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Quantitative *in vitro* Renaturation of Subtilisin BPN' without the Aid of Pro-sequence

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We demonstrate for the first time *in vitro* refolding of subtilisin BPN'. The presence of 2 M potassium acetate in renaturation solution was found to recover about 30% of native enzyme activity. Further, supplementation with *Streptomyces* subtilisin inhibitor(SSI) to the refolding medium lead to quantitative refolding of subtilisin as measured by subtilisin-SSI complex formation.

The concept of self-assembly in protein structure formation implies that the three-dimensional structure of a globular protein is uniquely determined by its primary structure and that folding of the polypeptide chain is a spontaneous process.[1] Nevertheless, some proteins can not be renatured *in vitro* once they have been fully denatured. Although they are small bacterial serine proteases, subtilisin and α-lytic protease are two notorious examples difficult to refold *in vitro*.[2] It would therefore be of interest and of importance to accomplish the refolding of any one of these enzymes not only to understand the basic folding mechanism but also to develop novel refolding procedures.

Subtilisin BPN', an alkaline serine protease from *Bacillus amyloliquefaciens*, is produced *in vivo* as a large precursor containing a 30 amino acid pre-sequence, a 77 amino acid pro-sequence, and a 275 amino acid protease domain.[3,4] The pre-sequence is related to secretion, while the pro-sequence is essential for the proper folding of the enzyme.[5] The pro-sequence is proposed to function as an intramolecular chaperone for production of active subtilisin.[6] On the other hand, the *in vitro* restoration of the enzyme activity from the fully denatured subtilisin is indeed a difficult process. Certainly, the *in vitro* refolding of the pro-subtilisin proceeds easily,[7] but subtilisin lacking pro-sequence dose not. Ohta et al. reported that they achieved *in vitro* refolding of subtilisin at around 12% yields by coexistence of a synthetic 77 amino acid pro-peptide.[6] Without the pro-peptide, they never obtained an active subtilisin. Recent investigations by Eder et al.[8] demonstrated that there formed a metastable folding intermediate during the refolding of subtilisin and this state could not be converted to the native state without the aid of the pro-sequence. Therefore, it is a challenging problem to accomplish *in vitro* refolding of this enzyme.

In the present study, we examined a variety of refolding conditions and achieved for the first time the almost complete refolding of the enzyme from its fully denatured state. The presence of potassium acetate and *Streptomyces* subtilisin inhibitor(SSI), a strong inhibitor of bacterial serine proteases,[9] was found essential to achieve the quantitative refolding of subtilisin.

Subtilisin BPN' was purchased from Nagase Biochemicals. Mutated SSI, with the replacement of Met 73 to Asn (SSIM73N), were expressed in *Streptomyces lividans* and purified as described before.[10] Subtilisin BPN'(0.1 mg/ml) was denatured by treatment with 6 M guanidinium chloride(GdnHCl) solution at pH 2.0 for 2 h at room temperature. Refolding was initiated by mixing the denatured enzyme solution at 100 or 50-fold dilution into the refolding solution containing various salts at several concentration levels at resultant pH of 6.5 or 7.5, respectively. After the solutions were incubated at 4°C for various periods, the enzyme activity was assayed by use of the substrate *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide. The refolding yield of subtilisin was given on the basis of enzyme activity as percentages relative to the native subtilisin of an equimolar concentration. The influence of SSI on the refolding was evaluated by analyzing subtilisin-SSI complexes by gel filtration HPLC on a TSK G2000SW$_{XL}$ column (7.8 mm×30 cm), which was eluted by 0.20 M phosphate buffer(pH 7.0) at a flow rate of 0.5 ml/min and was monitored by the absorbance at 280 nm.

It has been generally recognized that subtilisin denatured in 6 M GdnHCl at acidic pH conditions could not be renatured by mere dilution of the denaturant.[5,8] However, here we disclose our finding that the fully denatured subtilisin could to some extent be renatured by dilution of the denaturant and incubation in the presence of high concentrations of some inorganic or organic salts. The effect of several salts examined by ourselves are listed in Table 1. There we found that potassium acetate and sodium acetate are the two most effective reagents among several salts we examined in assisting renaturation of the denatured subtilisin. Figure 1A shows the recovered activity of subtilisin from its fully denatured state against the varying concentrations of potassium chloride or potassium acetate in the refolding media after 24 h incubation. No positive effect of potassium chloride was observed at the concentrations below 1 M, whereas the restoration of the enzyme activity of 7% was observed at the concentrations of 2-3 M. Surprisingly, potassium acetate solution with the concentrations of 2-3 M led to the recovery of activity up to 28% of the native value originally possessed prior to denaturation. Figure 1B shows the time course of recovered activity in the renaturation solution containing 2 M potassium chloride or 2 M potassium acetate. The attainment of the maximum refolding yield required almost 2 h in both systems, and the recovered activities have been retained thereafter for several days long. The difference between potassium acetate and potassium chloride on the recovered activity suggested that the factor to promote the refolding of subtilisin may relate to the certain specific interactions between ions from the salt and the counter ions in the protein; especially the organic anion such as acetate ion can provide a favorable environment for the renaturation of denatured subtilisin.

In the course of these experiments, a low protein concentration, in this case, 1.0 μg/ml in the refolding

Table 1. Efficacy of various salts for renaturation of fully denatured subtilisin

Salt	Recovered activity / %		
	0.5 M	1.0 M	2.0 M
LiCl	0.0	0.0	0.0
NaCl	~0	~0	5.0
KCl	~0	~0	4.2
RbCl	0.0	~0	2.0
CsCl	0.0	0.0	0.0
NH_4Cl	0.0	0.0	—
$N(CH_3)_4Cl$	0.0	0.0	0.0
$MgCl_2$	0.0	0.0	0.0
$CaCl_2$	0.0	0.0	0.0
Na_2SO_4	0.0	0.0	0.0
$(NH_4)_2SO_4$	~0	2.8	—
$MgSO_4$	0.0	0.0	—
AcONa	~0	4.5	15.2
AcOK	~0	5.2	20.1

The concentration of subtilisin in renaturation solution(pH 7.5) was 2.0 μg/ml. The samples were incubated at 4 °C for 24 h and then assayed for subtilisin activity.

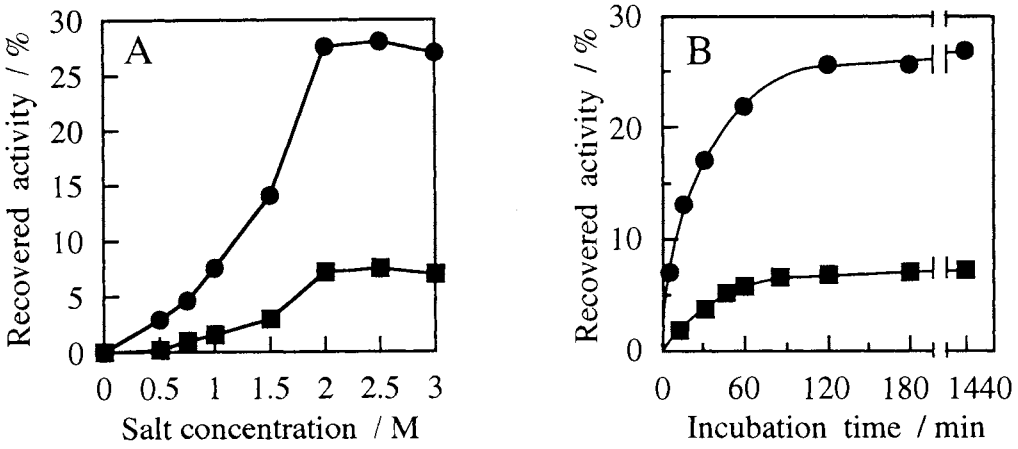

Fig. 1. (A)Effect of salts and their concentration on the recovered activity of subtilisin refolded from fully denatured state: Subtilisin was denatured by treatment with 6 M GdnHCl solution at pH 2.0 for 2 h at room temperature. The solution was diluted 100-fold with renaturation solution containing potassium chloride(■) or potassium acetate(●) at various concentrations at resultant pH 6.5. The concentration of subtilisin in renaturation solution was 1.0 μg/ml. The samples were incubated at 4 °C for 24 h and assayed for subtilisin activity.
(B)Time course of the recovered activity of subtilisin in the presence of 2 M potassium chloride(■)and 2 M potassium acetate(●).

buffer was adopted to avoid aggregation. As a matter of fact, the aggregation was observed at protein concentration of 20.0 μg/ml, and the recovered activity in 2 M potassium acetate and 2 M potassium chloride, pH 6.5, was only 7% and 3%, respectively. While, the recovered activity in 2 M potassium acetate solution retained approx.30% at the concentration below 1.0 μg/ml.

The next aim of our research is to increase the refolding yield higher than the level described above(approx.30%). For this purpose, we examined the effect of coexistence of SSI in the refolding media containing potassium acetate. In this experiment, we eventually used SSIM73N because physicochemical properties including the inhibition constant for subtilisin were almost same with that of wild type SSI.[10] Refolding studies in the presence of SSIM73N was monitored by gel filtration HPLC. Two contradictory experimental conditions should be compromised, *i.e.*, the low subtilisin concentration is preferred against aggregation, and at the same time high protein concentration is needed for the reliable HPLC analysis. Considering these situations, the refolding experiment was performed according to the step-wise refolding procedure of Fischer et al.[11] with slight modifications. Namely, every 2 h, the denatured subtilisin at concentration of 1.56 μg/ml was introduced to the renaturation solution containing 2 M potassium acetate and 25.0 μg/ml SSIM73N at pH 6.5 and 4°C. After ten cycles of this procedure, a final subtilisin concentration of 15.6 μg/ml was attained, which was enough to be analyzed precisely by gel filtration HPLC. Figure 2B shows the gel filtration HPLC pattern of the complex between subtilisin refolded in 2 M potassium acetate solution and SSIM73N. The three peaks with retention times of 15.5 min, 16.7 min and 19.0 min corresponded to E_2I_2, EI_2 and I_2(E: subtilisin, I: SSIM73N), respectively, which was confirmed by the chromatogram of a control sample shown in Fig.2A, obtained by injecting a mixture of SSIM73N and native subtilisin in 2 M potassium acetate. Quantitative estimation of the peak areas revealed that these peaks contained subtilisin as much as 90% of the initial amount. Thus, we predicted that the quantitative refolding from the fully denatured subtilisin was attained in the presence of 2M potassium acetate and SSI, provided that SSI couldn't form complexes with the

incorrectly folded subtilisin.

The reason why potassium acetate solution prevailed other salts as the refolding media is not understood by now. It has been shown recently that some salts such as potassium chloride, sodium chloride or sodium sulfate influenced slightly the refolding of the mutant subtilisin, lacking the high-affinity calcium binding site[12] or carboxypeptidase Y.[13] We believe that potassium acetate might be much more effective than such salts also in these cases. Another important problem remained unsolved is the role of SSI in the present experiments. SSI functioned to induce correct and solid structure of subtilisin even in the absence of pro-sequence by means of its tightly-fitted template effect. However, we noticed recently diverse small peaks in reverse-phase HPLC chart implying occurrence of autoproteolytic digestion in those experiments without the use of SSI. A further detailed study is needed to elucidate the role(s) of SSI, an accelerating template effect and/or an autoproteolysis preventing effect, in our quantitative *in vitro* refolding process of subtilisin.

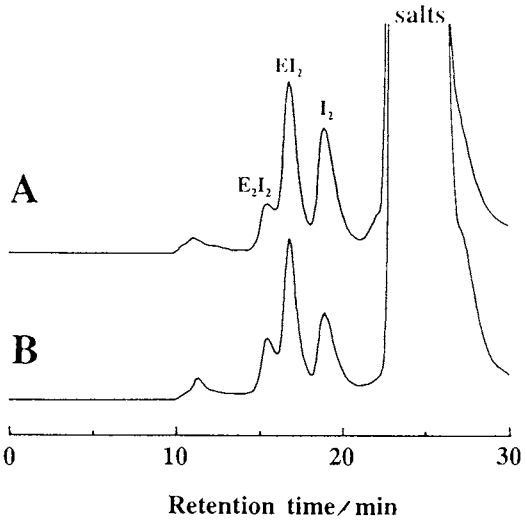

Fig. 2. Analysis by gel filtration HPLC of the refolded subtilisin in the presence of SSIM73N and 2 M potassium acetate. (A)Complex formation between native subtilisin and SSIM73N. (B)Complex formation between refolded subtilisin and SSIM73N.

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