Journal of Chromatography B, 879 (2011) 2971–2977

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

On-column refolding of denatured lysozyme by the conjoint chromatography composed of SEC and immobilized recombinant DsbA

Man Luo, Yi-Xin Guan*, Shan-Jing Yao

Department of Chemical and Biological Engineering, Zhejiang University, Hangzhou 310027, PR China

ARTICLE INFO

Article history:
Received 3 June 2011
Accepted 26 August 2011
Available online 31 August 2011

Keywords:
Conjoint column
DsbA
Immobilization
Lysozyme
Chromatographic refolding
Protein refolding *in vitro*

ABSTRACT

DsbA (disulfide bond formation protein A) located in the periplasm of *Escherichia coli* is a disulfide isomerase, which is vital to disulfide bonds formation directly affecting the nascent peptides folding to the correct conformation. In this paper, recombinant DsbA was firstly immobilized onto NHS-activated Sepharose Fast Flow gel. Then Sephadex G-100 gel was sequentially packed on the top of recDsbA Sepharose Fast Flow, and a so-called conjoint chromatography column composed of SEC and immobilized recombinant DsbA was constructed. Denatured lysozyme was applied on the conjoint column. The effect of SEC volume, flow rate, loading amount and volume, pre-equilibrium mode and KCl concentration in the buffer on lysozyme refolding were investigated in detail and the stability of DsbA immobilization was evaluated. Finally the reusability of the conjoint refolding column was also tested. When loading 2.4 mg denatured lysozyme in 0.5 ml solution, the activity recovery reached 92.7% at optimized experimental conditions, and the conjoint column renaturation capacity decreased only 7.7% after six run reuse due to the use of SEC section in the chromatographic refolding process. The conjoint chromatography offers an efficient strategy to refold proteins *in vitro* with high productivity and column reusability.

1. Introduction

The *Escherichia coli* recombinant system is generally employed in genetically engineered proteins production because of its easy manipulation and high productivity. When proteins are expressed in heterologous cells, the recombinant proteins are often present in the form of inclusion bodies [1,2], which are insoluble protein aggregates with no biological activities. Inclusion bodies can occur in both the cytosolic and periplasmic location in bacteria, which is dependent of the expression level of the target proteins and the environment parameters, including richness of the medium, micronutrients, pH, the level of aeration, growth temperature, *etc*. Specially, most of eukaryotic proteins are expressed in the form of inclusion bodies in prokaryotic system due to the lack of the post-translational modifications, *e.g.* glycosylation, as in the eukaryotic system. How to refold the inclusion bodies has become a problem in genetically engineered proteins production in large scale. In order to refold inclusion bodies to biologically active recombinant proteins, it is necessary to develop an efficient procedure for refolding these proteins *in vitro* [3].

Till now, many studies have showed that proteins folding *in vivo*, especially with high concentrations, are mediated by helper proteins, such as molecular chaperones [4,5] and foldases [6,7]. From the classical 'self-assembly' principle to the new recognized 'assisted assembly' theory, this undergoing viewpoint has made more clear about the protein folding mechanism in *de novo* protein synthesis in cells. Even *in vitro* protein refolding at high concentration has also been enlightened from it by introducing these helper proteins into former spontaneous systems [8,9].

As the rate-limiting step in the folding process, disulfide bonds formation is usually assisted by protein disulfide isomerase (PDI), which is the typical representative of foldases among the helper proteins. DsbA (disulfide bond formation protein A) with a molecular weight of 21.1 kDa is one kind of PDI existing in *E. coli* periplasm. The active site of DsbA is Cys^{30}–Pro^{31}–His^{32}–Cys^{33}, which could not only oxide the free thiols to disulfide bonds but also isomerase the mismatched disulfide bonds to correct ones, and accelerate the folding procedure. The *dsbA* gene and its product DsbA, the first found member of Dsb family, were identified by two independent research groups, almost simultaneously *via* two totally different approaches. In 1991, research results by Bardwell [10] indicated that pulse-labeled β-lactamase, alkaline phosphatase and OmpA could be secreted but severely defective in disulfide bond formation in *dsbA* mutant *E. coli* cells; while Kamitani [11] in 1992 found that the formation of correctly folded alkaline phosphatase was impaired in Tn5 insertion mutant *E. coli* cells, where insertion occurred at the *ppfA* (periplasmic phosphatase formation) gene which is identical with *dsbA*, confirmed by Plunkett [12] in 1993. Several *in vitro* investigations indicated high performance of DsbA

* Corresponding author. Tel.: +86 571 87951982; fax: +86 571 87951982.
E-mail address: guanyx@zju.edu.cn (Y.-X. Guan).

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.08.032

in assisting the refolding and renaturation of bovine pancreatic ribonuclease A (RNase A) [11], bovine pancreatic trypsin inhibitor (BPTI) [13], and alkaline phosphatase (PhoA) [11]. Recent studies also revealed that DsbA had intrinsic activity as a molecular chaperone besides its isomerase activity in the catalysis of native disulfide formation [14,15]. Immobilized system of DsbA on an agarose gel column together with minichaperone GroEL and peptidyl-prolyl isomerase had achieved a 87% yield of protein with 100% biological activity in the refolding of denatured and reduced scorpion toxin Cn5 [16].

Although immobilized system of helper proteins has achieved good results in protein refolding *in vitro*, the lacking of both stability and reusability has to be taken into account in the implementation. In the refolding with immobilized minichaperone GroEL [17,18], high concentration of denaturant in the loading buffer inactivated the assistant proteins and shortened the lifetime of the column. On the other hand, since denaturant concentration fell sharply, lots of folding intermediates would be prone to aggregating at the entrance, blocking the column and increasing the operation pressure. Urea gradient size exclusion chromatography (SEC) [19,20] is a well established on-column refolding system and could be introduced to solve above problem. Lysozyme of large molecules immigrates faster than urea of small molecules in the porous medium of SEC, so concentration of urea is decreasing along the column. Besides, urea gradient SEC could effectively but not sharply dilute the high concentration of urea and provide a much gentler environment for subsequent refolding process. Based upon that, a column containing of 1 cm height of Sephadex G 200 at the top and packed with immobilized mini-GroEL has been designed and applied successfully to refolding of recombinant human interferon-γ inclusion bodies by our group [21].

In this work, a conjoint refolding chromatography was established by packing the Sephadex G-100 gel (SEC section) on the top of recDsbA Sepharose Fast Flow in one column. On-column refolding of denatured lysozyme was carried out with this conjoint column to investigate the stability and reusability of immobilized DsbA refolding system. The refolding conditions were optimized and refolding mechanism by the conjoint chromatography was discussed.

2. Experimental

2.1. Plasmid and strain

Plasmid pAVD63, encoding the sequence of DsbA and a signal peptide, was a gift from Prof. Fersht in Centre for Protein Engineering MRC, Cambridge. Host strain *E. coli* BL21 (λ DE3) [F^-, ompT, hsdSB, $r_B{}^-$, $m_B{}^-$ (λ Ci857, ind1, Sam7, nin5, lacUV5-T7.AL)] pLysS was stored in our lab. DsbA was induced by IPTG under the control of the strong T7 promoter and solubly expressed as cytoplasmic protein from plasmid pAVD63, where a *par* locus (originally from pSC101 *via* pMTL21P) was added in order to stabilize the plasmid.

2.2. Chemicals and reagents

Lysozyme substrate micrococcus *lysodeiktisus*, coomassie brilliant blue R-250, tris (hydroxymethyl) aminomethane (Tris) and L-arginine were purchased from Sigma (St. Louis, MO, USA). Hen egg white lysozyme, tryptone, yeast extract, dithiothreitol (DTT), nitro-thiocyanobenzoic acid (NTCB) and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from BBI (Markham, Ontario, Canada). All other chemicals were of analytical grade. Buffers were prepared with deionized water filtrated by 0.22 μm membrane (Millipore, Billerica, MA, USA). Q Sepharose Fast Flow gel, Sephadex G-100 and NHS-activated Sepharose Fast Flow gel were obtained from GE Healthcare (Piscataway, NJ, USA).

2.3. Reduction and denaturation of lysozyme

Lysozyme was fully unfolded in the presence of DTT and high concentration of denaturants. The typical denaturing buffer contained 8 M urea, 30 mM DTT, 50 mM Tris–HCl at pH 8.5. Lysozyme was added to a final concentration of 10 mg/ml and shaken in an incubator under 37 °C, 220 rpm for 90 min.

2.4. Production of recombinant DsbA

Recombinant DsbA protein was produced as our previous work [22]. After harvesting, the cells were collected by centrifugation at 8000 × *g* for 10 min at 4 °C and resuspended with 1/10 broth volume of Tris–HCl buffer (pH 8.3, 16.7 mM). Followed disrupting the cells by ultrasonication, the supernatant were recollected by centrifugation. Then, 9.25 ml cells extract was directly loaded to a Q Sepharose Fast Flow column (16 mm × 100 mm) at 2.5 ml/min with a column volume (CV) of 20 ml and monitored by absorbance at 280 nm. Finally DsbA protein was eluted by 4 CVs step elution buffer composed of 5.88% high salt concentration, *i.e.* pH 8.3, 16.7 mM Tris–HCl, 117.6 mM KCl. This purification process was performed on ÄKTA Explorer 100 (GE Healthcare, Piscataway, NJ, USA). The purified DsbA protein was concentrated by Amicon Ultra-4 10,000 MWCO (Millipore, Billerica, MA, USA), and stored at −20 °C for later use. The concentration of DsbA protein was calculated based upon its absorbance at 280 nm using the extinction coefficient of 1.1 mg/ml for the native oxidized protein [16].

2.5. Immobilization of DsbA

The purified DsbA protein was immobilized onto the NHS-activated Sepharose Fast Flow gel followed by Altamirano's description [16], which was named recDsbA Sepharose Fast Flow. Firstly the thiols at the active site of DsbA were protected by reversible block reaction using NTCB, which was carried out under nitrogen atmosphere. The blocked DsbA was concentrated to 10 mg/ml. Then the concentrated protein was rapidly diluted into the coupling buffer pH 8.3 (50 mM $NaHCO_3$ and 500 mM NaCl) and mixed with the gel end-over-end for 6 h at 25 °C, followed by washing with the coupling buffer. After immobilization, the blocked thiols at the active site of DsbA were reoxidized to disulfide bond. The buffers in the whole immobilization procedure were gathered to monitor the loss of DsbA using Bradford's assay in order to evaluate the immobilization efficiency.

The stability of immobilization was also measured by soaking the recDsbA Sepharose Fast Flow in the refolding buffer of 50 mmol/l PBS with pH 8.5 containing 0.15 M KCl, 1 mM EDTA, 1.5 M Urea, 0.5 mM GSSG, 1 mM GSH, 0.25 M arginine for 24, 48, 72 and 96 h respectively.

2.6. Preparation of the conjoint refolding chromatography column

5 ml recDsbA Sepharose Fast Flow were packed into a chromatography column with sectional area of 1 cm^2. After sedimentation, a certain volume of Sephadex G-100 gel functioning as the size exclusion chromatography was sequentially packed onto the top of the recDsbA Sepharose Fast Flow in the column. Namely, SEC gel and the immobilized DsbA were packed into a conjoint refolding chromatography column, as shown in Fig. 1.

2.7. On-column refolding of denatured lysozyme

After the conjoint column was constructed, denatured lysozyme was loaded onto the column and refolded as it went through the column, *i.e.* on-column refolding. Prior to on-column refolding, the

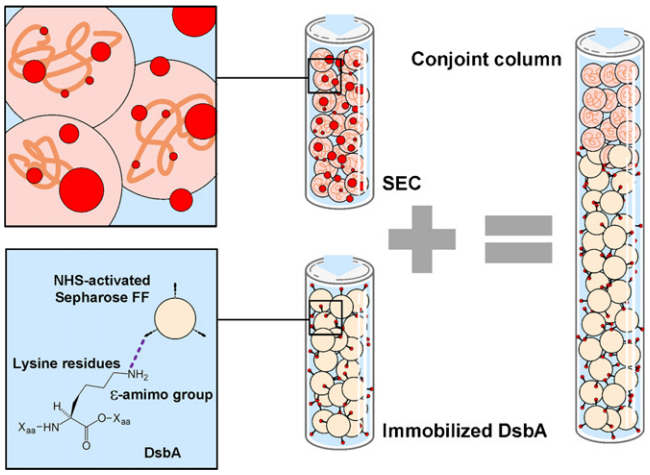

Fig. 1. The scheme of the conjoint refolding chromatography column composed of SEC and immobilized recombinant DsbA.

conjoint column was pre-equilibrated. Then solubilized lysozyme was applied to the column at a relatively high flow rate of 1 ml/min. Finally, lysozyme was eluted with the refolding buffer at a slow flow, while all factions were collected and enzymatically assayed. The whole refolding procedure was carried out on ÄKTA chromatographic system.

The conjoint column could be pre-equilibrated in three modes (Fig. 2), where buffer A was the same ingredients as the refolding buffer and buffer B was buffer A containing 8 M urea. Pre-equilibrium mode I: the whole conjoint column was equilibrated by buffer A, *i.e.* the urea concentration was uniform at 1.5 M throughout the whole column. Pre-equilibrium mode II: a linear gradient of urea concentration from 8 M to 1.5 M along the SEC section was established by mixing buffer A and B, and urea concentration of 1.5 M in the immobilized DsbA section. Pre-equilibrium mode III: similar as mode II with urea gradient from 4 M to 1.5 M.

The effects of the volume of SEC section, elution flow rate, loading amount and volume of denatured lysozyme, pre-equilibrium mode, and KCl concentration in the refolding buffer on the activity recovery of lysozyme were studied in detail, and the reutilization of conjoint column was also tested.

2.8. Assay

Cells growth was monitored by optical density at 600 nm using Ultraspec 3000 spectrophotometer (GE Healthcare, Piscataway, NJ, USA), and proteins were detected by SDS-PAGE with 12% of acrylamide for running gel and 4% for stacking gel, respectively [23]. The semi-quantitation and purity of proteins were analyzed by the GD 2000 gel documentation system (Bio-rad, Hercules, CA, USA). The exact protein concentration was determined by a modified Bradford's method using bovine serum albumin (BSA) as a reference [24]. The activity of native and refolded lysozyme was determined by the modified turbidimetric assay [25] using Micrococcus *lysodeikticus* as substrate. Correspondingly, the activity recovery was calculated dividing the measured enzymatic activity by the native lysozyme activity in a 10 μl sample solution. In this work, all experiments and measurements were carried out in triplicates, and the averages were adopted in the data analysis.

3. Results and discussion

3.1. Preparation of recombinant DsbA protein

Fig. 3 showed the chromatogram of ion-exchange chromatography elution profile of recombinant DsbA by Q Sepharose Fast Flow and SDS-PAGE of the corresponding fractions respectively. No further desalting procedure was required after the ion-exchange chromatography because of the low salt concentration at elution. Totally, 377.9 ± 1.74 mg recombinant DsbA per liter fermentation broth was obtained with the purity 95%. The molecular weight of DsbA was 23.1 kD with the signal peptide.

3.2. Immobilization of DsbA on NHS-activated Sepharose Fast Flow gel

After reversibly blocking the thiols at the active site of DsbA, DsbA protein was immobilized onto the NHS-activated Sepharose Fast Flow gel *via* the ε-amino groups of lysine residues. Data from Bradford's assays indicated the immobilization capacity of about 5.3 mg DsbA/ml swollen gel.

In stability test, the immobilized DsbA presented excellent stability when soaked in the refolding buffer containing some extreme reagents such as urea, which simulated the microenvironment in the refolding procedure. After 4 days test, only about 6% DsbA desorbed, as shown in Fig. 4.

3.3. On-column refolding of lysozyme by the conjoint column

Sephadex G-100 gel was packed into the column followed by the recDsbA Sepharose Fast Flow gel. Before applied to on-column refolding of denatured lysozyme, the conjoint column was pre-equilibrated in mode III by refolding buffer with a urea gradient [20], as shown in Fig. 2. The operation parameters of on-column refolding of denatured lysozyme were initially set as follows: 2.4 mg denatured lysozyme dissolved in 0.5 ml aqueous solution

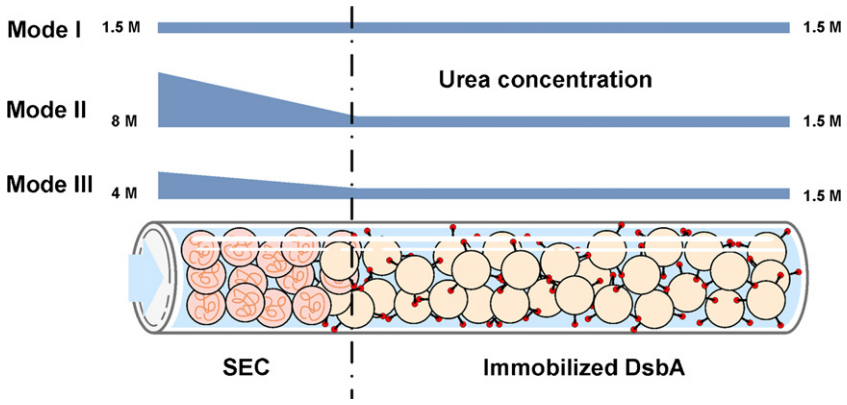

Fig. 2. Three pre-equilibrium modes for the conjoint column.

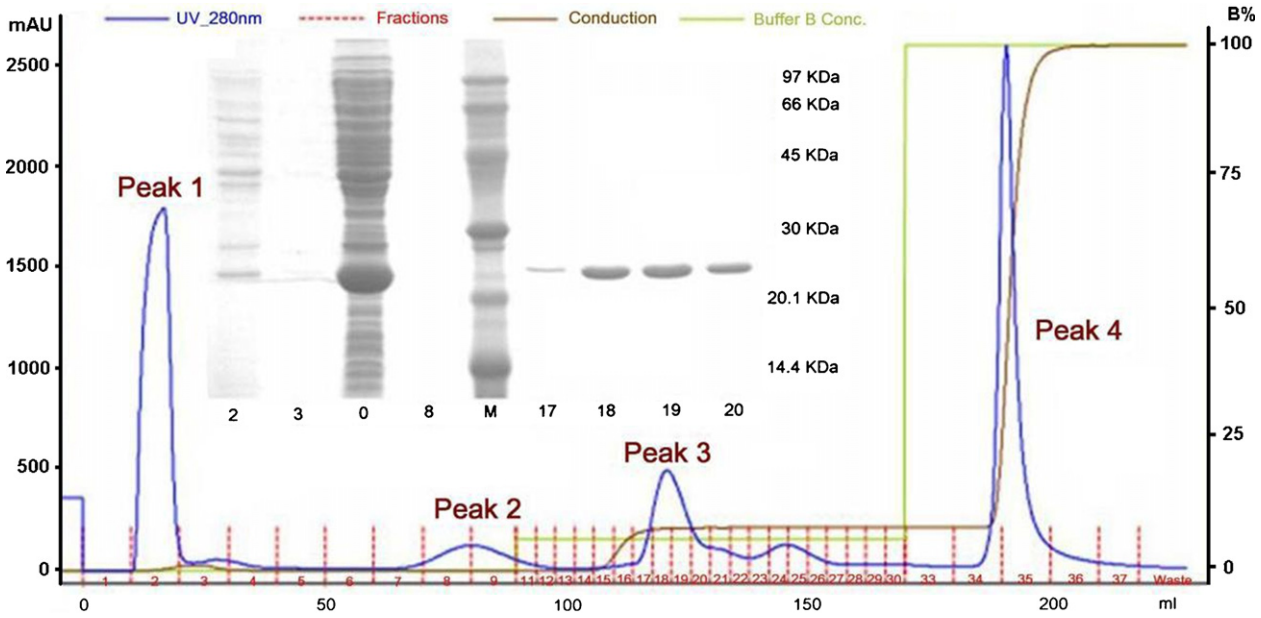

Fig. 3. The elution profile of purification of DsbA protein by ion-exchange chromatography using Q Sepharose Fast Flow and SDS-PAGE of the corresponding fractions of the eluate. Equilibration buffer: 16.7 mM Tris–HCl, pH 8.3, and elution buffer: 16.7 mM Tris–HCl with 117.6 mM KCl, pH 8.3. (Lanes 2 and 3, peak 1; lane 0, supernatant after cell disruption; lane 8, peak 2; lane M, marker; lanes 17, 18, 19 and 20, peak 3.)

was loaded at a flow rate of 1 ml/min, and refolded lysozyme was eluted at 0.1 ml/min by refolding buffer in the conjoint column stacked by 1 ml Sephadex G-100-packed SEC and 5 ml recDsbA Sepharose Fast Flow in the same chromatography column.

3.3.1. *Effect of the conjoint SEC volume on lysozyme refolding*

For the conjoint chromatography refolding, the conjoint SEC section acted both as the protection and pre-folding in the on-column refolding procedure of denatured lysozyme. Generally denatured lysozyme or other inclusion bodies were solubilized in denaturants of high concentration, such as 6 M guanidine hydrochloride or 8 M urea, which would severely destroy the active conformation of the immobilized DsbA, even denature it, and shorten the longevity of the immobilized DsbA column if loaded directly. These denaturants would be diluted by the SEC section on the top of the conjoint column and then a slightly gentle working environment was offered for the immobilized DsbA. On the other hand, disulfide bonds formation reactions were widely recognized as the rate limiting step in protein refolding process, while DsbA could specifically speed up these reactions. Moreover, the biological activity of molecular chaperone was also embedded within DsbA molecules. Totally denatured proteins achieved the pre-folding steps in SEC section and refined folding assisted by immobilized DsbA.

Effects of different SEC volume on lysozyme refolding were compared, as shown in Fig. 5. We can see that the activity recovery increased 25% when 1 ml SEC was conjoined than that no SEC gel involved. In the on-column refolding of the conjoint chromatography, while denatured proteins entering the column, high concentration denaturants were partially diluted in SEC section and denatured proteins could spontaneously fold to some intermediates conformation. At this stage, aggregations would be observed because of the hydrophobic interactions among the intermediates, in which some basic structures such as α-helix and β-sheet had already formed while disulfide bonds were not posed or mismatched. Whereafter in the immobilized DsbA, aggregations were scattered by the molecular chaperone activity of DsbA, meanwhile disulfide bonds were catalyzed by the strong oxidizability of the disulfide bond of active site of DsbA and mismatched disulfide bonds were isomerized by the isomerase activity of DsbA correspondingly. On the contrary, when SEC volume further increased, the activity recovery dropped from 96.7% to 92.7%, 82.4%, 75.1%

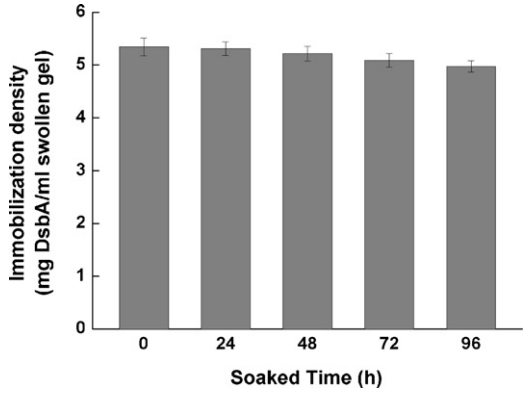

Fig. 4. Stability test for immobilized DsbA on NHS-activated Sepharose Fast Flow gel.

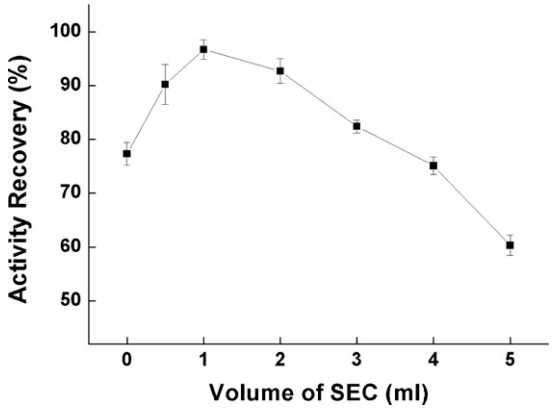

Fig. 5. Effect of the SEC volume on the refolding of lysozyme using the conjoint column.

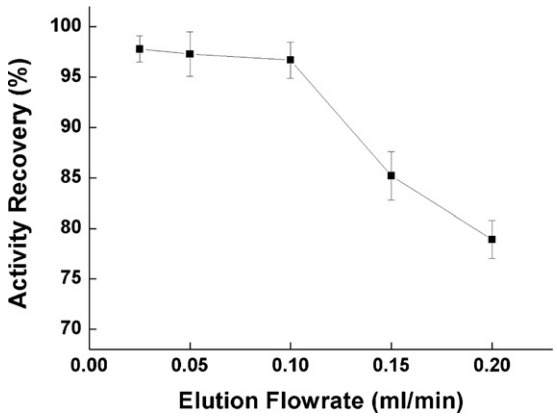

Fig. 6. Effect of flow rate on the refolding of lysozyme using the conjoint column.

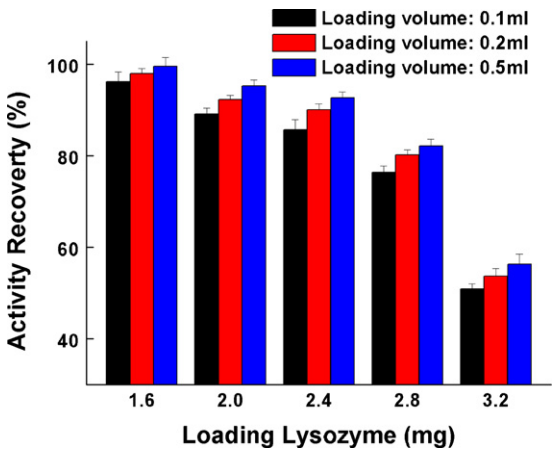

Fig. 7. Effect of loading amount and volume in refolding buffer on the refolding of lysozyme using the conjoint column.

and 60.3% for 2, 3, 4 and 5 ml SEC, respectively. Unacceptable low urea concentration caused by excessive SEC length led to more mismatched disulfide bonds and aggregations with the absence of the appropriate redox microenvironment provided by DsbA, and activity recovery dropped accordingly.

3.3.2. *Effect of the flow rate on lysozyme refolding*

The main functions of DsbA are to oxidize the free thiols into disulfide bonds or isomerize the mismatched disulfide bonds in the refolding. These redox reactions are all time-consuming processes, so the residence time of denatured lysozyme, or to some extent, the contact time between denatured lysozyme molecules and DsbA could be an important parameter in the on-column refolding using the conjoint chromatography, which would be definitely influenced by the operation flow rate. Macroscopic shearing force brought by the flux of the mobile phase could always be stronger than microscopic intermolecular forces between DsbA and lysozyme. Hence, flow rate should also be taken into account when lysozyme was applied to the conjoint column for refolding.

Elution flow rate on lysozyme refolding was studied and the results are in Fig. 6. It was shown that at low flow rate, there was no significant difference on the activity recovery and the activity recovery could reach about 97%. Usually relatively low flow rate led to longer residence time, longer contact time and also the higher possibility of disulfide bonds exchanging between immobilized DsbA and lysozyme molecules. Taking time efficiency and production cost into account, flow rate of 0.1 ml/min would be a better choice.

3.3.3. *Effect of loading amount and loading volume on lysozyme refolding*

Loading amount directly influences the molar ratio of lysozyme to DsbA on the conjoint column, while loading volume would alter the concentration of urea at the inlet of immobilized DsbA. All these two factors could affect on the activity recovery of refolded lysozyme and should be investigated. The effects of loading amounts with different loading volumes are shown in Fig. 7.

For a fixed conjoint column, large loading amounts would result in the troubles for the column capacity. With excessive denatured lysozyme molecules, the SEC section of the conjoint column might not be able to assist every denatured lysozyme molecule in prefolding. Also, the subsequent lysozyme intermediates pre-folded in the SEC section would have been passing through the column with incorrect conformation due to the insufficient binding capacity of the immobilized DsbA for disulfide formation and isomerization. On the contrary, small loading amount would lead to the unacceptable low throughput. Thus, suitable loading amount should be confirmed. In Fig. 7, we can see that the activity recovery dropped about 50% when denatured lysozyme loaded was increased to 3.2 mg from 1.6 mg with different loading volume. But loading of 2.8 mg denatured lysozyme led to only about 20% decrease compared with 1.6 mg sample. So considering column capacity and productivity, loading amount of 2.4 or 2.8 mg denatured lysozyme would be much favorable.

It is well known that same loading amount with different loading volume would result in different protein concentration. With low concentration of denatured lysozyme, as the injection pulled through the urea gradient and immobilized DsbA, a much more favorable micro-environment could be achieved for denatured lysozyme to refold into native conformation. From Fig. 7, we know that the activity recovery in 0.5 ml loading volume would be higher than that in 0.1 ml with different loading amount of denatured lysozyme respectively. The results also demonstrated the importance of the SEC section on the top of the conjoint column. For 2.8 mg denatured lysozyme, the activity recovery of the refolded lysozyme increased to 82.1% with loading volume of 0.5 ml.

3.3.4. *Effect of pre-equilibrium mode*

Usually urea acted as both a folding promoter and denaturant simultaneously in protein refolding. As the folding promoter, aggregation inhibitor urea weakened the intermolecular hydrophobic interactions of protein molecules, which resulted in the aggregations among the folding intermediates [26,27]. Meanwhile, as the denaturant, urea competed hydrogen bonds with polypeptide chains and thus destructed the secondary structure domain of the proteins, resulting in denaturation. The activity recoveries of lysozyme under three different pre-equilibrium modes were studied and the results are presented in Fig. 8. From Fig. 8, we know that mode III obtained the best results with lysozyme activity recovery of 96.7%, about 33% and 8.5% higher than mode I and mode II, respectively.

In mode I, 1.5 M urea was homogeneously distributed from the entrance to the exit of whole conjoint column. When denatured lysozyme entered the column, urea concentration was directly diluted from 8 M to 1.5 M, which would weaken the aggregation suppressing effect of urea. Hence urea molecules existed were not enough to offer the hydrophobic interactions interference for intermediates refolding to correct conformation, leading to lower activity recovery. For pre-equilibrium modes II and III, urea concentration gradually decreased from 8 M to 1.5 M or 4 M to 1.5 M along the SEC section of the conjoint column respectively. Denatured lysozyme, relatively larger molecules, migrated faster than the linear urea gradient which was formed by the smaller urea molecules in the refolding column. Thus, the denatured lysozyme molecule

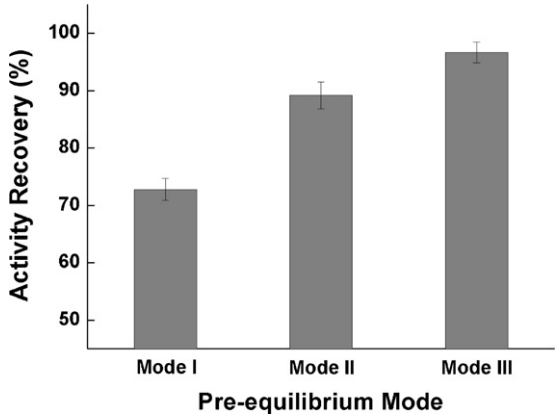

Fig. 8. Effect of pre-equilibrium mode on the refolding of lysozyme using the conjoint column. Mode I: a uniform urea concentration of 1.5 M throughout the whole conjoint column. Mode II: a linear gradient of urea concentration from 8 M to 1.5 M along the SEC section and urea concentration of 1.5 M in the immobilized DsbA section. Mode III: similar as mode II with urea gradient from 4 M to 1.5 M.

underwent a gradually decreased gradient of urea concentration along the SEC section of the conjoint column. When passing by the SEC section, denatured lysozyme refolded into some intermediates and entered the immobilized DsbA for further folding. It was also reported that urea gradient size exclusion chromatography achieved high refolding efficiency [19]. However, mode II spread 8 M urea through the column, which would slightly damage the immobilized DsbA. In mode III, denatured lysozyme exactly experienced a two-step urea dilution refolding, firstly a dilution refolding with 4 M urea followed by a 4–1.5 M urea gradient refolding. In this way, better refolding effect was obtained.

3.3.5. *Effect of KCl concentration on lysozyme refolding*

The ionic strength in the refolding buffer, *i.e.* concentration of KCl, was investigated at different levels, as shown in Fig. 9. Ionic strength played a significant role not only in inhibiting aggregation of refolding intermediates of lysozyme but also in disulfide bonds formation. Excess low or high concentration of KCl had a negative effect on lysozyme activity recovery. The optimal concentration of KCl was at 0.15 M, under which activity recovery of refolded lysozyme was about 22% higher than that at concentration of 0.05 M KCl. In contrast, the optimum KCl concentration for immobilized DsbA refolding column alone was 0.20 M (unpublished data). The difference might be due to the extra SEC section in the conjoint chromatography: actual local concentration of KCl in SEC section would be higher than that in the bulk buffer since

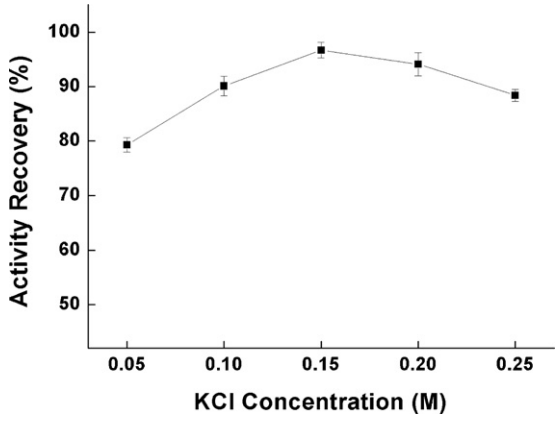

Fig. 9. Effect of KCl concentration in refolding buffer on the refolding of lysozyme using the conjoint column.

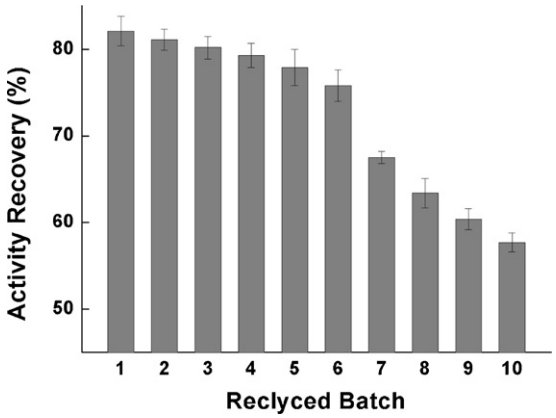

Fig. 10. Reutilization of the conjoint column for denatured lysozyme refolding.

small molecules of KCl went through longer way across the SEC column. When loaded on the conjoint column, denatured lysozyme pre-folded in the SEC section with higher local KCl concentration. Hence, lower KCl was required in a conjoint column system.

Ionic strength could result in the nonspecific action on protein conformation, so lysozyme and immobilized DsbA might be affected by KCl concentration in the system. DsbA activity might change and indirectly influence the disulfide bond formation in lysozyme and its chaperone activity for preventing protein aggregation. Direct effect of ionic strength on lysozyme could be as follows: at low ionic strength, ions adsorbed onto protein (also peptide or folding intermediates) surfaces increased the electronic repulsive force between different molecules and reduced aggregation; while at high concentration, hydration effect peeled off the hydrated layer on the hydrophobic area of protein and thus increased the interactions between hydrophobic area [28], finally led to a low protein recovery due to aggregation. Moreover, ionic strength would also act on the disulfide bonds formation. Higher ionic strength would be favorable to disulfide bonding [29].

3.4. *Reutilization of the conjoint column*

In the assistant-mediated *in vitro* refolding field, how to recover the used media becomes a key problem. These assistants are usually artificial synthesized molecules [30–34] or recombinant assistant proteins [21,16], both of which require fine technical controls with high cost of production. In the DsbA-mediated batchwise refolding system, it is difficult to recycle free DsbA protein with biological activity. Immobilization of DsbA settled this tough problem to a great extent. However, high concentration of denaturants, such as urea (almost 8 M) in the denatured buffer might denature the immobilized DsbA at the entrance of the column, damage the column permanently and shorten the longevity. The conjoint SEC section in this work greatly improved the reusability of immobilized DsbA column.

Investigating the reusability, the conjoint column was repeatedly used at least 10 batches, and the loading amount was set as 2.8 mg denatured lysozyme with a loading volume of 0.5 ml, achieving a higher throughput. No significant drop in activity recovery of refolded lysozyme was observed in the first six usages with the protection of foregoing SEC section, as shown in Fig. 10, while only first three run could obtain steady activity recovery in the immobilized DsbA column alone (unpublished data). There was an obvious drop in the seven run, about 18% off than the first use. After ten run, refolded lysozyme still could gain 57.7% activity recovery, about 70% of the first use. Overall, easier recovery, higher stability and repeated reutilization of the conjoint column were achieved than batch or on-column refolding using immobilized DsbA.

Compared with the normal SEC refolding system, much higher throughput and refolding efficiency were achieved in the conjoint chromatography due to the introduction of immobilized DsbA into the refolding system. Helper protein of DsbA with both molecular chaperone and disulfide bonds isomerase activity could facilitate the protein molecules to refold, especially for those, *e.g.* lysozyme, with disulfide bonds in its steric structure. Herein, the comparatively small SEC volume could dilute the denaturants in the loading sample and functions in two aspects. On one hand, with SEC section, the concentration of denaturants was relatively low at the entrance of immobilized DsbA; thus reduced its damage to foldase. On the other hand, with lower concentration of denaturants, denatured lysozyme began to refolding to some intermediates conformation. Under the optimal condition, the activity recovery of refolded lysozyme in the conjoint chromatography reached about 92.7% and the refolded lysozyme with a concentration of 542 μg/ml in 4.2 ml was fractionated.

4. Conclusions

A novel conjoint refolding chromatography column with high stability and batches reutilization was reported in this paper. The conjoint column was composed of 1 ml Sephadex G-100-packed SEC and 5 ml recDsbA Sepharose Fast Flow and successfully applied to lysozyme *in vitro* refolding. After pre-equilibrated by the refolding buffer with a profile of urea gradually decreased from 4 M to 1.5 M along the SEC section and homogeneously 1.5 M for the immobilized DsbA section, 2.4 mg denatured lysozyme with a loading volume of 0.5 ml was loaded on the conjoint refolding column at a flow rate of 0.1 ml/min and the concentration of KCl in the refolding buffer at 0.15 M. The activity recovery of refolded lysozyme reached about 92.7% with the productivity of 151 mg active lysozyme per hour per liter column volume.

With the protection of SEC section, the immobilized DsbA in the conjoint column was much more stable than that with the absence of SEC section. To some extent, the urea concentration in the denatured lysozyme was diluted by the SEC section at the entrance of recDsbA Sepharose Fast Flow section, accordingly less loss of biological activity of DsbA and much longer longevity of the conjoint column were achieved. For six batches usage, the activity recovery showed only 5% loss compared with the first usage, while the ten run could also obtain 57.7% recovery. On-column refolding, as one of the newly focused research, presents great potential in reusability with higher recovery and productivity and will be an inviting research orientation.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (No. 20876138). We would like to thank Professor Fersht in Centre for Protein Engineering MRC, Cambridge very much for the gift of the DsbA plasmid pAVD63.

References

[1] F.A.O. Marston, Biochem. J. 240 (1986) 1.
[2] S.E. Radford, Trends Biochem. Sci. 25 (2000) 611.
[3] S. Misawa, I. Kumagai, Biopolymer (Pept. Sci.) 51 (1999) 297.
[4] F.U. Hartl, Nature 381 (1996) 571.
[5] F.U. Hartl, M. Hayer-Hartl, Science 295 (2002) 1852.
[6] G. Fischer, H. Bang, C. Mech, Biomed. Biochim. Acta 43 (1984) 1101.
[7] R.B. Freedman, Cell 57 (1989) 1069.
[8] Q. Wei, Y.S. Kim, J.H. Seo, W.S. Jang, I.H. Lee, H.J. Cha, Appl. Environ. Microbiol. 71 (2005) 5038.
[9] E.R. LaVallie, J.M. McCoy, Curr. Opin. Biotechnol. 6 (1995) 501.
[10] J.C.A. Bardwell, K. McGovern, J. Beckwith, Cell 67 (1991) 581.
[11] S. Kamitani, Y. Akiyama, K. Ito, EMBO J. 11 (1992) 57.
[12] G. Plunkett, V. Burland, D.L. Daniels, F.R. Blattner, Nucleic Acids Res. 21 (1993) 3391.
[13] A. Zapun, L. Cooper, T.E. Creighton, Biochemistry 33 (1994) 1907.
[14] C.C. Wang, C.L. Tsou, FEBS Lett. 425 (1998) 382.
[15] C.C. Wang, Ann. N. Y. Acad. Sci. 864 (1998) 9.
[16] M.M. Altamirano, C. Garcia, L.D. Possani, A.R. Fersht, Nat. Biotechnol. 17 (1999) 187.
[17] Y.X. Guan, Z.Z. Fei, M. Luo, T. Jin, S.J. Yao, J. Chromatogr. A 1107 (2006) 192.
[18] M.M. Altamirano, R. Golbik, R. Zahn, A.M. Buckle, A.R. Fersht, PNAS 94 (1997) 3576.
[19] Z.Y. Gu, Z.G. Su, J.C. Janson, J. Chromatogr. A 918 (2001) 311.
[20] M. Li, Z.G. Su, J.C. Janson, Protein Expr. Purif. 33 (2004) 1.
[21] Y.G. Gao, Y.X. Guan, S.J. Yao, M.G. Cho, Biotechnol. Prog. 19 (2003) 915.
[22] M. Luo, Y.X. Guan, S.J. Yao, Chin. J. Biochem. Mol. Biol. 21 (2005) 376.
[23] U.K. Laemmli, Nature 227 (1970) 680.
[24] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[25] D. Shugar, Biochim. Biophys. Acta 8 (1952) 302.
[26] J. Buchner, R. Rudolph, Nat. Biotechnol. 9 (1991) 157.
[27] R. Rudolph, H. Lilie, FASEB J. 10 (1996) 49.
[28] M. Dixon, E.C. Webb, Adv. Protein Chem. 16 (1961) 197.
[29] Y.D. Livney, E. Verespej, D.G. Dalgleish, J. Agric. Food Chem. 51 (2003) 8098.
[30] D. Rozema, S. Gellman, J. Biol. Chem. 271 (1996).
[31] D. Rozema, S. Gellman, Biochemistry 35 (1996) 15760.
[32] L. Vuillard, D. Madern, B. Franzetti, T. Rabilloud, Anal. Biochem. 230 (1995) 290.
[33] H. Shimizu, K. Fujimoto, H. Kawaguchi, Colloids Surf. B Biointerfaces 18 (2000) 137.
[34] H. Shimizu, K. Fujimoto, H. Kawaguchi, Colloids Surf. A Physiochem. Eng. Aspects 153 (1999) 421.