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A Novel Member of the Subtilisin-Like Protease Family from *Streptomyces albogriseolus*

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We previously isolated three extracellular endogenous enzymes from a *Streptomyces albogriseolus* mutant strain which were targets of *Streptomyces* subtilisin inhibitor (SSI) (S. Taguchi, A. Odaka, Y. Watanabe, and H. Momose, Appl. Environ. Microbiol. 61:180–186, 1995). In the present study, of the three enzymes the largest one, with a molecular mass of 45 kDa (estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis), termed SAM-P45, has been characterized in detail. The entire gene encoding SAM-P45 was cloned as an approximately 10-kb fragment from *S. albogriseolus* S-3253 genomic DNA into an *Escherichia coli* host by using a shuttle plasmid vector. The amino acid sequence corresponding to the internal region of SAM-P45, deduced from the nucleotide sequence of the gene, revealed high homology, particularly in three regions around the active-site residues (Asp, His, and Ser), with the amino acid sequences of the mature domain of subtilisin-like serine proteases. In order to investigate the enzymatic properties of this protease, recombinant SAM-P45 was overproduced in *Streptomyces coelicolor* by using a strong SSI gene promoter. Sequence analysis of the SAM-P45 gene and peptide mapping of the purified SAM-P45 suggested that it is synthesized as a large precursor protein containing a large C-terminal prodomain (494 residues) in addition to an N-terminal preprodomain (23 and 172 residues). A high proportion of basic amino acids in the C-terminal prodomain was considered to serve an element interactive with the phospholipid bilayer existing in the C-terminal prodomain, as found in other membrane-anchoring proteases of gram-positive bacteria. It is noteworthy that SAM-P45 was found to prefer basic amino acids to aromatic or aliphatic amino acids in contrast to subtilisin BPN′, which has a broad substrate specificity. The hydrolysis by SAM-P45 of the synthetic substrate (*N*-succinyl–L-Gly–L-Pro–L-Lys–*p*-nitroanilide) most preferred by this enzyme was inhibited by SSI, chymostatin, and EDTA. The proteolytic activity of SAM-P45 was stimulated by the divalent cations Ca^{2+} and Mg^{2+}. From these findings, we conclude that SAM-P45 interacts with SSI and can be categorized as a novel member of the subtilisin-like serine protease family.

The interaction of proteases and their cognate inhibitors is a typical physiological regulation system involved in important biological processes such as blood coagulation (20), the cell cycle (8), and developmental processes (1) in mammals. It is generally considered that protease inhibitors modulate protease activities and control a variety of the critical protease-mediated processes mentioned above. However, relatively little is known about the biological role of protease-protease inhibitor interactions in microorganisms. Serine proteases produced by *Streptomyces lactamdurans* (7) and *Streptomyces peucetus* (6) were reported to coordinately regulate the cellular protein turnover associated with secondary metabolism and morphogenesis. These organisms are also well known to be producers of protease inhibitors, including both low-molecular-mass (42) and proteinaceous (10) compounds.

In our recent studies, we found that proteinaceous protease inhibitors, *Streptomyces* subtilisin inhibitor (SSI [reviewed in reference 10])-like proteins (SIL proteins), were ubiquitous in *Streptomyces* (27–29). Comparative studies on the primary structures and inhibitory properties of isolated SIL proteins revealed that these proteins have similar molecular masses, form dimers, exhibit sequence homology in conserved regions essential for maintaining their tertiary structures, and exhibit strong correlation between the P1 reactive-site residue of inhibitors and substrate specificity of exogenous target proteases (12, 30, 31, 37–40). In addition, to clarify the physiological role of SIL proteins, we identified three endogenous target proteases in the culture supernatant of an SSI-nonproducing mutant strain using an affinity column to which SSI was bound. One of the target proteases, termed SAM-P20, was classified as a novel member of the chymotrypsin superfamily (25), and its proteolytic activity was strongly inhibited by tight complex formation with SSI (34, 35). All of the SSI-nonproducing mutant strains derived from *Streptomyces albogriseolus* S-3253 exhibited several common pleiotropic properties: slightly slow growth even in a rich medium, a marked decrease in aerial mycelium-forming ability, and a remarkable increase in the extracellular activity and/or productivity of proteases (34). The molecular and biochemical characterization of these target proteases is an essential step for understanding the physiological significance of their interaction with SSI in the cell. In particular, it is of interest to elucidate the molecular mechanism for the secretion, processing, and activation of extracellular proteases in *Streptomyces*, considering the possibility of the involvement of SSI in these processes.

Here we report on the second target protease, termed SAM-P45, which was isolated by taking advantage of its affinity for SSI and give results from the following: (i) the molecular cloning and nucleotide sequence analysis of the SAM-P45

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gene, (ii) the determination of the C-terminal end of the mature domain of overproduced recombinant SAM-P45 by peptide mapping, and (iii) a detailed enzymatic characterization of SAM-P45. The results of this study indicate that SAM-P45 can be categorized as a novel member of the subtilisin protease family due to its large C-terminal prodomain, presumably for membrane-anchoring, and its exhibition of trypsin-like proteolytic activity.

MATERIALS AND METHODS

Genetic manipulation and culture conditions. Genetic manipulation methods applicable to *Escherichia coli* and *Streptomyces coelicolor* were performed as described by Sambrook et al. (23) and Hopwood et al. (11), respectively. Standard media, culturing methods, and transformation procedures for *Streptomyces* spp. were as described previously (33). For the selection of *S. coelicolor* transformants, thiostrepton (50 mg/liter) was used in liquid culture. *E. coli* transformants were grown in Luria-Bertani medium containing ampicillin (50 mg/liter).

Cloning of the SAM-P45-encoding gene. Total chromosomal DNA from *Streptomyces albogriseolus* prepared by the method of Saito and Miura (22) was digested with several restriction enzymes and then separated by 0.8% agarose gel electrophoresis. The separated DNA fragments were subjected to Southern blotting. The Southern blotting and hybridization methods used were performed as previously described (11). The synthetic antisense DNA oligomer, 5′-GCCT TC(G)GGG(C)GTG(C)CCGATCTG-3′, corresponding to seven amino acids of the N-terminal sequence of SAM-P45, was used as the hybridization probe (probe 1). An approximately 10-kbp *Xho*I fragment, which gave a positive signal on Southern hybridization, was extracted from the agarose gel by using the Sephaglass band preparation kit (Pharmacia) and inserted into the *Sal*I site of plasmid pUC18. After transformation of *E. coli* JM109 with this ligation mixture, white colonies on Luria-Bertani medium containing ampicillin (50 mg/liter) 5-bromo-4-chloro-3-indolyl-β-galactoside (40 mg/liter) and 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) were selected to generate a library. Positive clones carrying the SAM-P45 gene were isolated 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 reaction method with double-stranded plasmids as templates (17) and with a commercially available sequence kit (BcaBEST; Takara Shuzou) and [α-^{32}P]dCTP. The sequence was determined for both strands by using overlapping fragments.

Construction of an expression vector for the SAM-P45 gene. A shuttle vector for expression of the SAM-P45 gene in both *E. coli* and *Streptomyces* was constructed by the following three ligation steps (see Fig. 2). First, two linear DNA fragments derived from pBluescript II KS+ (Toyobo) digested at the *Pst*I and *Sac*I sites and pAP45-4 carrying the SAM-P45 gene digested at the *Pst*I and *Sac*I sites were ligated to generate pBP45. Second, to locate the SAM-P45 structural gene in the proximate downstream region of two promoters of the SSI gene (17), pSI205 (32) digested with *Eco*RI and *Nae*I and pB-P45 digested with *Eco*RI and *Sma*I were ligated to generate pSIP45. Finally, the expression vector was constructed by ligating pUJSAM and pSIP45 digested with *Eco*RI and *Hin*dIII, and the resulting plasmid was designated pUJP45.

Purification of recombinant SAM-P45. *S. coelicolor* transformants carrying pUJP45 were initially cultivated in 20 ml of tryptic soy broth medium at 30°C for 2 days, and then cultivation was continued in 3.0 liters for 3 days. After cells were removed from the culture medium by filtration, ammonium sulfate was added to 60% saturation, and this solution was stirred at 4°C overnight. The proteins thus precipitated were harvested by centrifugation at 12,000 rpm (HIMAC SCR20B; Hitachi Co. Ltd.) for 20 min. The precipitate was dissolved in 100 ml of 10 mM citric acid-sodium citrate buffer (pH 5.4) and dialyzed against the same buffer at 4°C overnight. The protein solution was then subjected to ion-exchange chromatography (column size, 3 by 45 cm) on DEAE-cellulose, and fractions containing only SAM-P45 were obtained. Finally, these fractions were dialyzed against distilled water and lyophilized. The purity and size of proteins in each purification step were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (14). The protein samples were precipitated with trichloroacetic acid at a final concentration of 8% before loading. Proteins in the gel were stained with 2% (wt/vol) Coomassie brilliant blue R-250.

Amino acid sequence determination of SAM-P45. Purified SAM-P45 was inactivated by precipitation with 10% trichloroacetic acid (final concentration) and then dissolved in buffer containing 2 M urea for enzymatic digestion. Digestion of the inactivated SAM-P45 by proteases was carried out by procedures described previously (30). Amino acid sequences of intact SAM-P45 and digested peptides were determined with an Applied Biosystems model 476A protein sequencer.

Protein concentration. Protein concentration was determined by the method of Bensadoun and Weinstein, with bovine serum albumin as a standard (2).

Assay for proteolytic activity. All synthetic substrates used in this study were purchased from Sigma Co. Ltd. The activity of the recovered SAM-P45 in each purification step was measured by monitoring the increase of absorbance at 410 nm resulting from the release of *p*-nitroaniline due to the enzymatic hydrolysis of *N*-succinyl–L-Phe–L-Val–L-Arg–*p*-nitroanilide by SAM-P45. All synthetic substrates used for the measurement of the substrate specificity of the SAM-P45 are listed below (see Table 2). Each hydrolysis reaction was started by the addition of 100 nM SAM-P45 into a solution composed of 100 μM substrate, 100 mM Tris-HCl (pH 8.5), 0.1% dimethyl sulfoxide, and 10 mM $CaCl_2$ to a final volume of 1 ml. The specific activity of SAM-P45 was expressed as the amount (in micromoles) of *p*-nitroanilide liberated by 1.0 mg of SAM-P45 in 1 s under the above-described conditions.

Effect of inhibitors. Protease inhibitors used and their concentrations are presented below (see Table 3). All assays were performed by the procedure described above, with 100 μM *N*-succinyl–L-Gly–L-Pro–L-Lys–*p*-nitroanilide (GPK) as the substrate.

Effect of temperature and pH on enzyme activity. The hydrolysis of GPK by purified SAM-P45 was assayed at different temperatures (1 to 90°C) with a temperature-controlled cuvette holder attached to a recirculating water bath. For each assay, the temperature of the buffer containing the substrate was adjusted 5 min prior to the addition of enzyme. The effect on protease activity of adding 10 mM calcium ion was also tested. The effect of pH on GPK hydrolysis was determined with the following buffers at 50 mM: 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 Gly-NaOH (pH 9.0 to 11.0). The assays at different pH values were carried out at 25°C.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, GenBank/EMBL, and NCBI nucleotide sequence databases under the accession number D83672.

RESULTS

Molecular cloning of the SAM-P45 gene. As described in our previous paper (34), three SSI-interacting proteases were isolated by affinity chromatography using a Sepharose column to which SSI was bound. The major protease among them, SAM-P20, was clearly demonstrated to be an endogenous target of SSI by complex formation analysis (35).

In this study, SAM-P45, represented by the 45-kDa protein band on an SDS-PAGE gel, was electroblotted onto a polyvinylidene difluoride membrane; the region of the membrane containing SAM-P45 was then cut out, and SAM-P45 was directly subjected to N-terminal amino acid sequence analysis as described previously (34). The sequence of the first 13 amino acids was determined to be Leu-Asp-Thr-Ser-Val-Gly-Gln-Ile-Gly-Thr-Pro-Lys-Ala. In the cloning of the SAM-P45 gene, this sequence was used, considering the codon usage bias of GC-rich *Streptomyces* genome DNA (50), to design a mixture of 20-bp oligonucleotide probes (probe 1) with eightfold degeneracy. By Southern blotting, probe 1 was shown to hybridize with approximately 10-kbp fragments generated by *Xho*I digestion of *S. albogriseolus* S-3253 chromosomal DNA. These DNA fragments were then purified from the agarose gel following electrophoresis and ligated to the *Sal*I site of the pUC18 vector to generate a gene library. The gene library thus established was used to screen for positive clones by colony hybridization. Putative positive clones were detected with a high frequency of 2×10^{-3} under stringent hybridization conditions. One of the positive recombinant plasmids, termed pAP45-4, was subjected to DNA sequencing analysis.

Nucleotide sequence analysis. The nucleotide sequence of the SAM-P45 gene, including the 5′- and 3′-flanking sequences, was determined in both directions. The pertinent area sequenced is shown in Fig. 1. A computer analysis based on the G+C content of the nucleotide positions within each codon for *Streptomyces* genome DNA (50) indicated the presence of an open reading frame of 3,309 nucleotides with a high G+C content in the third position in the sequence. A putative ribosome binding site which showed moderate complementarity to the 3′ end of the 16S rRNA of *Streptomyces lividans* (3) was found to precede a GTG start codon. In addition, a potential stable stem-loop terminator structure for the mRNA was found in the sequence directly downstream of the SAM-P45 gene (data not shown). The coding sequence is characteristic

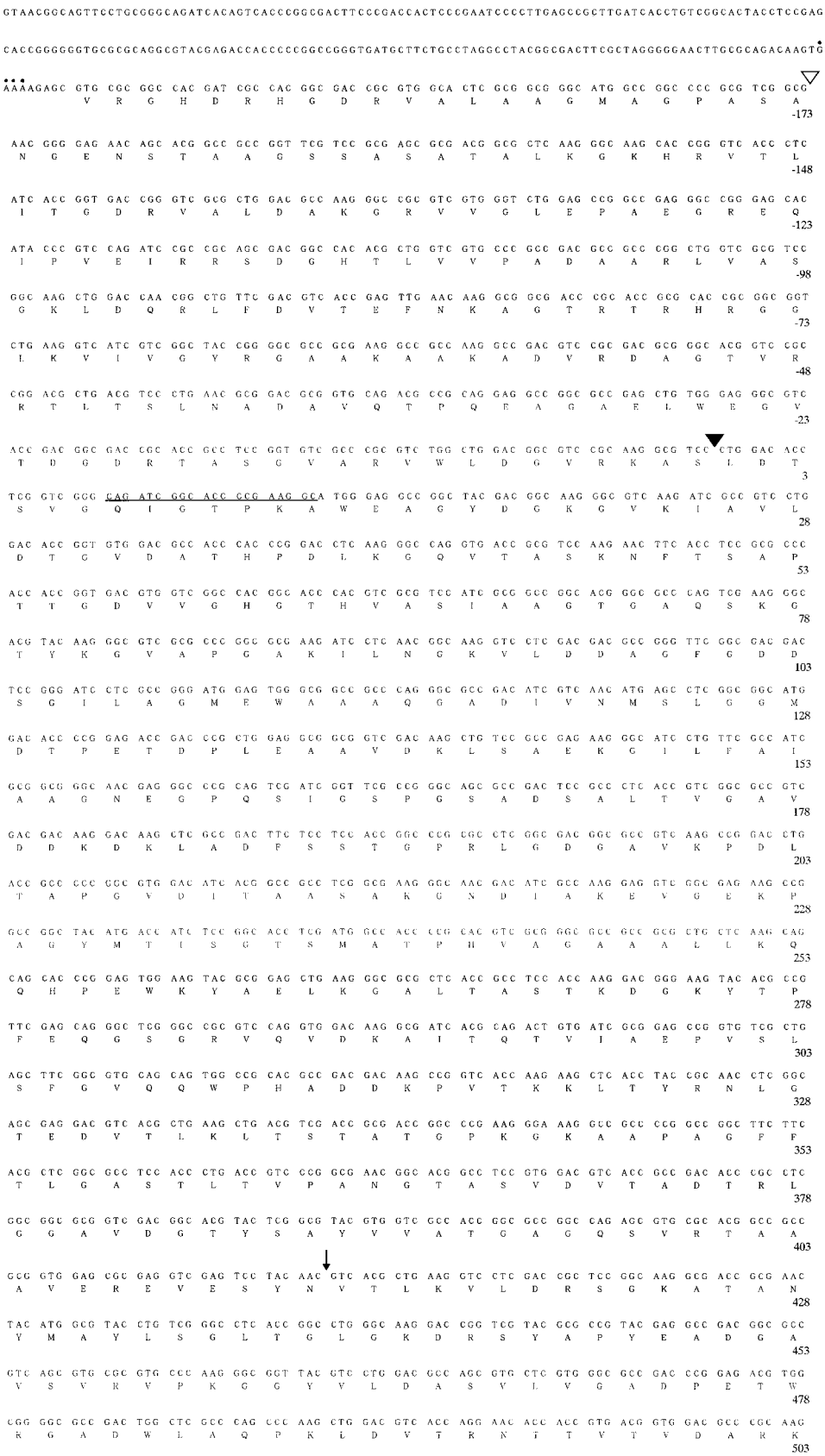

FIG. 1. Nucleotide sequence and deduced amino acid sequence of SAM-P45. The numbering of the deduced amino acid sequence, counting from the N-terminal amino acid residue of the mature protein, is shown below the DNA sequence. The termination codon is indicated by an asterisk. The putative ribosome binding site is indicated above the DNA sequence by dots. The DNA sequence hybridized to the oligonucleotide probe 1 is underlined. The open and closed triangles indicate the cleavage site predicted by signal peptidase and the identified cleavage site of the prodomain, respectively. The C-terminal end amino acid residue of secreted SAM-P45 protein is indicated by a downward arrow.

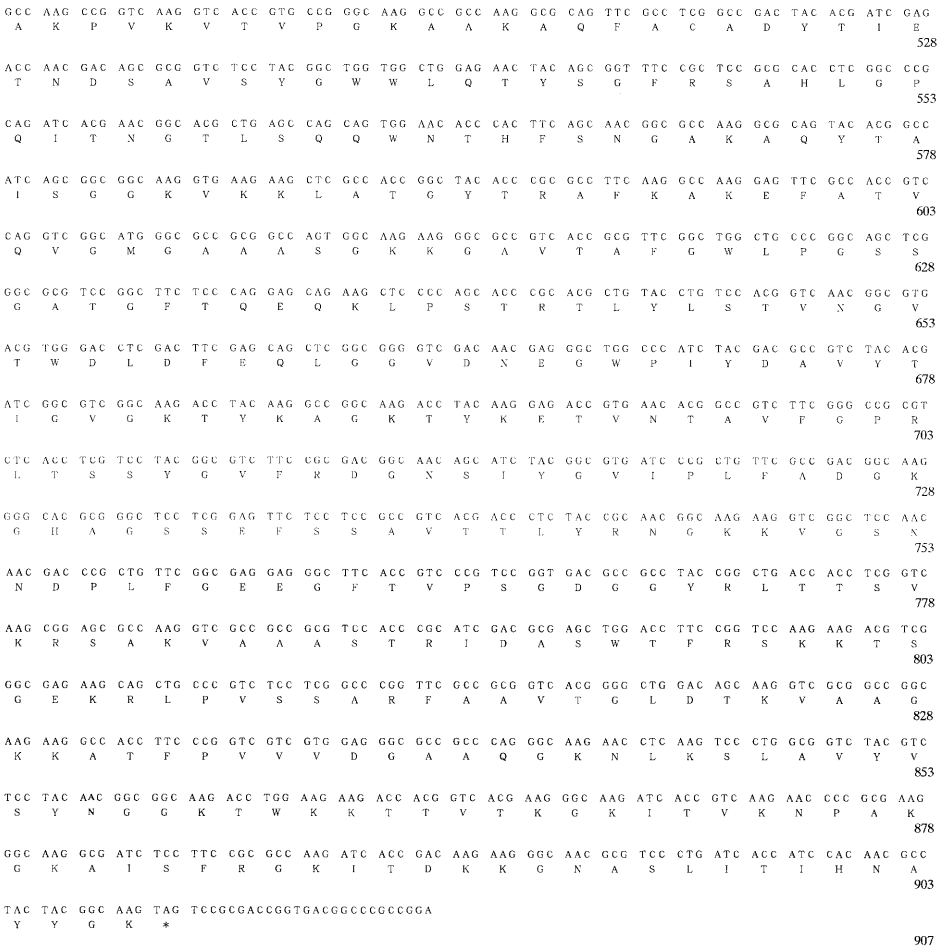

FIG. 1—*Continued.*

of many *Streptomyces* genes in that it has a high overall G+C content (70.8 mol%) and an extremely strong tendency (98.2 mol%) to utilize codons that have G or C in the third position.

This deduced open reading frame has the capacity to encode a large protein of 1,102 amino acids with a calculated molecular mass of 115 kDa, as shown in Fig. 1. The sequence of the first 13 amino acids of the native SAM-P45 could be determined for the deduced proteins. It indicates that the SAM-P45 protease would initially be synthesized as a large precursor proprotein.

Secretory overproduction and purification of SAM-P45. When the shuttle expression vector pUJP45 (Fig. 2) was introduced into the heterologous host, *S. coelicolor*, clear-zone formation due to the degradation of skim milk could be clearly observed around transformant colonies on plates (data not shown). This suggests that a combination of the SSI gene promoter and the secretory machinery of SAM-P45 itself might efficiently function in *S. coelicolor*. The clear-zone formation due to the protease activity of the secreted SAM-P45 was completely masked by the addition of SSI to the plate (data not shown). The results of the purification of SAM-P45 produced by *S. coelicolor* transformant carrying pUJP45 are summarized in Table 1. By a combination of salting out proteins with ammonium sulfate and performing column chromatography, SAM-P45 protein could be purified to homogeneity, as evidenced on an SDS-polyacrylamide gel (Fig. 3). Recombinant SAM-P45 protein was finally purified (32.7-fold) from 2.6 liters of culture supernatant with 38.4% activity recovery. The amount of SAM-P45 produced by the system employed was estimated to be 27.0 mg/liter of the culture supernatant.

Complete amino acid sequence of SAM-P45. That the molecular mass of the secreted SAM-P45 was much smaller than that calculated from the deduced sequence shown in Fig. 1 indicated processing of the precursor of SAM-P45 in the C-terminal region in addition to that in the N-terminal region. Therefore, in order to determine a C-terminal residue of the secreted SAM-P45 and to confirm its amino acid sequence deduced from the nucleotide sequence of the gene, purified SAM-P45 was digested by proteases and digested peptides were subjected to amino acid sequence analysis. Digestion of SAM-P45 by *Staphylococcus aureus* V8 protease and subsequent analysis of the digestion products by reverse-phase high-performance liquid chromatography resulted in nine peaks on the chromatogram, and the sequences of the peptides which gave rise to these peaks were determined, as shown in Fig. 4. Peptide V1 possessing an Asn residue as the C-terminal residue was considered to be a C-terminal peptide of SAM-P45. The amino acid sequences of the C-terminal regions of peptides V6 and V9 were determined by sequence analysis of peptides generated by digestion with arginylendopeptidase and lysylendopeptidase, respectively. Digestion of SAM-P45 by lysylendopeptidase produced four peptides which corresponded to the C-terminal region of SAM-P45, as shown in Fig. 4. Two peaks obtained by arginylendopeptidase digestion of peptide

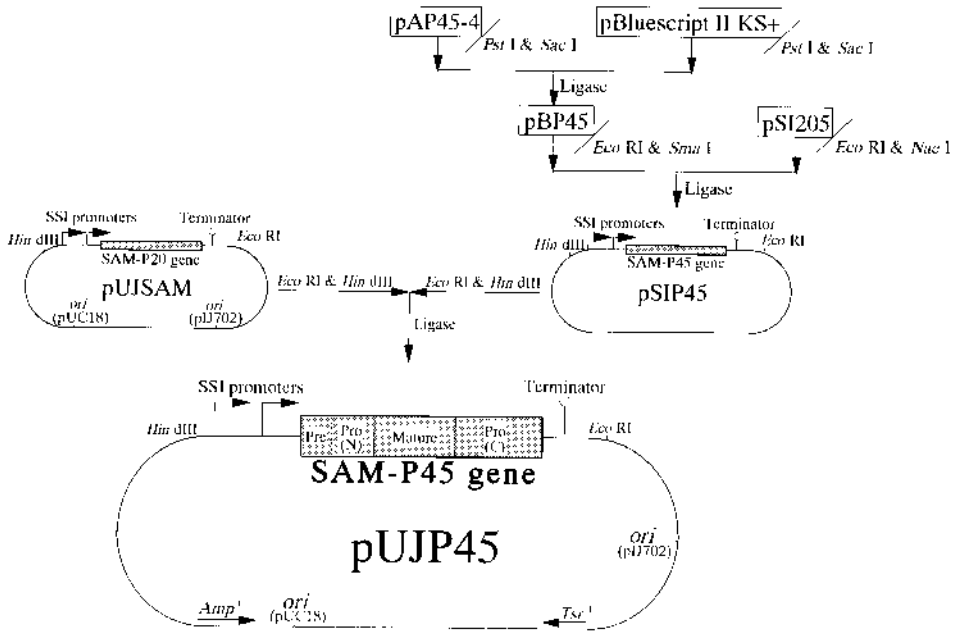

FIG. 2. Construction of recombinant SAM-P45 secretory vector system. The construction of a plasmid, pUJP45, for secretory expression of SAM-P45 in *S. coelicolor* is shown. A detailed explanation is given in Materials and Methods. *Amp*^{r} and *Tsr*^{r} indicate the ampicillin resistance gene and thiostrepton resistance gene, respectively. The pre-region (Pre), mature domain, and N- and C-terminal [Pro(N) and Pro(C), respectively] prodomains of SAM-P45 are indicated.

L17 clarified its remaining sequence. The amino acid sequence of peptide L17/A1 possessing an Asn residue as the C-terminal residue was consistent with that of peptide V1. Thus, the primary structure of SAM-P45 deduced from its nucleotide sequence was confirmed by amino acid sequence analysis, and it was concluded that the C-terminal residue of the secreted SAM-P45 was Asn413 and that SAM-P45 did not undergo any posttranslational modifications. It was concluded that SAM-P45 might be secreted in an active form carrying a C-terminal extension approximately 120 amino acid residues longer than those of class I subtilisins categorized by Siezen et al. (26).

Taken together with the nucleotide sequence of the SAM-P45 gene, it was concluded that the SAM-P45 gene product possesses additional portions consisting of 195 and 494 amino acid residues at the N-terminal and C-terminal ends of the mature domain, respectively.

Comparison of the mature region of SAM-P45 with those of other proteases. The determined amino acid sequence of the mature region of SAM-P45 was compared with other potential protein sequences by using the DNASIS program (Takara Shuzo Co., Ltd.). A computer search revealed that the mature region of SAM-P45 exhibits sequence similarity to the family of subtilisin-like serine proteases (termed subtilases by Siezen et al. [26]); in particular, it exhibits 32.3% identity with subtilisin BPN′ (48) and 33.1% identity with thermitase (15). The best-studied member among over 60 members of this subtilase family, subtilisin BPN′, was used for sequence alignment with SAM-P45. As shown in Fig. 5, the amino acid sequence of SAM-P45 was arranged to give a good alignment between the

TABLE 1. Purification of recombinant SAM-P45 from *S. coelicolor* carrying pUJP45

Purification step	Vol (ml)	Total amt (mg)	Total activity (U)[a]	Sp act (U/mg)	Activity recovery (%)
Culture supernatant	2,600	2,260	138	0.06	100.0
Precipitate by $(NH_4)_2SO_4$	120	770	96	0.12	69.6
DEAE-cellulose	150	27	53	1.96	38.4

[a] One unit of activity equals 1.0 μmol of *p*-nitroanilide released per min.

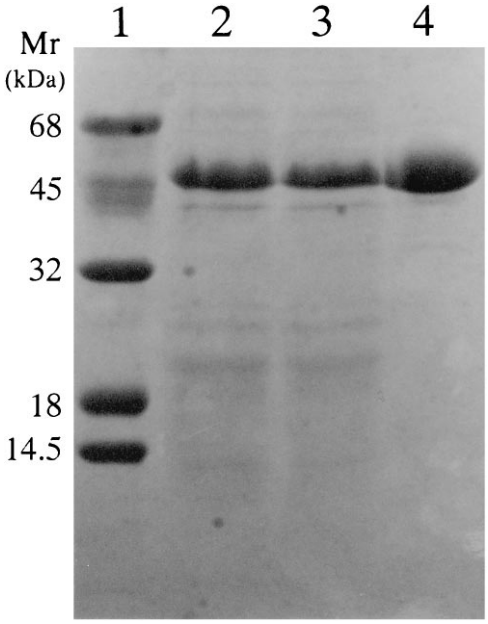

FIG. 3. SDS-PAGE pattern at each step of purification of recombinant SAM-P45. Lane 1, molecular mass markers; lane 2, culture supernatant of *S. coelicolor* carrying pUJP45; lane 3, ammonium sulfate (60% saturation)-precipitated sample; lane 4, sample purified by chromatography on a DEAE-cellulose column.

FIG. 4. Complete amino acid sequence and sequencing strategy of secreted SAM-P45. 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; L, lysylendopeptidase; A, arginylendopeptidase.

sequences of the two proteases. Potential catalytic triad residues, Asp, His, and Ser, which are common in subtilisin-like proteases, are conserved at the positions corresponding to Asp29, His61, and Ser238 (SAM-P45 numbering).

Substrate specificity. The substrate specificity of SAM-P45 was investigated with a series of chromogenic substrates possessing the P1 site residues Lys, Arg, Phe, Leu, Ala, and Glu. As is evident from Table 2, SAM-P45 exhibited significant

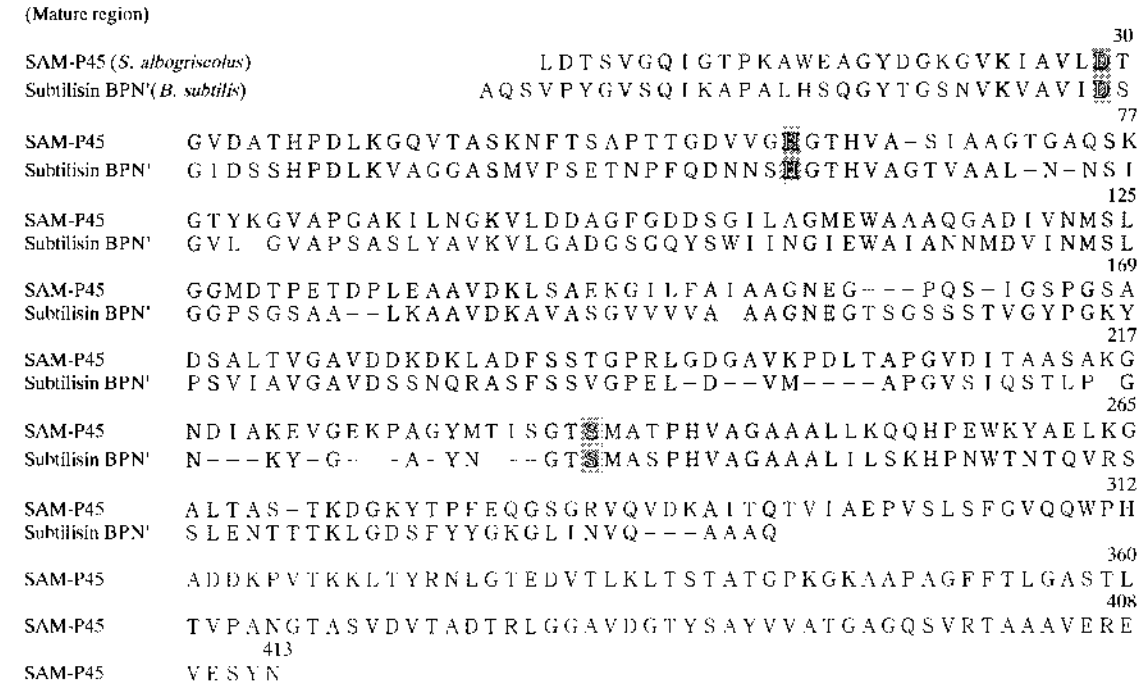

FIG. 5. Comparison of SAM-P45 and subtilisin BPN′ amino acid sequences. The single-letter code for amino acids is used. SAM-P45 is used as a criterion for numbering. The outlined letters are the three amino acid residues forming the catalytic traid (putative for SAM-P45 and identified for subtilisin BPN′). Identical amino acid residues are shaded. Deletions for maximum alignment are indicated by dashes.

TABLE 2. Substrate specificity of SAM-P45 and subtilisin BPN′

Substrate[a]	Sp act (mmol min^{-1} g^{-1} [%])	
	SAM-P45	Subtilisin BPN′
Suc–L-Bly–L-Pro–L-Lys–*p*-NA	460 (100.0)	1 (0.2)
Suc–L-Gly–L-Pro–L-Arg–*p*-NA	160 (35.0)	0 (0.0)
Tos–L-Phe–L-Val–L-Arg–*p*-NA	200 (43.0)	3 (1.9)
*N*α-Benzoyl–L-Arg–*p*-NA	0 (0.0)	0 (0.0)
Suc–L-Ala–L-Ala–L-Pro–L-Phe–*p*-NA	71 (15.0)	167 (100.0)
Suc–L-Ala–L-Ala–L-Pro–L-Leu–*p*-NA	24 (5.2)	36 (21.3)
Suc–L-Ala–L-Ala–L-Val–L-Ala–*p*-NA	4 (1.0)	2 (1.0)
Tos–L-Ile–L-Ile–L-Glu–*p*-NA	0 (0.0)	0 (0.0)

[a] The boxed residues occupy the P1 site. Abbreviations: Suc, succinyl; Tos, tosyl; NA, nitroanilide.

hydrolytic activity towards tripeptide substrates whose P1 site residues were the basic amino acids Lys and Arg. In contrast to SAM-P45, subtilisin BPN′ preferred substrates possessing the hydrophobic amino acids Phe and Leu at the P1 site (47).

The kinetic parameters K_m, k_{cat}, and k_{cat}/K_m of SAM-P45 for the most preferable substrate, GPK, were estimated to be 130 μM, 6.7 s^{-1}, and 50 mM^{-1} s^{-1}, respectively.

Effect of protease inhibitors on SAM-P45 activity. As shown in Table 3, the hydrolysis of GPK by SAM-P45 was strongly inhibited by SSI, chymostatin, EDTA, and antipain and was slightly inhibited by leupeptin, phenylmethylsulfonyl fluoride (PMSF), and Zn^{2+} but was not inhibited by E-64, pepstatin, and bestatin. Surprisingly, the serine protease inhibitors TPCK (*N*-tosyl-L-phenylalanine chloromethyl ketone) and TLCK (*N*–-*p*-tosyl-L-lysine chloromethyl ketone) did not affect the proteolytic activity of SAM-P45.

Optimal pH and optimal temperature for SAM-P45 activity. SAM-P45 was most active at pH greater than 10.0 in the hydrolysis of GPK, indicating that SAM-P45 is a typical alkaline protease. The optimum temperature for the proteolytic activity of SAM-P45 was 70 or 80°C in the absence or presence of 10 mM Ca^{2+}, respectively, which indicates the effect of Ca^{2+} on the thermal stability of SAM-P45 activity (data not shown).

DISCUSSION

To investigate an endogenous extracellular protease, SAM-P45, acting as a potential target enzyme interactive with SSI, the corresponding gene was cloned from the *S. albogriseolus* genome. The amino acid sequence deduced from the nucleotide sequence of the SAM-P45 gene was composed of 1,102 residues, with a potential 23-residue signal peptide and a 172-residue prodomain in the N-terminal region of the SAM-P45 precursor protein. The putative signal peptide possesses five positively charged amino acids [Arg(−194) to Arg(−186)] in the N-terminal region followed by a central hydrophobic stretch [Val(−185) to Gly(−177)] and C-terminal small side chain amino acids. The junction sequence between Ala(−173) and Asn(−172) would be a potential cleavage site for the signal peptidase according to the [Ala(−3), Ala(−1)] rule (43, 44).

The nucleotide sequence of the SAM-P45 gene, together with the peptide mapping of recombinant SAM-P45 synthesized by *S. coelicolor*, also suggested that precursor SAM-P45 protein possesses a 494-residue C-terminal domain. A serine protease of *Serratia marcescens*, a gram-negative bacterium, was found to be generated as a large precursor composed of three functional domains: an N-terminal signal sequence, the mature protease, and a large C-terminal prodomain. The prodomain of this protease is processed during the secretory process and has an essential role in the export of the mature protease through the outer membrane (24). A number of cell wall proteases capable of associating with the cell envelope were found in gram-positive bacteria such as *Lactococcus lactis* (41, 45). These proteases commonly share sequences for membrane-anchoring domains, i.e., proline-rich regions, a hydrophobic α-helix, and an 18- to 20-amino-acid hydrophilic tail in the C-terminal end (45). Although the C-terminal prodomain of SAM-P45 does not possess this membrane-anchoring motif, it is very characteristic in the sense that several stretches of hydrophobic amino acid residues and many mono- or dibasic amino acids (13.5%) are present in the C-terminal prodomain. If the SAM-P45 C-terminal domain functions in membrane anchoring, the basic amino acids may serve as an effective stop-transfer signal and prevent transport of the protein through the cell membrane by interaction with the negatively charged phosphate groups of the phospholipid bilayer (46).

It is noteworthy that SAM-P45 prefers substrates possessing basic amino acids at the P1 site in contrast to subtilisin BPN′, as shown in Table 2, although both proteases exhibit broad substrate specificity. The hydrolytic activity of SAM-P45 toward basic amino acids is considered to be due to the interaction with Glu158 (36). This residue corresponds to the Glu156 of subtilisin BPN′, and their electrostatic interaction was shown to be the reason for the activity of subtilisin BPN′ toward basic amino acids by extensive mutational analysis of the subtilisin BPN′ gene (47–49) and X-ray crystallographic analysis of subtilisin BPN′ (13, 19) and its complexes with mutated forms of SSI (36). A similar interaction may occur in SAM-P45. Aromatic and aliphatic amino acids are better substrates of subtilisin BPN′ than are basic amino acids. In contrast, SAM-P45 prefers basic amino acids to the others. As a reason for such specificity differences between SAM-P45 and subtilisin BPN′, we propose a one-residue insertion (Ile153) and a four-residue deletion (Thr158 to Gly160 and Thr164) in SAM-P45. The region Thr158 to Thr164 of subtilisin BPN′ is a connecting region between two regions (Ala151 to Gly157 and Val164 to Gly169) which form the S1 pocket walls, and Ile153

TABLE 3. Effects of inhibitors on SAM-P45[a]

Protease inhibitor	Specificity(ies) of inhibitor	Concn	Residual activity (%)
SSI	Serine proteases such as subtilisin and chymotrypsin	600 nM	26.0
Chymostatin	Chymotrypsin	50 μM	1.8
Aprotinin	Serine proteases	100 nM	85.5
PMSF	Serine proteases	50 μM	69.1
TPCK	Chymotrypsin, papain, and bromelain	50 μM	98.2
TLCK	Trypsin, plasmin, and thrombin	50 μM	90.9
EDTA	Metalloproteases	5 mM	7.3
Phosphoramidon	Metalloendopeptidases such as thermolysin	50 μM	87.3
Bestatin	Aminopeptidases	50 μM	85.5
E-64	Cysteine proteases	50 μM	85.5
Leupeptin	Serine and cysteine proteases	50 μM	31.6
Antipain	Papain, trypsin, cathepsin A and B	50 μM	7.3
Pepstatin	Aspartate proteases	50 μM	92.7
$CaCl_2$		1 mM	110.0
$MgCl_2$		1 mM	110.0
$ZnSO_4$		1 mM	80.0

[a] Enzyme (200 nM) was incubated on ice with each inhibitor in 25 mM citrate buffer (pH 5.5) containing 10 mM $CaCl_2$ for 10 min. After the incubation, the residual activity was assayed in 100 mM Tris-HCl (pH 8.5) containing 10 mM $CaCl_2$ with 100 μM GPK as a substrate at 25°C. In this assay, the final enzyme concentration was 20 nM.

of SAM-P45 is considered to be located in this pocket wall. Therefore, these deletions and this insertion would result in structural changes of the S1 pocket which in turn would result in the side chains of aromatic and aliphatic amino acids in the substrates being less accommodative. Structural analysis of SAM-P45 or its complex with an inhibitor will clarify this point.

The mammalian subtilisin-like proprotein convertases, including human furin, have a specific cleavage site after dibasic or multibasic residues (18, 46) and are categorized as subfamily members of the family of subtilisin-like proteases (26). SAM-P45 shares features with these members in terms of the Ca^{2+} dependence (1 to 10 mM) of proteolytic activity and insensitivity toward typical serine protease inhibitors such as PMSF, TLCK, and TPCK (9, 16, 21). In this sense, SAM-P45 can be considered to be between the primitive bacterial subtilisins and highly diversified eukaryotic proteases in the evolutionary process.

Recently, an extracellular tripeptidyl aminopeptidase homologous to subtilisin BPN′, but distinctly different from SAM-P45 in terms of the absence of a C-terminal prodomain and no exhibition of trypsin-like endoproteolytic activity, was isolated from *S. lividans* 66 (5). Consequently, we first isolated from *Streptomyces* a novel member of the subtilisin-like serine protease family based on its affinity for endogenous target inhibitor SSI. Although the physiological role of cell membrane-anchoring proteases such as SAM-P45 in *Streptomyces* is unclear, Binnie et al. reported that two mycelium-anchoring aminopeptidases might be involved in activating various hydrolytic enzymes based on the gene disruption experiment (4). SSI-nonproducing mutant strains exhibited changes in growth and aerial mycelium formation (34). The complexed form of SAM-P45 with SSI was also identified in the culture supernatant of the original *S. albogriseolus* strain (data not shown). With respect to morphological differentiation, the interaction between SSI and membrane-anchored SAM-P45 precursor on the cell surface is of great interest. Further investigations, including site-directed mutagenesis, will be carried out to elucidate the molecular mechanisms for the posttranslational processing, activation, and localization of SAM-P45 and to understand the relationship between SSI production and these processes.

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