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Refolding of an unstable lysozyme by gradient removal of a solubilizer and gradient addition of a stabilizer

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Keisuke Kohyama, Toshihiko Matsumoto and Taiji Imoto*

Faculty of Biotechnology and Life Science, Sojo University, Ikeda 4-22-1, Kumamoto 860-0082, Japan

*Taiji Imoto, Faculty of Biotechnology and Life Science, Sojo University, Ikeda 422-1, Kumamoto 860-0082, Japan, Tel: +81-96-326-3934, Fax: +81-96-323-1330, E-mail: imoto@bio.sojo-u.ac.jp

Earlier, we formally established an effective refolding procedure for a protein by gradient removal of a solubilizer such as urea [Maeda *et al.* (1995) Effective renaturation of reduced lysozyme by gentle removal of urea. *Protein Eng*. 8, 201–205]. However, this procedure was less effective for unstable proteins. We developed here an excellent method to add protein stabilizer so as to get reasonable amounts of folded protein under the concentration of solubilizer where the unstable protein does not form aggregate. We examined many stabilizers and found that 60% of a concentrated (2.5 mg/ml) unstable protein can be refolded using 40% glycerol as the best stabilizer. This procedure can be widely applicable for the refolding of unstable proteins.

Keywords: dialysis/refolding/solubilizer/stabilizer/unstable protein.

Abbreviations: 3SS lysozyme, unstable lysozyme derivative that has reduced and carboxymethylated one SS bridge (Cys6–Cys127).

The development of protein engineering methods enabled researchers to produce many proteins in reasonable quantities. However, the proteins are produced as denatured proteins in many cases and we have to refold these proteins effectively to study and utilize them. For this purpose, we previously developed an effective refolding procedure by gradient removal of a solubilizer (*1*). The point of our gradient dilution method is to let the protein pass the best denaturant concentration where the protein can fold but still keep sufficient denaturant present to prevent aggregation of denatured protein. In this way, we can refold high concentration of protein (5 mg/ml) effectively (more than 80% yield). We found using immunoglobulin that this method is especially effective for the refolding of oligomeric proteins that require high protein concentrations for the refolding to form stable oligomeric structure (*2*).

While this procedure was very effective for stable proteins, it was less effective for unstable proteins. In the case of stable protein, the folding starts in a high concentration of solubilizer where the aggregation of denatured proteins is still suppressed. On the other hand, in the case of unstable protein, this concentration becomes considerably low and the aggregation of denatured protein becomes dominant. In this article, we show that the concentration of solubilizer where the folding of unstable protein is initiated is increased by addition of protein stabilizers.

As an unstable protein, we employed destabilized hen egg-white lysozyme (3SS lysozyme). 3SS lysozyme was prepared by reducing the outmost disulfide bridge (Cys6–Cys127) and carboxymethylating the produced SHs (*3*). This derivative is less stable than the native lysozyme by 7.2 kcal/mol (*4*) and is a good protein to examine the refolding of an unstable protein in comparison to the native lysozyme.

Materials and methods

Materials

Recrystallized (five times) hen egg-white lysozyme was the product of Seikagaku Kogyo Co. Tokyo, Japan. Oxidized glutathione, dithiothreitol, iodoacetate, sucrose, ammonium sulfate, L-arginine mono hydrochloride and ribonuclease A (bovine pancleas) were purchased from Nacalai Tesque, Kyoto, Japan. Guanidine hydrochloride, Tris, urea, 2-mercaptoethanol, glycerin, sarcosine, and *Micrococcus luteus* were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. All other chemicals used were of the highest quality commercially available. CM-Tyopearl 650M was obtained from TOSOH Co. Ltd, Japan.

Equipment

A Spectrophotometer U-2900 equipped with a thermal cell-holder (Hitachi High-Technologies Co.) was employed.

Preparation of 3SS lysozyme

3SS lysozyme was prepared following the method of Radford *et al.* (*3*) with slight modifications. Hen egg-white lysozyme (200 mg) was dissolved in 10 ml of 0.1 M Tris-HCl buffer (pH 7.8) and incubated for 7 min at 20°C. Dithiothreitol (7.7 mg: final 5 mM) was then added and mixed thoroughly. The mixture was incubated for 70 min at 20°C. To this reduced solution was added 1 ml of iodoacetate solution (0.25 mmol: 5 mol equivalents of dithiothreitol) and the carboxymethylation reaction was performed for 60 min at 20°C. Iodoacetate solution was prepared by dissolving 51.1 mg of iodoacetic acid in 1.1 ml of 0.25 M NaOH. The reaction mixture was centrifuged ($7000 \times g$, 30 min) and the supernatant was subjected to chromatography using CM-Toyopearl.

Lytic activity

Eleven concentrations (from $A_{280} = 0$ to 0.1) of enzyme solution in 0.05 M phosphate buffer (pH 7.0) were prepared. To the *Micrococcus luteus* suspension preincubated at 40°C ($A_{350} = \sim 1$ in the same buffer above, 2.5 ml), 100 µl of the enzyme solution was added and the decrease of A_{350} was determined after 10 min at 40°C. The decreases of A_{350} were plotted against enzyme concentrations and the tangent of the straight line portion was compared with that of the native lysozyme.

Measurement of thermal stability

Protein solution (3 ml, $A_{280} = \sim 1$) in 0.1 M acetate buffer (pH 3.8) was prepared. UV spectra from 320 to 240 nm were measured at

temperatures from 20 to 80°C in 5°C divisions. The temperature was controlled with thermal cell-holder checking cell temperature. The spectra were converted to difference spectra after comparison with the spectrum at 20°C and ΔAs ($A_{291} - A_{300}$) were plotted against temperature.

Reduction and oxidation of protein

Sample protein was dissolved (2.5 mg/ml) in 8 M urea solution (8.13 M urea, 0.59 M Tris buffer pH 8.6, 5.37 mM EDTA) and the protein concentration was determined by A_{280} using a portion of the solution. 10 µl of 2-mercaptoethanol was added to 2 ml of the protein solution under a nitrogen gas atmosphere and the tube was sealed with a glass stopper. The tube was incubated for 1 h at 40°C to reduce disulphide bonds (reduction solution). Oxidized-glutathione (32.4 mg) was added to the reduction solution (redox solution). This redox solution was employed for the refolding procedure.

In the case of refolding using guanidium hydrochloride (GuHCl) as a solubilizer, 5 M GuHCl instead of 8 M urea was employed.

Folding procedure

We basically followed the former refolding procedure (*1*). The redox solution obtained above was placed in dialysis bag and dialyzed against 100 ml (outer solution) of 8 M urea redox solution (8 M urea, 0.1 M Tris–HCl pH 5.0, 3.6 mM 2-mercaptoethanol, 1.3 mM oxidized glutathione, 1 mM EDTA) at 8°C under stirring. To the outer solution, 400 ml of 0 M urea redox solution (stabilizer, 0.1 M Tris–HCl pH 8.0, 3.6 mM 2-mercaptoethanol, 1.3 mM oxidized glutathione, 1 mM EDTA) (solubilizer diluting solution) was added using a tubing pump at the flow rate of 0.1 ml/min. The outer solution was removed to keep the volume of outer solution at 100 ml.

Results and discussion

Preparation of destabilized lysozyme (3SS lysozyme)

Hen egg-white lysozyme (200 mg) was partially reduced at 20°C following the method of Radford *et al.* (*3*). Reexamination of the reduction time revealed that reduction for 70 min gave the best 3SS yield. The reduced protein was carboxymethylated and centrifuged. The supernatant was applied to a column of CM-Toyopearl equilibrated with 0.1 M phosphate buffer (pH 7.0) and eluted by a linear gradient of NaCl from 0 to 0.5 M in the same buffer (Fig. 1). The last peak to elute was the native lysozyme that was most basic and the elution volume accorded with that of standard native lysozyme. The second peak was estimated to be that of 3SS lysozyme having two carboxymethylated SH groups. Since this protein was quantitatively eluted at the same position under the same chromatographic conditions, we recognized that this protein was a lysozyme derivative containing no free SH groups. The yield of this protein was ~30 mg (15%).

After the protein recovered in second peak was desalted and lyophilized, it was determined to be a 3SS lysozyme. Its lytic activity was 35% of native lysozyme. The 3SS lysozyme is devoid of one SS bond out of four and is less stable than the native enzyme. Moreover, it has two extra carboxymethyl groups. Thus, it is reasonable that it is less active than native lysozyme. Previously, it was reported that the lytic activity (58%) was greater than that of our product (*3*). This result might be due to different conditions used for lytic activity measurement. We examined the heat stability of our product (Fig. 2) and it showed almost the same stability as was reported previously for 3SS lysozyme (*3*). Thus, we are confident that the preparation used in this study is 3SS lysozyme. It is less stable than native lysozyme and we have previously reported that 3SS lysozyme is less stable by 7.2 kcal/mol than native lysozyme (*4*).

Refolding equipment, method to add stabilizers, and refolding yield

The equipment for refolding by a gradient dilution of solubilizer and the method of refolding were basically the same as the former ones (*1*). We employed the equipment shown in Fig. 3. A protein was dissolved in a buffer containing solubilizer and reduced. After adding oxidant, the redox solution was placed into a dialysis bag and dialyzed against redox solution containing solubilizer (bottle B). Then, a redox solution without solubilizer in bottle A (400 ml) was gradually (0.1 ml/min) pumped in bottle B (100 ml). Protein concentration was 5 mg/ml in the former trials (*1*). It was kept in 5 mg/2 ml in these trials because around 30% of the volume of the inner tube solution was decreased by the presence of additives leading to a decrease in the folding yield.

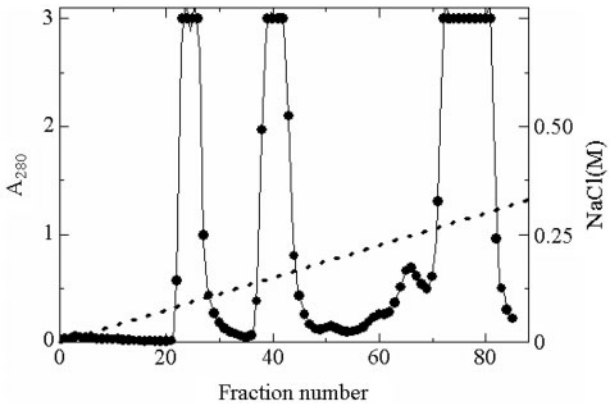

Fig. 1 CM-Toyopearl chromatography of partially reduced and carboxymethylated lysozyme. The sample was eluted from the column (1.7 × 42 cm) by 0.1 M sodium phosphate buffer (pH 7.0) with NaCl linear gradient from 0 (200 ml) to 0.5 M (200 ml). 3SS lysozyme was eluted as the second peak.

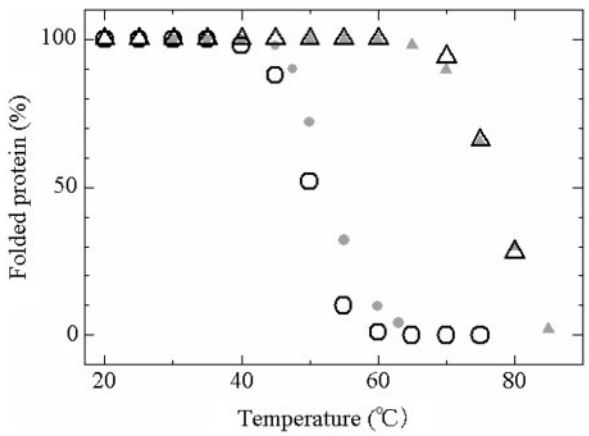

Fig. 2 Thermal unfolding of 3SS lysozyme (circle) and native lysozyme (triangle). The data were normalized after correcting the base line. Gray points are those redrawn from reference (*3*).

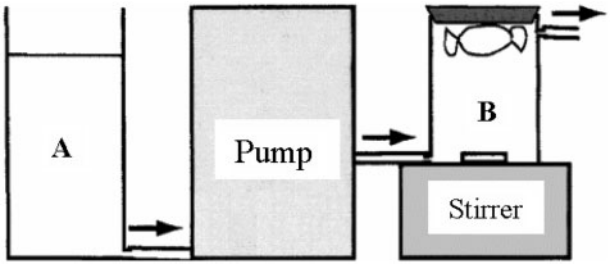

Fig. 3 Systematic renaturation device. Redrawn from reference (*1*). The sample in dialyzing tube was placed in the dialyzing bottle (B). The sample was dialyzed against 100 ml of dialyzing buffer containing solubilizer (8 M urea or 5 M GuHCl) with stirring. The dilution buffer (400 ml) containing additives in bottle A was added to the dialyzing bottle at a flow rate of 0.1 ml/min by a tubing pump at 8°C.

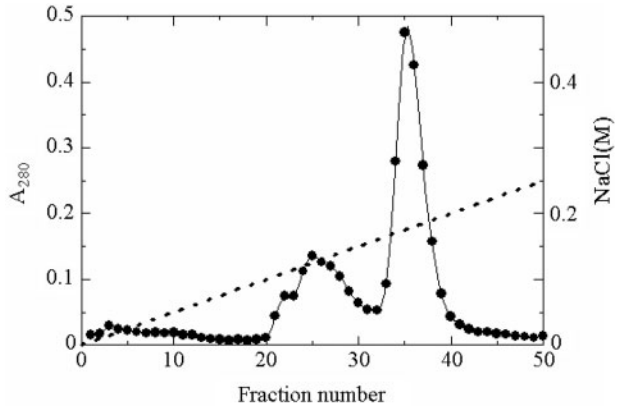

Fig. 4 CM-Toyopearl chromatography of 3SS lysozyme renatured by gentle removal of urea in the presence of glycerol (45%). Refolded protein was eluted from the column (1.2 × 25 cm) by 0.1 M sodium phosphate (pH 7.0) with a NaCl linear gradient from 0 (200 ml) to 0.5 M (200 ml). The last peak was the correctly refolded protein.

Since it is difficult to add a high concentration of stabilizer in the presence of high concentrations of solubilizer, the additives were added to the solubilizer diluting solution (bottle A). As a result, the concentration of additives increased as the concentration of the solubilizer decreased. The change in the concentration of additive depending on the flow rate was described in our previous paper (*1*).

The yield of refolded protein was determined from the elution peak area of the supernatant of dialyzed solution after ion-exchange chromatography (Fig. 4). The last peak occurred at the elution position of authentic 3SS lysozyme. In several refolding trials, specific activities were checked for the proteins eluted in the last peaks. Full activities were obtained in all cases tested. The standard deviation of the refolding yields (3SS-urea-no additive) was ~3%.

The effects of stabilizers

When 8 M urea was employed as a solubilizer, the refolding yield of 3SS lysozyme was 30% in the absence of additives. Under the same conditions, the refolding yield of native lysozyme was about 80%. This means that the refolding yield of 3SS

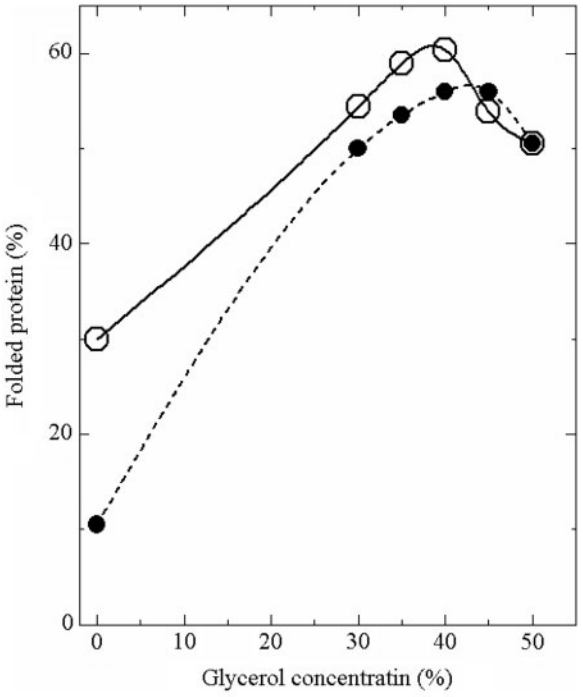

Fig. 5 Refolding yield of 3SS lysozyme renatured by gentle removal of urea (open circle) or GuHCl (closed circle) in the presence of glycerol. Starting solubilizer concentrations were 8 M for urea and 5 M for GuHCl. It was reported that even native lysozyme completely denatured in 5 M GuHCl (*9*).

lysozyme is substantially decreased owing to its instability and that this protein is a suitable model protein to be employed here.

Glycerol stabilizes protein by destabilizing denatured proteins and is widely employed as a stabilizer of proteins (*5–8*). In the first place, glycerol was employed as an additive and 8 M urea as a solubilizer. We examined the refolding of 3SS lysozyme at several glycerol concentrations (Fig. 5) and obtained a refolding yield of 60% at a 40% glycerol concentration. We also examined the effects of glycerol when employing 5 M GuHCl as a solubilizer (Fig. 5). In this case, the refolding yield in the absence of additives was only 11% and the best refolding yield of 56% was obtained at 45% glycerol. We know urea is the better solubilizer than GuHCl for lysozyme refolding. It is very interesting that refolding yield was best at around 40% glycerol concentration. The actually effective glycerol concentration should be less because glycerol concentration was gradually increased. We are now investigating on these interesting points.

The results for other potential stabilizers are shown in Table 1. We have reported that sucrose is an excellent polyol stabilizer devoid of reducing power (*10*). However, it produced only a 40% refolding yield. Sarcosine, another protein stabilizer (*8*,*11*), was effective in refolding native lysozyme (*8*). However, sarcosine gave only a 34% refolded yield. Ammonium sulfate (*8*, *12*) gave a very low refolding yield of 11%. This salt is believed to be a stabilizer of protein but also a good salt for protein precipitation. In fact, almost all proteins were precipitated after refolding

Table 1. Refolding yields of 3SS lysozyme renatured by gentle removal of solubilizers in the presence of various stabilizers.

Stabilizer (conc.) [solubilizer]	Refolded yield (%)
None (−) [Urea]	30
None (−) [GuHCl]	11
Glycerol (40%) [Urea]	60
Glycerol (45%) [GuHCl]	56
Sucrose (0.5 M) [Urea]	40
Sucrose (1 M) [Urea]	33
Sarcosine (2 M) [Urea]	30
Sarcosine (4 M) [Urea]	34
Ammonium sulfate (2 M) [Urea][a]	11
Arginine (0.5 M) [Urea]	6
Arginine (0.5 M) + glycerol (40%) [Urea]	39
Arginine (0.5 M) + glycerol (45%) [GuHCl]	22

As solubilizers, 8 M urea [Urea] and 5 M guanidium hydrochloride [GuHCl] were employed.
[a]The dialyzate was redialyzed against deionized water for 1 day to desalt and then applied to the ion exchange column.

Table 2. Refolding yields of native lysozyme and ribonuclease A renatured by gentle removal of solubilizers in the presence of various stabilizers.

Protein: stabilizer (conc.) [solubilizer]	Refolded yield (%)
Native lysozyme: none (−) [Urea]	80
Native lysozyme: Arginine (0.5 M) [Urea]	51
Native lysozyme: Arginine (0.5 M) [GuHCl]	40
Ribonuclease A: none (−) [Urea]	94
Ribonuclease A: arginine (0.5 M) [Urea]	100

As solubilizers, 8 M urea [Urea] and 5 M guanidium hydrochloride [GuHCl] were employed.

procedure using 2M ammonium sulfate and we had to desalt to evaluate folding yield. This precipitation power might hinder the refolding.

The addition of arginine, which has been widely employed to improve the refolding yield (*13*), decreased the refolding yield here. It was also true even in the presence of glycerol. It did not improve the situation when we employed 5 M GuHCl as the solubilizer instead of 8 M urea. We examined the effects on native lysozyme (Table 2). Arginine declined the folding yields both in urea and GuHCl. We further examined the effect on ribonuclease A and found that it did not decline the folding yield. Thus, we concluded that the declining effect of arginine did not arise from the method we employed but from lysozyme itself. The declining effect of arginine seems to be protein-dependent and we are examining the reason further.

Conclusions

To examine the improvement on refolding yield of an unstable protein, 3SS lysozyme was prepared and its character was confirmed. 3SS lysozyme is much more unstable than native lysozyme. Since 3SS lysozyme differed only in stability from native lysozyme, it was a suitable protein to examine the refolding efficiency of this unstable protein when compared with that of native lysozyme.

We developed a method to add stabilizers efficiently so that sufficient amounts of unstable protein are able to fold in concentrations of solubilizer where a denatured unstable protein is still soluble. We employed the refolding equipment that had been utilized previously (Fig. 3). Since the stabilizer was dissolved in the solubilizer diluting solution, the concentration of the stabilizer increased as the concentration of solubilizer decreased. Thus, we could effectively refold rather high concentration (2.5 mg/ml) of unstable protein.

We examined refolding of unstable (3SS) lysozyme employing various potential stabilizers and we obtained a 60% refolding yield when we employed 40% glycerol as the stabilizer and 8 M urea as solubilizer. This has a 2-fold better refolding yield than that in the absence of additives (~30%).

The refolding of unstable proteins is a difficult challenge. By the use of this method, the efficient refolding for unstable proteins can be attained under appropriate conditions.

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Conflict of interest

None declared.

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