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High Performance in Refolding of *Streptomyces griseus* Trypsin by the Aid of a Mutant of *Streptomyces* Subtilisin Inhibitor Designed as Trypsin Inhibitor

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Refolding of reduced and denatured *Streptomyces griseus* trypsin (SGT) was investigated. In the standard buffer of 50 mM Tris-HCl, the refolding yield of 1 μg/ml of SGT did not exceed 15%. This low yield was assumed to be due mainly to autoproteolysis and/or aggregation occurring concurrently with refolding. On the basis of this assumption, SGT was immobilized on agarose gel in order to suppress such intermolecular interactions, and various refolding media were examined for their ability to minimize misfolding. As a result, 1 M Tris, 1 M diethanolamine, and 1 M triethanolamine were chosen, and their application to the solution system increased the refolding yield considerably, to *ca.* 45%. A further dramatic increase in yield, to 85%, was observed when a mutant *Streptomyces* subtilisin inhibitor (SSI, C71SM73KC101S), engineered as a temporary inhibitor of SGT, was added to the solution system to suppress autoproteolysis during refolding. The application of a temporary inhibitor may be greatly effective in not only improvement of yield but also selection of media for the refolding of protease.

Key words: mutant *Streptomyces* subtilisin inhibitor, protein refolding, refolding media selection, *Streptomyces griseus* trypsin, temporary inhibitor.

Recently, large-scale production of useful proteins has become possible by use of recombinant DNA techniques (*1-3*). In the application of these techniques, the target protein is often accumulated in the host cells as inactive precipitates, so-called inclusion bodies (*4-6*). The refolding or renaturing of the protein molecule from such precipitates into its intrinsic bioactive structure is a significant step in the downstream processes of protein engineering (*7-12*).

The central theme in the refolding procedure is the selection of environmental conditions after solubilization of the precipitates, especially the optimum refolding solution or medium (*13-15*). We previously found that 2 M potassium acetate solution was excellent for the refolding of mature subtilisin BPN′, which had been extremely difficult to refold (*16*). However, restricting improvement of the refolding process to medium selection may lead to misjudgement about whether the denatured protein molecule concerned can be essentially refolded, especially in case of protease. In the case of subtilisin BPN′, vigorous autoproteolysis was found to occur during refolding (*17*). However, if *Streptomyces* subtilisin inhibitor (SSI) was present during the refolding of subtilisin, quantitative renaturation could be achieved by prevention of the autoproteolysis (*16*). Furthermore, application of a digestible mutant SSI engineered specially as a temporary inhibitor allowed the complete recovery of fully active subtilisin BPN′ (*17*). These results indicate that the activity of this enzyme could not be recovered without steps to prevent its self-degradation, although its refolding is essentially possible.

In the present work, *Streptomyces griseus* trypsin (SGT), a subtilisin-like serine protease comprised of a single polypeptide chain, was employed as a globular protein to be refolded. This enzyme (M_r: 23,000) has three S-S bonds (*18-20*), unlike subtilisin, which has no S-S bond. No more than 10% of the activity of SGT can be recovered from its disulfide-reduced form by reoxidation in the standard buffer solution.

Therefore, the renaturation of SGT from its fully reduced form was examined from three aspects: (i) preliminary selection of refolding media by the immobilization method, *i.e.*, the suspension system, developed by the authors (*15, 21-23*); (ii) further selection from among these media for use in the solution system; and (iii) the effect of the presence of a digestible mutant SSI engineered for SGT.

MATERIALS AND METHODS

Enzyme—*S. griseus* trypsin (SGT) was purified from pronase E purchased from Sigma Chemical. The purification method is described later. The concentration of SGT was determined spectrophotometrically by use of the molar extinction coefficient of 39,600 $cm^{-1} \cdot M^{-1}$ (*18*).

Substrate—*N*-α-Benzoyl-DL-arginine-*p*-nitroanilide

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Abbreviations: GdmCl, guanidinium chloride; SGT, *Streptomyces griseus* trypsin; SSI, *Streptomyces* subtilisin inhibitor.

hydrochloride (Wako Pure Chemical Industries) was employed as the substrate (*18*). The substrate stock solution was prepared by dissolving this reagent in dimethylsulfoxide to a concentration of 50 mM.

Mutant SSI—At first, a mutant SSI (M73K) was engineered that would exhibit an inhibitory effect on SGT by replacement of M73 at the reactive site of SSI with Lys, which is susceptible to trypsin. Removal of a disulfide bond near the reactive site of SSI was shown to convert SSI into a temporary inhibitor (*24*). Therefore, the same mutation was introduced into the mutant SSI (M73K), with the expectation that the mutated SSI would be a temporary inhibitor of SGT. Removal of a disulfide bond near the reactive site by replacement of Cys71 and Cys101 of SSI (M73K) with Ser residues was carried out by essentially the same procedures as described for SSI (C71SC101S) (*24*).

Purification of Enzyme—SGT was purified by affinity chromatography with Benzamidine Sepharose 4B (Pharmacia) from the commercially available pronase E. Two milliliters of pronase E solution at *ca.* 25 mg/ml was loaded on the column (1.6×12 cm) packed with the gel described above. The elution rate was 0.5 ml/min. The other details were similar to the literature (*25*).

Enzyme Activity Assay—The activity of native, denatured, or renatured (recovered) enzyme was evaluated from the rate of enzymic hydrolysis of the substrate at 37°C. The assay was carried out by measurement of the change in absorbance at 410 nm caused by release of *p*-nitroaniline (*18*). In practice, 50 μl of the enzyme solution to be assayed was introduced into the mixture of 2.92 ml of 50 mM Tris-HCl buffer (pH 8.2) containing 20 mM $CaCl_2$ and 30 μl of the substrate stock solution in a UV-cuvette. The resulting concentration of SGT and the substrate in the assay solution were 0.73 nM and 500 μM, respectively. Recovered activity was expressed as a percentage relative to the activity exhibited by the equimolar protein concentration of native SGT.

Denaturation Including Reduction of S-S Bond—Denaturation without reduction of any of the S-S bonds in the SGT molecule, *i.e.*, denaturation of intact SGT, was carried out with 6 M guanidinium chloride (GdmCl) at pH 2. On the other hand, denaturation with reduction was started by pre-incubation of intact SGT in 6 M GdmCl at pH 2 for 30 min. The pH of the solution was then shifted to 8.5 with 0.5 M NaOH, dithiothreitol (DTT) was added to a concentration of 4 mM, and the reduction of SGT was allowed to proceed at 37°C for 2 h. The concentration of SGT during the reduction was 0.2 mg/ml (*ca.* 8.7 μM). The reaction was stopped by lowering the pH of the solution to *ca.* 4.5 with 1 M HCl. SGT showed no activity after such treatment.

Renaturation—Renaturation was attempted in various solutions at pH 8.2 containing 3 mM glutathione (GSH), 0.3 mM oxidized glutathione (GSSG), and 20 mM $CaCl_2$ at room temperature. After denaturation with reduction, the SGT solution was diluted 200-fold with various renaturation solutions such as 50 mM Tris-HCl, 1 M Tris-HCl, 1 M potassium chloride, 1 M triethanolamine, *etc.* The resulting SGT concentration during the renaturation was 1 μg/ml (*ca.* 44 nM).

In this work, a specially devised procedure was applied. The digestible mutant SSI was added to the renaturing SGT solution so that a molar ratio of the mutant to SGT was three, the mixture was incubated at room temperature for 24 h, then at 37°C to accelerate digestion of the coupled mutant SSI.

The immobilized SGT used for the preliminary selection of renaturation media was prepared as described previously (*21-23*).

RESULTS AND DISCUSSION

Since completion of the correct refolding should guarantee the recovery of the whole activity, the terms "refold" and "renature" or "recover" are used interchangeably in this article.

Influence of GdmCl Concentration in Assay Solution—As SGT was denatured with 6 M GdmCl, the refolding solution contained 30 mM GdmCl after 200-fold dilution of the denatured SGT. On measurement of the recovered activity of SGT in such refolding solution, some amount of GdmCl was inevitably introduced into the assay solution. The SGT activities exhibited were dependent largely on the concentration of GdmCl contained in the assay solution, as shown in Fig. 1. Similar dependencies were observed and discussed previously in the case of activity measurement of lysozyme (*26, 27*). Proper activity measurement is guaranteed at the GdmCl concentration below 2 mM, as seen in Fig. 1. In the present experiments, the amount of GdmCl introduced into the assay solution from the refolding solution system was reduced below 1 mM.

Preliminary Media Selection—Quantitative refolding cannot be achieved in many cases in spite of attempts to arrange the environment for refolding. In such cases, the failure is due to misfolding and/or aggregation, or degradation by autoproteolysis in the case of protease (*15*). Aggregation and autoproteolysis are caused by the interaction between different protein molecules, while misfolding occurs unimolecularly with respect to the protein molecule. Consequently, in a preliminary survey of optimum refolding media, it is useful to employ protein that is immobilized to agarose gel to exclude the mutual interaction of protein molecules (*23*). By use of such a suspension system, it is possible to evaluate the suitability of media to minimize misfolding and accelerate correct refolding (*21-23*). Use of

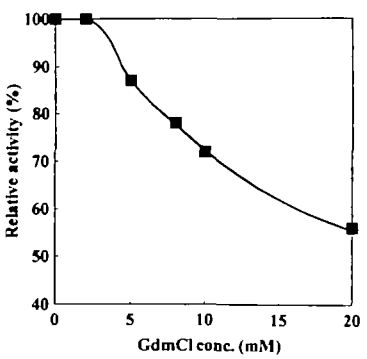

Fig. 1. **Dependence of SGT activity on GdmCl concentration in the assay solution.** The concentrations of SGT and substrate were 0.726 nM and 500 μM, respectively, in 50 mM Tris-HCl buffer (pH 8.2) containing 20 mM $CaCl_2$. The assay was carried out as described in "MATERIALS AND METHODS."

the immobilized SGT revealed that aqueous solutions of 1 M Tris-HCl, 1 M triethanolamine, and 1 M diethanolamine were excellent for refolding of this enzyme, each bringing about a refolding yield of *ca.* 100% relative to the activity present before denaturation. On the other hand, 2 M potassium acetate and 2 M potassium chloride, both of which were very effective for refolding of the immobilized subtilisin (*23*), were found to be ineffective for refolding of the immobilized SGT. Conversely, 1 M Tris-HCl could hardly refold the immobilized subtilisin.

In a separate experiment using the immobilized SGT without reduction of S-S bonds, almost 100% recovery of activity could be attained in 50 mM or 1 M Tris-HCl, 1 M diethanolamine, or 1 M triethanolamine.

Media Selection in the Solution System—The optimum media for use in the solution system without immobilization were chosen from among the media selected by use of the immobilized SGT. Figure 2 shows the time courses of renaturation of S-S intact SGT in 50 mM Tris-HCl buffer and of reduced SGT in 50 mM Tris-HCl buffer, 1 M diethanolamine, and 2 M potassium acetate, the last being an excellent medium for refolding of subtilisin (*16, 17, 21, 22*). In the solution system, the refolding yield of S-S intact SGT did not exceed *ca.* 80% even by use of 50 mM Tris-HCl, which had been found to be effective in the suspension system. It can be seen that the refolding from reduced SGT is much more difficult in the solution system. The recovered activities or the refolding yields of reduced SGT in the various media after 24 h are listed in Table I. The yields did not exceed 15% in 50 mM Tris-HCl buffer or 2 M potassium acetate. Such media as 1 M Tris, 1 M triethanolamine, and 1 M diethanolamine were revealed to be more favorable to the refolding of SGT than inorganic salts or organic acids. However, the reactivation did not exceed 40 and 45% in 1 M triethanolamine and 1 M diethanolamine, respectively, in the solution system. Also, the appropriate refolding media are quite different from those for subtilisin. Although the reason for this difference is still unclear, several facts offer some suggestions. Thus, maximum refolding yield was obtained at pH 6.5 with subtilisin and at pH 8–9 with SGT. The pI value of SGT may be inferred to be around 10.5 from its similarity to bovine trypsin, while that of subtilisin BPN′ is 7.8 (*28*). There is an analogous difference between the optimum refolding pH and the pI value. This does not mean that refolding is dependent only on pH: it may suggest the existence of a suitable reagent species that can refold these enzymes effectively at the respective optimum pH. Autoproteolysis may be also suppressed at high concentration of a reagent that possesses an amino group in the case of SGT and an organic acid group in the case of subtilisin. Consequently, the effect of autoproteolysis on refolding yield may be relieved.

In addition, these results indicates the concurrence of autoproteolysis and/or aggregation even with S-S intact SGT during refolding in the solution system, because complete refolding was achieved with the immobilized SGT without reduction.

Deactivation of Native SGT—To confirm the occurrence of autoproteolysis and/or aggregation in the solution system, deactivation of native SGT in the various solutions used for refolding was investigated. The time courses of

TABLE I. **Effect of various refolding reagents on the renaturation of denatured and reduced SGT.**

Refolding medium		Recovered activity (%)
50 mM Tris	$(CH_2OH)_3CNH_2$	15
1 M Tris	$(CH_2OH)_3CNH_2$	29
1 M monoethanolamine	$(CH_2CH_2OH)NH_2$	25
1 M diethanolamine	$(CH_2CH_2OH)_2NH$	42
1 M triethanolamine	$(CH_2CH_2OH)_3N$	42
2 M diethanolamine	$(CH_2CH_2OH)_2NH$	50
2 M triethanolamine	$(CH_2CH_2OH)_3N$	49
2 M potassium acetate	CH_3COOK	10
2 M sodium acetate	CH_3COONa	0
2 M potassium chloride	KCl	0
2 M lithium chloride	LiCl	0

The denatured and reduced SGT at 0.2 mg/ml was diluted 199-fold with each reagent (pH 8.2) containing 20 mM $CaCl_2$ at 25°C. After 1 min, reoxidation was initiated by addition of GSH and GSSG to initial concentrations of 3 and 0.3 mM, respectively, at 25°C. The final concentration of SGT in the renaturation solution was 1 μg/ml (*ca.* 44 nM).

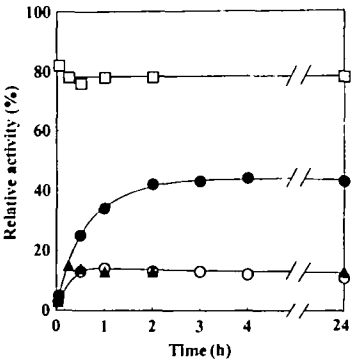

Fig. 2. **Time course of renaturation of SGT.** Squares (□) indicate renaturation of the denatured S-S intact SGT in 50 mM Tris-HCl buffer, while the other symbols indicate renaturation of the denatured and reduced SGT in different refolding media: (●), 1 M diethanolamine; (▲), 50 mM Tris-HCl buffer; (○), 2 M potassium acetate. Denaturation and renaturation procedures with and without reduction and reoxidation are described in detail in "MATERIALS AND METHODS."

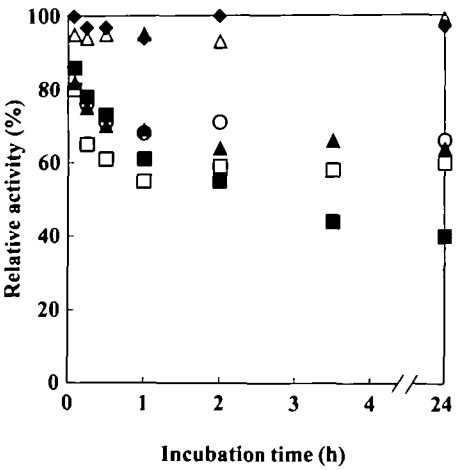

Fig. 3. **Deactivation of SGT in various solutions at pH 8.2 and 4°C.** The stock solution of native SGT (0.25 mg/ml) was diluted with various solutions: (◆), 1 M triethanolamine; (△), 1 M ethanol; (○), 1 M Tris-HCl buffer; (▲), 1 M potassium acetate; (□), 1 M potassium chloride; (■), 50 mM Tris-HCl buffer. SGT concentration was 1 μg/ml.

deactivation of native SGT in the various solutions are shown in Fig. 3. This result gives interesting information on the selection of refolding media. SGT is considerably deactivated with 1 M or 50 mM Tris-HCl, although these solutions were found to be useful for refolding in the preliminary selection. On the other hand, 1 M ethanol, which is disadvantageous for refolding itself, suppresses the deactivation. For minimizing both the misfolding and the deactivation due to autoproteolysis and/or aggregation, the most appropriate of the solutions employed here was 1 M triethanolamine.

Renaturation in Coexistence of Mutant SSI—In the solution system, the deficiency in the recovery is probably attributable mainly to degradation of refolded or refolding molecules by the reactivated molecule of SGT. On the basis of this assumption, an engineered digestible mutant SSI (C71SM73KC101S) was added to the system to temporarily trap the SGT molecules that had recovered the proteolytic activity. Before the mutant SSI was used in the renaturation experiment, the exhibition and elimination of its inhibitory activity were examined. Figure 4 illustrates the features of the temporary inhibition by the mutant SSI of the activity of native SGT at room temperature for one day and subsequent digestion of the mutant SSI by native SGT. To accelerate the digestion, the temperature of the mixed solution was elevated to 37°C after one day. After mixing of the mutant SSI with SGT, the enzymic activity of SGT was suppressed immediately; and after three days, SGT digested all of the mutant SSI and completely recovered its activity. The inhibition constant of the digestible mutant SSI (C71SC101S) toward subtilisin BPN′ was measured as $K_i = 9$ nM, while that of the mutant SSI (C71SM73KC101S) employed here was observed to be 300 nM toward SGT. The inhibitory activity of the mutant SSI adopted for SGT is 33 times lower than that of the mutant previously used with subtilisin (*17*). This explains why the mutant SSI does not appear to cause 100% inhibition in the assay solution in Fig. 4. That is, although the mutant SSI couples with the native or the reactivated SGT molecule sufficiently to exhibit the inhibitory effect in the mixed solution described above or in the refolding solution, part of the mutant SSI coupled with SGT can be dissociated by 60-fold dilution in the assay in the presence of a large excess of the substrate ($K_m = 150$ μM).

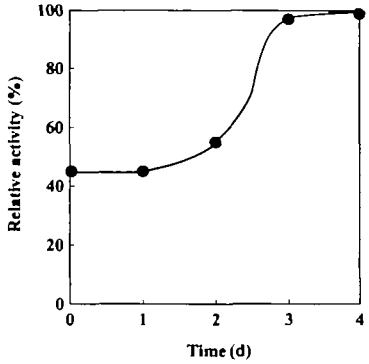

Fig. 4. **Digestion of a mutant SSI by SGT.** SGT at 1 μg/ml (*ca.* 44 nM) was mixed with a 3-fold molar excess of a mutant SSI (C71SM73KC101S) in 1 M diethanolamine (pH 8.2) containing 20 mM $CaCl_2$. The mixture was incubated at room temperature for 1 day, and thereafter at 37°C to accelerate the digestion. Although SGT was completely inhibited by the mutant SSI in the mixed solution, *ca.* 40% of activity was expressed in the assay system because of competitive inhibition in the presence of a large excess of substrate. $K_i = 300$ nM; substrate concentration, 500 μM; $K_m = 150$ μM.

Figure 5, a and b, shows the time course of recovery of activity in the presence of the mutant SSI and other proteins such as wild-type SSI, bovine serum albumin, and hen egg-white lysozyme, when a molar ratio of the mutant or other proteins to SGT was three in the effective refolding solutions such as 1 M triethanolamine or 1 M diethanolamine. Figure 5a shows the features of refolding in the first day at room temperature. Figure 5b shows the subsequent changes over several days at 37°C. The maximum yield in the solution system was increased dramatically to *ca.* 85% in the presence of the mutant. No influence was observed in the presence of the other proteins or in the absence of the mutant. The presence of the mutant SSI thus suppressed the autoproteolysis caused by the reactivated SGT molecules, as intended. The recovered activity did not increase from day 2 through day 5 if the temperature was not raised to 37°C or if the mutant was used in the 100-fold molar excess, probably because digestion of the mutant could not be easily completed. Some deficiency still remains, probably due to autoproteolysis and/or aggregation that could not be completely repressed. In the present work, the formation of aggregates in the denatured SGT solution was not detectable even by spectroscopy. On the other hand, in a separate experiment where 1.6 M calcium acetate was employed as a refolding medium, the recovery of activity

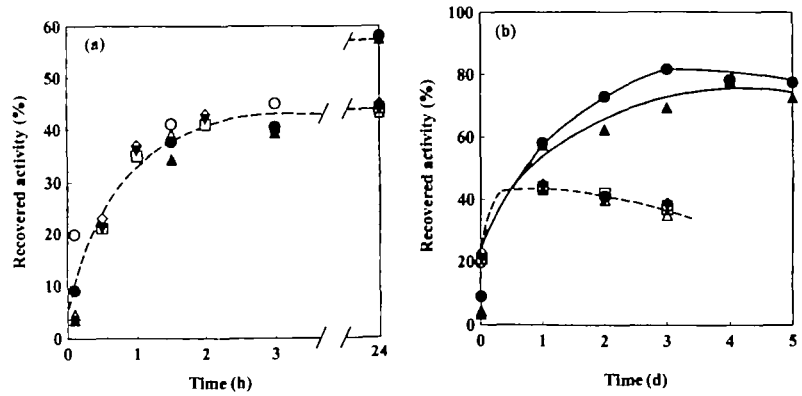

Fig. 5. **Renaturation of denatured and reduced SGT in the presence of mutant SSI (C71SM73KC-101S) in 1 M diethanolamine or 1 M triethanolamine.** Denatured and reduced SGT at 0.2 mg/ml was diluted with 1 M diethanolamine or triethanolamine containing 20 mM $CaCl_2$ with and without 3-fold molar excess of a mutant SSI (C71SM73KC-101S) at pH 8.2. The concentration of SGT in the renaturation solution was 1 μg/ml (*ca.* 44 nM). Incubation time: 1 day at room temperature (a), subsequent 5 days at 37°C (b). Renaturation solution: 1 M diethanolamine (●) or 1 M triethanolamine (▲) with the mutant SSI; 1 M diethanolamine (○) or 1 M triethanolamine (△) without the mutant SSI. Reference renaturation in 1 M diethanolamine in the presence of 3-fold molar excess of proteins other than the mutant SSI: bovine serum albumin (◇); hen egg-white lysozyme (□); wild type SSI (▼).

did not exceed *ca.* 15%, and visible aggregates were formed. Since the reactivated SGT molecule is immediately trapped by the mutant SSI molecule, it cannot degrade other SGT molecules. Therefore, in the presence of the mutant, autoproteolysis of SGT can be suppressed. Consequently, the loss of yield should be attributed exclusively to aggregation caused by the interaction between partially renatured and/or denatured protein molecules during the refolding process. To achieve complete renaturation, further investigation is necessary into a solution that can more effectively minimize misfolding and thereby reduce aggregation.

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