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A Study on the Refolding of Lysozyme Using Fed-Batch and Step-Addition Strategies

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Abstract—The refolding step is essential for industrially produced protein aggregates to restore their biological activities. Various strategies, including the direct dilution method, have been utilized to enhance the performance of the refolding process; however, refinement and improvement on these methods are ongoing. We have used two different operating schemes, fed-batch and step-addition, independently and collaboratively to refold denatured lysozymes. Our research shows that the fed-batch and the step-addition schemes overcame the aggregation formation and favored the refolding step at higher feeding concentrations in comparison with the typical dilution method. Moreover, with relatively longer folding time intervals, the step-addition scheme provided higher activity recovery, while the fed-batch scheme was advantageous in terms of productivity. Finally, a combined scheme was proposed to optimize the refolding process. This work demonstrates the feasibility of applying fed-batch and/or step-addition operation schemes to the renaturation of enzyme activity.

Key Words : Lysozyme, Refolding, Fed-batch, Step-addition

INTRODUCTION

The expression of genetically engineered proteins in bacteria has been widely used in the pharmaceutical industry and in biochemical research. Large quantities of genetically engineered proteins can be produced in rapid and inexpensive fermentation processes. However, these proteins of interest often form aggregation of inactive deposits inside the cells (*i.e.*, inclusion bodies) during the production process (Buchner *et al.*, 1992). The procedure for converting inclusion bodies into active biological proteins typically consists of washing, isolation, solubilization, and renaturation/refolding steps (Clark, 2001). While the efficiency of the first two steps can be fairly high, the main challenge is to develop optimum processes whereby the formation of inactive misfolded aggregated species can be prevented during the renaturation/refolding step.

The aggregation pathway is governed by intermolecular interactions between hydrophobic patches on various protein molecules, and therefore, is highly dependent on protein/polypeptide concentrations. This unwanted aggregation phenomenon follows a higher order kinetic scheme that competes with the first order reaction of refolding. Thus, extensive efforts have been directed toward preventing such an unwanted side reaction in the refolding step.

The most direct means of reducing aggregates is to inhibit intermolecular interaction by decreasing the protein concentration. Therefore, techniques such as dilution (Goldberg *et al.*, 1991), dialysis with buffer-exchange (West *et al.*, 1998), gel filtration chromatography (Batas and Chaudhuri 1996; Hamaker *et al.*, 1996; Fahey *et al.*, 1999; Liu and Chang, 2003), and immobilization of the denatured protein onto a solid support (Kim *et al.*, 1997; Negro *et al.*, 1997) have been proposed as probable methods to renature/refold the solubilized protein by removing the excess denaturants. However, as none of these techniques are perfect, refinement of them is still underway.

In the current work, with lysozymes utilized as a model system, attempts were made not only to assess the feasibility of applying the fed-batch and/or step-addition strategies to the enzyme refolding step, but also to determine the optimum operating conditions. Our results show that, by allowing adequate time for renaturation and by maintaining a low concentration profile of the denatured proteins, we can obtain a high yield under a high final protein concentration through both strategies. We believe that the results from this study may facilitate the development of a more effective treatment strategy for refolding processes.

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EXPERIMENTAL

Materials

Hen egg white lysozyme (HEWL, EC 3.2.1.17), hydrochloric acid (HCl), and di-potassium hydrogen phosphate (K_2HPO_4) were purchased from Merck (Germany). Dithiothreitol (DTT), *Micrococus lysodeikitus* dried cells, oxidized glutatione (GSSG), reduced glutathione (GSH), and tris-(hydroxymethyl) aminomethane were purchased from Sigma (U.S.A.). Urea, EDTA, and potassium di-hydrogen phosphate (KH_2PO_4) were obtained from Hayashi Pure Chemical Ind., Co., Ltd (Japan). All other chemicals were of analytical grade.

Preparation of the denatured lysozyme sample solution

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 at a pH of 8.6 (0.1 M Tris-HCl, 10 M urea, 12.5 mM DTT, and 1.25 mM EDTA) and a volume ratio of 1 to 4. The activity of the denatured lysozyme sample solutions was observed to reach zero after storage at room temperature for 24 hours, and the mixture was then used in the following refolding experiments.

Lysozyme concentration and activity assays

The lysozyme concentration was measured spectrophotometrically at a wavelength of 280 nm. The activity of the lysozyme sample solutions was determined by measuring the decrease in absorbance at 450 nm. Under a pH of 6.2 at 25°C, a 2.5 mL volume of *M. lysodeikticus* suspension (0.2 mg/mL) in 0.05 M potassium phosphate was used as the substrate solution to react with 40 μL of protein sample. One unit of activity corresponded to an absorbance decrease of 0.001 per minute. The measurement of absorbance was performed using the Spectronic Genesys 5 spectrophotometer (Spectronic Instrument, U.S.A.).

Refolding of fully reduced lysozyme

Three methods, direct (batch) dilution, fed-batch, step-addition, were used to perform renaturation in this study. The performance of the refolding process, determined by the refolding efficiency, was assessed based on the ratio of the total activity recovered after refolding to that of the native lysozyme before denaturation.

Direct dilution method

Following denaturation, the renaturation step was initiated by a rapid 6.7-fold to 200-fold dilution of the denatured lysozyme sample solution (0.5-10 mg/mL) in the refolding buffer at pH 8.2, consisting of 0.1 M Tris-HCl, 1 mM EDTA, 3 mM GSH, and 0.3 mM GSSG with various concentrations of urea (0, 1.5, or 2 M). The activity of the lysozyme sample solution was monitored during different periods of refolding.

Fed-batch method

After denaturation, the lysozyme sample solution was transferred at a speed of 0.05-0.5 mL/min into the beaker with the refolding buffer at pH 8.2, consisting of 0.1 M Tris-HCl, 1 mM EDTA, 3mM GSH, and 0.3 mM GSSG with various amounts of urea (0, 1.5, or 2 M). The activity of the lysozyme sample solution was measured during different periods of refolding. The experimental setup was composed of three parts: a vessel with denatured lysozyme solution, a microfeeder/peristaltic pump, and a vessel on a magnetic stirrer for collecting the outlet liquid.

Step-addition method

Different volumes (100 or 200 μL) of various initial concentrations of denatured lysozyme sample solution were injected into the refolding buffer in a stepwise manner. The time interval between consecutive injections was varied (3, 5, 10, or 20 min). The change in enzyme activity was monitored at different time intervals.

For all the measurements, the operating temperature, the rotating speed, and the initial volume of refolding buffer solution in use was set at 25°C, 100 rpm, and 30 mL, respectively.

RESULTS AND DISCUSSION

Refolding of denatured lysozyme at lower concentrations via fed-batch method

Effect of flowrate

The yield of lysozyme renaturation in the fed-batch operation was monitored during the time course of refolding at different feeding flowrates. As there were two mechanisms, intrachain (folding route) and interchain (aggregation route) interactions, competing for the intermediate species during the refolding process, the extent of restored enzyme activity was chosen as the indicator of overall yield in the refolding process. The data were reported as the percentage of total lysozyme activity recovered after the refolding step relative to the enzyme activity before denaturation. Our data (Fig. 1) showed that, when comparing conditions at higher flowrates, the operating flowrate at 0.05 mL/min gave the best

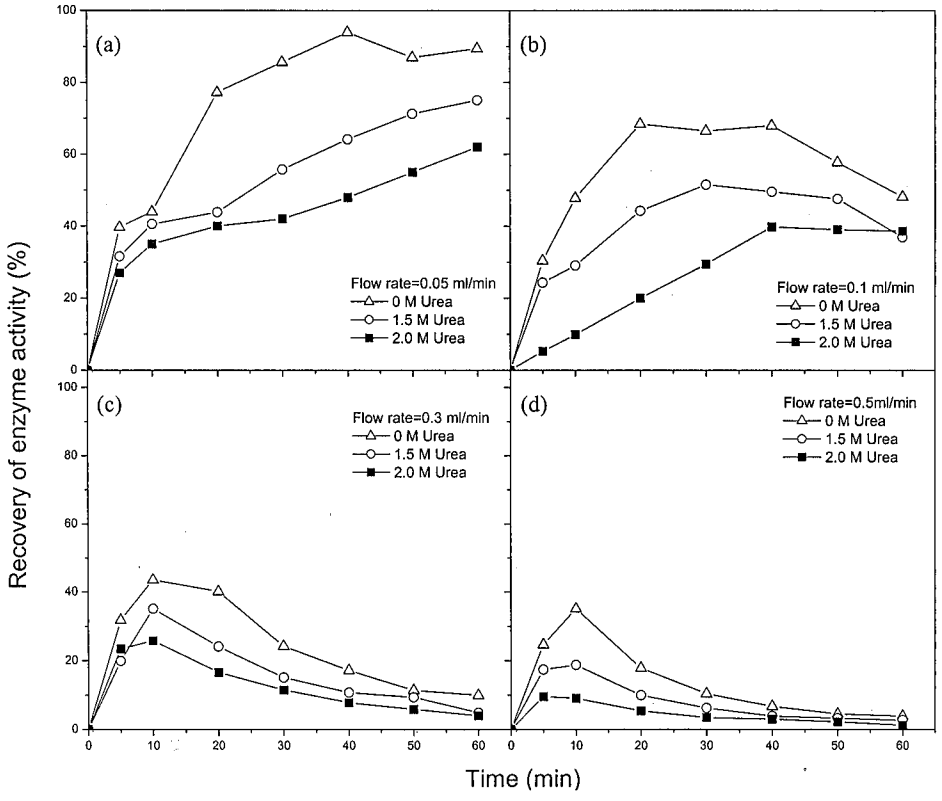

Fig. 1. Effect of the urea concentration in the refolding buffer on the enzyme refolding efficiency via fed-batch method. The recovery of lysozyme activity was determined as a function of time at different urea concentrations (0, 1.5, 2.0 M). The data were reported as the percentage of the total lysozyme activity recovered after the refolding step relative to the enzyme activity before denaturation. The denatured lysozyme sample solution at 0.5 mg/mL was fed into the refolding buffer solution (pH 8.2, 0.1 M Tris-HCl, 1 mM EDTA, 3 mM GSH, and 0.3 mM GSSG) without (0 M) or with urea (1.5 and 2 M) at four different feeding flowrates: (a) 0.05 mL/min; (b) 0.1 mL/min; (c) 0.3 mL/min; (d) 0.5 mL/min.

activity recovery when denatured lysozyme sample solution was used at an initial concentration of 0.5 mg/mL. Moreover, the level of activity recovered dropped off noticeably after 20 min at higher flowrates of 0.3 and 0.5 mL/min. For all cases examined in our study, the recoveries of enzyme activity increased with the operation duration during the early stage. However, the recoveries of enzyme activity for feeding flowrates of 0.05, 0.10, 0.30, and 0.50 mL/min began to drop within 40, 20, 10, and 10 min of refolding, respectively. Our results suggested that, due to the collision with the incoming denatured protein molecules, the conditions at higher flowrates made it more difficult for the existing denatured protein molecules to convert back to the correct conformation even in a proper renaturation environment.

The recovery of lysozyme activity in the fed-batch operation was plotted as a function of the final denatured enzyme concentration at different flowrates during the course of the refolding process (Fig. 2). As can be seen in Fig. 2, the data show an optimum curve for all flowrates. In addition, the percentage of lysozyme recovery at all flowrates decreased as the final concentration of denatured lysozyme exceeded the approximate quantity 0.04 mg/mL, implying that the system was governed by two different mechanisms before and after 0.04 mg/mL of the final lysozyme concentration.

Effect of the urea concentration

To investigate how different urea concentrations in the refolding buffer affected lysozyme renaturation in the fed-batch mode, we performed the refolding experiments by varying the urea concentrations (0, 1.5, 2 M). Our data (Fig. 1) indicated that, regardless of the feeding flowrate of denatured lysozyme, the recovery of enzyme activity decreased as the urea concentration increased in the refolding buffer. It is believed that a trace amount of urea not only prevented the formation of aggregated species, but also retarded the refolding of denatured lysozyme (Matsubara *et al.*, 1993). Our results showed that the addition of urea contributed more to the destruction of refolding than to the formation of aggregates at lower concentrations of lysozyme.

Refolding of denatured lysozyme at higher concentrations via fed-batch method

Results from the previously mentioned experi-

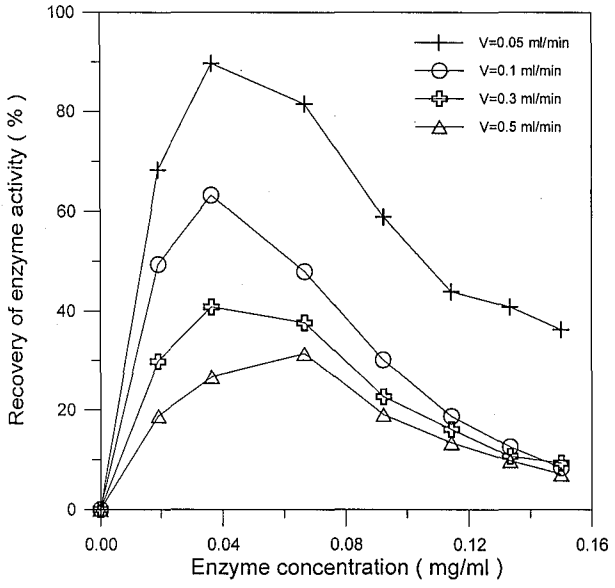

Fig 2. The relationship between the activity recovery of enzyme and final enzyme concentration when the fed-batch refolding method was used. The recovery of lysozyme activity was determined as a function of the final lysozyme concentration at different feeding flowrates (0.05, 0.1, 0.3, 0.5 mL/min). The data were reported as the percentage of the total lysozyme activity recovered after the refolding step relative to the enzyme activity before denaturation. The denatured lysozyme sample solution at 0.5 mg/mL was fed into the refolding buffer solution (pH 8.2, 0.1 M Tris-HCl, 1 mM EDTA, 3 mM GSH, and 0.3 mM GSSG).

ments indicated that the highest refolding efficiency occurred at the lowest feeding rate (0.05 mL/min). Therefore, we examined the effect of higher initial concentrations of denatured enzyme on the refolding performance at this specific flowrate.

Effect of the urea concentration and initial denatured lysozyme concentration

As shown in Table 1, the performance of recovery was elevated at lower initial concentrations of denatured lysozyme. For example, the recovered activity of the denatured lysozyme solutions at concentrations ranging from 0.5 mg/mL to 10 mg/mL, dropped from 90% (75% with 1.5 M urea) to 6% (20% with 1.5 M urea) about 1 hour after the initiation of refolding. Moreover, the optimum enzyme recovery was observed at a lysozyme concentration of 0.5 mg/mL in the absence of urea.

After 1 hour of the fed-batch mode refolding process, we incubated the denatured lysozyme sample solutions for another 24 hours and continued to monitor the recovered enzyme activity. As indicated in Table 1, an initial lysozyme concentration of 5 mg/mL (the percentage of active protein recovered was 50%) was not as efficient as 0.5 mg/mL (the percentage of active protein recovered was 75%) within 60 min of refolding. However, the level of activity recovered at 5 mg/mL (the percentage of activity recovery was 102%) became higher than that at 0.5 mg/mL (the percentage of activity recovery was 94%), implying that concentration-dependent aggre gation could be prevented by the fed-batch refolding operation.

Table 1. Effect of the urea concentration in the refolding buffer and the initial concentration of denatured enzyme on the refolding efficiency via fed-batch method. The recovery of lysozyme activity was determined as a function of time at different initial lysozyme concentrations (0.5, 5, 10 mg/mL) or at different urea concentrations (0, 1.5 M). The data were reported as the percentage of the total lysozyme activity recovered after the refolding step relative to the enzyme activity before denaturation. The denatured lysozyme sample solution was fed into the refolding buffer solution (pH 8.2, 0.1 M Tris-HCl, 1 mM EDTA, 3 mM GSH, and 0.3 mM GSSG) without (0 M) or with urea (1.5 M) at a feeding flowrate of 0.05 mL/min. After the renaturation step continued for 60 min, activity measurement of the refolding mixture was conducted immediately or after 24 h of undisturbed incubation.

Concentration Applied (mg/mL)	Operating Time (min)	Final Concentration (mg/mL)	Total Protein Applied (mg)	Recovery of Enzyme Activity (%)		Active Protein Recovered (mg)	
				0 M Urea	1.5 M Urea	0 M Urea	1.5 M Urea
0.5	60 (without incubation)	0.045	1.5	90	75	1.35	1.12
	60 (with 24 h of incubation)				94		1.41
5	60 (without incubation)	0.45	15	20	50	3	7.5
	60 (with 24 h of incubation)				102		15
10	60 (without incubation)	0.90	30	6	20	1.8	6
	60 (with 24 h of incubation)				7		2.1

It has been reported in the literature that the presence of a trace amount of urea helps increase the recovery efficiency by reducing the amount of aggregates that form (Katoh, 1999). Our data also indicated that, under higher initial concentrations of denatured lysozyme, low concentrations of urea in the refolding buffer significantly prevented the formation of aggregated species and further enhanced the recovery performance. However, the opposite trend was observed for lower feeding concentrations of denatured lysozyme. A conclusion that can be drawn from this and our previous findings is that the addition of a trace amount of urea favors the enhancement of refolding efficiency under higher initial concentration of denatured lysozyme.

Refolding of denatured lysozyme at lower concentrations via step-addition method

The major disadvantage of utilizing the fed-batch method is that the formation of aggregates takes place before correct protein folding can be completed. Evidence has indicated that, with a lower volume requirement of refolding buffer, a higher final enzyme concentration and enzyme recovery can be obtained by using the step-addition strategy, thus overcoming the aggregation problem often encountered with the fed-batch method.

Effect of the injection volume of denatured enzyme and the urea concentration

Figure 3 shows, via the step-addition method, the renaturation behavior of denatured lysozyme solutions under different concentrations of urea in the refolding buffer and different injection volumes of denatured lysozyme. As can be seen in the figure, about 10 min after the first injection, the recovered activity reached a maximum of approximately 160% at an injection volume of 100 μL. Regardless of the amount of injected liquid (or the actual concentration of the denatured lysozyme), the recovery of lysozyme activity reached a plateau at 60%-80% after 20 min of operation, which represented a greater yield than that obtained with the fed-batch mode operation at higher flowrates (~40%). Our results suggest that the evidence that the sufficient time of low concentration protein folding, not the denatured enzyme concentration, is the contributing factor to the enhancement of enzyme activity. Meanwhile, similar to the fed-batch operation results, in the case of step-addition operation at lower lysozyme concentrations, the recovery of activity decreased as the concentration of urea in the refolding buffer increased.

Effect of the time interval between injections

It has been shown that completely denatured lysozyme has the capability of refolding back to its intermediate species with one or two disulfide bonds within 2 ms (Dobson *et al.*, 1994). In addition, it takes 2 s for denatured lysozyme to fold back to its native state with a stable tertiary structure. To understand the mechanism and/or effect from the aspect of time, we investigated the effect of the injection interval on the refolding performance of lysozyme with the step-addition method.

The change in activity recovery resulted from the variation of the injection interval along with the injection volume was examined in this study. When 100 μL of denatured lysozyme was injected, there was no significant difference in the activity recovered for 10 and 20 min injection intervals (Table 2). However, the activity recovered was compromised with shorter injection intervals. It was also noted that 3 min between sequent injections was not long enough for denatured protein molecules in the solution to refold appropriately. When the injection volume was increased from 100 to 200 μL a drop in the refolding performance was perceived, possibly due

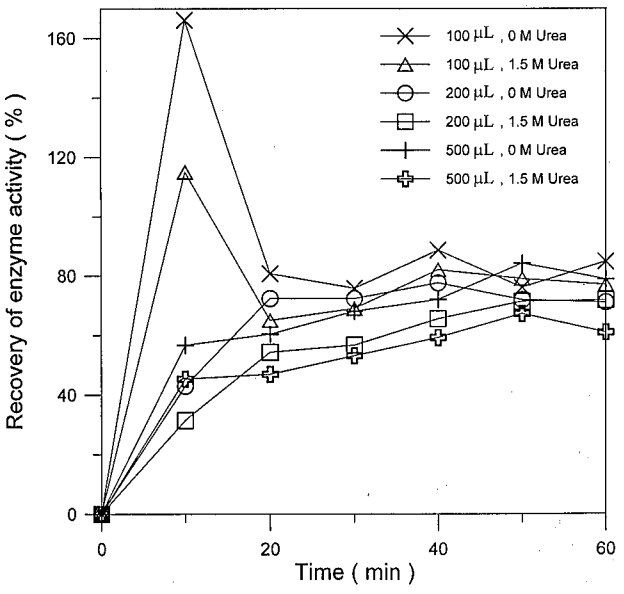

Fig 3. Effect of the injection volume of denatured enzyme and the urea concentration in the refolding buffer on the refolding efficiency via step-addition method. The recovery of lysozyme activity was determined as a function of time at different injection volumes of denatured lysozyme (100, 200, and 500 μL) or at different urea concentrations (0 and 1.5 M). The data were reported as the percentage of the total lysozyme activity recovered after the refolding step relative to the enzyme activity before denaturation. The denatured lysozyme sample solution at 0.5 mg/mL was stepwise injected into the refolding buffer solution (pH 8.2, 0.1 M Tris-HCl, 1 mM EDTA, 3 mM GSH, and 0.3 mM GSSG) without (0 M) or with urea (1.5 M) every 10 min.

Table 2. Summary of the effects of the time interval, injection volume, and initial lysozyme concentration on the performance of the refolding process when the step-addition method was used. A volume of 100 or 200 μL of denatured lysozyme solution were stepwise injected, with time intervals of 3, 5, 10, or 20 min between injections, into the refolding buffer (pH 8.2, 0.1 M Tris-HCl, 1 mM EDTA, 3 mM GSH, and 0.3 mM GSSG) with (1.5 M) or without urea (0 M). After the renaturation step continued for 60 min, the refolding mixture was left undisturbed and all the measurements were carried out 24 h later.

Concentration Applied (mg/mL)	Volume of Each Addition (μL)	Interval Time (min)	Total Protein Applied (mg)	Recovery of Enzyme Activity (%), 60 min		Activity Recovery (%) after 24 h incubation	
				0 M urea	1.5 M urea	0 M urea	1.5 M urea
0.5	100	3	1.05	62.3		70.2	
		5	0.65	66.5		73.3	
		10	0.35	84.2		80.0	
		20	0.2	91.2		88.6	
	200	5	1.3	72.2		92.9	
		10	0.7	71.5		94.9	
		20	0.4	102.0		105.8	
5	100	5	6.5		79.5		101.2
		10	3.5	72.0	85.0	82.4	104.3
10	100	10	7	42.0	56.0	54.6	84.9
	200	10	14	55.0	73.6	60.3	93.5

to the increased number of injected denatured enzyme molecules indirectly interfering with the interactions between the lysozyme molecules in the solution. A summary of results obtained at low initial enzyme concentrations is listed in Table 2.

Refolding of denatured lysozyme at higher concentrations via step-addition method

As mentioned earlier, the step-addition operation achieves higher recovery of activity by allowing adequate time for correct folding. In addition to maximizing the percentage of recovered activity, we also focused on enhancing the quantity of recovered active enzyme by increasing the initial concentration of denatured lysozyme via the step-addition method for protein refolding.

Effect of the injection volume of denatured enzyme and the urea concentration

The yield of lysozyme renaturation with the step-addition method was monitored as a function of the operating time period for the refolding process at higher initial concentrations of denatured enzyme. As can be seen in Table 2, the higher the initial lysozyme concentration, the lower the recovered activity, indicating the effect of the initial concentration on the refolding performance. Our data suggest that the initial concentration is an important factor influencing the recovery process even though its effect is significantly reduced in the case of the step-addition method. Again, the addition of urea had a positive effect on activity recovery as shown in Table 2.

We also examined the combined effect of the injection volume of denatured lysozyme and the urea concentration on the recovery of activity under a higher initial lysozyme concentration (10 mg/mL) (Table 2).

After the refolding process via the step-addition protocol proceeded for one hour, the denatured lysozyme sample solutions were allowed to incubate for another 24 h before the recovery of activity was determined. As detailed in Table 2, the level of activity recovered at a concentration of 5 mg/mL was 104%, which was still higher than the 85% recovery achieved at a concentration of 10 mg/mL. Thus, the conclusion can be drawn that at higher initial lysozyme concentrations, concentration-dependent aggregation is not completely hindered by applying the step-addition approach to protein refolding.

Effect of the time interval between consecutive injections

Using 5 and 10 min as reference time intervals between 100 μL injections, we examined the recovery of denatured lysozyme starting at a concentration of 5 mg/mL. We found that the recoveries at 5 and 10 min intervals were about 60% and 80%, respectively, approximately 40 min after the initiation of refolding (data not shown). We hypothesize that this discrepancy can be attributed to the effect of concentrtion-dependent aggregation. After 24 h of incubation following the refolding process, regardless of the time interval used, almost 100% of the denatured lysozyme molecules have their activity restored. The same holds true for either operating condition (Table 2). Moreover, we found that the 5-min injection interval resulted in a higher total amount of recovered enzyme (Table 2).

Comparisons between refolding processes in fed-batch and step-addition modes

We further compared the results of the refolding processes obtained using the fed-batch and step-addition strategies under the same experimental conditions (an identical initial concentration of denatured lysozyme and the same refolding buffer system). The summary of the results of the three important indices, that is the activity recovery, productivity, and total amount of active protein recovered, under both modes of operation is given in Tables 3(b) and 3(c).

As shown in the tables, based on an identical final enzyme concentration (0.45 or 0.90 mg/mL), the duration of operation required was shorter for the fed-batch mode of refolding when the initial lysozyme concentration was 5 or 10 mg/mL. Our results showed that the step-addition method was superior to the fed-batch method with respect to the activity recovery and the total amount of active protein recovered (Table 3(b)). However, the level of productivity achieved using the step-addition method was relatively lower due to its longer operating time (Table 3(b)).

Refolding of denatured enzyme via impulse feeding operation

With the aims of maximizing recovered activity and enhancing productivity, we took the advantages from both fed-batch and step-addition modes of

Table 3. A comparison between fed-batch and step-addition modes of refolding: (a) the experimental conditions; (b) fed-batch and step-addition methods with an initial lysozyme concentration of 5 mg/mL were compared on the basis of identical final lysozyme concentration of 0.45 mg/mL; (c) fed-batch and step-addition methods with an initial lysozyme concentration of 10 mg/mL were compared on the basis of identical final lysozyme concentrations of 0.90 mg/mL. The denatured lysozyme was added into the vessel containing refolding buffer with the components and compositions stated in (a). The feeding flowrate used with the fed-batch method was 0.05 mL/min, while the injection volume and time interval between injections were 100 μL and 5 min, respectively. After the renaturation step via fed-batch and step-addition methods, activity measurement of refolding mixture was performed immediately or after 24 h of undisturbed incubation.

(a)

Feeding Concentration (mg/mL)	Refolding Method	Operating Time (min)	Final Concentration (mg/mL)	Refolding Buffer
5	fed-batch	60	0.45	3 mM GSH + 0.3 mM GSSG + 1 mM EDTA + 1.5 M Urea + 0.1 M Tris-HCl (pH 8.2)
	step-addition	145	0.45	
10	fed-batch	60	0.90	
	step-addition	145	0.90	

(b)

Refolding Method	Operating Time (min)	Recovery of Enzyme Activity (%)	Active Protein Recovered (mg)	Productivity (mg/min)
Fed-batch	60 (without incubation)	58.2	8.73	0.145
	60 (with 24 h of incubation)	102	15	0.01
Step-addition	145 (without incubation)	73.5	11.03	0.076
	145 (with 24 h of incubation)	107	15	0.01

(c)

Renatured Method	Operating Time (min)	Recovery of Enzyme Activity (%)	Active Protein Recovered (mg)	Productivity (mg/min)
Fed-batch	60 (without incubation)	12.3	3.69	0.062
	60 (with 24 h of incubation)	7	2.1	0.001
Step-addition	145 (without incubation)	13.4	4.02	0.027
	145 (with 24 h of incubation)	10	3	0.002

refolding and developed a modified renaturation scheme to recover denatured lysozyme. The impulse refolding method, based on the cyclic operation scheme, was carried out by, first, conducting the fed-batch operation for 5 min, then stopping the operation for 5 min, and finally feeding denatured lysozyme solution for another 5 min. A comparison between the results obtained under different feeding flowrates (or velocities) and an identical final enzyme concentration of 0.45 mg/mL is shown in Figs. 4(a) and 4(b). The same level of final lysozyme concentration could be accomplished via the impulse refolding method even at a higher flowrate of feeding relative to the one used in the fed-batch method (Fig. 4(a)). In addition, as shown in Fig. 4(b), using the identical feeding flowrate to aim for the same final concentration of lysozyme recovered, the operation duration required (15 min) with the fed-batch method was shorter than that for the impulse feeding method (30 min). We also measured the recovered activity of denatured lysozyme to explore the performance of both fed-batch and impulse feeding methods (see Fig. 5 and Table 4). Results suggested elevations in the productivity, recovery of enzyme activity, and active protein recovered when the repetitive and impulse methods were applied with the fed-batch and step-addition protocols. Therefore, application of the impulse feeding method proposed in this study, along with the optimal operating conditions, provides a feasible approach to the refolding/renaturation processes.

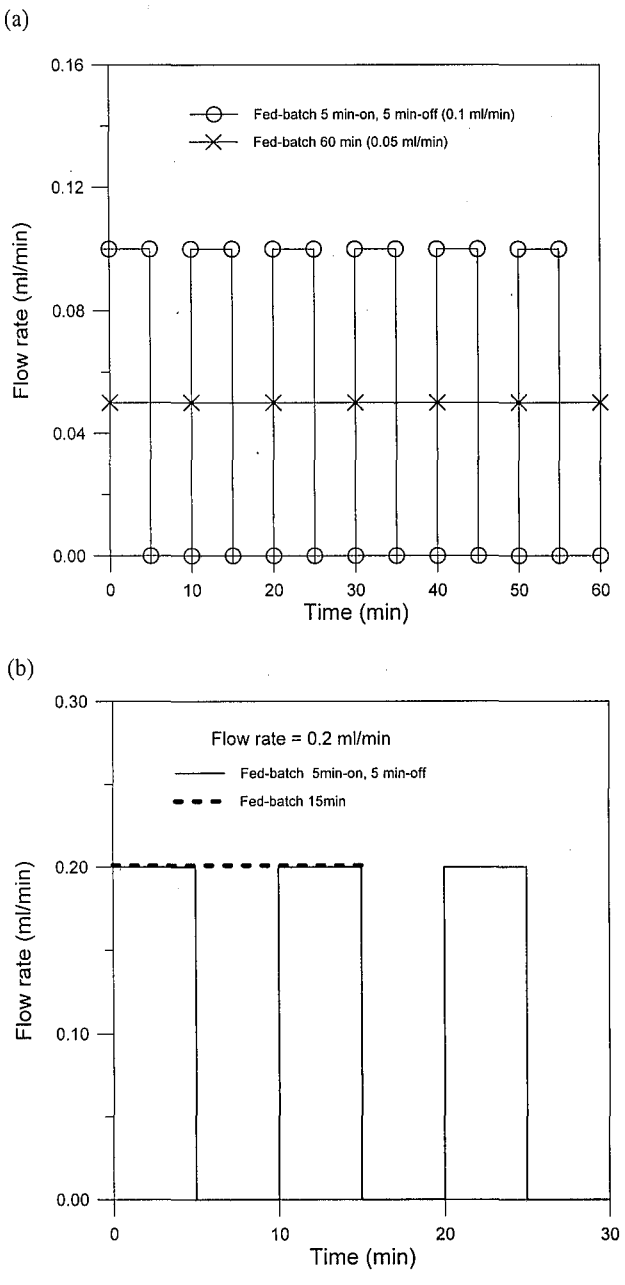

Fig. 4. Feeding velocity profiles of two different operational schemes, fed-batch and impulse feeding methods: (a) same feeding duration; (b) same feeding velocity. The denatured lysozyme sample solution at 5 mg/mL was fed into the refolding buffer solution (pH 8.2, 0.1 M Tris-HCl, 1 mM EDTA, 3 mM GSH, and 0.3 mM GSSG) with a urea concentration of 1.5 M. A comparison was made on the basis of an identical final enzyme concentration at 0.45 mg/mL.

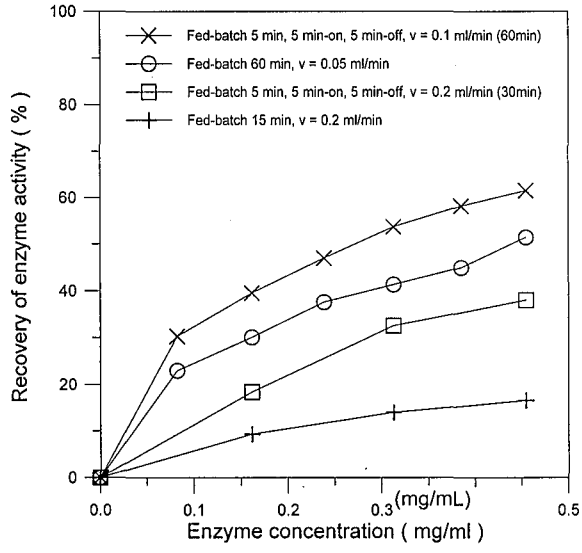

Fig. 5. Comparison between fed-batch and impulse feeding methods. The data were reported as the percentage of the total lysozyme activity recovered after the refolding step relative to the enzyme activity before denaturation. The denatured lysozyme sample solution at 5 mg/mL was fed into the refolding buffer solution (pH 8.2, 0.1 M Tris-HCl, 1 mM EDTA, 3 mM GSH, and 0.3 mM GSSG) with a urea concentration of 1.5 M. The operating conditions, including the feeding flowrate, time interval between injections, and operation time duration, are shown in the figure.

Table 4. A comparison between fed-batch and impulse feeding modes of refolding. Two methods were compared on the basis of an identical final lysozyme concentration (0.45 mg/mL). Denatured lysozyme at 5 mg/mL was added into the vessel containing refolding buffer (pH 8.2, 0.1 M Tris-HCl, 1 mM EDTA, 3 mM GSH, and 0.3 mM GSSG) with urea (1.5 M). The operating conditions are shown in Figs. 4(a) and 4(b). After the renaturation step continued for 60 min, activity measurement of the refolding mixture was conducted immediately or after 24 h of undisturbed incubation.

Refolding Method	Operating Duration (min)	Recovery of Enzyme Activity (%)	Active Protein Recovered (mg)	Productivity (mg/min)
Fed-batch (v = 0.05 mL/min)	60 (without incubation)	51.4	7.71	0.129
	60 (with 24 h of incubation)	96.2	14.43	0.010
Impulse feeding method (v = 0.1 mL/min, 5 min-on, 5 min-off)	60 (without incubation)	61.5	9.22	0.154
	60 (with 24 h of incubation)	98.4	14.76	0.006
Fed-batch (v = 0.2 mL/min)	15 (without incubation)	16.5	2.47	0.165
	15 (with 24 h of incubation)	23.2	3.48	0.002
Impulse feeding method (v = 0.2 mL/min, 5 min-on, 5 min-off)	30 (without incubation)	40.0	6.03	0.201
	30 (with 24 h of incubation)	91.7	13.76	0.009

CONCLUSION

The following conclusions may be drawn from this work:

(1) In the case of refolding processes at higher initial concentrations of denatured lysozyme, a moderate concentration of urea in the refolding buffer is suggested to inhibit aggregate formation due to the retardation of hydrophobic interaction between intermediates. However, in the case of refolding processes with lower feeding concentrations, the presence of urea in the refolding buffer will reduce the collapse rate or disrupt the structure of the refolded protein, resulting in a relatively low refolding yield.

(2) When the feeding rate is controlled, the fed-batch of refolding method has the advantage of high productivity. However, the step-addition method of refolding results in higher recovery of enzyme activity under a longer duration of operation.

(3) The proposed combination scheme (impulse feeding method), which combines the advantages of fed-batch and step-addition methods, results in an additional 10-20% increase in the percentage of activity recovery, in the amount of recovered protein, and in productivity.

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饋料式與間歇式復性法對溶菌杭復性行為之研究

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摘　要

利用基因重組技術生產蛋白質的過程中，蛋白質的再摺疊程序是將不具活性或不可溶解性之聚合體再恢復至原有生物活性的必經過程。直接稀釋法是復性程序中最簡單的方法，但如何提高其復性效率仍在持續研究中。本研究中，我們利用饋料式與間歇式不同的復性方法，以獨立地或結合地方式進行溶菌酶之復性。由實驗結果顯示，饋料式與間歇式復性法相較於稀釋法已可有效地避免聚集體之生成及提高最終的操作濃度。此外，由於間歇式較饋料式提供更充足的摺疊時間間隔，故可獲得較高的活性回收率，而饋料式則具有高產率之優點。最後本研究中，結合饋料式與間歇式復性法之操作策略，可更有效率地提高其復性效率。