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An Endogenous Target Protease, SAM-P26, of *Streptomyces* Protease Inhibitor (SSI): Primary Structure, Enzymatic Characterization, and Its Interaction with SSI[1]

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We have been focusing on the potent involvement of the molecular interaction between a protease and a protease inhibitor in the physiological or morphological regulation of *Streptomyces* cells producing them [Taguchi *et al.* (1995) *J. Bacteriol.* 177, 6638-6643; Suzuki *et al.* (1997) *J. Bacteriol.* 179, 430-438]. In this study, an extracellular protease, termed SAM-P26, was isolated as a target of endogenous protease inhibitor (SSI) from the culture medium of an SSI non-producing mutant strain derived from *Streptomyces albogriseolus* S-3253. Complete amino acid sequence determination revealed that SAM-P26 is identical to a protein encoded by the SAM-P20D gene, which was previously found to be located downstream of the gene for SAM-P20, another target protease of SSI. Based on the sequence homology, SAM-P26 was categorized as a member of the chymotrypsin family like SAM-P20. Sequence similarity between SAM-P26 and SAM-P20 was immunologically demonstrated by Western blot analysis using anti-SAM-P20 antiserum. The molecular mass (26 kDa) of SAM-P26 estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was much higher than that calculated from the amino acid sequence of SAM-P26 (18,376.8 Da) and that of the *S*-pyridylethylated form (18,808.4 Da) of SAM-P26 determined by Matrix-assisted Laser Desorption/Ionization-Time of Flight/Mass Spectrometry. Analytical gel-filtration analysis revealed that SAM-P26 exists as a monomer (18.8 kDa) in the native state. The results as to substrate specificity and inhibitor sensitivity indicated SAM-P26 exhibits chymotrypsin-like activity. For the proteolytic activity, the optimal pH was 10.5 and the optimal temperature was 60°C. The complex formation of SAM-P26 with SSI was confirmed by native-PAGE analysis.

Key words: chymotrypsin-type protease, complex formation, endogenous target proteases, gene duplication, *Streptomyces* proteinaceous protease inhibitor.

It is generally considered that mammalian protease inhibitors modulate a variety of critical protease-mediated processes such as blood clotting cascades, the cell cycle, activation of precursor proteins and developmental processes (*1-3*). In *Streptomyces*, extracellular serine proteases were reported to coordinately regulate cellular protein turnover associated with secondary metabolism and morphogenesis (*4, 5*). Among various mechanisms related to the regulation of protease activities, inhibition of the activities by protease inhibitors seems to be direct and effective (*3, 6, 7*). Previously, we discovered that proteinaceous protease inhibitors, which are homologous to subtilisin inhibitor SSI (*8*), are distributed widely as a family (termed SIL proteins) in *Streptomyces* (*9-12*). In addition, a strong correlation was found to exist between the structure around the reactive center region of SIL proteins and their inhibition specificities toward proteases (*13-16*).

During the course of investigating the biological significance of SIL proteins, we have identified at least three endogenous target proteases of SSI in the culture supernatant of an SSI non-producing mutant strain (M1) derived from *Streptomyces albogriseolus* S-3253 using an SSI-bound affinity column (*17*). The 20-kDa protease termed SAM-P20 is the smallest member of the chymotrypsin family and its proteolytic activity was found to be stoichiometrically inhibited by tight complex formation with SSI (*18*). The 45-kDa target protease termed SAM-P45 is characteristic in that it can be categorized as a novel member of the subtilisin family like mammalian proprotein convertases such as human furin (*19*), but prefers basic amino acids to aromatic or aliphatic amino acids. In the present study, we isolated the third target protease, termed SAM-P26, from the culture supernatant of the M1 mutant strain in order to perform a comparative study with other SSI target proteases, SAM-P20 and SAM-P45. The results indicate that the amino acid sequence of this protease is completely identical to that deduced from the

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nucleotide sequence of the SAM-P20D gene, which was found downstream of the SAM-P20 gene (*20*). The difference between the molecular mass calculated from the sequence and that determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis is also discussed on the basis of the data obtained on MALDI-TOF-MS and gel-filtration FPLC.

MATERIALS AND METHODS

Materials and Bacterial Strain—The proteolytic enzymes for peptide mapping, *i.e.* trypsin (TPCK-treated), endoproteinase Asp-N and *Staphylococcus aureus* V8 protease, were purchased from Sigma, Takara Shuzo and Boehringer Mannheim, respectively. All the synthetic substrates listed in Table II were products of Sigma. Protease inhibitors were purchased as an assay kit from Boehringer Mannheim. DEAE-cellulose (type II) and Sephacryl S-200 (High Resolution) were from Wako Pure Chemical and Pharmacia LKB, respectively. All other chemicals were of the best grade for biochemical research. SSI non-producing mutant strain M1 (*17*) derived from *Streptomyces albogriseolus* S-3253 was used as the source for the isolation of target proteases of SSI.

Identification of Proteins Interacting with SSI—Affinity chromatography involving SSI as a ligand was used to identify potent proteins interacting with SSI. First, approximately 1 mg of purified SSI was immobilized on a 0.5-ml cyanogen bromide-activated Sepharose 4B column by means of a coupling reaction. The column (0.7×1.5 cm) of SSI-bound Sepharose 4B was preequilibrated with 0.1 M phosphate buffer (pH 7.0). The culture supernatant of strain M1 was concentrated as a starting sample for application to the affinity column by salting out with 80% saturated ammonium sulfate. After this concentrated sample had been applied to the column, it was washed with a sufficient volume of the same phosphate buffer. The proteins adsorbed were eluted with 5 ml of 0.01 M HCl (pH 2.5) and then rapidly neutralized with 1 M NaOH. Aliquots of the eluted proteins were separated by SDS-PAGE (with an 18.8% polyacrylamide gel). The electrophoresed gel was washed with 10 mM CAPS (cyanohexylaminopropanesulfonic acid) buffer (pH 11.0) and 10% methanol, and then subjected to electroblotting (380 mA, 120 min, 4°C) onto a highly hydrophobic polyvinylidene difluoride membrane (Immobilon-P transfer membrane) by the method of LeGendre and Matsudaira (*21*). The blotted proteins were stained with Coomassie Brilliant Blue R-250, and the stained bands corresponding to SSI-interacting proteins were cut out from the polyvinylidene difluoride membrane and directly subjected to amino acid sequencing.

Purification of SAM-P26—A spore suspension of the SSI non-producing mutant strain derived from *S. albogriseolus* S-3253 was inoculated into 200 ml of YEME medium (*11*) in each batch (total 25 Erlenmeyer's flasks), followed by cultivation for 7 days at 30°C. Cells were removed by filtration with Whatman 3MM chromatography paper and then ammonium sulfate was added to approximately 5 liters of the filtrated culture medium to give 80% saturation. The precipitate was collected by centrifugation, dissolved in 20 mM Tris-HCl (pH 7.5), and then dialyzed against the same buffer for 2 days. The dialysate was applied to a DEAE-cellulose column (3×45 cm). Subsequently, the protein was eluted by applying a linear gradient of NaCl, from 0 to 0.5 M, in the same buffer. The fractions containing SAM-P26 were pooled, dialyzed against distilled water and then lyophilized. The lyophilisate was dissolved in 5 ml of 0.1 M Tris-HCl (pH 7.0) and 0.5 M NaCl, applied to a size exclusion column (TOSOH TSK-GEL 2000SWXL 0.1 Å), and then eluted with the same buffer using a HPLC system (Gilson). The fractions containing SAM-P26 were dialyzed against 20 mM phosphate buffer (pH 6.0) and then rechromatographed on another DEAE-cellulose column (3×15 cm) using the same linear NaCl gradient program as for the first chromatography.

Polyacrylamide Gel Electrophoresis (PAGE)—Sodium dodecyl sulfate (SDS)-PAGE was carried out according to the method of Laemmli (*22*) with a gel concentration of 18.8%. The protein samples were precipitated with trichloroacetic acid at a final concentration of 8% before loading to prevent auto-proteolysis of the protease during the processes of denaturation and electrophoresis. Native-PAGE was performed with a 10% polyacrylamide gel in the same manner, except that the heat treatment, and the addition of SDS and the reducing reagent were omitted. Proteins in the gel were stained with 2% (w/v) Coomassie Brilliant Blue R-250.

Immunological Analysis—The homolog of SAM-P26, SAM-P20 (*20*), was once inactivated with phenylmethanesulfonyl fluoride and was raised in a rabbit. The prepared anti-SAM-P20 antiserum was used for Western blot analysis as described previously (*23*).

S-Pyridylethylation of Cystine Residues and Enzymatic Digestion for Peptide Mapping—The cystine residues of the purified SAM-P26 were reduced with dithiothreitol and then *S*-pyridylethylated with 4-vinylpyridine under the same conditions as described previously (*14*), and the modified SAM-P26 was purified by reverse-phase HPLC on a C4 column (Asahipak C4P-50, 4.6×150 mm). Portions of 5 nmol of the *S*-pyridylethylated SAM-P26 were digested with trypsin or endoproteinase Asp-N at 37°C overnight at an enzyme/substrate ratio of 1:100 in 1 M urea/50 mM Tris-HCl/1 mM $CaCl_2$ (pH 8.2) or 0.5 M urea/50 mM sodium phosphate (pH 8.0), respectively. Digestion of the peptide with *S. aureus* V8 protease was carried out at 37°C for 4 h at an enzyme/substrate ratio of 1:100 in 0.1 M NH_4HCO_3 (pH 7.8). The digested peptides were separated by reverse-phase HPLC on a C18 column (L-column ODS, 4.6×150 mm), and designated by a serial number, in order of elution on HPLC, and a prefix letter representing the type of digestion: T, trypsin; D, endoproteinase Asp-N; V, *S. aureus* V8 protease.

Amino Acid Sequence Analysis—The amino acid sequences of the modified SAM-P26 and its digested peptides were analyzed using an Applied Biosystems model 476A protein sequencer.

Molecular Mass Estimation by Gel-Filtration—Analytical gel-filtration to estimate the molecular mass in the native state was performed on a Superdex 75 pg column (1×35 cm) in an FPLC system. The eluent was 0.1 M Tris-HCl/0.5 M NaCl (pH 7.5) and the flow rate was 0.7 ml/min. The molecular mass markers used were bovine pancreatic trypsin inhibitor (6.5 kDa), cytochrome *c* (12.5 kDa), soybean trypsin inhibitor (20.1 kDa), and bovine serum albumin (69.0 kDa).

Mass Spectrometric Analysis—Matrix-assisted Laser Desorption/Ionization-Time of Flight/Mass Spectrometry (MALDI-TOF-MS) was performed with a Bruker Reflex II (Bruker, Bremen, Germany) laser desorption time of flight mass spectrometer operating in the positive linear mode with pulsed ion extraction. The purified peptide samples (1 μl for each one) were deposited on a thin layer of α-cyano-4-hydroxycinnamic acid matrix (in 33% acetonitrile containing 0.07% CF_3CO_2H) made by fast evaporation of a saturated solution in acetone. One microliter of this peptide matrix solution was transferred to the stainless steel target. The droplet was allowed to dry under gentle vacuum before introduction into the mass spectrometer. Mass spectra were obtained by averaging 150 shots. External mass calibration was provided by the $[M+H]^+$ ion and $[M+2H]^{2+}$ of carbonic anhydrase II (29,024.74 Da). The analytical precision of the system was 0.03% in molecular mass.

Protein Concentration—The concentration of SAM-P26 was determined by the method of Bensadoun and Weinstein (*24*) with bovine serum albumin as a standard. The SSI subunit concentration was determined spectrophotometrically with a molar absorption coefficient constant (ε_{280nm}) of 9,330 M^{-1}.

Enzyme Activity Assay—The activity of SAM-P26 recovered at each purification stage was measured by monitoring the change in A_{410nm} caused by the release of *p*-nitroaniline due to the enzymatic hydrolysis of succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (sAAPF*p*NA). All the synthetic substrates (Sigma) used for the measurement of the substrate specificity of SAM-P26 are listed in Table II. Each hydrolysis reaction was started by adding 20 nM SAM-P26 to a solution comprising 100 μM substrate, 100 mM Tris-HCl (pH 8.5), 0.1% dimethylsulfoxide, and 10 mM $CaCl_2$, to give a final volume of 1.0 ml. Within 1 min of reaction at 25°C, the absorbance of each liberated chromogenic group was measured at each specific wavelength. The specific activity of SAM-P26 was expressed as the amount (in micromoles) of the chromogenic group liberated by 1.0 mg of SAM-P26 in 1 s under the above-described conditions.

Protease Inhibitor Sensitivity—The species and concentrations of the protease inhibitors used here are presented in Table III. All assays were performed with 100 μM sAAPF*p*NA as the substrate by the procedure described above.

Effects of Temperature and pH on Enzyme Activity—The proteolytic activity of the purified SAM-P26 was assayed at different temperatures (1 to 75°C) using sAAPF*p*NA with a temperature-controlled cuvette holder attached to a recirculating water bath. For each assay, the buffer containing the substrate was warmed for 5 min prior to addition of the enzyme. The effect of adding 10 mM calcium ions was also examined at the same time. The effect of pH on sAAPF*p*NA hydrolysis was measured with the following buffers at 100 mM, supplemented with 10 mM $CaCl_2$: citric acid (pH 3.0 to 5.5), sodium phosphate (pH 6.0 to 7.0), Tris-HCl (pH 7.5 to 8.5), and glycine-NaOH (pH 9.0 to 11.0). Assays at different pHs were carried out at 25°C.

RESULTS

Identification of Proteins Interacting with SSI—With the SSI-bound affinity column, four SSI-interacting proteins in the extracellular fraction of SSI non-producing mutant strain M1 could be detected on the gel, as shown in Fig. 1. Their molecular masses were 20, 26, 40, and 45 kDa. Under stringent conditions such as elution with 10 mM HCl, SSI was found to be coeluted with the adsorbed proteins. This suggests high binding affinity between the target proteins and SSI. Among them, the two stained proteins with molecular masses of 20 and 45 kDa have been demonstrated to be endogenous target proteases (termed SAM-P20 and SAM-P45, respectively) of SSI at the molecular level (*18, 19*). In this study, we tried to identify the third candidate target SSI-interacting protease corresponding to the 26 kDa band detected on the gel. Protein samples separated on the gel were subsequently electroblotted onto a polyvinylidene difluoride membrane (data not shown), and then cut out and directly subjected to N-

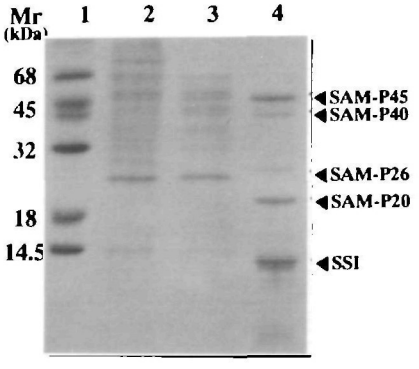

Fig. 1. **Identification of endogenous target proteins of SSI.** SDS-PAGE analysis at different target protein purification steps was performed. Lane 1, size marker proteins (bovine serum albumin, 68,000; oval- bumin, 45,000; carbonic anhydrase, 32,000; β-lactoglobulin, 18,000; lysozyme, 14,500); lane 2, culture supernatant sample of strain M1; lane 3, ammonium sulfate-precipitated sample; lane 4, sample purified by affinity chromatography on an SSI-bound Sepharose 4B column. Proteins interacting with SSI detected on the gel are designated as SAM-P20, SAM-P26, SAM-P40, and SAM-P45 according to their molecular masses, as indicated by arrows.

TABLE I. **Purification steps for SAM-P26.**

Purification step	Volume (ml)	Total amount (mg)	Total activity (units)	Specific activity (units/mg)	Activity recovery (%)
Culture supernatant	4,600	740	1100	1.5	100.0
Precipitated by $(NH_4)_2SO_4$	200	150	460	3.1	42.0
DEAE-cellulose (1st)	250	53	430	8.1	39.0
Gel-filtration	20	4.4	170	38.6	15.0
DEAE-cellulose (2nd)	54	2.1	90	42.8	8.2

One unit of activity equals 1.0 μmol of *p*-nitroaniline released from Suc-L-Ala-L-Ala-L-Pro-L-Phe-*p*-NA per minute.

terminal amino acid sequence analysis. The first 22 amino acid residues of this protein sequence were determined to be IAGGDAITGNGGRCSLGFNVTK. This sequence is significantly homologous to that of the N-terminus of SAM-P20 and completely identical to that deduced from the nucleotide sequence of the putative N-terminal of the SAM-P20D gene product (*20*). Here we designated this 26 kDa protein as SAM-P26.

Purification of SAM-P26—Purification was carried out in order to characterize the SAM-P26 protein, particularly in comparison with SAM-P20. The purification processes for SAM-P26 are summarized in Table I. By a combination of salting out of proteins with ammonium sulfate and several column chromatographies, SAM-P26 was purified approximately 28.5-fold, in specific activity, from the culture medium (4.6 liters) with an activity recovery of 8.2%. The homogeneity of the final preparation was confirmed by SDS-PAGE (data not shown). The final amount of SAM-P26 was approximately 2.1 mg. Therefore, the production level of SAM-P26 was calculated to be greater than 5.4 mg/liter of culture medium.

Complete Amino Acid Sequence of SAM-P26—Initially, the amino acid sequence of the purified *S*-pyridylethylated SAM-P26 was determined from its amino terminus up to the 28th residue, as shown in Fig. 2. Then the modified SAM-P26 was digested with trypsin, and six peptides were isolated by reverse-phase HPLC. Their amino acid sequences were determined, and the sequences of five peptides, the exception being peptide T6, could be determined up to their carboxyl termini, as shown in Fig. 2. The sequence of peptide T6 was determined up to the 33rd residue, the remaining sequence being revealed by sequence analysis of peptide T6/V1 obtained on digestion of peptide T6 with *S. aureus* V8 protease. The amino acid sequence of the undigested SAM-P26 revealed the order, T1-T2-T4, in the amino-terminal region. To obtain the connectivities of the tryptic peptides, *S*-pyridylethylated SAM-P26 was digested with endoproteinase Asp-N, and sequence analysis of peptides D8, D10, and D4 confirmed the connectivities of T4-T3, T3-T5, and T5-T6, respectively, as shown in Fig. 2. Peptide T6, which does not possess Lys and Arg residues, was thus concluded to be a carboxy-terminal peptide.

From these results, it was concluded that SAM-P26 is identical to a protein encoded by the SAM-P20D gene, which was found downstream of the SAM-P20 gene (*17*). SAM-P26 exhibited significant similarity in the mature region to those of SAM-P20 (60.0% identity) and *S. griseus* proteases (*25*), SGPA (57.6% identity), SGPB (59.0% identity), and SGPD (73.7% identity) as shown by the sequence alignment in the previous paper (*20*). Based on the primary sequence, SAM-P26 was categorized as a member of the chymotrypsin superfamily, like SAM-P20. The tertiary structural similarity between SAM-P26 and SAM-P20 was supported by the finding that the anti-

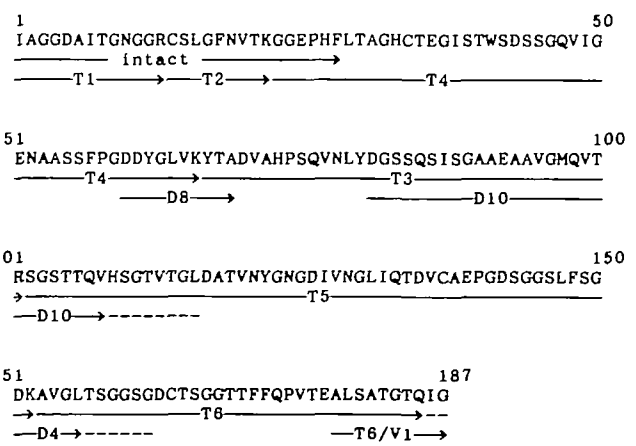

Fig. 2. **Complete amino acid sequence of and sequencing strategy for the purified SAM-P26.** Arrows show the amino acid sequence identified with the sequencer, and dashed lines indicate the remaining regions. Peptides are designated by a serial number prefixed by a letter which represents the type of digestion. Abbreviations for digestion types: V, *Staphylococcus aureus* V8 protease; T, trypsin; D, endoproteinase Asp-N.

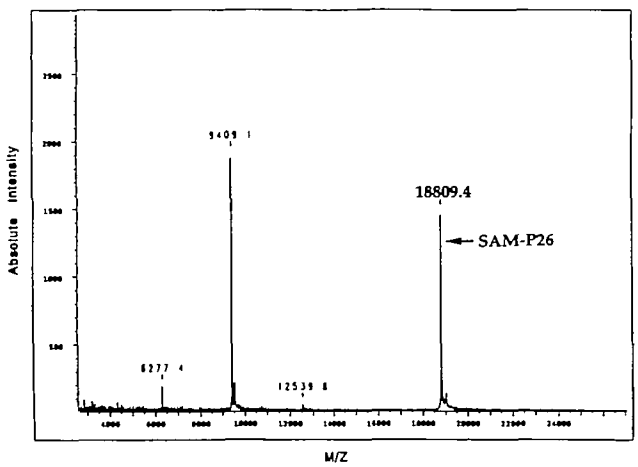

Fig. 3. **Mass spectrometric analysis.** *S*-Pyridylethylated SAM-P26 was subjected to MALDI-TOF-MS and then analyzed by the procedure described under "MATERIALS AND METHODS." The peak with 9,409.1 mass and that with 18,809.4 mass are derived from *S*-pyridylethylated SAM-P26s protonated with two protons and one proton, respectively.

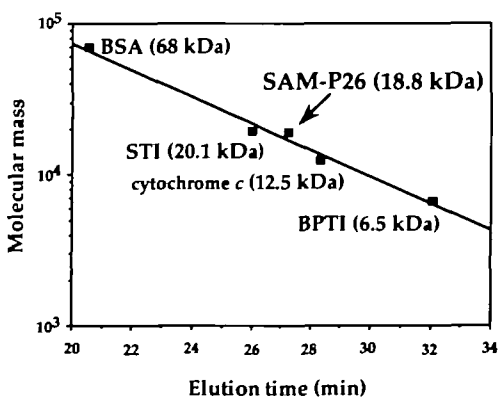

Fig. 4. **Estimation of the molecular mass of SAM-P26 by gel-filtration.** SAM-P26 or molecular mass markers were applied to a Superdex 75 pg column (1×35 cm) in an FPLC system, and elution was carried out under conditions described under "MATERIALS AND METHODS." The molecular mass markers used were bovine pancreatic trypsin inhibitor (6.5 kDa), cytochrome *c* (12.5 kDa), soybean trypsin inhibitor (20.1 kDa), and bovine serum albumin (68.0 kDa).

SAM-P20 antiserum was cross-reactive with SAM-P26 (data not shown).

Molecular Mass of SAM-P26—At first, we estimated the molecular mass of SAM-P26 in the denatured state to be approximately 26 kDa by SDS-PAGE using five standard proteins (data not shown). On the other hand, this molecular mass was not coincident with the value (18,376.8) calculated from the determined sequence of SAM-P26. The *S*-pyridylethylated form of SAM-P26 was subjected to mass spectral analysis to determine the actual molecular mass of SAM-P26. The mass determined for the *S*-pyridylethylated form of SAM-P26 (18,808.4 Da, Fig. 3) was 431.6 Da higher compared to the mass (18,376.8 Da) calculated from the amino acid sequence of the native SAM-P26, indicating that the four cysteines might be engaged in two intramolecular disulfide bridges and that there is no additional modification such as glycosylation in SAM-P26. The molecular mass in the native state was estimated to be 18.8 kDa from the results of analytical gel-filtration (Fig. 4). Thus, it was concluded that SAM-P26 exists in a monomeric form with no modification.

Comparison in the Substrate Specificity of SAM-P26 with Those of SAM-P20 and SAM-P45—The specificity of SAM-P26 as to synthetic substrates was examined with a series of chromogenic substrates possessing P1 sites of Phe, Arg, Leu, Lys, Ala, and Glu, respectively, as listed in Table II. A similar tendency in substrate specificity was observed for SAM-P26 and SAM-P20, as expected from their high structural homology to each other, in contrast with SAM-P45. SAM-P26 exhibited high hydrolytic specificity to sAAPF*p*NA among the substrates used the same as SAM-P20 did.

Effects of Protease Inhibitors on SAM-P26 Activity—The inhibition profile of SAM-P26 with various protease inhibitors was examined using sAAPF*p*NA, as shown in Table III. SAM-P26 was strongly inhibited by SSI, chymostatin, and PMSF, and weakly by leupeptin and antipain, but not at all by aprotinin, EDTA, phosphoramidon, bestatin, or pepstatin, which are specific inhibitors of serine proteases, metalloproteases, aminopeptidases, and aspartic proteases, respectively. Trypsin inhibitor of egg white was effective for SAM-P26, being consistent with the fact that SAM-P26 preferably cleaves Arg at the P1 site of the substrate (Table III). TPCK, a well-known specific inhibitor for chymotrypsin, caused non-perfect inhibition of SAM-P26 (42.9%), as for SAM-P20 (22.2%). The inhibition sensitivity of SAM-P26 toward PMSF, E-64, and leupeptin were different from those for SAM-P20.

Optimal pH and Optimal Temperature for SAM-P26 Activity—SAM-P26 was most active at around pH 10.5 for the hydrolysis of sAAPF*p*NA, indicating that SAM-P26 is a typical alkaline serine protease. The optimum temperature for its proteolytic activity was approximately 60°C in both the absence and presence of 10 mM calcium ions (data not shown). This result differs from the fact that an enhancing effect of calcium ions on the thermal stability was observed for SAM-P20 (*18*).

In Vitro Complex Formation of SAM-P26 with SSI—To determine whether or not SAM-P26 is a potent target protease of SSI, the interaction of SAM-P26 with SSI was analyzed by native-PAGE under the condition that SAM-P26 was mixed with SSI in an excess molar ratio (5:1) of

TABLE II. **Comparison of the substrate specificities of proteases interacting with SSI.**

Substrate	Specific activity (μmol·s^{-1}·mg^{-1}[%]) SAM-P26	SAM-P20	SAM-P45
P1 site			
Suc-L-Ala-L-Ala-L-Pro-L-**Phe**-*p*-NA	7.3(100)	41(100)	1.2(15.2)
Tos-L-Phe-L-Val-L-**Arg**-*p*-NA	5.0(68.8)	11(26.8)	3.3(43.3)
Suc-L-Ala-L-Ala-L-Pro-L-**Leu**-*p*-NA	0.7(9.1)	11(26.8)	0.4(5.2)
Suc-L-Gly-L-Pro-L-**Arg**-*p*-NA	0.4(5.2)	N.T.	2.7(35.2)
Suc-L-Gly-L-Pro-L-**Lys**-*p*-NA	0.4(5.2)	0.42(1.0)	7.7(100)
Suc-L-Ala-L-Ala-L-Val-L-**Ala**-*p*-NA	0.1(1.3)	1.9(4.6)	0.08(1.0)
Tos-L-Leu-L-Leu-L-**Glu**-*p*-NA	0.1(1.3)	N.T.	0(0)
Nα-Benzoyl-L-**Arg**-*p*-NA	0(0)	N.T.	0(0)

Abbreviations: Suc, *N*-succinyl; Tos, *N*-*p*-tosyl; NA, nitroanilide; N.T., not tested. Amino acid residues in bold letters correspond to the P1 site. The kinetic parameters, k_{cat}/K_m, of SAM-P20 and SAM-P45 for the most preferable substrates, AAPF and GPK, were 170 mM^{-1}·s^{-1} and 50 mM^{-1}·s^{-1}, respectively (*18, 19*).

TABLE III. **Inhibitor sensitivity of proteases.**

Protease inhibitor	Concentration	Residual activity (%) SAM-P26	SAM-P20	SAM-P45
SSI	500 nM	1.8	0.0	34.5
Aprotinin	500 nM	100.0	100.0	85.5
Chymostatin	50 μM	0.9	0.0	1.8
PMSF	50 μM	5.4	87.2	69.1
TPCK	50 μM	57.1	77.8	98.2
TLCK	50 μM	95.5	100.0	90.9
Pefabloc SC (6)	50 μM	63.4	85.8	92.7
EDTA	5 mM	100.0	100.0	7.3
Phosphoramidon	50 μM	100.0	100.0	87.3
Bestatin	50 μM	81.3	100.0	85.5
E-64	50 μM	57.1	93.8	85.5
Leupeptin	50 μM	38.4	64.2	31.6
Antipain-dihydrochloride	50 μM	22.3	35.2	7.3
Pepstatin	50 μM	100.0	100.0	92.7
Trypsin inhibitor (soybean)	500 nM	50.9	–	90.0
Trypsin inhibitor (egg white)	500 nM	16.1	–	76.4

Pefabloc SC(6) is an inhibitor of serine proteases such as trypsin and chymotrypsin.

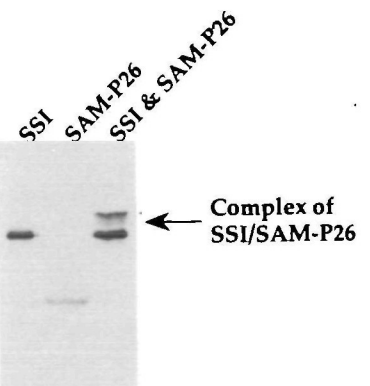

Fig. 5. **Analysis of the interaction of SAM-P26 with SSI.** SAM-P26 was mixed with SSI in the molar ratio of approximately 1:5 (protease:SSI subunit) in the buffer solution (100 mM Tris-HCl (pH 8.5) and 10 mM $CaCl_2$), and then subjected to native-PAGE. Lane 1, SSI; lane 2, purified SAM-P26; lane 3, SAM-P26 and SSI. The black arrow indicates the protein band corresponding to the complex of SAM-P26 with SSI.

SSI to SAM-P26 for 30 min. As shown in Fig. 5, a band corresponding to the expected band for the complexed form of SAM-P26 and SSI (lane 3) was detected in the upper part of the gel than that for the SSI free from the SSI/SAM-P26 complex.

DISCUSSION

To understand the physiological significance of proteinaceous protease inhibitor SSI, the studies on endogenous target proteases of SSI are essential as well as a gene disruption experiment on SSI. Our search for SSI-interacting proteases was triggered by the observation that the deletogenic loss of the SSI gene from the terminal region of the chromosome (*26*) causes a remarkable increase in extracellular activity and/or productivity protease(s) (*17*). At the same time, marked decreases in the growth rate and aerial mycelium formation were observed for all SSI non-producing mutant strains, *i.e.* including M1 (*17*). The use of an SSI-bound affinity column was effective for identification of target SSI-interacting proteases. In fact, SAM-P20 and SAM-P45 were isolated and proved to be targets for SSI from their *in vitro* interactions with SSI (*18, 19*). In this study, the third target, SAM-P26, was purified and characterized.

In the initial stage of the present study, from the sequence data, we were interested in clarifying whether or not SAM-P26 is identical to a putative protein encoded by the SAM-P20D gene, which was identified as a homolog of the SAM-P20 gene (*20*). The completely identical sequence of the N-terminal 22 residues of both SAM-P26 and the putative SAM-P20D gene product suggested that the two proteins are the same, but could not account for the large difference in molecular mass between the 26 kDa for SAM-P26 estimated on SDS-PAGE and the 18,376.8 Da for the SAM-P20D gene product calculated from its nucleotide sequence. As the result of peptide mapping of the purified SAM-P26, SAM-P26 was proved to be the SAM-P20D gene product (Fig. 2). Mass spectrometry and gel-filtration analyses revealed that intact SAM-P26 is approximately 18.4-18.8 kDa in mass and exists in a monomeric form with no modification. The calculated isoelectric points were 4.0 for SAM-P26 and 5.4 for SAM-P20, respectively, suggesting that the two proteins do not differ from each other in net-charge. *Streptomyces griseus* protease, SGPD, which exhibits 73.7% identity with SAM-P26, was reported to show extraordinary behaviour on SDS-PAGE, suggesting tight dimer formation of SGPD molecules *via* metal chelation or an intermolecular salt bridge (*25*). However, in our case, these possibilities were excluded by the gel-filtration data.

Previously, the SAM-P26 (formerly termed SAM-P20D) gene was identified downstream of the SAM-P20 gene, and both genes were indicated to be transcribed independently by their own promoters (*20*). These promoters were found to operate regulatorily for the SAM-P20 gene and constitutively for the SAM-P26 gene, respectively (unpublished data). Therefore, this tandemly arranged gene organization for SAM-P26 and SAM-P20 is of interest with respect to evolutionary mechanisms, in particular, such as gene duplication for these type proteases. In enzymatic properties, the two proteases are very similar to each other. This might be feasible from the finding that in the two proteases potential catalytic triad residues (His-33, Asp-61, and Ser-143, numbering for SAM-P26), which are common in various chymotrypsins, are highly conserved in the region surrounding each catalytic site residue (*20*). At present, we have no clear idea why the SSI-producing strain requires the production of these two target proteases similar to each other in structure and activity. It might be argued that the possession of enzymes of overlapping specificity might confer a selective survival advantage, particularly in a relatively competitive natural soil environment, where both chemical inhibitors and mutagenic events are likely to be encountered.

The production of a chymotrypsin-like protease, although not isolated, was reported to be related to morphological differentiation, mycelium growth, in *S. albidoflavus* SMF301 (*27*). We have also observed that the expression level of the SAM-P20 gene varied in response to the proteinaceous nitrogen nutrients (unpublished data). The complex formation between SSI and SAM-P26 as well as SAM-P20 (*18*) suggests that the physiological role of this type of proteases is modulated by internal inhibitor SSI. In this context, studies on the level and timimg of gene expression for SAM-P26 and SAM-P20 should be essential for clarifying the physiological significance of their production, considering the possibility of the involvement of SSI in some physiological processes.

To date, three target proteases, including SAM-P26, have been isolated. In contrast, all of the SIL proteins, such as SSI, isolated so far were the only molecular species in a strain. In this sense, it is possible to consider that the broad inhibitory specificity of SSI toward target proteases can be advantageous in that it interacts, as one species of molecule, with multiple target proteases with various substrate specificities.

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