

Preparation of Fluorescence-Labeled and Cross-Linked Subtilisin

Kazutaka TANIZAWA,* Tsutomu MANO, and Yuichi KANAOKA

Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-ku, Sapporo 060, Japan. Received June 30, 1989

Streptomyces subtilisin inhibitor (SSI) is a protein characterized by both its potent inhibitory activity toward subtilisin and its structure, composed of two homologous subunits. It binds two molecules of subtilisin to form a tetrameric complex.

Intermolecularly cross-linked subtilisin is expected to form a polymeric complex with SSI. This could provide a useful model of protein-protein association. Therefore, preparation of fluorescence-labeled and cross-linked subtilisin was carried out.

Keywords subtilisin; chemical modification; cross-linking; fluorescence label; protein-protein interaction; 2-aminothiolane; disulfide exchange reaction; subtilisin inhibitor

Streptomyces subtilisin inhibitor (SSI) is a protein proteinase inhibitor produced by *Streptomyces albogriseolus* S-3253.¹⁾ It is characterized by potent inhibitory activity toward serine proteases of the subtilisin family.²⁾ It has been demonstrated that the inhibitor is a dimeric protein of homologous subunits, and it binds two molecules of subtilisin BPN'.^{3,4)} The enzyme-inhibitor complex has been extensively examined as a model of specific protein-protein interactions, and the results obtained have been reviewed.⁵⁾

We are interested in the intrinsic character of SSI, which is composed of two homologous subunits. It is expected that a linear protein aggregate constituted by SSI and subtilisin will be formed if chemically modified dimeric subtilisin is allowed to interact with SSI. Analysis of this polymerization process will be of interest from the viewpoint of protein-protein interactions. Fluorescence-labeled cross-linked subtilisin is expected to be suitable for this purpose.

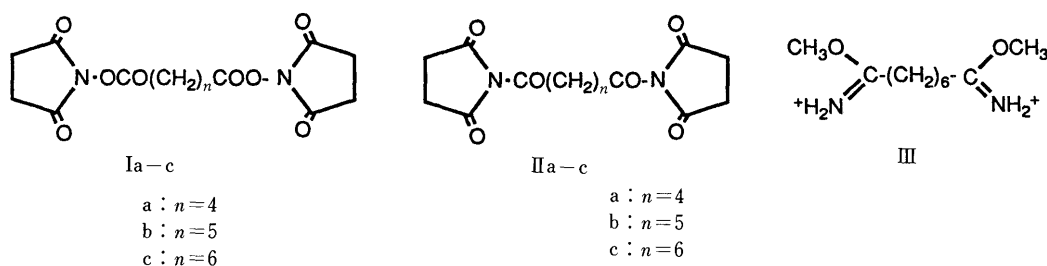


Fig. 1. Divalent Cross-Linking Reagents

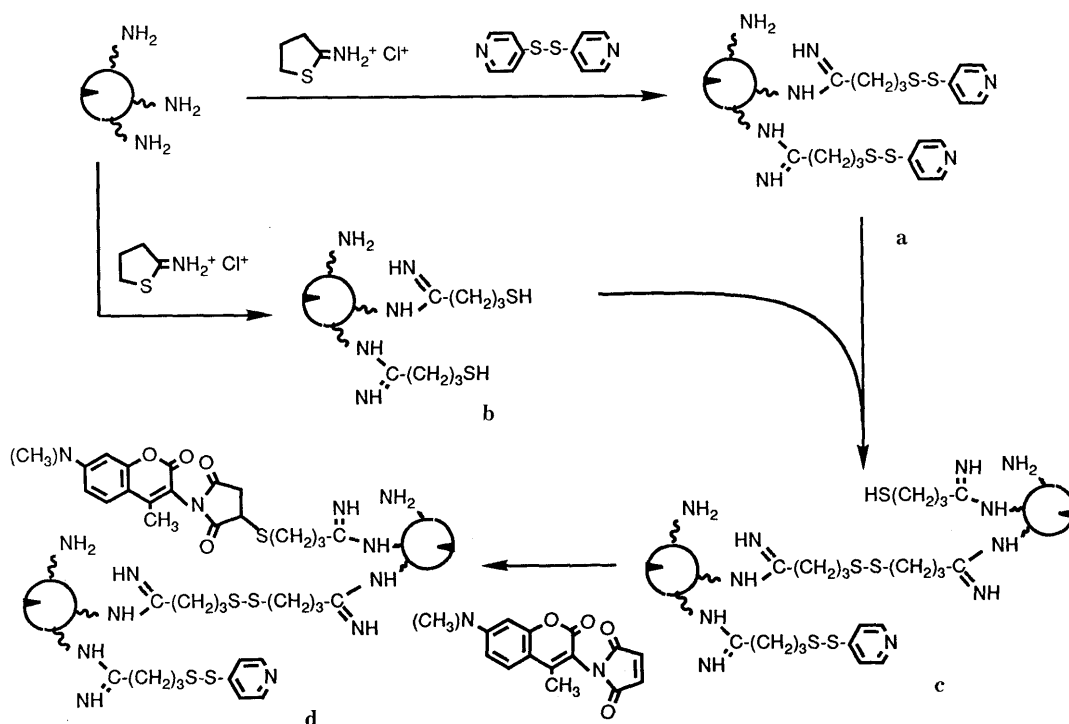


Fig. 2. Schematic Presentation of the Synthetic Route to Fluorescence-Labeled and Cross-Linked Subtilisin

Studies on the preparation of fluorescence labeled and cross-linked subtilisin were started by using amino-specific homobifunctional cross-linking reagents, I, II, and III (Fig. 1), since it is known that the chemical modification of lysine residues of subtilisin does not affect the catalytic activity.⁶⁾ All attempts to cross-link subtilisin by means of these reagents under various conditions were unsuccessful. No product of higher molecular weight than subtilisin was detected by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Variation of the length of the spacer group was ineffective for the purpose. It may be concluded, therefore, that a one-step cross-linking reaction by way of amino group modification is not feasible for subtilisin. The enzyme has as many as 11 lysine residues⁶⁾ and this could result in predominant intramolecular coupling.

The procedure shown in Fig. 2 was designed on the basis that a two-step coupling can eliminate the intramolecular reaction. Both the modified subtilisin samples **a** and **b** prepared in separate batches will be coupled and disulfide exchange reaction will occur intermolecularly. The process is advantageous for the subsequent fluorescence labeling. It is expected that the unreacted sulfhydryl groups will be utilized for the reaction with the sulfhydryl specific fluorescence reagent, *N*-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide (DACM).⁷⁾ 2-Aminothioline is expected to result in the site-specific modification of lysine residues.⁸⁾

Results and Discussion

Preparation of Modified Subtilisins (a) and (b) For the preparation of dimeric conjugate (**c**), it is necessary to provide both modified enzymes **a** and **b** in which limited numbers of lysine residues are modified. The effects of the reagent concentration and the reaction period on the number of modified residues were studied. The conditions were selected to afford the modified enzyme in which average number of introduced groups is slightly larger than 1.0. Under the conditions described in the experimental section, introduction of 1.6 residues of dithiopyridyl group and 1.3 residues of thioalkyl group was achieved in **a** and **b**, respectively. It is clear that not all the components derived from the preparations **a** and **b** will be converted to the fluorescent conjugate (**d**). Not every dimeric subtilisin is necessarily substituted with the fluorescent group. It is important to keep the number of introduced residues as low as possible to minimize the possible formation of oligomeric subtilisin which will lead to two-dimensional polymer formation with SSI. It should be noted that the observed number of introduced residues is an average value. Therefore, the dimeric subtilisin preparation may be contaminated by small amounts of monomeric and oligomeric subtilisins.

Coupling of Modified Subtilisins and Fluorescence Labeling Solutions of **a** and **b** in borax buffer (pH 9.0) were mixed at 4°C. After 1 h, DACM was added. It was reported that the thiol-disulfide interchange reaction is a relatively fast process.⁹⁾ Incubation of **a** and **b** for a prolonged period (3 h) did not cause an increase of the dimeric subtilisin.

Separation of Coupling Products by Gel Filtration Elution of the reaction mixture was carried out on a Sephadex G-75 column using 50 mM ammonium acetate,

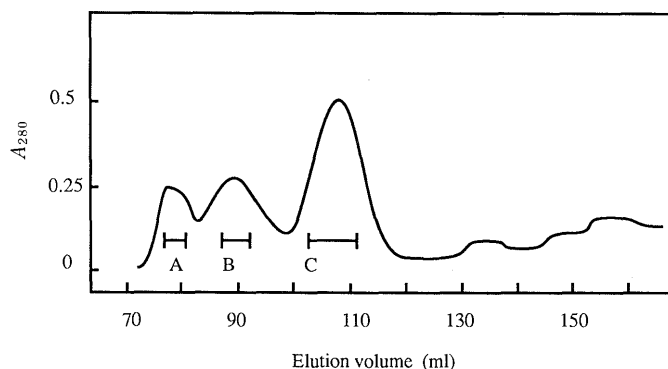


Fig. 3. Separation of a Coupling Mixture of **a** and **b** after Treatment with DACM on a Sephadex Column

The sample containing 0.264 μ mol of enzyme was applied to a column of Sephadex G-75 (5 \times 10 cm), and the column was eluted with 50 mM ammonium acetate (pH 5.7) at a flow rate of 8 ml per h. Cuts A, B, and C were each lyophilized and freeze-dried.

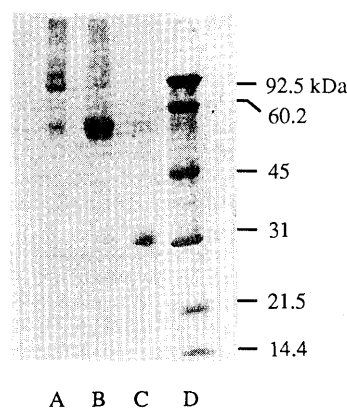


Fig. 4. SDS-Polyacrylamide Gel Electrophoretic Pattern of Coupling Products of **a** and **b**

Lanes A, B and C are cuts A, B and C (Fig. 3), respectively. Lane D is a standard protein mixture.

pH 5.7. The elution profile consists of three peaks as shown in Fig. 3. SDS polyacrylamide-gel electrophoresis revealed that cut A is a mixture of oligomeric subtilisins, cut B consists mainly of the dimeric product and cut C contains unreacted monomeric subtilisin (Fig. 4). Based on the electrophoretic mobilities of standard proteins, the molecular weights of major bands from cuts B and C were estimated to be 60 kDa and 30 kDa, respectively. Cuts A, B and C were each collected and lyophilized.

Extent of Modification and Coupling Products Distribution The isolation yield of the dimer was 24% when the modified subtilisins **b** (1.3 residue per mol) and **a** (1.6 residues per mol) were used. As shown in Fig. 3, the major component of the reaction products was unreacted monomeric subtilisin. Thus, the preparation of **a** and **b** with higher extents of modification was carried out. Preparation of **b** with 2.5 residues of the thiol group was performed by treatment with a 7.5 mM solution of 2-iminothiolane for 100 min. Introduction of 2.6 residues of dithiopyridyl group into **a** was achieved when subtilisin was reacted with 17 mM 2-iminothiolane and 15 mM dithiopyridine at 15°C for 80 min. Coupling of the two components yielded monomer, dimer and oligomers (polymer) in a ratio of 1:1:1. However, in this case separation of dimer from oligomers was unsatisfactory.

The absorption spectra of the lyophilized samples of A, B and C were measured. The number of fluorescent groups introduced group was determined from the optical densities at 280 and 390 nm. The values were analyzed based on the reported molar extinction coefficients of subtilisin⁴⁾ and *N*-(7-dimethylamino-4-methyl-3-coumarinyl)succinimide (DACS).¹⁰⁾ Average numbers of the fluorescent group introduced into the subtilisin molecule (per 27.5 kDa) were calculated to be 0.16, 0.18 and 0.20 for A, B and C, respectively.

Fluorescence Spectra of Cross-Linked Subtilisin Fluorescence spectra of A, B and C were measured with excitation at 394 nm in 50 mM ammonium acetate buffer, pH 6.9, at 25°C. The emission spectra were the same for these three samples, exhibiting emission maxima at 480 nm. The emission maximum is identical to that of DACS in aqueous solvent. This is in contrast to the previous observation that the emission maximum of DACM-treated egg albumin is 460 nm.¹¹⁾

Experimental

Materials Subtilisin BPN' was purchased from Nagase Co., Osaka. Using *p*-nitrophenyl acetate as a titrant,⁴⁾ the operational normality of the enzyme preparation was determined to be 84% of the amount based on the absorbancy at 280 nm. Dimethyl suberimide dihydrochloride III was purchased from Aldrich Chem. Co., Inc. Other chemicals were products of Nacalai Tesque Inc.

Synthesis of Ia, Ib and Ic *N*-Hydroxysuccinimide and dibasic acid were coupled using *N,N*-dicyclohexylcarbodiimide following the standard procedure for peptide synthesis. Ia; colorless needles from methanol, mp 160–162°C. *Anal.* Calcd for C₁₄H₁₆N₂O₈: C, 49.41; H, 4.74; N, 8.23. Found: C, 49.53; H, 4.73; N, 8.18. Ib; colorless needles from ethanol, mp 158–160°C (lit.¹²⁾ mp 159–163°C). Ic; colorless crystalline powder from ethanol, mp 153–155°C. *Anal.* Calcd for C₁₈H₂₄N₂O₈: C, 54.54; H, 6.10; N, 7.07. Found: C, 54.48; H, 6.13; N, 7.04.

Synthesis of IIa, IIb and IIc A solution of *N*-hydroxysuccinimide and adipic acid chloride in tetrahydrofuran (THF) was refluxed for 3 h. Recrystallization from THF of the residue obtained from this solution afforded IIa; colorless needles, mp 158–160°C. *Anal.* Calcd for C₁₄H₁₆N₂O₆: C, 54.54; H, 5.23; N, 9.09. Found: C, 54.67; H, 5.28; N, 9.05. In the same manner IIb and IIc were prepared. IIb; colorless needles from THF, mp 142–144°C. *Anal.* Calcd for C₁₆H₂₀N₂O₆: C, 57.13; H, 5.99; N, 8.33. Found: C, 57.10; H, 6.04; N, 8.37. IIc; colorless needles from benzene, mp 111–114°C. *Anal.* Calcd for C₁₈H₂₄N₂O₆: C, 59.33; H, 6.64; N, 7.69. Found: C, 59.11; H, 6.76; N, 7.59.

Reaction of Subtilisin with Ia—c and IIa—c Subtilisin was treated with Ia—c in 100 μl of 10 mM phosphate, pH 7.1, containing 2% dimethylsulfoxide at 25°C for 6 h. Reactions were performed at various concentrations of reagents and subtilisin in the range of 7 μM–7 mM and 7 μM–1 mM, respectively. After incubation, an aliquot was subjected to SDS polyacrylamide-gel electrophoretic analysis.

Reaction of Subtilisin with III Reaction with III was carried out at 25°C for 4 h at pH 5, 7.1, 8.5 and 9.5. Reactions at each pH were performed by changing the concentrations of reagents and subtilisin in the ranges of 7 μM–7 mM and 7 μM–1 mM, respectively. Aliquots were subjected to SDS polyacrylamide-gel electrophoretic analysis.

Synthesis of 2-Iminothiolane Hydrochloride Following the reported procedure¹³⁾ 4-isothioureido-butyronitrile hydrochloride was synthesized from thiourea and 4-chlorobutyronitrile. Treatment of the isothioureido derivative with aqueous sodium hydroxide solution and subsequently with 6N sulfuric acid afforded 2-iminothiolane hydrochloride. Recrystallization from dimethylsulfoxide-ethyl acetate mixture gave a colorless powder: mp 195°C (dec.) (lit.¹²⁾ mp 202–203°C). *Anal.* Calcd

for C₄H₈ClNS: C, 34.91; H, 5.86; Cl, 25.76; N, 10.18; S, 23.30. Found: C, 34.77; H, 5.88; Cl, 25.73; N, 10.22; S, 23.31.

Preparation of b Subtilisin (3.3 mg, 120 nmol) and 323 μg of 2-iminothiolane hydrochloride were dissolved in 470 μl of 25 mM borax buffer (pH 9.0) and allowed to react at 25°C for 1 h. The concentrations of subtilisin and 2-iminothiolane hydrochloride were 0.25 and 5 mM, respectively. The reaction mixture was applied to a Sephadex column (G-25, 0.6 × 25 cm) and eluted with 100 mM phosphate buffer (pH 6.6) containing 1 mM Na₂EDTA. The number of thiol groups present was determined by spectrometric titration of the eluate with 4,4'-dithiopyridine (1 mM) in phosphate buffer (pH 6.6).¹³⁾ The release of 4-thiopyridone was analyzed at 324 nm taking $\epsilon_{324\text{ nm}} = 1.98 \times 10^4$.

Preparation of a A solution of subtilisin (3.3 mg, 120 nmol) in 270 μl of 25 mM borax buffer (pH 9.0) was mixed with a solution of 4,4'-dithiopyridine (1.1 mg in 80 μl of methanol) at 0°C. To this solution, a solution of 2-iminothiolane (0.69 mg in 178 μl of 25 mM borax, pH 9.0) was added. The concentrations of subtilisin, 2-iminothiolane hydrochloride and 4,4'-dithiopyridine were 0.25, 10 and 10 mM, respectively. The reaction mixture was kept at 0°C for 1 h, then applied to a Sephadex column in the same manner as described for b. The number of dithiopyridyl groups in the eluent was determined from the release of 4-thiopyridone at 324 nm on addition of β-mercaptoethanol (6 mM) in pH 6.6 phosphate buffer in the same manner as for b.

Formation of c from a and b Solutions of a and b (33 μM) in 25 mM sodium borax (pH 9.0) were prepared, and mixed (4 ml each) were mixed at 4°C. After 1 h, a solution of DACM (1.3 mmol in 1.3 ml acetone) was added the mixture was kept for 20 min at 25°C. The resulting reaction mixture was dialyzed against distilled water and lyophilized. The lyophilized sample was applied to a Sephadex G-75 column (5 × 10 cm).

SDS Polyacrylamide-Gel Electrophoresis Slab electrophoresis in polyacrylamide gel was carried out following the method of Laemmli.¹⁴⁾ Prior to the electrophoresis, denaturation of the enzyme sample was carried out with one tenth volume of 6M hydrochloric acid for 10 min at 25°C. The sample was applied to 10% SDS-polyacrylamide slab gel in 25 mM Tris–190 mM glycine (pH 8.5) containing 0.1% SDS. The gel was stained with Coomassie brilliant blue.

Measurement of Fluorescence Spectra Lyophilized fraction B was dissolved in 50 mM ammonium acetate, pH 6.9, to give a 0.645 μM solution. Corrected fluorescence spectra were recorded on a Hitachi 650-40 spectrofluorometer equipped with a 650-0178 data processor using rhodamine B as a photon counter. Measurement was carried out at the excitation wavelength of 394 nm.

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