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Effect of sample loop dimension on lysozyme refolding in size-exclusion chromatography

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Abstract

The formation of misfolded protein aggregates, in particular inclusion bodies, has been widely considered as the major hindrance of good yield in refolding processes. To enhance the performance of protein refolding, extensive efforts were directed toward seeking out methods or means to reduce the aggregate production during the refolding process. Since simultaneous refolding and separation can be feasibly achieved within the packing matrices, size-exclusion chromatography (SEC) has been regarded as an efficient buffer exchange method to enhance protein refolding performance As of now, the effect of the process or operating parameters has yet to be thoroughly investigated. The present work is aimed at understanding how aggregate formation, as well as renaturation yield, varied with the diameter or length of sample loop in size-exclusion chromatography refolding process. Our results showed that not much difference was found in the patterns of aggregate formation for the contraction and the control cases. However, the formation of an additional peak was observed in the expansion cases. In addition, the amount of aggregates was not dependent on the sample loop diameter or length, but instead, influenced by injection volume and protein concentration. It was further concluded that a sample with large volume and low concentration was preferable for refolding process. We believe that the outcome from this work may shed light on the development of a more effective strategy for refolding processes.

Keywords: Lysozyme; Refolding; Sample loop; Size-exclusion chromatography

1. Introduction

With the advent of genetic engineering, the expression of valuable proteins in bacterial hosts has been widely employed in the pharmaceutical industry as well as the biochemical research. Large quantities of proteins of interest can be produced in efficient and inexpensive fermentation processes. Nevertheless, the high expression rates of these proteins in bacteria frequently lead to the generation of intracellular proteinacious deposits, such as inclusion bodies, during the production process [1]. The proteins in the form of inclusion bodies are insoluble, misfolded, and inactive; therefore, the refolding procedure is of primary importance to inclusion bodies for regaining their activities.

The procedure for converting inclusion bodies into proteins with biological activity typically consists of washing, isolation, solubilization, and renaturation/refolding [2]. In most cases, various denaturant species, for instance, guanidine hydrochloride and urea, are used to solubilize aggregated species. While the efficiency of the first two steps can be fairly high, the foremost challenge is to develop optimum processes whereby the formation of inactive, misfolded, aggregated species can be prevented during the renaturation/refolding step. Owing to its simplicity and scale-invariant feature, the direct dilution of denatured proteins with proper refolding buffer remains the most commonly used method in the majority of the refolding studies [3–6]. Apart from direct dilution refolding, other techniques such as dialysis [7], immobilization of the denatured protein onto a solid support [8,9], size-exclusion chromatography (SEC) [10–14], ion-exchange chromatography [15–18], hydrophobic interaction chromatography [19] and their modified versions have been proposed as potential means to renature/refold the solubilized protein by removing the excess denaturants or decreasing the denaturant concentration.

It has been widely recognized that protein refolding is a kinetically competitive process between the undesired intermolecular aggregation formation, a higher order kinetic pathway, and the correct folding reaction, a first order pathway [3,20]. As a result, suppression of the aggregation pathway via decreasing

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protein concentration is the key to elevate protein renaturation yield. In the attempt to hamper such unwanted side reaction, numerous methods have been developed to improve renaturation yield. These methods include the addition of low molecular weight compounds to prevent aggregations [2,21,22], the use of polyethylene glycol to stabilize refolding intermediates [20,23], and the technique of passage through a column with immobilized helper proteins termed "molecular chaperones" [24,25].

Since simultaneous refolding and separation can be feasibly achieved within the packing matrices, SEC has been regarded as an efficient buffer exchange method to enhance protein refolding performance even at high loading concentrations [10,26]. Several proteins have been demonstrated to refold successfully via SEC [10–14]. As of now, despite significant numbers of reports focusing on SEC refolding, the effect of the process or operating parameters has yet to be thoroughly investigated and elucidated. Some efforts have also been made to refine the size-exclusion chromatography refolding process [12–14]. For example, we have demonstrated that the undesired protein aggregates appeared before SEC column inlet. It could attribute to the immediate contact (mixing) between refolding buffer (mobile phase) and denatured protein (injected sample). Also, these aggregates obviously hinder refolding performance in a SEC protein refolding operation. Although we have illustrated some feasible strategies to alleviate this problem successfully, the mechanism of aggregation formation around sample injector is not clear yet.

In our current work, using hen egg-white lysozymes as a model system, we further examine how aggregate formation, as well as renaturation yield, varies with the dimension (diameter or length) of sample loop in SEC refolding process. Our results showed that the second aggregation peak became obvious when the length of sample loop increased and an additional third peak appeared for sample loop had larger diameters than the connecting tube (expansion case). However, it was interesting to find that the total amount of aggregates was not strongly dependent on the sample loop dimension (either diameter or length), but instead, influenced by injection volume and protein concentration. It was further concluded that a sample with large volume and low concentration was preferable for refolding process.

2. Materials and methods

2.1. Materials

Hen egg-white lysozyme (HEWL; EC 3.2.1.17), hydrochloric acid (HCl) and dipotassium hydrogenphosphate (K_2HPO_4) were purchased from Merck (Darmstadt, Germany). Dithiothreitol (DTT), *Micrococcus lysodeikitus* dried cells, reduced and oxidized forms of glutathione (GSH and GSSG, respectively), and tris(hydroxymethyl)aminomethane were purchased from Sigma (St. Louis, MO, USA). Urea, EDTA, and potassium dihydrogenphosphate (KH_2PO_4) were obtained from Hayashi Pure Chemical Ind., Co., Ltd. (Osaka, Japan). All other chemicals were of analytical grade.

2.2. Preparation of denatured lysozyme

Different amounts of HEWL lysozyme were dissolved in 0.1 M Tris–HCl solution to prepare lysozyme stock solutions. Denatured lysozyme sample solutions were made by mixing the lysozyme stock solution with the buffer solution (0.1 M Tris–HCl, pH 8.6 containing 8 M urea, 1 mM EDTA and 0.01 M DTT). The activity of the denatured lysozyme sample solutions was observed to reach zero after storing at room temperature for 24 h and the mixture was then used for the following refolding experiments.

2.3. Determination of aggregate formation between injector and column inlet

Turbidity measurements provided a means to monitor formation of insoluble aggregates of lysozyme. To investigate the effect of sample application on the aggregate formation between the injector and column inlet, stream before column inlet was directly connected to the detector to monitor the aggregates. That is, the SEC column was temporarily removed and the detector was set at 450 nm for aggregates measurement. The denatured protein sample (1, 5, 10 and 20 g/l) was loaded though a sample loop which had been previously equilibrated with refolding buffer (0.1 M Tris–HCl, pH 8.2, 1.2 mM GSH, 1.2 mM GSSG, 1.5 M sodium chloride, 1 mM EDTA and 2 M urea). The sample loop dimension was divided into two types of standards for sample loading: one is fixed internal diameter (0.25 mm) but variable tubing length (10.2–400 cm). The other is fixed tubing length (25.5 cm) but variable internal diameter (0.17, 0.5, 0.75 and 1.0 mm). The amount of insoluble aggregated protein was determined by integrating chromatograms. It was hypothesized that the integral area is proportional to the amount of aggregate formation.

2.4. Refolding of lysozyme using size-exclusion chromatography

The high performance liquid chromatography (HPLC) system (Waters, Milford, MA, USA) was equipped with a dual λ absorbance detector (Waters 2487) and a binary HPLC pump (Waters 1525). All eluents were filtrated by nitrocellulose membrane (0.2 μm) and degassed beforehand. A 20 μl sample of denatured lysozyme (5 g/l) was injected onto a Superdex 75 HR 10/30 column (Amersham Pharmacia Biotech, Bjorkgatan, Sweden) previously equilibrated with refolding buffer (0.1 M Tris–HCl, pH 8.2, 1.2 mM GSH, 1.2 mM GSSG, 1.5 M sodium chloride, 1 mM EDTA and 2 M urea) and eluted with 0.5 ml/min mobile phase flow rate at room temperature. Sample fractions were collected and analyzed for the enzyme activities.

2.5. Enzyme activity analysis

The activity of lysozyme sample solutions was determined by measuring the decrease in absorbance at 450 nm. Under the pH of 6.2 and 25 °C, a 2.5 ml volume of *M. lysodeikticus* suspension (0.2 mg/ml) in 0.06 M potassium phosphate was used as

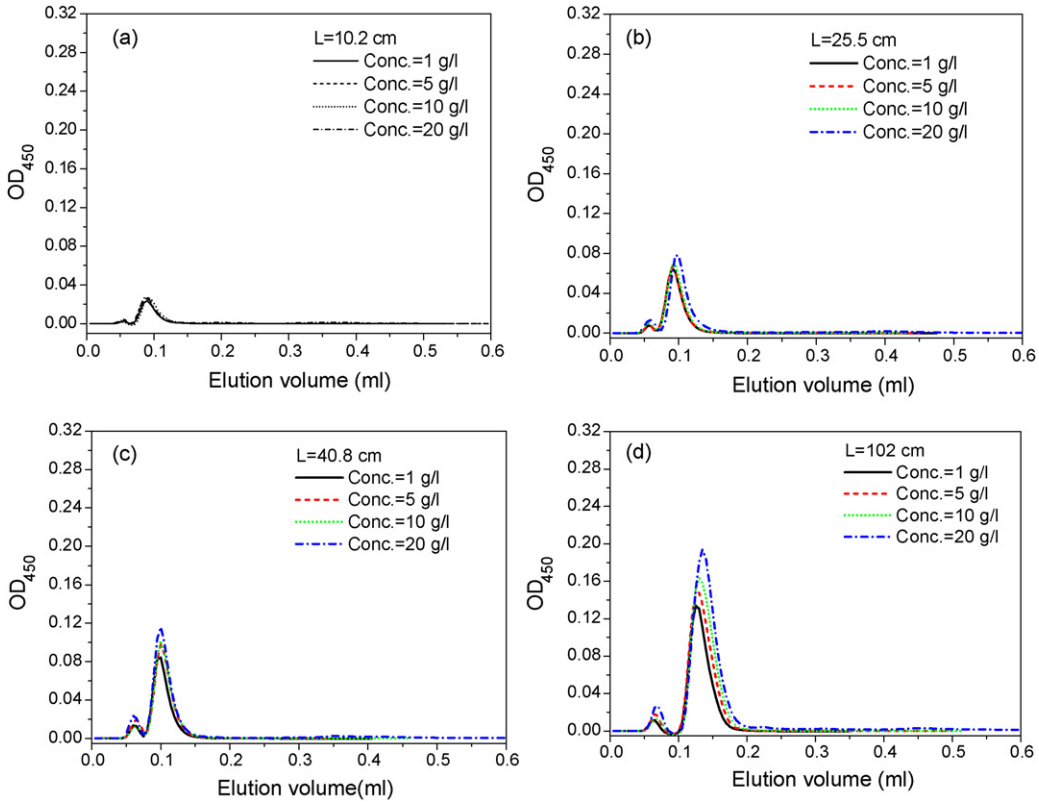

Fig. 1. Aggregate formation of lysozymes as a function of the length of sample loop measured at 450 nm. The diameter of sample loop was set at 0.25 mm and the denatured lysozyme fed was set at 1, 5, 10, and 20 g/l.

the substrate solution to react with 40 μl of protein sample. One unit of activity corresponds to an absorbance decrease of 0.001 per minute. The absorbance measurement was performed using the Spectronic Genesys 5 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

2.6. Efficiency indices of renaturation/refolding

Fractions were collected and analyzed for total activity of the refolded lysozyme. The mass recovery is defined as the ratio of amount of eluted lysozyme to that of injected denatured sample, which was integrated from chromatograms. Specific activity is therefore calculated as total activity divided by the amount of lysozyme eluted.

3. Results

3.1. Effect of sample loop dimensions on aggregate formation

To investigate the effect of sample application on aggregate formation and to avoid aggregates clogging the column packing, injector was directly connected to the UV–vis detector set at 450 nm. The denatured lysozyme samples were applied through various dimensions of sample loops for turbidity measurements at 450 nm without the SEC column. Two types of sample loops were tested (i.e. different lengths and different diameters, details see Section 2). The turbidity of protein sample recorded by UV–vis detector at the wavelength of 450 nm was monitored as a function of the length and diameter of sample loop at various protein concentrations as depicted in Fig. 1 (various lengths) and Fig. 2 (various diameters), respectively. The absorbance at 450 nm would reflect the amount of aggregate presumably. It is observed in Fig. 1a–d, loop length from 10.2 to 102 cm, that two peaks (aggregates) evolved gradually, although Fig. 1a might be interpreted as one peak. As noted, the second peak (higher elution volume) became obvious and completely resolved as the length increased. Also it can be noted that the peak area increased with increasing protein concentration and loop length, especially the second one. The resolution of two peaks may attribute to the aggregates formed at two contact points of sample (denature protein) and mobile phase (refolding buffer), namely, front and tail of the sample. The distances between two aggregate peaks were roughly proportional to the length of sample loop. That is, as the length of sample loop increased, the difference of migration (traveling) time for these two aggregates at two contact points increased and accordingly the resolution of the peak became obvious. Also noted in Fig. 1, the first peak appeared at similar elution volume while the appearance of the second peak delayed as the loop length increased. These support the hypothesis that aggregates are primarily formed at the contact points of samples and refolding buffer at the injector. In addition, it is interesting to note that the second peaks were always larger, implying the mixing intensity would be more significant at the tail of injected sample. In Fig. 2a (loop length of 25.5 cm), the aggregation profiles are depicted for contraction case (I.D. = 0.17 mm) and

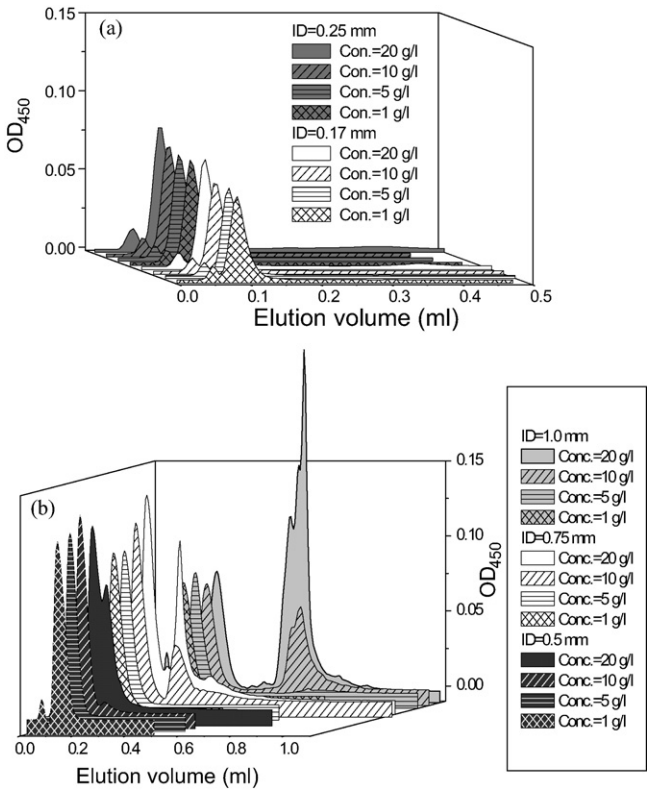

Fig. 2. Aggregate formation of lysozymes as a function of the diameter of sample loop measured at 450 nm. The length of sample loop was set at 25.5 cm and the denatured lysozyme fed was set at 1, 5, 10, or 20 g/l. (a) Contraction and control cases; (b) expansion cases.

control (I.D. = 0.25 mm, same as the rest connecting tubing) for various protein concentrations. It seems that there is not much difference in the patterns of aggregate formation for the contraction and the control cases. On the contrary, for the cases of expansion (Fig. 2b, I.D. = 0.5, 0.75, 1.0 mm), the patterns look very different from those shown in Fig. 2a. Specifically, the appearance of the additional aggregate peaks (the third peaks) was significantly noted for the case of I.D. = 1.0 mm and 20 g/l, the out-of-range one, as well as others in Fig. 2b. The formation of the third peak may attribute to the expansion of sample loops. The effect became significant with high expansion coefficient (diameter ratio of sample loop to connecting tubing) and protein concentration.

3.2. *Effect of injection volume on total amount of aggregates before the column inlet*

The experimental data of Figs. 1 and 2 were integrated as the indication of aggregate amount. And the total amount of aggregates (including all the peaks) for each case is plotted as a function of injection volume in Fig. 3 for either different lengths or different diameters of sample loops. The results of this figure seem to suggest that the total aggregate amount was not dependent on the sample loop dimension (diameter or length), but instead, influenced by injection volume and protein concentration. For lower protein concentrations up to 10 g/l, the aggregation increased with the increasing sample volume to some saturation values. However, for the case of 20 g/l, the saturation was not reached under our experimental conditions

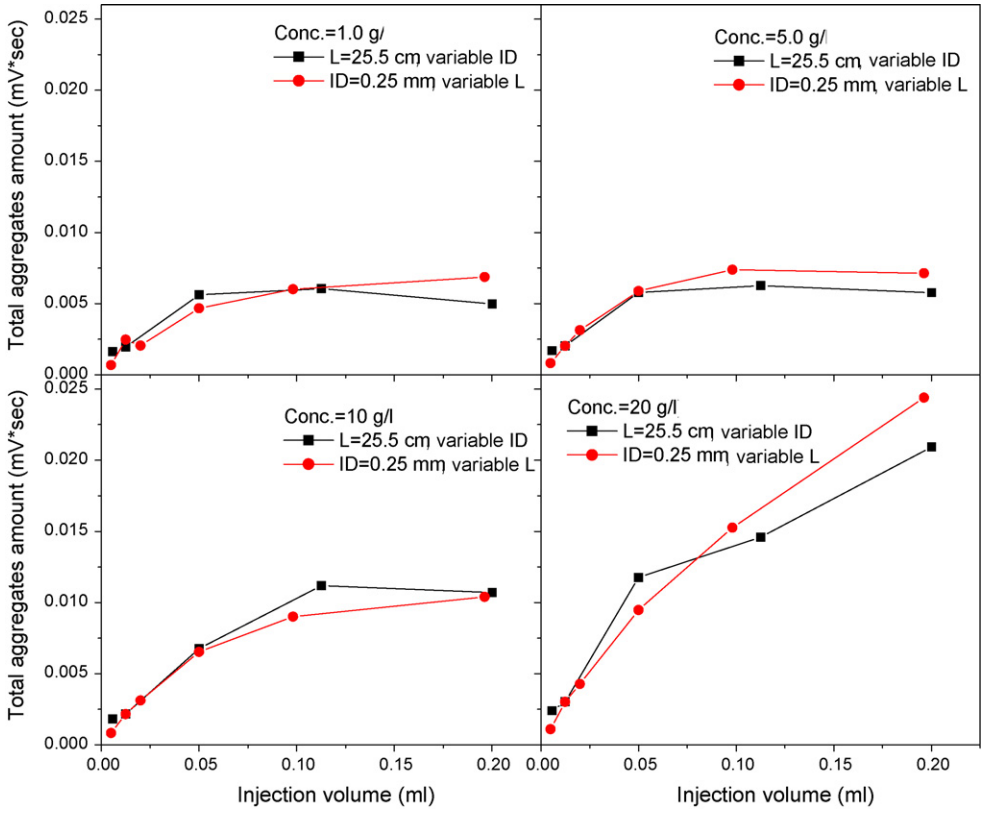

Fig. 3. Effect of injection volume of denatured lysozymes on total amount of aggregates. The denatured lysozyme fed was set at 1, 5, 10, or 20 g/l.

and increased significantly with sample volume. For example, at 1 g/l and 5 g/l of denatured lysozyme, the maximum amount of aggregate formation was achieved at about 0.05 ml injection volume. This seems to indicate that although the migration time of denatured protein between the sample loop and sample inlet was the key for aggregate formation, the total aggregate amount gradually reached equilibrium. Also noted in Fig. 3, the injection volume corresponding to saturated aggregates increased with an increase of protein concentration. For example, for 5 and 10 g/l of protein concentrations, the aggregation reached equilibrium at roughly 0.05 and 0.1 ml of injection volume, respectively. However, at higher protein concentration (20 g/l), an approximately linearly proportional relationship was observed between the amount of aggregates and the injection volume. This implied that higher protein concentration could accelerate aggregate formation, perhaps attributing to fact that higher-order concentration dependence of aggregation kinetics. In other words, at high protein concentrations there is an increased probability of collisions between folding intermediates at the point of sample application, resulting in an increase in aggregation. However, it is interesting to note that the numbers of aggregates as well as the patterns of aggregates might be different for various lengths and diameters of sample loop. The total amount of aggregates, which would be directly related to refolding performance, might not be significantly affected by the dimension of sample loop. On the other hand, the sample volume (migrating time) of denatured proteins as well as protein concentration seems to be the crucial factors for the total aggregates formation during sample application. Further, the similarity of the aggregate amount for both sample application conditions (different length and different diameter) in Fig. 3 reveals that the formation of aggregate may be determined mainly by injection volume, in addition to protein concentrations. Also when compared with Fig. 2, although the third peak appeared in the cases of expansion, the total aggregate was not affected significantly by the expansion, at least, for the range of our experiments.

3.3. Effect of sample loop dimension on chromatograms during refolding the denatured lysozyme in SEC

Fig. 4 (sample loops of various lengths) and Fig. 5 (sample loops of various diameters) show the chromatograms of refolding lysozyme monitored by UV 280 nm, indicating the quantities of eluted protein. A filter was installed before the column to avoid damage by the aggregate blocking. Therefore, these aggregates detected at 450 nm without the column in previous section may be filtered out in these refolding experiments, especially the large aggregates. In these two figures, the first peaks are proteins and the second peaks are compounds of small molecular weights, mainly denaturants. As noted the peaks increase with increasing sample loading, including longer sample loop, wider sample loop and higher protein concentration. The integrated area of the first peaks (eluted protein) divided by the original amount of protein injected to the system is termed as mass recovery. Primary

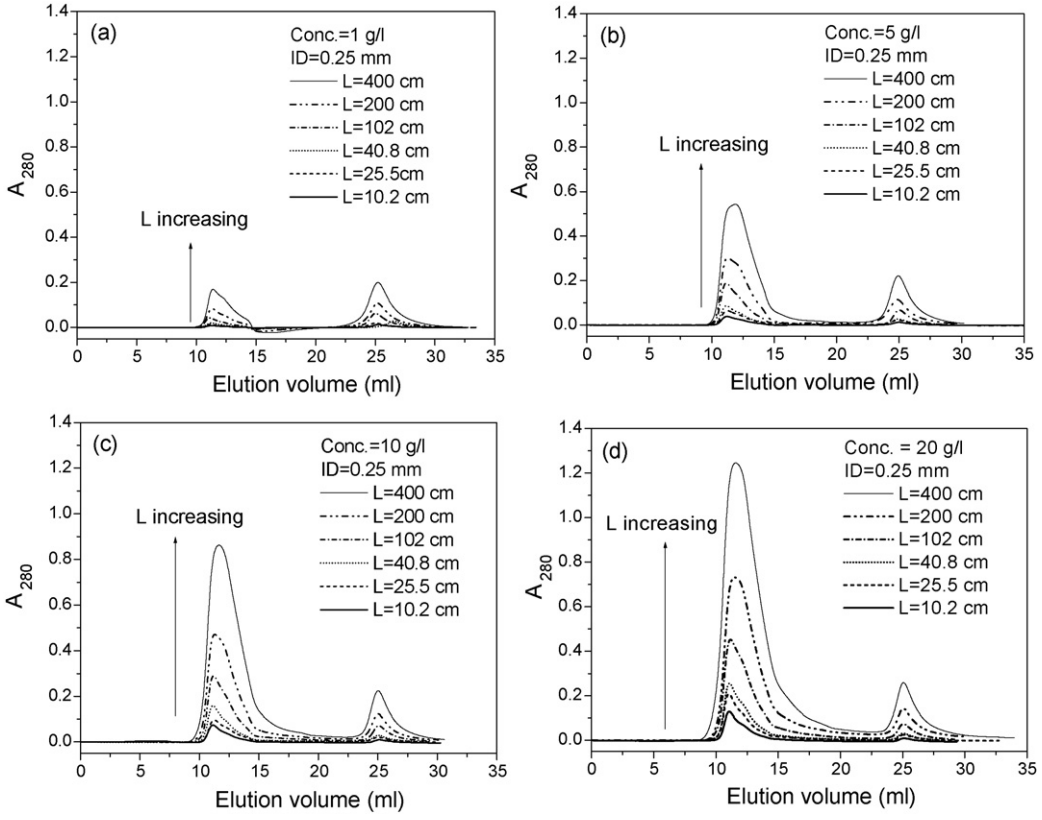

Fig. 4. Effect of sample loop length on chromatograms during refolding the denatured lysozyme in SEC. The diameter of sample loop was set at 0.25 mm. Four different lysozyme concentrations, 1, 5, 10, and 20 g/l, were used in our SEC refolding experiments.

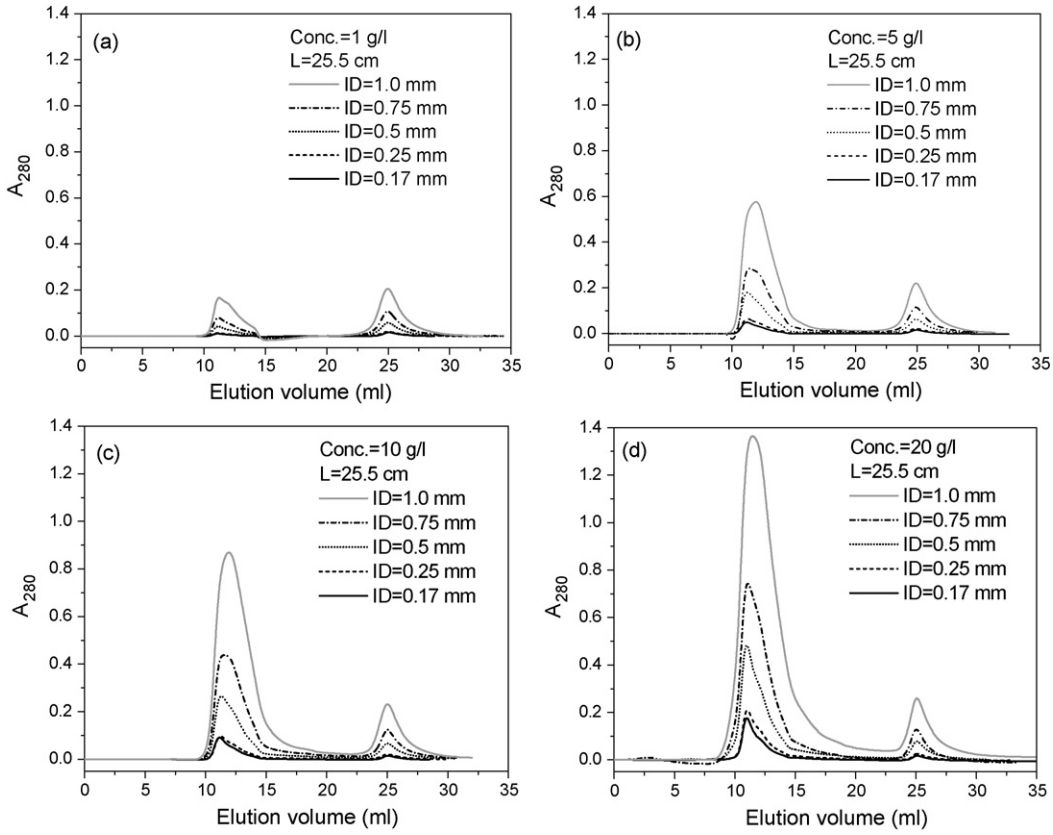

Fig. 5. Effect of sample loop diameter on chromatograms during refolding the denatured lysozyme in SEC. The length of sample loop was set at 25.5 cm. Four different lysozyme concentrations, 1, 5, 10, and 20 g/l, were used in our SEC refolding experiments.

loss of protein were those aggregates retained on the filter before the column, and therefore, more aggregate observed in previous section would result in lower mass recovery. The eluted protein may be active if fully folded during flowing through the SEC column, inactive if not properly folded. The activity measurements had to be carried out for each fraction polled out from chromatographic operations. The activity recoveries were represented by the ratio of sum activity of all the fractions to the original activity of injected denatured lysozyme samples. These two indices, mass recovery and activity recovery, are then summarized and present in the following section.

We have also performed the size-exclusion chromatography refolding experiments on native lysozymes before denaturation. For example, the elution volume of the peak of native lysozymes was around 13.5 ml at $L = 40.8$ cm. As can been seen in Figs. 4 and 5, the peaks of the refolded protein exhibited smaller elution volume (<13.5 ml), indicating that the protein, indeed, was not fully folded into its native form. Our results showed that with increasing protein concentration, the peaks gradually shifted to the right. Moreover, the displacement of peaks to the right observed in the chromatograms (Figs. 4 and 5) was perhaps due to the conformational changes, or the non-linear partition of proteins between the mobile and stationary phases. It should be pointed out that, since only protein activity and quantity measurements were made, the conformational interpretations is not available at this stage.

3.4. Effect of injection amount on refolding of denatured lysozyme

For preparative application of protein refolding by SEC column, the loading capacity of denatured protein on the column is an important factor to be considered. Because it was noted that the amount of aggregates is mainly dependent on loading amount as shown in Fig. 3, the refolding performances are presented in terms of injection amount (injection volume × concentration) as shown in Fig. 6 (mass recovery) and Fig. 7 (activity recovery). Not surprising, both recoveries decreased with increasing protein loading onto the column. This is because that at higher protein there is an increase in probability of collision between folding intermediates from the point of sample application till the inlet of the column, resulting in an increase in aggregation and a decrease in refolding yield.

4. Discussion

According to our previous results [12] showing that aggregation mainly occurred between the injector and column inlets (the sample loop), we were interested in exploring protein refolding by adjusting the tubing length and diameter of the sample loop in this research work. Our results pointed out that the expansion (larger tubing diameter) and the contraction (smaller tubing diameter), which corresponds to distinctive flow patterns, resulted in fairly different results. There was not

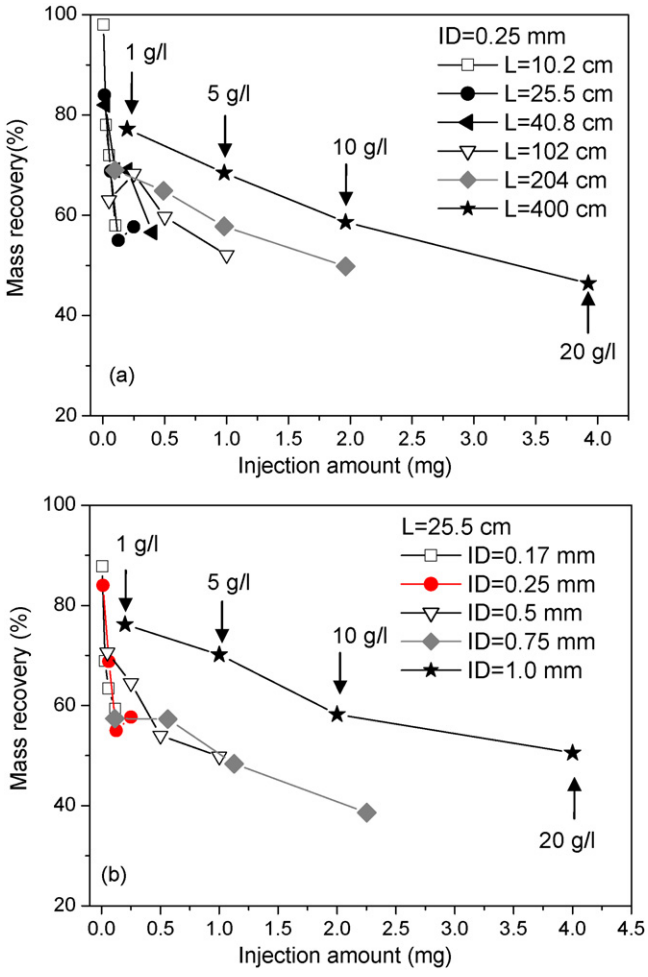

Fig. 6. Effect of injection amount on percentage of mass recovery via SEC refolding method. (a) Constant diameter (0.25 mm) and various lengths of sample loop were used in the experiments; (b) constant length (25.5 cm) and various diameters of sample loop were used in the experiments.

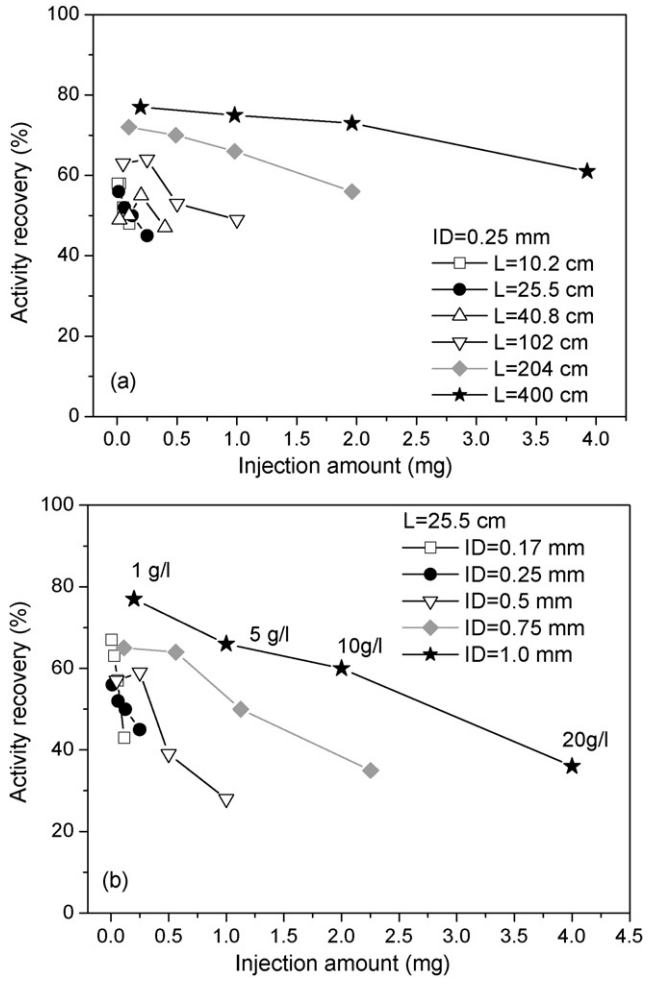

Fig. 7. Effect of injection amount on percentage of activity recovery via SEC refolding method. (a) Constant diameter (0.25 mm) and various lengths of sample loop were used in the experiments; (b) constant length (25.5 cm) and various diameters of sample loop were used in the experiments.

much difference in the patterns of aggregate formation between the contraction and the control cases. However, a third peak appeared on the chromatogram in the expansion case compared with only two peaks observed in its contraction counterpart. Importantly, after a careful examination of our data, we have concluded that the total amount of aggregates was markedly affected by the protein concentration but not the dimension of sample loop within the experimental conditions considered in our investigation.

Based on the previous discussion and experimental results, another important problem related to refolding process arises. For a sample with specific amount of denatured protein, is the sample with large volume and low protein concentration preferable to the sample with small volume and high protein concentration in order to obtain a high refolding yield? This question was answered by the comparison of various samples with same amount of protein mass but different volumes and concentration. The results in Figs. 6 and 7 suggest that a higher recovery (including mass and activity) could be obtained with samples of large volumes and low protein concentration than those of small volumes and high concentration. This increased refolding yield with increased injection volume indicated that the extent of aggregation could be reduced by either increasing the sample loop length or diameter. Although increasing length or loop would prolong the migration time of denatured protein between the injector and column inlet, the aggregation would eventually reach equilibrium (Fig. 3) and increase the refolding yield. Although samples of low concentration are recommended to design for SEC refolding (the activity recovery is inversely proportional to the injection protein concentration), which is in agreement with the batch dilution refolding, it demonstrated again that aggregates formed during sample application may be determined mainly by the mixing of the denatured lysozyme and refolding buffer, where no SEC packing assistance is available. And mixing prior to the column would result in aggregation, thus significantly affect the performance of entire protein refolding process with SEC, including mass recovery and activity recovery. Therefore, reduction of aggregation formation between injector and column inlet by SEC is crucial for proper protein refolding.

Acknowledgement

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