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# Molecular Characterization of a Gene Encoding Extracellular Serine Protease Isolated from a Subtilisin Inhibitor-Deficient Mutant of *Streptomyces albogriseolus* S-3253

SEIICHI TAGUCHI,* AYAKO ODAKA, YOSHIHARU WATANABE, AND HARUO MOMOSE

*Department of Biological Science and Technology, Science University of Tokyo, 2641 Yamazaki, Noda-shi, Chiba 278, Japan*

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**An extracellular serine protease produced by a mutant, M1, derived from *Streptomyces albogriseolus* S-3253 that no longer produces a protease inhibitor (*Streptomyces* subtilisin inhibitor [SSI]) was isolated. A 20-kDa protein was purified by its affinity for SSI and designated SAM-P20. The amino acid sequence of the amino-terminal region of SAM-P20 revealed high homology with the sequences of *Streptomyces griseus* proteases A and B, and the gene sequence confirmed the relationships. The sequence also revealed a putative amino acid signal sequence for SAM-P20 that apparently functioned to allow secretion of SAM-P20 from *Escherichia coli* carrying the recombinant gene. SAM-P20 produced by *E. coli* cells was shown to be sensitive to SSI inhibition.**

Proteinaceous inhibitors of proteolytic enzymes have been found to be produced by a wide variety of animals, plants, and microorganisms (13, 27, 41). Most of the extracellular inhibitory proteins discovered to date have been isolated from *Streptomyces* species and classified as members of the *Streptomyces* subtilisin inhibitor (SSI) family on the basis of their similar structures and protease inhibitory specificities (17). SSI is the first proteinaceous protease inhibitor isolated from the culture filtrate of *Streptomyces albogriseolus* S-3253 and shows strong inhibitory activity against alkaline serine proteases such as subtilisin (21). Although its structure-function relationship has been studied extensively by various physicochemical techniques (9), its biological significance in nature is little known.

Since, at the initial stage, SSI protein was screened and used as an excellent proteolysis-resistant substrate analog for investigating the molecular catalytic mechanism of an external target protease, subtilisin BPN′ of *Bacillus amyloliquefaciens*, attention has never been focused on it from a physiological viewpoint. Therefore, to clarify the physiological significance and evolutionary process of SSI protein in *Streptomyces* spp., we attempted to isolate and characterize an intrinsic extracellular protease(s) acting as a true target enzyme(s) interactive with SSI.

We describe here the following: (i) the isolation and characterization of an extracellular chymotrypsin-type protease, designated SAM-P20, which was found in an SSI-nonproducing mutant of *S. albogriseolus* S-3253 and which interacts with SSI; (ii) the gene cloning and sequencing of the protease; and (iii) the secretory expression of the protease in *Escherichia coli*. To our knowledge, this report provides the first and original findings on the interaction between extracellular inhibitors and their target enzymes in *Streptomyces* spp.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The *Streptomyces* strains used were *S. albogriseolus* S-3253 (21), its SSI-nonproducing mutant M1, and *Streptomyces lividans* 66 (10). *E. coli* JM109 (43) was used for experiments on gene cloning and expression. Plasmid pJS177 (36), constructed previously for SSI gene expression in *S. lividans* 66, was used for a transformation test of the mutant strain M1. Plasmid pUΔS16-1 (39), constructed previously for subtilisin BPN′ gene expression in *E. coli* JM109, was used as a control vector for examining functional expression of the SAM-P20 gene in *E. coli* JM109.

**Analytical procedures.** The purity and size of proteins were analyzed electrophoretically in an 18.8% polyacrylamide gel (by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) in the presence of 0.1% SDS by the method described by Laemmli (15). Immunoblotting and immunodiffusion analysis with anti-SSI antiserum was done for identification of SSI production by a method described previously (23, 36). Production of proteases by SAM-P20 producers (both natural and recombinant) was qualitatively detected on the basis of the pattern of clear zones formed around colonies grown on two types of 2% skim milk-containing plates, namely, S plates (3% Trypticase soy broth medium plus 1.5% agar) and lactose plates (1% lactose and 0.1% yeast extract plus 1.5% agar). The former plates were used for *Streptomyces* natural strains, and the latter were used for recombinant strains of *E. coli* JM109. The N-terminal amino acid sequences were determined by automated Edman degradation with an Applied Biosystems 470A gas-phase protein sequencer (35).

**Purification of extracellular proteases.** We applied two purification procedures (A and B) to the isolation of an extracellular SSI-interacting protease(s) from the culture medium of strain M1. All operations were carried out at 4°C. Procedure A consisted of three steps, namely, salting out with 80% saturated ammonium sulfate, ion-exchange chromatography on a DEAE-cellulose column (3 by 38 cm) with a linear gradient of NaCl (0 to 1.0 M), and gel filtration on a Sephacryl S-200 column (3 by 95 cm), which was essentially the same system applied to the purification of SSI protein (35, 38). Active fractions interacting with SSI were identified by measuring the inhibition of proteolytic activity by SSI. Proteolytic activity of each fraction was detected initially by a convenient skim milk-hydrolyzing plate assay (39) at 30°C and subsequently by monitoring the release of *p*-nitroaniline from a synthetic substrate, *N*-Succinyl-L-Ala-L-Ala-L-Pro-L-Pro-L-Phe-*p*-nitroanilide (AAPF; Sigma). The enzymatic release of the pigment from 20 μM AAPF in 0.1 M Tris-HCl buffer (pH 8.5) was measured spectrophotometrically at 410 nm in the presence and absence of SSI.

Procedure B was carried out by applying affinity chromatography using SSI as a ligand to a concentrated protein sample with ammonium sulfate (80% saturation) from 6.5 liters of culture supernatant of mutant M1. About 1 mg of purified SSI was immobilized on a 0.5-ml cyanogen bromide-activated Sepharose 4B column by a coupling reaction. A column (0.7 by 1.5 cm) of SSI-bound Sepharose 4B was preequilibrated with 0.1 M phosphate buffer (pH 7.0). After a sample was applied to the column, it was washed with a sufficient volume of the buffer described above. 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 eluted proteins were separated by SDS-PAGE (with an 18.8% polyacrylamide gel). The electrophoresed gel was washed with 10 mM CAPS (cyclohexylaminopropanesulfonic acid) buffer (pH 11.0) and 10% methanol and 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 et al. (18). 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 subjected directly to amino acid sequencing.

**Effects of various inhibitors on protease.** The following proteinaceous and

* Corresponding author. Mailing address: Department of Biological Science and Technology, Science University of Tokyo, 2641 Yamazaki, Noda City, Chiba JP-278, Japan. Phone: (0471) 24-1501, ext. 4428. Fax: (0471) 25-1841.

nonproteinaceous protease inhibitors were tested for their effect on AAPF hydrolysis: SSI, aprotinin (trypsin inhibitor), chymostatin, EDTA, and monoiodoacetic acid. The enzyme samples were preincubated at room temperature with each inhibitor and then assayed at 25°C with AAPF (final concentration, 20 μM) as a substrate in 0.1 M Tris-HCl buffer (pH 8.5).

**DNA manipulations and isolation.** Restriction enzyme mapping and subcloning were carried out by standard procedures (26). Chromosomal DNA from *Streptomyces* spp. was prepared by the method of Saito and Miura (25). Small-scale plasmid DNA preparation of *Streptomyces* spp. or *E. coli* was performed as described by Kieser (14). The preparation and transformation of *Streptomyces* protoplasts were essentially the same as that described by Hopwood et al. (10). Transformation of *E. coli* was carried out by standard procedures (26). All reagents and enzymes used were of the highest grade available.

**Cloning of SAM-P20-encoding gene.** Two DNA oligomers, 5′-GCGTAGATC (G)GCGTCC(G)CCC(G)CCCTGGAT-3′ (probe 1) and 5′-CGCTGC(T)TCC (G)CTC(G)GGC(G)TTCAACGT-3′ (probe 2), which correspond to the first nine amino acids of the N-terminal sequence of SAM-P20 determined by amino acid sequence analysis and an identical sequence found in the N-terminal regions (positions 13 to 20) of both the SGPA and SGPB genes, respectively, were synthesized (see Fig. 4). Total DNA of *S. albogriseolus* S-3253 was digested with several restriction enzymes and then separated by 0.8% agarose gel electrophoresis. The separated DNA fragments were subjected to the Southern blotting technique (26) with a slight modification: 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]) was replaced with 0.1 M Tris-HCl (pH 10.5) containing 0.9 M NaCl. The blotted nylon membranes were subjected to hybridization with probes prepared as described above (ca. $10^6$ cpm) in 0.1% SDS–6× SSC–1× Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 25 mM sodium phosphate buffer [pH 7.0]) at 55°C overnight, washed three times with 1× SSC containing 0.1% SDS for 15 min at 42°C, and dried, and the blots were visualized on an imaging analyzer (FUJIX model BAS2000) or by autoradiography (37).

The 3.3-kb *Pst*I and 1.9-kb *Bam*HI fragments, which showed positive signals by Southern hybridization, were fractionated from the agarose gel with a Sephaglas band prep kit (Pharmacia) and inserted into the *Pst*I and *Bam*HI sites, respectively, dephosphorylated with bacterial alkaline phosphatase, on plasmid pUC18. *E. coli* JM109 was transformed with the ligation mixture. White, ampicillin-resistant colonies of recombinants were selected on Luria-Bertani agar plates containing ampicillin (50 μg $ml^{-1}$), 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (40 μg $ml^{-1}$), and 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to generate a library. Positive clones containing the SAM-P20 gene were screened from the gene libraries by colony hybridization with probe 1 under the same conditions as those used for Southern hybridization.

**DNA sequencing.** The DNA sequence was determined by the dideoxy chain termination method with double-stranded plasmids as templates (7), a commercially available kit (the BcaBEST sequence kit [Takara Shuzo Co.]), [α-$^{32}$P]dCTP, DNA probes (1 and 2), and three new synthetic DNAs as primers. Nucleotide sequences were determined completely for both strands by using overlapping fragments.

The construction of deletion mutants of the SAM-P20 gene for nucleotide sequence determination was carried out with exonuclease III and mung bean nuclease as described previously (22). Several DNA fragments containing the SAM-P20 gene isolated from the initially constructed plasmid pSAM1 (Fig. 3) were digested by appropriate restriction enzymes, repaired with Klenow fragment, and inserted into the *Sam*I site of pUC18 in both orientations. The resultant plasmids were digested with *Xba*I and *Pst*I and treated with exonuclease III at 37°C with sampling at appropriate time intervals within 30 min. Successively, the resulting linear plasmids were treated with mung bean nuclease at 37°C for 30 min, treated with Klenow fragment, and, finally, ligated with T4 DNA ligase. *E. coli* JM109 was then transformed with the heterogeneously deleted plasmids.

**Construction of expression vectors for SAM-P20 gene in *E. coli*.** Expression of the SAM-P20 gene in *E. coli* was carried out with the two initially constructed plasmids, pSAM1 and its control plasmid, pSAM2; the isogenic *Pst*I-*Pst*I DNA fragment was inserted into the pUC18 vector so that the reading direction of the SAM-P20 gene was the same as (pSAM1) or opposite to (pSAM2) that of the *lac* promoter in pUC18. An expression assay of *E. coli* JM109 transformants harboring the constructed plasmids, pSAM1 and pSAM2, was done on lactose plates containing 2% skim milk, ampicillin (final concentration, 50 μg/ml), and IPTG (final concentration, 0.5 mM) as described previously (39). The inhibitory effect of SSI on SAM-P20 expressed in this system was assayed with the same plates without or with SSI supplementation (concentration, 0.1 μg/ml of medium), and the *E. coli* expression system of subtilisin BPN′ was used as a control (39).

## RESULTS

**Isolation of SSI-nonproducing mutants.** In the course of establishing a new *Streptomyces* host-vector system for heterologous protein production with the SSI high producer *S. albogriseolus* S-3253, we noticed that SSI-nonproducing mutant strains could be obtained at high frequency. These strains were confirmed not to retain the production of SSI by sensitive immunoblot analysis (data not shown). These mutants appeared merely through protoplast formation and regeneration and were detected by immunoreaction test (36) on a plate with anti-SSI antiserum (38a). They exhibited several common pleiotropic properties: slightly slow growth in the rich medium, a marked decrease in sporulation activity, and a remarkable increase in extracellular activity and/or productivity of a protease(s). These facts suggest that SSI is not essential for growth but might have some effects on morphological differentiation and the activity and/or productivity of an extracellular protease(s). Addition of SSI (1 μg/ml of medium) to these mutant cultures did not affect the recovery of sporulation on the plate. In contrast, introduction of the expression vector for the SSI gene (pJS177) into these strains led to a decrease in the activity and/or productivity of the extracellular protease(s), as judged from the decrease in size of a clear zone around the colonies on a skim milk-containing plate. However, the recovery of SSI production by the plasmid introduction did not completely suppress protease production, as determined in comparison with the protease production pattern of the parent strain. This difference in protease production between the two strains was probably caused by the difference in SSI production, which was 500 mg/liter in the parent strain and 20 to 30 mg/liter in the transformant M1/pJS177 (data not shown). These findings strongly suggest that there exists potentially an extracellular protease(s) interactive with SSI.

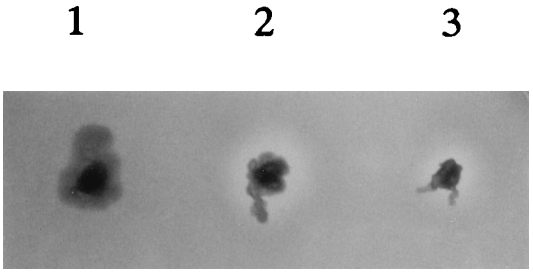


FIG. 1. Plate assay for protease. Zones: 1: wild-type *S. albogriseolus* S-3253, 2: mutant M1, 3: M1 transformant carrying pJS177 (SSI gene expression vector; see Results).

Figure 1 shows a typical demonstration of the phenomenon mentioned above in which the parent strain, *S. albogriseolus* S-3253, its representative mutant strain, designated M1, which is completely devoid of SSI productivity, and its transformant strain carrying pJS177 (M1/pJS177) were subjected to the clear zone-forming plate assay for detecting protease production. The transformant strain M1/pJS177 was found to secrete large amounts of SSI into its culture media (data not shown).

**Purification and partial characterization of SSI-interacting protease.** To search for and isolate the newly appeared protease(s) due to the defect in SSI production, we attempted to apply two purification procedures (see Materials and Methods). Figure 2 shows the elution pattern of gel filtration employed in procedure A. This procedure was carried out to isolate a target protease(s) with activity in response to SSI. Fractions (around no. 90) with proteolytic activity inhibited by SSI were clearly obtained. However, the peak obtained by measuring the $A_{280}$ appeared slightly deviated from that of proteolytic activity, and a major protein band with a molecular mass of 20 kDa was observed on SDS-PAGE (data not shown). The fractionated solution exhibited preferential specificity for AAPF and was strongly inhibited by SSI (78.0 μM) and chymostatin (41.0 mM), a typical inhibitor of chymotrypsin. A

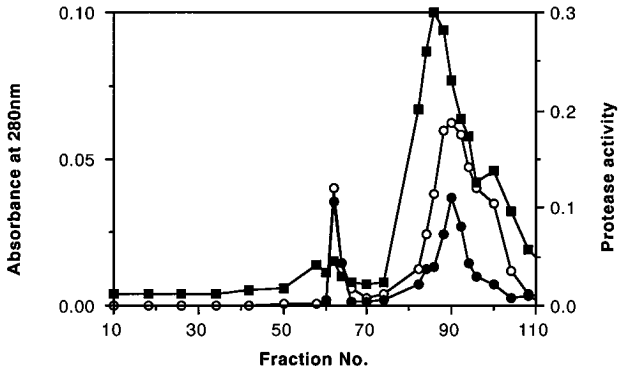


FIG. 2. Gel filtration profile and proteolytic activity of an SSI-interacting protease(s). Symbols: ■, $A_{280}$ for protein; ○ and ●, protease activities in the absence and presence, respectively, of 180 ng of SSI. Protease activity of each fraction was measured by the procedure described in Materials and Methods.

metalloprotease inhibitor, EDTA (25.5 mM), and a thiol protease inhibitor, monoiodoacetic acid (25.5 mM), showed no inhibition of its proteolytic activity of the fractionated solution.

Upon affinity chromatography with an SSI-bound Sepharose column, an SSI-interacting protease(s) could be eluted only under stringent conditions such as elution with 0.01 M HCl. Under these conditions, SSI was found to be coeluted with the proteases by immunoblot analysis (data not shown). This implied that the binding affinity between the target protease(s) and SSI was too high. The protease(s) neutralized with NaOH retained slight proteolytic activity. Several (at least three) SSI-interacting proteins purified by an affinity column were detected by SDS-PAGE (data not shown). On the basis of these results and those of purification procedure A, of the three proteins, the most major one that corresponded to the stained band with a molecular mass of 20 kDa was considered to be a candidate for the target SSI-interacting protease and was therefore designated SAM-P20. Protein samples separated on a gel were subsequently electroblotted onto a polyvinylidene difluoride membrane (data not shown) and then cut out and directly to N-terminal amino acid sequence analysis. The first 20 amino acid residues of the SAM-P20 sequence revealed high homology with those of *S. griseus* proteases A and B.

**Molecular cloning of the SAM-P20 gene.** To clone the SAM-P20 gene, total DNA from *S. albogriseolus* S-3253 was digested with *Pst*I and with *Bam*HI. Probe 1 was shown to hybridize with 3.3-kb *Pst*I-*Pst*I fragments and 1.9-kb *Bam*HI-*Bam*HI fragments (data not shown). These DNA fractions were then purified from the agarose gel after electrophoresis and ligated to the *Pst*I and *Bam*HI cloning sites of the pUC18 vector to generate gene libraries. Gene libraries thus established were applied to the screening of positive clones by colony hybridization with probe 1. Restriction enzyme mapping showed that two positive clones isolated from the *Pst*I library were identical. We designated these two clones pSAM1 (from the *Pst*I library) and pSAM3 (from the *Bam*HI library).

**Nucleotide sequence and analysis of the SAM-P20 gene.** The 3.3-kb *Pst*I and 1.9-kb *Bam*HI fragments from the clones described above were mapped with a variety of six-base recognition restriction enzymes. The results are presented as a combined map in Fig. 3. These two fragments share a common region of about 1 kb from the *Pst*I site to the *Bam*HI site. The template DNAs were either defined subclones or exonuclease III deletion clones. Sequencing was started with probes 1 and 2 and new synthetic DNA oligomers as primers as well as with universal primers reading from the pUC18 vector into the inserts by use of the sequencing strategy presented in Fig. 3.

Figure 4 shows the nucleotide sequence of the coding region for SAM-P20 and the deduced amino acid sequence. A single open reading frame consisted of 906 nucleotides (starting with a GTG initiation codon and terminating with a TAG translational stop codon) coding for a polypeptide chain of 302 amino acid residues. A putative ribosome binding site which showed

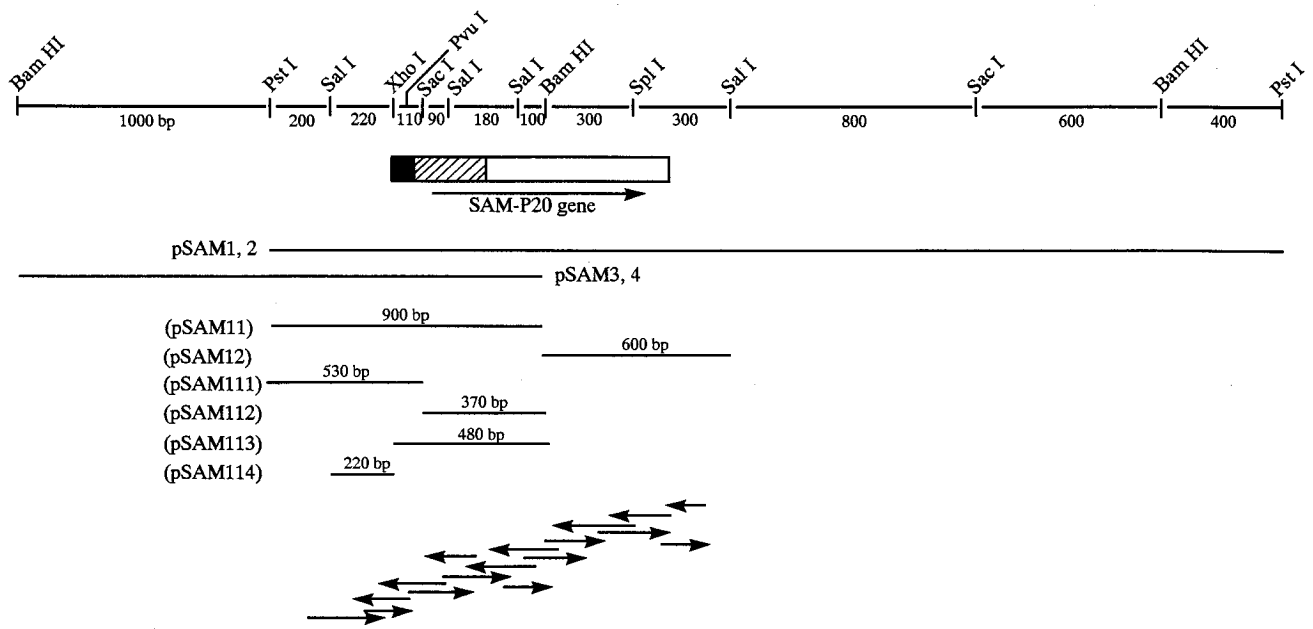


FIG. 3. Restriction enzyme cleavage map of the cloned fragments containing the SAM-P20 gene and the sequencing strategy employed. The 3.3-kb *Pst*I and 1.9-kb *Bam*HI cloned inserts were mapped with a variety of restriction enzymes. The position of the gene is deduced from the sequencing data. Blackened, striped, and open portions of the box represent the sequences encoding the putative pre, putative pro, and mature portions of SAM-P20, respectively. Plasmids of pSAM series are presented under the restriction map. Arrows denote the length and direction for reading the nucleotide sequence of the SAM-P20 gene-containing DNA fragments.

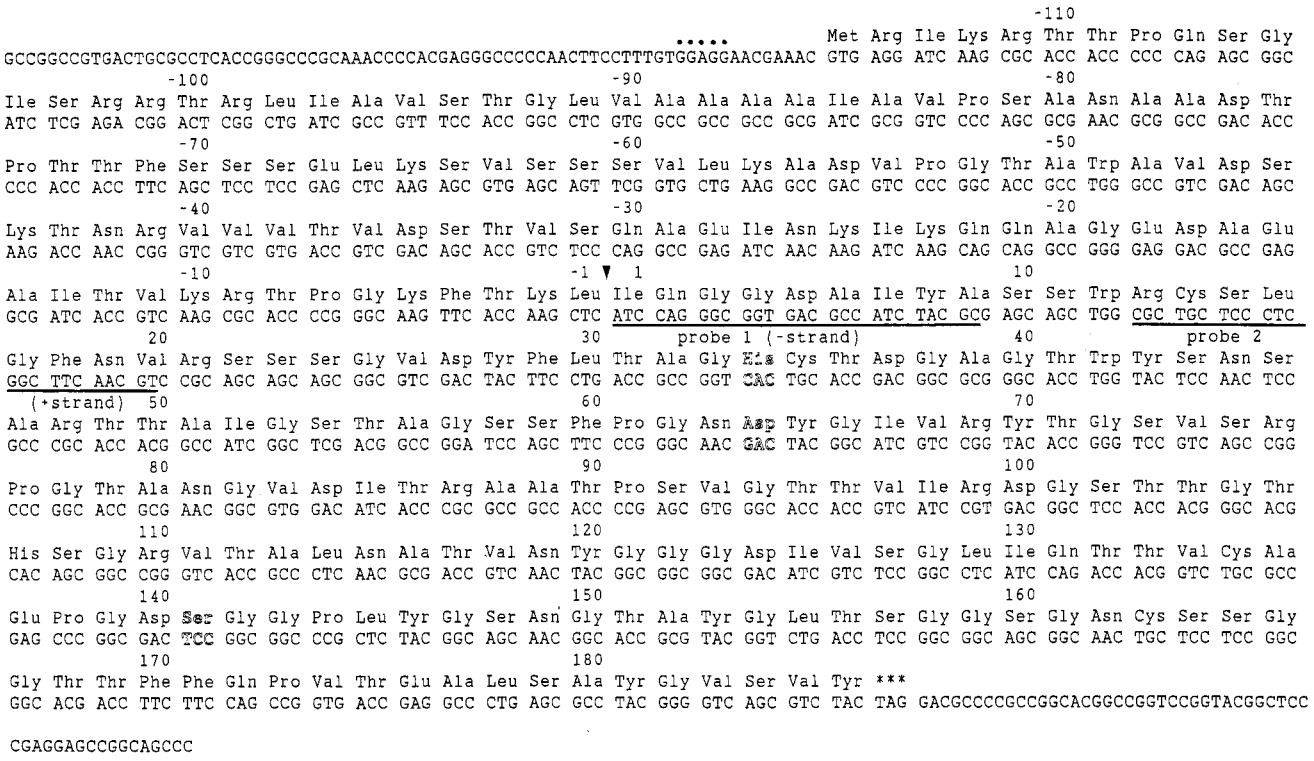


FIG. 4. Nucleotide sequence of the SAM-P20 gene and its flanking regions. The deduced amino acid sequence, which is numbered from the N terminus of the mature protein, is shown above the DNA sequence. The termination codon is indicated by asterisks. The putative ribosome binding site is indicated above the nucleotide sequence by dots. DNA sequences which hybridized to the oligonucleotide probes 1 and 2 are underlined. The downward arrowhead indicates the processing site for the maturation of SAM-P20 protease precursor.

complementarity to the 3′ end of the 16S rRNA of *S. lividans* (2) was found to precede the start codon. The coding sequence is characteristic of many *Streptomyces* genes in that it has a high overall G+C content (70 mol%) and a strong tendency (97 mol%) to utilize codons that have G or C in the third position (3). The nucleotide sequence data reported here have been submitted to the DDBJ database of the National Institute of Genetics, Mishima, Japan, and are available under accession number D29744.

The amino acid sequence deduced from the nucleotide sequence of the SAM-P20 gene revealed significant homology with those of *S. griseus* proteases (8), SGPA (49.6% identity in the prepro region, 57.3% identity in the mature region, and 55.6% identity in total), and SGPB (58.6% identity in the prepro region, 75.5% identity in the mature region, and 69.1% in total) (Fig. 5). Possible secondary cleavage at the position between Leu(−1) ↓ Ile(+1) would yield a mature protein (18.5 kDa), which is in reasonable agreement with the molecular size estimated by SDS-PAGE.

**Expression of the SAM-P20 gene in *E. coli*.** To elucidate whether the isolated SAM-P20-encoding region has expression ability, the initially cloned plasmid, pSAM1, was introduced directly into *E. coli* JM109. In this plasmid, the reading direction of the SAM-P20 gene was designed to be oriented in the same direction as that of the *lac* promoter on the pUC18 vector. As shown in Fig. 6, a discrete clear zone indicating degradation of skim milk could be detected around the transformant-growing cells carrying pSAM1. On the other hand, plasmid pSAM2, in which the reading direction of the SAM-P20 gene was designed to be opposite that of the *lac* promoter in pUC18, formed no clear zone. These results indicate that the cloned *Pst*I-*Pst*I DNA fragment is capable of secretory expression of active SAM-P20 under the control of *lac* promoter alone.

It is interesting that the addition of SSI did not completely inhibit the clear-zone formation around transformant cells carrying pSAM1 (expressing SAM-P20) under the experimental conditions adopted. In contrast, SSI completely inhibited the clear-zone formation around the transformant carrying pUΔS16-1, as was expected, which expressed subtilisin BPN′ (Fig. 6, lane 4, lower zone). This difference seems to be due to the difference in the modes of interaction of SSI and the two target proteases mentioned above, SAM-P20 and subtilisin BPN′: the former protease ultimately inactivates SSI and the latter does not.

## DISCUSSION

The aim of the present study was to isolate and characterize the internal true target enzyme(s), if any, which specifically interacts with the proteinaceous protease inhibitor, SSI, produced by *S. albogriseolus* S-3253. We considered this to be a key step essential for investigating the physiological role(s) and evolutionary process of SSI and SSI-like inhibitor proteins which were found by us to be widespread in *Streptomyces* spp. (31–33). In this respect, isolation of the SSI-nonproducing mutant strain M1 derived from *S. albogriseolus* S-3253 enabled us to isolate one of the candidate target enzymes, designated SAM-P20, and to gain a basis for studying the biological significance of SSI in the original cell.

That mutants such as M1, deficient in SSI, could be isolated at a high frequency merely through a simple protoplast forma-

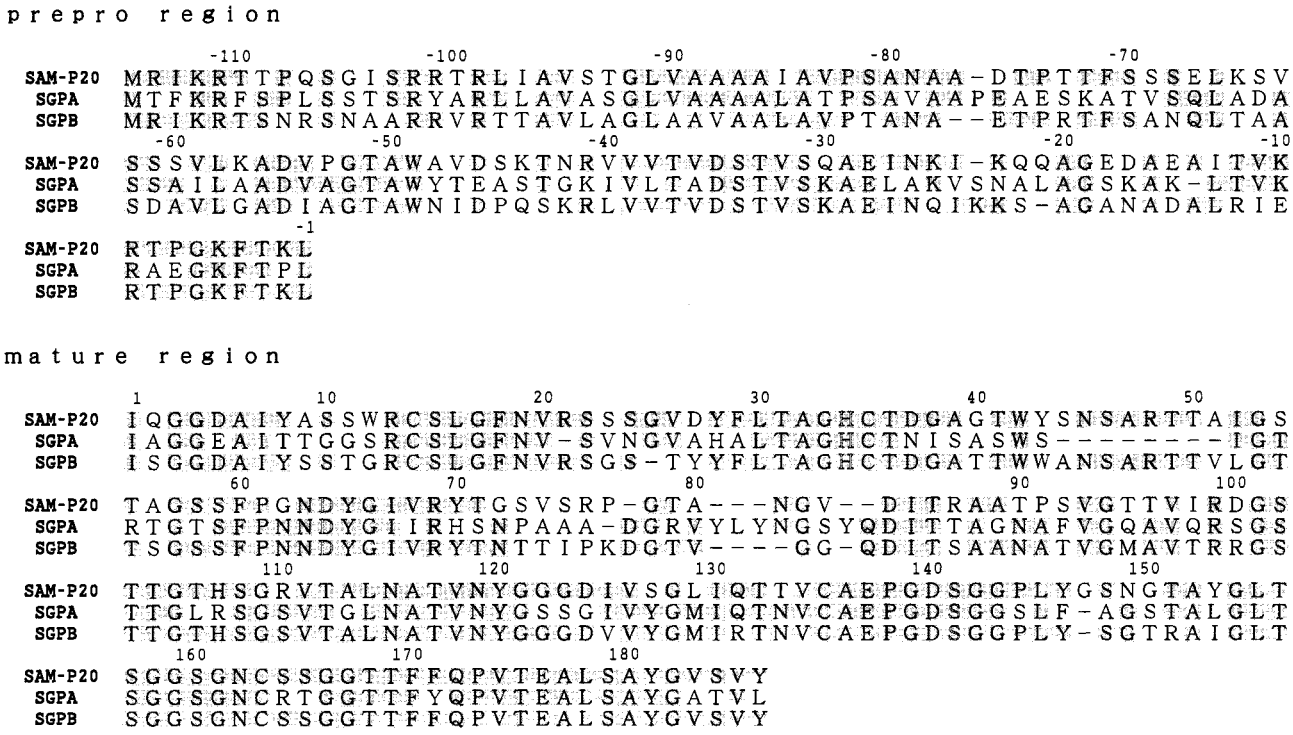


FIG. 5. Alignment of deduced amino acid sequences of SAM-P20 and the *S. griseus* proteases SGPA and SGPB. The single-letter code for amino acids is used. SAM-P20 is used as a criterion for numbering. The outlined letters indicate the three putative amino acid residues (His-34, Asp-64, and Ser-141) forming the catalytic triad found in serine proteases. Deletions for maximum alignment are indicated by dashes. Identical amino acid residues among three proteases are shaded.

tion-regeneration procedure (10) suggests that the gene coding for the SSI might be located on an extrachromosomal element. By Southern hybridization analysis with probes specific for the SSI gene, no hybridization band was detected in restriction enzyme-treated total DNAs from M1 (data not shown). This strongly suggests that elimination of either an extrachromosomal or chromosomal deletogenic (24) element containing the SSI gene occurred readily. This hypothesis can also explain why the SIL proteins are so widely distributed among *Streptomyces* strains, if these elements are transferable, e.g., in the form of plasmids, under some physiological condition. At present, we found that the extra DNA band hybridized with the SSI gene probe only in the *S. albogriseolus* parent strain by pulsed-field gel electrophoretic analysis (unpublished data).

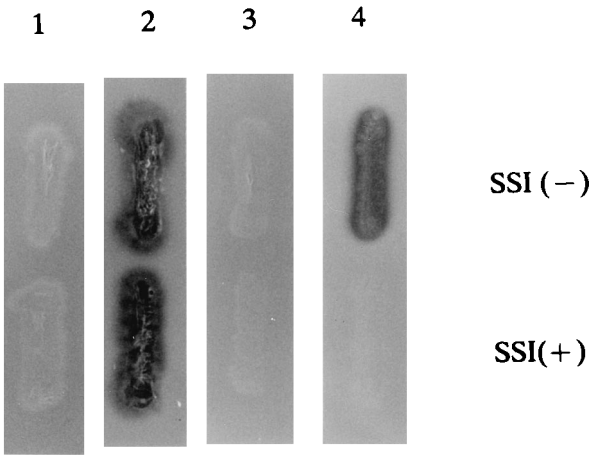


FIG. 6. Plate assay for identification of functional expression of the SAM-P20 gene in *E. coli*. Zones: 1, *E. coli* JM109; 2, *E. coli* JM109 with pSAM1; 3, *E. coli* JM109 with pSAM2; 4, *E. coli* JM109 with pUΔS16-1 containing the subtilisin BPN′ gene. Each strain was grown on a plate containing 2% skim milk, 1% lactose, and 0.1% yeast extract at 37°C for 24 h. The temperature was then shifted to 25°C, and incubation was continued for 120 h. The assay plate was not supplemented [SSI(−)] or was supplemented [1 μg/ml of medium; SSI(+)] with SSI.

The newly discovered target protease SAM-P20 was found to have significant structural homology, in both its N-terminal amino acid and whole nucleotide sequences (Fig. 4), with the already well-characterized proteins SGPA and SGPB from *Streptomyces griseus* (8). Also, to date, production of α-chymotrypsin-type serine proteases with molecular sizes ranging from 14 to 20 kDa from several *Streptomyces* species has been reported, although their structures have not been well characterized (1, 8, 19, 42). These findings strongly suggest that a variety of proteases of this type might be distributed ubiquitously, just like SSI-like inhibitor proteins, among the genus *Streptomyces*, although some might be genetically masked as, e.g., in *S. albogriseolus* S-3253. Very recently, we also isolated a gene for SAM-P20-like protease from a mutant strain of *Streptomyces griseoincarnatus* Kto-250, designated GM (unpublished data). Therefore, these microorganisms might possess a set of specific interacting systems between SSI-like inhibitors and their targets, SAM-P20-like proteases. The same strategy as that employed here using an affinity column conjugated with an SSI-like inhibitor(s), originating from each *Streptomyces* strain, would be applicable for hunting an individual cognate target protease(s).

Microbial endolytic proteases have been divided generally into four major families, serine or alkaline, metal or neutral, thiol or cysteine, and acid or aspartic acid proteases, on the basis of their mechanisms of action (6). Recently, however,

Kajiwara et al. and Tsuyuki et al. (11, 40) have demonstrated that the metalloproteases SGMPI and SGMPII, isolated from pronase P of *S. griseus*, are strongly inhibited by serine protease inhibitors such as SSI, alkaline protease inhibitor (API-2c′) (29), and plasminostreptin (12) as well as a metalloprotease inhibitor (SMPI from *Streptomyces nigrescens* TK-24) (20). Therefore, grouping of proteases and their inhibitors on the basis of their individual genus or species specificities should also be considered from the viewpoint of their physiological functions.

Figure 5 shows a comparison of the deduced amino acid sequence of SAM-P20 with those of SGPA and SGPB from *S. griseus*. In the overall homology based on amino acid identity, the primary structure of SAM-P20 is more similar to that of SGPB (69.1%) than to SGPA (55.6%). Potential catalytic triad residues, His, Asp, and Ser, which are common for various serine proteases, are conserved at the positions corresponding to His-34, Asp-64, and Ser-141 (SAM-P20 numbering), and the regions of catalytic site topographies of the three proteases show significant homologies. Also, SAM-P20 has four cysteine residues (Cys-14, Cys-35, Cys-135, and Cys-163) in positions identical with those of SGPA and SGPB, suggesting that the formation of disulfide bridges of SAM-P20 may occur in a manner similar to those of SGPA and SGPB. These structural similarities suggest that SAM-P20 is categorized as a chymotrypsin-type serine protease of *Streptomyces* spp. and that further discovery of its homologs will enable us to promote comparative genetic and biochemical studies and to establish unified physiological and evolutionary views on them.

The 115 amino acid residues upstream of the N terminus of the mature protease probably constitute a long prepro-type leader sequence, as has been found with most *Streptomyces* extracellular proteases (4, 5, 8, 16). The processing of pro-SAM-P20 is probably autocatalytic, because the amino acid sequence near the scissile bond, Leu(−1) ↓ Ile(+1), would provide a favorite substrate for the chymotrypsin-type protease. The pro-region may play an essential role in guiding the folding of the pro-SAM-P20 molecule into its proper conformation necessary for activity, as observed with subtilisin (44) and α-lytic protease (28).

The expression experiments revealed that, in *E. coli* JM109, the cloned SAM-P20 gene was expressed and secreted (Fig. 6). The inhibitory effect of SSI showed contrasting patterns toward the cognate and noncognate targets, SAM-P20 and subtilisin BPN′, respectively (Fig. 6). Also, a more temporary inhibition by SSI of the hydrolyzing activity of SAM-P20 produced by *E. coli* was caused in parallel by decreasing the amount of SSI contained in the medium (data not shown). The API-2c′ producer, *S. griseoincarnatus* KTo-250, has been reported to produce a structurally unknown 28-kDa protease capable of degrading API-2c′ and being inhibited by SSI (30). SSI and API-2c′ are the most homologous among recently characterized SIL proteins (34) in terms of structural similarity (90%) and presence of the same amino acid, Met, at the reactive site P1. These characteristics suggest that SSI might exhibit temporary inhibition of the cognate target SAM-P20 and show gradual degradation by SAM-P20 over a wide time range for some internal physiological advantage, although other advantages through interaction with the external environment are not ruled out.

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