REFOLDING OF SUBTILISIN BPN' ACHIEVED ALMOST QUANTITATIVELY BY COVALENT IMMOBILIZATION ON AN AGAROSE GEL

Tetsuya HAYASHI,* Mamoru MATSUBARA, Eiji KURIMOTO, Daisuke NOHARA, and Tomoya SAKAI

Department of Chemical Reaction Engineering, Faculty of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya 467, Japan

Renaturation of subtilisin BPN' could successfully be attained in the case of subtilisin immobilized covalently on an agarose gel in the medium containing 2 M potassium acetate or other salts. The maximum yield of renaturation based on enzymatic activity was as high as 80% in the presence of 2 M potassium acetate from the fully denatured immobilized subtilisin. Preliminary denaturation in 6 M guanidine hydrochloride at pH 2.4 for 4 h rendered 20-30% of immobilized subtilisin to release from the agarose gel, which was estimated by reverse-phase high performance liquid chromatography as well as by electrophoresis. We concluded that, in the presence of 2 M potassium acetate, we could achieve almost quantitative refolding of subtilisin by immobilization.

Next, the rate of renaturation of denatured immobilized subtilisin was compared among several media containing several salts at the concentration of 2 M, at 25℃. The times for 50% renaturation, $t_{1/2}$'s, in the presence of potassium acetate, potassium chloride, and lithium chloride were about 24, 63, and 153 min with apparent ultimate yields of 80, 71, and 40%, respectively. Higher rates and yields resulted also in the presence of the other organic salts such as dipotassium succinate and potassium propionate.

KEYWORDS subtilisin BPN' ; immobilization; renaturation; potassium acetate

Subtilisin BPN' is a 275 amino acid serine protease secreted by *Bacillus amyloliquefaciens*. Subtilisin is initially secreted as a proenzyme with the 77 amino acid pro-sequence attached to mature subtilisin which is indispensable for *in vivo* folding.[1-4] *In vitro*, however, it is generally recognized at present that the mature subtilisin denatured in 6 M guanidine hydrochloride (GdnHCl) at acidic pH condition could never be renatured by mere dilution of the denaturant.[4-6] Ohta et al. tried *in vitro* refolding of subtilisin in the coexistence of synthetic 77 amino acid pro-peptide to attain the refolding yield of about 12%.[7] Recent investigations by Matsubara et al. revealed that the presence of 2 M potassium acetate in renaturation solution (pH 6.5, 4℃) was effective to recover about 30% of native enzymatic activity.[8] Further, they demonstrated that supplementation of *Streptomyces* subtilisin inhibitor (SSI) to the renaturation medium gave almost quantitative refolding of subtilisin because they obtained an equivalent amount of the subtilisin-SSI complex peaks on the high performance liquid chromatography (HPLC) chart.[8] However, it is not clear whether the subtilisin molecule bound to SSI has been completely active or not.

We attempted the renaturation of subtilisin immobilized covalently on an agarose (Sepharose 4B) gel by preventing autoproteolysis of subtilisin. Almost quantitative refolding of subtilisin from the denatured state could be attained in the case of immobilized subtilisin and in the refolding medium containing various organic or inorganic salts at high concentrations.

MATERIALS AND METHODS

Materials Subtilisin BPN' was purchased from Nagase Biochemicals. Cyanogen bromide (CNBr)-activated sepharose 4B was from Pharmacia. Synthetic substrate, *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide was purchased from Sigma. All other chemicals were of analytical grade.

Preparation of Immobilized Subtilisin The immobilized subtilisin was prepared by using the procedure of Svensson[9] with a slight modification. A CNBr-activated Sepharose 4B gel (4 g) was swollen and washed with 200 ml of 1 mM HCl per g-solid gel to remove stabilizers and then washed again with 5 ml/ml-settled gel of 0.1 M sodium carbonate buffer containing 0.5 M sodium chloride (pH 10.0). Subtilisin BPN' dissolved in deionized water (*ca.* 0.8 mg/ml), 0.125 ml, was added to

30 ml of the 33% (v/v) gel suspension of the same buffer (pH 10.0) to get an immobilized subtilisin gel of 0.01 mg-protein/ml-gel which meant almost 100% coupling. The mixture was stirred gently for 24 h at 4℃ and then filtrated, followed by washing with 10 ml/ml-gel of the buffer described above to remove possibly remaining free protein molecules. It was not necessary, however, in this case. On the contrary, remaining active sites on the gel were coilapsed by hydrolysis in 0.1M Tris-HCl buffer (pH 8.0) with 2ml/ml-gel for 6 h at 4℃. The filtrated gel of immobilized subtilisin was then thoroughly washed with solutions of 5 ml/ml-gel each in the following sequence: 0.1 M sodium hydrogen carbonate (pH 8.2), 1 M sodium chloride, 0.1 M sodium acetate buffer (pH 5.4), and finally with deionized water. The suspension was stored at 4℃ in the deionized water. The amount of immobilized subtilisin was determined to be 0.01 mg/ml-gel, because no sign of protein was detected for all filtrates arising in all procedures after concentration by optical density measurements at 280 nm.

Activity Measurements Activity of the immobilized subtilisin was determined by use of *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide as a substrate. The substrate was dissolved in dimethyl sulfoxide at the concentration of 0.50 mg/ml. To the mixture of 8.7 ml of 50 mM Tris-HCl buffer containing 1 mM $CaCl_2$ (pH 8.5) and 0.050 ml of 80% (v/v) gel suspension, a 1.25 ml of substrate solution was introduced under gentle agitation at 25℃. After reaction for 1, 3, and 5 min, the suspension was rapidly filtrated. The amount of *p*-nitroanilide released in the filtrates was determined by measurement of the absorbance at 410 nm. Activity of 69±2% relative to that of the dissolved free subtilisin was retained for the immobilized subtilisin. The recovered activity by renaturation was evaluated on the basis of enzymatic activity as a percentage of that of the immobilized subtilisin which was stored without denaturation.

RESULT AND DISCUSSION

The immobilized subtilisin was denatured by stirring gently in 6 M GdnHCl solution (pH 2.4) at 2 ml/ml-gel for 4 h at 25℃ or in 5 mM HCl solution (pH 2.4) at 2 ml/ml-gel for 4-6 h at 25℃. Mere replacement of the denaturant solution with 50 mM sodium acetate buffer (pH 6.5) was not effective to recover the activity (Fig. 1). For renaturation, denatured immobilized subtilisin should be subjected to filtration and washing with about 10 ml/ml-gel of renaturation solutions containing various salts at high concentration levels at pH 6.5. The 33% (v/v) gel suspensions in the renaturation solutions were stirred gently at 4 or 25℃.

Figure 1 shows the rate of renaturation from the denatured immobilized subtilisin at 4 or 25℃, in the presence of a 2 M salt such as potassium acetate, potassium chloride, or lithium chloride. The presence of 2 M potassium acetate gave a yield as high as 80% after 24 h at pH 6.5 and at 25℃. Subtilisin molecules adsorbed non-specifically might be released from the gel surface. Indeed, leaching of a certain amount of subtilisin from gel surface during 4 h denaturation was observed by reverse-phase HPLC as well as by sodium dodecyl sulfate-polyacrylamide gel electrophpresis. These results indicated that, in the presence of 2 M potassium acetate, almost quantitative refolding of subtilisin by immobilization could be achieved.

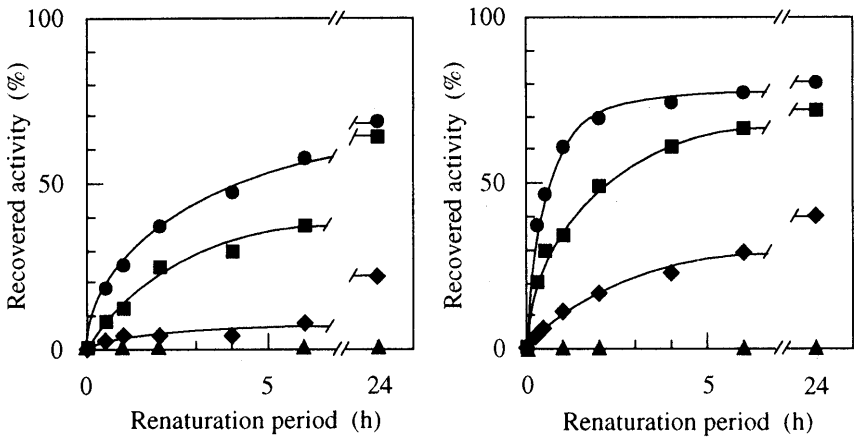

Fig. 1. Renaturation of Immobilized Subtilisin

Immobilized subtilisin was denatured with 6 M GdnHCl at pH 2.4 for 4 h at 25℃. The renaturation media contained 2 M potassium acetate (●), 2 M potassium chloride (■), 2 M lithium chloride (◆), and 50 mM sodium acetate buffer (▲) at pH 6.5, and renaturations were carried out at 4℃ (left) or 25℃ (right).

The amount of immobilized subtilisin carried on the gel, 0.01 mg/ml-gel, implied that autoproteolysis was not likely to occur. The surface concentration was roughly estimated to be 7.4 nmol/m^2 or 230 nm^2/molecule, assuming the gel as a sphere of 90 μm diameter with the packing porosity of 26%. Since the molecular shape of subtilisin is approximately spherical with a diameter of about 4.2 nm,[10] a contact between molecules could not be expected, assuming uniform surface coverage. In fact, no digestion product was detected in the filtrates.

Moreover, the immobilization of subtilisin rendered it possible to study the effectiveness of each salt on renaturation more distinctly than in the case of free subtilisin accompanying autoproteolysis.[8] It should be noted that in the medium containing 2 M potassium chloride, the rate of renaturation was considerably slower than that of 2 M potassium acetate, although the renaturation yields were similar (63-71%) in distinct contrast with the results of Matsubara et al. (10%). The times for 50% renaturation, $t_{1/2}$'s, were 24, 63, and 153 min, in the 2 M potassium acetate, potassium chloride, and lithium chloride, respectively. Furthermore, in the case of 2 M lithium chloride, the yield attained by immobilization was 40% in contrast to 0% which was observed without the immobilization.[8] It should be emphasized, in line with the conclusion of Matsubara et al., that potassium acetate was distinctively effective on renaturation of immobilized subtilisin.

Table I. Effect of Several Salts on the Recovered Activity of Immobilized Subtilisin

Salt	concentration (M)	recovered activity (%)
LiCl	2	41
KCl	2	67
KSCN	2	0
CH_3COOK	2	69
CH_3COONa	2	64
$(CH_3COO)_2Mg$	2	69
$(CH_3COO)_2Ca$	1.6	79
CH_3CH_2COOK	2	77
CH_2COOK | CH_2COOK	1	74
COOK | COOK	1	55
CH_2COOK | C(OH)COOK | CH_2COOK	0.7	44

The immobilized subtilisin was denatured with 5 mM HCl at pH 2.4 for 4-6 h at room temperature. Renaturation of immobilized subtilisin was performed in the media containing several salts at pH 6.5 for 72 h at 25℃.

Several other organic salts at high concentration were examined (Table I). It was revealed that the effectiveness of the acetate anion was excellent irrespective of the counter cations, and that organic salts such as dipotassium succinate and potassium propionate had a similar or more favorable effect on the renaturation of subtilisin.

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