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Refolding of Lysozyme at High Concentration in Batch and Fed-batch Operation

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Abstract−Based on optimization of denaturing conditions and the character of time course of protein refolding, renaturation by dilution in batch and fed-batch operation to improve yield as well as the initial and final protein concentrations has been studied. The optimum DTT in denatured solution was 30 mM. Urea can suppress protein aggregation to sustain pathway of correct refolding at high protein concentration. Fed-batch operation was better than batch dilution with comparison of yield recovery in large-scale downstream processes. Under our research condition in fed-batch operation, lysozyme was successfully refolded from initial protein concentration of up to 40 mg/mL by dilution 20-fold, the yield recovery was nearly 60%.

Key words: Protein Refolding, Lysozyme, Disulfide Bond, Dilution, Fed-batch Operation

INTRODUCTION

Recombinant proteins over-expressed in *Escherichia coli*, which is the main host-cell in the protein expression system, are often produced as inactive inclusion bodies. These aggregates have no biological activity and there is a need to refold them into the native structure. Protein refolding from inclusion bodies is a series of operations that involves isolating the inclusion bodies, dissolving them into strong denaturants and recovering the biological activity by the controlled removal of the denaturant or reduction of the concentration of the denaturant. Because protein refolding is a limiting step for the bioprocess industry, it has been a focus for basic research and application [Eliana, 2001].

It is known that the refolding yield decreases with the increasing of the concentration of denatured proteins because of the kinetic competition between the protein aggregation and protein refolding [Batas and Chaudhuri, 1996]. The loss of activity at high protein concentration is strongly linked to aggregates, the main formation of incorrectly folded species, resulting from the denatured protein molecules which have their hydrophobic core exposed to solvent. However, refolding at low protein concentration often leads to the requirement of large refolding reactor and quantities of buffer, also the increasing difficulty in protein recovery. There have been many attempts to improve the efficiency of protein refolding at high concentration [Batas and Chaudhuri, 1996; Müller and Rinas, 1999; Fahey and Chaudhuri, 2000]. The diffusion and partition coefficient of protein are related to the protein concentration, salt and polymer concentration in aqueous solution; recently protein refolding combined with separation of unfolded and refolded protein using aqueous two-phase systems has made progress [Kim and Myerson, 1996; Kuboi et al., 2000]. As a weak chaotropic agent, urea not only may weaken intermolecular interactions of the denatured protein leading to aggregation, but also influence the solubility and the stability of the native, denatured, intermediate states during refolding; thus the use of optimum urea is a very efficient strategy to suppress aggregation [Eliana, 2001; Cardamone et al., 1995; Maeda et al., 1996]. Moreover, the price of urea is very cheap and it is relatively easy to remove when refolding is complete. Protein refolding in a fed-batch mode with addition of optimum urea in renaturation buffer is the effective method and easy to operate, because the portion of the proteins refolded at low concentration contributed to higher overall efficiency in the fed-batch operation [Katoh et al., 1997].

Denatured lysozyme refolding has been a subject of interest for many years; recently lysozyme has been becoming popular for evaluation of new refolding strategies [Rozema and Gellman, 1996]. In this work, based on optimization of denaturing conditions and the character of time course of protein refolding, we will present the results on refolding of denatured lysozyme at high concentration in a fed-batch operation with optimum urea in renaturation buffer as dilution additives.

MATERIALS AND METHODS

1. Materials

Hen egg white lysozyme, dried Micrococcus lysodeikticus cells, reduced glutathione (GSH), oxidized glutathione (GSSG), dithiothreitol (DTT) and Trizma base were purchased from Sigma. A prepackaged gel chromatography column (Hi Trap™ Desalting, Amersham Phamacia Biotech) was employed to recover lysozyme for titration of free SH-residues. Other chemicals were of analytical grade.

2. Preparation of Denatured Lysozyme

Native lysozyme was denatured by incubating in 0.1 M Tris-HCl, pH 8.5 containing 8 M urea and certain DTT for 4 h at room temperature, then the denatured lysozyme was aliquoted into an eppendorf tube and stored at –20 °C as stock solution. Thereafter a series of concentrations of denatured lysozyme could be obtained by diluting the stock solution.

3. Refolding of Lysozyme in Batch Operation

Denatured lysozyme was diluted 10-fold with renaturation buffer (0.1 M Tris-HCl, pH 8.0 containing 1 mM EDTA, 0.15 mM NaCl

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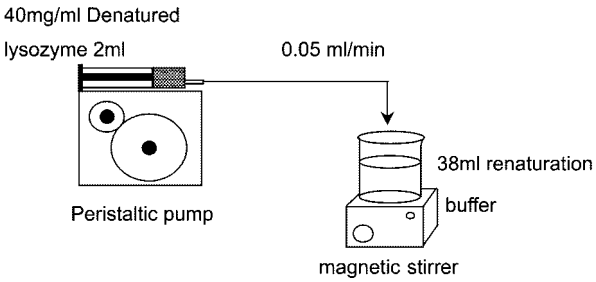

Fig. 1. Schematic show of fed-batch operation.

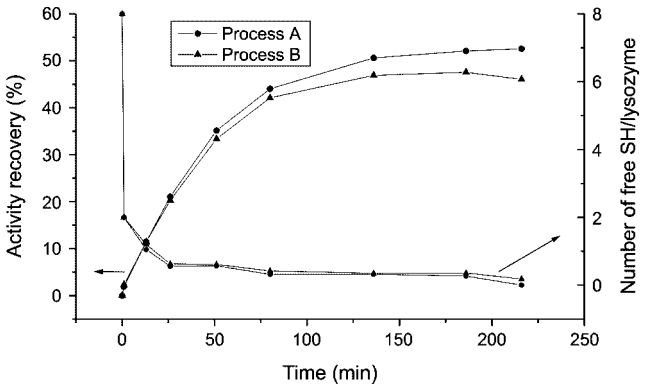

Fig. 2. Influence of DTT in denatured solution on time course of lysozyme refolding by dilution (3 M urea in renaturation buffer, Process A: directly diluted the denatured lysozyme solution; Process B: dilution refolding after removal of DTT).

and a defined urea) by rapidly vortexing 2 min later, the renaturation buffer with GSH and GSSG was added to the solution, the total dilution factor was 20-fold and the final concentrations of GSH and GSSG were 3 mM and 0.3 mM, respectively.

4. Refolding of Lysozyme in Fed-batch Operation

Denatured lysozyme was supplied with peristaltic pump at a flow rate of 0.05 ml/min into 38 ml of the renaturation buffer by gentle stirring. The feed volume was 2 ml at regular intervals; the total fed-feed time was 50 min. The experimental equipment is shown as Fig. 1.

5. Titration of Free SH Residues

The number of free SH residues contained in the denatured lysozyme and refolding sample was determined by the titration according to Ellman's method [Terashima et al., 1996].

6. Enzyme Activity and Protein Assays

Lysozyme activity was measured by the method of F.I.P [Stellmach and Qian, 1992], and protein concentration was determined by a modified Coomassie Brilliant Blue assay [Brandford, 1976].

RESULTS AND DISCUSSION

1. DTT Concentration in Denatured Solution

During protein folding, the formation of disulfide bond was the key step. Using lysozyme as a model protein, we had to break all disulfide bonds to make them fully reduced so that the refolding strategies could be really evaluated. Reducing agent (such as dithiothreitol, dithioerythritol, β-mercaptoethanol, etc.) containing free SH group can possess that effect; usually dithiothreitol (DTT in brief) was put to use. The DTT added into denatured protein solution could provide free SH and change redox potential of the solution to reduce all disulfide bonds of the protein; however, there was little research reported on the influence of DTT concentration in denatured protein solution on breakage of disulfide bond and directly dilution refolding at low dilution factor. One lysozyme molecule contains 4 pairs of disulfide bonds and no free SH; if the numbers of free SH is 8, it means that the lysozyme is fully reduced [Martin et al., 2000]. If denaturing solution did not contain DTT or contained 10 mM DTT, it was shown that the lysozyme was not fully reduced and the lysozyme was not suitable to evaluate the refolding strategies. While the concentration of DTT was 80 mM and 150 mM and the dilution factor was 20, the refolding rate of denatured lysozyme was very slow and the final activity recovery was much lower that of 30 mM DTT, if no removal of DTT before refolding (See Table 1), because an excess of DTT will convert oxidized glutathione to the reduced form due to Eq. (1).

$$\text{DTT (reduced)} + \text{GSSG} \rightarrow \text{DTT (oxdized)} + 2\ \text{GSH} \quad (1)$$

According to Table 1, the optimum concentration of DTT was 30 mM, and at this concentration, the influence of DTT on time course of recovery of enzyme was shown as Fig. 2; there is no evident difference in the activity recovery between Process A and Process B. After dilution by 20-fold, the concentration of DTT existing in denatured lysozyme solution is very low (only 1.5 mM), and at this concentration DTT has little effect on the disulfide bond formation of lysozyme. Under the denatured condition of 30 mM DTT in additive, it is unnecessary to remove DTT in the denatured lysozyme by dialysis or gel filtration, which is costly and time consuming on an industrial scale; this is very helpful for an industrial process to lower cost.

Table 1. Influence of DTT on the free SH of denatured lysozyme and activity recovery (denatured lysozyme concentration 40 mg/ml)

DTT concentration (mM)	Number of free SH/ denatured lysozyme	Enzymatic activity (U/mg)	Refolding directly by 20-fold dilution for 18 h activity recovery (100%)	Refolding directly by 20-fold dilution for 42 h activity recovery(100%)
0	0	2057	/	/
10	4	0	42.5	39.6
30	8	0	46.9	45.7
80	8	0	0	30.2
150	8	0	0	3.9

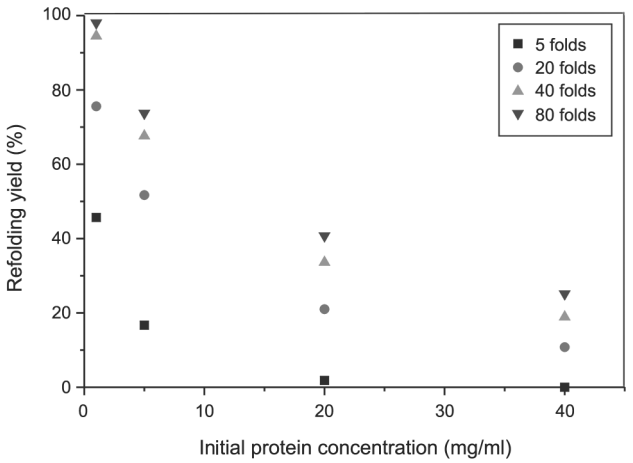

Fig. 3. Effect of initial protein concentration and dilution factor on refolding of lysozyme by dilution (The initial protein concentration was 1 mg/ml, 5 mg/ml, 20 mg/ml and 40 mg/ml respectively, renaturation buffer containing 2 M urea, refolding at 20 ℃ O/N).

Changes in the number of free SH residues in Fig. 2 indicated that three pairs of disulfide bonds out of four were formed immediately after renaturation buffer addition and one pair of disulfide bond was formed gradually. Lysozyme activity was recovered almost simultaneously with the formation of the last one disulfide bond, suggesting that some critical conformation near the active site must be correctly refolded for full enzymatic activity. It is known that disulfide bond contributes to formation and stabilization of protein conformation, so there would exist at least one pair of disulfide bonds to sustain the critical conformation of lysozyme for activity, and the correct formation of that was the limited step for lysozyme refolding process.

2. Refolding of Lysozyme by Batch-dilution

The refolding yield of denatured lysozyme diluted 5-fold, 20-fold, 40-fold and 80-fold with renaturation buffer is illustrated as Fig. 3. Almost full activity can be recovered at the lowest initial protein concentration of 1 mg/ml by diluted 80-fold, which fell to 25% at the initial protein concentration of 40 mg/ml. While the initial protein increased, the refolding yield clearly dropped at the same dilution factor. Dilution factor also has an important impact on the refolding yield. The refolding yield increased with the increasing of the dilution factor. However, the dilution factor was 80-fold, the refolding yield only was 47% and 25% corresponding to initial protein concentration of 20 mg/ml and 40mg/ml. The results show that both the initial protein concentration and dilution factor affected the refolding yield when denatured lysozyme was refolded by dilution. The initial unfolded protein concentration was relatively low at high dilution factor of 80-fold, the probability of molecule collision decreased and incorrect interaction of interchain became weak, as the main cause for aggregation [Eliana, 2001], so the refolding yield was the best. However, if the final protein concentration was too low after dilution, it would be even more difficult to recover the active protein. It should be noted that it is uncommon for dilution factors of 100-fold or greater to be employed to refold inclusion bodies proteins from *Escherichia coli* [Batas and Chaudhuri, 1996]; therefore, it is necessary to improve the recovered protein concentration for dilution refolding at high initial protein concentration and low dilution factor.

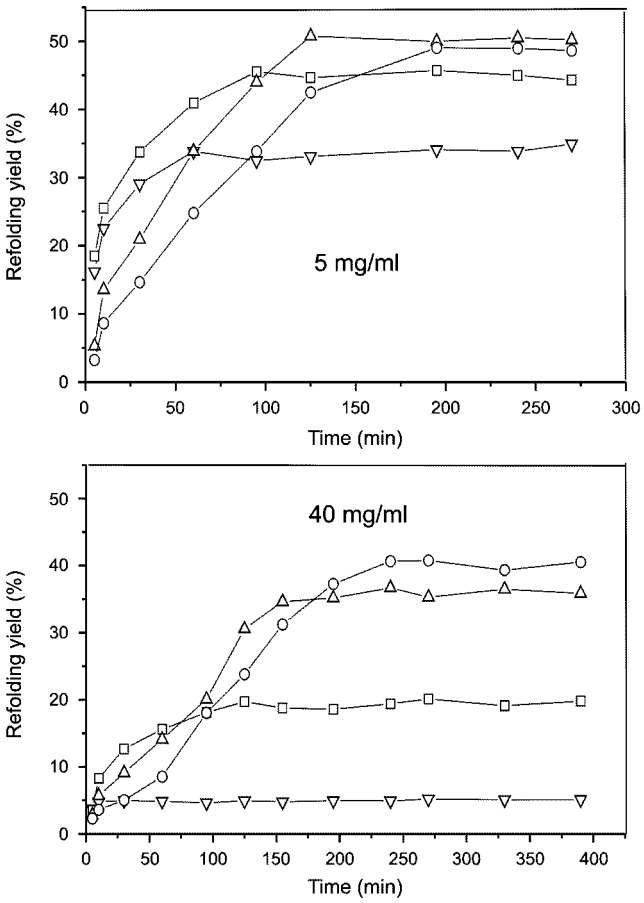

Fig. 4. Effect of urea on time course of lysozyme refolding at low (5 mg/ml) and high (40 mg/ml) initial protein concentration (▽: 1 M Urea, □: 2 M Urea, △: 3 M Urea, ○: 4 M Urea).

Urea has great impact on both yield and rate of the lysozyme refolding (see Fig. 4). In order to obtain high recovery, the optimum urea concentration is increased with the increasing of denatured lysozyme concentration. During refolding, two types of interactions occur: correct intrachain and incorrect interchain interactions--the former leads to protein refolding and the latter results in protein aggregation. Kinetic competition exists between these interactions; the rate of aggregation increases faster than that of refolding if the protein concentration is increased [Batas and Chaudhuri, 1996]. In our research, dilution refolding by lower folds and at higher protein concentration, higher concentration urea was needed to suppress the protein aggregation, accordingly the rate of aggregation decreased faster than that of refolding. This effect can maintain high concentration of unfolded protein to continue to fold; therefore, refolding was promoted and refolding yield was improved.

3. Refolding of Lysozyme in Fed-batch Operation

The refolding yield decreases with the increase of initial protein concentration due to protein aggregation at high concentration during refolding pathway. After addition of native lysozyme in renaturation mixture to increase initial protein concentration, the refolding yield was depicted in Fig. 5. Taking the refolding yield of no

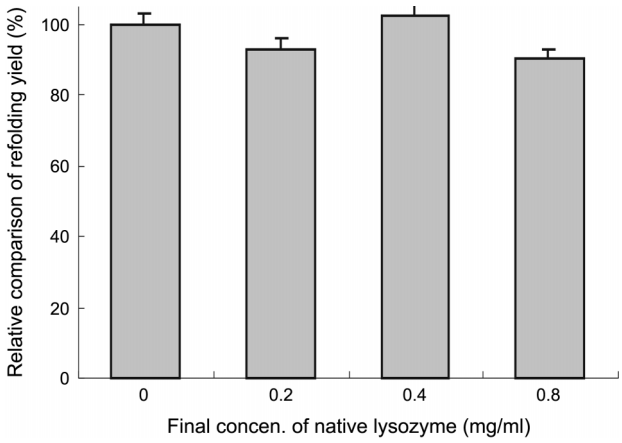

Fig. 5. Relative comparison of refolding yield after addition of native lysozyme in renaturation mixture (Directly diluted 40 mg/ml denatured lysozyme at 20-flod with renaturation buffer, 0.1 M Tris-HCl, pH 8.0 containing 1 mM EDTA, 0.15 mM NaCl, 3 M urea and defined native lysozyme, 20 ºC O/N).

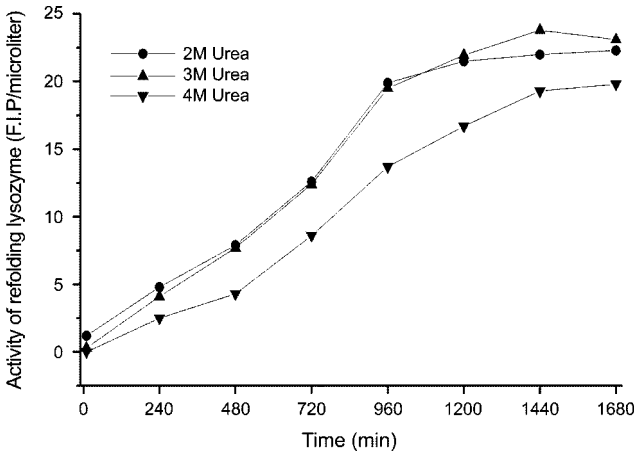

Fig. 6. Effect of urea on time course of recovery of lysozyme activity in fed-batch operation.

addition of native lysozyme as 100%, the relative refolding yield was 93.2%, 102.3% and 90.2% at the addition of native lysozyme to the final concentration of 0.2 mg/ml, 0.4 mg/ml and 0.8 mg/ml, respectively. Even if at the beginning of refolding native lysozyme concentration was as high as 0.8 mg/ml, the refolding yield did not evidently decrease; this showed that lysozyme with correct conformation was not involved in the protein aggregation (the main cause leads to low refolding yield). Because denatured lysozyme refolded gradually and fully refolding lysozyme had little effect on protein coprecipitation, the refolding yield would be improved by addition of denatured lysozyme in fed-batch operation.

According to Fig. 4, the time that the activity of refolding lysozyme became stable was about 4 hours, so in fed-batch operation, denatured lysozyme was added into renaturation buffer for 16 h with 4 steps. The activity of refolding lysozyme gradually increased with addition of denatured lysozyme; however, with the addition of 2 M or 3 M urea in renaturatin buffer, the final activity of refolding was higher than that of addition of 4 M urea, and there was no distinct difference between 2 M urea and 3 M urea (Represented as Fig. 6). The initial concentration of denatured lysozyme decreased in fed-batch operation; correspondingly the amount of urea in renaturatin buffer decreased compared with refolding of 40 mg/ml lysozyme by batch dilution. The time meeting the requirement of recovery of lysozyme activity with 4 steps was about 24 h; in order to shorten the time, 2 steps and 3 steps were also studied. The result showed that there was no obvious increase in activity recovery among 2 steps, 3 steps and 4 steps, but the required time was shortened with 2 steps and 3 steps, i.e., 8 h and 15 h.

CONCLUSIONS

The recovery of bioactive proteins from inclusion bodies is a complex process; above all, optimum conditions should be adopted to solubilize inclusion bodies. Lysozyme is a model protein; the results here illustrated that DTT in the denatured buffer was 30 mM, 40 mg/ml lysozyme was reduced fully; moreover there is no need to remove DTT prior to refolding by dilution at 20-fold. During lysozyme refolding, one pair of dislufide bond in or near the active site gradually formed, and incorrect intrachain interaction leading to protein aggregates took place quickly. Therefore, renaturation buffer additive of urea is a very useful strategy to improve activity recovery and protein concentration while refolding by dilution. However, the optimum concentration of urea varies with the changes of starting protein concentration.

Refolding by batch dilution was easy to operate in laboratory, but there are some difficulties in uniform mixing in large-scale stirred tanks that will cause heterogeneity. This can be solved in fed-batch operation, so the comparison between batch and fed-batch operation was shown in Table 2. Addition of denatured lysozyme in fed-batch operation, although the final concentration of recovered protein was 1.8 mg/ml, the concentration of denatured lysozyme in solution decreased during refolding, so the optimum of urea concentration decreased and the yield recovery improved.

Table 2. Comparison between batch and fed-batch operation during lysozyme refolding

	Batch operation	Fed-batch operation with 2 steps
Refolding time (h)	4	8
Operation requirement to scale-up	Difficulty in uniform mixing and continuous operation, 3-4 M urea	Homogeneous mixing, flexibility to meet various requirements, 2-3 M urea
Denatured protein conc. (mg/ml)	40	40
Recovered protein conc. (mg/ml)	1.5	1.8
Maximum yield recovery (%)	44	59.7

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