Dansylation of *Streptomyces* Subtilisin Inhibitor: Spectrometric Analysis of the Fluorescence-Labeled Inhibitor

Kazutaka TANIZAWA,* Katsuhiro MORIYA, and Yuichi KANAOKA

Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan. *Received March 22, 1990*

Specific labeling of tyrosine residues of *Streptomyces* subtilisin inhibitor (SSI) was carried out by dansyl chloride. Analysis revealed that two tyrosine residues out of three in SSI were modified. The resulting fluorescent SSI was fully active as a subtilisin inhibitor. Fluorescence spectra of the modified SSI were investigated. Efficiency of energy transfer from intrinsic tryptophan residues of SSI to the introduced dansyl residue was found to be influenced by the complex formation of SSI with subtilisin.

Keywords fluorescence labeling; *Streptomyces* subtilisin inhibitor; dansyl chloride; protein–protein interaction; fluorescent reporter group; energy transfer

Streptomyces subtilisin inhibitor (SSI) is a protein proteinase inhibitor of microbial origin, and it inhibits serine proteases of the subtilisin family.[1,2] The inhibitor is composed of two homologous subunits, and binds two molecules of subtilisin BPN′.[3,4] The enzyme-inhibitor complex is of special interest in studies of the physicochemical aspects of specific protein–protein interaction, and preparation of fluorescence-labeled and biologically active inhibitor is expected to be meaningful for this purpose. In our previous paper[5] specific labeling of Lys-89 residue of SSI was carried out with fluorescein isothiocyanate. It is necessary to extend the investigation further by preparing a variety of SSI samples in which particular amino acid residues are modified specifically with fluorescent reagent.

1-Dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride) is one of the most useful reagents for fluorescent labeling of proteins. It can react with a variety of nucleophiles of protein molecules such as α-amino, ε-amino, thiol, imidazole and phenolic hydroxy groups. Purpose of the present investigation was to prepare biologically active modified SSI in which a limited number of amino acid residues are labeled. It was pointed out that SSI is heterogeneous with respect to the amino-terminal residue since the amino-terminal region is susceptible to proteolysis.[6] Therefore, we planned to modify residue(s) other than α-amino terminal, and planned protection of the amino terminal prior to fluorescence-labeling.

Results and Discussion

Protection of Amino-Terminal Residue α-Amino group is one of the most reactive nucleophiles among protein functional groups. Preferential protection of the amino-terminal residue of SSI was expected to be successfully carried out by electrophilic modification reagent. The reaction was carried out using maleic anhydride as reported in our previous work.[5] When maleic anhydride (15 eq to total amino groups) was added to SSI in five portions at five minute intervals, polyacrylamide-gel electrophoresis (PAGE) of the reaction product afforded an excellent single band. Analysis of the number of the modified residue revealed that it was nearly 1 mol per subunit of SSI by titration with 2,4,6-trinitrobenzenesulfonate (TNBS).

The modified residue was confirmed to be the amino-terminal residue by the following observation: The maleylated SSI was reacted exhaustively with *O*-methylisourea and subsequently with formaldehyde and sodium boronhydride. The amount of remaining amino group of the resulting modified SSI was analyzed as low as 0.4 residues per subunit by TNBS titration. The modified SSI was hydrolyzed and subjected to amino acid analysis. A decrease of 1.9 residues of lysine per subunit was observed for the hydrolysate of the modified SSI in comparison with that of maleylated SSI. SSI subunit contains two lysine residues: guanidinated residue and methylated residue are resistant to acid hydrolysis whereas maleylated residue is hydrolyzed to regenerate lysine residue. It is therefore apparent that preferential protection of the amino-terminal residue has been successfully carried out by the reaction with maleic anhydride.

Fluorescence Labeling of Protected Subtilisin Introduction of dansyl residue was conducted at pH 9.5, and dansyl chloride (60 molar excess) was added in three portions at 60 min intervals. The resulting modified SSI afforded a clear single band on PAGE. Mobility of the fluorescent SSI was the same as that of maleyl-SSI which was detected by dye-staining. The modified sample was subjected to diethylaminoethyl (DEAE)-cellulose column chromatography, and the elution pattern showed that the sample consisted of a single component. The eluent was analyzed spectrophotometrically and shown to contain 2.25 residues of dansyl group per subunit of SSI. Amino acid composition of the dansylated SSI is shown in Table I. Hydrolysis of

TABLE I. Amino Acid Analysis of Hydrolysates of Native-SSI and Dansylated Maleyl-SSI

Amino acid	Native SSI	Dns Mal SSI
Asp (9)	8.9	9.4
Thr (8)	6.9	7.0
Ser (9)	7.1	6.8
Glu (6)	7.6	8.1
Gly (11)	10.6	10.6
Ala (18)	16.7	16.2
Cys (4)	3.2	2.7
Val (13)	9.7	11.2
Met (3)	2.4	1.7
Ile (0)	0.8	1.2
Leu (9)	9.0	9.0
Tyr (3)	2.7	0.6
Phe (3)	2.7	3.3
Lys (2)	2.6	2.1
His (2)	1.8	1.5
Arg (4)	3.8	3.5
Pro (8)	8.1	7.7

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the protein was carried out by 6M hydrochloric acid at 110 °C for 24 h. Sulfonylated products of lysine and tyrosine are known to be stable to acid hydrolysis under the conditions used. As shown in Table I only the amount of tyrosine was substantially reduced with the modification. The observed decrease, 2.1 residues per subunit, is in good accord with that determined spectrophotometrically. Therefore, it may be concluded that two tyrosine residues of the total of three in SSI were modified.

The positions of tyrosine residues on the primary structure of SSI are known to be Tyr-7, Tyr-75 and Tyr-93,[7] and one of them is resistant to nitration with tetranitromethane.[8] Spectroscopic titration study revealed that one tyrosine had very high pK_a of 12.42.[9] Crystallographic study demonstrated that tyrosin-93 is buried in the protein molecule.[10] Therefore, it was estimated that the buried Tyr-93 is the residue with high p*K*a value and is resistant to the nitration reaction. We may assume that the dansylated tyrosine residues in our experiment are Tyr-7 and Tyr-75, though there is no direct evidence at the present.

Deprotection of Amino-Terminal Residue Removal of maleyl group was tested for maleylated SSI. The reaction was carried out in a medium containing 5% ammonium formate (pH 3.5) at 25 °C. Progress of the deprotection reaction was monitored by subjecting each aliquot of the incubate to PAGE analysis. After 48 h the aliquot indicated a single band: the electrophoretic mobility was identical to that of native SSI. Titration of amino group with TNBS revealed that the resulting preparation did not contain any maleyl group, that is, 3.15 amino groups per subunit were titrated. The inhibitory activity of the deprotected SSI toward subtilisin was identical to that of native SSI.

Inhibitory Activity of Fluorescence-Labeled SSI toward Subtilisin BPN′ Effect of the fluorescence-labeled SSI sample on the catalytic activity of subtilisin was determined using *N*-acetyl-L-tyrosine ethyl ester (ATEE). Catalytic activities of subtilisin (113 nM) in the presence of native SSI (37 nM) and fluorescence-labeled SSI (37 nM) were 34.5 and 37.6% of that in their respective absence. Therefore, it was concluded that the dansylated SSI in which two tyrosine residues were modified was fully active as an inhibitor. Our observation is in accord with that of Satoh[8] who found that nitration of two tyrosine residues caused no change in the inhibitory activity of SSI.

The results further support that, upon modification, the dimeric structure of SSI was not destroyed, since it was reported that inhibitory activity of SSI was lost when SSI was dissociated into monomeric form.[4] The stoichiometry of subtilisin and the modified dimeric SSI was shown to be 2:1 as well.

Effect of Binding of Subtilisin on Fluorescence Energy Transfer Efficiency of Modified SSI Crystallographic studies have shown that upon binding with subtilisin, the subunit of SSI undergoes a global induced-fit conformational change.[11] The change is most remarkable at the subunit–subunit interface region. Crystallographic data show that Tyr-75 situates near the subtilisin-binding site and Tyr-7 at the terminal position of the subunit–subunit interface. SSI subunit has a single tryptophan residue (Trp-86) and its position is very close to Tyr-7. The distance between two aromatic rings of the tyrosine and tryptophan risidues is within 10 Å, and the distance between Trp-86 and Tyr-75 is more than 30Å.

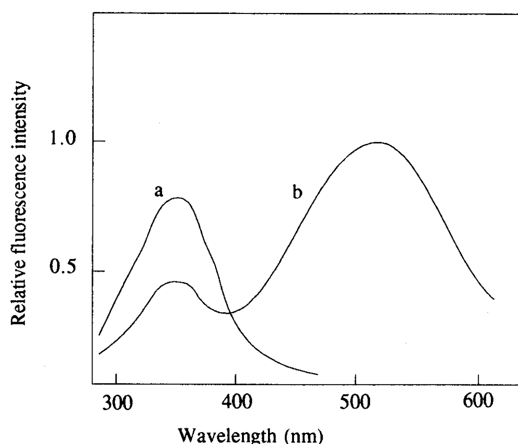

Fig. 1. Emission Spectra of Modified SSI and Native SSI

Samples were dissolved in 1 ml of 1.0 M Tris–HCl buffer (pH 7.0) at the concentration of 560 nM on the basis of monomer subunits. Measurements were carried out at the excitation wavelength 295 nm. a, native SSI; b, modified SSI.

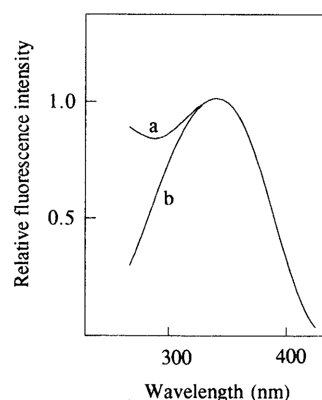

Fig. 2. Excitation Spectra of Modified SSI and Dansyl Fluorophore

Measurements were performed at the emission wavelength 510 nm following the method stated in Fig. 1. a, modified SSI; b, dansyl chloride.

It is, therefore, of interest to determine the fluorescence energy transfer efficiency between the intrinsic tryptophan and the introduced dansyl group in the presence and absence of subtilisin. Figure 1 shows the fluorescence spectra of the modified SSI and native SSI excited at 295 nm (excitation maximum of tryptophan residue). Native SSI exhibited an emission maximum at 350 nm due to the tryptophan residue. The spectrum of the modified SSI shows two emission maxima due to tryptophan and dansyl group. Dansyl fluorophore is effectively excited at 350 nm but not at 295 nm. Therefore, dansyl group is excited by emission light from tryptophan which is excited at 295 nm. The reduced emission at 350 nm and the appearance of emission at 510 nm in Fig. 1 are due to the energy transfer from the tryptophan residue to the dansyl group. Figure 2 shows fluorescence excitation spectra of dansyl chloride and the modified SSI emitted at 510 nm. The modified SSI exhibits intense efficiency at the wavelength around 295 nm in connection with the emitting process at 510 nm (dansyl fluorophore), and dansyl chloride lacking tryptophan is seen to be less efficient at that wavelength.

Energy transfer efficiency (*T*) of tryptophan to dansyl group was calculated by Eq. 1.[12] to be 0.12 for the modified

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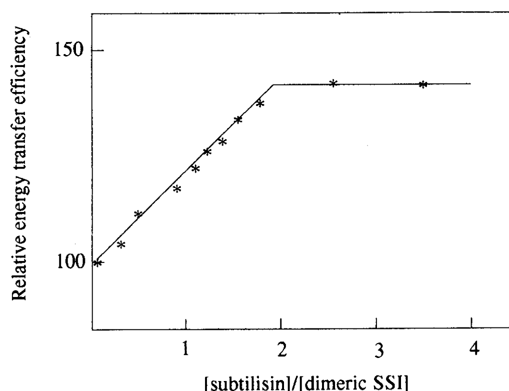

Fig. 3. Effect of Subtilisin on the Energy Transfer Efficiency of Fluorescence-Labeled SSI

An appropriate amount of subtilisin was added to a solution of the modified SSI (16 μg/ml) in 1.0 M Tris–HCl buffer (pH 7.0) at 25 °C. Measurement of fluorescence spectra was carried out after allowing to stand for 20 min at 25 °C. Dependence of the relative energy transfer efficiency on the ratios of subtilisin and the modified dimeric SSI was plotted.

SSI sample.

$$T=(E_{295}/E_{350}-\varepsilon_{295}(\mathrm{DNS})/\varepsilon_{350}(\mathrm{DNS}))\times\varepsilon_{350}(\mathrm{DNS})/\varepsilon_{295}(\mathrm{SSI}) \quad (1)$$

The value E_{295}/E_{350} was determined to be 0.86 for the relative fluorescence intensities at 295 and 350 nm in the excitation spectrum of the modified SSI (Fig. 2). The experimentally determined values, 1200, 2500 and 8000 were used for molar absorption coefficients, ε_{295} (DNS), ε_{350} (DNS) and ε_{295} (SSI), respectively. The addition of subtilisin to the modified SSI solution resulted in an increase of the efficiency proportional to the amount of subtilisin added until saturation of the modified SSI with subtilisin. Dependence of the relative efficiency was plotted as a function of the ratio of modified SSI and subtilisin. As shown in Fig. 3, the molar ratio of subtilisin and modified SSI is 2:1, the same result as found in analysis of the catalytic activity of subtilisin. Taking the reported crystallographic study into account, it can be presumed that the present observation reflects an induced-fit conformational change occurring at the subunit–subunit interface region near Tyr-7. We cannot, however, state unequivocally that the above explanation applies, since the possibility cannot be excluded that proximity of tryptophan residue(s) of subtilisin to the dansyl group of SSI may cause the enhancement of the energy transfer.

Experimental

Materials Partially purified SSI and crystalline subtilisin BPN′ were gifts from Professors B. Tonomura and K. Hiromi, Kyoto University. Purification of SSI was carried out by means of Sephadex G-100 column chromatogtaphy and DEAE-cellulose column chromatography according to the reported procedure.[13] Concentration of a subunit of SSI was determined based on the reported value, $E^{0.1\%}$ (280 nm); 0.81 and molecular weight; 11500.[14] Concentration of subtilisin was determined based on the active site titration method[15] using *trans*-cinnamoyl imidazole. The titrated normality of the active enzyme is 80% based on the concentration calculated from $E^{1\%}=11.7$ (280 nm). Molecular weight of the enzyme was counted as 27500. ATEE was purchased from Peptide Institute Inc. All other chemicals were products of Nacalai Tesque Inc. or Wako Pure Chemicals.

Modification of SSI with Maleic Anhydride SSI (5 mg) was dissolved in 2 ml of 0.1 M phosphate buffer (pH 7.0). To this, 15 μl of a solution of maleic anhydride in dioxane (1 M) was added in five portions over a 25 min period at 0 °C. The reaction mixture was applied to Sephadex G-25 column (1.2 × 40 cm) using 20 mM ammonium bicarbonate (pH 8.1) as a eluent, and the protein fraction was collected and lyophilized.

Removal of Maleyl Group Maleyl-SSI 0.6 mg was suspended in 5 ml of 5% ammonium formate solution (pH 3.5), and the solution was incubated at 37°C. Time course of the demaleylation was monitored by applying aliquots of the incubate to polyacrylamide gel electrophoresis. After incubating for 48 h the incubate solution was adjusted to pH 7 with 5 N NaOH, applied to Sephadex G-25 column (1.2 × 40 cm) using 20 mM ammonium bicarbonate (pH 8.1) as an eluent, and the protein fraction was lyophilized.

Disc Electrophoresis Polyacrylamide gel electrophoresis (10% acrylamide) was carried out at pH 9.5 using Tris–glycine buffer. The gel was stained with Coomassie brilliant blue.

Quantitation of Free Amino Groups Titration of free amino groups was carried out according to the method of Haynes *et al.*[16] The number of trinitrophenylated amino groups was calculated based on $\varepsilon_{335}=1.4\times10^4\,\mathrm{M}^{-1}\,\mathrm{cm}^{-1}$.

Preparation of Guanidinated Maleyl-SSI The guanidination of protein was done according to the method of Kimmel.[17] Maleyl-SSI 3 mg (130 nmol) was dissolved in 2.5 ml of water. To this solution 2.5 ml of 1 M *O*-methylisourea (pH 10.5) was added and reacted for 7 d at 4 °C. The reaction mixture was applied to Sephadex G-25 column (1.2 × 40 cm) using 20 mM ammonium bicarbonate (pH 8.1) as an eluent, and the protein fraction was lyophillized.

Reductive Methylation of Guanidinated Maleyl-SSI The reductive methylation of protein was carried out according to the method of Means and Feeney.[18] Guanidinated maleyl-SSI 1 mg (43 nmol) was dissolved in 2 ml of 0.2 M borate containing 8 M urea (pH 9.2). To this solution, 2 mg (53 mmol) of sodium boronhydride was added and 5 ml (6 mmol) of formaldehyde solution (18%) was added in five portions at 5 min intervals at 0 °C. The reaction mixture was applied to Sephadex G-25 column (1.2 × 40 cm) using 20 mM ammonium bicarbonate (pH 8.1) as an eluent, and the protein fraction was lyophilized. The obtained sample was subjected to the same procedure again to complete the reductive methylation reaction.

Preparation of Dansylated Maleyl-SSI Maleyl-SSI 1.6 mg (70 nmol) was dissolved in 3 ml of 0.5 M carbonate buffer (pH 9.5). To this, a solution of dansyl chloride in acetone (4.1 μmol in 0.375 ml) was added to three portions at 60 min intervals. After 24 h the reaction mixture was applied to Sephadex G-25 column (1.2 × 40 cm) using 20 mM ammonium bicarbonate (pH 8.1) as an eluent, and the protein fraction was lyophilized. The number of dansyl groups coupled to SSI was calculated based on $\varepsilon_{330}=3400\,\mathrm{M}^{-1}\,\mathrm{cm}^{-1}$. Protein concentration of dansylated maleyl-SSI was determined using native SSI as a standard according to the method of Lowry *et al.*[19]

Measurement of Inhibitory Activity of SSI Inhibitory activity of native and modified SSI samples was determined by measuring the catalytic activity of subtilisin BPN′ toward ATEE. Assay was carried out in a medium containing 0.1 M KCl and 10% dioxane at pH 8.0 using a pH-stat (Radiometer TTT2) at 25 °C. The titrant used was 0.02 M sodium hydroxide. Concentrations of substrate and enzyme were 5 mM and 113 nM, respectively. Concentration of native or modified SSI was 37 nM (on the basis of monomer subunits).

Amino Acid Analyses Hydrolysis of the native and modified SSI was performed in 6 N HCl and 110 °C for 24 h, and the hydrolysate was subjected to a Hitachi-835 amino acid analyzer.

Measurement of Catalytic Activity of Subtilisin Catalytic activity of subtilisin BPN′ in the presence of SSI sample was determined using ATEE as a substrate. The rates were analyzed potentiometrically using a Radiometer pH-stat model TTT2b. The reaction was carried out in a medium containing 0.1 M KCl and 10% dioxane at pH 8.0, 25 °C. Concentrations of substrate and enzyme were 5 mM and 55 nM, respectively. Concentration of native or modified SSI was varied within 0—30 nM (on the basis of monomer subunits).

Measurements of Fluorescence Spectra Corrected fluorescence spectra were recorded on a Hitachi fluorescence spectrophotometer 650-60 equipped data processor 650-0178 using rhodamine B as a photon counter.

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