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Protein Aggregation and Adsorption upon *In vitro* Refolding of Recombinant *Pseudomonas* Lipase

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Recombinant *Pseudomonas* lipase was used to study protein aggregation and adsorption upon *in vitro* refolding. Protein adsorption as well as aggregation was responsible for major side reactions upon *in vitro* refolding as a function of protein concentration. The optimal range of protein concentration was determined by the relative contribution of protein aggregation and adsorption. Above the optimal range, the yield of active lipase inversely correlated with protein aggregation, showing a competition between folding and aggregation. However, adsorption of protein rather than protein aggregation is thought to contribute as a major side reaction of the refolding process at sub-optimal concentrations at which the formation of aggregates should be more reduced. Protein aggregation was influenced by the amount of guanidine hydrochloride in the refolding solvent. The refolding temperature was a critical factor determining the extent of protein aggregation. The refolding yield was also affected by the dilution fold and dilution mode, which suggests that the refolding process might kinetically compete with the rate of mixing.

The formation of protein aggregates is frequently observed as a major side reaction of protein refolding (4, 5, 11, 12). The yield of native protein on *in vitro* refolding is determined by kinetic competition between the rate of folding and the rate of aggregation. For monomeric proteins, restoration of native conformation is an intramolecular process but protein aggregation is an intermolecular process. Therefore, the rate of protein aggregation exhibits a reaction order equal to more than two and protein concentrations have the most important effect on aggregation (7).

Although the kinetics of the refolding process based on competition between folding and protein aggregation promises that the lower protein concentration would give the better refolding yield, the yield is sometimes optimal within a narrow range of protein concentration and decreases at sub-optimal range at which the susceptibility of protein aggregation would be more reduced (2, 10). Furthermore, most studies about *in vitro* refolding have been focused only on protein aggregation as a side reaction. Undesirable effects, not only with very dilute concentration of protein, but also with concentrated solutions of denatured protein, have not been well elucidated.

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A recombinant lipase from *Pseudomonas fluorescens* SIK W1 which was expressed as an inclusion body in *Escherichia coli* (3) was used to study protein aggregation and adsorption upon *in vitro* refolding. This lipase is monomeric thermostable and stable at wide range of pH (8). This lipase is a good model protein to study side reactions of *in vitro* refolding because it has the property of binding to a hydrophobic interface which may favor susceptibility to protein aggregation and adsorption. We report here that the adsorption of protein into the wall of a container exerts a detrimental influence on the refolding process at a very dilute concentration of proteins, resulting in the reduction of the refolding yield. In addition, we show that overall refolding kinetics are affected by the kinetics of mixing depending on dilution.

MATERIALS AND METHODS

Expression of Lipase

E. coli BL21 containing plasmid pTTY2 was grown overnight in 6 separate flasks containing 100 ml LB medium supplemented with ampicillin (100 μg/ml). These cultures were inoculated to 5 liters of the same medium in a jar fermentor. After growth for 1 h at 37°C, the cells were induced with 0.5 mM isopropyl thiogalactoside (IPTG) and further grown for 3 h.

Preparation of Denatured Protein

The cells in which the *P. fluorescens* lipase were expressed were harvested (24.7 g wet wt) and resuspended in 100 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 5% (v/v) glycerol, 2 mM EDTA, 1 mM dithiothreitol (DTT) and 0.2 M NaCl). The cells were lysed by a combination of lysozyme treatment (9) and further sonication for 1 min. After disrupting the cells, inclusion bodies were separated by centrifugation at $6000 \times g$ for 10 min. The pelleted inclusion bodies were washed 3 times with lysis buffer containing 0.5% (v/v) Triton X-100.

The inclusion bodies were denatured and reduced by 6 M guanidine hydrochloride (GdnHCl) and 0.1 M DTT, and the components not solubilized were removed by centrifugation at $13{,}000 \times g$ for 15 min. The denatured protein adjusted to 3 mg/ml protein concentration under 6 M GdnHCl and 10 mM DTT was used for the refolding experiments.

Purification of *P. fluorescens* Lipase Using FPLC System

Recombinant *P. fluorescens* lipase was purified to homogeneity through FPLC system (Pharmacia LKB Biotechnology, Sweden) using three steps of column chromatography, sequentially Phenyl-Superose (hydrophobic interaction chromatography), Superose 12 (gel filtration) and Mono-Q (ion-exchange chromatography). The refolding of the lipase was performed under optimal conditions determined in this study; 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 10 mM $CaCl_2$ and 0.8 M GdnHCl at 10°C. After centrifugation of the refolded sample in order to remove aggregates, the sample was applied to Phenyl-Superose column chromatography. The buffers for binding and washing in all chromatography procedures contained 50 mM Tris-HCl (pH 7.5), 5 mM DTT and 10 mM $CaCl_2$. The bound lipase was eluted with a linear gradient of 0 to 2.4% Triton X-100 and 0 to 0.8 M NaCl in Phenyl-Superose and Mono-Q chromatography, respectively. Purity of samples during purification was monitored by non-reducing SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Non-reducing SDS-PAGE was performed on 9% gel and the gel was stained with silver nitrate (8).

Refolding Procedure

Refolding of the lipase was carried out by dilution of unfolded lipase to a predetermined protein concentration with refolding solvent containing 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 10 mM $CaCl_2$ and 0.8 M GdnHCl at 10°C, unless otherwise indicated. Refolding yield was measured by assaying lipase activity using the pH-stat method (8) and the formation of aggregates was monitored by turbidity at 340 nm.

Protein Assay

Protein concentration was determined by the dye reagent method, as supplied by Bio-Rad using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Evaluation of Refolding Efficiency

To determine specific activity of native recombinant *P. fluorescens* lipase, we carried out the purification of the lipase as shown in Fig. 1. Specific activity of the pure lipase was 12,918 unit/ mg protein. The purity of inclusion body preparation which was used for *in vitro* refolding was determined as 62.76% (lane 2), when silver-stained gel was scanned by Laserdensitometer (Pharmacia LKB Biotechnology, Sweden). Therefore, the maximum specific activity of the lipase achievable from *in vitro* refolding of the inclusion body preparation was 8,107 unit/ mg protein ($12{,}918 \times 62.76\%$). Refolding yield was calculated on the basis of the specific activity, dividing the specific activity of refolding medium by the maximum achievable specific activity.

Protein Aggregation and Adsorption as a Function of Protein Concentration

When *in vitro* refolding of *Pseudomonas* lipase was carried out with a variation of protein concentrations in two different concentrations of GdnHCl, the yield of native protein was highly dependent on protein concentration as shown in Fig. 2. The yield was optimal within a narrow range of protein concentration. The optimum was influenced by the amount of GdnHCl, 20-50 μg/ml at 0.2 M GdnHCl and 40-100 μg/ml at 0.8 M. The GdnHCl used for refolding experiments (up to 1 M) had no effect on activity of native protein (data not shown). Above the optimal range, higher concentrations of protein led to a greater tendency for protein aggre-

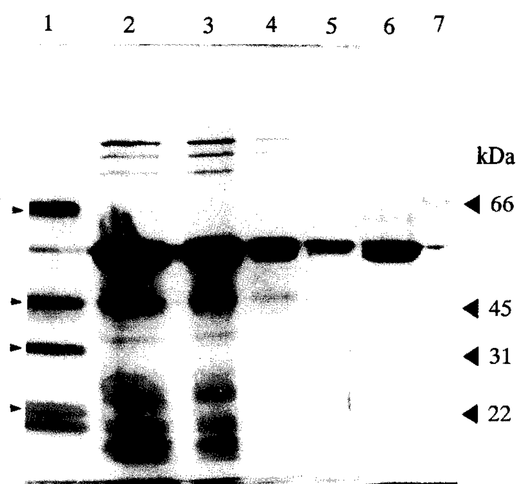

Fig. 1. Analysis of purified lipase by SDS-polyacrylamide gel electrophoresis under non-reducing condition.
Samples during purification was subjected on 9% gel and stained with silver nitrate. lane 1 & 7, molecular weight standards; lane 2, refolded sample; lane 3, Phenyl-Superose elute; lane 4, Superose 12 elute; lane 5, Mono-Q elute; lane 6, another run of purified lipase after Mono-Q.

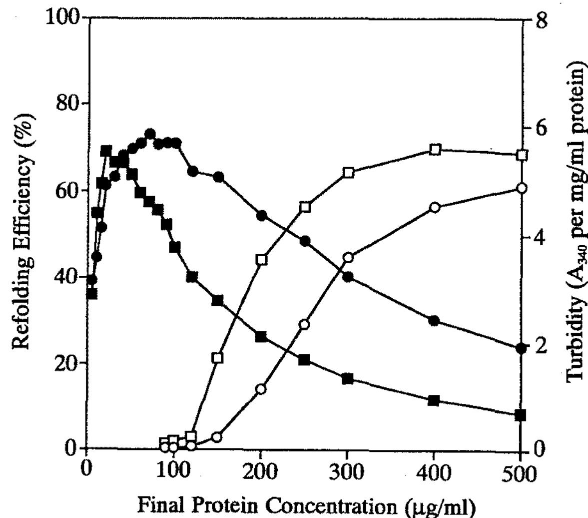

Fig. 2. Refolding and aggregation depending on protein concentration.

Refolding was carried out by rapidly diluting the unfolded lipase with refolding buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 10 mM $CaCl_2$ in the indicated concentrations of protein at 10°C. Refolding was performed at two different final concentrations of GdnHCl (0.2 M and 0.8 M). Total refolding volume was 900 μl in eppendorf tube. Refolding yield was monitored by lipase activity at 15 h after dilution. Lipase activity was assayed by pH-stat method using olive oil emulsion as a substrate. Formation of aggregates was measured by turbidity at 340 nm. Dilution folds (buffer to unfolded lipase) were 6.5 (v/v) and 14 (v/v) under 0.8 and 0.2 M GdnHCl, respectively. Closed symbols, refolding efficiency; open symbols, turbidity at 340 nm; circles, under 0.8 M GdnHCl; squares, under 0.2 M GdnHCl. All data are averages obtained from duplicate experiments.

gation, resulting in a decrease in refolding efficiency concomitant with the increase of turbidity. However, the refolding yield increased in proportion to protein concentration at sub-optimal range, although it was expected, based on kinetic competition between folding and aggregation that lower protein concentrations would further reduce susceptibility to protein aggregation. This result cannot be explained by kinetic competition between folding and protein aggregation convincing us that the lower protein concentration leads to a better recovery of native protein. This suggests that factors other than protein aggregation, such as stability or adsorption of protein might exert an unfavorable influence on the refolding process, especially at very dilute concentrations.

In order to explain how sub-optimal concentrations of protein resulted in the decreased refolding yield, we focused on stability and adsorption of the lipase at very dilute concentrations. Stability and adsorption of the native form of the lipase was analyzed under the same conditions as used for the *in vitro* refolding experiment. Under assay conditions the enzymatic activity of lipase was linear with the amount of enzyme added. At high protein concentration of 120 μg/ml, specific activity was

Table 1. Adsorption and stability of soluble lipase under refolding condition.

Protein concentration	Specific activity (Relative activity, %)	
	15 h	40 h
Control (700 μg/ml)	8,984±101 (100%)	
120 μg/m	8,585±282 (95.6%)	8,649±201 (96.3%)
60 μg/m	7,587±213 (84.5%)	7,640±162 (85.0%)
24 μg/m	6,110± 96 (68.0%)	6,128±113 (68.2%)
12 μg/m	3,602±452 (40.1%)	3,313±522 (36.9%)

Soluble lipase was prepared as follows; The lipase refolded under optimal condition was concentrated by ultrafiltration and dialyzed in the refolding buffer. Protein concentration of the concentrated lipase was 700 μg/ml and had a specific activity of 8984 unit/mg protein. The lipase was diluted in refolding buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 10 mM $CaCl_2$ and 0.8 M GdnHCl to the predetermined concentrations and incubated at 10°C. The total volume of soluble lipase incubated was 900 μl. Lipase activity was measured after incubation for 15 h and 40 h. All data are expressed as mean ± S.D. from triplicate experiments.

similar to the control in which protein adsorption would be negligible (Table 1). Specific activity decreased in proportion to protein concentration at low protein concentrations. A decrease in specific activity was irrelevant to incubation time and was not caused by inactivation of the protein. The decrease was dependent only on protein concentration. As inactivation of protein should increase with time but the adsorption isotherm of the protein is dependent on the concentration, this result strongly suggests that the decrease in specific activity may be caused by adsorption of protein to the wall of the container. Therefore, the decrease in refolding yield at sub-optimal concentrations of protein at which protein aggregation should be minimized probably resulted from adsorption of the protein to the wall of the container. Unfortunately, however, the protein concentrations studied were too low to confirm this by protein assay. The coating of the container by siliconizing did not overcome the decrease of refolding yield at low protein concentrations, suggesting that the siliconization made the wall of the container more hydrophobic (data not shown).

The effect of adsorption was more prominent at lower concentrations because the portion of proteins adsorbed to the wall of the container was much higher than the proteins in the solution at low concentrations (Fig. 2). At high protein concentrations, most of the protein existed in solution and thus the adsorption was negligible. Lipases have the property of binding to hydrophobic interfaces (1) so that adsorption of their native forms to hydrophobic interfaces such as the wall of a container can be so remarkable. Moreover, most of the refolding intermediates also have hydrophobic residues exposed to solvent that is involved in protein aggregation. The intermediates seem to be susceptible to adsorption as well.

The decrease in refolding yield at very dilute concentrations has been observed in refolding of proteins other than lipase (2, 10). We propose here that adsorption of refolding intermediates may be a general phenomenon of *in vitro* refolding, even though we showed only adsorption of native protein in this study.

Refolding Yield Depending on Refolding Conditions

Fig. 2 shows that the susceptibility to protein aggregation was influenced by the amount of GdnHCl and the aggregation was more susceptible at lower concentrations of GdnHCl. A feature of refolding depending on other refolding conditions was studied at protein concentrations where protein adsorption was negligible and a major side reaction of refolding process was represented by the aggregation. Protein aggregation was very sensitive to refolding temperature (Fig. 3). The aggregation proportionally increased with refolding temperature. The lower temperatures, below 10°C, were essential to suppress protein aggregation. The recovery yield of native lipase was inversely proportional to protein aggregation, clearly showing the competition between folding and aggregation in the *in vitro* refolding process. This result is coincident with the fact that a major driving force in protein aggregation is hydrophobic interaction which is enhanced by an increase in temperature.

The refolding rate of the lipase was dependent on the dilution method as shown in Table 2. At low dilution fold, in which initial concentration of denatured protein should be low, there was no difference in dilution mode in the refolding efficiency. However, the efficiency was decreased at high dilution folds, which was more significant with higher final concentrations. Regarding the dilution mode, the dilution of the buffer to denatured protein was better than that of the reverse. There was a drastic decrease of refolding yield at 120 μg/ml, 50:1 (or 100:1) dilution and unfolded to buffer dilution. We thought that the yield of about 34% might be low limit of refolding yield in this experimental conditions. Therefore, the lower dilution fold, i.e. the lower concentration of denatured protein led to improved refolding efficiency. This result suggests that the refolding process was influenced by the rate of mixing and controlled by the initial concentration of denatured protein and the dilution mode. Also the difference in the refolding yield with dilution mode seemed to suggest that the refolding process might proceed simultaneously with the start of dilution and that the competition between folding and protein aggregation should start prior to the solvent and protein reaching equilibrium.

For monomeric proteins, *in vitro* folding generally takes place within the time scale of seconds to minutes (6). The rate of protein aggregation is observed to be faster than that of folding. The start of dilution forces a denatured protein to instantly proceed into the folding process by changing the surrounding solvent properties. Protein aggregation kinetically competes with folding simultaneously with dilution. Therefore, the time taken for equilibration of a concentrated protein in the solvent is not negligible, compared to the rate of aggregation.

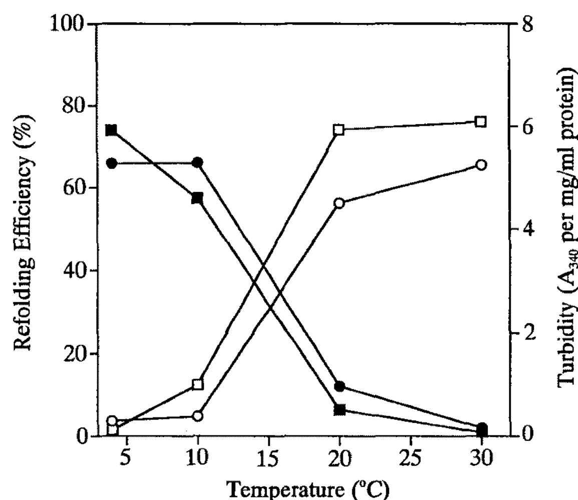

Fig. 3. Effect of temperature on refolding of lipase.
Refolding was carried out at the predetermined temperatures (4 , 10, 20, and 30°C). Two different final protein concentrations, 60 and 120 μg/ml were chosen to study the effect of temperature. Volume ratio of the unfolded lipase to the buffer was 1 to 49 and 1 to 24 under 60 and 120 μg/ml protein concentration, respectively. Total refolding volume was 900 μl in eppendorf tube. The refolding yield and the formation of aggregates were measured by lipase activity and turbidity at 340 nm after incubation of 15 h, respectively. Closed symbols, refolding efficiency; open symbols, turbidity at 340 nm; circles, at 60 μg/ml protein concentration; squares, at 120 μg/ml protein concentration. All data are averages obtained from duplicate experiments.

Table 2. Effect of dilution fold and dilution mode on refolding of lipase.

Final protein concentration	Dilution fold (buffer: unfolded)	Specific activity (Refolding efficiency) unfolded → buffer	buffer → unfolded
60 μg/ml	10:1	5,513±275 (68.0%)	5,659±253 (69.8%)
	50:1	5,355±288 (66.1%)	5,785±263 (71.4%)
	200:1	3,359±222 (41.4%)	4,257±334 (52.5%)
120 μg/ml	10:1	5,711±139 (70.4%)	5,807± 44 (71.6%)
	50:1	2,755±373 (34.0%)	5,106±177 (63.0%)
	100:1	2,800±133 (34.5%)	3,830±268 (47.2%)

Refolding was carried out by rapid dilution of the unfolded lipase in refolding buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM DTT and 10 mM $CaCl_2$ and 0.8 M GdnHCl at 10°C. Rapid dilution was performed by pipetting and immediately several invertings of the container, eppendorf tube. Two different concentrations, 60 and 120 μg/ml were chosen and total refolding volume was 900 μl. Dilution folds represent volume ratio of buffer to the unfolded lipase. Two different dilution orders, unfolded lipase (small volume) to buffer (large volume) and buffer to unfolded lipase were investigated under several dilution folds. All data are expressed as mean ± S.D. from triplicate experiments.

Protein aggregation can be more prominent when the protein destined to fold is localized transiently. This effect will be significant at high concentrations of initial denatured proteins and with inefficient mixing. Thus the dependence of refolding yield on initial protein concentrations of denatured protein and dilution modes seems to reflect competition between folding and aggregation prior to complete mixing.

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