Characteristics of Protease Inhibitor Produced by *Streptomyces fradiae* SMF9

Hyoung Tae Kim[1,2], Joo Won Suh[2,3], and Kye Joon Lee[1,2*]

[1]*Department of Microbiology, Seoul National University, Seoul 151-742, Korea,*
[2]*Research Center for Molecular Microbiology, Seoul National University, Seoul 151-742, Korea and* [3]*Institute of Biological Science, Myoung Ji University, Youngin 449-728, Korea*

(Received February 21, 1995/Accepted May 19, 1995)

***Streptomyces fradiae* protease inhibitor (SFI) was purified effectively by preparative isoelectric focusing and hydroxyapatite chromatography. The molecular weight of SFI was estimated to be 1.7 kDa by SDS-PAGE and 1.8 kDa by molecular sieving HPLC. One hundred and sixty amino acid residues were determined from which molecular weight of SFI was calculated to be 17,064 Da and carbohydrate residue was not detected. SFI was a monomeric protein with two reactive site, of which isoelectric point was pH 4.1. N-terminal amino acid sequence of SFI had homology with SSI (*Streptomyces* subtilisin inhibitor) and other protease inhibitors produced by *Streptomyces*.**

Key words: *Streptomyces fradiae* SMF9, protease inhibitor, N-terminal sequence, amino acid composition

Streptomyces subtilisin inhibitor (SSI) was the first serine protease inhibitor isolated from bacteria (13, 14) and other inhibitors were isolated from other species of *Streptomyces* (6, 16, 17, 21). All of them were dimeric proteins consisting of two identical subunits, each of which was composed of about 100 amino acids. These inhibitors inhibited proteases by forming a tightly bound inhibitor-protease complex in a molar ratio of 2 : 2 (4).

Streptomyces fradiae SMF9 produced extracellularly a protease inhibitor (SFI) (2). SFI inhibited only alkaline serine proteases including subtilisin, α-chymotrypsin and pronase E but not trypsin (2). Growth rate, dissolved oxygen tension, and pH were the important factors regulating the SFI production. The inhibitory mode of the purified SFI against α-chymotrypsin was competitive ($K_i = 5.5 \times 10^{-7}$ M) and one mole of SFI could bind two moles of α-chymotrypsin and the complex had very low dissociation constant (10).

In the present study, very effective purification method for obtaining large amount of SFI was developed and chemical characteristics of the purified protein were determined. The characters were compared with other protease inhibitors produced by *Streptomyces* spp.

*To whom correspondence should be addressed.

Materials and Methods

Microorganism and culture condition

The microorganism used in this study was *Streptomyces fradiae* SMF9, a tylosin non-producing variant of *Streptomyces fradiae* NRRL 2702 (2, 10). Stock culture medium was prepared with 0.1% (w/v) yeast extract, 0.1% beef extract, 0.2% casein hydrolysate, 1% glucose and 1.8% agar (pH 7.0). Seed culture medium was formulated with 3% glucose, 2.2% soytone, 0.3% peptone and 4% of $CaCO_3$. Main culture medium was composed of 1% glucose, 2% peptone, 0.3% yeast extract, 0.3% NaCl, 0.1% KH_2PO_4, 0.34% K_2HPO_4, 0.002% $FeSO_4 \cdot 7H_2O$, and 0.002% $ZnSO_4 \cdot 7H_2O$.

The strain was maintained by transferring on agar slopes of stock culture media each month and storing at 10℃. Spores formed on the stock culture agar medium were harvested and suspended in stock culture liquid medium containing glycerol (20%, v/v), then kept in a deep freezer (−70℃). Frozen spore suspensions were thawed in a water bath (30℃) then inoculated into 50 ml of seed culture medium. The seed culture was carried out using 500 ml baffled flasks and a rotary shaking incubator (200 rpm) for 3 d at 30℃. The seed was inoculated into the main culture medium to give a 5% (v/v) inoculum size. The main culture was performed

in 4.5 L culture vessels (Korea Fermentor Co.) where agitation speed and aeration rate were 250 rpm and 0.1 vvm, respectively. Culture temperature was maintained at 30℃ and pH was maintained at 6.8 by automatic addition of 1 N HCl or 1 N NaOH.

Purification of protease inhibitor

After 60 h of cultivation, culture broth was centrifuged (10,000 *g* at 4℃) and the cell free culture broth was concentrated by ultrafiltration (NMWL 10,000, Millipore) to 1/5 volume. Ammonium sulfate was added to the concentrated broth to make 40% saturation and the precipitate formed was separated by centrifugation (10,000 *g*, 30 min). Ammonium sulfate was added to the supernatant to make 60% saturation. The precipitate collected from 40~60% saturation was dissolved in 0.05 M phosphate buffer (pH 7.5) and loaded on gel permeation chromatography with Sephadex G-75 (Pharmacia) column (2.8×100 cm) equilibrated with the same buffer. The elution rate was 18 ml/h. The active fraction of the gel permeation chromatography was collected and loaded on ion-exchange column (5×10 cm) packed with DEAE-Sephadex A-50 (Pharmacia) and washed with 2 bed volumes of 0.05 M K-phosphate buffer (pH 7.5). The following elution was carried out with NaCl gradient (from 0 to 0.75 M) in the same buffer. Elution rate was 60 ml/h. The active fraction of the DEAE-Sephadex A-50 was collected, dialyzed and applied to preparative isoelectric focusing apparatus (Rotofor, BioRad) then ampholyte of pH 3~10 (Pharmacia) was added to give final concentration of 1%. pH gradient was formed by applying 12 W constant current for 4 h. Fractions of the preparative isoelectric focusing apparatus was dialyzed against distilled water and SFI activity was determined. Dialyzed active fraction obtained from the preparative isoelectric focusing apparatus was loaded on column (2.5×25 cm) packed with hydroxyapatite (BioRad) equilibrated with 0.005 M of Na-phosphate buffer (pH 7.0) and washed with 2 volumes of the same buffer. The following elution was carried out with the same buffer of increasing phosphate concentration from 0.005 M to 0.6 M. The flow rate was 30 ml/h.

Analytical methods

Protein concentration was determined by the dye-binding method (1). Purity of SFI was examined by SDS-polyacrylamide gel electrophoresis (15%) with discontinuous gel system (9). The protease inhibitor activity toward α-chymotrypsin was measured by the same procedure described previously (2). The activity of protease was estimated by measuring tyrosine liberated after hydrolysis of Hammarsten casein (Merck) at 37℃ for 15 min in 0.05 M K-phosphate buffer (pH 7.5). One unit of protease activity was defined as the amount of protease needed for the production of 1 μg of tyrosine per min. The protease inhibitor activity was calculated as follows: % inhibition$=100\times(A-B)/A$, where A is the protease activity without inhibitor and B is the protease activity with inhibitor. One unit of inhibitory activity was defined as the amount of inhibitor needed for the 50% inhibition of 1 unit of α-chymotrypsin (Sigma Co.).

Amino acid analysis was performed on an amino acid analyzer (PICO.TAG, Waters) after hydrolysis with 6 M HCl for 24 h at 115℃ (12). Half-cystine was determined as cysteic acid with oxidation with performic acid before hydrolysis (12). Tryptophan was determined after alkaline hydrolysis (5). N-terminal amino acids of protease inhibitor were sequenced by a modified phenylthiohydantoin (PTH) method (11, 20) by using amino acid sequencer (Prosequencer 6600, Milligen/Biosearch).

Molecular weight of denatured SFI was determined by 15% SDS-PAGE. Molecular weight of native SFI was determined by molecular sieving HPLC with Protein PAK 125 column (Waters). SFI and molecular markers: α-lactoglobulin (18,400 Da), pepsin (34,700 Da) and ovalbumin (45,000 Da), were applied to a Protein PAK 125 column on an HPLC system, and elution was carried out with 0.1 M sodium phosphate (pH 6.0) at a flow rate of 0.6 ml/min. Void volume was determined by blue-dextran (2,000,000 Da). All of size markers and blue-dextran was purchased from Sigma Co.

Isoelectric point of SFI was determined by electric focusing with preparative isoelectric focusing apparatus (Rotofor, BioRad). Mixture of ampholyte pH 2.5~5 and ampholyte pH 3~10 with ratio 9 : 1 was added to the purified SFI solution in distilled water. pHs of the focused fraction were determined with pH electrode after fractionation. SFI activity of each fraction was determined after dialysis against distilled water for 24 h.

Carbohydrate content was determined by the phenol/sulfuric acid method (3). 200 μg of purified SFI was dissolved in 200 μl of distilled water and 200 μl of phenol solution (5% v/v in water) was added with gentle mixing. 1.0 ml of concentrated sulfuric acid was added and mixture was stood for 10 min before shaking vigorously. The absorbances at 490 nm were determined after a further 30 min. Carbohydrate standard was 200 μl solution of 30 μM~2 mM of glucose in distilled water.

Results and Discussion

Purification and protein chemical characteristics

SFI was purified through gel permeation chromatography, anion-exchange chromatography, preparative isoelec-

Table 1. Purification of SFI

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)	Fold
Culture broth	583,400	7,820	74.6	100.0	1.0
Ultrafiltration	543,150	5,900	92.1	93.1	1.2
$(NH_4)_2SO_4$ precipitation	292,500	2,050	142.7	50.1	1.9
Sephadex G-75	202,700	1,390	145.8	34.7	2.0
DEAE Sephadex A-50	156,100	920	169.7	26.8	2.3
Preparative isoelectric focusing	143,300	670	213.9	24.6	2.9
Hydroxyapatite	121,100	439	276.0	20.8	3.7

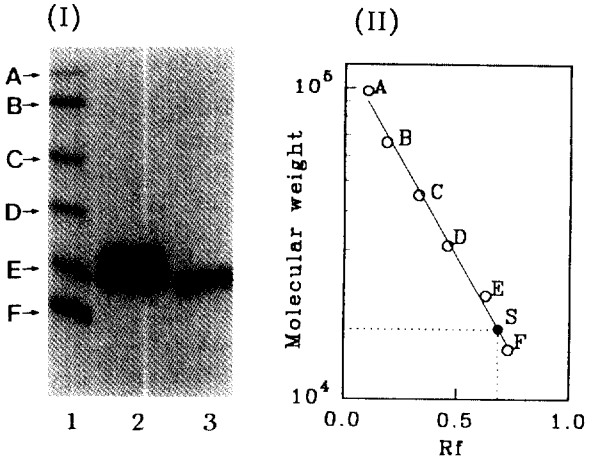

Fig. 1. SDS-polyacrylamide gel electrophoresis of SFI and molecular weight estimation. (I) Purified SFI was analyzed by SDS polyacrylamide gel electrophoresis with a gel concentration of 15%. Lane 1, molecular weight markers: rabbit muscle phosphorylase b (A. 97,400 Da), bovine serum albumin (B. 66,200 Da), hen egg white ovalbumin (C. 45,000 Da), bovine carbonic anhydrase (D. 31,000 Da), soybean trypsin inhibitor (E. 21,500 Da) and hen egg white lysozyme (F. 14,400 Da); Lane 2, active fraction of preparative isoelectric focusing; Lane 3, SFI purified by hydroxyapatite column. (II) moleuclar weight estimation. SFI (S).

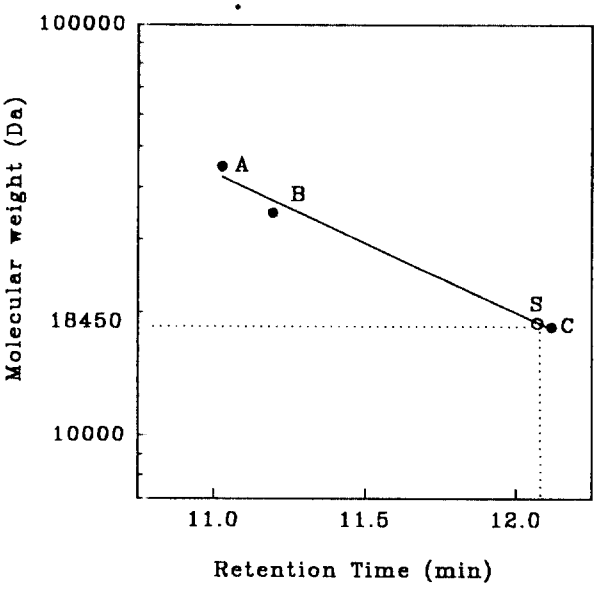

Fig. 2. Estimation of the molecular weight of native SFI by molecular sieving HPLC. SFI or molecular weight markers were applied to a Protein PAK 125 (Waters) column on an HPLC system, and elution was carried out with 0.1 M Na-phosphate (pH 6.0) at a flow rate of 0.6 ml/min. Molecular weight markers were A: ovalbumin (45,000 Da), B: pepsin (34,700 Da) and C: α-lactoglobulin (18,400 Da). S: SFI.

tric focusing, and hydroxyapatite chromatography (Table 1). Preparative isoelectric focusing and hydroxyapatite chromatography were found to be essential steps to remove the contaminated proteins which were carried with SFI in gel permeation chromatography (Sephadex G-75) and anion-exchange chromatography (DEAE-sephadex A-50) (2).

The molecular weight of the denatured SFI was estimated to be 16,800 Da as estimated by SDS-PAGE (Fig. 1), but that of native SFI was determined to be 18,450 Da by HPLC using Protein PAK 125 column (Waters) (Fig. 2). These results suggested that SFI was a monomeric protein. The isoelectric point of SFI was determined by using preparative isoelectric focusing apparatus to be pH 4.1, since SFI activity was detected only in the fraction of pH 4.1 (data not shown). That was similar to that of SSI and other subtilisin inhibitors except plasminostreptin (6). Carbohydrate content in the SFI was lower than 0.5%, indicating that SFI was not a glycoprotein.

Amino acid composition

The amino acid composition of SFI and other protease inhibitor produced by *Streptomyces* spp. are compared in Table 2. It was evident that SFI contained a relatively greater portion of isoleucine, arginine and glx (glutamic acid and glutamine) than other SSI-family protease inhibitors but only one residue of methionine and no lysine. SFI consisted of 160 amino acid residues and molecular weight calculated from amino acids composition was 17,064 Da. Hence it was thought that SFI was a monomeric protein.

Molar stoichiometry of SFI binding to α-chymotrypsin was found to be 1 : 2 (10), indicating that SFI had two reactive site per molecule. However SSI-like protease inhibitors had one reactive site to each monomer (2 : 2). Larger molecular weight of SFI compared to other SSI-like protease inhibitor might supply the space for two reactive sites.

Subtilisin inhibitors including SSI, API-2b, API-2c, and

Table 2. Amino acid compositions of SFI and other protease inhibitors isolated from various *Streptomyces* spp.

Amino acid	Protease inhibitors						
	SFI	API-2b	API-2c	SLPI	SSI	Plasminostreptin	SI-1-72
Ala	20	18	16	24	19	15	10
Val	17	13	13	11	13	13	7
Leu	12	10	9	7	9	7	7
Ile	3	1	1	0	0	0	3
Pro	13	9	8	6	8	5	10
Phe	6	2	2	3	3	5	5
Trp	2	1	1	1	1	1	0
Met	1	3	3	1	3	2	2
1/2 Cys	4	4	4	4	4	4	0
Gly	15	11	10	7	11	11	11
Ser	5	10	9	8	8	5	8
Thr	11	8	8	10	8	11	6
Tyr	4	3	3	3	3	3	3
Lys	0	1	1	1	2	3	5
Arg	9	4	4	6	4	5	10
His	3	2	2	3	2	2	9
Asx[1]	14	9	9	5	9	10	10
Glx[2]	21	8	8	7	6	7	12
Total number[3]	160	117	111	107	113	109	118
Mr[4]	17,064	11,872	11,162	10,445	11,698	11,395	13,101

SSI (*Streptomyces* subtilisin inhibitor from *S. albogriseolus* S-3253: 13, 14), Plasminostreptin (from *S. antifibrinolyticus*: 6), API-2b and API-2c (alkaline protease inhibitor from *S. griseoincarnatus*: 16, 17), SLPI (*S. lividans* protease inhibitor from *S. lividans* 66: 21), and SFI (*S. fradiae* protease inhibitor from *S. fradiae* SMF9: 2, 10, this study). SI-1-72 (*Streptomyces* sp.; 8). 1. Asx: Asparagine and aspartic acid, 2. Glx: Glutamine and Glutamic acid, 3. Total residues of amino acids, 4. Mr: Molecular weight calculated from the amino acid composition.

AP-I had methionine in their P_1 of reactive site. The inhibitory activity of SSI against subtilisin BPN' was not affected by the replacing methionine at P_1 site with polar and basic amino acid or alanine or leucine. And a mutated SSI, of which methionine at P_1 site was replaced with aromatic amino acids, showed new inhibitory activity against chymotrypsin (7), whereas the standard SSI did not inhibit chymotrypsin. Therefore, it is possible that SFI might contain tyrosine or tryptophan residue at P_1 site instead of methionine, since SFI had a strong inhibitory activity against α-chymotrypsin. Further study about this will be carried out.

Trypsin inhibitors including plasminostreptin, STI1 and STI2 had a lysine or arginine residue at the P_1 site (15, 16). And a genetically engineered SSI which contained either a lysine or an arginine residue at P_1 site instead of methionine residue also showed a new inhibitory activity to trypsin (7). Those results indicated that lysine or arginine residue at P_1 site was essential to give inhibitory activity to trypsin.

SFI had two reactive sites and one methionine in the molecule, indicating that methionine was not the residue of the P_1 in reactive site or that the structure of the two reactive sites might be different. And lysine was not the P_1 site residue of SFI, because trypsin was not inhibited by SFI (2) and lysine was absent in the SFI molecule. Therefore it was thought that SFI was different from other subtilisin inhibitor of *Streptomyces* spp. in reactive site architecture.

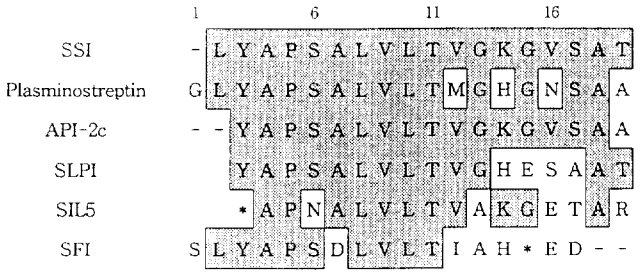

Fig. 3. Homology among N-terminal amino acid residues of the protease inhibitors from *Streptomyces* spp. Comparison was carried out for the 19 amino acid residues from aligned N-terminal. Amino acids identical to SSI were shaded. *: undetermined amino acid residue.

N-terminal amino acid sequence

N-terminal amino acid sequence of SFI was determined as Ser-Leu-Tyr-Ala-Pro-Ser-Asp-Leu-Val-Leu-Thr-

Table 3. Properties of SFI and protease inhibitors from various *Streptomyces* spp.

	API-2b	API-2C	SLPI	SSI	PS[1]	SFI
Molecular weight						
Gel filtration	36,000	32,000	19,000	27,000	26,000	18,000
SDS-PAGE	12,000	11,000	9,500	12,000	12,000	12,000
Amino acid						
Number of A.A.[2]	117	111	107	113	109	160
Lysine	1	1	1	2	3	0
Isoelucine	1	1	0	0	0	3
Methionine	3	3	1	3	2	1
1/2 cystine	4	4	4	4	4	4
NH_2-terminal	Ala	Asp	Tyr	Asp	Gly	Asp
Isoelectric point	3.4	3.3	–	4.3	6.3	4.1
Protease inhibition						
Subtilisin	+	+	+	+	+	+
α-Chymotrypsin	–	–	NT[3]	–	–	+
Trypsin	–	–	+	–	+	–

1. PS=Plasminostreptin, 2. A.A.: amino acids, 3. NT=Not determined.

Ile-Ala-His-*-Glu-Asp-····. From the comparison of N-terminal amino acid sequence to those of various protein protease inhibitor of *Streptomyces* spp., it was clear that SFI from *S. fradiae* SMF9 was closer to SSI and other SSI-family protease inhibitors (Fig. 3). Most of amino acids in N-terminal of SFI were identical to or same category with those of SSI-like protease inhibitors. In this region SFI had 60% homology with SSI and three hydrophobic residues were replaced with the same category amino acids *ie*, at 12 (Met → Ile) site, at 13 (Gly → Ala) and at 14 (Lys → His) site. But three acidic residues replaced the hydrophobic at 7 (Ala → Asp) site, 16 (Val → Glu) site and one hydrophilic amino acids at 17 (Ser → Asp) site. N-terminal amino acid analysis of SSI-like protease inhibitors showed that amino acid residues in β-strand were very conserved (18, 19), which was also conserved in SFI (4~15 site).

According to Taguchi *et al.* (18, 19) SSI-like protease inhibitors are distributed widely in streptomycetes. In their work, it was reported that *S. fradiae* Y059 also produced SSI-like protease inhibitor (SIL5) but its N-terminal amino acid sequence was different from that of SFI. And SIL5 inhibited subtilisin, trypsin but did not inhibit α-chymotrypsin activity. This result was also different from inhibition profile of SFI as compared with other inhibitors produced by *Streptomyces* presented in Table 3.

Acknowledgements

This work was supported by a research grant from the Research Center for Molecular Microbiology (RCMM) sponsored by the Korea Science and Engineering Foundation (KOSEF). We thank Korea Basic Science Center (KBSC) for amino acid analysis and N-terminal amino acid analyses.

References

1. **Bradford, M.M.,** 1976. A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **72**, 248-254.
2. **Chung, Y.H., B.K. Lee, and K.J. Lee,** 1990. Characters of protease inhibitor isolated from *Streptomyces fradiae*. *Kor. Jour. Microbiol.* **28**, 65-70.
3. **Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith,** 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **28**, 350.
4. **Hiromi, K., S. Akasaka, Y. Mitsui, B. Tonomura and S. Murao (eds),** 1985. Protein protease inhibitor—The case of *Streptomyces* Subtilisin Inhibitor (SSI). Elsevier, Amsterdam.
5. **Hugli, T.E. and S. Moore,** 1972. Determination of the tryptophan content of proteins by ion exchange chromatography of alkaline hydrolysates. *J. Biol. Chem.* **247**, 2828-2834.
6. **Kakinuma, A., H. Sugino, N. Moriya, and M. Isono,** 1978. Plasminostreptin, a protein proteinase inhibitor produced by *Streptomyces antifibrinolyticus*. *J. Biol. Chem.* **253**, 1529-1537.
7. **Kojima, S., Y. Nishiyama, I. Kumagai, and K. Miura,** 1991. Inhibition of subtilisin BPN' by reaction site P1 mutants of *Streptomyces* subtilisin inhibitor. *J. Biochem.* **109**, 377-382.
8. **Kourteva, Y. and R. Boteva,** 1989. A novel extracellular subtilisin inhibitor produced by a *Streptomyces* sp. *FEBS Lett.* **247**, 468-470.
9. **Laemmli, U.K.,** 1970. Cleavage of structural proteins dur-

ing assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

10. **Lee, B.K., Y.H. Chung, and K.J. Lee,** 1990. The fermentation kinetics of protease inhibitor production by *Streptomyces fradiae*. *Kor. Jour. Microbiol.* **28**, 264-267.
11. **Levy, W.P.,** 1981. Manual Edman sequencing techinques for proteins and peptides at the nanomol level. In *Methods in Enzymol.* Prestka, S. (ed.). Academic Press Inc., New York, **79**. 27-31.
12. **Moore, S.J. and W.H. Stein,** 1963. Chromatographic determination of amino acids by the use of automatic recording equipment. In *Methods in Enzymol.* Colowick, S.P. and N.O. Kaplan (eds.). Academic Press Inc., New York, **6**, 819-831.
13. **Murao, S. and S. Sato,** 1972. S-SI, a new alkaline proteinase inhibitor from *Streptomyces albogriseolus* S-3252. *Agric. Biol. Chem.* **36**, 160-163.
14. **Sato, S. and S. Murao,** 1973. Isolation and crystallization of microbial alkaline protease inhibitor, S-SI. *Agric. Biol. Chem.* **37**, 1067-1074.
15. **Strickler, J.E., T.M. Berka, J. Gorniak, J. Fornwald, R. Keys, J.J. Rowland, M. Rosenberg, and D.P. Taylor,** 1992. Two novel *Streptomyces* protein protease inhibitors. *J. Biol. Chem.* **267**, 3236-3241.
16. **Suzuki, K., M. Uyeda, and M. Shibata,** 1978. API-2c, a new alkaline protease inhibitor produced by *Streptomyces griseoincarnatus* strain No. KTo-250. *Agric. Biol. Chem.* **42**, 1539-1543.
17. **Suzuki, K., M. Uyeda, and M. Shibata,** 1981. Partial amino acid sequence of an alkaline protease inhibitor, API-2 (b and c). *Agric. Biol. Chem.* **45**, 629-634.
18. **Taguchi, S., H. Kikuchi, M. Suzuki, S. Kojima, M. Terabe, K. Miura, T. Nakase, and H. Momose,** 1993a. *Streptomyces* subtilisin inhibitor-like proteins are distributed widely in Streptomycetes. *Appl. Environ. Microbiol.* **59**, 4338-4341.
19. **Taguchi, S., H. Kikuchi, S. Kojima, I. Kumagai, T. Nakase, K. Miura, and H. Momose,** 1993b. High frequency of SSI-like protease inhibitors among *Streptomyces*. *Biosci. Biotech. Biochem.* **57**, 522-524.
20. **Tarr, G.E.,** 1977. Improved manual sequencing methods. In *Methods in Enzymol.* Hirs, C.H.W. and S.N. Timasheff (eds). New York, Academic Press, **47**, 335-337.
21. **Ueda, Y., S. Kojima, K. Tsumoto, S. Takeda, K. Miura, and I. Kumagai,** 1992. A protease inhibitor produced by *Streptomyces lividans* 66 exhibits inhibitory activities toward both subtilisin BPN' and trypsin. *J. Biochem.* **112**, 204-211.