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Size exclusion chromatography with an artificial chaperone system enhanced lysozyme renaturation

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Abstract

Protein renaturation is of importance in the recovery of inclusion-body protein produced by recombinant microbial cells. It has been recognized that the artificial chaperone system, Cetyltrimethylammonium bromide and β-cyclodextrin, are effective in enhancing protein renaturation. Using chicken egg white lysozyme as a model protein, this work studied protein renaturation by size exclusion chromatography (SEC) incorporating with the artificial chaperone system. At first, a cooperative effect of the artificial chaperones and guanidinium chloride (GdmCl) on the protein renaturation was confirmed, and it was concluded that the artificial chaperone system promoted the renaturation of lysozyme (1.05 mg/ml) in the presence of 1 mol/liter GdmCl. Using the SEC (29.5 $\times$ 2.6 cm I.D., packed with Sephacryl S-100 h gel) incorporating with the artificial chaperones, higher renaturation yield was obtained at high flow rate (0.8–2.2 ml/min). In contrast, using the SEC without the artificial chaperones, very low flow rate (i.e. $<$ 0.4 ml/min) should be used to receive a comparable renaturation yield. Thus, use of the SEC incorporating with the artificial chaperone system would greatly benefit in reaching a high refolding productivity.

Keywords: Size exclusion chromatography; Artificial chaperone; Lysozyme; Oxidative renaturation

1. Introduction

Heterologous proteins produced by recombinant microbial organisms often form inactive aggregates known as inclusion bodies. In order for the recovery of biologically active protein, the aggregates must be separated from cell debris and solubilized by exposing them to a strong denaturant. Decreasing the denaturant concentration by dialysis or direct dilution can initiate the unfolded polypeptide chains to refold to their native state. However, only a fraction of the native-state protein can be recovered in this process since the unfolded protein tends to re-aggregate by intermolecular interactions. This is because of the fact that protein renaturation is a kinetically competitive process between the folding and aggregation [1–3]. As a unimolecular process, protein folding is a first-order reaction, while the aggregate formation is higher order because it is caused by intermolecular interactions [4]. Due to the kinetic nature of protein refolding process, suppression of the aggregating reaction is the key point to enhance protein renaturation yield.

Protein folding in vivo is mediated by helper proteins termed "molecular chaperones" [5]. These proteins bind to nascent or unfolded polypeptides and/or the folding intermediates, preventing improper polypeptide chain interactions that lead to aggregation, thus facilitating correct folding [6]. Therefore, molecular chaperone proteins also facilitate in vitro protein refolding. The chaperonin, GroEL, and its co-chaperonin, GroES, from *Escherichia coli*, have been extensively investigated. The function of GroEL is to bind to partially folded intermediates, while GroES helps the substrate protein to release in an ATP-dependent manner [7,8]. Inspired by the mechanism of the GroEL/ES system, Rosema and Gellman developed an artificial chaperone system for controlling the competition between renaturation and aggregation [9–12]. The system consists of a detergent and a cyclodextrin. In a renaturation process, the detergent is introduced in the dilution step to bind the denatured

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protein and thus preventing intermolecular interactions that lead to aggregation, then the cyclodextrin is added to strip the detergent and release the protein, initiating the protein folding. By the artificial chaperoning method, proteins such as carbonic anhydrase B [9,10], lysozyme [11] and citrate synthase [12] can regain their activity at favorable yields, for example, over 80% for 0.2 mg/ml of lysozyme [11].

In addition to the use of the chaperones, alternative protein refolding techniques have been attempted. Among those, size exclusion chromatography has been found to be efficient in regaining protein activity with high yield at high loading concentrations [13,14]. Recently, this method has been extended to the renaturation of inclusion body proteins, and favorable results were achived [15–17]. It was concluded that protein diffusion into the porous gel media helped protein refolding by reducing intermolecular interactions [13,18].

It is clear that the size exclusion chromatography and the chaperone systems mediate protein refolding by different mechanisms. Thus, it is expected that the combination of these two techniques would further give rise of protein-refolding performance. The purpose of work is to examine the oxidative renaturation of lysozyme by size exclusion chromatography incorporating with the artificial chaperones. Cetyltrimethylammonium bromide was used as the detergent and β–cyclodextrin solution as the stripping agent (elution buffer). GdmCl–unfolded/dithiothreitol–reduced lysozyme was used as a model protein since it has been widely used for refolding studies with the artificial chaperone system [11] and size exclusion chromatography [13,14, 18].

2. Materials and methods

2.1. Materials

Chicken egg white lysozyme, *Micrococcus lysodeikticus*, guanidinium chloride (GdmCl), dithiothreitol (DTT), reduced glutathione (GSH) and oxidized glutathione (GSSG) were purchased from Sigma. Cetyltrimethylammonium bromide (CTAB) was obtained from Wako (Osaka, Japan). Beta-cyclodextrin (β–CD) was a product of Huabei Special Chemicals Company (Tianjin, China). Sephacryl S–100 h gel was received from Amersham Pharmacia Biotech (Sweden). Other chemicals were all commercially available reagents of analytical grade. All chemicals and reagents were used as received.

2.2. Denaturation-reduction of lysozyme

Lysozyme at 30–80 mg/ml was denatured and reduced in 0.1 mol/liter Tris sulfate buffer (pH 8.5) containing 6 mol/liter GdnHCl and 30 mmol/liter DTT at 37°C for 80 min. This procedure yielded a fully denatured-reduced lysozyme [19]. The denatured-reduced lysozyme was stored at 4°C and used within two weeks.

2.3. Oxidative renaturation by batch dilution

In order to find an appropriate condition for lysozyme renaturation by SEC with the molecular chaperones, we first studied the renaturation of denatured lysozyme by batch dilution with or without the artificial chaperones. The experimental procedure for artificial chaperone-assisted lysozyme renaturation was similar to that reported by Rozema and Gellman [11]. Briefly, the denatured–reduced lysozyme (30 mg/ml) was diluted to 0.1 mol/liter Tris sulfate buffer containing definite amount of CTAB, GdmCl, EDTA, GSH and GSSG (pH 8.5, 37°C) at a proper volume ratio. After 10 min, 16 mmol/liter β-CD stock solution in 0.1 mol/liter Tris sulfate buffer (pH 8.5, 37°C) was added to the above solution to bring the final concentrations of 1.05 mg/ml lysozyme, 0.1 mol/liter Tris sulfate, 4 mmol/liter GSH, 0.4 mmol/liter GSSG, 1 mmol/liter EDTA and GdmCl at a desired value. The final volume of the renaturation system was 1 ml. The renaturation system was incubated at 37°C in a shaking incubator and samples were withdrawn at different time intervals for lysozyme activity assay.

In the renaturation experiments without the artificial chaperones (by simple dilution), the denatured–reduced lysozyme solution (30 mg/ml) was rapidly diluted to 0.1 mol/liter Tris sulfate solution of definite concentrations of GdmCl, EDTA, GSH and GSSG (pH 8.5, 37°C) at a proper volume ratio to give the final concentrations of 1.05 mg/ml lysozyme, 0.1 mol/liter Tris sulfate, 4 mmol/liter GSH, 0.4 mmol/liter GSSG, 1 mmol/liter EDTA and GdmCl at a desired value. It was incubated at 37°C, and the renaturation experiments thereafter were conducted as described above. In all the experiments described above, lysozyme renaturation yield was expressed as the ratio of the specific activity of renatured lysozyme to that of the native lysozyme (relative activity).

2.4. Oxidative renaturation by SEC

Fig. 1 shows the experimental setup for the refolding by size exclusion chromatography (SEC). It was performed on the GradiFrac chromatography system (Pharmacia Biotech, Sweden). A glass column (40 × 2.6 cm I.D.) with a water jacket was packed with 155 ml of Sephacryl S-100 h gel (29.5 cm settled-bed height). The column temperature was kept at 37°C by circulating thermostated water to the column jacket. To the top of the column a 0.8-ml mixer was connected with a tube of 1 mm I.D. and 10 cm long. Prior to each renaturation batch, the column was equilibrated with the corresponding elution buffer (see below).

In the SEC experiments with the artificial chaperones, the denatured–reduced lysozyme (80 mg/ml) was first diluted to CTAB solution to give final concentrations of 40 mg/ml lysozyme, 10 mmol/liter CTAB, 4.6 mol/liter Gd-

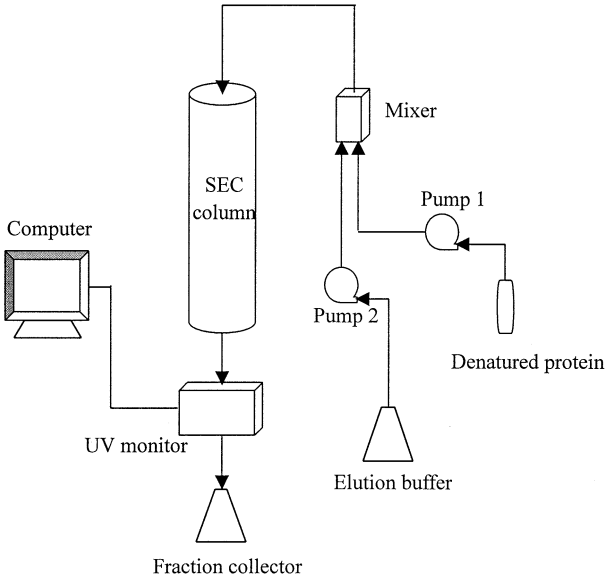

Fig. 1. Experimental setup for lysozyme renaturation by size exclusion chromatography

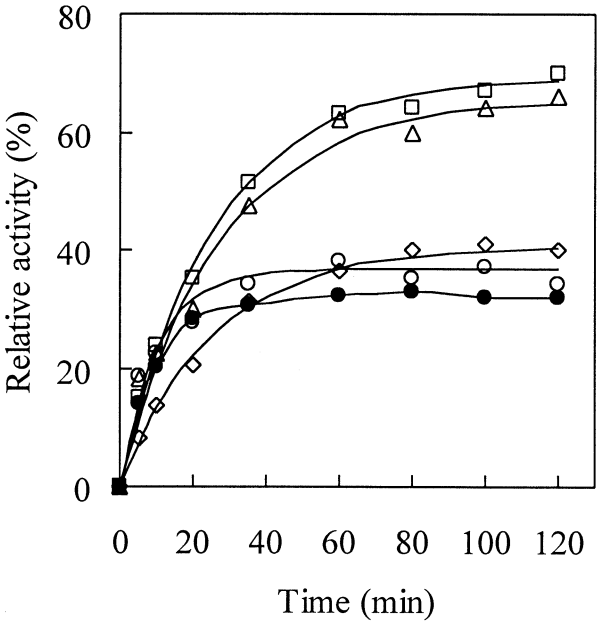

Fig. 2. Lysozyme renaturation at 1.05 mg/ml by (●) simple dilution and (○, □, △, ◇) in the presence of the artificial chaperones (1.2 mmol/liter CTAB, 4.8 mmol/liter β-CD). GdmCl concentration was (●, ○) 0.724, (□) 0.98, (△) 1.2 and (◇) 2.02 mol/liter.

mCl, 1 mmol/liter EDTA, 0.4 mmol/liter GSH and 4 mmol/liter GSSG. The elution buffer consisted of 16 mmol/liter β–CD, 1 mol/liter GdmCl, 1 mmol/liter EDTA, 0.4 mmol/liter GSH and 4 mmol/liter GSSG (in 0.1 mol/liter Tris sulfate buffer pH 8.5). To initiate a refolding experiment, the denatured-reduced protein sample and the elution buffer were supplied into the column via the mixer at flow rates of 0.5 and 1.5 ml/min, respectively. This was continued for 1 min to load 20 mg of the denatured protein at 10 mg/ml. Thereafter, the protein sample was rapidly transported into the column top by supplying the elution buffer at 2 ml/min for 1 min, then the column was developed with the elution buffer at a desired flow rate (0.15–6 ml/min).

In the control SEC experiments without the artificial chaperones, the denatured-reduced lysozyme (80 mg/ml) was diluted to 10 mg/ml lysozyme solution containing 6 mol/liter GdmCl, 1 mmol/liter EDTA, 0.4 mmol/liter GSH and 4 mmol/liter GSSG. The denatured protein sample (2 ml) was loaded to the column via the peristaltic pump at 2 ml/min, then immediately developed with the elution buffer (without β-CD) at a desired flow rate (0.3–6 ml/min).

In all the SEC experiments, the effluent from the column was detected by a flow UV monitor and the data acquired by a computer. The eluted protein peak was pooled and, depending on flow rate, incubated at 37°C to ensure a total refolding time (including the residence time in the column and the incubation time if any) longer than 2 h. Then, the collected sample was subjected to protein concentration and activity determinations.

2.5. Analytical methods

Lysozyme concentration was determined spectrophotometrically at 280 nm with an extinction coefficient of 2.63 ml/mg.cm [20]. Lysozyme activity was measured at 25°C by following the absorbance decrease at 450 nm using 0.25 mg/ml *Micrococcus lysodeikticus* in 60 mmol/liter phosphate buffer (pH 6.2) as the substrate [13]. In the assay, 0.1 ml of lysozyme sample was added to 1.5 ml of the substrate, and the absorbance decrease was recorded for 2 min in 15 s of the mixing. The aggregation of the denatured/reduced lysozyme was detected by measuring the optical density of protein-containing solution at 450 nm [21].

3. Results and discussion

3.1. Oxidative renaturation by batch dilution

It has been reported that the artificial chaperone system, CTAB and methyl-β-CD mediated the renaturation of 0.2 mg/ml lysozyme to a yield of over 80% [11]. In the case of higher lysozyme concentration (1 mg/ml), however, a renaturation yield of 57% was obtained even with very high CTAB and methyl-β-CD concentrations (12 and 150 mmol/liter, respectively). This value is smaller than the lysozyme renaturation yield achieved at high GdmCl concentrations [19]. Because GdmCl of proper concentration has been recognized as a stabilizer of the molten globule state [22] and to enhance protein renaturation yield [19], we first investigated the effect of GdmCl concentration on the oxidative renaturation of lysozyme in the presence of the artificial chaperones. Fig. 2 represents the results. It is found that with 0.724 mol/liter GdmCl, the renaturation yield was only a little enhanced by the artificial chaperones at con-

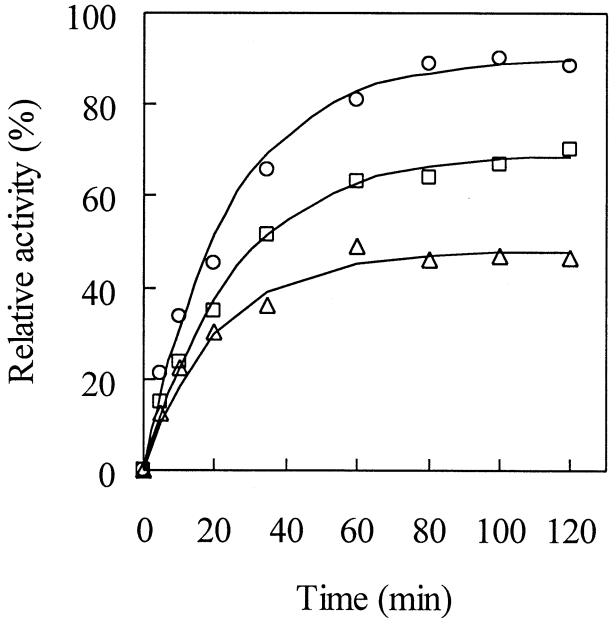

Fig. 3. Effect of the artificial chaperone concentrations on lysozyme renaturation at 1.05 mg/ml. CTAB and β-CD concentrations were: (△) 0 and 0, (□) 1.2 and 4.8, and (○) 2.4 and 9.6 mmol/liter, respectively. GdmCl concentration was 0.98 mol/liter.

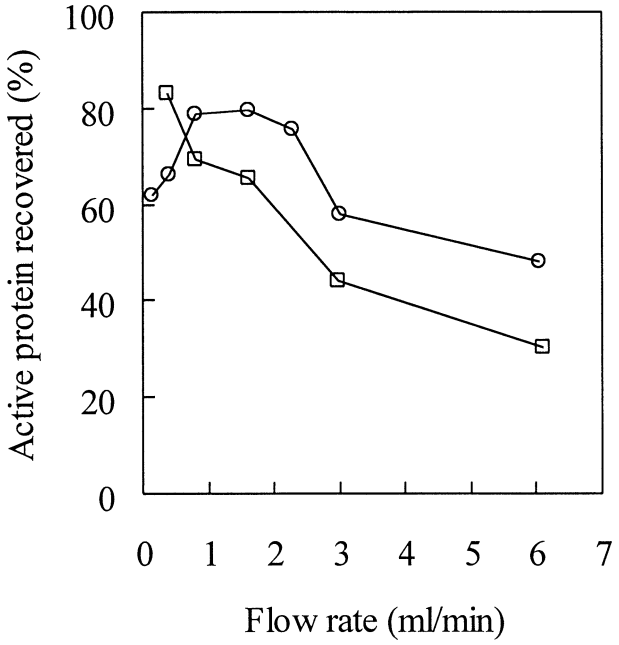

Fig. 4. Effect of elution flow rate on the recovery yield of lysozyme renaturation by (○) SEC with the artificial chaperones and (□) SEC without the artificial chaperones. The recovery yield of active protein was the average value of three to five measurements, and the deviations between the measurements were less than 5%.

centrations of 1.2 mmol/liter CTAB and 4.8 mmol/liter β-CD. Keeping the artificial chaperone concentrations unchanged, we found that the renaturation yield was significantly increased when GdmCl concentration was raised to 0.98–1.2 mol/liter, and the further increase of GdmCl to 2.02 mol/liter suppressed the renaturation of lysozyme. This result implies that a GdmCl concentration of about 1 mol/liter would be favorable to mediate lysozyme renaturation.

Thus, the effect of CTAB and β-CD concentrations at 0.98 mol/liter GdmCl was examined, as shown in Fig. 3. Clearly, at this GdmCl concentration, the presence of the artificial chaperones resulted in more significantly enhanced renaturation yield as compared to that at 0.724 mol/liter (Fig. 2). When the artificial chaperone concentrations were doubled, the renaturation yield increased further. The results shown in Figs. 2 and 3 indicate that the artificial chaperones and GdmCl of a proper concentration have a cooperative effect on enhancing the renaturation yield of lysozyme, and it can be conclude that the artificial chaperone system promoted lysozyme renaturation together with 1 mol/liter GdmCl. Thus, in the renaturation experiments with SEC, a buffer containing 1 mol/liter GdmCl and 16 mmol/liter β-CD, which is the solubility of β-CD, was employed to elute (refold) the denatured-reduced lysozyme.

3.2. Oxidative renaturation by SEC

A control experiment was first carried out to check if any protein renaturation and aggregation occurred between the mixer and the top of the column in the SEC experiments with the artificial chaperones. It was found that by the experimental protocols described in Section 2.4, the protein sample was supplied into the column within 2 min, and no lysozyme activity was detected within the time scale. This is in agreement with the results shown in Figs. 2 and 3, where lysozyme renaturation yield was less than 5% within a refolding time of 2 min. Moreover, no aggregation was observed within 2 min, which was confirmed by measuring the turbidity of the sample at 450 nm.

The influence of elution flow rate on lysozyme renaturation by SEC was investigated. The retention time of protein decreases with increasing flow rate, therefore, as mentioned above, the eluted protein from the column at flow rates higher than 1.6 ml/min was incubated at 37°C to give a total refolding time of 2 h. During the incubations, lysozyme activity increased with time. However, a time longer than 2 h did not further improve the renaturation. As shown in Fig. 4, lysozyme renaturation with and without the artificial chaperones depended on elution flow rate in different ways. Without the artificial chaperones, a high renaturation yield was achieved at the low flow rate, 0.38 ml/min (i.e. 0.7 mm/min), and it decreased monotonically with the flow rate. This has also been observed by Gu et al. [14] and Fahey et al. [17] It is considered due to the inadequate retention time of the substrate protein in the SEC column [13]. With the artificial chaperones, however, there existed a flow rate range (0.8–2.2 ml/min) that maximized the renaturation yield at about 80%; neither lower nor higher flow rates gave rise of the renaturation yield. This can be explained in terms of the refolding mechanism involved in the artificial chaperone-assisted protein refolding [11]. Before the denatured

protein is loaded to SEC column, it has been captured by the detergent CTAB (see Materials and methods). After mixing with the elution buffer and loading to the column, β-CD interacts with the denatured lysozyme-CTAB complex to strip the detergent from the complex and to initiate lysozyme refolding. It has been found that rapid stripping of the detergent from its protein complex is required to yield efficient protein refolding [11]. Hence, lower elution flow rate may not give rapid stripping of the detergent because of the lack of fresh β-CD. At higher flow rate, however, fast elution of protein from the SEC column did not give the protein adequate retention time to fully refold in the column. For example, at the flow rates of 3 and 6 ml/min, lysozyme activity increased by about 15 and 40% respectively during the post-elution incubation, and the turbidity of the collected sample increase was also observed during the incubation process. At these high flow rates, the elution time was less than 40 min. Thus, the role of SEC was not fully brought into play at higher flow rates. Consequently, there existed an optimal flow rate range that gave the highest renaturation yield. In the presence of the artificial chaperones, however, elution at high flow rate always gave higher renaturation yield than that without the artificial chaperones. This is an advantage of the SEC incorporating with the artificial chaperone system, which allows a high renaturation yield at high elution flow rate, and thus benefits in reaching a high refolding productivity.

The results described above showed the additive effect of the SEC and the artificial chaperone system on enhancing lysozyme renaturation. It is considered due to the difference in the mechanisms of the two methods in facilitating protein refolding. When the protein sample is applied to the column, it is in the state of protein-detergent complex. This suppresses the intermolecular interactions of the denatured lysozyme, which may lead to significant protein aggregation at a high concentration. After loading to the column, β-CD interacts with the detergent-protein complex to strip the detergent and initiate lysozyme refolding. In this process, the detergent-protein complex and the transiently folding protein molecules diffuse into the gel beads, and the detergent stripping by β-CD also occurs in the gel beads. The gel matrix works to prevent the aggregate formation by isolating the transiently folding molecules dynamically [13]. Thus, the artificial chaperone system and the size exclusion method separately help the denatured protein to correctly refold to its native state, leading to higher renaturation yield than that achieved by using SEC without the artificial chaperones.

It is known that the elution volume of protein in an SEC decreases with increasing the mobile phase flow velocity, since less time is available for the protein to achieve diffusion equilibrium as the flow velocity increases [23]. This behavior has also be observed in the lysozyme renaturation using SEC, as indicated in Fig. 5. Moreover, it is interesting to note that the elution volume by the SEC with the artificial chaperones was smaller than that without the artificial chaperones. This demonstrated the complex formation of the detergent CTAB and the denatured protein. Because of the formation of CTAB and denatured-reduced lysozyme complex, leading to a distinct increase in size of the detergent-complexed protein, the protein-CTAB complex moved faster in the early stage of the size exclusion chromatography, so the protein was finally eluted at less retention volume.

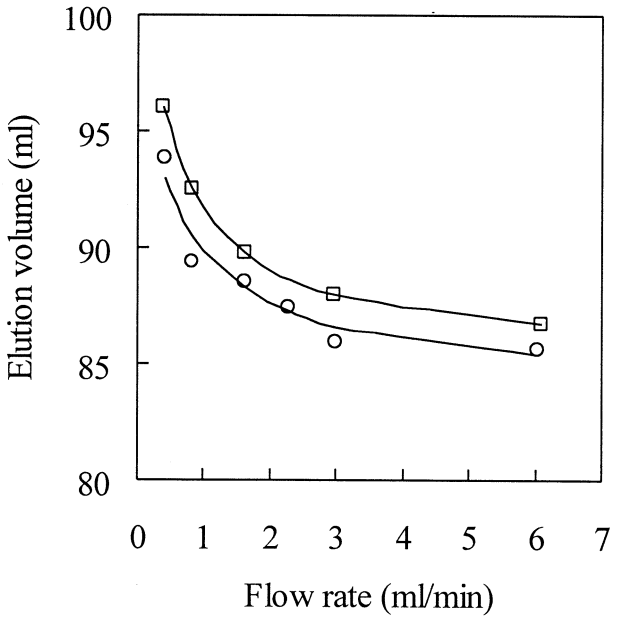

Fig. 5. Retention volume as a function of elution flow rate in lysozyme renaturation by (○) SEC with the artificial chaperones and (□) SEC without the artificial chaperones.

Because the column dimensions and/gel matrix were different from those reported earlier [13,14], the results herein cannot be directly compared with the previous results obtained using SEC. However, parallel comparison of the results obtained in this work definitely indicated the advantage of the SEC incorporating with the artificial chaperones in its applicability at high flow rates. Moreover, this technique can be optimized to further improve its protein refolding performance. This may be done in terms of the refolding mechanism by the artificial chaperones. For example, to rapid stripping the detergent from the complex, elution flow rate can be higher at the initial stage. Then a slower flow rate can be used to give the protein time to fully refold and to increase the column resolution [13]. In addition, choice of the gel medium in accordance with the protein to be refolded may also give rise of the renaturation ability [13,16]

Acknowledgment

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