 ml) and Hitrap Phenyl Sepharose HP column (1 ml)) respectively pre-equilibrated with the refolding buffer (100 mM Tris, 1 mM EDTA, 3 mM GSH, 0.3 mM GSSG, pH 8.5) and then was eluted by refolding buffer at 0.2 ml/min flow rate on AKTA Purifier. The fractioned peaks were incubated overnight at room temperature and their activity and protein concentration were assayed.

200 μg/ml of final protein concentration. Table 1 shows the comparison of refolding efficiency by various HIC columns and by dilution.

For dilution refolding, large amount of precipitation was observable and soluble protein recovery of dilution refolding in supernatant upon centrifugation of refolded solution was only 32.6%. Comparatively, the pooled fractions from the HIC columns were transparent although their protein concentrations (more than 300 μg/ml) were all higher than that of dilution refolding. Soluble protein recovery of packed Poros PE column (1 ml), Hitrap Butyl Sepharose FF (1 ml), Hitrap Octyl Sepharose FF (1 ml) and Hitrap Phenyl Sepharose HP (1 ml) was 69.7%, 79.5%, 81.2% and 89.2%, respectively, indicating decreasing amount of aggregate. The hydrophobic strength of those columns was found to sequentially increase from packed Poros PE column (1 ml), Hitrap Butyl Sepharose FF (1 ml), Hitrap Octyl Sepharose FF (1 ml) to Hitrap Phenyl Sepharose HP (1 ml) owing to elevating retention volume of native lysozyme by them (not data shown). Thus commercially available HIC columns are able to suppress aggregation likely thanks to hydrophobic interaction between ligands and protein molecules. Moreover, increasing hydrophobicity of media enhance this capability.

However, specific activity recovery of refolded lysozyme with above HIC columns was lower than that of dilution refolding although total activity yield of the former was two times higher than that of the latter. Specific activity recovery of refolded lysozyme in supernatant by dilution was 82.2%. Comparatively, specific activity recovery of refolded products with above HIC media was 74.7%, 70%, 69.8% and 62.7%, respectively. The low specific activity of pooled fractions indicated the formation of non-native structures.

Theoretically, refolded product can recover its native activity since normal protein folding is a spontaneous process driven by Gibbs free energy between unfolded and native protein involving intra-molecular hydrophobic interaction [21–23]. The hydrophobic interaction between ligands and protein molecules likely restrains intra-molecular hydrophobic association of some amino acids of proteins that is necessary for correct refolding, thus causing the exposure of some inappropriate hydrophobic clusters. Therefore, it is suspected that commercially available HIC columns tend to prevent normal folding process resulting in misfolded structure and misfolding extent aggravates with increasing hydrophobic strength of HIC media.

3.2. HIC enhances lysozyme correctly refolding when assisted by glycerol

Here, glycerol, a typical osmolyte, was attempted to assist commercially available HIC column in refolding lysozyme. Unfolded lysozyme was loaded to Poros PE HIC column (128 mm × 10 mm i.d.) at high salt concentration followed by elution with refolding buffer. Two ways was adopted to introduce glycerol: for the first, glycerol was mixed with pooled fraction from HIC column; for the second, glycerol was added into eluent. In a control experiment, dilution refolding was conducted in the presence of glycerol. Table 2 shows the effect of glycerol addition on refolding efficiency.

For glycerol-assisted dilution refolding, precipitation was observable, similar to that in the absence of glycerol. Under the optimum condition, the activity yield increased a little to 32.9% at 20% (v/v) glycerol in refolding buffer although around 90% of specific activity recovery was obtained. Thus, glycerol is likely able to partially enhance lysozyme correctly refolding but fails to prohibit aggregate. For HIC-assisted refolding, bound protein to the HIC column was unable to be eluted by decreasing salt concentration according to our

Table 2
Effect of glycerol addition on refolding efficiency with HIC

Refolding ways	Glycerol addition ways	Activity yield (%)	Soluble protein recovery (%)	Specific activity recovery (%)
Dilution	As an additive	32.9	36	91
HIC[1]	No	50.9	80	59
HIC[2]	In pooled fraction	58.8	80	68
HIC[3]	In the eluent	86.3	85	100

For dilution refolding, 20 μl denatured lysozyme at the protein concentration of 10 mg/ml was directly diluted into 1 ml refolding buffer (100 mM Tris–HCl, 1 mM EDTA, 3 mM GSH, 0.3 mM GSSG, pH 8.5) with the addition of 20% (v/v) of glycerol. HIC[1], 100 μl unfolded lysozyme of 10 mg/ml was adsorbed to the Poros PE HIC column (128 mm × 10 mm i.d.) at high salt concentration. A urea gradient was used to release bound protein followed by elution with the refolding buffer (100 mM Tris–HCl, 1 mM EDTA, 0.4 M $(NH_4)_2SO_4$, 0.01% (v/v) β-mercaptoethanol, pH 8.5); HIC[2], 40% (v/v) of final glycerol concentration was mixed with pooled fraction according to the step described in HIC[1]; HIC[3], the steps described for HIC[1] were performed with 50% (v/v) of final glycerol concentration to the refolding buffer.

observation, implying multiple-site hydrophobic interaction between the denatured peptide chains and the solid media. This kind of interaction is too strong for the peptide chains to refold freely. The introduction of urea gradient could efficiently release bound protein resulting in 80% mass recovery. Nevertheless, the activity yield was only 50.9% with 59% specific activity recovery, even lower than that of passing-through way with the small column as described above. This implies HIC, although it minimizes aggregation, promotes further misfolding when using adsorption–elution way with a long column than when using passing-through way with a short column. Compared to the absence of glycerol, immediate addition of glycerol into pooled fraction from the column leaded to slight improvement of activity yield. Under the optimum condition, it was 58.8% with 68% specific activity recovery at 40% of glycerol concentration, indicating that direct mixing of glycerol with pooled lysozyme could partially enhance refolding. Glycerol is thus considered to be unable to intrigue the re-arrangement of already misfolded structure and only strengthen compactness of local hydrophobic regions.

When glycerol acted as the eluent for HIC, activity yield continuously rose with increasing glycerol concentration. 86.3% activity yield and 85% mass recovery were obtained at 50% (v/v) of glycerol concentration. Furthermore, specific activity recovery reached 100% demonstrating the formation of native structure. Therefore, participation of glycerol into total refolding process is supposed to be necessary for the formation of final correct structure. In addition, a gradient of denaturant to glycerol provides a gradually strengthened hydrophilic environment to avoid a sudden shock to the protein. During elution process of bound protein, released and partially refolding protein that has many exposed hydrophobic groups can avoid of aggregation due to its hydrophobic interaction with HIC media. The successful refolding of lysozyme with our strategy lies in the combination of advantages of glycerol and HIC.

To further probe into the role of glycerol on refolding efficiency, refolded lysozyme by HIC column with or without glycerol addition was characterized with SEC. As Fig. 2 indicated, multiple lysozyme peaks were found in the profile of refolded lysozyme in the absence of glycerol indicating the presence of soluble aggregate and/or misfolded structure. In the case of 50% of glycerol in eluent, only one lysozyme peak of which retention volume was identical to the native was seen, which indicates that eluted lysozyme has been successfully refolded.

3.3. Proposed procedure for refolding lysozyme with HIC assisted by glycerol

As shown in Fig. 3a, when unfolded lysozyme passed through Poros PE HIC column (128 mm × 10 mm i.d.) filled with refolding buffer, two peaks were observable indicating two structures of different hydrophobic surfaces. Moreover, absorbance at 280 nm was higher than that at 254 nm for the

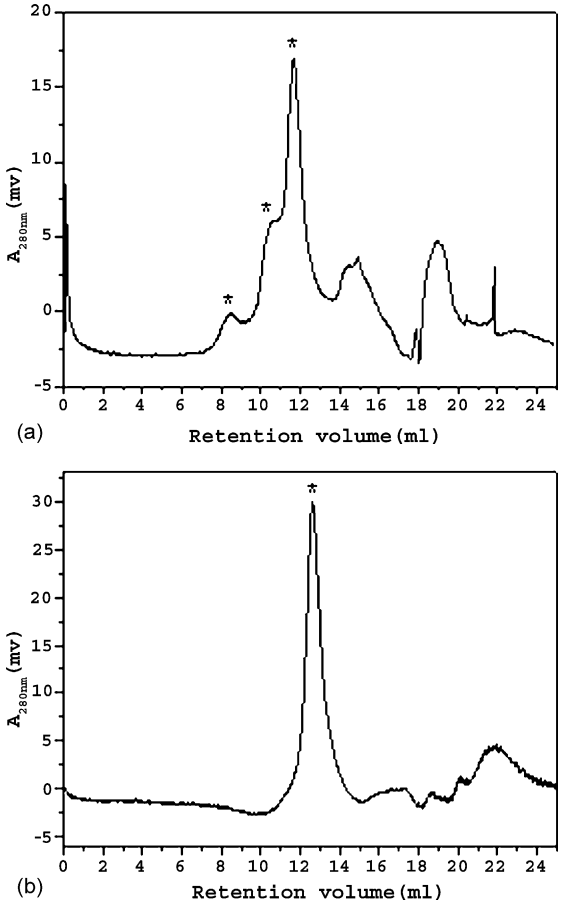

Fig. 2. Effect of glycerol addition on refolding efficiency by HIC. Fifty microlitres refolded lysoyzme was applied to Superdex 75 HR 10/30 (300 mm × 10 mm i.d.) equilibrated with mobile phase (4 M urea, 20 mM Na_2HPO_4, pH 7.0, 0.16 M NaCl) followed by elution with above buffer at flow rate of 0.5 ml/min. Sample were prepared with following methods: 100 μl unfolded lysozvme of 10 mg/ml was adsorbed to the Poros PE HIC column (128 mm × 10 mm i.d.) at high salt concentration. A urea gradient was used to release bound protein followed by elution with the refolding buffer containing glycerol of different concentrations: (a) none of glycerol; (b) 50% (v/v) glycerol. (*) Lysoyzme peaks.

first peak and nevertheless lower for the second peak. The second peak was thus assumed to be an abnormal protein implying refolding intermediate. Fig. 4a displays enzymatic reactivation as a function of time for eluted lysozyme passing through HIC column. A little activity of these two eluted peaks was immediately obtained accounting for the formation of partially active structure during elution. The two curves appeared two phases: activity yield of eluted peaks quickly increased within initial 10 min, but becoming flat during later incubation. The activity of the second peak was a little higher than that of the first.

Previous study demonstrated that refolding of lysozyme in solution was subject to two routes: the fast track involves the formation of near native structure containing both α and β domains; the slow track is responsible for the production of refolding intermediate containing α domain [24]. It can be speculated that the application of unfolded protein to the

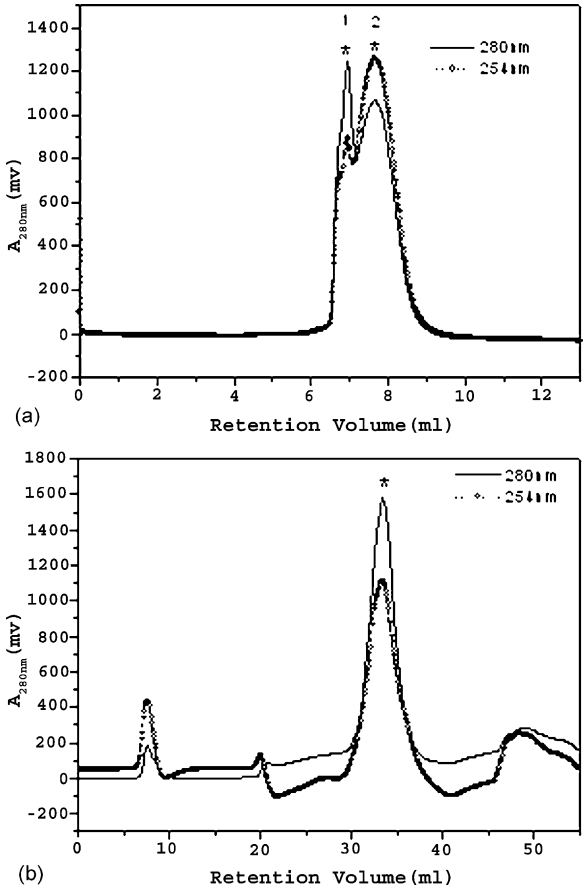

Fig. 3. Curves for lysozyme to be refolded by HIC with passing and adsorption–elution way. Solid curves, monitoring at 280 nm; dotted curves, at 254 nm. (a) Refolding by passing-through way with the HIC column: 100 μl unfolded lysozyme of 10 mg/ml was applied to the Poros PE (128 mm × 10 mm i.d.) pre-equilibrated by refolding buffer (100 mM Tris, 1 mM EDTA, 3 mM GSH, 0.3 mM GSSG pH 8.5) followed by elution with the same buffer at 0.2 ml/min; (b) refolding by adsorption–elution way with the HIC column assisted by glycerol: 100 μl unfolded lysozyme of 10 mg/ml was adsorbed to the Poros PE HIC column (128 mm × 10 mm i.d.) at high salt concentration. A urea gradient was used to release bound protein followed by elution with the refolding buffer containing 50% glycerol. (*) Lysozyme peaks.

HIC column filled with refolding buffer leads to an immediate dilution process of denaturant and simultaneously initiates primary refolding. Thus, two initial refolding intermediates similar to those of dilution refolding were possibly produced. Our data suggests that HIC potentially functions as a tool to characterize refolding intermediates. These two refolding intermediates were likely dynamic-controlled and thus produced a slight initial activity due to quick hydrophobic collapse. However, they failed to form native structure due to the effect of hydrophobic environment of HIC in the end.

Fig. 3b is the chromatogram for lysozyme refolding by HIC adopting adsorption–elution way in the presence of 50% glycerol in refolding buffer. One peak was eluted compared to above result. Furthermore, its absorbance at 280 nm was higher than at 254 nm, consistent with normal protein. This result suggests that refolded proteins with homogeneous hydrophobicity are likely produced unlike above case. On the other hand, the kinetic curve for its activity recovery versus time was distinctly different from that for passing-through way (Fig. 4b). Initial post-column activity yield of eluted peak was almost absent. It continuously rose with time and reached 86% 10 h later. It can be assumed that enzymatic reactivation process of obtained lysozyme by adsorption–elution way with HIC mediated by glycerol is much slower than passing-through way. This strategy is speculated to involve the pre-formation of a productive refolding intermediate that can finally recover native activity during post-column incubation.

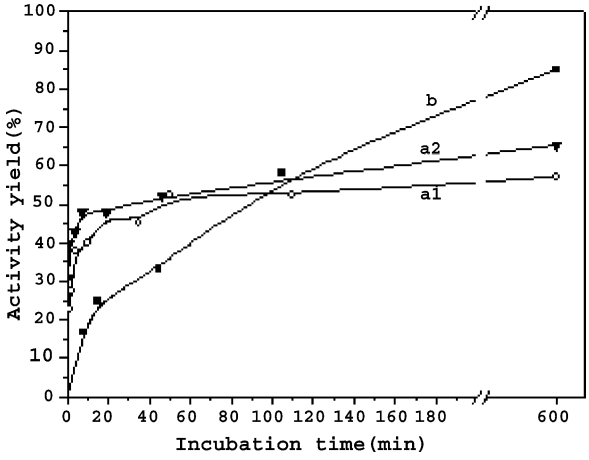

Fig. 4. Kinetic curves of reactivation for eluted lysozyme from HIC column with different methods: (a1) corresponding to the first peak of Fig. 3a; (a2) corresponding to the second peak of Fig. 3a; and (b) corresponding to the peak of Fig. 3b.

It is thus assumed that single refolding route distinct from dilution refolding was adopted resulting in a productive refolding intermediate that could reactivate. Furthermore, this process was possibly thermodynamic-controlled and relatively slow. Our refolding system is thus considered to vary the refolding pathway of lysozyme resulting in a decreased refolding rate. However, it does not interfere with the final formation of correct structure of target protein.

4. Conclusion

The combination of commercially available HIC media and glycerol was first introduced to refold denatured protein. Commercially available HIC columns were able to suppress aggregation and nevertheless prevented correct refolding resulting in non-native structure with low specific activity. The side effect of misfolding was exacerbated with increasing hydrophobicity of media. Correct refolding could be achieved when glycerol was present in refolding buffer as the eluent for HIC column.

Two refolding intermediates of lysozyme were separated, suggesting that HIC can be used to a potent tool to monitor refolding process. Glycerol-HIC refolding system

was considered to produce a thermodynamic-controlled on-pathway refolding intermediate that can slowly recover native activity likely through a route different from that for dilution refolding.

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