Fluorescence Labeled and Cross-Linked Subtilisin: Kinetic Characteristics and Binding to *Streptomyces* Subtilisin Inhibitor

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In the preceding paper, the preparation of fluorescent cross-linked subtilisin was described. In this paper we present the catalytic and binding properties of the modified enzyme. Kinetic analysis showed that the cross-linked dimeric subtilisin retained both catalytic activity and binding affinity toward synthetic substrates. These kinetic characteristics of the modified enzyme were nearly identical to those of the native enzyme. The modified enzyme also exhibited a specific interaction with *Streptomyces* subtilisin inhibitor (SSI) with 1 to 1 stoichiometry. The formation of a polymeric complex, which is the expected product with cross-linked subtilisin, was demonstrated.

Keywords cross-linking; subtilisin dimer; protein–protein interaction; catalytic activity; subtilisin-*Streptomyces* subtilisin inhibitor complex; polymerization; fluorecence label

Many studies have been made on the structure and function of *Streptomyces* subtilisin inhibitor (SSI) on account for its unique properties.[1] SSI is a dimer of homologous subunits, and it binds two molecules of subtilisin BPN′ to form a tetrameric $\alpha_2\beta_2$ type complex.[2,3] We are interested in the development of an artificial polymerization system of proteins making use of dimeric subtilisin, which should spontaneously form a linear protein aggregate with SSI. For this purpose, preparation of fluorescence-labeled cross-linked subtilisin BPN′ was carried out, and the results were reported in the preceding paper.[4]

The present paper describes the kinetic characteristics of the modified subtilisins and the applicability of the enzyme preparations to a spontaneous polymerization system of proteins. Some physicochemical properties of the polymeric complex with SSI are also described.

Results and Discussion

As reported in our previous work,[4] fluorescence labeled and cross-linked subtilisin was prepared by thiol–disulfide interchange reaction between thiol containing subtilisin and dithiopyridyl group containing subtilisin, and subsequent treatment with *N*-(7-dimethylamino-4-methyl-3-coumarinyl) maleimide (DACM). Separation of dimeric subtilisin from the by-products, monomeric and oligomeric subtilisins, was achieved by gel filtration of the coupling products.

Catalytic Activity of the Modified Enzyme Preparations The modified subtilisin samples described in the preceding paper[4] were subjected to active site titration using *p*-nitrophenyl acetate.[5] Titrated values on the basis of monomeric subtilisin unit (27.5 kDa) were determined. As shown in Table I, a reasonable titrated value was obtained for the dimer fraction. The titrated active enzyme concentration was 63% on the basis of the absorbance $A^{1\%}$ (280 nm) = 10.41.[6] The value for the original enzyme itself was 84%. Even for the oligomer fraction, quite a large titrated value was obtained. Kinetic parameters in the hydrolyses of *p*-nitrophenyl acetate and tripeptide methylcoumarinamide (Z–Asp–Pro–Leu–MCA)[7] were determined as shown in Table I. It was found that the dimeric enzyme exhibited a marked similarity to native subtilisin in K_m and k_{cat} values with ester substrate and amide substrate. Therefore it was concluded that the cross-linking reaction of subtilisin in the present study had been accomplished in the desired manner.

Stoichiometry in the Interaction of Cross-Linked Subtilisin with SSI and Estimation of K_i The molar ratio of complexation of the modified subtilisins with SSI was studied. The interaction was analyzed by measuring the catalytic activity of the enzyme preparation as a function of SSI concentration added. The catalytic activity was measured using Z–Asp–Pro–Leu–MCA as a substrate. A decrease of the catalytic activity of the dimeric subtilisin proportional

TABLE I. Kinetic Parameters for Subtilisin- and Modified Subtilisin-Catalyzed Hydrolyses[a]

Subtilisin preparation	*p*-Nitrophenyl acetate			Z–Asp–Pro–Leu–MCA	
	K_m (M)	k_{cat} (s^{-1})	N (%)	K_m (M)	k_{cat} (s^{-1})
Native	6.8×10^{-4}	0.20	84	2.9×10^{-3}	4.7×10^{-2}
Modified monomer[b]	6.4×10^{-4}	0.22	70	2.5×10^{-3}	4.3×10^{-2}
Modified dimer[c]	6.5×10^{-4}	0.22	63	3.1×10^{-3}	4.7×10^{-2}
Modified oligomer[d]	6.3×10^{-4}	0.22	59	3.3×10^{-3}	5.0×10^{-2}

a) Hydrolysis was carried out in 50 mM tris containing 7.5% DMF, pH 8.2, at 37 °C. Determination of kinetic parameters was carried out based on the enzyme concentration titrated by *p*-nitrophenyl acetate. *b*) Fluorescence labeled monomeric subtilisin, fraction C.[4] *c*) Monomeric preparation, fraction B.[4] *d*) Mixture of oligomeric enzymes, fraction A.[4]

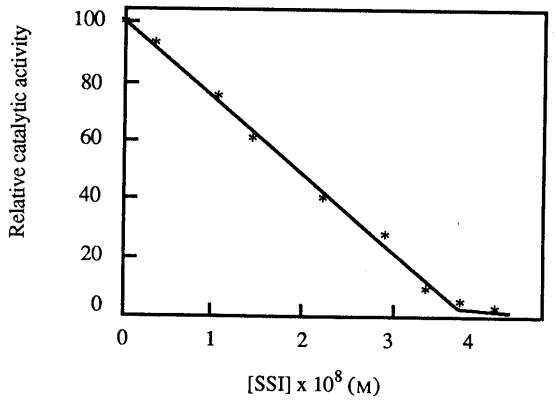

Fig. 1. Inhibition of Dimeric Subtilisin-Catalyzed Hydrolysis by SSI

Catalytic activity of the modified enzyme was assayed using Z–Asp–Pro–Leu–MCA as a substrate in 50 mM tris buffer (pH 8.2) at 37 °C as a function of SSI concentration. Concentration of the enzyme used was 36 nM. Concentration of SSI-monomer (molecular weight 11.5 kDa) is given on the abscissa.

to the added amount of SSI was observed as shown in Fig. 1. Extrapolation of the plot to the abscissa indicates the saturation concentration of SSI in the complexation with the enzyme. Analyses were carried out for samples of native subtilisin, and modified monomeric and oligomeric subtillisins, and results similar to those in Fig. 1 were obtained. The ratios of the monomeric enzyme unit (27.5 kDa) per subunit of SSI (11.5 kDa) were 0.96, 1.03, 1.03 and 1.01 for native, modified monomeric, modified dimeric and oligomeric subtilisins, respectively. Thus in all cases the stoichiometries of complexation of SSI and subtilisin samples were analyzed to be essentially 1 to 1 on the basis of single SSI subunit *vs.* subtilisin monomer unit.

Estimation of the K_i values for the modified subtilisin–SSI complex is of interest. It is known that native subtilisin forms a very tight complex with SSI, and the dissociation constant of the complex is so small that it can not be determined by conventional rate assay methods. The value was found by a single photon counting technique to be 7×10^{-11} M.[8] As shown in our previous work, Z–Asp–Pro–Leu–MCA is a subtilisin-specific fluorogenic substrate which permits micro scale analysis of subtilisin.[7] Kinetic determination of very small K_i values is possible only by the use of very sensitive substrates which permit analysis at very low concentrations of enzyme. In the present study using Z–Asp–Pro–Leu–MCA, the catalytic activity of the dimeric subtilisin at the concentrations as low as 36 nM could be detected, and the enzyme activity in the presence of 36 nM SSI was found to be completely lost following 1 to 1 stoichiometry. This means that the K_i value for dimeric subtilisin–SSI complex is much smaller than 36 nM. Thus, K_i for cross-linked subtilisin against SSI is still exceedingly small, and it can be concluded that the cross-linking did not cause significant interference with the complexation process.

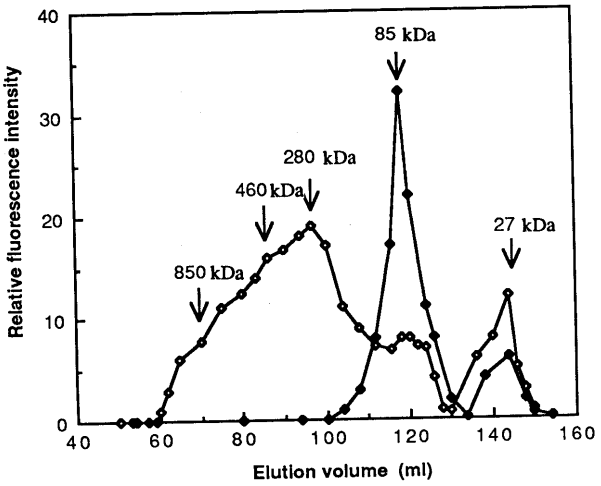

Fig. 2. Elution Diagram of Dimeric Subtilisin–SSI Incubate

Elution of dimeric subtilisin–SSI incubate was carried out on a Bio-gel A 1.5 m (200—400 mesh) column (1.5 × 80 cm), pH 7.6, at 4 °C with a flow rate of 4 ml/h (◇—◇), A control experiment was carried out using unmodified subtilisin under the same conditions (◆—◆). The protein concentration of the eluate was determined fluorometrically. The molecular weights were estimated from the calibration plot in Fig. 3.

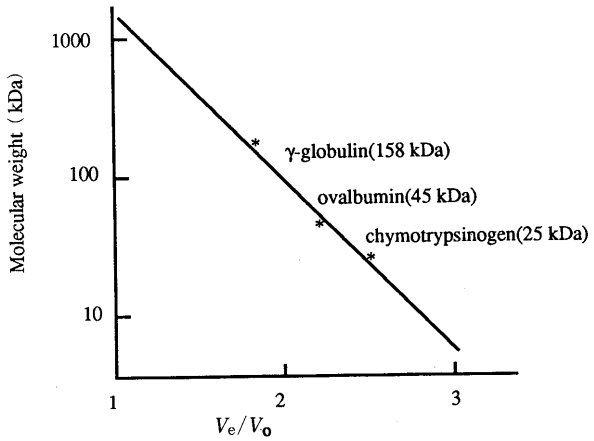

Fig. 3. Calibration of Molecular Weight

Elution volumes (V_e) of standard proteins, γ-globulin (158 kDa), egg albumin (45 kDa) and trypsinogen (25 kDa) were determined using a Bio-gel column under the conditions used in Fig. 2. Ratios of V_e to V_o (elution volume of Blue dextran-200; 2000 kDa) were plotted as a function of molecular weight.

Characterization of Product from Dimeric Subtilisin and SSI Two equivalents of SSI was mixed with modified dimeric subtilisin in 50 mM tris buffer (pH 7.5), and incubated at 37 °C for 30 min. Formation of polymerized products was analyzed on a Bio-gel A 1.5 m column (Fig. 2). Unmodified subtilisin was incubated with SSI and the resultant incubate was subjected to the same chromatographic procedure as a control. As expected, in the case of the modified subtilisin with SSI, polymerized product was detected. Elution of protein started at the void column volume (V_o), which was determined by the elution of blue dextran as shown in Fig. 2. For the incubate with unmodified subtilisin, in contrast, protein appeared at the expected volume for expecting $\alpha_2\beta_2$ complex. The molecular weights of the eluted samples were estimated using the plot shown in Fig. 3. [The values of V_e/V_o (ratio of elution volume *vs.* void volume) for standard proteins, γ-globulin (158 kDa), ovalbumin (45 kDa) and chymotrypsinogen (25 kDa), were determined using the same column, and a linear calibration plot was obtained.] Estimated molecular weight of the main fraction of the dimeric subtilisin–SSI incubate was 200—450 kDa. Eluates at the positions of 850,

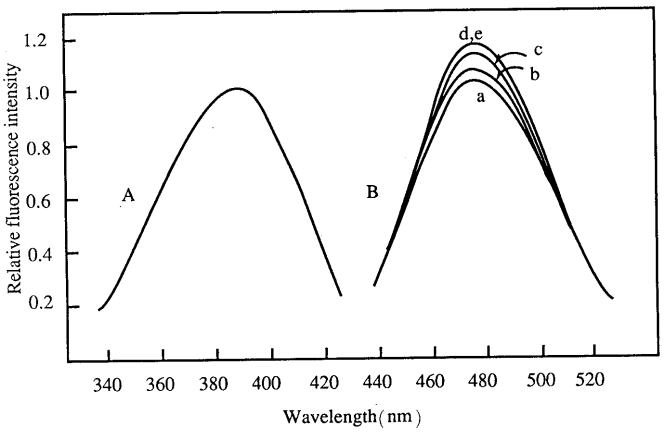

Fig. 4. Fluorescence Spectra of Modified Monomeric Subtilisin

Corrected fluorescence spectra of the modified monomeric subtilisin (3.5 μM) were recorded using rhodamine B as a photon counter in 50 mM ammonium acetate, pH 6.9. The preparation contains 0.20 residue of dimethylaminocoumarin group per monomeric subtilisin[4] (molecular weight 27.5 kDa). The effect of the addition of the SSI on the fluorescence spectra was analyzed at SSI subunit concentrations of 1.00—5.05 μM. A, excitation spectrum of monomeric subtilisin in the absence of SSI. B, emission spectra of monomeric subtilisin in the absence (a), in the presence of 1.00 μM (b), 2.35 μM (c), 4.34 μM (d) and 5.05 μM (e) SSI. The emission wavelength of 480 nm was used for spectrum A. For spectra B the excitation wavelength of 394 nm was used.

280 and 85 kDa were collected and their catalytic activities toward Z–Asp–Pro–Leu–MCA were analyzed. None showed any appreciable activity, in accord with the result of Fig. 1. This indicates that each complex irrespective of its degree of polymerization is composed of equimolecular amounts of SSI and subtilisin. A peak at the elution volume of 115—125 ml was considered to be due to tetrameric $\alpha_2\beta_2$ complex formed from contaminating monomeric subtilisin. The main fraction of unmodified subtilisin and SSI incubate was seen at the elution volume of 118 ml. A slow-eluting peak, observed for both samples, was considered to be due to excess SSI. Estimated molecular weight for the eluate from native subtilisin and SSI incubate was about 85 kDa, which is similar to that of tetrameric $\alpha_2\beta_2$ complex (78 kDa).

Effect of Complex Formation on Fluorescence Spectra As reported previously,[4] fluorescence spectra of oligomeric, dimeric and uncoupled monomeric subtilisins were identical and they exhibited an excitation maximum and an emission maximum at 394 and 489 nm, respectively. These enzyme preparations were expected to cast light on physicochemical aspects of protein–protein interaction. Thus, the effect of the addition of SSI on the fluorescence spectra of the modified subtilisin samples was studied.

No spectral change was observed on the addition of SSI to modified dimeric subtilisin. However, the fluorescence intensity of the monomeric subtilisin was increased by the addition of SSI though the extent was not large (18%). The intensity increased proportionally to the added amount of SSI and no further enhancement was observed after the addition of a saturating amount of SSI to subtilisin (Fig. 4). The result is in contrast with that for dimeric subtilisin. The fluorescence intensity of dimeric subtilisin was unaffected by the addition of SSI. No simple explanation can be offered for the observation at the present stage. Cross-linking may cause some structural change of the subtilisin molecule. This may alter the flexibility of the subtilisin molecule, and this would influence the fluorescence process. We may expect that the single $\alpha_2\beta_2$ complex formed from the monomeric subtilisin and cluster of $\alpha_2\beta_2$ units formed from the dimeric subtilisin are completely different morphologically. This difference should be reflected in certain properties such as the relaxation of polarized fluorescence. The rapid polymerization of the dimeric subtilisin and SSI is of special interest and experiments are now being designed to study this step by using a stopped flow fluorometer.

Experimental

Materials Modified subtilisins were prepared according to the preceding report.[4] The enzyme concentration was determined based on the titrated value using *p*-nitrophenyl acetate as a titrant.[5] A three times crystallized SSI was a generous gift from Professor S. Murao and Dr. S. Sato. Concentration of SSI was calculated on the absorbancy basis taking $A^{1\%}$ (276 nm) = 8.29 and molecular weight = 11500 for the subunit.[8] γ-Globulin, egg albumin and chymotrypsinogen were products from Sigma, Nutritional Biochem. Co., and Worthington Biochem. Co., respectively. The subtilisin substrate Z–Asp–Pro–Leu–MCA was prepared as previously reported.[7]

Kinetic Analysis Subtilisin-catalyzed hydrolysis of Z–Asp–Pro–Leu–MCA was followed by monitoring the fluorescence intensity at 460 nm using a Hitachi 650—40 spectrofluorometer. Catalytic rates were calculated based on the fluorescence intensity of a standard solution of 4-methylumbelliferone. The enzyme concentration titrated by *p*-nitrophenyl acetate was used for the rate assay. Kinetic parameters were determined from Lineweaver plots. Excitation wavelength was 380 nm. The reaction was carried out in 50 mM tris buffer (pH 8.2) containing 7.5% dimethylformamide (DMF) at 37 °C.

Determination of Active Enzyme Concentration Active subtilisin concentration was determined by the active site titration method using *p*-nitrophenyl acetate as a titrant. Analysis was carried out in 0.04 M pyrophosphate, pH 8.5 containing 3.3% isopropanol at 20 °C, monitoring the absorbance change at 405 nm. The observed burst was analyzed as a function of titrant concentration and the operational normality of the enzyme was determined taking $\Delta\varepsilon$ 405 nm = 17600 M^{-1} cm^{-1}.[3,5] Titrant concentration was 0.6—1.2 mM. Enzyme concentration used in the analysis was 3.2—4.8 μM on the basis of absorbancy at 280 nm, taking $A^{1\%} = 10.41$.[3]

Interaction with SSI Stoichiometry of Inhibition of Subtilisin Samples with SSI: Inhibitory activity of SSI against subtilisin preparations was assayed using Z–Asp–Pro–Leu–MCA under the conditions used for the kinetic analysis described above. The enzyme and the inhibitor were incubated first in 50 mM tris buffer (pH 8.2) at 37 °C for 5 min. Then a solution of the substrate in DMF was added, and the catalytic activity was determined. Concentrations of enzyme, SSI, substrate and DMF were 36 nM, 3.0—45 nM, 0.20 mM and 7.5%, respectively.

Gel-Filtration: Modified dimeric subtilisin (0.5 mg) and SSI (0.4 mg, 2.4 eq) were incubated in 50 mM tris buffer, pH 7.6, containing 145 mM sodium chloride and 4 mM ethylenediaminetetraacetic acid (EDTA) at 37 °C for 30 min, and applied to a Bio-gel A 1.5 m (200—400 mesh) column (1.5 × 80 cm). Elution was carried out using the same buffer at 4 °C with a flow rate 4 ml/h. A control experiment was carried out using unmodified subtilisin under the same conditions. Elution of standard proteins, γ-globulin (158 kDa), egg albumin (45 kDa) and trypsinogen (25 kDa), was performed in the same manner. Monitoring of the elution of protein was carried out by measuring fluorescence intensity at 347 nm (excitation at 293 nm). Elution of Blue dextran-200 (mean molecular weight 2000 kDa) was monitored at 630 nm.

Fluorescence Spectra: Modified subtilisins were dissolved in 50 mM ammonium acetate buffer, pH 6.9, to give 3.5 μM solutions. Corrected fluorescence spectra were recorded on a Hitachi 65-40 spectrofluorometer equipped with a 650-0178 data processor using rhodamine B as a photon counter at the excitation wavelength of 394 nm. The effect of the addition of SSI on the fluorescence spectra was analyzed at the concentration of the SSI subunit = 1.00—5.05 μM.

References

1) K. Hiromi, K. Akasaka, Y. Mitsui, B. Tonomura, and S. Murao, "Protein Proteinase Inhibitor—The Case of *Streptomyces* Subtilisin Inhibitor (SSI)," Elsevier Biomedical, Amsterdam, 1987.
2) S. Sato and S. Murao, *Agric. Biol. Chem.*, **38**, 587 (1974).
3) K. Inouye, B. Tonomura, K. Hiromi, S. Sato, and S. Murao, *J. Biochem.* (Tokyo), **82**, 961 (1977).
4) K. Tanizawa, T. Mano, and Y. Kanaoka, *Chem. Pharm. Bull.*, **38**, 467 (1990).
5) M. L. Bender, M. L. Begúe-Cantón, R. L. Blakley, L. J. Brubacher, J. Feder, C. R. Gunter, F. J. Kézdy, J. V. Killheffer, Jr., T. H. Marshall, C. G. Miller, R. W. Roeske, and J. K. Stoops, *J. Am. Chem. Soc.*, **88**, 5890 (1966).
6) K. Inouye, B. Tonomura, K. Hiromi, S. Sato, and S. Murao, *J. Biochem.* (Tokyo), **82**, 961 (1977).
7) Y. Kanaoka, T. Takahashi, H. Nakayama, and K. Tanizawa, *Chem. Pharm. Bull.*, **33**, 1721 (1985).
8) Y. Uehara, B. Tonomura, and K. Hiromi, *J. Biochem.* (Tokyo), **84**, 1195 (1978).