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Lysozyme refolding with immobilized GroEL column chromatography

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Abstract

A refolding chromatography with immobilized molecular chaperonin GroEL was studied for the reactivation of denatured-reduced lysozyme. The effect of denaturant concentration (guanidine hydrochloride, 0.1–1.5 *M*) in the elution buffer, the elution flow-rate, and the loading concentration and volume of the substrate protein on the reactivation yield was studied. All the operating parameters showed minor effects on the recovery yield of lysozyme mass, which remained at 90–100%, but exhibited relatively notable influences on the specific activity of the recovered lysozyme. For example, there existed an optimum denaturant concentration of about 1 *M* at which the highest yield of specific activity (up to 97%) was obtained. Using the immobilized GroEL column, 3 ml of the lysozyme (1 mg/ml) per batch could be refolded at an overall yield of 81%, which corresponded to a refolding productivity of 54 mg per l gel per h. At comparable reactivation yields (over 80%), this value of productivity was over four-times larger as that of the size-exclusion refolding chromatography reported previously (12 mg per l gel per h), indicating the advantage of the present system for producing a high throughput in protein refolding operations.

Keywords: Immobilized chaperonins; Lysozyme; Proteins

1. Introduction

Molecular chaperones are cellular heat-shock proteins widely present in prokaryotic and eukaryotic cells. 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 [1]. Among the variety of molecular chaperones, the *Escherichia coli* chaperonins GroEL and GroES are mostly investigated [2]. GroEL is made of two stacked seven-membered rings of M_r 57 000, while GroES is a single heptameric ring of M_r 10 000, which helps GroEL in promoting protein refolding. In vitro experiments indicate that the chaperonin GroEL has a broad specificity for the folding of proteins; in the presence of the chaperonin(s), denatured enzymes and proteins can regain their activity in an adenosine triphosphate-dependent manner [3–5].

Recently, methods for extending the application of the molecular chaperones in facilitating protein reactivation to bioseparation processes have been studied. It has been shown that the GroEL can be recycled by recovery with ultrafiltration [6,7], and the immobilized GroEL/ES can be used for reac-

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tivating thermally denatured enzymes [8]. Most importantly, a protein refolding chromatography with an immobilized peptide fragment of GroEL (i.e., GroEL minichaperone) has been demonstrated by Altamirand et al. [9]. They have additionally developed an oxidative refolding chromatography with three molecular chaperones (i.e., GroEL minichaperone, DsbA and peptide prolyl isomerase) immobilized to agarose gel to fold the scorpion toxin Cn5 [10].

The authors have studied the reactivation of denatured-reduced lysozyme in the presence of immobilized GroEL and determined the optimum operating conditions such as pH (7.8) and temperature (37°C) [11,12]. Our results indicate that the immobilized chaperonin can be repeatedly used for protein reactivation. As a further investigation, we here report the results of lysozyme refolding with a packed-column chromatography using the immobilized GroEL as the stationary phase. Our purpose includes quantifying the productivity of the system as a large-scale protein refolding procedure. The effects of denaturant concentration in the elution buffer, the elution flow-rate, and the loading volume and concentration of the substrate protein on the reactivation yield has been discussed. Furthermore, the results are compared with that of lysozyme refolding in a suspended reactivation system using the immobilized GroEL and that using a gel filtration chromatography reported by Batas and Chaudhuri [13].

2. Materials and methods

2.1. Materials

Chicken egg white lysozyme, bovine serum albumin (BSA), Mg^{2+} salt of adenosine triphosphate (ATP), guanidine hydrochloride (Gdn·HCl), and dithiothreitol (DTT) were purchased from Sigma (St. Louis, MO, USA). Formyl-Cellulofine gel of 125 to 210 μm was a product of Chisso (Tokyo, Japan); the matrix was based on a porous cellulose gel activated with formyl groups (10 μmol/ml) via a long spacer (manufacturer's specifications). Other chemicals were all commercially available reagents of analytical grade.

The *E. coli* strain harboring the plasmid pND5 [14] was utilized to produce GroEL. Detailed procedures for GroEL purification and immobilization have been described earlier [11].

2.2. Reactivation in suspended system

Lysozyme (5 mg/ml) was denatured-reduced in 50 m*M* Tris·HCl buffer (pH 8.0) containing 6 *M* Gdn·HCl, 10 m*M* DTT and 2 m*M* EDTA at 4°C for 40 min. The denatured/reduced lysozyme solution was added to the refolding buffer (50 m*M* Tris·HCl, 20 m*M* KCl, 10 m*M* $MgCl_2$, 2 m*M* EDTA, 2 m*M* ATP, pH 7.8) to initiate reactivation experiments. In GroEL-assisted reactivation experiments, the refolding buffer contained additionally definite amount of free GroEL or the GroEL-immobilizing gel. In all suspended reactivation experiments, the final Gdn·HCl concentration in the refolding buffer was 60 m*M*. In addition, the molar ratios of free and immobilized GroEL (tetradecamer) to the substrate lysozyme were 2 and 10, respectively [11]. The reactivation system was incubated at 37°C in a shaking incubator. During the reactivation process, liquid supernatant was sampled to measure the change of lysozyme activity with the incubation time. The reactivation yield was expressed as the percentage of specific activity of reactivated lysozyme relative to that of the native lysozyme.

2.3. Reactivation with immobilized GroEL chromatography

The refolding experiments with the packed column of immobilized GroEL were carried out with the GradiFrac chromatography system (Pharmacia Biotech, Uppsala, Sweden). A chromatography column (200×16 mm I.D.) with a water jacket was packed with 20 ml of the GroEL-immobilizing gel (coupling density 12.8 mg/ml gel). The column temperature was kept at 37°C by circulating thermostated water to the column jacket. Prior to each refolding batch, the column was equilibrated with the corresponding elution buffer (see below). A definite amount of the denatured-reduced lysozyme described above was diluted to 3 *M* Gdn·HCl and was directly applied to the column top. Then the column was immediately developed with an elution buffer com-

posed of the refolding buffer (see above) containing, unless stated otherwise, 1.0 *M* Gdn·HCl. The effluent from the column was detected by a UV monitor and the data acquired by a computer. The eluted protein peak was pooled and subjected to protein concentration and activity determinations.

2.4. Analytical methods

The concentrations of proteins were analyzed according to Bradford [15]. GroEL concentration was determined using BSA as the standard. Lysozyme activity was assayed by the method proposed by Imoto and Yagishita [16].

3. Results

3.1. Reactivation in suspension system

The reactivation of the substrate lysozyme was performed in shaking flasks with or without GroEL (Fig. 1). Under the operating condition, about 45% of the lysozyme activity was restored by the simple dilution method (spontaneous refolding). In the presence of free GroEL, the reactivation yield increased rapidly with the operation time; the activity yield of lysozyme reached 95% in 30 min. In contrast, however, the activity increase in the presence of the immobilized GroEL was much slower than that in the presence of the free GroEL; an operation time longer than 150 min was required to enable the reactivation yield to approach 90%. This is considered due to the intraparticle diffusion resistance of the matrix to the protein and the inactivation of GroEL upon immobilization to the gel matrix [8,11]. Namely, both the mass transfer resistance and the GroEL inactivation could result in the decrease of the apparent activity of GroEL for facilitating protein refolding.

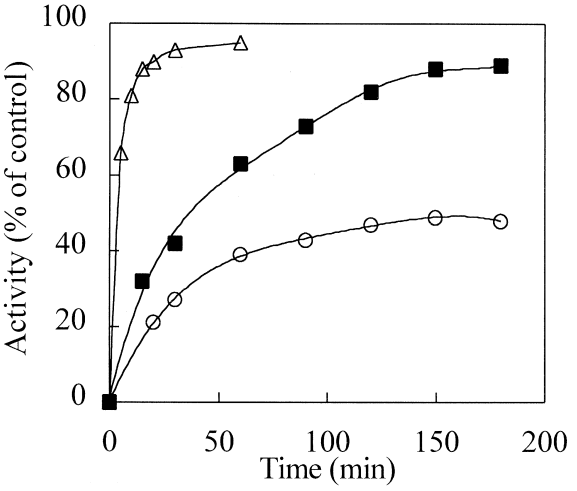


Fig. 1. Lysozyme reactivation in the absence (○) and presence of free GroEL (△) and immobilized GroEL (■). Lysozyme concentration: 30 μg/ml. GroEL coupling density: 12.8 mg/g gel.

3.2. Reactivation with immobilized GroEL chromatography

3.2.1. Effect of Gdn·HCl concentration

Denaturant concentration in the refolding buffer is an important factor for mediating the reactivation of denatured-reduced lysozyme [17,18]. Hevehan and Clark have reported that a refolding buffer with about 1 *M* Gdn·HCl minimizes the aggregate formation of lysozyme at high concentrations [19]. It has also been claimed that a refolding buffer containing 2 *M* urea gives the highest reactivation yield for lysozyme [20] and Batas and Chaudhuri used the refolding buffer at the high-concentration loading of denatured enzymes in the size-exclusion chromatography [13]. In this refolding chromatography, the effect of Gdn·HCl concentration in the elution buffer was first investigated to find out its proper concentration for the efficient lysozyme reactivation. Fig. 2 shows the experimental results. Lysozyme mass of 90 to 95% was recovered and the elution profiles (not shown) from the packed column presented little difference from each other at the Gdn·HCl concentration range (0.1–1.5 *M*). In contrast, the restored specific activity of lysozyme exhibited a maximum value at about 1 *M* Gdn·HCl. This is similar to the literature data at high concentrations mentioned above, but different from that obtained in the suspended reactivation system, in which the reactivation yield of lysozyme (0.03 mg/ml) increased with decreasing the Gdn·HCl concentration until 0.06 *M* [12]. The result is likely attributed to the higher loading protein concentration in the column chromatography. That is, higher denaturant concentration was needed to avoid the incorrect

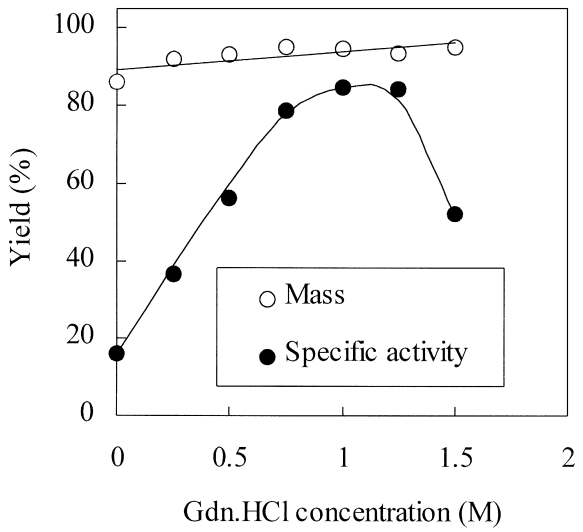


Fig. 2. Effect of Gdn·HCl concentration in elution buffer on reactivation yields. Lysozyme loading: 3.0 ml at 1.0 mg/ml. Elution flow-rate: 0.045 cm/min.

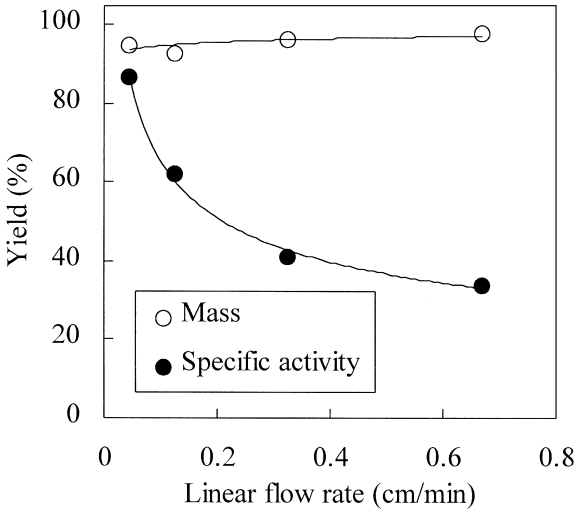


Fig. 3. Effect of elution flow-rate on reactivation yields. Lysozyme loading: 3.0 ml at 1.0 mg/ml.

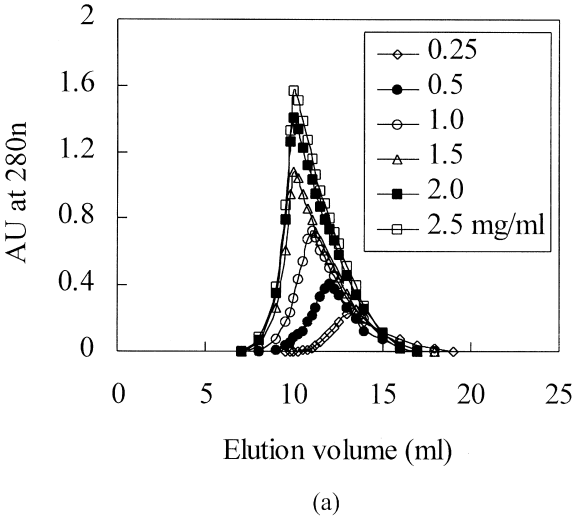
folding of the denatured lysozyme after it had been loaded onto the column. The decrease of the reactivation yield at the high denaturant concentration (1.5 *M*) might be due to the inhibition of the denaturant to the chaperoning activity of GroEL [21].

3.2.2. Effect of elution flow-rate

Based upon the results shown in Fig. 2, the following packed-column reactivation experiments were carried out using the elution buffer consisting of 1.0 *M* Gdn·HCl. To know whether a high reactivation yield could be obtained at higher flow-rates, we performed the refolding chromatography at higher elution flow-rates than that in Fig. 2. As shown in Fig. 3, the mass yields remained at a high level and increased somewhat with increasing the flow-rate, while the specific activity of the recovered protein decreased rapidly when the elution flow-rate was increased. At flow-rates higher than 0.3 cm/min, the reactivation yields was even less than the spontaneous refolding in the suspended system (see Fig. 1). This was due to the high protein concentration in the packed column (the recovered protein concentration was increased by increasing the flow-rate, data not shown). The results indicated that a residence time long enough was necessary to gain a high reactivation yield. At the lowest flow-rate shown in Fig. 3, that is, 0.045 cm/min, the average residence time of the protein peak in the column was 133 min. This was approximately the same as the refolding results in the suspended system shown in Fig. 1, in which an incubation time over 150 min was needed for the reactivation yield to reach 90%. Thus, the following experiments were carried out at 0.045 cm/min.

3.2.3. Effect of substrate loading amount

The influence of the loading volume and concentration of the denatured lysozyme was investigated to determine the refolding capacity and productivity of the immobilized GroEL column. Fig. 4 shows the elution curves at different loading concentrations (Fig. 4a) and volumes (Fig. 4b) of the substrate lysozyme. Obviously, only one protein peak was detected in each case. This was similar to the protein refolding using the size-exclusion chromatography with which an aggregate profile before the refolded protein peak was observed only at very high loaded protein concentrations [13]. Hence, quite high protein mass yields, 90–100%, were obtained in the substrate loading range investigated (Fig. 5). In contrast, the activity yield decreased distinctly with increasing the loading concentration (Fig. 5a) as well



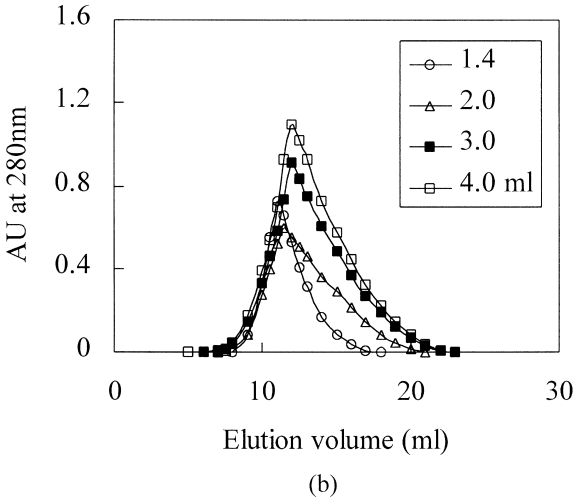
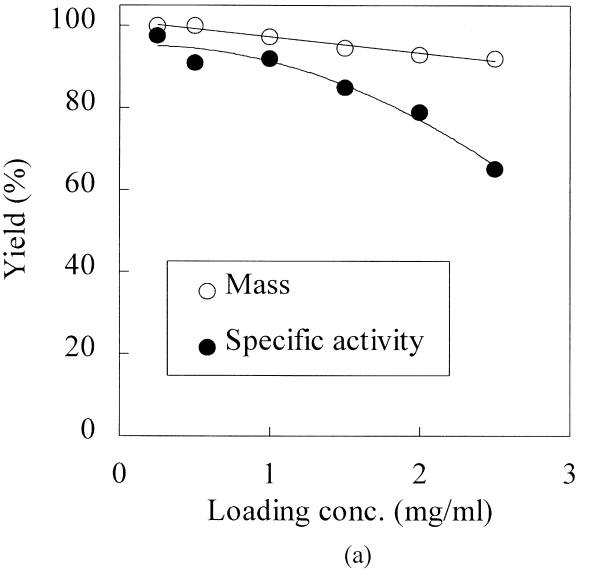


Fig. 4. Elution profiles of refolded lysozyme, (a) at different loading concentrations (loading volume: 1.4 ml), and (b) at different loading volumes (loading concentration: 1.0 mg/ml) of the denatured/reduced lysozyme.



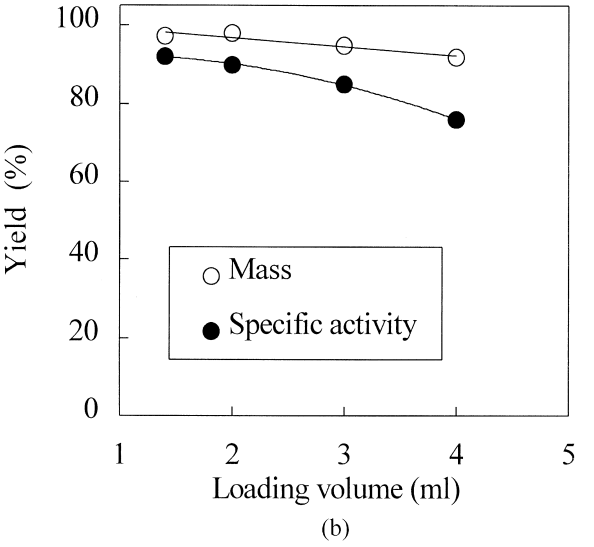


Fig. 5. Dependence of mass and activity yields, (a) on the loading lysozyme concentration at constant loading volume (1.4 ml), and (b) on the loading volume at constant loading concentration (1.0 mg/ml).

as the loading volume (Fig. 5b). However, at a low lysozyme mass loading, the specific activity yield up to 97% was achieved.

Fig. 6 describes the effect of protein loading amount on the overall activity yield of lysozyme, which was calculated by multiplying the mass yield and the specific activity yield indicated in Fig. 5a and b. As expected, to increase protein mass loading, increasing its loading volume at a constant concentration was beneficial in obtaining higher overall reactivation yield. Using the present immobilized GroEL column, 3 ml of 1 mg/ml lysozyme per batch could be reactivated at an overall yield of 81%.

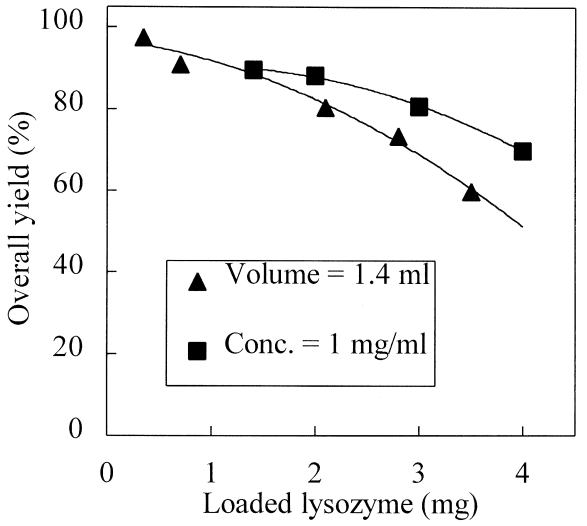


Fig. 6. Overall yield of lysozyme as a function of loaded lysozyme mass.

4. Discussion

The present immobilized GroEL column contained a packed gel volume of 20 ml with a voidage of 0.31, corresponding to the void volume of 6.2 ml. According to the retention theory of linear chromatography, the following equation is written:

$$V_R = V_0 + K(V_t - V_0) \quad (1)$$

or

$$K = \frac{V_R - V_0}{V_t - V_0} \quad (2)$$

where V_t, V_0 and V_R are the total gel volume in the column, void volume of the packed column and the average retention volume of the refolded lysozyme profile, respectively, and K is the partition coefficient of lysozyme. With the retention volumes shown in Fig. 4 and the column parameters, it is calculated from Eq. (2) that the partition coefficient K ranges from 0.3 to 0.5, decreasing with increasing the substrate loading concentration (Fig. 4a).

It has been reported that in the refolding chromatography with immobilized minichaperone the partition coefficients of the folded indole 3-glycerol phosphate synthase lacking residues 1–48 and cyclophilin A were 1.7 and 1.43, respectively, which indicated the binding of the proteins to the stationary phase [9]. In the present system, however, due to the very low partition coefficient of lysozyme, it is likely that the substrate retention by the GroEL-immobilizing gel was mainly attributed to the gel filtration effect. In other words, the denatured or partially folded lysozyme in the column was hardly retarded by binding to the immobilized GroEL. The results imply the rapid binding and release of the substrate protein promoted by ATP present in the elution buffer. To demonstrate this explanation, we performed a control experiment using the elution buffer without addition of ATP. The results of the refolding chromatography using the elution buffer with and without ATP are provided in Table 1. Obviously, the partition coefficient increased more than twice when ATP was not included in the elution buffer, indicating the binding of lysozyme to the immobilized GroEL during the chromatography process. Moreover, the reactivation yield of lysozyme was lowered when ATP was excluded. This phenomenon is the same as that found in the suspended reactivation systems for lysozyme [11] and other enzymes [6,8] with free and immobilized GroEL or GroEL/ES.

The immobilized GroEL column had been utilized for the lysozyme refolding study for 50 days. During the period, 27 refolding experiments were performed and the column was washed several times with 0.1 *M* sodium hydroxide for the cleaning of the column. As a result, no significant change in the refolding ability of the column was observed, indicating a high

Table 1
Effect of ATP in elution buffer on the retention and refolding of lysozyme (loading amount: 3 ml of 1 mg/ml substrate protein)

ATP (m*M*)	V_R (ml)	*K*	Specific activity yield (%)	Mass yield (%)	Overall yield (%)
2	12.0	0.42	85	95	81
0	18.3	0.88	54	80	43

Table 2
Comparison of lysozyme refolding results by immobilized GroEL chromatography, the suspended system using the immobilized GroEL, and size-exclusion chromatography (SEPROS)

System	Suspended system	Chromatography		SEPROS[a]
Gel volume (ml)[b]	6.4 (4.5)[c]	20	20	467
Lysozyme loading:				
Mass (mg)	0.15	2.0	3.0	14.5
Concentration (mg/ml)	0.03	1.0	1.0	9.6
Volume (ml)	5.0	2.0	3.0	1.5
Incubation/elution time (min)[d]	150	128	133	132
Recovery:				
Mass yield (%)	96	98	95	83
Specific activity (%)	89	90	85	101
Overall activity yield (%)	85	88	81	84
Concentration (mg/ml)	0.011	0.16	0.18	0.18
Productivity (mg per l gel per h)[e]	8.0	42	54	12

[a] SEPROS refers to a size-exclusion protein refolding system [13]. Data calculated from Ref. [13].
[b] Settled gel volume in a packed column.
[c] Data in parentheses: the suction dried mass of the gel.
[d] Incubation time for the reactivation in suspension system, while the elution time for the other two column systems.
[e] Productivity based on the renatured lysozyme activity.

stability of the immobilized GroEL sustaining repeated uses over a long period.

This paper is concerned with the performance of protein refolding in the immobilized GroEL column chromatography, aiming at quantifying the potential productivity of the system from the standpoint of practical application. Therefore, it is of significance to compare our results with that of lysozyme refolding with the size-exclusion protein refolding system (SEPROS) proposed by Batas and Chaudhuri [13], which was also aimed at the large-scale protein renaturation. Together with the result in the suspended reactivation performed in this work, the reactivation data displaying comparable activity yield at higher protein loading conditions achieved using the immobilized GroEL chromatography (two batches) and the SEPROS are summarized in Table 2. It is found that the suspended reactivation system was far less effective than the packed-column refolding chromatography; very low product concentration and protein-refolding productivity was realized. At achieving comparable reactivation yields, the productivity of the present refolding chromatography was up to four-times greater than that of the SEPROS, indicating the advantage of the present system at gaining high throughput in the protein refolding. Moreover, it is considered that an immobilized molecular chaperone system can be used for the refolding of a variety of proteins, especially of those difficult to be refolded, as demonstrated by Altamirand et al. [10]. However, the SEPROS was a simpler method because it was based on the principle of gel filtration chromatography. Thus, in order to better compare the two systems as large-scale protein refolding approaches, they should be evaluated by comprehensively taking into account various aspects that affect a cost-effective reactivation process.

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