Refolding of Denatured/Reduced Lysozyme at High Concentration with Diafiltration

Hidefumi YOSHII,* Takeshi FURUTA, Takahiro YONEHARA, Daisuke ITO, Yu-Yen LINKO,** and Pekka LINKO**

Department of Biotechnology, Tottori University, Tottori 680-8552, Japan
**Department of Chemical Technology, Helsinki University of Technology. FIN-02015, P.O.Box 6100 HUT, (Espoo), Finland

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Refolding of reduced and denatured protein in vitro has been an important issue for both basic research and applied biotechnology. Refolding at low protein concentration requires large volumes of refolding buffer. Among various refolding methods, diafiltration is very useful to control the denaturant and red/ox reagents in a refolding solution. We constructed a refolding procedure of high lysozyme concentration (0.5–10 mg/ml) based on the linear reduction of the urea concentration during diafiltration under oxygen pressure. When the urea concentration in the refolding vessel was decreased from 4 M with a rate of 0.167 M/h, the refolding yields were 85% and 63% at protein concentrations, 5 mg/ml and 10 mg/ml, respectively, after 11 h. This method gave a high productivity of 40.1 μM/h of the refolding lysozyme. The change in refolding yields during the diafiltration could be simulated using the model of Hevehan and Clark.

Key words: diafiltration; protein refolding; lysozyme

Protein refolding from an inclusion body is an important process in recombinant protein production. There have been many reports on suitable refolding conditions. These include dilution with a refolding buffer,1 dialysis against a refolding buffer,3 buffer exchange by gel chromatography,13 and the use of a mixed cellulose solid phase,6 hollow-fiber ultrafiltration,2 and diafiltration.4 In the diafiltration and dialysis methods, the solubilized, denatured inclusion body proteins are diafiltrated against a refolding buffer to reduce the denaturant concentration and to introduce a reductive/oxidation environment. Prevention of aggregation is the important factor to get a higher recovery yield during the refolding.

Clark et al.7 suggested that diafiltration is the more practical membrane-based alternative because the rate of removal of the denaturant is not diffusion limited but they pointed out that accumulation of the denatured protein on the membrane may limit its application. The refolding yield is a strong function of the initial concentration of the denatured protein. Varnerin et al.3 studied gram-scale refolding of Escherichia coli-derived human leptin inclusion bodies by gradually reducing the denaturant level by diafiltration. Maeda et al.9 reported high lysozyme refolding yields with dialysis against a buffer solution of decreasing urea concentration and found that slow dialysis of urea provides higher refolding yields at concentrations as high as 5 mg/ml. The decrease in the refolding yield at a high concentration of reduced lysozyme has been explained by the kinetic competition between refolding and aggregation.3 A quantitative model of the kinetic competition between refolding (first-order reaction) and aggregation (second-order reaction) has been presented by Kiefhaber et al.10 More recently, Hevehan and Clark11 investigated quantitatively the refolding of lysozyme on the basis of the kinetic competition model and demonstrated that the aggregation is best described by third-order kinetics. They estimated the kinetic constants for refolding and aggregation as the function of the concentration of the denaturant such as guanidine chloride or urea.

Renaturation at a low protein concentration requires large volumes of refolding buffer. Most previous research on protein refolding has been limited to a low protein concentration (<0.2 mg/mL), although some attempts1,11,12 have also been made to improve the refolding yields at higher protein levels. We constructed a refolding procedure of high lysozyme concentration (up to 750 μM, 10 mg/ml) by means of a diafiltration method to reduce the urea concentration under oxygen pressure. The oxidation method used oxygen without heavy metals. The refolding yields during the diafiltration were simulated using the model of Hevehan and Clark13. The diafiltration method developed with a linearly con-

* To whom correspondence should be addressed FAX: +81-857-31-5272; E-mail: yoshii@bio.tottori-u.ac.jp
trolled urea concentration gave a high productivity of 40.1 μM/h of the refolding lysozyme.

Materials and Methods

Materials. Hen egg-white lysozyme, six times crystallized, was obtained from Seikagaku Corporation (Tokyo). Micrococcus lysodeikticus cells were from Sigma. A prepacked gel chromatography column (Hi Trap Desalting, Pharmacia Biotech) was used to recover lysozyme for the titration of free SH-residues. All other chemicals were of reagent grade from Wako Pure Chemicals (Tokyo).

Denaturation/reduction. Lysozyme at 10 mg/ml was denatured and reduced in a solution of 8 M urea containing 130 mM 2-mercaptoethanol (2-ME) in 0.1 M Tris-HCl, pH 8.0. The solution was incubated in a tube under nitrogen at 25°C for 15 h to obtain a fully denatured and reduced lysozyme solution. The number of free SH-groups of the unfolded lysozyme in 8 M urea as measured by the method of Terashima et al. was nearly 8 (7.5 to 7.9), indicating complete unfolding. The urea concentration was measured by a refractive index meter (Atago, Kyoto).

Enzyme assay. The lysozyme activity assay was a modification of the method used by Jolles. The Micrococcus lysodeikticus suspension was prepared in 60 mM sodium phosphate buffer, pH 6.2. Ten μl of a protein solution 20 μg/ml concentration was added and mixed well to a 1-ml cuvette (1.0 cm path length).

The reaction was done at 25°C and the decrease in the turbidity was measured at 450 nm in a Shimadzu UV-2200 spectrophotometer. The refolding yield was defined as the relative activity to the native lysozyme.

Refolding procedure with a diafiltration method. Figure 1 shows the diafiltration equipment. The diafiltration vessel, SM16526 (Sartorius, Germany) consisted of a water jacket, a magnetic stirrer and a circular disk type ultrafilter membrane (Polysulphon, MWCO10,000, Sartorius). The filter membrane was immersed in pure water for 1 h to remove glycerin. The water temperature in the jacket was controlled at a desired temperature (4–35°C), usually at 30°C. The denatured and reduced lysozyme (750 μM, 10 mg/ml) was diluted to a desired concentration in 50 ml and put into the vessel, followed by pressurizing at 2 atm by oxygen. The use of oxygen as an electron acceptor is cheaper in reoxidizing disulfides and is able to do slow down the rearrangement of disulfides. The mass of the filtrate was measured by an electric balance (FB-200, A&D, Tokyo) connected via an RS-232C communication line to a computer (NEC, PC-9801V/M, Tokyo). The computer was also used to control the flow rate of the mixture of the renaturation buffer (0.1 M Tris/HCl, pH 8.0, including 1 mM EDTA) and the buffer containing the urea with the HPLC pump (LC-9A, Shimadzu). The presence of EDTA and the slow refolding rate together with the slow removal of urea by dialysis was necessary in order to control protein aggregation and to obtain a high refolding yield at high protein concentrations.

![Fig. 1. Experimental Apparatus.](image-url)
In most earlier work, the rapid refolding rate was related to low protein concentration. This method maintained a constant volume of the solution and controlled the urea concentration in the vessel. One hundred µl of solution in the vessel was sampled regularly to measure lysozyme activity and urea concentration.

To decrease the urea concentration linearly with time, first, the filtration rate was measured at several lysozyme concentrations at a constant urea concentration in the vessel. The filtration rates, F, at different urea concentrations could be approximately correlated with the following linear function (1) of the urea concentration, C,

\[ F = \alpha C + \beta \]  

where \( \alpha \) and \( \beta \) are constant values. The mass balance of the urea in the vessel was given by equation 2, assuming complete mixing,

\[ C_{in} \cdot F - C_{t} \cdot F = V(dC_{t}/dt) \]  

where \( C_{in} \) and \( C_{t} \) are the urea concentrations in the input flow and in the vessel, respectively, and \( V \) is the volume of the solution in the vessel. To decrease the urea concentration linearly with time (i.e., \( C_{t} = C_{0} - at \)), \( C_{in} \) should be controlled as in Eq. 3:

\[ C_{in} = C_{0} - a(t + V/F) \]  

where \( a \) is the urea decrement rate. Substituting Eq. 1 into Eq. 3 gives Eq. 4:

\[ C_{in} = C_{0} - a(t + V/\alpha(C_{0} - at) + \beta) \]  

The input urea concentration, \( C_{in} \), was controlled with a HPLC pump according to Eq. 4. Figure 2 shows a controlled example of the urea decrement rate, 0.52 m/h and the initial urea concentration of 8 m. The urea concentration in the vessel could be well controlled and decreased linearly with time according to Eq. 4.

**Estimation model of Lysozyme refolding.** In vitro folding of monomeric proteins is generally a unimolecular reaction and obeys first order kinetics\(^{19}\). According to Hevehan and Clark the kinetic competition between the refolding and aggregation of lysozyme can be described with the simplified model as shown by the Eqs. 5 and 6:

\[ -dD/dt = k_{1}D + k_{2}U_{0}D^{3} \]  

\[ dY/dt = k_{1}D \]  

where D is the dimensionless concentration of unfolding lysozyme, \( U/U_{0} \) and \( U_{0} \) are the concentrations of the initial unfolding lysozyme, respectively, \( Y \) is the dimensionless concentration of refolding lysozyme divided by \( U_{0} \), \( k_{1} \) is the kinetic constant for refolding and \( k_{2} \) is the kinetic constant for aggregation. The kinetic constants, \( k_{1} \) and \( k_{2} \), could be assumed by the Eqs. 7 and 8 in analogy of Hevehan and Clark\(^{16, 17}\),

\[ k_{1} = \alpha_{1}(1 + C) - 6.34 \]  

\[ k_{2} = \alpha_{2}(1 + C) - 12.56 \]  

where \( C \) is the urea concentration (M). The rate constants of lysozyme refolding were fitted to the equation\(^{10}\); \( \log k = \log k_{0} + (\text{constant}) \times (\text{concentration of denaturant}) \). When the urea concentration in the vessel was controlled as a linear gradient with time, the refolding yield, \( Y \), could be estimated/simulated with the numerical calculation from Eqs. 5 and 6 by changing the parameters \( \alpha_{1} \) and \( \alpha_{2} \).

**Results and Discussion**

**Linear decrease of urea removal**

Figure 3 shows the effects of the control of urea concentration on the refolding process of the denatured and reduced lysozyme of 350 µM (5 mg/ml) at the initial urea concentration of 8 m and 30°C. When the urea concentration was not controlled by feeding additional urea with an HPLC pump, the urea concentration decreased rapidly to about 3 m in about 2 h and then decreased gradually to about 1 m for 7 h. On the other hand, when the rate of the decrease of the urea concentration was controlled at 0.48 m/h or 1.26 m/h, the maximum refolding yield could be increased from about 35% in 4-6 h to about 60% in 7 h and to about 75% in 10 h. These results suggested that the slow removal of urea in the refolding vessel was very effective to obtain a high refolding yields at relatively high protein concentrations.

The effect of the initial urea concentration in the refolding buffer on the refolding yield was also investigated. The refolding yields at 5 mg/ml lysozyme

![Fig. 2. Time Profile of Urea Concentration of the Linear Decre-ment at 0.52 m/h from 8 m, the Initial Urea Concentration.](image-url)
were plotted against time for four initial urea concentrations, 8 M, 6 M, 4 M, and 2 M (the respective urea decrement rate were 0.5, 0.334, 0.167 or 0 m/h), as shown in Fig. 4. These initial refolding profiles were almost identical for the first 3 h, and afterwards the refolding yield increased at the initial urea concentration of 4 M to about 85% in 11 h. This is an about 10% increase in the maximum refolding yield in comparison to that obtained with the initial 8 M urea concentration, and as much as 17% increase in comparison to 6 M urea. The solid lines in Figure 4 represent the calculated results based on the competitive kinetics between the refolding and aggregation. The values of \( \alpha_1 \) for \( k_1 \) and \( \alpha_2 \) for \( k_2 \) used in the calculation were 0.717 (1/s) and 0.0478 (1/s/\( \mu g \times \text{protein} \)), respectively. These parameters were determined with the fitting of the refolding data of the initial urea concentration for 4, 6 and 8 M. The calculated line for the initial urea concentration of 4 M together with the rate of urea removal of 0.167 m/h was in a good agreement with the experimental results, except for the data in the initial five hours. This difference between the calculated values and the results might be attributed to the presence of 2-mercaptoethanol. Other estimated lines show the characteristics of the refolding yield in the latter half of the refolding time. The results show that the course of lysozyme refolding could be satisfactorily estimated with the kinetic model represented by Eqs. 6 and 7, using the modified parameters \( \alpha_1 \) and \( \alpha_2 \).

Figure 5 shows the replots of the refolding yields from Fig. 4 against the urea concentration. The refolding yield began to increase markedly at a urea concentration of about 4 M and reached a maximum when the urea concentration decreased below about 3 M for all refolding conditions used. Batas et al.\(^3\) reported that the percentage of denatured lysozyme gradually decreases as the urea concentration decreases until 4 M. Therefore, the decrease of the urea concentration from 4 M to 2 M could be expected to give a high refolding yield of lysozyme.

**Effect of temperature on refolding yield**

Figure 6 shows the effect of temperature on the time course of the refolding yield at 350 \( \mu \text{M} \) (5 mg/mL) for the urea decrement rate of 0.167 m/h, starting from the initial urea concentration of 4 M. At
Table 1. Comparison of the Refolding Yield, Operation Time, and Productivity in the Various Refolding Methods

<table>
<thead>
<tr>
<th>Refolding method</th>
<th>Lysozyme conc. (µM)</th>
<th>Time (h)</th>
<th>Refolding yield (%)</th>
<th>Productivity (µM/h)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel Filtration</td>
<td>50</td>
<td>2.7</td>
<td>46</td>
<td>8.5</td>
<td>Batas et al., 1995</td>
</tr>
<tr>
<td>Flow-type</td>
<td>84</td>
<td>1.67</td>
<td>44</td>
<td>22.1</td>
<td>Terashima et al., 1995</td>
</tr>
<tr>
<td>Fed-batch Method</td>
<td>139</td>
<td>2.8</td>
<td>56</td>
<td>27.8</td>
<td>Katoh et al., 1997</td>
</tr>
<tr>
<td>Dialysis Method</td>
<td>350</td>
<td>66</td>
<td>82</td>
<td>4.4</td>
<td>Maeda et al., 1995</td>
</tr>
<tr>
<td>Dilution Method</td>
<td>350</td>
<td>24</td>
<td>82</td>
<td>12.0</td>
<td>Hevehan and Clark, 1997</td>
</tr>
<tr>
<td>Difiltration Method</td>
<td>700</td>
<td>11</td>
<td>63</td>
<td>40.1</td>
<td>This work</td>
</tr>
</tbody>
</table>

30°C, the refolding yield was the highest throughout the refolding time. The refolding yields for the initial 2 h indicated almost identical behavior, whereas the refolding yield increased from 2 to 4 h in proportion to the temperature, with the exception of 35°C. In the case of 35°C, the refolding yield increased steadily for about 7 h and then decreased at 10 h. For example, the refolding yield at 4°C increased gradually during the total refolding period, reaching an about 70% at 14 h.

Dependence on Lysozyme concentration

The effect of lysozyme concentration on the refolding yield was investigated with the decrease of the urea concentration from 4 M to 2 M for 12 h (the decrement rate of the urea concentration was 0.167 M/h) at 30°C. Figure 7 shows the time course of the refolding yields for the lysozyme concentration ranging from 35 µM to 700 µM. The refolding yield was strongly dependent on lysozyme concentration. With this method, the maximum refolding yield at a lysozyme concentration of 35 µM was almost 100%. Even at 700 µM lysozyme concentration, the maximum refolding yield of about 63% was obtained. In all lysozyme concentrations investigated, the maximum refolding yield could be reached in about 11 h.

The refolding time and yield in lysozyme refolding with various methods was also compared under conditions of high lysozyme concentration. Table 1 shows the refolding yield at a high lysozyme concentration with various refolding methods, the operation time and the productivity (the production rate per hour). The dialysis method reported by Maeda et al., gave a high refolding yield of 82% at 350 µM, 40°C. However, this method required a long refolding time of about 66 h. Recently, Hevehan and Clark obtained an 82% refolding yield in 24 h at 350 µM with the dilution method with 1.75 M guanidine chloride, 2 mM dithiothreitol, and 5 mM oxidized glutathione in 50 mM Tris buffer at 25°C. The dialfiltration method used in this study gave an 85% refolding yield at 350 µM, and 30°C in only 11 h. In the case of human carbonic anhydrase, Vick and Clark obtained a 70% refolding yield at 0.284 mg/ml in 30 min by dialfiltration and West et al., a 90% activity recovery upon reactivation with the recovery of 55% at 5 mg/ml in about 1 h by hollow-fiber membrane dialysis. The comparison of the productivity for the various methods indicated that our dialfiltration method gave the highest productivity of 40.1 µM/h. In the fed-batch method of Katoh
et al.,\textsuperscript{19} the productivity was only 27.8 \( \mu \text{M/h} \) in spite of the low refolding yield of 56\%. These results suggest that the diafiltration method with the linear-decrement control of the urea concentration in the refolding vessel is an effective refolding method at high lysozyme concentration.

Figure 8 shows the effect of lysozyme concentration on the refolding yield for the linear decrement of urea concentration at 0.167 \( \text{M/h} \) and no control experiments of urea concentration in the diafiltration vessel. In Figure 8, the calculated solid lines of the maximum refolding yield were also plotted for the initial urea concentrations, 5, 4 and 3 \( \text{M} \). When the initial urea concentration increased from 3 \( \text{M} \) to 5 \( \text{M} \), the calculated time required to reach the maximum refolding yield increased from 5 h to 9 h. These results suggested that the controlling of the denaturant concentration in the vessel is very important and the controlling method proposed in this study is useful to obtain a high refolding yield at high concentration of refolding protein. In this study, the oxidation of the reduced protein was carried out under oxygen. However, the optimum method for oxidation should be further studied. We are currently investigating such reagents as the oxidized glutathione and cystine in this context.

**Conclusion**

In the diafiltration method presented, the denaturant urea concentration in the refolding vessel could be easily controlled with a HPLC pump, and the flux of the denaturant could be measured by the weight balance. The linear removal of urea in the refolding solution resulted in an 85% refolding yield at 350 \( \mu \text{M} \) lysozyme, 30°C in 11 h, with a productivity of 40.1 \( \mu \text{M/h} \). It suggests that this diafiltration method is very efficient in refolding proteins at high concentrations.

**References**


