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Streptomyces Serine Protease (SAM-P20): Recombinant Production, Characterization, and Interaction with Endogenous Protease Inhibitor

SEIICHI TAGUCHI,[1]* MASAYUKI SUZUKI,[1] SHUICHI KOJIMA,[2]
KIN-ICHIRO MIURA,[2] AND HARUO MOMOSE[1]

Department of Biological Science and Technology, Science University of Tokyo, 2641 Yamazaki, Noda-shi, Chiba 278,[1] and Institute for Biomolecular Science, Gakushuin University, Tokyo 171,[2] Japan

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Previously, we isolated a candidate for an endogenous target enzyme(s) of the *Streptomyces* subtilisin inhibitor (SSI), termed SAM-P20, from a non-SSI-producing mutant strain (S. Taguchi, A. Odaka, Y. Watanabe, and H. Momose, Appl. Environ. Microbiol. 61:180–186, 1995). In this study, in order to investigate the detailed enzymatic properties of this protease, an overproduction system of recombinant SAM-P20 was established in *Streptomyces coelicolor* with the SSI gene promoter. The recombinant SAM-P20 was purified by salting out and by two successive ion-exchange chromatographies to give a homogeneous band by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Partial peptide mapping and amino acid composition analysis revealed that the recombinant SAM-P20 was identical to natural SAM-P20. From the results for substrate specificity and inhibitor sensitivity, SAM-P20 could be categorized as a chymotrypsin-like protease with an arginine-cleavable activity, i.e., a serine protease with broad substrate specificity. For proteolytic activity, the optimal pH was 10.0 and the optimal temperature was shifted from 50 to 80°C by the addition of 10 mM calcium ion. The strong stoichiometric inhibition of SAM-P20 activity by SSI dimer protein occurred in a subunit molar ratio of these two proteins of about 1, and an inhibitor constant of SSI toward SAM-P20 was estimated to be 8.0×10^{-10} M. The complex formation of SAM-P20 and SSI was monitored by analytical gel filtration, and a complex composed of two molecules of SAM-P20 and one dimer molecule of SSI was detected, in addition to a complex of one molecule of SAM-P20 bound to one dimer molecule of SSI. The reactive site of SSI toward SAM-P20 was identified as Met-73–Val-74 by sequence analysis of the modified form of SSI, which was produced by the acidification of the complex of SSI and SAM-P20. This reactive site is the same as that toward an exogenous target enzyme, subtilisin BPN′.

Streptomyces spp. are filamentous gram-positive soil bacteria widely known as producers of a variety of hydrolytic enzymes as well as of commercially important antibiotics. A commercial crude sample, pronase (13), prepared from *Streptomyces griseus* is a mixture of several extracellular proteolytic enzymes and has been widely used in biological studies for a long time. Serine proteases produced by *Streptomyces lactamdurans* (5) and *Streptomyces peucetius* (4) were reported to coordinately regulate the cellular protein turnover associated with secondary metabolism and morphogenesis. This organism is also well known to produce protease inhibitors, including low-molecular-mass (26) and proteinaceous (6) compounds. A protease inhibitor might be considered a modulator molecule for the proteolytic activities of some enzymes involved in physiological processes, such as cell differentiation and secondary metabolism, as suggested by Aoyagi (1). However, the biological roles of the interactions between protease and protease inhibitors in the producer strains have not been well identified. *Streptomyces* subtilisin inhibitor (SSI) is one of the few well-characterized microbial proteinaceous protease inhibitors and is a stable dimer (I_2) composed of two identical subunits, each with an M_r of 11,500 (12). It strongly inhibits a microbial serine protease, subtilisin BPN′ (E), by forming an E_2I_2 complex with an M_r of 79,000 at a molar ratio of 2:2. This specific interaction between subtilisin BPN′ and SSI has been studied extensively as a suitable model not only for protease-protease inhibitor interaction but also for protein-protein interaction (6).

Recently, we have demonstrated that SSI-like proteins are widely distributed among *Streptomyces* spp. (16–18) and that a strong correlation exists between the structure around the reactive region of SSI-like proteins and their inhibition specificities toward proteases (19, 25). In this situation, the isolation and characterization of an endogenous extracellular protease(s) acting as a true target enzyme(s) interactive with SSI would enable us to explore the physiological role and evolutionary process of the interaction system between protease and a protease inhibitor in *Streptomyces* spp. We have actually identified the existence of one of the endogenous target enzymes (termed SAM-P20) capable of interacting with SSI. The molecular characterization of the SAM-P20 gene and an in vivo interaction analysis of the functionally expressed SAM-P20 with SSI was also performed (23). Further biochemical investigations of the in vitro interaction of SAM-P20 with SSI should be an important step to clarify the biological roles of protease inhibitors such as SSI-like proteins and their physiological interaction with target enzymes.

Here we describe (i) the establishment of an overproduction system for recombinant SAM-P20, (ii) physicochemical and enzymatic properties of the expressed SAM-P20, and (iii) an in vitro investigation of the interaction of SAM-P20 with SSI.

* Corresponding author. Mailing address: Department of Biological Science and Technology, Science University of Tokyo, 2641 Yamazaki, Noda-shi, Chiba JP-278, Japan. Phone: 81 471 24 1501 (ext. 4428). Fax: 81 471 25 1841.

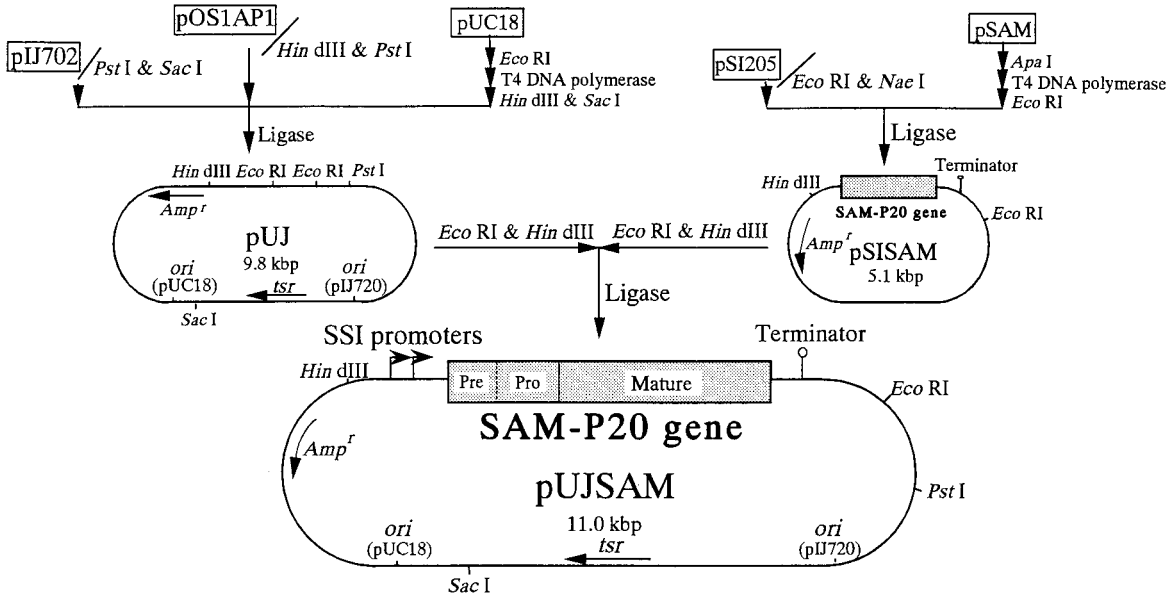


FIG. 1. Construction of a plasmid, pUJSAM, for the secretory expression of SAM-P20 in *S. coelicolor*. A detailed explanation is given in Materials and Methods. Bent arrows and pins denote SSI gene promoters and terminators, respectively. *Amp*^{r}, ampicillin resistance gene; *tsr*, thiostrepton resistance gene.

MATERIALS AND METHODS

Bacterial strains and materials. *Escherichia coli* JM109 (28) was used as a host for the construction of various plasmids. *Streptomyces coelicolor* KCC-S006 for the recombinant host strain was kindly provided by T. Nakase at the Institute of Physical and Chemical Research, Riken. Restriction endonucleases and DNA-modifying enzymes were purchased from Takara Shuzo, Nippon Gene, Toyobo, or Boehringer Mannheim. Thiostrepton was kindly supplied by Asahi Chemical Industries. Proteolytic enzymes, subtilisin BPN′ and *S. griseus* protease B (SGPB) were generous gifts from Nagase Biochemicals and T. Kumazaki at Hokkaido University, respectively. All other chemicals were of reagent grade for biochemical research.

Genetic manipulation and culture conditions. The gene manipulation methods applicable to *E. coli* and *Streptomyces* spp. were as described by Sambrook et al. (15) and Hopwood et al. (7), respectively. Standard media, methods of culture, and transformation procedures for *Streptomyces* spp. were as described previously (20). For *Streptomyces* transformant selection, thiostrepton was used at 50 μg/ml on agar plates and at 1 μg/ml in liquid culture. *E. coli* transformants were grown in Luria-Bertani medium containing 50 μg of ampicillin (Sigma) per ml.

Construction of the SAM-P20 gene expression vector. The shuttle expression vector of the SAM-P20 gene, to be functional in both *E. coli* and *Streptomyces* spp., was constructed via the following three ligation steps (refer to the diagram illustrated in Fig. 1). First, three linear DNA fragments derived from pIJ702 (8) digested at *Pst*I and *Sac*I sites, pOS1AP1 (21) digested at *Hin*dIII and *Pst*I sites, and pUC18 (28) (with a disrupted *Eco*RI site) digested at *Hin*dIII and *Sac*I sites were ligated to generate pUJ. Thus, the constructed plasmid contains two replication origins for both *E. coli* and *Streptomyces* spp. Second, to locate the SAM-P20 structural gene in the proximate downstream region of the two promoters (22) of the SSI gene, pSI205 (22) digested with *Eco*RI and *Nae*I and pSAM (23) digested with *Eco*RI and *Apa*I (modified with T4 DNA polymerase to create a blunt end) were ligated to generate pSISAM. Finally, the expression vector of the SAM-P20 gene was made by ligating pUJ and pSISAM at *Eco*RI and *Hin*dIII sites and was designated pUJSAM.

Purification of the recombinant SAM-P20. *S. coelicolor* transformants carrying pUJSAM were initially cultivated in 20 ml of trypton soya broth at 30°C for 2 days, and then cultivation was continued on a large scale (with 4.75 liters) for 7 days. Cells were removed from the culture medium by centrifugation at 6,000 rpm for 20 min. Ammonium sulfate was added to 65% saturation, and then proteins were precipitated by centrifugation at 12,000 rpm for 20 min. The precipitate was dissolved in 50 mM Tris-HCl buffer (pH 7.0) and dialyzed against the same buffer at 4°C for 2 days. The SAM-P20 was purified by applying combinational ion-exchange chromatography with a DEAE-cellulose column (3 by 45 cm) connected to a carboxymethyl cellulose column (3 by 20 cm) previously equilibrated with 50 mM Tris-HCl buffer (pH 7.0). Active SAM-P20 was eluted with the same buffer, and the pass-through fraction was dialyzed against distilled water and lyophilized. All steps were performed at 4°C.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (10) with a gel concentration of 16.5%. Proteins in the gel were stained with 2% (wt/vol) Coomassie brilliant blue R250.

Immunological analysis. SGPB was once inactivated by phenylmethanesulfonyl fluoride and was raised in a rabbit. Prepared anti-SGPB antiserum was used for double immunodiffusion analysis as described previously (21).

Partial peptide mapping of the recombinant SAM-P20. Partial peptide mapping of the purified SAM-P20 was carried out by S pyridylethylation of cystine residues and by the digestion of S-pyridylethylated SAM-P20 with arginyl endopeptidase, as described previously (19).

Amino acid composition and sequence analysis. Amino acid composition of an intact or S-pyridylethylated SAM-P20 was analyzed with a Hitachi model L-8500 amino acid analyzer after hydrolysis in 5.7 N HCl containing 0.1% phenol at 110°C for 24 h. Automated sequence analysis by Edman degradation was performed with an Applied Biosystems model 476A protein sequencer.

Protein concentration. The protein concentration was determined spectrophotometrically with an $A_{280}^{1\%}$ of 11.7 for subtilisin BPN′ (11), an $A_{280}^{1\%}$ of 8.1 for SGPB (3), and a molar absorption coefficient constant (ε_{280}) of 9,330 M^{-1} for a subunit of SSI. The concentrations of SAM-P20 were determined by the method of Bensadoun and Weinstein (2) and amino acid composition analysis.

Assay for enzyme activity. The activity assay of the purified SAM-P20 was essentially as described previously (24): the activity of recovered SAM-P20 in each purification process was measured by monitoring the A_{410} resulting from 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 synthetic substrates used for

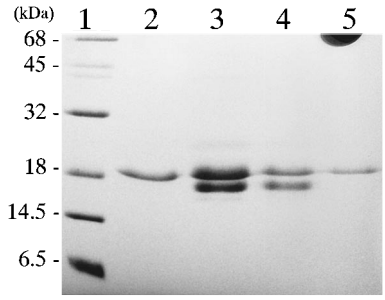


FIG. 2. SDS polyacrylamide gel at each step of purification of recombinant SAM-P20. Lane 1, molecular mass markers: BSA (68 kDa), ovalbumin (45 kDa), carbonic anhydrase (32 kDa), β-lactoglobulin (18 kDa), lysozyme (14.5 kDa), aprotinin (6.5 kDa); lane 2, authentic SAM-P20; lane 3, culture supernatant of *S. coelicolor* KCC-S006 harboring pUJSAM; lane 4, ammonium sulfate (65% saturation)-precipitated sample; lane 5, purified sample by chromatographies on a DEAE-cellulose column connected to a carboxymethyl cellulose column.

TABLE 1. Purification of SAM-P20 from *S. coelicolor* carrying pUJSAM

Purification step[a]	Vol (ml)	Total amt (mg)	Total activity (units)[b]	Sp act (units/g)[b]	Activity recovery (%)
Culture supernatant	4,750	398	22.2	0.06	100.0
Precipitate with ammonium sulfate	116	319	18.1	0.06	81.5
Flowthrough fraction by combination of DEAE and carboxymethyl celluloses	2,800	46	10.0	0.22	45.0
Dialyzed sample	2,700	35	8.4	0.24	37.8

[a] The purification procedure is described in detail in Materials and Methods.
[b] One unit of activity equals 1.0 μmol of *p*-nitroaniline released per s.

the measurement of substrate specificity of SAM-P20 are listed in Table 2. Each hydrolysis reaction was started by adding 20 nM SAM-P20 to the solution composed of 100 μM substrate, 100 mM Tris-HCl (pH 8.5), 0.1% dimethyl sulfoxide and 10 mM $CaCl_2$ to make 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-P20 was expressed as the amount (in micromoles) of the chromogenic group member liberated by 1.0 mg of SAM-P20 in 1 s under the above-described conditions.

Determination of kinetic constants. The K_m and catalytic constant (k_{cat}) values for three substrates, sAAPF*p*NA, benzoyl-Phe-Val-Arg-*p*-nitroanilide, and succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide, were determined from a Hanes-Woolf plot generated from the initial reaction velocities obtained with substrate concentrations between 40 and 800 μM and 100 nM purified SAM-P20. All experiments were carried out at least in triplicate, and the results were expressed as the means of these data.

Effects of inhibitors. Protease inhibitors used and their concentrations are presented in Table 4. 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. Substrate hydrolysis of sAAPF*p*NA by purified SAM-P20 was assayed at different temperatures (1 to 80°C) with a temperature-controlled cuvette holder attached to a recirculating water bath. For each assay, the buffer containing substrate was warmed for 5 min prior to the addition of enzyme. At the same time, the effect of adding 10 mM calcium ion was tested. The effect of pH on sAAPF*p*NA hydrolysis was determined with the following buffers at 100 mM, supplemented with 10 mM $CaCl_2$: citric acid (pH 3.0 to 6.8), Tris-HCl (pH 6.8 to 8.8), and glycine-NaOH (pH 8.8 to 10.5). The assays for optimal pH were carried out at 25°C.

Measurement of inhibitory activity. The inhibitory activity of SSI against SAM-P20 was measured with 100 μM sAAPF*p*NA as the synthetic substrate, by a procedure described previously (9).

Determination of inhibitor constant. Residual activities of SAM-P20 (294 μM) in the presence of SSI at various concentrations were measured with 100 μM sAAPF*p*NA in 100 mM Tris-HCl (pH 8.5) at 25°C, and an inhibitor constant of SSI toward SAM-P20 was determined from this inhibition profile by the procedures described previously (9).

Estimation of molecular mass by analytical gel filtration. Analytical gel filtration high-performance liquid chromatography (HPLC) to estimate the molecular mass in the native state was performed with a TSK-GEL2000SW column (0.75 by 60 cm) by the HPLC system (Gilson model 305). The eluent was 0.1 M Tris-HCl–0.5 M NaCl (pH 7.0), and the flow rate was 0.7 ml/min. Molecular mass markers used were bovine serum albumin (BSA; 68 kDa), SSI (dimeric form; 23 kDa), and aprotinin (6.5 kDa).

Identification of the reactive site of SSI toward SAM-P20. A mixture of SAM-P20 (25 μg, 1.35 nmol) and a twofold molar excess of SSI (31 μg, 2.70 nmol) in 150 μl of 10 mM Tris-HCl (pH 8.0) were incubated at 37°C for 10 min to form a complex. Then, an equal volume of ice-cold 0.4 M Gly-HCl (pH 2.5) was added and incubated at 4°C for 5 min, after which proteins were precipitated with a final concentration of 20% trichloroacetic acid. The precipitate was dissolved in 0.1% trifluoroacetic acid and subjected to reverse-phase HPLC on a C_{18} column under the conditions described previously (19).

TABLE 2. Substrate specificity of SAM-P20

Substrate[a]	Protease activity ($\mu mol \cdot s^{-1} \cdot mg^{-1}$)
Succinyl-Ala-Ala-Val-[Ala]-*p*-nitroanilide	1.90
Succinyl-Ala-Ala-Pro-[Phe]-*p*-nitroanilide	41.00
Succinyl-Ala-Ala-Pro-[Leu]-*p*-nitroanilide	11.00
Benzoyl-Phe-Val-[Arg]-*p*-nitroanilide	11.00
Benzoyl-[Arg]-*p*-nitroanilide	0.00
Tosyl-Gly-Pro-[Lys]-*p*-nitroanilide	0.42

[a] The amino acid residues of substrates in boxes correspond to P1 sites.

RESULTS AND DISCUSSION

Overproduction of recombinant SAM-P20. To investigate detailed enzymatic properties of SAM-P20 that are interactive with SSI, we tried to establish a secretory overproduction system of recombinant SAM-P20 in *Streptomyces* spp. When the constructed shuttle expression vector, pUJSAM (Fig. 1), was cloned to the heterologous host, *S. coelicolor*, large clear zones indicating the degradation of skim milk could be detected around the transformant colonies (data not shown). By SDS-PAGE analysis (Fig. 2, lane 3), the recombinant SAM-P20 was found to accumulate as the most major protein in the extracellular proteins from the culture fluid of transformants. Therefore, the strong promoters of the SSI gene (22) and the signal sequence of SAM-P20 might be efficiently functional in *S. coelicolor* for the secretory expression of the SAM-P20 gene. As a consequence of overexpression of the SAM-P20 gene, reduced sporulation and red color pigmentation were observed for the transformants of *S. coelicolor* on a poor-medium-containing agar plate, although no cell lysis appeared.

Purification of SAM-P20. The results of the purification of recombinant SAM-P20 are summarized in Table 1. By a combination of ammonium sulfate precipitation and two column chromatographies, recombinant SAM-P20 protein was purified about fourfold from the culture supernatant (4.75 liters) with an activity recovery of 38%. The homogeneity of the final preparation was proved by SDS-PAGE (Fig. 2, lane 5). The amount of the purified protease finally obtained was about 35 mg. This yield implies that the level of productivity of recombinant SAM-P20 was 19.5 mg/liter of the culture.

Characterization of SAM-P20. The molecular mass of the recombinant SAM-P20 was estimated to be 18.5 kDa by SDS-

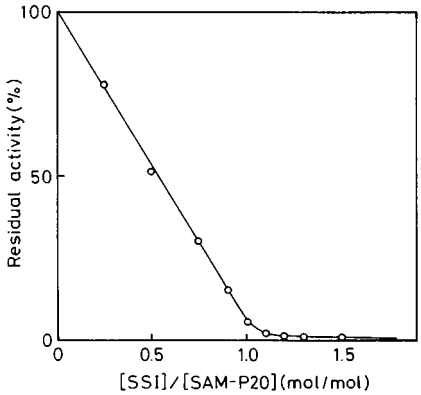


FIG. 3. Inhibition profile of SAM-P20 by SSI. SAM-P20 and various amounts of SSI were incubated in 100 mM Tris-HCl (pH 8.5) at 25°C for 20 min, and residual activities of SAM-P20 were measured with 100 μM sAAPF*p*NA. The concentration of SAM-P20 in the reaction mixture was 294 nM. The amount of SSI shown is that of the subunit.

TABLE 3. Kinetic parameters of SAM-P20 for substrate hydrolysis

Substrate	Mean K_m ± SD (μM)	Mean k_{cat} ± SD (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Succinyl-Ala-Ala-Pro-Phe-*p*-NA[a]	420 ± 36	71.0 ± 3.8	170
Benzoyl-Phe-Val-Arg-*p*-NA	230 ± 8.1	8.9 ± 0.3	37
Succinyl-Ala-Ala-Pro-Leu-*p*-NA	660 ± 50	28.0 ± 1.9	42

[a] NA, nitroanilide.

PAGE (Fig. 2). This value agreed well with that calculated from the amino acid sequence deduced from the gene and with that of authentic SAM-P20 previously purified from mutant strain M1 of *Streptomyces albogriseolus* S-3253 (23). The 15 amino acid residues in the N-terminal region were found to be identical to those of authentic SAM-P20. This indicates that the SAM-P20 gene product was secreted through processing at the same site (−1Leu ↓ +1Ile) as the authentic SAM-P20. In a manner similar to that of the original strain (*S. albogriseolus*), the maturation of the recombinant precursor SAM-P20 could occur to generate an active form of SAM-P20 in *S. coelicolor*. Also, three internal amino acid sequences (amino acid residues 22 to 36, 70 to 87, and 111 to 139) were identified by sequence analysis of the peptide fragments obtained by arginyl endopeptidase digestion of S-pyridylethylated SAM-P20. From this result and the amino acid composition (data not shown), it was confirmed that the SAM-P20 gene was faithfully translated.

SAM-P20 has a much higher identity level to SGPB (75.5%) than to SGPA (57.3%) on the basis of the primary structures (23). This was supported by the evidence that anti-SGPB antiserum was cross-reactive only with SAM-P20 (data not shown).

The specificity of SAM-P20 for the synthetic substrate was examined with a series of chromogenic substrates possessing the P1 sites of Ala, Phe, Leu, Arg, and Lys, respectively. As is evident from Table 2, SAM-P20 showed a high level of reactivity to sAAPF*p*NA and was weakly cleaved at Leu (a P1 site) in the substrate possessing the same backbone as that of sAAPF*p*NA. Interestingly, in this study, not negligible trypsin-like activity was found for SAM-P20 with succinyl-Phe-Val-Arg-*p*-nitroanilide as the substrate. The kinetic values, K_m, k_{cat}, and k_{cat}/K_m, for the three substrates are summarized in Table 3. These results indicate that SAM-P20 has broad substrate specificity. It is no wonder that SAM-P20 recognizes hydrophobic amino acids with small and large side chains and positively charged amino acids, because such broad substrate specificity was also observed for subtilisin BPN′ (27). In the case of subtilisin BPN′, structural origins of broad specificity were discussed on the basis of its three-dimensional structure determined by X-ray crystallography (14) and the results obtained from mutational analyses by using protein engineering techniques (27).

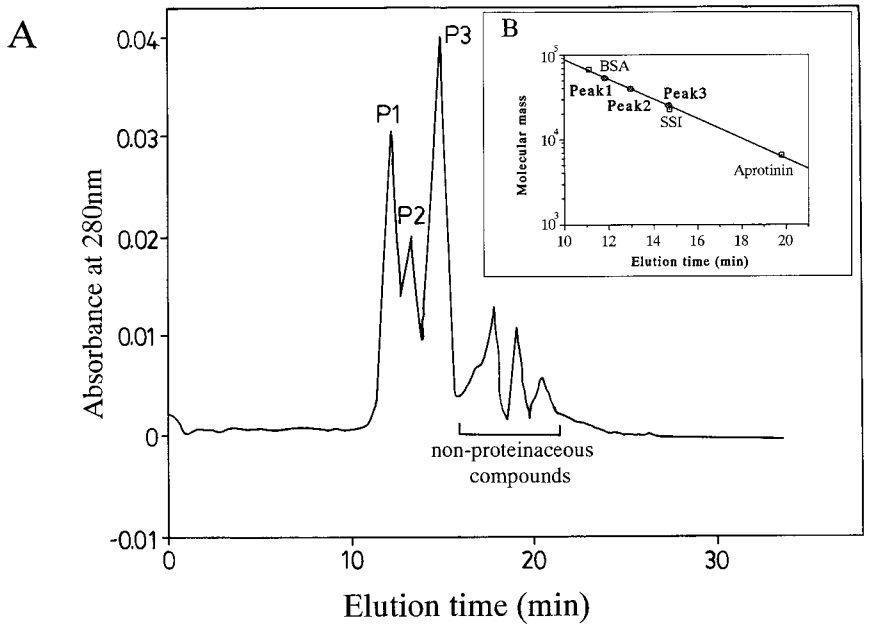
As for the inhibition profiles of various inhibitors, the hydrolysis of sAAPF*p*NA by SAM-P20 was markedly inhibited by chymostatin and SSI and weakly inhibited by leupeptin and antipain but not inhibited by phosphoramidon, bestatin, or pepstatin, which are specific inhibitors of metalloproteases, aminopeptidases, and aspartic proteases, respectively, as shown in Table 4. Also, SSI and chymostatin completely inhibited the hydrolysis of both succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide and succinyl-Phe-Val-Arg-*p*-nitroanilide by SAM-P20 under the conditions described above (data not shown). These data and the primary structure deduced from the gene show that SAM-P20 belongs mainly to the chymotrypsin-like protease family rather than the subtilisin-like proteases of the serine protease family, as suggested in a previous communication (23). However, the conflicting substrate specificities for Phe and Arg residues at P1 sites should be clarified by solving the three-dimensional structure of the SAM-P20–substrate complex.

SAM-P20 was most active near pH 10.0 for the hydrolysis of sAAPF*p*NA, indicating that SAM-P20 is a typical alkaline protease. The optimum temperature for proteolytic activity was about 50°C or 80°C in the absence or presence of 10 mM calcium ion. The addition of 10 mM calcium ion would have an effect on the heat resistancy of SAM-P20 activity.

In vitro interaction of SAM-P20 with SSI. Initially, the inhibitory profile of SAM-P20 by SSI was examined by measuring residual activities of SAM-P20 in the presence of SSI at various protease inhibitor ratios. As shown in Fig. 3, the activity of SAM-P20 was inhibited by SSI almost completely at a molar ratio of about 1. Since SSI exists in a dimer form, two molecules of SAM-P20 become inhibited by a dimer molecule of SSI. From this profile, the inhibitor constant of SSI toward SAM-P20 was estimated to be 8.0×10^{-10} M. This value is comparable to that of an exogenous target enzyme, subtilisin BPN′ (3.6×10^{-10} M) (6). Temporary inhibition, which is defined as a time-dependent decrease of inhibitory activity

TABLE 4. Effects of inhibitors on SAM-P20

Protease inhibitor	Specificity(ies) of inhibitor	Concn	% Residual activity
SSI	Serine proteases such as subtilisin and chymotrypsin	100 nM	0.0
Chymostatin	Chymotrypsin	50 μM	0.0
Phenylmethylsulfonyl fluoride	Serine proteases	50 μM	87.2
N-Tosyl-L-phenylalanyl chloromethyl ketone	Chymotrypsin, papain, and bromelain	50 μM	77.8
Nα-*p*-tosyl-L-lysine chloromethyl ketone	Trypsin, plasmin, and thrombin	50 μM	100.0
EDTA	Metalloproteases	5 mM	100.0
Phosphoramidon	Metalloendopeptidases such as thermolysin	50 μM	100.0
Bestatin	Aminopeptidases	50 μM	100.0
E-64	Cysteine proteases	50 μM	93.8
Leupeptin	Serine and cysteine proteases	50 μM	64.2
Antipain	Papain, trypsin, and cathepsin A and B	50 μM	35.2
Pepstatin	Aspartic proteases	50 μM	100.0



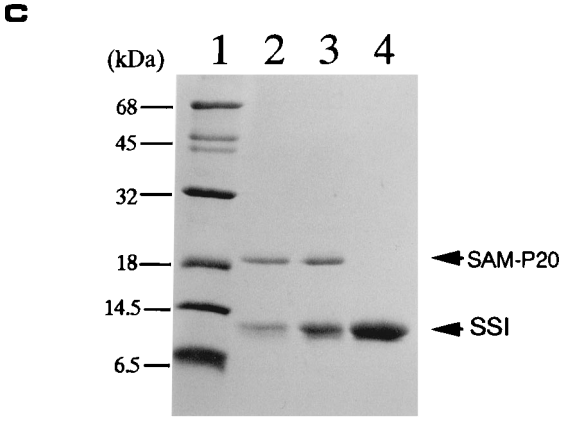


FIG. 4. Analysis of the interaction of SAM-P20 with SSI. (A) Gel filtration HPLC analysis. After mixing of SAM-P20 and SSI in an excess molar ratio (fivefold) of SSI to SAM-P20, the sample solution was applied to a TSK-GEL2000SW column. Molecular-mass-marker proteins were also applied independently. Elution was performed by HPLC as described in Materials and Methods. P1 to P3 indicate peak numbers 1 to 3, respectively. (B) An estimation of the molecular masses of protein species contained in peak fractions was done with a calibration curve. Molecular mass markers used were BSA (68 kDa), SSI (dimeric form; 23 kDa), and aprotinin (6.5 kDa). (C) SDS-PAGE analysis. Lane 1, molecular mass markers; lane 2, peak 1 fraction; lane 3, peak 2 fraction; lane 4, peak 3 fraction. Samples from peaks 1 and 2 were loaded into lanes 2 and 3 by adjusting them to the same density as the Coomassie brilliant blue R250-stained band corresponding to SAM-P20. The authentic SSI used here contains a small number of the minor N-terminal residues of truncated species resulting from processing during secretion from *S. albogriseolus* S-3253.

under a molar excess of an inhibitor relative to a protease, was not observed for this SSI–SAM-P20 system, indicating the stable complex formation.

Next, the stoichiometry of the complex formation of SAM-P20 and SSI was examined by analytical gel filtration. Figure 4A shows an HPLC profile of a mixture of SAM-P20 and a fivefold molar excess of SSI. Three peaks were observed. An estimation of molecular mass from the elution time (Fig. 4B) and SDS-PAGE analysis (Fig. 4C) indicated that the peak 1 fraction contained a complex with a molecular mass of 58 kDa that was composed of equimolar amounts of SAM-P20 and SSI. Since SSI is a dimer protein with a molecular mass of 23 kDa, the protein molecule in peak 1 was a complex of one dimer molecule of SSI and two molecules of SAM-P20 (with a calculated molecular mass of 60 kDa). In a similar way, it was concluded that the protein molecule with a molecular mass of 39 kDa in peak 2 was a complex of one dimer molecule of SSI and one molecule of SAM-P20 (with a calculated molecular mass of 41.5 kDa). A free dimer molecule of SSI was found to be contained in peak 3 on the basis of its elution time and SDS-PAGE analysis. The assignments to three species were also confirmed by amino acid composition analysis (data not shown).

Finally, in order to identify the reactive site of SSI toward SAM-P20, a twofold molar excess of SSI was treated with SAM-P20 under acidic conditions and then subjected to reverse-phase HPLC to isolate a modified form of SSI in which the peptide bond at the reactive site was specifically cleaved. Figure 5 shows a chromatogram. A sequence analysis of peak A shows a sequence, Val-Tyr-Asp-Pro-Val-Leu, corresponding to positions 74 to 79 of SSI, in addition to the sequence from the amino terminus. The amino acid composition of peak A was almost identical to that of intact SSI (data not shown). These results indicate that the reactive site of SSI toward SAM-P20 was Met-73–Val-74, which is the same as that reactive toward subtilisin BPN′. Peak B was found by sequence analysis to contain an intact SSI.

We have already isolated three SSI-interacting proteins, including SAM-P20 from the culture of non-SSI-producing mutant strain M1 (unpublished data). Mutant strains such as M1 exhibited several common pleiotropic properties: slightly slow growth, a marked decrease in sporulation activity (observed in a scanning electron micrograph), and a remarkable increase in the level of extracellular activity and/or productivity of a protease(s). Therefore, it is possible that SSI molecules may be involved in physiological regulatory processes such as cell dif-

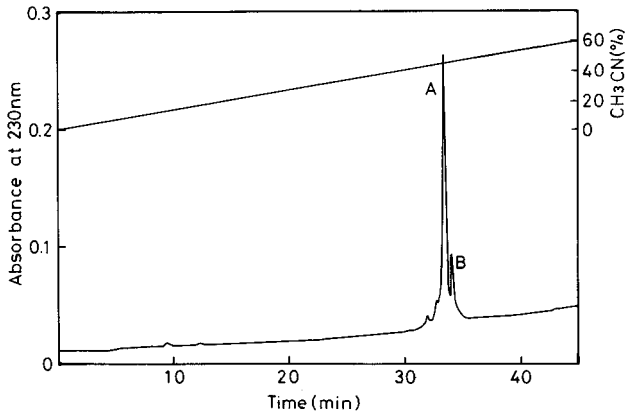


FIG. 5. Reverse-phase HPLC profile of SAM-P20-treated SSI under acidic conditions. A twofold molar excess of SSI was treated with SAM-P20 at 4°C for 5 min under acidic conditions, and proteins were precipitated with trichloroacetic acid. The precipitate was dissolved in 0.1% trifluoroacetic acid and subjected to HPLC on a C_{18} column (L-column ODS; 0.46 by 15 cm).

ferentiation by modulating activities of multiple target enzymes (at least three proteins) in a natural culture environment. Two other possible target enzymes should also be investigated in detail, as was done for SAM-P20 in this study.

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