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Fluorescence-Labeled *Streptomyces* Subtilisin Inhibitor: Analysis of the Interaction with Subtilisin and *Streptomyces griseus* Proteases

KAZUTAKA TANIZAWA,[1] SINYA WATANABE,[2] AND YUICHI KANAOKA

Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan

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Specific labeling of the Lys-89 residue of *Streptomyces* subtilisin inhibitor (SSI) was carried out by fluorescein isothiocyanate. The dimeric structure of SSI, which is composed of identical subunits, was not changed upon modification and the resulting fluorescent SSI was fully active as an inhibitor. Induced conformational change in the vicinity of the modified residue of SSI followed by complexation with subtilisin was evidenced by fluorescence spectra. Dissociation of the subunits was also shown to be associated with the change in the fluorescence spectra.

INTRODUCTION

Streptomyces subtilisin inhibitor (SSI) is a protein proteinase inhibitor produced by *Streptomyces albogriseolus* S-3253 (*1*). The inhibitor exhibits potent activity against serine proteases of the subtilisin family (*2*). It is a dimeric protein of homologous subunits, and binds two molecules of subtilisin BPN′ (*3*, *4*). The enzyme–inhibitor complex has received much attention as a model of specific protein–protein interactions. Preparation of fluorescence-labeled and catalytically active subtilisin is expected to be useful in analyzing aspects of the SSI–subtilisin interaction.

It is reported that each subunit of SSI contains three amino groups, i.e., an amino-terminal and two lysine residues (*5*), and that they are not responsible for the inhibitory activity (*6*). It is pointed out that SSI is heterogeneous with respect to the amino-terminal residue since the amino-terminal region is susceptible to proteolysis (*5*). It is also known that two lysine residues situate in different environments; lysine-89 is exposed to the surface of the molecule and accessible to solvent, whereas lysine-18 is buried in the molecule, being involved in an electrostatic interaction with Asp-76 (*7*, *8*). In this respect we planned preferential modification

[1] To whom correspondence should be addressed.

[2] Present address: Tsukuba Research Laboratories, Fujisawa Pharmaceutical Co., 5-2-3 Tokodai, Tsukuba, Ibaraki 300-26, Japan.

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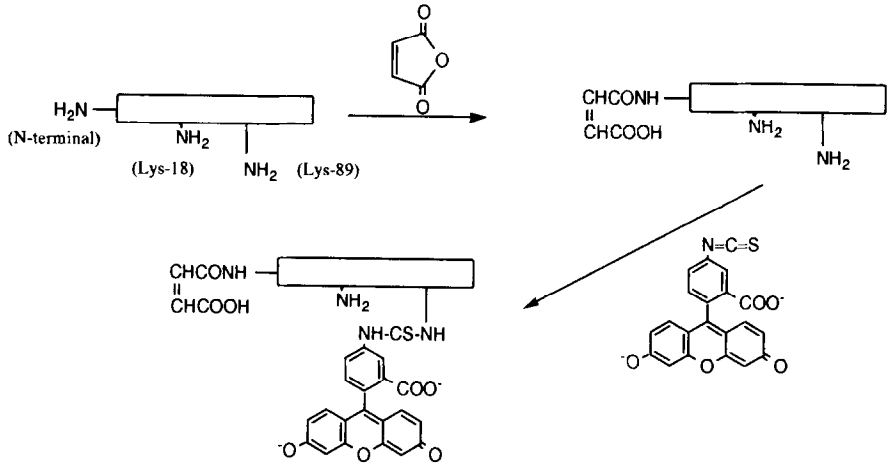

FIG. 1. Preparation of lysine-89-modified SSI by means of fluorescein isothiocyanate.

of the lysine-89 residue with the fluorescent reagent fluorescein isothiocyanate, after protection of the amino-terminal residue as outlined in Fig. 1.

RESULTS AND DISCUSSION

Protection of the Amino-Terminal Residue

It must be noted that the α-amino group is generally more reactive than the ε-amino group toward electrophilic modification reagents. Therefore, protection of the amino-terminal was carried out by using maleic anhydride prior to fluorescence labeling. Optimum conditions for the introduction of a single maleyl residue per subunit of SSI were determined by analyzing each reaction product with SDS–polyacrylamide gel electrophoresis (SDS–PAGE). When the reaction was carried out at pH 7.0 and 0°C with maleimide being added (15 eq. to total amino groups) in five portions at 5-min intervals, SDS–PAGE of the reaction product resulted in an excellent single band. The number of residues modified was taken to be nearly one mole per subunit of SSI on the basis of the following observation: Reaction of the resulting maleyl SSI with 2,4,6-trinitrobenzenesulfonate (TNBS) afforded incorporation of 2.1 residues of the trinitrophenyl group per subunit whereas that of native SSI afforded incorporation of 3 residues.

The site of the modification was confirmed as follows: Maleylated SSI was reacted exhaustively with *O*-methylisourea and subsequently with formaldehyde and sodium borohydride. The amount of amino group remaining on the resulting modified SSI was analyzed by TNBS titration to be as low as 0.4 residues per subunit. The modified SSI was hydrolyzed by 6 M hydrochloric acid at 110°C for 24 h and subjected to amino acid analysis. A decrease of 1.9 residues of lysine was observed for the hydrolysate of the modified SSI compared to that of native

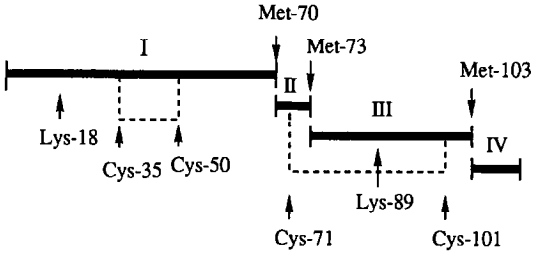

FIG. 2. Fragmentation of SSI subunit expected for cyanogen bromide cleavage. Position of the disulfide bond is indicated by dotted lines.

SSI. The guanidinated residue and the methylated residue are resistant to acid hydrolysis whereas the maleylated residue is hydrolyzed to regenerate the lysine residue. Therefore, it is obvious that protection of the amino-terminal residue was successfully carried out by the reaction with maleic anhydride.

Fluorescence Labeling of Protected Subtilisin

Treatment of maleyl-SSI with a seven molar excess of fluorescein isothiocyanate (FITC) at pH 10.5 afforded a clear single band with Coomassie brilliant blue on SDS–PAGE. A single fluorescent band was observed on unstained SDS–PAGE and corresponded to that on the dye-stained gel. TNBS titration of the reaction product revealed that 0.9 amino group residue per subunit had been reacted with FITC, i.e., the titrated amino groups were 2.1 and 1.2 mol of maleyl-SSI and fluorescence-labeled maleyl-SSI (FTC–maleyl-SSI), respectively.

Determination of Fluorescence-Labeled Amino Acid Residues

The subunit of SSI is composed of three methionine residues (Met-70, Met-73, and Met-103) (*5*). Cyanogen bromide cleavage of the protein may yield four peptide fragments, Peptides I–IV. As shown in Fig. 2, Lys-18 and Lys-89 are included in Peptide I and Peptide III, respectively.

FTC–maleyl-SSI was reduced with 2-mercaptoethanol and subsequently alkylated with monoiodoacetic acid. The reduced and alkylated sample was treated with 100 molar excess of cyanogen bromide in 70% formic acid. The elution pattern of the resulting reaction mixture on a Sephadex G-75 column is shown in Fig. 3. Both peak A and peak B exhibit an absorption maximum at 496 nm, which is intrinsic to fluorescein residue. It was suggested that peak A was a mixture of peptides, and cut *a* was further purified on a DEAE-cellulose column. As shown in Fig. 4, the sample was separated into two components, A-1 and A-2. The fluorescein residue was included in A-2 but not in A-1. Cuts *b*, *a-1*, and *a-2* were subjected to amino acid analysis. Table 1 shows that cut *b* corresponds to Peptide III (74–103) and nonfluorescent *a-1* corresponds to Peptide I (1–70). Cut *a-2* (data not shown) was analyzed to be the partially degraded peptide fragment 1–103. At this point it was clearly shown that the labeling occurred at Lys-89 specifically.

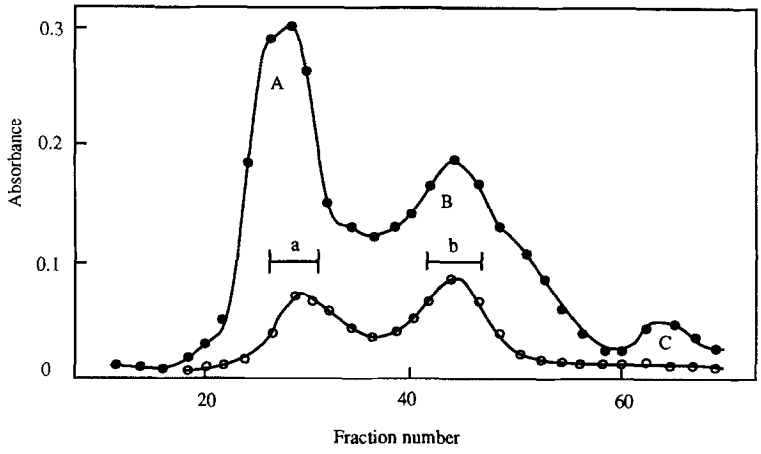

FIG. 3. Separation of cyanogen bromide peptides of FTC–maleyl-SSI on Sephadex G-75 column (1.8 × 115 cm) with 10 mM ammonium carbonate (pH 8.0). Flow rate, 10 ml/h. The absorbancy of each fraction (4 ml) was mentioned at 230 nm (●) and 496 nm (○) for the peptide bond and fluorescein residue, respectively.

Amino acid analysis data of cut *b* are informative regarding the purity of the sample. SSI is alanine rich protein, containing as many as 18 residues. None of them are included in Peptide III. Peptides I and IV include 16 and 2 alanine residues, respectively (*5*). Therefore amino acid analysis data of cut *b* will provide a sensitive indication for the purity of the sample. In the amino acid analysis of cut *b*, only the value for alanine is in conflict with the composition of Peptide III.

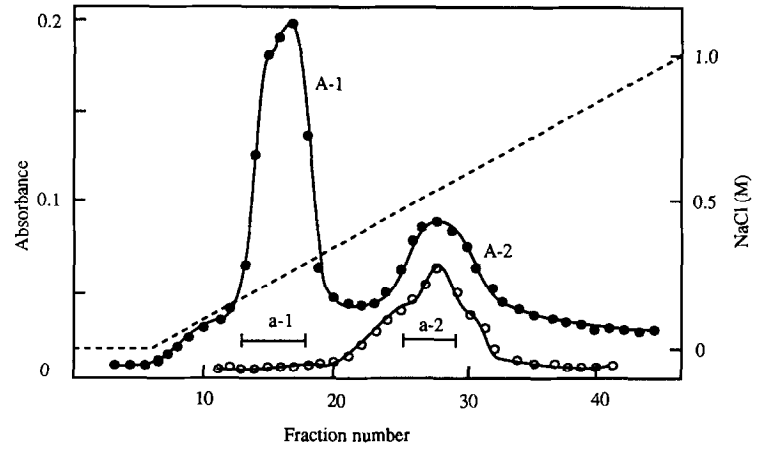

FIG. 4. Further separation of cut *a* by a DEAE-cellulose column (2.2 × 15 cm) with 10 mM phosphate (pH 7.0) and a linear gradient of 0–1.0 M sodium chloride. Flow rate, 100 ml/h. Absorbancy of each fraction (10 ml) was monitored at 230 nm (●) and 496 nm (○).

TABLE 1

Amino Acid Composition of Cut *a-1* and Cut *b*

	Cut *a*-1		Cut *b*	
	Found (theoretical for peptide I)		Found (theoretical for peptide III)	
Asp	5.0	(5)	3.0	(3)
Thr	6.5	(7)	1.4	(1)
Ser	4.5	(5)	2.0	(2)
Glu	2.1	(2)	3.8	(4)
Pro	6.9	(6)	1.5	(1)
Gly	8.8	(8)	2.7	(2)
Ala	16.0	(16)	1.8	(0)
Cys/2	0.7	(0)	0.2	(0)
Val	6.0	(6)	5.0	(6)
Met	0.0	(0)	0.1	(0)
Leu	6.8	(7)	2.3	(2)
Ile	0.0	(0)	0.3	(0)
Tyr	0.9	(1)	1.4	(2)
Phe	0.0	(0)	1.1	(1)
Trp	—	(0)	—	(1)
Lys	1.1	(1)	1.0	(1)
His	0.9	(1)	0.2	(0)
Arg	2.0	(2)	1.7	(2)
CM-Cys[a]	1.2	(2)	0.8	(1)
homo-Ser	0.3	(1)	0.3	(1)

[a] Carboxymethylcysteine.

This discrepancy could be caused by the contamination of Peptide I into Peptide III. The contamination, even if only 10%, will result in a substantial difference in the value for the alanine residue. Contamination of Peptide I into Peptide III is conceivable to some extent judging by the separation pattern in Fig. 3. Amino acid analysis data of cut *a-1* are in excellent agreement with theoretical values for Peptide I.

Estimation of the Molecular Weight of Fluorescence-Labeled SSI

The behavior of the modified SSI on Sephadex G-100 column was studied. Elution volumes for modified SSI and native SSI were compared to those for standard proteins. The modified sample emerged from the column at the same elution volume as native SSI, corresponding to a mass of 40 kDa. This value is higher than that expected from amino acid composition (23 kDa). This deviation may be attributable to the structure of SSI. The facts further support the idea that the modification did not cause appreciable differences in not only the dimeric structure of SSI but also in its gross structure and shape.

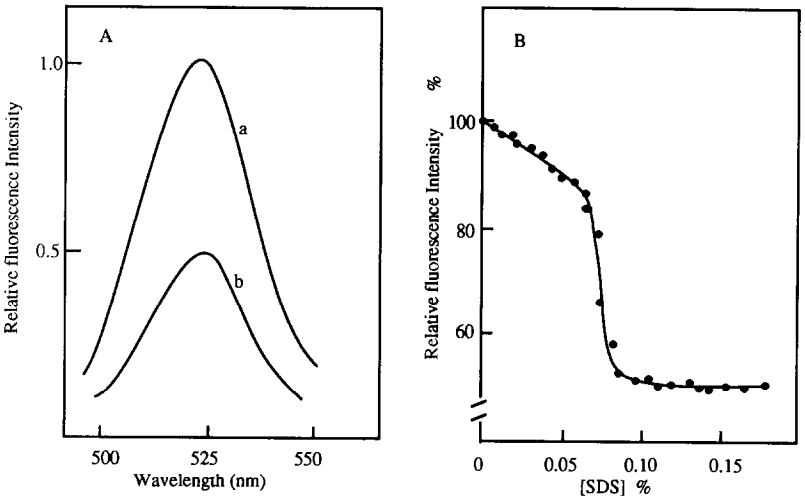

FIG. 5. Effect of SDS on the fluorescence spectra of FTC–maleyl-SSI in 20 mM phosphate (pH 7.0) at 25°C. The concentration of the modified SSI is 0.1 μM on the basis of the monomer subunit. Fluorescence spectra were measured at the excitation wavelength 496 nm. (A) Spectra in the absence (a) and presence (b) of 0.1% SDS are shown. (B) Dependence of the relative fluorescence intensity on SDS concentration was plotted.

Inhibitory Activity of Fluorescence-Labeled SSI Toward Subtilisin BPN'

The effect of the modified inhibitor on the catalytic activity of subtilisin was determined using acetyltyrosine ethyl ester and fluorescent tripeptide methylcoumarinylamide (*Z*-Gly–Gly–Leu-MCA) (*9*) as substrates. The inhibitory activity of the modified inhibitor was analyzed and found to be fully preserved. The results further support that upon modification the dimeric structure of SSI was not destroyed, since it was reported that the inhibitory activity of SSI was lost when SSI was dissociated into monomers (*4*). The stoichiometry of subtilisin and the modified dimeric SSI was determined to be 2 : 1 analyzing the residual activity of subtilisin in the presence of various concentrations of SSI. As is known, determination of very small K_i values is only possible when very low concentrations of enzyme solution are used. The sensitive fluorescent substrate, Z-Gly–Gly–Leu-MCA made it possible to carry out the kinetic analysis of subtilisin activity at concentrations as low as 2 nM. The K_i value for the modified inhibitor was determined from a Scatchard plot to be 0.7×10^{-10} M; this value is comparable to that of native SSI, 0.6×10^{-19} M.

Effect of SDS on Fluorescence Spectra of Modified SSI

Fluorescence spectra of the modified SSI are shown in Fig. 5. The addition of SDS caused a decrease in fluorescence intensity without changing the emission wavelength. In Fig. 5 the dependence of the fluorescence intensity on the SDS concentration was shown. At concentrations of 0.07–0.08% SDS, a sharp change

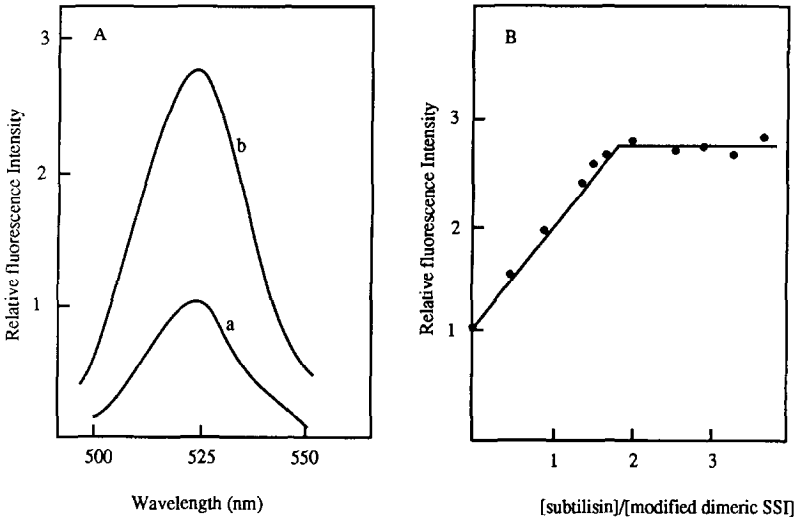

FIG. 6. Effect of subtilisin BPN′ on the fluorescence spectra of FTC–maleyl-SSI in 20 mM phosphate (pH 7.0) at 25°C. The measurement was carried out under the conditions stated in Fig. 5. (A) Spectra in the absence (a) and presence (b) of 0.2 μM subtilisin BPN′ were shown. (B) Dependence of the relative fluorescence intensity on the ratios of subtilisin and the modified dimeric SSI was plotted.

in fluorescence intensity was observed. It is known that dimeric SSI dissociates into monomers upon addition of SDS at concentrations higher than 0.08% (*10*). The result suggests that the modified SSI is dissociated by SDS in the same manner as native SSI, and the fluorescent characteristic of monomeric modified SSI is different from that of dimeric SSI.

Effect of the Binding of Subtilisin on the Fluorescence Spectra of Modified SSI

The effect of the binding of subtilisin on the fluorescence spectra of the modified SSI has been analyzed. Addition of subtilisin resulted in an increase in the fluorescence intensity of the modified SSI and no spectral shift of the emission maximum was observed. As shown in Fig. 6, the increase in fluorescence intensity was proportional to the amount of subtilisin added up to the molar ratio of subtilisin and the modified dimeric SSI, 2 : 1. The observation is in accord with the stoichiometry of the modified SSI and subtilisin determined from the catalytic activity of subtilisin. Therefore, the observed change in the fluorescence intensity was due to the specific interaction between subtilisin and the modified SSI. Crystallographic study revealed that each subunit of the dimeric inhibitor has a five-stranded antiparallel β-sheet and that the Lys-89 residue is a constituent of the β-sheet (*11*). It was also discovered that the subunit–subunit interface is formed as a stack of the β-sheets and that this region is distant from the region in contact with subtilisin. Crystallographic study further revealed that upon binding with subtilisin, the subunit of SSI undergoes a global induced-fit movement apart from the binding site, which was found to occur markedly at the β-sheet region (*12*). It may be considered

that the observed enhancement of the fluorescence intensity reflects the structural change around the Lys-89 residue as a result of the movement. Also, the destruction of the dimeric structure by SDS causes structural changes in each subunit that are reflected in the decrease in fluorescence intensity.

It is known that the fluorescein group is less useful as a reporter group since the fluorescence intensity is not significantly influenced by the polarity change of the microenvironment in which the group resides (*13*). Thus, one of possible explanations for the present observation is the change of relative positioning between the introduced fluorescein group and an intrinsic amino acid residue acting as a quencher. We have no proof of the presence of such a residue, but it would have an aromatic ring and reside near the β-sheet. Trp-86 is one of the most plausible candidates.

Interaction with Streptomyces griseus Proteases

It is known that *Streptomyces griseus* protease A (SGPA) and protease B (SGPB) are chymotrypsin-like enzymes which are inhibited very effectively by SSI (*14*). Fluorescence-labeled SSI was found to inhibit SGPA and SGPB specifically as well. It was also found that the fluorescence intensity of the modified SSI was increased by complexation with SGPA or SGPB. The stoichiometry and extent of the fluorescence enhancement were almost identical to those with subtilisin. It is interesting that SGPA and SGPB are specifically inhibited by SSI, though they are classified as proteases of the trypsin family and are structurally different from proteases of the subtilisin family such as subtilisin BPN′. A computer simulation study suggested that the structure of the contact regions of SGPA and SGPB with SSI are highly complementary to the surface structure of SSI (*14*). Present observations suggest that a conformational change in SSI by the interaction of SGPA and SBPB is taking place in a manner similar to that of subtilisin. SGPA and SGPB belong to a different family than subtilisin, and bear little resemblance to it in global structure. These three enzymes (SGPA, SGPB, and subtilisin) are, however, common in the sense that they are highly specific to SSI. We may assume that the induced conformational transitions of SSI in the interaction with SGPA, SGPB, and subtilisin could be similar and reflect the tight binding of K_i values ranging between 10^{-11} and 10^{-12} M.

EXPERIMENTAL PROCEDURES

Materials

Partially purified *Streptomyces* subtilisin inhibitor (SSI) was a gift of Professors. B. Tonomura and K. Hiromi, Kyoto University. Further purification was carried out by means of Sephadex G-100 column chromatography (eluent, 10 mM phosphate, pH 7.0) and DEAE-cellulose column chromatography (eluent, 10 mM phosphate, pH 7.0 with a linear gradient from 0 to 1 M of sodium chloride) according to the reported procedure (*15*). The concentration of the subunit of SSI was determined on the basis of the reported value, $E^{0.1\%}$ (280 nm): 0.81 (*16*). Subtilisin

BPN′ was a gift of Professor K. Hiromi. *S. griseus* protease A and protease B were a gift of Professor S. Ishii, Hokkaido University. The concentrations of these enzyme were determined by the active site titration method (*17*) using *trans*-cinnamoyl imidazole and *p*-nitrophenyl acetate,for subtilisin and *S. griseus* proteases, respectively. The titrated normalities of the active enzymes were 80, 50, and 43% for subtilisin BPN′, and *S. griseus* proteases A and B, respectively. Chymotrypsinogen A was obtained from Worthington Biochemical. Bovine serum albumin was a product of Nacarai Tesque. Carbobenzyloxydiglycyl-L-leucine-*N*-methyl-coumarinylamide was prepared according to the reported procedure (*9*). Fluorescein isothiocyanate was purchased from Dojin Kagaku Co. All other chemicals were products of Tokyo Kasei or Wako Pure Chemicals and were of analytical grade.

Modification of SSI with Maleic Anhydride

SSI (14 mg) was dissolved in 4 ml of 0.1 M phosphate buffer (pH 7.0). To this, 180 μl of a solution of maleic anhydride in dioxane (0.1 M) was added in five portions during a period of 25 min at 0°C. The reaction mixture was applied to a Sephadex G-25 column (2.2 × 22 cm) equilibrated with 20 mM ammonium bicarbonate and the protein fraction was freeze-dried.

Guanidination of Maleyl-SSI

O-Methylisourea (0.43 g) in 2.5 ml water was adjusted to pH 10.5 by the addition of sodium hydroxide solution. To this, a solution of maleyl-SSI in water (3 mg in 2.5 ml) was added and kept at 4°C for 7 days. The solution was adjusted to pH 7 with hydrochloric acid and applied to a Sephadex G-25 column (1.2 × 40 cm) equilibrated with 20 mM ammonium bicarbonate, and the protein fraction was freeze-dried.

Reductive Methylation of Guanidinated Maleyl-SSI

A guanidinated sample (1.0 mg) was dissolved in 2 ml of 2 M borate buffer (pH 9.2) containing 8 M urea and 2 mg of sodium borohydride was added. To this 1 μl of 18% aqueous formaldehyde solution was added at 4°C and the addition was reported five times at 5-min intervals. After being stirred for 30 min, the reaction mixture was applied to a Sephadex G-25 column (1.2 × 40 cm) equilibrated with 20 mM ammonium bicarbonate and the eluted protein fraction was freeze-dried.

Modification with FITC

Maleyl-SSI (8 mg) was dissolved in 4 ml of 0.1 M carbonate (pH 10.5). To this solution, FITC (3.8 mg) was added and stirred at 20°C for 3 h. The reaction mixture was separated by a Sephadex G-25 column (2.2 × 22 cm: 20 mM ammonium bicarbonate) and the protein fraction was freeze-dried. The concentration of the modified SSI was determined by the Folin–Lowry method (*18*).

Chemical Cleavage with Cyanogen Bromide

Prior to the treatment with cyanogen bromide, fluorescence-labeled SSI was subjected to reductive carboxymethylation as follows. A sample (4 mg) was dissolved in 3 ml of 0.5 M Tris (pH 8.3) containing 8 M urea and 0.2% EDTA. The reaction vessel was filled with nitrogen gas, 47 μl of 2-mercaptoethanol was added, and the solution was stirred at 20°C for 4 h. Sodium iodoacetate (138 mg) was added portion wise and kept at 20°C for 15 min protected from light. The sample was applied to a Sephadex G-25 column (20 mM ammonium carbonate, pH 8.0) and the protein fraction was freeze-dried.

The freeze-dried sample (2.3 mg) was dissolved in 0.5 ml of 70% formic acid and a solution of cyanogen bromide in 70% formic acid (6.3 mg/28 μl) was added. After being stirred for 24 h at 20°C, 9 vol of water was added and the solution was freeze-dried.

Separation of Degradated Peptides

Sephadex G-75 column: the freeze-dried sample was eluted as stated in the legend to Fig. 3. DEAE-cellulose column: freeze-dried cut *a* (Fig. 3) was applied to a DEAE-cellulose column as stated in Fig. 4.

Measurement of Inhibitory Activity

Inhibitory activity of native and modified SSI samples were determined by measuring the catalytic activity of subtilisin BPN′ toward *N*-acetyl-L-tyrosine ethyl ester. The assay was carried out at 25°C in a medium containing 0.1 M KCl and 10% dioxane using a pH-stat (Radiometer TTT2) at pH 8.0. The concentrations of the substrate and enzyme were 5 mM and 55 nM, respectively. The concentration of native or modified SSI was varied within a range of 0–30 nM (on the basis of monomer subunits).

Determination of Inhibition Constant

Subtilisin BPN′ (2.4 pmol) was dissolved in 1 ml of Tris buffer (pH 8.0) and incubated with native or modified SSI (0–2.4 eq. to subtilisin) at 25°C for 2 h. A solution of carbobenzyloxydiglycyl-L-leucine-*N*-methyl-coumarinylamide in DMF (75 μl) was added to the incubate. The final concentration of the substrate was 0.2 mM. The increase of fluorescence intensity at 460 nm was monitored at the excitation wavelength of 380 nm using a Hitachi 650–60 spectrofluorometer following the reported procedure (*9*). Inhibition constants were determined from the slope of a Scatchard plot following the method of Bieth (*19*).

SDS–Polyacrylamide Gel Electrophoresis

Prior to electrophoresis, denaturation of the protein was carried out by heating the sample solution at 100°C for 3 min. The sample was applied to a 12% SDS–polyacrylamide slab gel in 25 mM Tris–190 mM glycine (pH 8.5) containing 0.1% SDS.

The gel was stained by Coomassie brilliant blue. Electrophoretic mobilities of SSI, maleyl-SSI and FTC–maleyl-SSI increased in this order.

Amino Acid Analysis

Hydrolysis was carried out with 6 M hydrochloric acid at 110°C for 24 h in a sealed test tube and hydrolysate was analyzed on a Hitachi Model 835 amino acid analyzer.

Quantification of Amino Group

Free amino group was titrated by trinitrobenzenesulfonate (TNBS) following the method of Hynes (*20*). The molar absorption coefficient at 340 nm, 1.4×10^4 $M^{-1}cm^{-1}$, was used.

Determination of Molecular Weight

A Sephadex G-100 column (1.3 × 45 cm) was equilibrated with 20 mM ammonium carbonate buffer (pH 8.0) and standard proteins were eluted separately with a flow rate 1 ml/h. The elution volume that affords the highest absorption at 280 nm was determined. Standard proteins used were *S. griseus* protease A (MW 18,000), chymotrypsinogen A (MW 25,000) and bovine serum albumin (MW 66,000).

Measurement of Fluorescence Spectra

Labeled SSI was dissolved in 20 mM phosphate buffer (pH 7.0) to give 0.1 μM solution (based on the monomeric form). Corrected fluorescence spectra were recorded on a Hitachi 650-40 spectrofluorometer equipped was a 650-0178 data processor using rhodamine B as a photon counter. Measurement was carried out at the excitation wavelength 496 nm. Fluorescence spectra were measured after incubating the labeled SSI with the enzyme for 1 h.

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