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Renaturation of the mature subtilisin BPN′ immobilized on agarose beads

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Abstract

We report here another example of renaturation of subtilisin BPN′(Sbtl) by using an immobilized preparation instead of applying a digestible mutant of *Streptomyces* subtilisin inhibitor (SSI), a proteinaceous inhibitor of Sbtl [M. Matsubara et al. (1994) FEBS Letters 342, 193–196]. The mature Sbtl was immobilized on agarose beads employing the amino group of the protein. After thorough washing, the immobilized Sbtl was subjected to denaturation in 6 M guanidine hydrochloride (GdnHCl) at pH 2.4 for 4 h, followed by renaturation in 2 M potassium acetate at pH 6.5 for 24 h. This denaturation/renaturation cycle was repeated five times. The recovered activity of the renatured immobilized Sbtl settled at a constant level after the third denaturation/renaturation cycle, demonstrating that almost 100% renaturation was attained by use of the immobilized Sbtl. This immobilized Sbtl preparation could well be utilized for the mechanistic study of protein folding. We then found that 2 M potassium acetate was superior to 2 M potassium chloride as a refolding medium and that the ability of SSI to induce the correct shape of the mature Sbtl was lacking in several refolding media in both thermodynamic and kinetic criteria. Thus the main cause for the increase of refolding yield of Sbtl by coexistence of SSI was prevention of the autolysis of Sbtl.

Key words: Protein folding; Subtilisin BPN′; Autoproteolysis; Immobilization; *Streptomyces* subtilisin inhibitor; Site-directed mutagenesis

1. Introduction

Subtilisin BPN′(Sbtl) is secreted in vivo as a proenzyme with 77-amino acid pro-sequence which is thought to be indispensable for folding[1–5] and is thereafter cleaved away to produce mature Sbtl. The mature Sbtl lacking the pro-sequence has been classified as one of proteins extremely difficult to refold in vitro [2–4]. Ohta et al. attained the in vitro refolding of Sbtl at a yield of about 12% in the presence of synthetic 77-amino acid pro-sequence [6]. Shinde et al. reported that GdnHCl-denatured Sbtl could be renatured with a yield of about 15% in the presence of the pro-sequence whereas the yield did not exceed 5% in the absence of the pro-sequence [7]. They claimed that the pro-sequence should act as an intramolecular chaperone to reduce the energy barrier of the rate-limiting step. Recently, we, Matsubara et al. [8], demonstrated that the refolding medium containing 2 M potassium acetate made it possible to renature the GdnHCl-denatured Sbtl at a yield of about 30% even in the absence of pro-sequence. Furthermore, we found that almost quantitative renaturation could be achieved in the presence of the mutated *Streptomyces* subtilisin inhibitor (SSI) which functioned as a temporary inhibitor for the refolding Sbtl in the medium containing 2 M potassium acetate [9]. These results imply that autolysis during the refolding process had impeded further increase in renaturation yield beyond 30%.

In this work, the refolding of Sbtl was attempted by use of Sbtl immobilized covalently employing its amino group on agarose beads [10]. Immobilized Sbtl retained about 68% activity to that of the wild Sbtl introduced. Denaturation of the immobilized Sbtl in 6 M GdnHCl resulted in a complete inactivity. Application of 2 M potassium acetate to the denatured immobilized Sbtl reactivated it at a level of 78% recovery based on the initial activity of the immobilized Sbtl. Repetition of denaturation/renaturation cycles more than three times brought forth the excellent immobilized Sbtl preparation which operated the denaturation/renaturation cycle almost reversibly. Thus, we demonstrate that we have established the almost quantitatively refolding immobilized Sbtl preparation.

Since concomitant autolysis and/or aggregation of the refolding Sbtl could be excluded by immobilization, this immobilized Sbtl preparation was utilized for the mechanistic study of protein folding, in the present case, to evaluate an intrinsically meaningful refolding medium without disturbance from aggregation or autolysis as well as to elucidate whether or not coexistence of SSI had operated as a shape-inducer for the refolding Sbtl in the previous study [9].

2. Materials and methods

2.1. Materials

Subtilisin BPN′ was purchased from Nagase Biochemicals. The gel employed was CNBr-activated Sepharose 4B from Pharmacia. Synthetic substrate, *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide,

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was from Sigma. Mutated SSI, with the replacement of Cys's 71 and 101 to Ser's (SSIC71SC101S), was expressed in *Streptomyces lividans* and purified as described before [11]. This mutant of SSI is a temporary or digestible inhibitor which is initially potent but is gradually degraded by the protease. All other chemicals were of analytical grades.

2.2. Preparation of immobilized subtilisin

The immobilized Sbtl was prepared by using the method of Svensson [10] with a slight modification as described previously [12]. Sbtl was dissolved in deionized water at a concentration of ca. 0.8 mg/ml. Several volumes ranging 0.06–1.25 ml of the Sbtl solution were added to 30 ml of the gel suspension containing 33% (v/v) gel in 0.1 M Na_2CO_3 buffer containing 0.5 M NaCl(pH 10.0). The mixture was gently agitated for 24 h at 4°C and then filtrated. The immobilized Sbtl gel was thoroughly washed with the buffer described above. The excess NH_2-sensitive CNBr groups remained on the gel surface, were desensitized in 0.1 M Tris-HCl buffer at pH 8.0 for 4 h at 4°C. Again, the gel was washed in succession with 0.1 M $NaHCO_3$ (pH 8.2), 1 M NaCl, 0.1 M sodium acetate buffer (pH 5.4), and deionized water. Finally, the gel was suspended in deionized water and stored at 4°C. The amount of Sbtl immobilized was estimated from the balance of introduced and leached Sbtl. In the present experiments, Any sign of protein leaching was not detected by measurement of optical density at 280 nm in the enriched solution of total filtrates and the washings. Therefore, the gel prepared in such a way immobilized 0.005–0.10 mg of Sbtl per ml of gel.

2.3. Denaturation and renaturation of immobilized subtilisin

Immobilized Sbtl was denatured in 6 M GdnHCl at pH 2.4 for 4 h at 25°C. This denatured Sbtl possessed no enzymatic activity at all. Low pH level was adopted because, in the case of free Sbtl solution, autoproteolysis proceeded to some extent at neutral pH levels even in 6 M GdnHCl solution. The procedure for renaturation of denatured immobilized Sbtl was carried out at pH 6.5 under 4°C or 25°C. These procedures were almost same to those of the preceding paper [9]. In order to measure the rate of refolding, a certain amount of the gel suspension was sampled at the appropriate time interval, filtrated, and washed with 50 mM sodium acetate buffer to remove the salt, and subjected to enzymatic activity assay. In the case of coexistence of SSIC71SC101S, the activities of immobilized Sbtl were measured after the inhibitory activities of bound SSI disappeared completely.

2.4. Activity measurement of the immobilized subtilisin preparation

The enzymatic activity of immobilized Sbtl preparation was measured photometrically by use of *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide as a substrate at 25°C. Detailed methods were described previously [12]. The retained activity of immobilized Sbtl was defined as percentages relative to the activity of the free dissolved Sbtl of the same molar amount. The activity recovered during renaturation of the once completely denatured immobilized Sbtl preparation was represented as a percentage to that of the initial immobilized Sbtl preparation.

In this paper, the term 'renaturation' means 'refolding evaluated by the enzymatic activity'. Structural informations, for example, far CD or NMR measurement cannot be expected in the case of immobilized proteins.

Table 1
Bound amounts and retained activities of immobilized subtilisin on agarose beads

Subtilisin (mg/ml gel)		Retained activity[a] (%)
Added	Bound	
0.10	0.10	50
0.05	0.05	52
0.02	0.02	55
0.01	0.01	68
0.005	0.005	67

[a]The retained activity of the immobilized subtilisin was defined as percentages relative to the activity of the free dissolved subtilisin of the same molar amount.

Table 2
Effect of several salts on the renaturation of subtilisin with and without immobilization

Salt	Concentration (M)	Recovered activity (%)	
		Free dissolved[a]	Immobilized
CH_3COONa	0.05	0.0	0
LiCl	2	0.0	40
KCl	2	7.2	71
CH_3COOK	2	28.3	80

Immobilized subtilisin was denatured in 6 M GdnHCl at pH 2.4 for 4 h at 25°C. Recovered activity was evaluated as the percentage to that of the initial value at pH 6.5 after 24 h incubation at 25°C.
[a]The recovered activities of 6 M GdnHCl-denatured subtilisin dissolved free in the refolding medium were taken from Matsubara et al. [9].

3. Results and discussion

3.1. Preparation of immobilized subtilisin

Table 1 shows results of immobilization of Sbtl on CNBr-activated Sepharose 4B. Each preparation listed in the Table 1 immobilized the whole quantity of protein introduced since no sign of protein leaching was detected at 280 nm in the enriched solution of total filtrates and the washings. The immobilization accompanied a substantial decrease in the enzymatic activity. The retained activity, however, increased with decrease in the concentration of immobilized Sbtl on the gel up to ca. 70%. Such trend in enzyme immobilization has often been noticed elsewhere [13]. Parts of Sbtl might be immobilized nonspecifically, accompanying some deformation at the active site of the protein. We adopted the immobilized subtilisin of 0.01 mg/ml gel in the present study. At this concentration, autoproteolysis is not likely to occur. The surface concentration of protein is estimated to be 14.2 nmol/m^2 or 120 nm^2/molecule, assuming that the agarose particle is spherical with a diameter of 90 μm and packed with a porosity of 50%. Since the Sbtl molecule is approximately spherical with a diameter of about 4.2 nm [14], the intermolecular interaction between the immobilized molecules could scarcely be expected. In fact, no autoproteolytic fragment was detected by HPLC in the filtrate arising from filtration of the gel suspension.

3.2. Renaturation of GdnHCl-denatured subtilisin in the presence of various salts with and without immobilization

Table 2 listed the recovered activities of immobilized and free Sbtl [9] from their GdnHCl-denatured states. Recovered activity was evaluated after 24 h incubation at 25°C in the refolding media containing various salts. In 2 M potassium acetate, the GdnHCl-denatured immo-

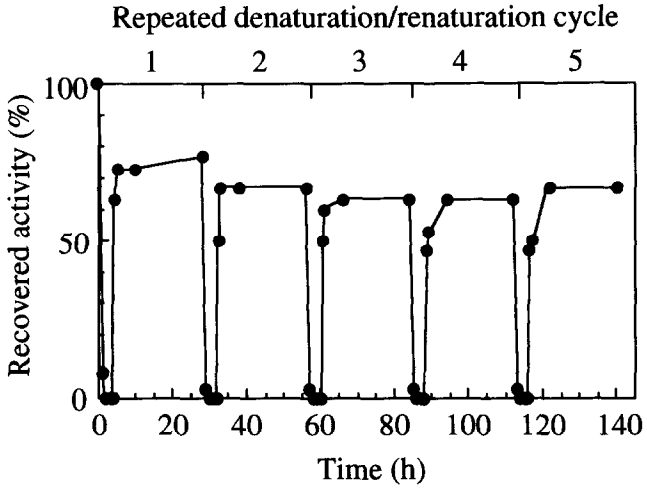

Fig. 1. Repeated denaturation/ renaturation treatments of the immobilized subtilisin. Immobilized subtilisin was denatured in 6 M GdnHCl at pH 2.4 for 4 h at 25°C. Renaturation was carried out in 2 M potassium acetate at pH 6.5 for 24 h at 25°C.

bilized Sbtl renatured at much higher recovered activities (80%) than the free Sbtl (28.3%). In the medium containing 2 M potassium chloride or lithium chloride, the yield with the immobilized Sbtl was 71% or 40%, respectively, in sharp contrast to those obtained with the free Sbtl, i.e. 7.2% or 0.0%. On the other hand, the GdnHCl-denatured immobilized Sbtl was never renatured at all when 50 mM sodium acetate buffer was applied as a refolding medium. From these results, it is concluded that the successful renaturation of Sbtl requires not only the suppression of autoproteolysis, for example by SSI or by immobilization, but also the presence of a certain salts like potassium acetate at high concentrations.

3.3. *Establishment of the quantitatively renaturing immobilized subtilisin preparation*

Fig. 1 shows the time course of recovered activity of the immobilized Sbtl preparation which was subjected to five successive denaturation/renaturation cycles. The renaturation was carried out in 2 M potassium acetate at 25°C for 24 h. The recovered activities were 77%, 67%, 63%, 63% and 67% in the succession of the cycle. Decrease in activities from the initial 100% may be due to an event that the Sbtl molecules immobilized nonspecifically are released from the gel surface during denaturation in 6 M GdnHCl. A remarkable evidence is that the recovered activity attains the level of the preceding cycle after the third cycle and afterward. These results imply that the GdnHCl-denatured immobilized Sbtl can be renatured almost quantitatively in the presence of 2 M potassium acetate due to the prevention of intermolecular interactions between refolding molecules. Thus, immobilized Sbtl preparation was established to operate denaturation/renaturation cycles reversibly. This preparation can be employed as a useful tool for an intrinsic evaluation of the various refolding reagents without concomitant autoproteolysis and/or aggregation.

3.4. *Examination of a possible shape-inducing action of SSI on renaturation of 6 M GdnHCl-denatured subtilisin*

Recently, a quantitative renaturation of free Sbtl dissolved in 2 M potassium acetate was achieved by ourselves in the presence of digestible SSI instead of the pro-sequence [9]. However, it has not been clarified whether or not the SSI played a role of a template to induce the molecular shape of Sbtl and to accelerate the refolding. The immobilized Sbtl preparation is an ingenious tool for examination of the role of SSI during refolding. Thus, the refolding experiments of GdnHCl-denatured immobilized Sbtl preparation were attempted in the presence or absence of SSIC71SC101S, applying 50 mM sodium acetate, 2 M lithium chloride, 2 M potassium chloride, or 2 M potassium acetate as refolding media at 4 and 25°C. Fig. 2 shows the results. Any noticeable effect of addition of SSI on renaturation was not observed for all media examined in both refolding rates and yields. These facts lead to a tentative conclusion that the role of SSI in the previous report [9] was rather a mere inhibitor for the proteolysis activity of free Sbtl to prevent autoproteolysis than an inducer of the shape of Sbtl from its random coil structure.

Fig. 3 illustrates three-dimensional structure of the Sbtl–SSI complex in a stereo image registered in Protein Data Bank [15]. Supposedly, fitting might be taking place between the almost refolded Sbtl and its comple-

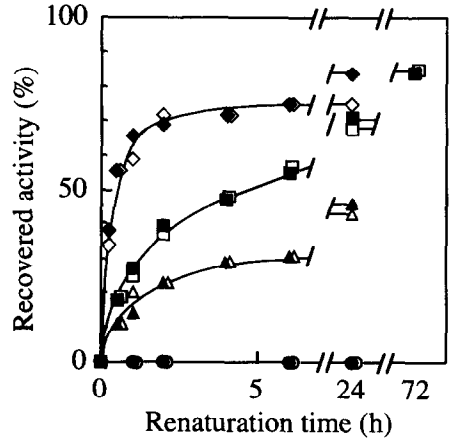

Fig. 2. Renaturation of the denatured immobilized subtilisin preparation in the presence or absence of SSIC71SC101S. Immobilized subtilisin preparation was denatured in 6 M GdnHCl at pH 2.4 for 4 h at 25°C. Renaturation was carried out at pH 6.5 in the medium containing 2 M potassium acetate at 4°C (open square), at 25°C (open diamond), 2 M lithium chloride at 25°C (open triangle), or 50 mM sodium acetate buffer (open circle) at 25°C. Closed symbol indicates the presence and open symbol and open symbol the absence of 0.58 μM SSIC71SC101S each in the renaturation medium.

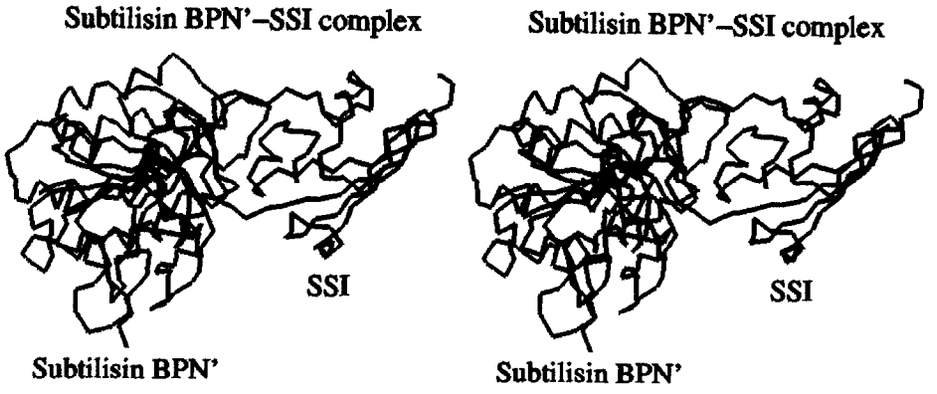

Fig. 3. Three dimensional structure of the subtilisin-SSI complex.

mentary SSI. It lacks assurance, however, to demonstrate that SSI exerted an inducing action on the shape formation of Sbtl from its random coil structure. The present results support an idea that the Sbtl refolds spontaneously by itself and that at a terminal stage of formation of the correct shape it is trapped by SSI mutant and its activity is quenched temporarily to prevent possible rapid digestion of the denatured Sbtl dissolved free in the medium. After all the Sbtl molecules present are tightly refolded and trapped by the SSI mutant, proteolysis of the digestible SSI proceeds and free authentic Sbtl molecules are produced. The rate of the autolysis of the tightly refolded or wild Sbtl is expected to be slow enough compared with that of the digestion of denatured Sbtl by an active Sbtl [16].

A remained problem is concerned with the role of the pro-sequence of Sbtl. It was suggested to be dual, i.e. inhibition of autoproteolysis [6] and induction of subtilisin refolding like chaperone [7]. Kobayashi et al. reported that ionic and hydrophobic regions of pro-sequence might interact complementarily with the counter regions of Sbtl fulfilling the dual task mentioned above [17]. In our experiments, SSI in the previous paper [9] or immobilization in the present work functioned to prevent autoproteolysis, while inorganic or organic salt, at high concentration, especially potassium acetate, effected to accelerate refolding of Sbtl. A detailed study is underway to elucidate the specific action of potassium acetate on refolding. In this respect, the immobilized Sbtl preparation will be helpful to evaluate several potential candidates of refolding medium effective as an artificial chaperone.

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