SUBTILOSIN A

A New Peptide Antibiotic Produced by *Bacillus subtilis* 168: Isolation, Structural Analysis, and Biogenesis.

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ABBREVIATIONS

HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid; DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); DABITC, 4-N,N-dimethylamino-azobenzene-4'-isothiocyanate; PITC, phenyl isothiocyanate; dansyl, 1-dimethylaminonaphthalene-5-sulphonyl; RCM, reduced and S-carboxymethylated; NSM, nutrient sporulation medium; MIC, minimum inhibitory concentration; FAB, fast atom bombardment; N-, amino-; C-, carboxyl-; m, molecular weight; z, electric charge; MES, 2-(N-morpholino)ethanesulfonic acid monhydrate.

SUMMARY

Subtilosin A, a new antibiotic produced by Bacillus subtilis 168, was extracted from culture medium with n-butanol and purified to homogeneity by a combination of gel filtration and thin-layer chromatography. Two methods (Methods A and B) were used for the isolation of subtilosin A. The yields according to the method A and B were 2 mg and 5.5 mg from a liter of culture, respectively. This antibiotic is hydrophobic, and has bacteriocidal activity against some gram-positive bacteria. Amino acid analysis and FAB mass spectrometry showed that it is a peptide with a molecular weight of 3398.9, consisting of 32 residues of usual amino acids and some non-amino acid residues. Methionine, tyrosine, phenylalanine, histidine and arginine were missing. Its amino- and carboxyl-termini were blocked. No D-amino acids and sugars were detected. By the analysis of the fragments obtained by partial acid hydrolysis, chymotryptic and thermolysin digestion of reduced and S-carboxymethylated samples and Achromobacter protease I digestion of performic acid-oxixized samples, the amino acid sequence was determined. The analyses on cross-linking structures revealed that there were linkages between the amino- and carboxyl-termini and between the Cys-19 residue and the Glx-28 residue through an unknown residue with a residue weight of 163. Consequently, subtilosin A was deduced to be a cyclic peptide antibiotic with a novel cross-linking structure.

The production of subtilosin A begins at the end of vegetative growth and finishes before spore formation. The antibiotic produced

was mostly secreted into medium. The maximal concentration was about 8 mg per liter of culture. Studies of the correlation between the production of subtilosin A and spore formation with decoyinine and with asporogenous mutants of B. subtilis 168 suggested that there was no close correlation between the two phenomena. The production of subtilosin A was repressed by inhibitors of protein and RNA syntheses in contrast to that of many other antibiotic peptides, suggesting that it is synthesized by the mechanism of usual protein synthesis. Glucose also suppressed the production of subtilosin A as well as spore formation. Subtilosin A-like antibiotics were found to be produced by B. natto and B. subtilis ATCC 6633.

INTRODUCTION

Microorganisms of genus Bacillus, which are useful in fermentation industries, are capable of entering into dormancy as spores when rapidly metabolizable nutritents such as glucose and amino acids are deprived from their growing environments. Sporulation of genus Bacillus species has been thought to be a model system for the studies of cell differentiation, and investigated extensively. As shown in Fig. 1 (1), cells of Bacillus species differentiate to spores in about 8 h after the end of vegetative growth in a sporulation medium. Sporulation was divided into seven stages on the basis of biochemical and morphological changes. In addition, during sporulation a number of substances such as antibiotics, and alkaline and neutral proteases are produced and excreted by the cells (2-4). Schaeffer (5) isolated many asporogenous mutants of Bacillus subtilis Marburg strain, and grouped these mutants on the basis of the producibility of antibiotics and proteases, and competence, that is, a potency to be transformed with DNA, as shown in Table I. Of these mutants, cells with spo0A and spo0B mutation, of which genes were thought to be involved in the initiation or an earlier process of sporulation, were found to be non-producer of antibiotics as well as of spores. From this result, Schaeffer suggested the possible correlation between the production of antibiotics and sporulation. The correlation between the formation or its physiological function of the well-defined antibiotics such as bacitracins, mycobacillin, gramicidin S, tyrocidines, or gramicidin A, and sporulation have been studied extensively by many workers (cf 4, 6). In some cases,

results that favor a function of antibiotic in sporulation were obtained as reported by Ristow and Paulas (7): Gramicidin A and tyrocidines promoted spore formation of the producer organism, but in others, the results that there was no direct cause and effect relationship between antibiotics and sporulation as shown in Table II were obtained. On the basis of the consideration of the work thus far reported, the use of a Marburg strain of Bacillus species, Bacillus subtilis 168, which is the most well-characterized strain, genetically and biochemically, among members of genus Bacillus and has been used extensively for the study of sporulation of bacterial cells (8-10), was thought to be most desirable for complete elucidation of the correlation between the production and the physiological function of antibiotics and sporulation, though there are little information about antibiotics produced by this strain. The close correlation between antibiotic production and spore formation in the Marburg strain of B. subtilis has been reported by Balassa et al. (11), Schmitt and Freese (12) and Spizizen (13). Schaeffer (5) reported that in the crude filtrate of the Spo^+ culture three distinct antibiotics were present. Schmitt and Freese (12) observed that there is no antibiotic activity during exponential growth, but that it greatly increases during the developmental period. They distinguished three major and two minor antibiotic components by thin-layer chromatography and column chromatography on Sephadex LH-20. However, none of them has been isolated or characterized further.

Recently, Freese and coworkers (14) have demonstrated that in

B. subtilis 168 sporulation can be intiated in the presence of excess glucose by conditions causing a partial deprivation of purine, in particular guanine nucleotides. In addition, by using such conditions they showed that some phenomena which accompany sporulation is not essential for sporulation itself. Their device permited to study more substantially sporulation phenomena.

Considering these facts, the author has attempted to study antibiotics produced by Bacillus subtilis 168. This thesis describes the isolation and structural analysis of subtilosin A, which is a new antibiotic peptide with a molecular weight of about 3400 and produced at the end of vegetative growth in the sporulation medium. Some properties, including the correlation with sporulation, are also discussed.

MATERIALS AND METHODS

Materials — The sources of materials used in this work were as follows: Nutrient broth, tryptone, yeast extract, vitamin assay casamino acids, and Bacto-agar from Difco laboratories; pre-coated TLC plates silica gel (5715 ,5721,11845 and 5745) from E. Merck; analytical TLC precoated plates KC_{18} from Whatman Inc.; Sephadex LH-20 and -60 (40-120 μm) and SP-Sephadex C-25 (40-120 μm) from Pharmacia Fine Chemicals, Inc.; EKICRODISC 13 from Gelman Sciences Japan, LTD; morpholinopropane sulfonate and 2-(N-morpholino)-ethanesulfonic acid monohydrate (MES) from Dojindo Laboratories; fluorescamine from Hoffmann-La Roche Inc.; rifamycin SV sodium salt (B grade) from Calbiochem-Behring; chloramphenicol from Sankyo, Co., LTD.; proline-specific endopeptidase from Seikagaku Kogyo Co., LTD.; α-chymotrypsin and carboxypeptidase A-DFP from Sigma Chemical Co.; thermolysin from Nakarai Chemicals, LTD.; dansyl chloride, phenyl isothiocyanate (PITC) and 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) from Wako Pure Chemical Industries; hydrazine (anhydrous), aminopeptidase M and 4-N,N-dimethylamino-azobenzene-4'-isothiocyanate (DABITC) from Pierce Chemical CO.; carboxypeptidase Y from Oriental Yeast Co., LTD.; polyamide layer sheets from Cheng Chin Trading Co., LTD.; liquid chromatography columns, Yanapak ODS-T (4 x 250 mm, 10 μm) from Yanagimoto MFG. Co., LTD. and Chemcopak (4.6 x 250 mm, C_8, 7 μm, and C_{18}, 5 μm) from Chemco Scientific CO., LTD. Decoyinine U-7984 was a gift from Dr. Joseph E. Grady of the Upjohn Co. and Achromobacter protease I was supplied by Dr. Kazuo Fujikawa of

University of Washington. All other chemicals were obtained commercially. Solvents for sequencing were of sequencing grade. Acetonitrile and 2-propanol were of high performance liquid chromatography (HPLC) grade.

Bacterial Strains — B. subtilis 168 obtained from Dr. S. Okubo was used as a producer organism of subtilosin A. Other B. subtilis sporulation mutants tested for the production of subtilosin A were obtaind from Drs. J. Spizizen and P. Schaeffer as described previously (15).

Media — Nutrient sporulation medium (NSM) is 2x SG medium of Korch and Doi (16) except that it contained double strengths of nutrient broth and glucose. S6C medium is the same as that described by Freese et al. (14) except for the omission of methionine. S6CG medium contained 1% glucose. LB medium contained 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl supplemented with 1 g of glucose per liter, pH being adjusted to 7.0 with NaOH. LB agar and soft agar were supplemented with 1.5% and 0.7% agar, respectively.

Thin-layer Chromatography and Detection of Subtilosin A — Subtilosin A was chromatographed on the following thin-layer plates with solvent systems A, B and C.

(1) Silica gel plates (Merck 5715, 11845 or 5745, 200 x 200 mm) with Solvent System A ($CHCl_3$: CH_3OH : H_2O = 60 : 25 : 4, v/v)

(2) Silica gel plates (Merck 5721, 200 x 200 mm) with Solvent System B ($CHCl_3$: DMFA : H_2O = 50 : 44 : 6, v/v) (DMFA denotes dimethylformamide)

(3) Reversed-phase plate (Whatman KC_{18}, 200 x 200 mm) with Solvent System C (CH_3OH : H_2O = 70 : 30, v/v)

Subtilosin A on silica gel plates was detected by UV, fluorescamine, the Ehrlich's reagent, and bioautography with Bacillus amyloliquefaciens H as an indicator organism according to the method of Schmitt and Freese (12). The Rf values were found to be around 0.5 and 0.65 on silica gel plates with the solvent system A and B, respectively, and 0.2 on the reversed-plate with the solvent system C.

Quantitative Analysis of Subtilosin A — An appropriate amount of culture removed at intervals was mixed with one fourth volume of n-butanol and vortexed for a few minutes. The organic phase was removed and centrifuged. One milliliter of the supernatant solution was evaporated to dryness. The residue was dissolved in 1 ml of 20% acetonitrile in 0.1% trifluoroacetic acid (TFA) and filtered through a EKICRODISC 13. The filtrate was subjected to HPLC (Yanaco liquid chromatograph L-4000W) on a reverse-phase column (C_{18}, 10 μm, 4 x 250 mm) equilibrated with 0.1% TFA. The elution was carried out with 40 ml acetonitrile with a linear gradient from 55% to 65% in 0.1% TFA at a flow rate of 1.5 ml/min and monitored with a Yanaco UV detector S-310A at 280 nm. Subtilosin A was eluted at 58% acetonitrile in 0.1% TFA. The amount of subtilosin A was estimated from the absorbance at 280 nm, using the molecular extinction coefficient of trypto-

phan 5690, because subtilosin A contains one residue of tryptophan per mol.

Fast Atom Bombardment (FAB) Mass Spectrometry — The molecular weight of subtilosin A was determined in a double focusing mass spectrometer (Jeol JMS-HX100) equipped with an FAB ion source and a mass data analysis system (Jeol JMA-3100 or DA-5000). Typical experimental conditions were carried out with a xenon atom beam source at 7-keV accelerating potential. Mass assignment was made using a mixture of CsI and KI as a mass reference. A sample solution containing 15-40 μg of subtilosin A was loaded on a stainless steel plate and mixed with glycerol and α-thioglycerol on the plate.

Analyses of Amino Acid Composition and Sequence— (1) Amino acid analyses: Amino acid analyses of peptides were performed on an amino acid analyzer (Hitachi 835-S) after hydrolysis with constant boiling HCl containing 0.2% phenol in evacuated, sealed tubes at 110°C for 24 h. No corrections were made for the destruction of amino acids during acid hydrolysis. Cysteine residues were determined as cysteic acid after oxidation of a sample with performic acid (17). Tryptophan residues were determined after hydrolysis of a sample with 4 M methane sulfonic acid containing 0.2% 3-(2-aminoethyl)indole (18).

(2) Methylamine treatment, reduction and carboxymethylation: Because reduction and S-carboxymethylation of subtilosin A could not

be achieved sufficiently by the standard method of Crestfield et al. (19), the following modification was devised. Subtilosin A was dissolved in methanol containing 40% methylamine and kept at room temperature for 1.5 h. After evaporation of the solvent, the method of Crestfield et al. (19) was followed. Salt was removed by gel filtration on Sephadex G-10 with 0.1% formic acid as a solvent.

(3). Partial acid hydrolyses: Reduced and S-carboxymethylated (RCM) subtilosin A (2.55 mg) was dissolved in 1 ml of 50% acetic acid containing 30 mM HCl and hydrolyzed at 105°C for 12 h. Intact subtilosin A (3 mg) was dissolved in 0.3 ml of iron-free concentrated HCl and hydrolyzed at 37°C for 17 h.

(4) Digestion of RCM-subtilosin A with proteases: Samples, 1.5 mg and 0.2 mg, were dissolved in 1 ml and 0.2 ml of 0.1 M NH_4HCO_3 containing 10 mM $CaCl_2$, pH 8.1 and digested with 50 µg of chymo= trypsin at 37°C for 6 h and with 10 µg of thermolysin at 60°C for 1 h, respectively.

(5) Digestion of performic acid-oxidized subtilosin A with Achromobacter protease I: Two samples (1.4 mg each) were dissolved in 0.14 ml of 0.1 M NH_4HCO_3 containing 10 mM $CaCl_2$. One sample was digested with 28 µg of Achromobacter protease I at 37°C for 2 h and the other with 3.5 µg of the enzyme at 37°C for 45 min.

(6) Peptide isolation: The digests or the hydrolyzates were applied to HPLC on reverse-phase columns (C_8, 7 μm; C_{18}, 10 μm or 5 μm) and peptide fragments were eluted with concentration gradients of acetonitrile in 0.1% TFA, or of a 7:3 mixture of 2-propanol and acetonitrile in 0.1% TFA. The amino (N)-terminal fragments yielded by chymotryptic or thermolysin digestion were isolated as flow-through fractions of ion-exchange chromatography on an SP-Sephadex C-25 column (12 x 50 mm) with 25 mM phosphoric acid as a solvent and purified by HPLC.

(7) Sequence determination: The dansyl-Edman procedure was performed as described by Chen (20) and Kimura (21). The DABITC/PITC double coupling method of Chang et al. (22) modified by Allen (23) was used. Digestion with carboxypeptidase A was performed on about 100 nmol of performic acid-oxidized samples in 100 μl of 0.2 M N-ethylmorphorine acetate buffer, pH 8.5. Carboxypeptidase A was used at an enzyme to substrate ratio of 1 : 23 by weight and incubation was at 37°C. Digestion with carboxypeptidase Y was performed on samples dissolved in 20 mM sodium phosphate buffer, pH 6.4, at a concentration of 0.1% (w/v). The enzyme to substrate ratio was 1 : 50 by weight and incubation was at 37°C. After lyophilizing, the digests were directly applied to an amino acid analyzer.

(8) Titration of thiol groups: Dried subtilosin A was dissolved in anhydrous hydrazine, left at room temperature for 5 min, evaporated to dryness and titrated with DTNB according to the method

of Kortt and Liu (24).

Isolation of Fragments with a Cross-linking Structure: Intact subtilosin A (1 mg) was suspended in 1.6 ml of 0.1 M MES buffer, pH 6.5, containing 10 mM $CaCl_2$, and digested with 255 µg of thermolysin at 37°C for 75 h. The fragments with a cross-linking structure were isolated from the digests by reverse-phase HPLC, and then dissolved in 50 µl of 50 mM sodium phosphate buffer, pH 6.5, and subjected to additional digestion with 60 µg of proline-specific endopeptidase at 37°C for 17 h. After digestion, fragments were isolated by reverse-phase HPLC.

RESULTS

Selection of an Indicator Strain for Bioassay of Antibiotics Produced by Bacillus subtilis 168 ——— Because antibiotics are well-known to have antibiotic spectra characteristic to themselves, an indicator strain suitable for bioassay of antibiotics produced by Bacillus subtilis 168 was selected. LB-soft agar cultures containing fresh cells of various bacteria were overlaid on the colonies of B. subtilis 168 grown on Schaeffer's agar medium plates, and the plates incubated at 37°C overnight. By inspection of the size of the growth inhibition zones formed around the colonies, Bacillus amyloliqefaciens H and Staphylococcus aureus were found to be more sensitive than the others examined. The growth inhibition zone of B. amyloliquefaciens H was clear, whereas that of S. aureus was turbid. Because it is a non-pathogenic microbe, in addition to the sensitivity and the convenience to define the boundary of growth inhibition zones, B. amyloliquefaciens H was selected as an indicator strain.

Selection of The Medium and Production of Antibiotics by B. subtilis 168 —— In preliminary experiments with several media, including sporulation and non-sporulation media, it was found that the patterns of antibiotic produced by this strain were dependent on the nature of the media used. One of the purpose of this work is to examine the correlation between the production of antibiotics and

sporulation in B. subtilis 168, so that a sporulation medium designated as NSM was selected for the production of antibiotics. This medium was derived from the schaeffer's sporulation medium. NSM contained twice as much nutrient broth and glucose as the 2 x SG medium of Korch and Doi (16). The modifications lead to the increase of the number of cells and the amounts of antibotics produced, but practically no change of the pattern of antibiotic production was observed under sufficient aeration.

As shown in Fig. 2, B. subtilis 168 produced more than 10 antibiotics in NSM. If some antibiotics have an effect on spore formation, they should be produced before the appearence of spore, that is, by 8 h after the end of vegetative growth. Accordingly, a major antibiotic which was produced at 4-8 h, and had the Rf value of about 0.7 in this bioautogram was selected as the subject of the research. This antibiotic was designated as subtilosin A.

Isolation of Subtilosin A —As shown by the diagram in Fig. 3, two methods (Method A and B) were used for the isoltion of subtilosin A, but the first three procedures of both methods were identical. Unless otherwise stated, operation was performed at room temperature. Cell growth was monitored by using Klett-Summerson colormeter with No. 66 filter.

Cells of B. subtilis 168 were inoculated from an overnight culture in LB-broth into a culture tube containing 30 ml of LB-broth, and incubated at 37°C in a water bath on a reciprocal shaker at a speed of 120 strokes per minute. When the turbidity of cells

attained 170 Klett units, 1 ml each of the culture was transferred into ten 2-liter Erlenmeyer flasks containing prewarmed 350 ml of NSM. Culture was incubated at 37°C on a New Brunswick rotatory shaker model 25 with vigorous shaking until 5 h after the end of vegetative growth, when the level of subtilosin A reached the maximum.

To the culture was added one fourth volume of n-butanol. The mixture was emulsified vigorously for 1 h and allowed to stand overnight at room temperature. The butanol layer was removed and evaporated to dryness under reduced pressure. The residue was dissolved in a minimum volume (less than 6 ml) of methanol and applied to a 2 x 47 cm column of Sephadex LH-20 equibrated with methanol. Elution was performed with methanol at a flow rate of 120 ml/h. Fractions of 3 ml were collected. Figure 4 shows the results of the Sephadex LH-20 column chromatography of the butanol extract. Subtisolin A eluted in the void fractions of this column chromatography was separated from most of other UV absorbing materials and antibiotics. To remove the antibiotic substances still contaminating in the subtilosin A fractions, the void fractions were concentrated in vacuo, and applied to one preparative TLC plate (Merck 5745) of silica gel followed by development with the solvent system A. After chromatography, subtilosin A fractions were scraped out from the plate, and extracted with methanol and concentrated in vacuo. Thin-layer chromatography on a silica gel plate (Merck 11845) with the solvent system A of the sample thus prepared revealed that there were still two minor UV absorbing impurities; one migrating in front of and the other trailing subtilosin A. They had no antibilotic

activities against B. amyloliquefaciens H, and were negative for the reaction with the Ehlrich's reagent. Therefore, the samples were further purified by two kinds of method as follows: Method A; after thin-layer chromtography on four silica gel plates (Merck 11845) with the solvent system A, subtilosin A fractions were passed through a 84.5 x 1.6 cm column of Sephadex LH-60 equilibrated with methanol. Elution was carried out with methanol at a flow rate of 32 ml/h. Fractions of 2 ml were collected. The eluates were monitored at 280 nm. In addition, the samples were chromatographed on three silica gel plates (Merck 5721) with the solvent system B and on Sephadex LH-60 column in the same manner as above.

Method B; the subtilosin A fractions obtained from the first TLC were chromatographed on three reversed-phase TLC plates with the solvent system C and on a Sephadex LH-20 column as above.

Table III shows the summary of isolation of subtilosin A by both methods. Subtilosin A thus obtained was homogeneous in HPLC on a reverse-phase column as shown in Fig. 5 and in mass spectrometry (data not shown). The concentration of subtilosin A in the culture was about 8 mg per liter, and the overall yield of the method A and B were 2 mg and 5.5 mg, respectively, from one liter of culture, or 25% and about 70%, respectively.

Later, in the small scale preparation, subtilosin A was found to be remarkably purified from the butanol extract by one reverse-phase HPLC. Figure 6 shows the chromatogram of the reverse-phase HPLC of the butanol extract.

Properties of Subtilosin A — Subtilosin A is a hydrophobic peptide as evidenced by its elution at 58% acetonitrile in 0.1% TFA on a reverse-phase columun (C_{18}) in HPLC (Fig. 5). It is soluble in methanol, glacial acetic acid, 70% formic acid, dimethylsulfoxide, but not in non-polar organic solvents such as ether or hexane. It is soluble in alkaline solutions, but labile and decomposed gradually. When subtilosin A was incubated in 0.1 M NH_4HCO_3 containing 10 mM $CaCl_2$, it was decomposed considerably in 17 h, and almost completely in 38 h as shown in Fig. 7, whereas it was degraded little at pH 6.5. However, the derivatives which were formed under the alkaline conditions had the same amino acid composition as that of subtilosin A and still the antibiotic activity. It is insoluble in acidic solutions. Its solubilities in 0.1 M NH_4HCO_3 containing 10 mM $CaCl_2$ and 0.1 M MES buffer containing 10 mM $CaCl_2$ (pH 6.5) were more than 1.6 mg/ml and less than 98.5 μg/ml, respectively. Subtilosin A gives positive reactions with fluorescamine and the Ehlrich's reagent, and shows a bacteriocidal activity against gram-positive bacteria such as Bacillus megaterium, B. amyloliquefaciens and Streptococcus faecium as shown in Table IV. It is also effective on B. subtilis 168 which is the producer organism of subtilosin A, but not on fungi tested.

Structural Analysis of Subtilosin A — Table V shows the amino acid composition of subtilosin A. It consists of 32 amino acid residues. Methionine, tyrosine, phenylalanine, histidine, and arginine are missing. No unusual ninhydrin-positive peaks were found in the amino acid analyses of performic acid-oxidized samples.

D-amino acids were not detected in acid hydrolyzates of subtilosin A with D-amino acid oxidase (data not shown) (27). The FAB mass spectrum of intact subtilosin A gave an intense signal at m/z 3399.9 as shown in Fig. 8 (m and z denote a molecular weight and an electric charge, respectively). Because the mass values of underivatized peptides were observed as $[m + H]^+$ in the positive mass spectra, the molecular weight of subtilosin A was estimated to be 3398.9. It was by 369.5-373.4 larger than the theoretical values (3025.5-3029.4) calculated from the amino acid composition. From this it was inferred that subtilosin A possesses other constituents than amino acids.

Figure 9 summarizes the strategy of amino acid sequencing of the peptide portion. Because preliminary experiments indicated that the N-terminal residue of this antibiotic was blocked, fragments with free amino groups were first obtained by partial acid hydrolysis of an intact sample with concentrated HCl, digestion of RCM-samples with chymotrypsin or thermolysin, partial acid hydrolysis of an RCM-sample with 0.03 M HCl in 50% aqueous acetic acid, or digestion of a performic acid-oxidized sample with Achromobacter protease I. The resultant fragments were purified by HPLC on a reverse-phase column. The N-terminal fragments were independently isolated by HPLC from the flow-through fractions from ion-exchange chromatography on a SP-Sephadex C-25 column of the chymotryptic and thermolysin peptides of RCM-samples. Details of isolation of fragments were shown in "Supplementary Materials". Table VI shows the amino acid composition of the fragments used for sequencing. Sequencing of these fragments

by the dansyl-Edman method, the DABITC/PITC double coupling method and the analysis with carboxypeptidase Y was repeated at least twice. The carboxyl (C)-terminal sequence was also determined by digestion with carboxypeptidase A or Y of performic acid-oxidized subtilosin A (Fig. 1S). As shown in Fig. 10, the complete amino acid sequence of subtilosin A was constructed from the above results. It is a single peptide with an N-blocked glycine residue at the N-terminus and with an alanine residue at the C-terminus. The γ-carboxyl group of the Glx residue at position 28 is substituted as will be described below. Therefore, it was not possible to determine whether this residue was derived from glutamic acid or glutamine.

Table VII summarizes the results of preliminary analyses of the possible modification of side chains of amino acid residues. The OH-groups of Serine and threonine residues were not substituted, because they were sensitive to oxidation with chromic acid (28). Dansyl chloride reacted with only the ε-amino group of the lysine residue. Two of the three Asx residues were concluded to be aspartic acid by analysis of the sample which was esterified with methanol-HCl followed by reduction with sodium borohydride (29). These results were also confirmed by sequencing of the fragments containing the relevant residues. As shown in Table V, subtilosin A has three cysteine residues determined as cysteic acids, but no thiol groups were titrated with DTNB, whereas they all were alkylated with iodoacetic acid after reduction (Table VI, CH-2). When the total thiol groups of this antibiotic were titrated with DTNB after treatment with anhydrous hydrazine, it was found that there were

three thiol groups as shown in Fig. 11. These results suggest that this antibiotic contains a modified cysteine residue in addition to cystine.

To verify the speculation and locate the modified cysteine residue, an intact subtilosin A was digested with thermolysin at pH 6.5. Five peptide fragments (Fig. 11), which were designated as Fragment I-V, were obtained from the digests by reverse-phase HPLC (Fig. 12). On the basis of the results of amino acid analysis (Table VIII) and sequencing (Fig. 11), Fragment I and II were found to have a novel cross-linking structure through the cysteine residue at position 19, whereas Fragment III and IV indicated the possibility of a linking between the N- and C-termini. Fragment V consisted of three peptide fragments which linked with each other through two cross-linking structures mentioned above.

The FAB mass spectrum of Fragment I gave two intense signals (Fig. 14, I): A parent ion signal at m/z 1362 and a fragment ion signal around m/z 1101. This fragmentation in FAB mass spectrometry will be considered below. The molecular weight of Fragment I was estimated to be 1361 from the parent ion signal. This value was by 145-146 larger than what was accounted for by the amino acid composition, suggesting that another constituent in addition to amino acids was present. This unknown residue was designated as Xu.

To analyse the cross-linking structure in Fragment I and locate Xu, further digestion of Fragment I with proline-specific endopeptidase pH 6.5 was carried out. A peptide from the digests was obtained by reverse-phase HPLC (Fig.15), and designated as Fragment

U. Based on the results of amino acid analysis and sequencing, Fragment U was found to consist of two fragments, the fragment with sequence from residue 17 through residue 19 Ala-Ala-Cys (chain A) and the fragment with the sequence from residue 25 through residue 29 Ile-Pro-Asp-Glx-Ile (chain B). As shown in Fig. 16, the FAB mass spectrometry of Fragment U again showed the fragmentation as demonstrated in Fragment I, and gave a parent ion signal at m/z 994 and a fragment ion signal around m/z 733. From the parent ion signal the molecullar weight of Fragment U was estimated to be 993, and again by 145-146 larger than the theoretical value calculated from the amino acid composition. From this result and amino aced sequencing, Fragment U was infered to have a new cross-linking structure which the Cys-19 linked with Glx-28 through Xu with a residue weight of 163. The fragment ion signal was of an irregular form, and its observed mass value was by 261-263 smaller than that of the parent ion signal. Since the molecular weight of chain A (Ala-Ala-Cys) is 263, the fragment ion signal is thought to correspond to the fragment consisting of chain B and Xu, which was derived from Fragment U by the cleavage of the Cys-Xu bond. The fragmentation in the FAB mass spectrometry of Fragment I was thought to occur in the same manner as above, because the observed mass value of the fragment ion signal (m/z 1101) was by 261-263 smaller than that of the parent ion signal (m/z 1362). As an example of fragmentation in FAB mass spectrometry, splitting of the disulfide bonds of cystine residues has already been reported (30).

To define which residue of chain B links with Xu, we digested

fragment U with aminopeptidase M, and analyzed its digests in FAB mass spectrometry. As shown in Fig. 17, after incubation in 0.1% NH_4HCO_3 (pH 7.9) at 37°C for 4 h, fragment U gave new intense signals at m/z 343, 406 and 748 in addition to signals at m/z 994 and 733, suggesting that fragment U is labile under alkaline conditions. This phenomenum was also found in an another fragment containing this cross-linking structure under the same conditions. When subtilosin A was digested with proteases under alkaline conditions, splitting of the peptide bond between Asp-27 and Glx-28 was often encountered as indicated by AP-1, CH-5 and -7, and TH-6 in Fig. 9. From this fact and fragmentation in FAB mass spectrometry, the signal at m/z 406 was infered to correspond to $[Glx(Xu)\text{-}Ile]^+$ ion. Figure 18 shows an FAB mass spectrum of the digest of fragment U with aminopeptidase M in 0.1% NH_4HCO_3 (pH 7.9) at 37°C overnight. Intense signals are present at m/z 229, 344, 406, 521, 538, 636 and 731, but the parent ion signal at m/z 994 and the signals at m/z 343 and 748 are absent. The signals at m/z 731, 521 and 406 are considered to be the fragment ions in FAB mass spectrometry, which were formed by splitting of the bond between Cys-19 and Xu, and to correspond to $[Ile\text{-}Pro\text{-}Asp\text{-}Glx(Xu)\text{-}Ile]^+$, $[Asp\text{-}Glx(Xu)\text{-}Ile]^+$ and $[Glx(Xu)\text{-}Ile]^+$ ions, respectively. Whereas signals at m/z 229 and 344 are thought to indicate $[Ile\text{-}Pro + H]^+$ and $[Ile\text{-}Pro\text{-}Asp + H]^+$, respectively. The above results, especially the presence of signals at m/z 344 and 406, indicate that Xu with a residue weight of 163 is present between Cys-19 and Glx-28 as shown in Fig. 19.

The FAB mass spectra of Fragment III and IV showed intense

signals m/z 1809.9 and 1979.8, respectively (Fig. 14, III and IV). No fragment ion signal was detected in both the FAB mass spectra as described in those of Fragment I and U. The molecular weights of both Fragment III and IV were 227.9 larger than the theoretical values calculated from the amino acid compositions. From the amino acid compositions and sequencings of these fragments, the N-terminus was suggested to link with the C-terminus, though the possibility that the C-terminus does not link with the N-terminus but with the Cys-10 and -13 residue as was seen in the cross-linkng structure of Fragment U cannot be excluded. However, from the concentrated HCl hydrolyzates of intact subtilosin A, fragments which had a Gly residue at the N-terminus and consisted of four residues as shown in Fig. 19 were obtained, though the yields of fragments were so low as about 2%. From the amino acid compositions and N-terminal analysis, these fragments are considered to be composed of the peptide with the N-terminal sequence of residues 1 and 2 and the peptide with the C-terminal sequence of residues 31 and 32. Therefore, I concluded that the N-terminus linked with the C-terminus. That C-terminus of intact subtilosin A was substituted was also suggested because no amino acids were detected when C-terminal analysis of intact subtilosin A was performed by the hydrazine method (31).

Consequently, from the above results the primary structure of subtilosin A was deduced as shown in Fig. 20.

Production of Subtilosin A in Relation to Sporulation — Since many reports on the correlation between the production of antibiotics

and sporulation in the members of the genus Bacillus have appeared (cf 4, 6, 7, 32)), it was attempted to examine the correlation between the production of subtilosin A and sporulation in B. subtilis 168. The time course of the production of subtilosin A in relation to cell growth and spore formation in the nutrient sporulation medium was shown in Fig. 21. The synthesis of this antibiotic began at 1 h after the cessation of vegetative growth. The antibiotic increased linearly with time for the first 2 h and reached a plateau followed by gradual decrease. The maximal concentration was about 8 mg per liter of culture. The antibiotic produced was mostly secreted into the medium. Heat-resistant spores appeared at 8 h after the end of vegetative growth, increased gradually in number and reached 20% of viable cells at 22 h. The production of subtilosin A had finished before the appearance of refractile spores.

As well as spore formation, the production of antibiotics is known to be senstive to catabolite repression (33). To determine whether the production of subtilosin A is controlled by catabolites, glucose at a concentration of 10 g per liter was added to the culture at the end of vegetative growth. As shown in Fig. 22, the addition of excess glucose resulted in promotion of cell growth and repression of both antibiotic production and spore formation. Under these conditions, small amounts of subtilosin A were produced, but no spores were formed.

Freese and his associates (14, 34) discovered that decoyinine, a specific inhibitor of GMP synthesis, is able not only to induce spore formation even in the presence of excess catabolites such as glucose

and ammonium ions, but also to lead some asporogenous mutants to initiate spore formation. To determine whether the production of subtilosin A as well as spore formation is induced with this drug in the presence of excess glucose, sporulation was induced with this drug, and the production of subtilosin A was followed under the similar conditions as described by Freese et al. (14). Figure 23 shows the effect of decoyinine on cell growth. As reported by Fresse et at. (14), this drug repressed cell growth and indeed induced sporulation to a normal level in the presence of glucose, but no production of subtilosin A was detected in either experiment with or without the addition of decoyinine as shown in Table IX, suggesting that the mechanism of repression of subtilosin A production and sporulation by glucose may be different and that the two phenomena have no correlation with each other.

Spore formation is a temporally ordered process (9, 10); consequently, if subtilosin A is indispensable for spore formation, it would not be produced by sporulation negative mutants, such as spo0A and spo0B, which are blocked at the earliest stage of sporulation, whereas the mutants, such as spoIII and spoIV, which are blocked at the later stages and known to produce some antibiotics, would be expected to produce subtilosin A. Table X summarizes the production of subtilosin A and other unidentified antibiotics by various asporogenous strains of B. subtilis 168. Of the strains tested only wild type and spo0C9V, of which mutation locates within the spo0A gene (Ikeuchi, T. Kudoh, J. and Kurahashi, K., manuscript submitted) and is less pleiotropic than spo0A mutations, produced

both subtilosin A and other antibiotics. As expected, spo0A and spo0B mutants did not produce subtilosin A as well as other antibiotics, but spoIII and spoIV mutants failed to produce subtilosin A, while the latter two produced other antibiotics.

Figure 24 shows the distribution of subtilosin A produced by the spo0C9V mutant and the wild type cells in the cells and media. Most of subtilosin A produced seems to be excreted. Although the amount of subtilosin A produced by the mutant is not so much as that produced by the wild type cells, the patterns of production and secretion were found to be similar in both strains.

On the basis of these results it is concluded that subtilosin A is not involved in sporulation, though the time course of the production of subtilosin A coincides with spore formation.

Effects of inhibitors of protein and RNA synthesis on the production of subtilosin A — Most of the peptide antibiotics so far studied have been reported to be synthesized by the multienzyme thio-template mechanism without the participation of either RNAs or ribosomes (cf 35). In contrast to usual protein synthesis, inhibitors of protein and RNA synthesis have little effects on their syntheses. To determine whether the synthesis of subtilosin A follows the similar mechanism or not, we examined the effects of inhibitors of protein and RNA synthesis on the production of subtilosin A. Figures 25, 26 and 27 show the effects of chloramphenicol, kanamycin and rifamycin SV, respectively. When chloramphenicol was added to the culture at a concentration of 20 μg/ml at 1 h after the

onset of production of subtilosin A, it completely repressed its production without marked effects on cell growth and its level remained constant thereafter. The results suggest that subtilosin A synthesis is ribosome-dependent and that its degradation is also prevented by the inhibitor. A similar result was obtained with kanamycin. Kanamycin was added to the culture at a concentration of 50 μg/ml. When rifamycin SV was used at a concentration of 30 μg/ml at the similar timing as above, it also inhibited the production (Fig. 27), but the level of subtilosin A increased with a decreased rate for the first 2 h after the addition of the inhibitor until it reached a plateau and remained constant thereafter. In this strain the minimum inhibitory concentration (MIC) of rifamycin SV was 3 μg/ml. In the above experiments the drug was used 10 times higher concentration than the MIC. Therefore, the reduced rate of production of subtilosin A observed after the addition of rifamycin SV is considered to be due to the synthesis of subtilosin A using the preformed mRNA which may have a longer half life than other prokaryotic mRNAs.

Production of Subtilosin A-like Antibiotics by Other Members of Genus Bacillus —— Whether subtilosin A was produced by other members of genus Bacillus than B. subtilis 168 was examined. As shown in Table XI, when cells of B. subtilis ATCC 6633 and B. natto IFO 13169 were cultivated under the same conditions as those of B. subtilis 168, they produced the antibiotics, which had the same retention time on a reverse-phase column in HPLC and the same amino acid composition

as that of subtilosin A (data no shown). Thus far, there is little information on the antibiotics produced by B. natto. B. megaterium and B. amyloliquefaciens H did not produce such a substance.

DISCUSSION

B. subtilis 168 was found to produce more than 10 antibiotics in the sporulation medium. Subtilosin A studied here is one of the major antibiotics produced in the early stages of sporulation.

When the pH of the culture was higher than 9, antibiotics were extracted from the culture with n-butanol without acidification of the culture to pH 2 or lower. Certainly, the butanol extraction under acidic conditions was more efficient. But the resultant extracts contained many undesirable substances to complicate the isolation of antibiotics, because cells were destroyed completely at acidic pHs and the cell debris were extracted together into the butanol layer. Most of subtilosin A was readily extracted from untreated cultures with n-butanol.

Two methods (Methods A and B) were used for the isolation of subtilosin A, but Method B was found better than Method A in many respects. During thin-layer chromatography on silica gel plates subtilosin A seems to be degraded considerably. Therefore, if possible, the procedure should be avoided for isolation of subtilosin A.

Subtilosin A consists of 32 amino acid residues of usual amino acids and two non-amino acid residues. No sugars were detected in subtilosin A by the phenol-sulfuric acid reaction (36). As shown in Fig. 28 and 29, small amounts of unknown ninhydrine-positive peaks were sometimes observed, but these peaks are absent in amino acid analyses of performic acid-oxidized samples, and if any, do not always appear quantitatively. Accordingly, these peaks are not

thought to indicate unknown amino acids contained in subtilosin A. They might be artifacts formed during hydrolysis with HCl of a compound like O-(γ-L-glutamyl)-L-serine (37).

Subtilosin A is a small peptide without unusual amino acids, but its structural analyses has encountered with many difficulties. Since both termini are blocked, it is necessary to obtain fragments with free amino groups for amino acid sequencing. When intact subtilosin A was subjected to digestion with various proteases, it was little degraded even in the presence of denaturants. The reduction and S-carboxymethylation of subtilosin A could not be achieved sufficiently by the usual method even if the reduction time was prolonged, as shown in the footnote of Table V. The resultant RCM-samples were also resistant to proteases. Later, pretreatment of subtilosin A with methylamine was found to be helpful for the reduction and S-carboxymethylation of this antibiotic. RCM-samples thus prepared were easily digested with proteases such as chymotrypsin and thermolysin. This finding enable me to determine the complete amino acid sequence of subtilosin A.

However, when I attempted to isolate the N-terminal fragments from thermolysin peptides of this RCM-sample, the reverse-phase HPLC of the N-terminal fragments gave many peaks, of which amino acid analyses showed only glycine as their components as shown in Fig. 9S and Table IXS, suggesting that the N-blocking group might have been destroyed during the following three processes: 1) Reduction and S-carboxymethlation, 2) digestion with thermolysin and 3) chromatography. In contrast to intact subtilosin A, the RCM-derivatives gave

rise to many heterogeneous peaks in reverse-phase HPLC, and gave broad weak signals in FAB mass spectrometry (data not shown). Therefore the first possibility seems very likely. However, by FAB mass spectrometry it was revealed that some N-terminal fragments changed in mass values but not in amino acid compositions during digestion with thermolysin in 0.1 M NH_4HCO_3 containing 10 mM $CaCl_2$ at 37°C or in 0.1 M MES buffer containing 10 mM $CaCl_2$, pH 6.5, at higher than 60°C (data not shown). These results show that subtilosin A is labile, particularly in alkali, suggesting that the above second possibility can not be ruled out. All these facts might explain that the reverse-phase HPLCs of the digests and the hydrolyzates, as shown in Fig. 2S-9S, gave complex chromatograms in spite of the small size of subtilosin A and that the yields of fragments isolated were low.

The N-terminus is blocked by an unknown residue X. There are unique cross-linking structures between the cysteine residue at position 19 and the Glx residue at positiion 28 through an unknown residue Xu, and between the N- and C-terminus (Fig. 19). Such a cross-linking structure between a cysteine residue and a glutamine or glutamic acid residue has already been reported in complement 3 (38) and α_2-macroglobulin (39). However, in contrast to that of subtilosin A, the thioester bonds are formed directly between the thiol group of the cysteine residue and γ-carboxyl group of the Glx residue.

The linking between the cysteine 19 and Xu may be considered to be through a thioester bond because of its lability in alkali treatment and performic acid oxidation, while the linkage between the

Glx-28 and Xu may be an O-ester bond. Because performic acid-oxidized subtilosin A has a free α-carboxyl group, the linking between the N- and C-termini is infered not to be an amide bond. To determine whether this antibiotic has S- and/or O-ester bonds, when intact subtilosin A was subjected to the alkaline hydroxylamine method (40), it gave a purple-brown color which indicates the formation of the corresponding ferric-hydroxamic acid complexes. As the resultant complexes were quantitated spectrophotometrically based on the calibration curve for the ferric-acetohydroxamic acid complex, its amount corresponded to 1.3 equivalent/mol. This result demonstrates that subtilosin A has one ester bond at least.

The method for determination of thiol groups used in this work is simple and very quantitative, and also gave the expected values on bovine trypsin and hen lysozyme in addition to bovine insulin, suggesting that hydrazine could split the disulfide bonds quantitatively. Therefore, no other residues with thiol groups than the three cysteine residues are considered to be present in subtilosin A.

Of the antibiotic peptides produced by species of Bacillus, of which structures and synthetic mechanisms have been clarified, only subtilin is similar to subtilosin A in size and the synthetic mechanism. Subtilin is a peptide antibiotic produced by B. subtilis ATCC 6633, and consists of 32 amino acid residues including unusual amino acids, such as methyllanthionine, lanthionine, dehydroalanine, and dehydrobutyrine. It was shown to be formed by processing of precursor proteins (26). Since subtilosin A was shown to be

synthesized by a mechanism dependent on mRNA-ribosome and it had no methionine residues, it may well be synthesized as a large precursor protein.

Ray and Bose (41) and Haavik and Thomassen (42) showed that the nonproducer mutants of mycobacillin and bacitracin were able to sporulate normally, suggesting that these antibiotics were dispens= able for spore formation. Our results showed that subtilosin A seems to have no direct correlation with sporulation. However, some other antibiotics produced by B. subtilis 168 were found to increase in amounts considerably during sporulation induced by decoyinine, while no subtilosin A was produced as described above. Whether these antibiotics have any effects on sporulation remains to be studied in more detail.

Further studies are required to elucidate the complete structure of subtilosin A. The blocking group at the N-terminus and the cross-linking structure between cysteine at residue 19 and Glx at residue 28 are being studied.

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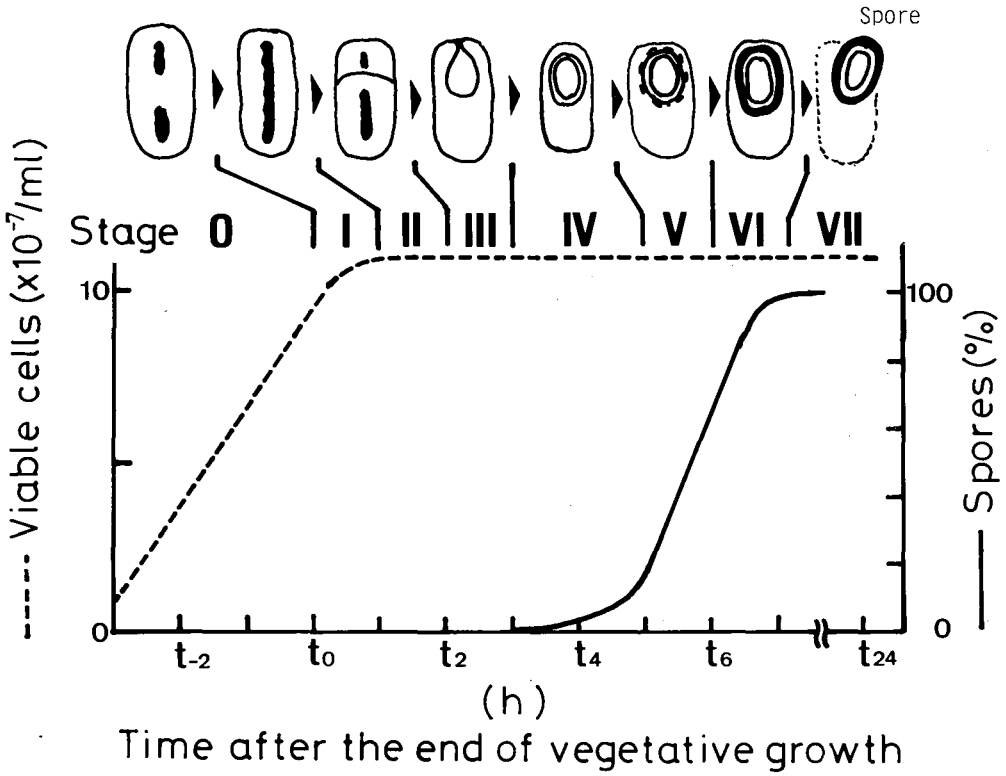

Fig. I. Summary of the correlation between sporulation and cell growth. The corresponding stages of sporulation are represented diagrammatically above the growth curve at appropriate times.

Stage I: Preseptation or axial chromatin stage
II: Septation of forespore
III: Protoplast development
IV: Cortex formation
V: Coat formation
VI: Maturation
VII: Free spore

Strain	Antibiotic production	Proteinase production	Competence
Wild	+	+	+
SpoOA	-	-	-
SpoOB	-	+	+
SpoOC	+	+	+
SpoI-V	+	+	+

(Schaeffer)

Table I. Properties of asporogenous mutants derived from Bacillus subtilis Marburg strain.

+ and - indicate production and non-production, respectively, or possession and non-possession of competence, respectively.

Strain	Antibiotic	Correlation
B. subtilis	Mycobacillin	None
B. licheniformis	Bacitracins	None
B. brevis	Gramicidin A	None
	Gramicidin S	None
	Tyrocidines	None

Table II. Correlation between sporulation and production of well-defined antibiotics.

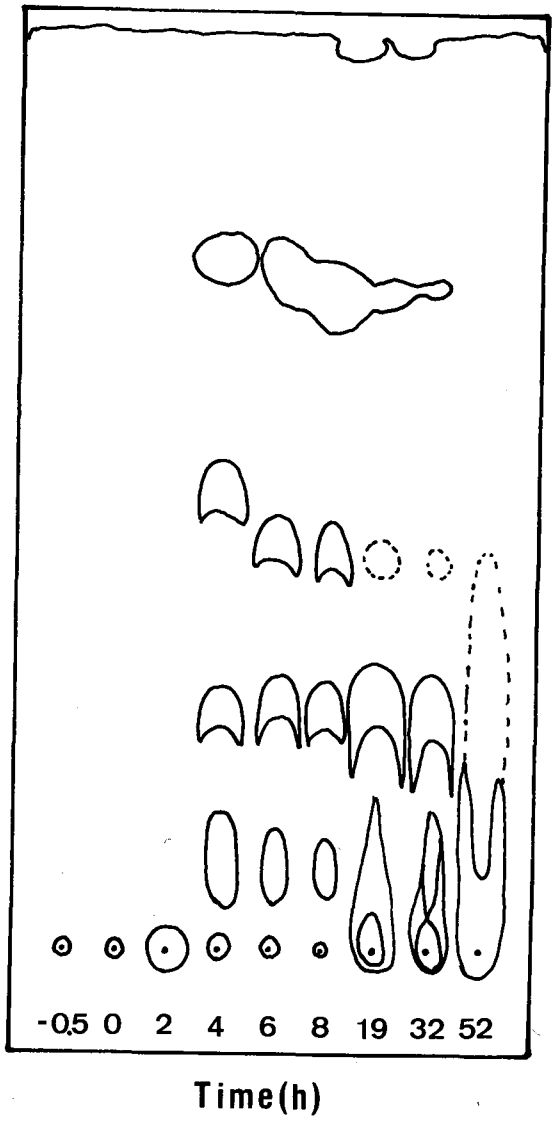

Fig. 2. Production of antibiotics by B. subtilis 168 in NSM.

Cells were grown in a 1 liter-Erlenmeyer flask containing 100 ml of NSM at 37°C in a reciprocal shaker at a speed of 120 strokes/min. To the samples (10 ml) removed, one fourth volume of butanol was added, and emulsified vigorously with a vortex mixer. After centrifugation, the butanol layer was removed and evaporated to dryness under vacuum. The residues were dissolved in 0.5 ml of methanol. A portion (20 µl) of the solution was chromatographed on a silica gel plate (Merck 5721) with a mixture of chloroform, methanol and water; 60: 35: 4, by vol.. The chromatogram was served for bioautography against B. amyloliquefaciens H. Areas marked by lines indicate antibiotics. Subtilosin A is an antibiotic with a Rf value of about 0.7 in this chromatogram. Times indicate the sampling times represented in hour after the end of vegetative growth.

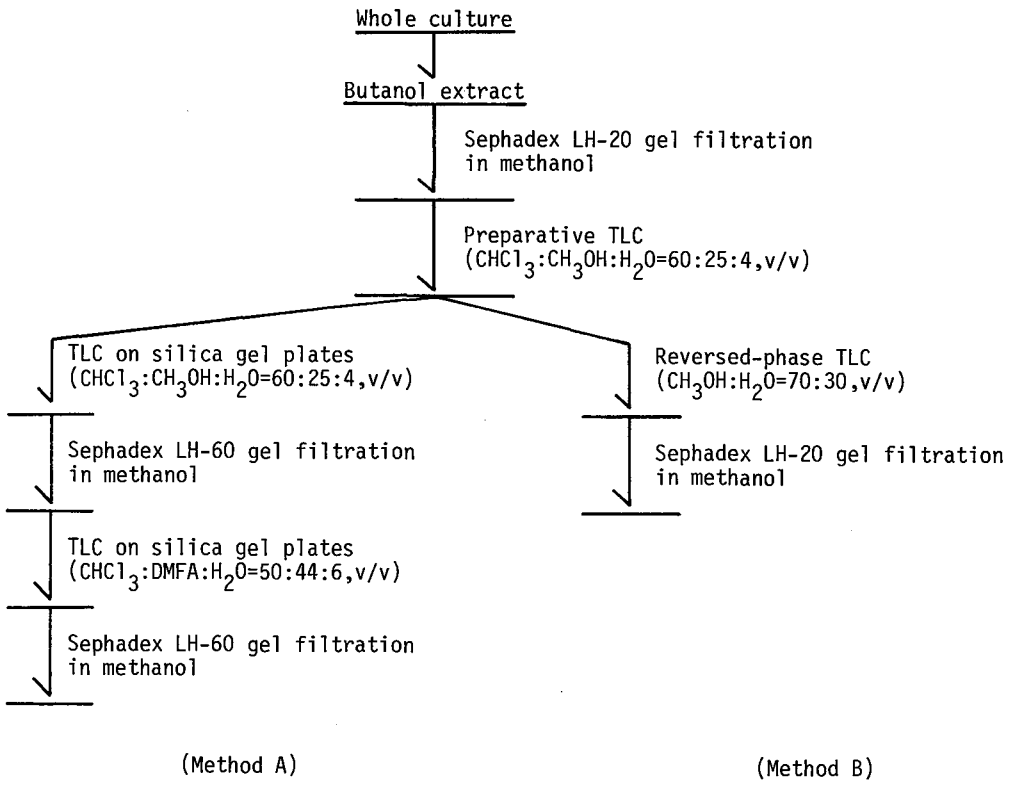

Fig. 3. Flow diagram of isolation procedures of subtilosin A. Details of both methods were described in the text. All procedures were carried out at room temperature. Samples were stored in methanol.

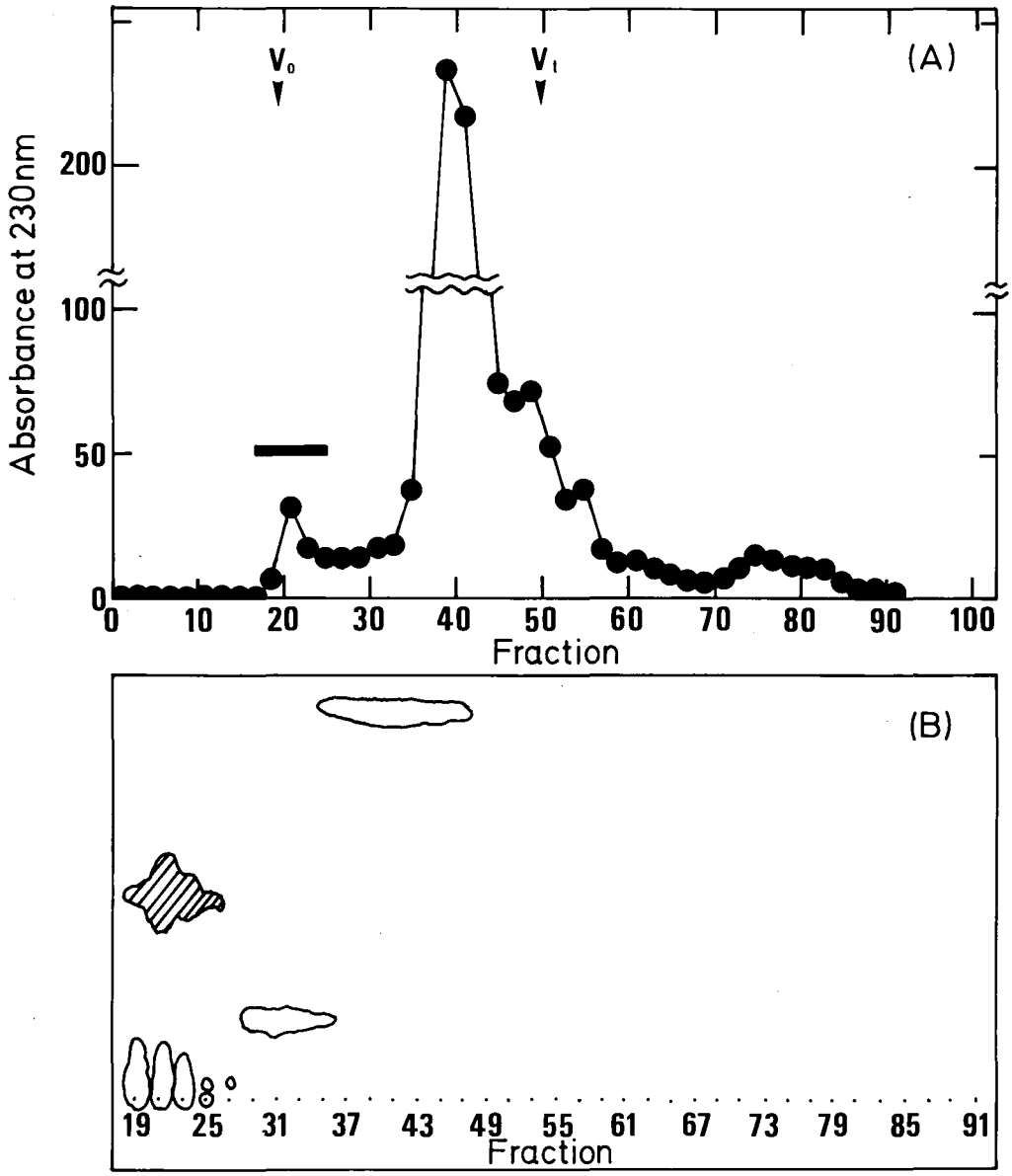

Fig. 4. (A) Sephadex LH-20 gel filtration of a butanol extract. The butanol extract from 3.5 liter of culture was subjected to Sephadex LH-20 gel filtration as described in the text. Closed circles, absorbance at 230 nm; a solid bar indicates the subtilosin A fractions pooled; and V_o and V_t indicate the positions of the void and total volumes of the column, respectively.

(B) Bioautography of the eluates for antibiotic activity. A portion (25 µl) of every other fraction was chromatographed on a silica gel plate (Merck 5715) with the solvent system A followed by bioautography as described in "Materials and Methods". The numbers of fractions correspond to those fractions in (A). The hatch area indicates subtilosin A (Rf = 0.48) and the area marked by lines the other antibiotics.

Method A

	Volume	Contents
Whole culture	3.5 l	
n-Butanol extract	ca. 0.8 l	
Sephadex LH-20 gel filtration		
Preparative TLC (silica gel)		32.2 mg
TLC (silica gel)		
Sephadex LH-60 gel filtration		16.5 mg
TLC (silica gel)		8.1 mg
Sephadex LH-60 gel filtration		7.0 mg

Method B

	Volume	Contents
Whole culture	3.5 l	
n-Butanol extract	ca. 0.8 l	
Sephadex LH-20 gel filtration		
Preparative TLC (silica gel)		32.2 mg
Reversed-phase TLC		
Sephadex LH-20 gel filtration		19.4 mg

Table III. Summary of isolation of subtilosin A.
Details of both methods were discribed in the text.
The contents of subtilosin A were estimated from the absorbance at 280 nm as described in "Materials and Methods".

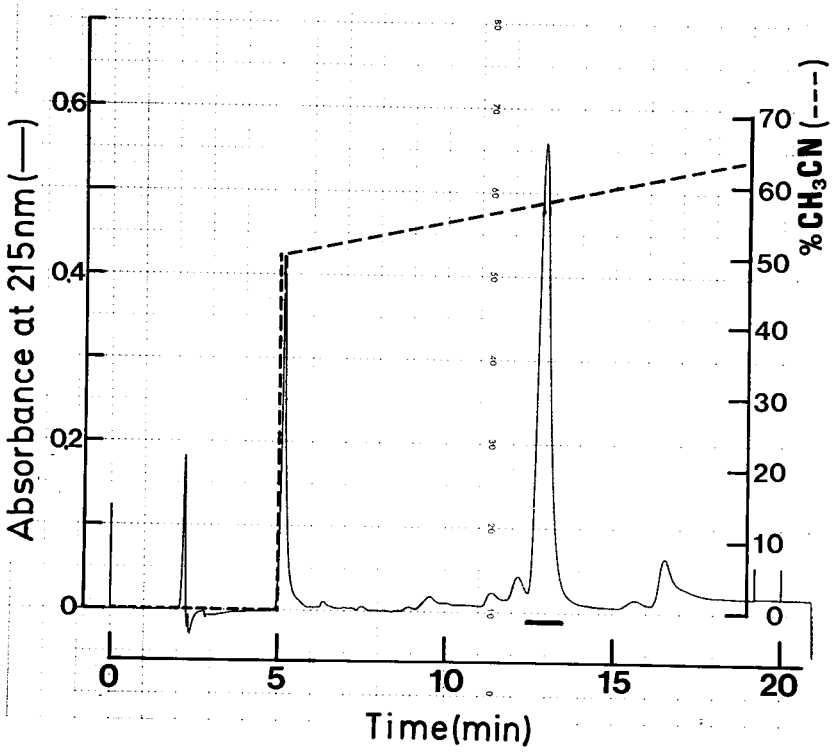

Fig. 5. Reverse-phase HPLC of purified subtilosin A.

Purified subtilosin A (37.5 μg) was subjected to HPLC on Yanapak ODS-T (4 x 250 mm, C_{18}, 10 μm). Elution was carried out with 40 ml of a linear gradient of acetonitrile in 0.1% TFA from 50% to 70% at a flow rate of 1.0 ml/mim. The eluates were monitored by absorbance at 215 nm. The solid bar indicates subtilosin A fractions. The other peaks are a background or show the perturbation of a solvent.

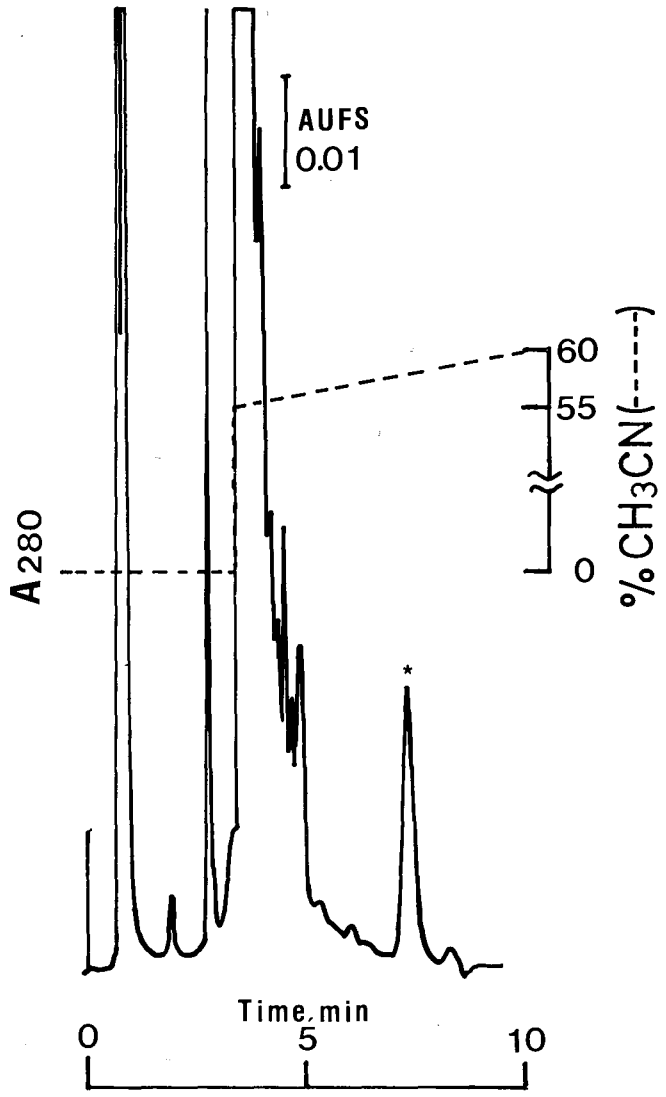

Fig. 6. Reverse-phase HPLC of butanol extract.

A butanol extract (1 ml) was evaporated to dryness. The residue was dissolved in 1 ml of 20% acetonitrile in 0.1% TFA and filtered through an EKICRODISC 13 filter. The filtrate was subjected to HPLC on a reverse-phase HPLC column (Yanapak ODS-T, 10 μm, 4 x 250 mm, C_{18}) equilibrated with 0.1% TFA. Elution was carried out with a 40 ml linear gradient of acetonitrile in 0.1% TFA from 55% to 65% at a flow rate of 1.5 ml/min, and monitored by absorbance at 280 nm. Subtilosin A fraction is indicated with an asterisk.

Microorganisms	Sensitivity	MIC (μg/ml)
Bacillus amyloliquefaciens H	++	13.5
Bacillus brevis ATCC 9999	-	
Bacillus licheniformis ATCC 10716	-	
Bacillus megaterium IFO 12108	++++	1.7
Bacillus natto IFO 13169	++	27
Bacillus polymyxa ATCC 10401	+	>108
Bacillus subtilis 168	+	>108
Bacillus subtilis ATCC 6633	-	
Lactobacillus casei IFO 3435	-	
Staphylococcus aureus IFO 12732	+	54
Streptococcus faecium IFO 3181	+++	3.4
Escherichia coli K-12 HB 101	-	
Aspergillus niger IFO 6341[a]	-	
Mucor juvanicus IFO 4570[a]	-	

[a] Grown at 28°C.

Table IV. Antibiotic spectrum of subtilosin A.

Cells of each microorganism except for Lactobacillus casei were grown on LB soft agar plates containing 0.2% Brij-58, and their sensitivities to subtilosin A were examined by a paper disc method (25). After incubation at 37°C for 18 h, diameters of the zone of inhibition, which was formed around paper discs (13 mm) containing 27 μg of subtilosin A, were measured. The sensitivity was designated by - and + as follows: - means no halo, +<16 mm, ++<18 mm, +++<20 mm, ++++≧20 mm. The minimum in hibitory concentration (MIC) was determined by an agar dilution method (25). L. casei was grown on a 0.7% agar plate of Lactobacilli medium (26) containing 0.2% Brij-58.

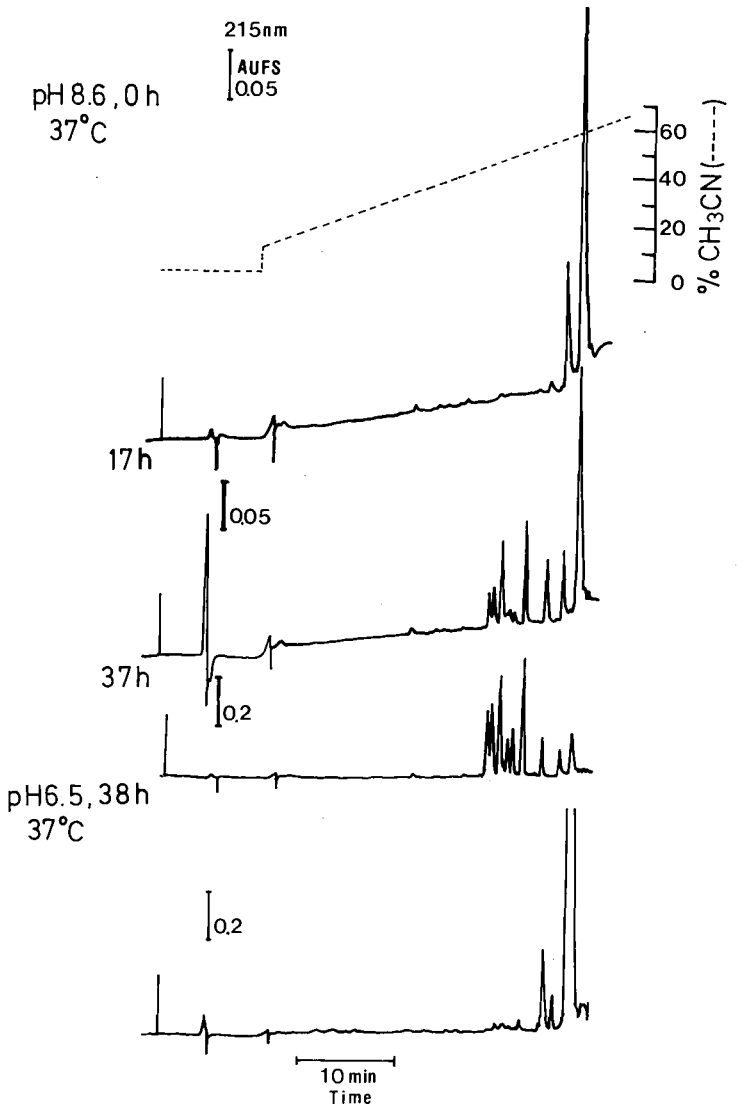

Fig. 7. Unstability of subtilosin A under alkaline conditions.

Intact subtilosin A (54 µg) was dissolved in 50 µl of 0.1 M NH_4HCO_3 containing 10 mM $CaCl_2$, pH 8.6, and incubated at 37°C. Samples of 10.8 µg, 10.8 µg and 32.4 µg at 0 h, 17 h and 37 h, respectively were removed, and subjected to reverse-phase HPLC on a Chemcopak $_5C_{18}$ column with a 40 ml linear gradient of acetonitrile in 0.1% TFA from 10% to 70%. The eluates were monitored by absorbance at 215 nm.

(The lowest figure) Intact subtilosin A (140 µg) was incubated in 1 ml of 0.1 M MES buffer containing 10 mM $CaCl_2$, pH 6.5, at 37°C for 38 h, and analysed in the same manner as above.

Amino acid	
Asx	3.0 (3)[a]
Thr	0.7 (1)
Ser	0.8 (1)
Glx	1.0 (1)
Pro	1.9 (2)
Gly	7.1 (7)
Ala	5.0 (5)
Cys[b]	2.5 (3)
Val	1.0 (1)
Ile	3.0 (3)
Leu	3.0 (3)
Lys	1.0 (1)
Trp[c]	0.5 (1)
Total residues	(32)

[a] values are expressed as residues per mol. Numbers in parentheses are residue values based on sequence.

[b] Cysteine was determined as cysteic acid. The S-carboxymethyl-cysteine contents were 2.0 and 0.8 mol/mol on RCM-subtilosin A with and without pretreatment with methylamine, respectively.

[c] Tryptophan content estimated from absorbance at 280 nm in methanol was 1.1-1.2 mol/mol.

Table V. Amino acid composition of subtilosin A.
Amino acid analyses were carried out as described in "Materials and Methods".

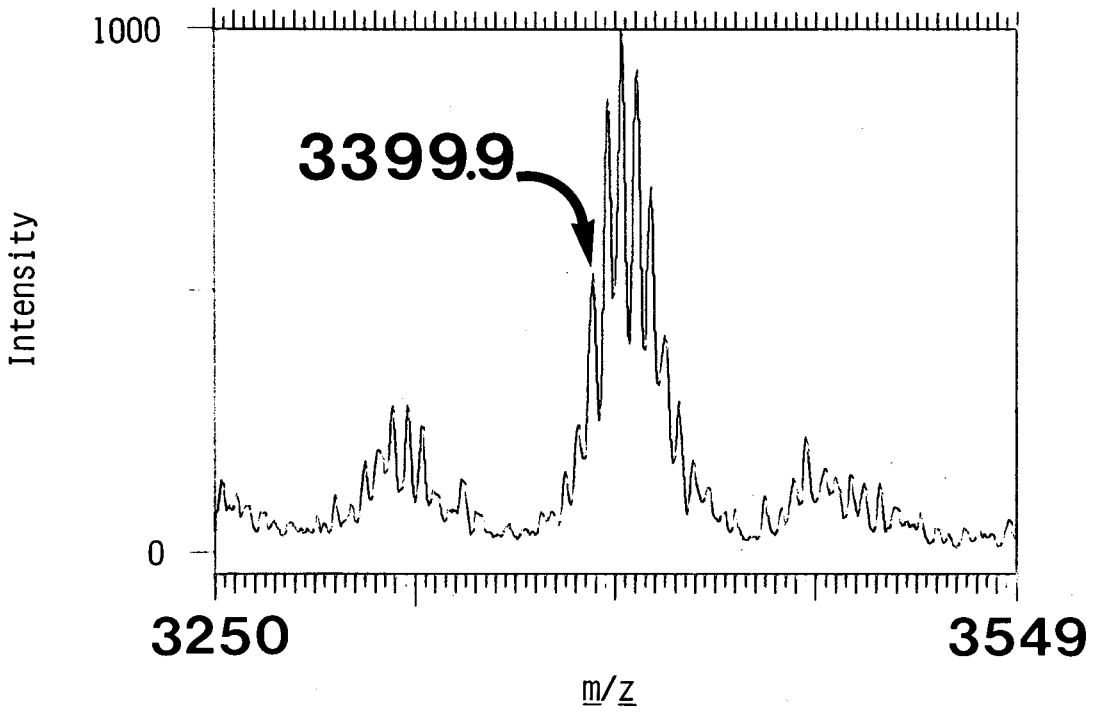

Fig. 8. FAB mass spectrum in the range from 3250 to 3549 of intact subtilosin A. The highest peak is present at m/z 3401.8, but the peak at m/z 3399.9 is thought to correspond to $[m + H]^+$ ion considering the presence of isotops.

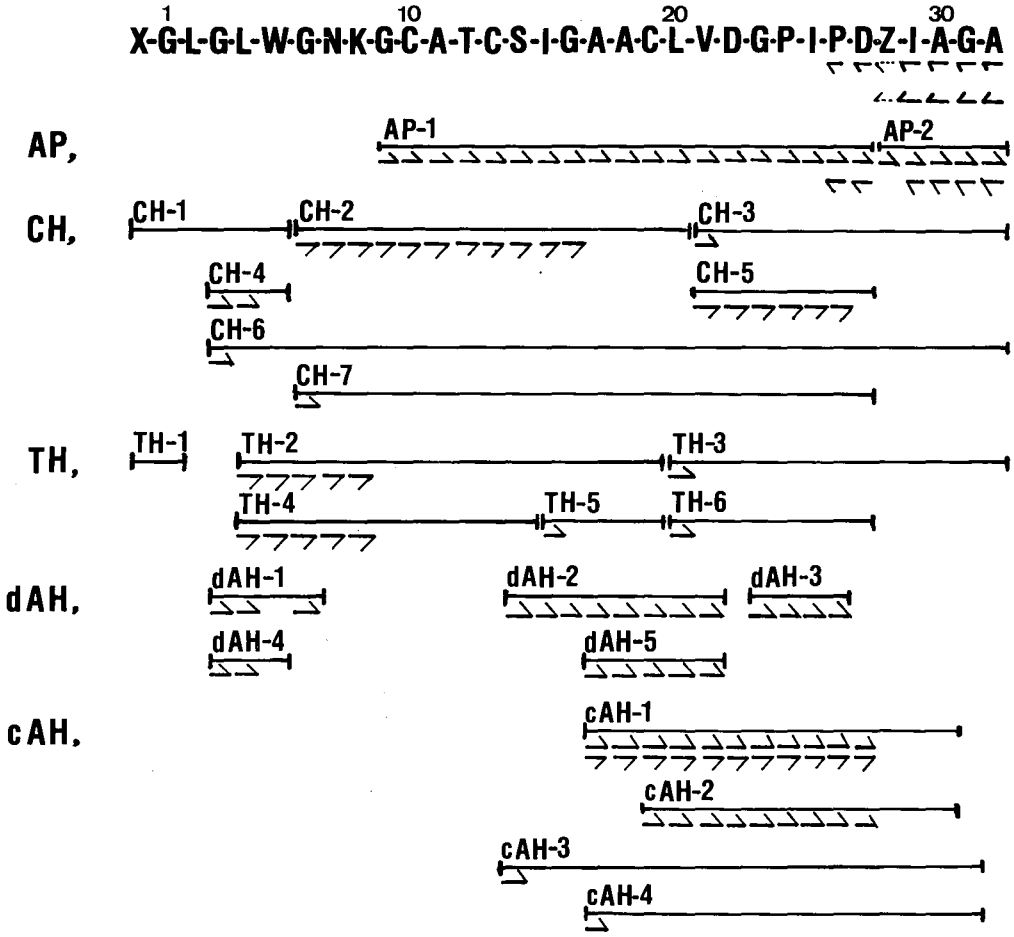

Fig. 9. Summary of strategy of amino acid sequencing of sutilosin A. Amino acid resisues were indicated by one-letter notation. CH, chymotryptic peptides of RCM-samples; TH, thermolysin peptides of RCM-samples; AP, digests of performic acid-oxidized samples with Achromobacter protease I; dAH, hydrolyzates of RCM-samples with dilute acid; cAH, hydrolyzates of intact samples with concentrated HCl. ⟵ , ⟵ , ⟶ , and ⟶ indicate sequencing by digestion with carboxypeptidase A, and Y, dansyl-Edman degradation, and the DABITC/PITC double coupling method, respectively.

	dAH-1	dAH-2	dAH-3	dAH-4	dAH-5	CH-1	CH-2	CH-3	CH-4	CH-5	CH-6	CH-7
Asx	0.2			0.2			1.0 (1)	1.9 (2)	0.1	1.9 (2)	3.0 (3)	2.6 (3)
Thr							0.9 (1)				0.9 (1)	0.8 (1)
Ser	0.3	0.9 (1)	0.1	0.2	0.1		0.9 (1)	0.1	0.1		1.0 (1)	1.0 (1)
Glx	0.3			0.2				1.0 (1)			1.0 (1)	0.3
Pro			2.1 (2)					1.9 (2)		1.9 (2)	2.1 (2)	1.9 (2)
Gly	2.2 (2)[a]	1.2 (1)	1.0 (1)	1.3 (1)	0.2	2.2 (2)	2.8 (3)	2.0 (2)	1.0 (1)	1.0 (1)	5.8 (6)	4.0 (4)
Ala	0.2	2.0 (2)	0.1	0.2	2.0 (2)		2.9 (3)	1.9 (2)	0.1		5.0 (5)	3.0 (3)
Cys		0.8 (1)[b]			0.8 (1)[b]		2.5 (3)[b]				1.8 (3)[b]	1.3 (3)[b]
Val		1.0 (1)			1.0 (1)			1.0 (1)		1.0 (1)	1.1 (1)	1.0 (1)
Ile		1.0 (1)	0.9 (1)				0.9 (1)	2.0 (2)		1.0 (1)	3.0 (3)	2.0 (2)
Leu	1.0 (1)	1.0 (1)		1.0 (1)	1.1 (1)	2.0 (2)	1.0 (1)		1.0 (1)		2.0 (2)	1.2 (1)
Lys		0.2			0.1		0.9 (1)				0.9 (1)	0.9 (1)
Trp	N.D.(1)			N.D.(1)		0.8 (1)			0.5 (1)		N.D.(1)	
Total	(4)	(8)	(4)	(3)	(5)	(5)	(15)	(12)	(3)	(7)	(30)	(22)
Yield (%)	1.4	3.7	20.0	1.5	6.6	7.5	16.6	11.0	4.0	12.5	3.0	1.0

[a] Values are expressed as residues per mol. Numbers in parentheses are residue values based on sequence.

[b] Determined as S-carboxymethyl-cysteine.

N.D., Not determined.

Table VI. Amino acid compositions of fragments used for sequencing. Notation of fragments is indicated in the legend to Fig. 9.

	cAH-1	cAH-2	cAH-3	cAH-4	AP-1	AP-2	TH-1	TH-2	TH-3	TH-4	TH-5	TH-6
Asx	1.9 (2)	1.8 (2)	2.0 (2)	1.8 (2)	2.0 (2)			1.1 (1)	2.0 (2)	1.0 (1)		2.0 (2)
Thr					0.8 (1)			1.0 (1)		0.9 (1)		
Ser			0.8 (1)	0.1	0.8 (1)			1.1 (1)		1.0 (1)		
Glx	1.0 (1)	0.9 (1)	1.1 (1)	1.0 (1)		1.1 (1)		0.2	1.0 (1)			
Pro	2.0 (2)	2.1 (2)	2.1 (2)	1.8 (2)	2.6 (2)				2.3 (2)			2.4 (2)
Gly	1.4 (1)	1.2 (1)	3.0 (3)	2.0 (2)	3.1 (3)	1.2 (1)	1.0 (1)	3.1 (3)	2.0 (2)	2.0 (2)	1.0 (1)	1.0 (1)
Ala	2.9 (3)	1.0 (1)	3.3 (3)	3.2 (3)	3.0 (3)	2.1 (2)		2.7 (3)	1.9 (2)	1.0 (1)	2.0 (2)	
Cys	1.0 (1)[c]	N.D.(1)	N.D.(1)	N.D.(1)	2.3 (3)[c]			2.8 (3)[b]		1.7 (2)[b]	0.9 (1)[b]	
Val	1.0 (1)	0.9 (1)	1.0 (1)	0.9 (1)	1.0 (1)				1.0 (1)			1.0 (1)
Ile	2.0 (2)	1.8 (2)	2.8 (3)	1.8 (2)	1.8 (2)	1.0 (1)		0.9 (1)	2.0 (2)		1.0 (1)	1.0 (1)
Leu	1.0 (1)	1.0 (1)	1.2 (1)	1.0 (1)	1.0 (1)			1.0 (1)	1.0 (1)	1.0 (1)		1.0 (1)
Lys								1.0 (1)		1.0 (1)		
Trp								N.D.(1)		0.6 (1)		
Total	(14)	(12)	(18)	(15)	(19)	(5)	(1)	(16)	(13)	(11)	(5)	(8)
Yield (%)	8.1	5.0	9.0	12.3	14.8	6.8	16.3	4.4	16.3	7.2	25.4	11.4

[c] Determined as cysteic acid.

Table VI. (continued)

1 10

X-Gly-Leu-Gly-Leu-Trp-Gly-Asn-Lys-Gly-Cys-

20

Ala-Thr-Cys-Ser-Ile-Gly-Ala-Ala-Cys-Leu-

30

Val-Asp-Gly-Pro-Ile-Pro-Asp-Glx-Ile-Ala-

Gly-Ala

Fig. 10. Amino acid sequence of subtilosin A

X indicates an unknown residue of a non-amino acid.

The γ-carboxyl group of the Glx-28 was substituted as described in the text.

NH_2-terminus	Blocked
COOH-terminus (intact)	Blocked
(after performic acid oxidation)	Ala residue
Sugar	None
Free SH-groups (intact)	None
(after NH_2NH_2 treatment)	3 mol/mol
OH-groups of Ser and Thr residues	Free
β-COOH groups	Asp, 2 mol/mol
ε-NH_2 group of Lys residue	Free
Optical configuration	L-form

Table VII. Chemical properties of subtilosin A.
Details are described in the text.

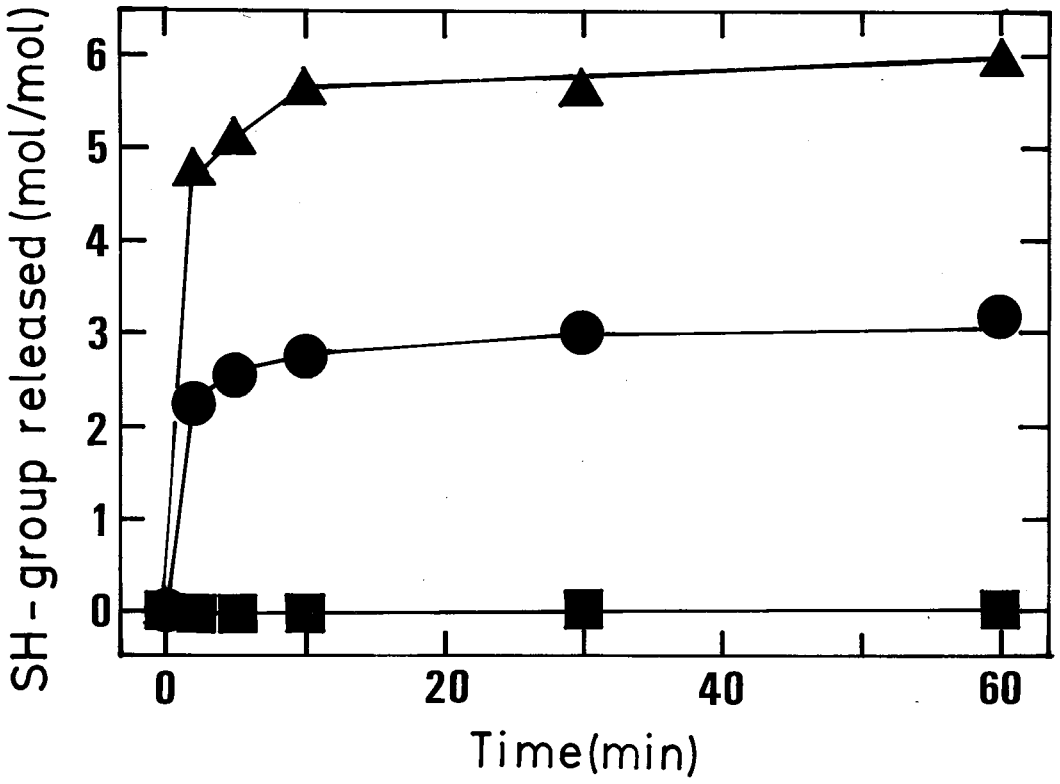

Fig. 11. Titration of the thiol groups released by treatment with anhydrous hydrazine of subtilosin A, bovine insulin and thermolysin.

Dried samples were dissolved in anhydrous hydrazine, and allowed to stand at room temperature. At various times, portions (16 nm/25 µl) of the solution were removed, evaporated to dryness, and titrated with DTNB according to the method of Kortt and Liu (24). Thermolysin and bovine insulin, which contain no and three cystine residues, respectively, were used as references. Closed triangles, circles, and squares indicate the amounts of the thiol groups titrated of bovine insulin, subtilosin A and thermolysin, respectively.

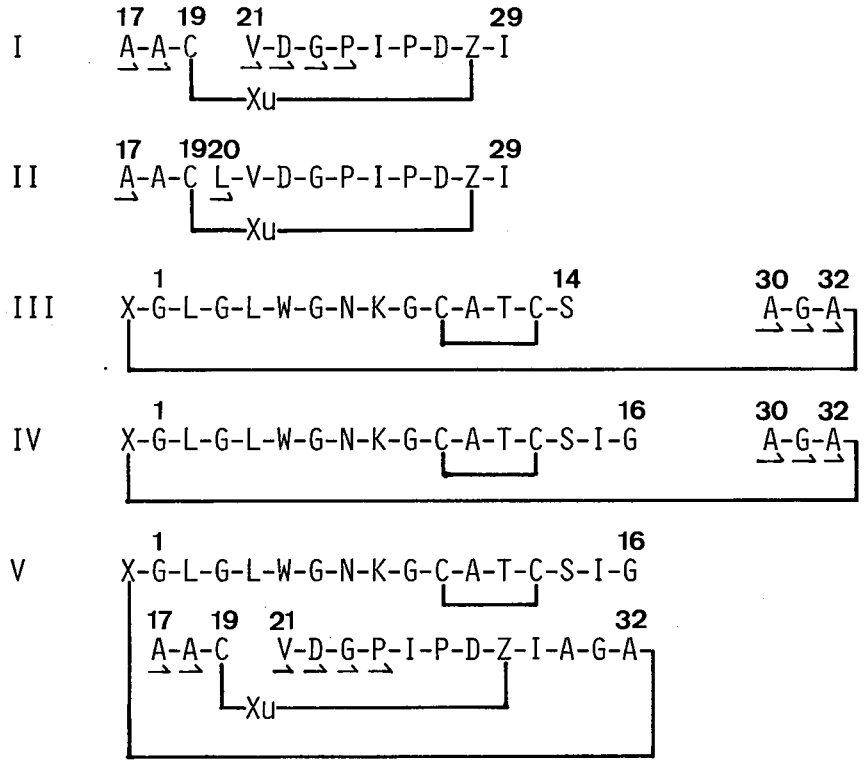

Fig. 12. Structures of thermolysin peptides of intact subtilosin A. I-V correspond to the structures of Fragment I-V, respectively. Isolation of peptides was described in the legend to Fig. 13. Amino acid sequence of each peptide was written in one-letter notation. $\longrightarrow$ indicates sequencing by the dansyl-Edman method. X and Xu are unknown residues.

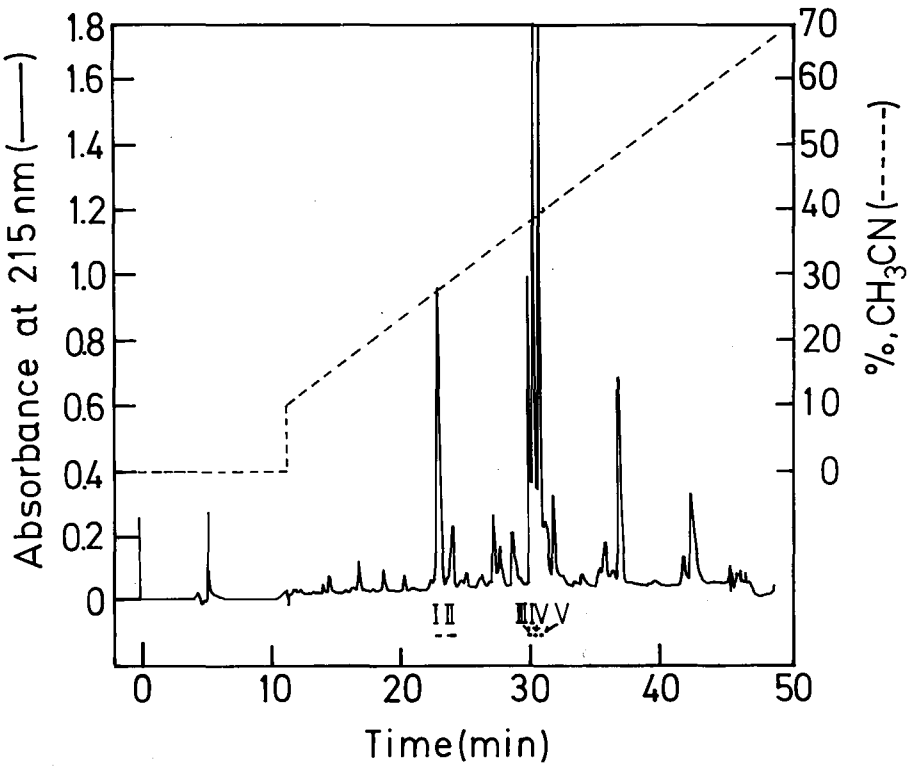

Fig. 13. Reverse-phase HPLC of thermolysin peptides of intact subtilosin A.

Digestion was performed as described in "Materials and Methods". After excluding precipitates from the digests by centrifugation, the supernatant was applied to a reverse-phase HPLC column (Chemcopak $_5C_{18}$, 4.6 x 250 mm). Elution was performed with a 40 ml linear gradient of acetonitrile in 0.1% TFA from 10% to 70% at a flow rate of 1 ml/min, and monitored by changes of absorbance at 215 nm. Each peak was further purified in the same manner as above, but with more shallow gradients of acetonitrile. The fragments derived from the peaks I-V were designated as Fragments I-V, respectively.

Amino acid	I	II	III	IV	V
Asx	2.1 (2)	2.3 (2)	1.2 (1)	1.3 (1)	3.1 (3)
Thr			1.0 (1)	1.0 (1)	1.0 (1)
Ser	0.1	0.2	0.9 (1)	0.9 (1)	0.9 (1)
Glx	1.2 (1)	1.3 (1)			1.0 (1)
Pro	2.3 (2)	2.0 (2)			2.2 (2)
Gly	1.1 (1)	1.4 (1)	4.7 (5)	6.0 (6)	6.9 (7)
Ala	2.0 (2)	2.0 (2)	2.8 (3)	3.0 (3)	5.0 (5)
Cys[a]	0.9 (1)	0.9 (1)	1.6 (2)	1.7 (2)	2.5 (3)
Val	1.0 (1)	1.1 (1)			0.9 (1)
Ile	2.0 (2)	2.0 (2)		1.0 (1)	2.8 (3)
Leu		0.7 (1)	2.0 (2)	2.1 (2)	2.1 (2)
Lys			1.0 (1)	1.1 (1)	1.1 (1)
Trp					N.D.(1)
Total	(12)	(13)	(16)	(17)	(31)

[a] Cysteine was determined as cysteic acid after performic acid oxidation.

Table VIII. Amino acid compositions of Fragment I-V.
I-V correspond to Fragment I-V, respectively.

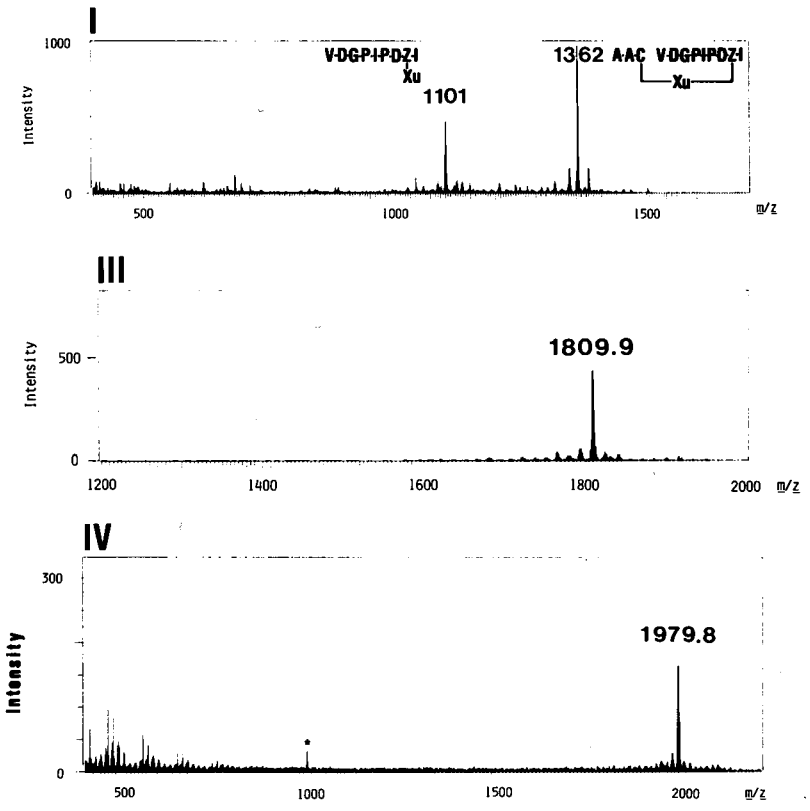

Fig. 14. FAB mass spectra of Fragment I, III and IV.

I, III and IV shows the FAB mass spectra of Fragment I, III and IV, respectively. From the observed mass value of the parent ion signal, the molecular weights of Fragment I, III and IV were estimated to be 1361, 1808.9 and 1978.8, respectively. In the FAB mass spectrum of Fragment I, the strucure of each signal is shown above the signal in one-letter notation of amino acids.

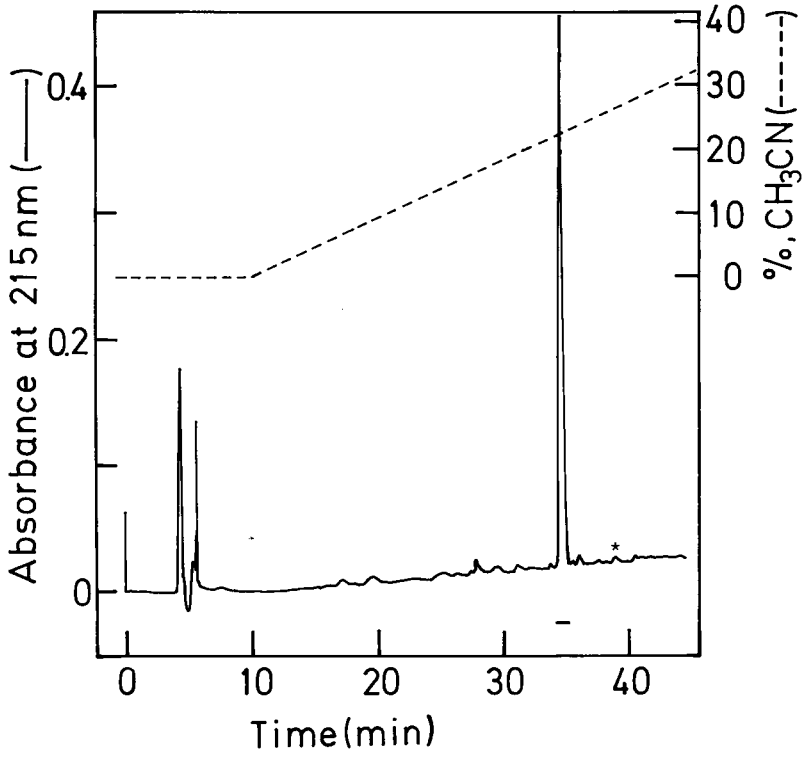

Fig. 15. Reverse-phase HPLC of the thermolysin digests of Fragment I for isolation of Fragment U.

Fragment I was digested as described in "Mateials and Methods". The digests were chromatographed by HPLC on a reverse-phase column (Chemcopak $_5C_{18}$) with a 40 ml linear gradient of acetonitrile in 0.1% TFA from 0% to 35%. Eluates were monitored by changes of absorbance at 215 nm. The bar and asterisk indicate the positions of Fragment U and I, respectively.

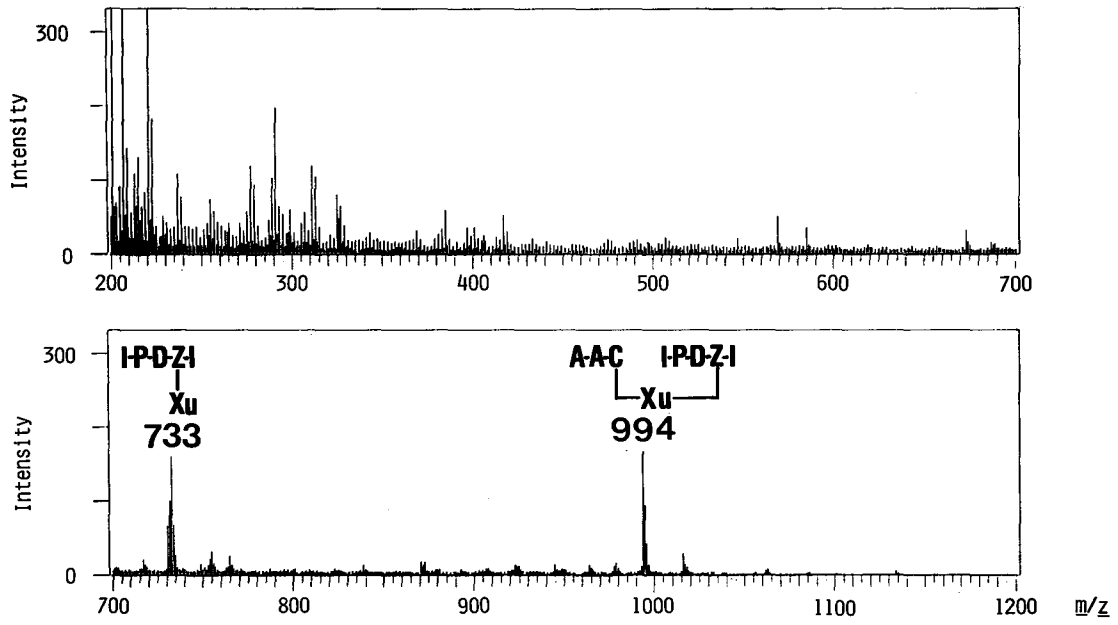

Fig. 16. FAB mass spectrum of fragment U in the range from 200 to 1200 atomic mass units.

The structure corresponding to each signal is shown above the signal in one-letter notation of amino acids.

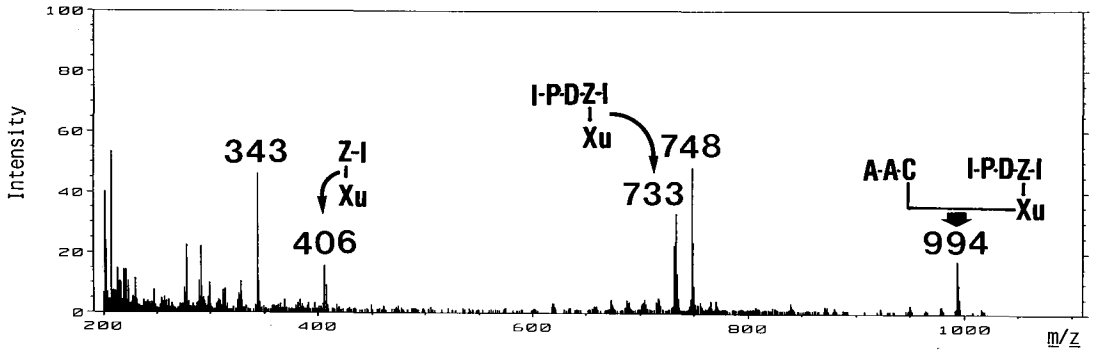

Fig. 17. FAB mass spectrum in the range from 200 to 1100 atomic mass units of Fragment U after incubation in 0.1% NH_4HCO_3 (pH 7.9) at 37°C for 4 h.

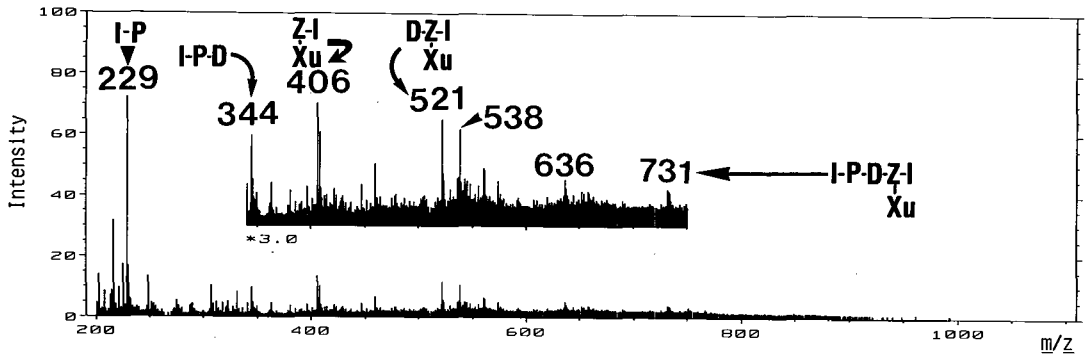

Fig. 18. FAB mass spectrum in the range from 200 to 1100 atomic mass units of the digests of Fragment U with aminopeptidase M.

Digestion was carried out in 0.1% NH_4HCO_3 (pH 7.9) at 37°C for overnight. The insertional figure is the FAB mass spectrum enlarged three-fold in the range from 350 to 750 atomic mass units.

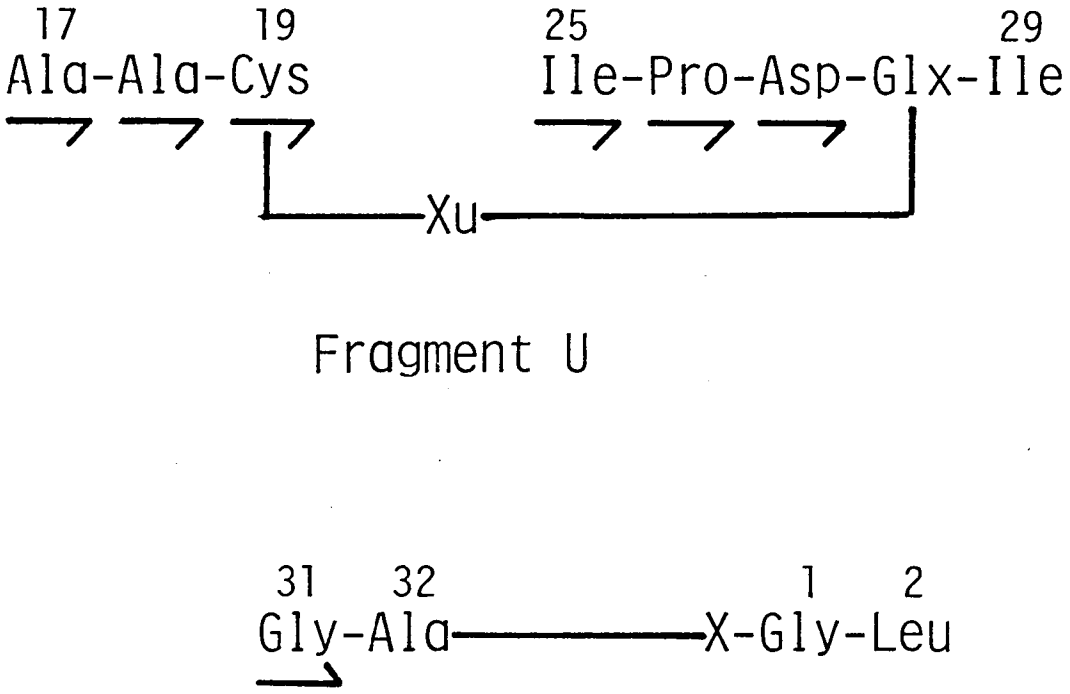

Fig. 19. (Upper) Structure of Fragment U with a cross-linking structure between Cys-19 and Glx-28.

The amino acid composition of Fragment U was as follows: $Asx_{1.0}$, $Glx_{1.1}$, $Pro_{1.0}$, $Ala_{2.0}$, $Cys_{0.1\ (1)}$, $Ile_{2.1}$. The molecular weight of Fragment U is 993. The molecular weight of chain A (Ala-Ala-Cys) minus H and chain B (Ile-Pro-Asp-Glx-Ile) minus OH or NH_2 are 262 and 568, respectively. The residue weight of Xu is estimated to be 163. $\longrightarrow$ indicates sequencing by the DABITC/PITC double coupling method.

(lower) Strucure of a fragment with a cross-linking structure between amino- and carboxyl-terminus.

The fragment was derived from the fraction IV or V of the hydrolyzates of intact subtilosin A with concentrated HCl (See Fig. 3S). $\longrightarrow$ indicates amino-terminal analysis by the dansylation metnod. The yield of this fragment was about 2%.

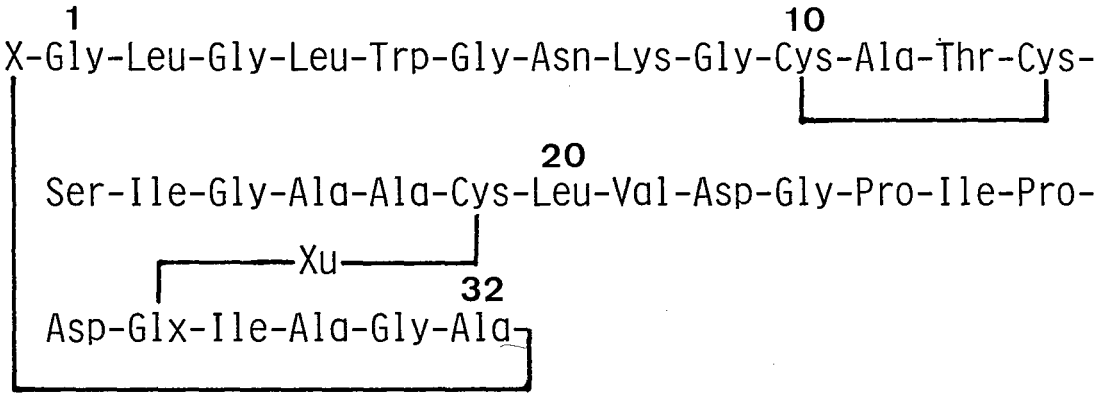

Fig. 20. Primary structure of subtilosin A.
X and Xu are unknown residues of residue weights of 246 and 163, respectively.

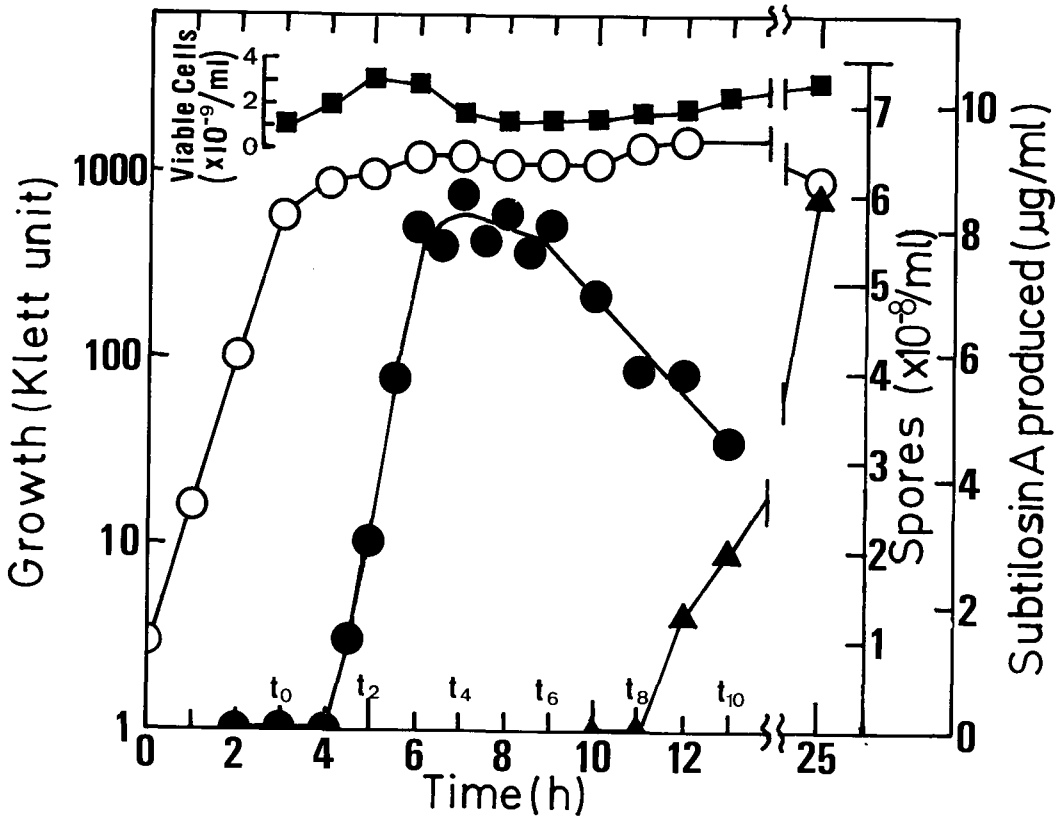

Fig. 21. Time course of production of subtilosin A, cell growth and sporulation.

Cells of B. subtilis 168 growing logarithmically in LB medium were transferred to a 2-liter Erlenmeyer flask containing 350 ml of NSM and incubated at 37°C on a New Brunswick rotatory shaker with sufficient aeration. To follow the production of subtilosin A, cell growth and sporulation, 10 ml-samples were removed at the indicated times. Cell growth was monitored by change of the turbidity at 660 nm in a Klett-Summerson photoelectric colormeter. The amount of subtilosin A was determined on 9 ml-samples as described in "Materials and Methods". Viable cells and heat-resistant spores were titrated by plating appropriate dilutions of the culture on LB agar plates before and after heating at 80°C for 30 min. Open circles, cell growth; closed circles, amounts of subtilosin A produced; closed squares, viable cell titers; closed triangles, heat-resistant spore titers; and t_0 - t_{10}, hours after the end of vegetative growth.

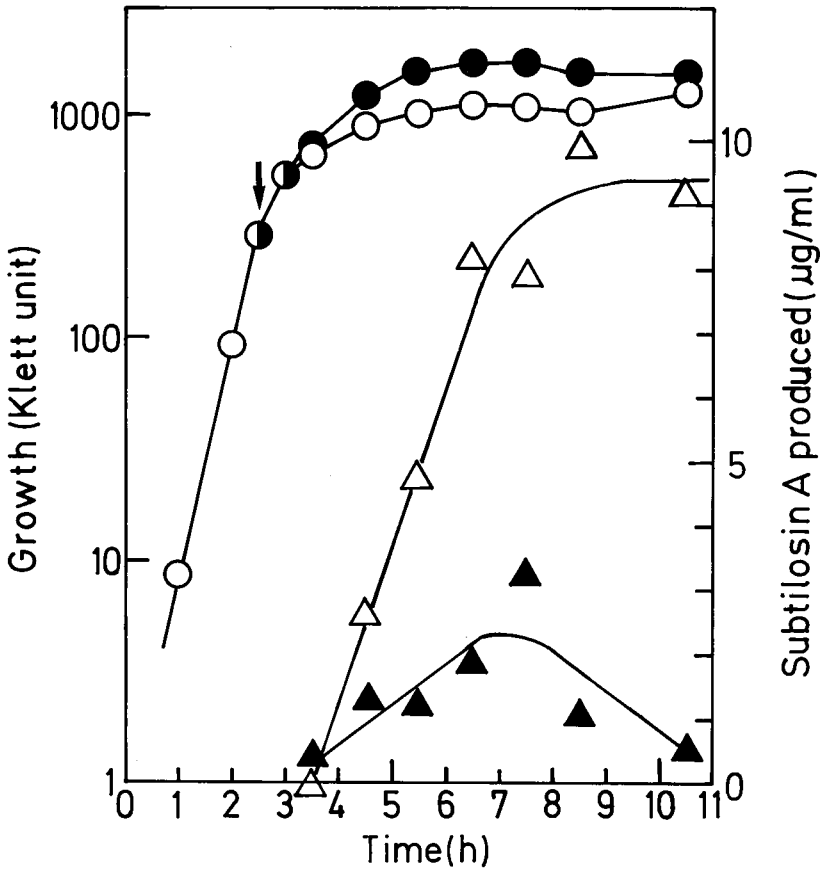

Fig. 22. Effect of glucose on production of subtilosin A.

Cells were grown in two 2-liter Erlenmeyer flasks containing 350 ml of NSM on a New Brunswick rotatory shaker at 37°C. The contents of the two flasks were mixed and divided equally at 10 min before the addition of glucose. Glucose was added to one flask at a concentration of 10 g per liter at the end of vegetative growth. Cell growth and the production of subtilosin A were followed as described in in the legend to Fig. 21. Open circles and triangles, cell growth and the amounts of subtilosin A, respectively, in the control culture; closed circles and triangles, cell growth and amounts of subtilosin A, respectively, in the culture with excess glucose; and the arrow indicates the time of glucose addition.

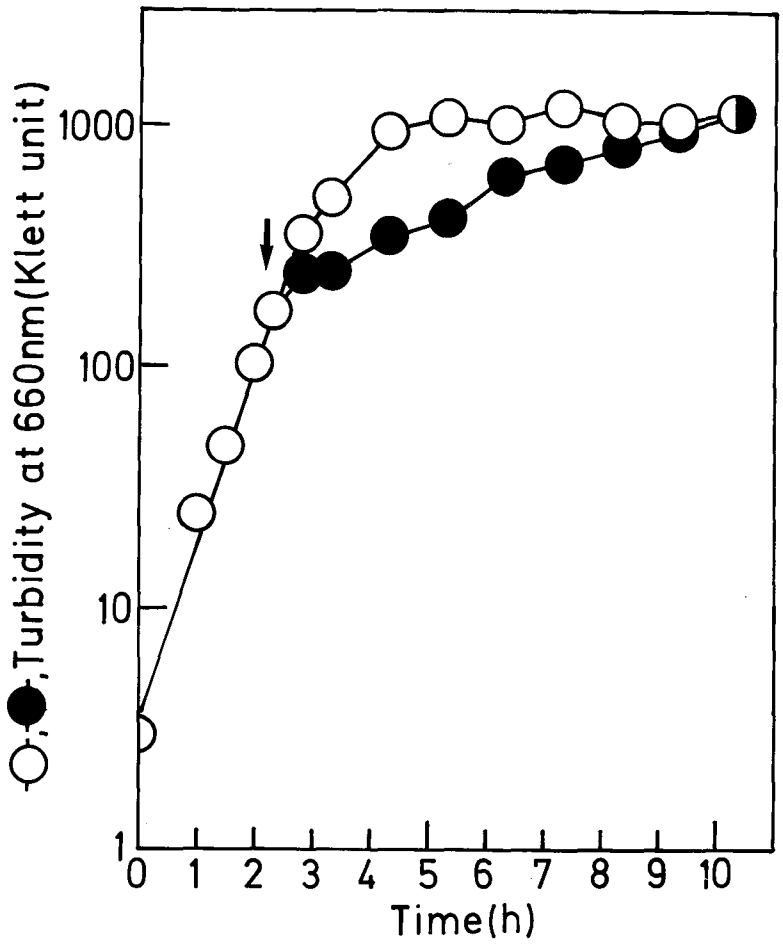

Fig. 23. Effect of Decoyinine on cell growth.

Cells were grown in two 2-liter Erlenmeyer flasks containing 350 ml of S6CG on a rotatory shaker at 37°C. When cells grew to 170 Klett units, decoyinine (0.6 mg/ml) was added to one flask. Cell growth was followed hourly as described in the legend to Fig. 21. Open and closed circles indicate cell growth in the absence and in the presence, respectively, of the drug. The arrow shows the time of drug addition.

	Decoyinine	
	Without	With
Viable cells (V)[a]	1.0×10^8	1.2×10^9
Spores (S)[a]	1.0×10^4	4.0×10^8
S/V x 100	<0.01	33
Subtilosin A production (μg/ml)	0.0	0.0

[a] Values were expressed as numbers per ml of culture.

Table IX. Effects of decoyinine on production of subtilosin A and sporulation.

Cells were grown as described in the legend to Fig. 23. Subtilosin A production was follwed hourly as described in the legend to Fig. 21. The numbers of spores and viable cell were determined at 10 h after the addition of the drug as described in the legend to Fig. 21.

Strain	Relevant genotype	Antibiotic production[a]	Subtilosin A production (μg/ml)[b]
168	spo^+	+	8.0
SR22	spo0A12	-	0.0
6Z	spo0B6Z	-	0.0
9V	spo0C9V	+	5.0
94UL	spoIII94U	+	0.0
11T	spoIV11T	+	0.0

[a] Production of antibiotics other than subtilosin A determined by autobiography.

[b] Determined at 5 h after the end of vegetative growth.

Table X. Production of subtilosin A by asporogenous mutants of B. subtilis 168.

Cells of each mutant were grown under the same conditions as those described in the legend to Fig. 21. Subtilosin A was determined as described in "Materials and Methods".

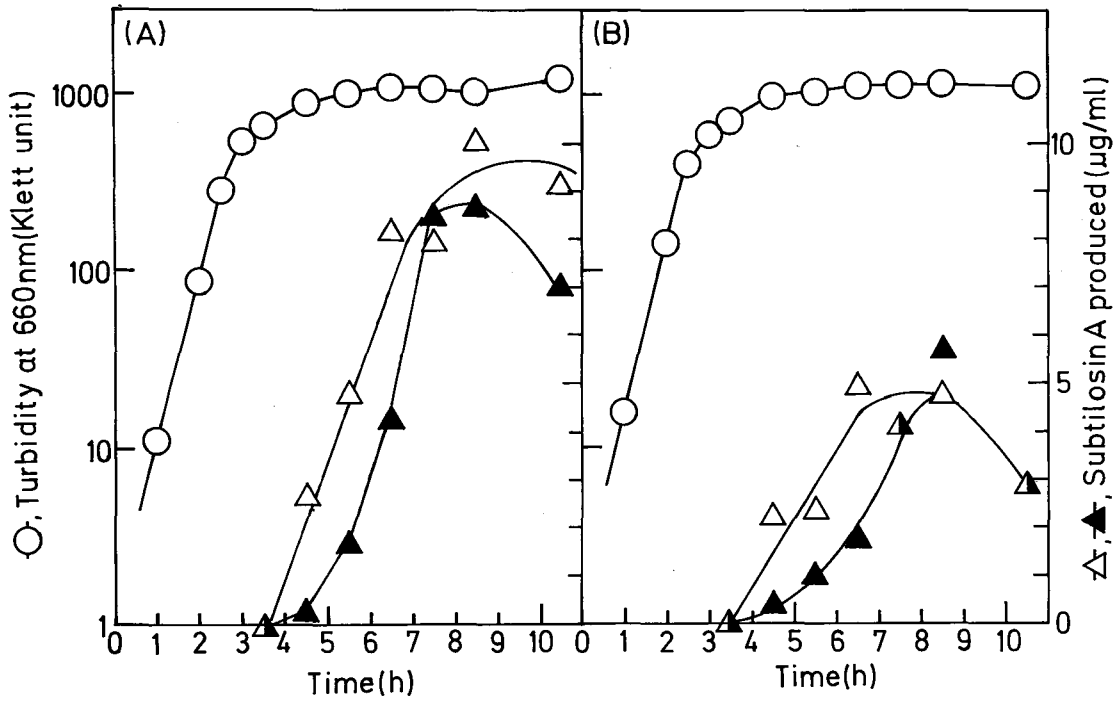

Fig. 24. Distribution of subtilosin A produced by B. subtilis 168 and spo0C9V mutant.

Cells of both strains were grown as described in the legend to Fig. 21. Two 10 ml samples were removed at various times. To determine the amounts of subtilosin A in medium, one sample was treated as follows: To exclude cells, samples were filtered through Millex-HA filters (0.45 μm) or centrifuged at 3500 rpm for 15 min. To the filtrate of the supernatant one fourth volume of n-butanol was added. The other procedures were carried out in the same manner as described in the ligend to Fig. 21. (A) and (B) show the results of B. subtilis 168 and the mutant, respectively. Open circles, cell growth; open and closed triangles, amounts of whole subtilosin A and amounts of subtilosin A in media, respectively.

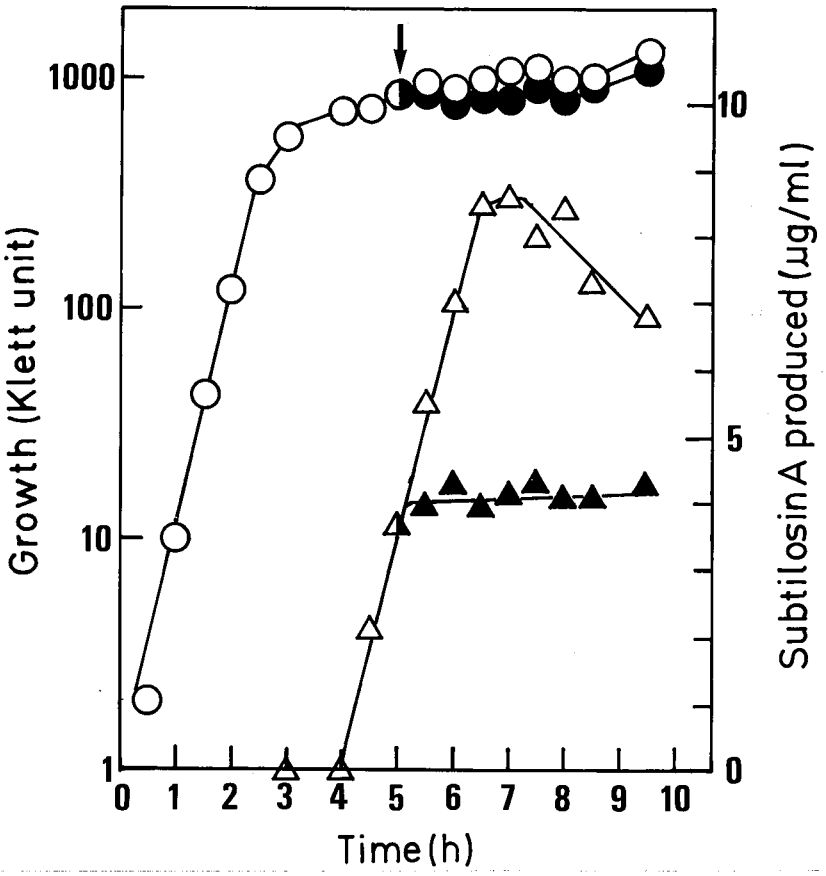

Fig. 25. Effect of chloramphenicol on subtilosin A production.

Cell culture and determination of subtilosin A were carried out as described in the legend to Fig. 22, but chloramphenicol (20 µg/ml) instead of glucose was added at two and one third hours after the end of vegetative growth. Open circles and triangles, cell growth and amounts of subtilosin A, respectively, in the absence of the inhibitor; closed circles and triangles, cell growth and amounts of subtilosin A, respectively, in the presence of the inhibitor. The arrow indicates the time of inhibitor addition.

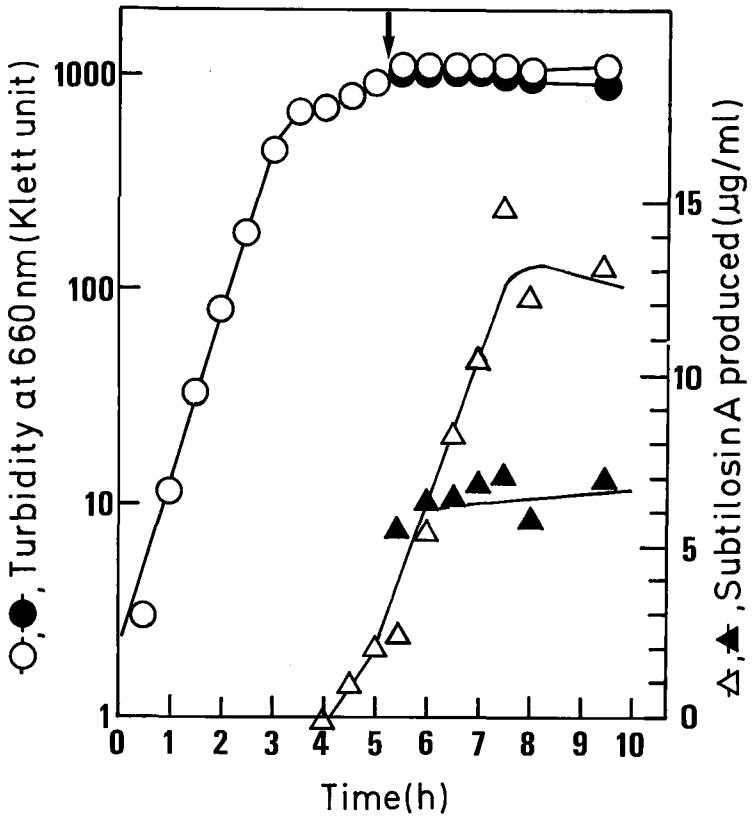

Fig. 26. Effect of kanamycin sulfate on subtilosin A production.

Cell culture and determination of subtilosin A were carried out as described in the legend to Fig. 25, but kanamycin sulfate (50 μg/ml) in place of chloramphenicol was added at the same time. Open circles and triangles, cell growth and amounts of subtilosin A, respectively, in the absence of the inhibitor; closed circles and triangles, cell growth and amounts of subtilosin A, respectively, in the presence of the inhibitor. The arrow indicates the time of inhibitor addition.

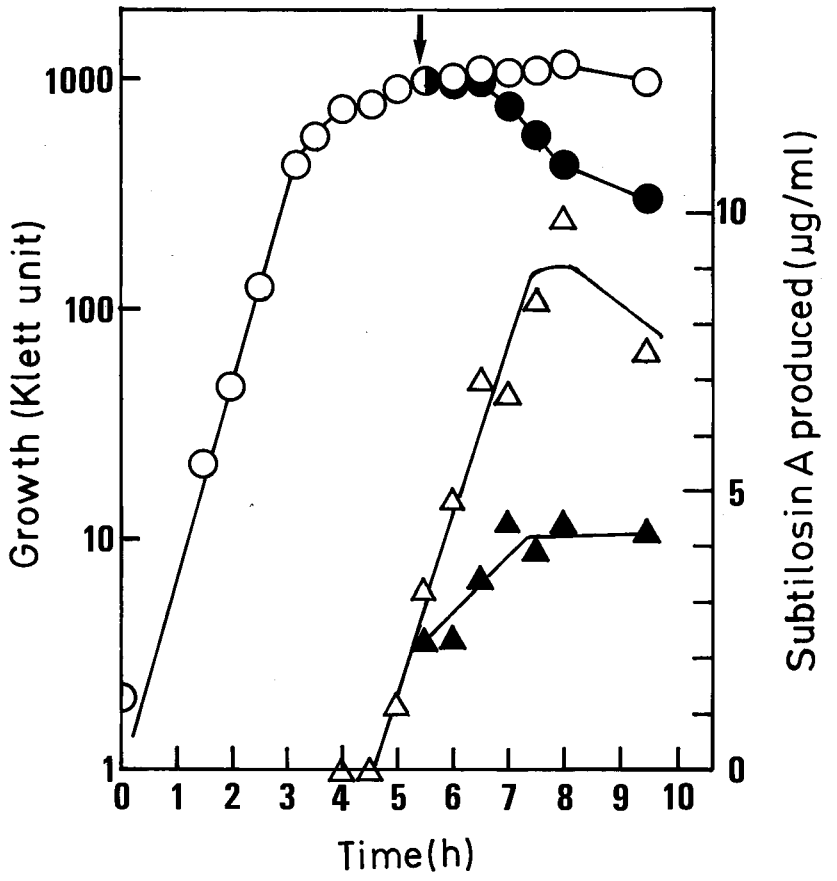

Fig. 27. Effect of rifamycin SV on subtilosin A production.

The cultivation of cells was carried out under the same conditions as those described in the legend to Fig. 25 except that rifamycin SV (30 μg/ml) was added in place of chloram= phenicol. Because rifamycin SV overlapped subtilosin A in the HPLC system for quantitative analysis of subtilosin A as described in "Materials and Methods", the method of subtilosin A estimation was modified as follows: Subtilosin A eluted from the HPLC system was converted to a fluorescamine-adduct and it was subjected to HPLC on the same column with a 40 ml linear gradient of acetonitrile in 0.1% TFA from 60% to 75%. The amount of subtilosin A was culculated from the absorbance of its fluorescamine-adduct. Open circles and triangles, cell growth and amounts of subtilosin A, respectively, in the absence of the inhibitor; closed circles and triangles, cell growth and amounts of subtilosin A, respectively, in the presence of the inhibitor; and the arrow indicates the time of inhibitor addition.

Strain	Subtilosin A-like antibiotic production
Bacillus subtilis 168	+
Bacillus subtilis ATCC 6633	+
Bacillus natto IFO 13169	+
Bacillus amyloliquefaciens H	-
Bacillus megaterium IFO 12108	-

Table XI. Production of subtilosin A-like antibiotics by various species of the genus Bacillus.

Cultivation and determination of subtilosin A were performed in the same manner as described in the legend to Fig. 21, except that the culture scale was one tenth. Samples (10 ml) were removed at 5 h after the end of vegetative growth. + and - indicate production and non-production, respectively, of subtilosin A-like antibiotics.

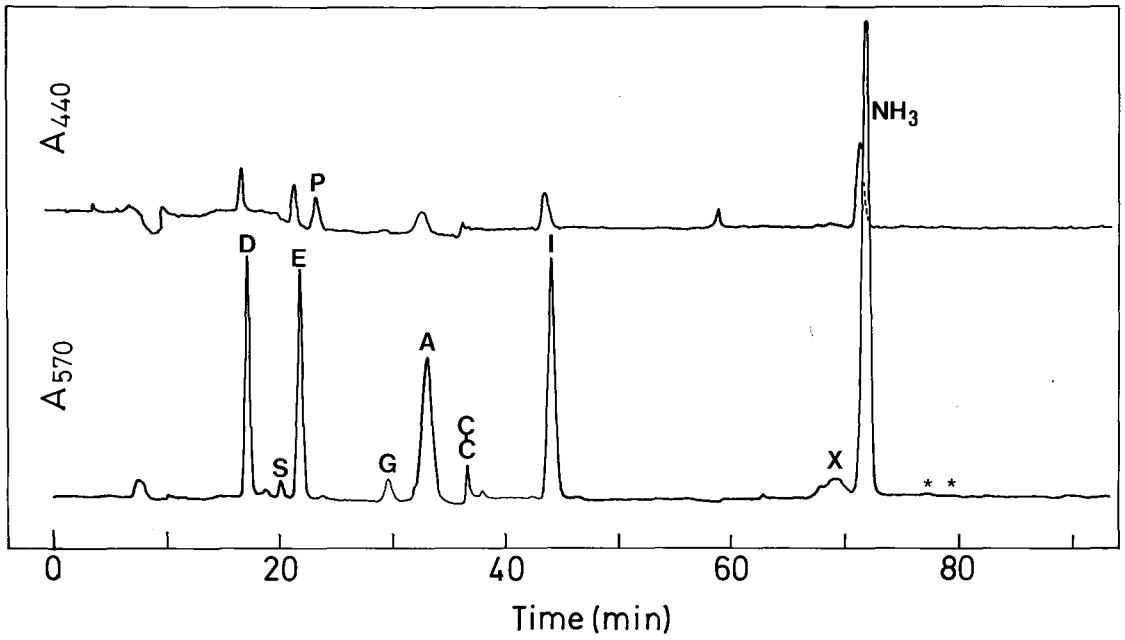

Fig. 28. Chromatogram of amino acid analysis of Fragment U.

Unknown ninhydrine-positive peaks were sometimes detected at the positions marked with X and asterisks in samples without performic acid oxidation. Amino acid analysis was performed as discribed in "Materials and Methods". The contents of amino acids were as follows (nmol): Asx (2.22), Glx (2.23), Pro (1.96), Ala (3.24), Ile (3.54), Ser (0.16), Gly (0.33), Cys (0.23).

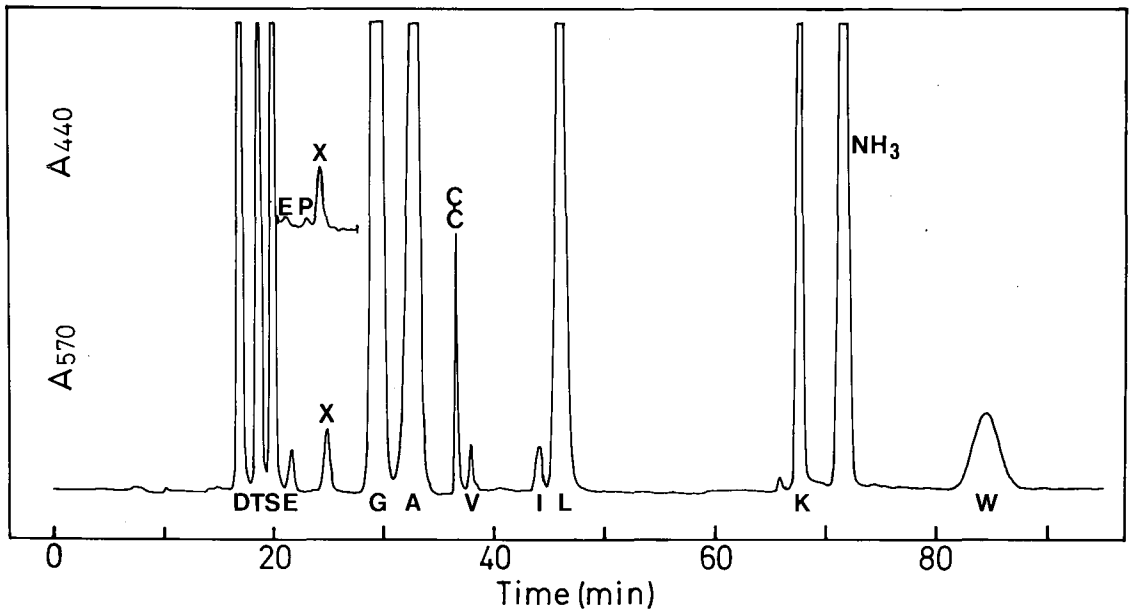

Fig. 29. Chromatogram of amino acid analysis of a fragment corresponding to Fragment III.

Amino acid analysis was performed as described in " Materials and Methods". An unknown ninhydrine-positive peak (X) was sometimes detected in samples without performic acid oxidation. The contents of amino acids were as follows (nmol): Asx (8.18), Thr (5.67), Ser (6.83), Glx (0.43), Gly (33.61), Ala (16.85), Cys (1.45), Val (0.37), Ile (0.58), Leu (16.40), Lys (7.49), Trp (6.33).

SUPPLEMENTARY MATERIALS

Table IS. One-letter notation and residue weight of the relevant amino acid.

Table IIS. Amino acid compositions of fragments isolated from the dilute acid hydrolyzates of RCM-subtilosin A.

Table IIIS. Amino acid compositions of fragments isolated from the concentrated HCl hydrolyzated of intact subtilosin A.

Table IVS. Amino acid compositions of fragments isolated from the digests of performic acid-oxidized subtilosin A with Achromobacter protease I. (I)

Table VS. Amino acid compositions of fragments isolated from the digests of performic acid-oxidized subtilosin A with Achromobacter protease I. (II)

Table VIS. Amino acid compositions of chymotryptic peptides of RCM-subtilosin A.

Table VIIS. Amino acid compositions of thermolysin peptides of RCM-subtilosin A.

Table VIIIS. Amino acid composition of an amino-terminal fragment isolated from the chymotryptic peptides of RCM-subtilosin A.

Table IXS. Amino acid compositons of the amino-therminal fragments isolated from thermolysin peptides of RCM-subtilosin A.

Fig. 1S. Digestion of a performic acid-oxidized subtilosin A with carboxypeptidase A-DFP and Y.

Fig. 2S. Reverse-phase HPLC of dilute acid hydrolyzates of RCM-subtilosin A.

Fig. 3S. Reverse-phase HPLC of concentrated HCl hydrolyzates of intact subtilosin A.

Fig. 4S. Reverse-phase HPLC of digests of performic acid-oxidized subtilosin A with Achromobacter protease I. (I)

Fig. 5S. Reverse-phase HPLC of digests of performic acid-oxidized subtilosin A with Achromobacter protease I. (II)

Fig. 6S. Reverse-phase HPLC of chymotryptic peptides of RCM-subtilosin A.

Fig. 7S. Reverse-phase HPLC of thermolysin peptides of RCM-subtilosin A.

Fig. 8S. Isolation of amino-terminal fragments from chymotryptic peptides of RCM-subtilosin A by reverse-phase HPLC.

Fig. 9S. Isolation of amino-terminal fragments from thermolysin peptides of RCM-subtilosin A by reverse-phase HPLC.

Fig. 10S. Amino acid sequences of fragments other than those shown in Fig. 9.

Figures 2S-9S show the first chromatographies for isolation of fragments used for amino acid sequencing of subtilosin A. Fractions pooled were further chromatographed on the same column with more shallow gradients.

Amino acid	One-letter notation	Residue weight
Asp	D	115.0
Asn	N	114.0
Asx	B	
Thr	T	101.0
Ser	S	87.0
Glu	E	129.0
Gln	Q	128.0
Glx	Z	
Pro	P	97.1
Gly	G	57.0
Ala	A	71.0
Cys	C	103.0
Val	V	99.1
Ile	I	113.1
Leu	L	113.1
Lys	K	128.1
Trp	W	186.1

Table IS. One-letter notation and residue weight of the relevant amino acid.

Amino acid	I	II	III	IV	V	VI
Asx		0.2		0.2		
Thr						
Ser	0.1	0.2	0.1	0.3		0.9 (1)
Glx	0.2		0.3			
Pro	2.1 (2)[a]					
Gly	1.0 (1)	1.3 (1)	0.2	2.0 (2)		1.2 (1)
Ala	0.1	0.2	2.0 (2)	0.2		2.1 (2)
Cys[b]			0.8 (1)		0.8 (1)	0.8 (1)
Val			1.0 (1)		1.0 (1)	1.0 (1)
Ile	0.9 (1)					1.0 (1)
Leu		1.0 (1)	1.1 (1)	1.0 (1)	1.0 (1)	1.0 (1)
Lys			0.1			0.2
Trp		N.D.(1)		N.D.(1)		
Total residues	(4)	(3)	(5)	(4)	(3)	(8)
Note[c]	dAH-3	dAH-4	dAH-5	dAH-1		dAH-2

[a] Values are expressed as residues per mol. Numbers in parentheses are residue values based on sequence.

[b] Cysteine was determined as S-carboxymethyl-cysteine.

[c] Indicated the notation of the fragments described in Fig. 9 and Table VI.

Table IIS. Amino acid compositions of fragments isolated from the dilute acid hydrolyzates of RCM-subtilosin A.

Fragments (I-VI) were derived from the fractions (I-VI) indicated by bars, respectively, in Fig. 2S.

Amino acid	I	II	III	IV	V	VI	VII	VIII	IX	X
Asx	1.1 (1)[a]	2.0 (2)				1.9 (2)	1.8 (2)	1.9 (2)	1.8 (2)	2.0 (2)
Thr	0.1									
Ser	0.1						0.1			0.8 (1)
Glx	0.1					1.0 (1)	1.0 (1)	1.0 (1)	0.9 (1)	1.1 (1)
Pro	2.0 (2)	1.5 (2)				2.0 (2)	1.8 (2)	2.0 (2)	2.1 (2)	2.1 (2)
Gly	1.3 (1)	2.0 (2)	2.0 (2)	2.0 (2)	2.1 (2)	2.3 (2)	2.0 (2)	1.4 (1)	1.2 (1)	3.0 (3)
Ala	0.2			1.0 (1)	0.9 (1)	2.5 (3)	3.2 (3)	2.9 (3)	1.0 (1)	3.3 (3)
Cys[b]						N.D.(1)	N.D.(1)	1.0 (1)	N.D.(1)	N.D.(1)
Val	0.1	1.1 (1)				0.8 (1)	0.9 (1)	1.0 (1)	0.9 (1)	1.0 (1)
Ile	1.0 (1)	1.0 (1)				1.8 (2)	1.8 (2)	2.0 (2)	1.8 (2)	2.8 (3)
Leu	0.2		0.9 (1)	1.3 (1)	1.0 (1)	1.2 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.2 (1)
Lys	0.2									0.1
Trp										
Total residues	(5)	(8)	(3)	(4)	(4)	(15)	(15)	(14)	(12)	(18)
Note[c]							cAH-4	cAH-1	cAH-2	cAH-3

[a] Values are expressed as residues per mol. Numbers in parentheses are residue values based on sequence.

[b] Cysteine was determined as cysteic acid.

N.D., Not determined.

[c] Indicated the notation of the fragments described in Fig. 9 and Table VI.

Table IIIS. Amino acid compositions of fragments isolated from the concentrated HCl hydrolyzates of intact subtilosin A

I-X were derived from the fractions I-X, respectively, indicated by bars in Fig. 3S.

Amino acid	I	II	III	IV
Asx	2.0 (2)[a]	2.3 (2)	2.0 (2)	0.3
Thr	1.0 (1)	1.0 (1)	0.8 (1)	
Ser	0.9 (1)	1.0 (1)	0.8 (1)	
Glx				1.2 (1)
Pro	2.8 (2)	3.2 (2)	2.6 (2)	
Gly	3.0 (3)	3.2 (3)	3.1 (3)	2.1 (2)
Ala	3.0 (3)	3.0 (3)	3.0 (3)	2.1 (2)
Cys[b]	3.4 (3)	3.0 (3)	2.3 (3)	
Val	1.0 (1)	1.1 (1)	0.9 (1)	
Ile	1.9 (2)	1.9 (2)	1.8 (2)	1.0 (1)
Leu	1.0 (1)	1.1 (1)	1.0 (1)	1.1 (1)
Lys				0.1
Trp				
Total residues	(20)	(20)	(20)	(7)
Note[c]			AP-1	

[a] Values are expressed as residues per mol. Numbers in parentheses are residue values based on sequence.

[b] Cysteine was determined as cysteic acid.

[c] Indicated the notation of the fragments described in Fig. 9 and Table VI.

Table IVS. Amino acid compositions of fragments isolated from the digests of performic acid-oxidized subtilosin A with Achromobacter protease I. (I)

I-IV were derived from the fractions I-IV, respectively, indicated by bars in Fig. 4S.

Amino acid	I	II	III	IV	V
Asx		3.0 (3)	3.0 (3)	0.1	3.0 (3)
Thr		0.9 (1)	1.1 (1)		0.8 (1)
Ser		0.9 (1)	1.3 (1)	0.1	0.9 (1)
Glx	1.1 (1)[a]	0.1		1.0 (1)	
Pro		2.2 (2)	3.0 (3)		3.6 (2)
Gly	1.2 (1)	4.0 (4)	4.5 (5)	1.8 (2)	4.7 (5)
Ala	2.0 (2)	3.0 (3)	3.1 (3)	2.1 (2)	2.8 (3)
Cys[b]		3.0 (3)	3.1 (3)		2.7 (3)
Val		1.0 (1)	1.0 (1)		0.9 (1)
Ile	1.0 (1)	1.9 (2)	1.9 (2)	1.0 (1)	1.7 (2)
Leu		1.4 (1)	1.7 (2)	1.0 (1)	2.1 (2)
Lys		0.7 (1)	0.8 (1)		0.7 (1)
Trp		N.D.(1)	N.D.(1)		N.D.(1)
Total residues	(5)	(23)	(26)	(7)	(25)
Note[c]	AP-2				

[a] Values are expressed as residues per mol. Numbers in parentheses are residue values based on sequence.

[b] Cysteine was determined as cystic acid.

[c] Indicated the notation of the fragments described in Fig. 9 and Table VI.

Table VS. Amino acid compositions of fragments isolated from the digests of performic acid-oxidized subtilosin A with Achromobacter protease I. (II)

I-V were derived from the fractions I-V, respectively, indicated by bars in Fig. 5S.

Amino acid	I	II	III	IV	V	VI	VII	VIII	IX
Asx	1.9 (2)[a]	1.7 (2)	1.0 (1)			1.9 (2)	3.0 (3)	0.3	2.6 (3)
Thr		0.1	0.9 (1)				0.9 (1)	0.2	0.8 (1)
Ser		0.2	0.9 (1)			0.1	1.0 (1)	0.2	1.0 (1)
Glx		0.1				1.0 (1)	1.0 (1)	0.2	0.3
Pro	1.9 (2)	1.9 (2)				1.9 (2)	2.1 (2)		1.9 (2)
Gly	1.0 (1)	1.2 (1)	2.8 (3)			2.0 (2)	5.8 (6)	2.4 (2)	4.0 (4)
Ala		0.2	2.9 (3)	1.0 (1)	1.0 (1)	1.9 (2)	5.0 (5)	0.7	3.0 (3)
Cys[b]			2.5 (3)				1.8 (3)	0.2	1.3 (3)
Val	1.0 (1)	1.0 (1)				1.0 (1)	1.1 (1)	0.1	1.0 (1)
Ile	1.0 (1)	1.0 (1)	0.9 (1)			2.0 (2)	3.0 (3)	0.4	2.0 (2)
Leu		0.1	1.0 (1)	1.0 (1)	1.0 (1)		2.0 (2)	2.0 (2)	1.2 (1)
Lys			0.9 (1)				0.9 (1)		0.9 (1)
Trp				0.5 (1)	N.D.(1)		N.D.(1)	1.0 (1)	
Total residues	(7)	(7)	(15)	(3)	(3)	(12)	(30)	(5)	(22)
Note[c]	CH-5		CH-2	CH-4		CH-3	CH-6		CH-7

[a] Values are expressed as residues per mol. Numbers in parentheses are residue values based on sequence.

[b] Cysteine was determined as S-carboxymethyl-cysteine.

N.D., Not determined.

[c] Indicated the notation of the fragments described in Fig. 9 and Table VI.

Table VIS. Amino acid compositions of chymotryptic peptides of RCM-subtilosin A.
I-IX were derived from the fractions I-IX, respectively, indicated by bars in Fig. 6S.

Amino acid	I	II	III	IV	V	VI
Asx		2.0 (2)	1.1 (1)	1.0 (1)	2.0 (2)	2.0 (2)
Thr			1.0 (1)	0.9 (1)		
Ser			1.1 (1)	1.0 (1)		0.1
Glx			0.2		1.0 (1)	1.0 (1)
Pro		2.4 (2)			2.3 (2)	2.2 (2)
Gly	1.0 (1)[a]	1.0 (1)	3.1 (3)	2.0 (2)	2.0 (2)	2.3 (2)
Ala	2.0 (2)		2.7 (3)	1.0 (1)	1.9 (2)	1.7 (2)
Cys[b]	0.9 (1)		2.8 (3)	1.7 (2)		
Val		1.0 (1)			1.0 (1)	1.4 (1)
Ile	1.0 (1)	1.0 (1)	0.9 (1)		2.0 (2)	2.0 (2)
Leu		1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.1 (1)
Lys			1.0 (1)	1.0 (1)		
Trp			N.D.(1)	0.6 (1)		
Total residues	(5)	(8)	(16)	(11)	(13)	(13)
Note[c]	TH-5	TH-6	TH-2	TH-4	TH-3	

[a] Values are expressed as residues per mol. Numbers in parentheses are residue values based on sequence.

[b] Cysteine was determined as S-carboxymethyl-cysteine.

[c] Indicated the notation of the fragments described in Fig. 9 and Table VI.

Table VIIS. Amino acid compositions of thermolysin peptides of RCM-subtilosin A.

I-VI were derived from the fractions I-VI, respectively, indicated by bars in Fig. 7S.

Amino acid	
Asx	
Thr	
Ser	
Glx	
Pro	
Gly	2.2 (2)[a]
Ala	
Cys	
Val	
Ile	
Leu	2.0 (2)
Lys	
Trp	0.8 (1)
Total residues	(5)
Note [b]	CH-1

[a] Values are expressed as residues per mol. Numbers in parentheses are residue values based on sequence.

[b] Indicated the notation of the fragments described in Fig. 9 and Table VI.

Table VIIIS. Amino acid composition of an amino-terminal fragment isolated from the chymotryptic peptides of RCM-subtilosin A.

This fragment was derived from the fraction indicated by a bar in Fig. 8S.

Amino acid	I	II	III	IV	V	VI
Asx						
Thr						
Ser	0.1	0.1				0.3
Glx	0.1	0.2				
Pro						
Gly	1.0 (1)[a]	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)
Ala						
Cys						
Val						
Ile						
Leu						
Lys						
Trp						
Total residues	(1)	(1)	(1)	(1)	(1)	(1)
Note[b]				TH-1		

[a] Values are expressed as residues per mol. Numbers in parentheses are residue values based on sequence.

[b] Indicated the notation of the fragments described in Fig. 9 and Table VI.

Table IXS. Amino acid compositions of the amino-terminal fragments isolated from thermolysin peptides of RCM-subtilosin A.

I-VI were derived from the fractions I-VI, respectively, indicated by bars in Fig. 9S.

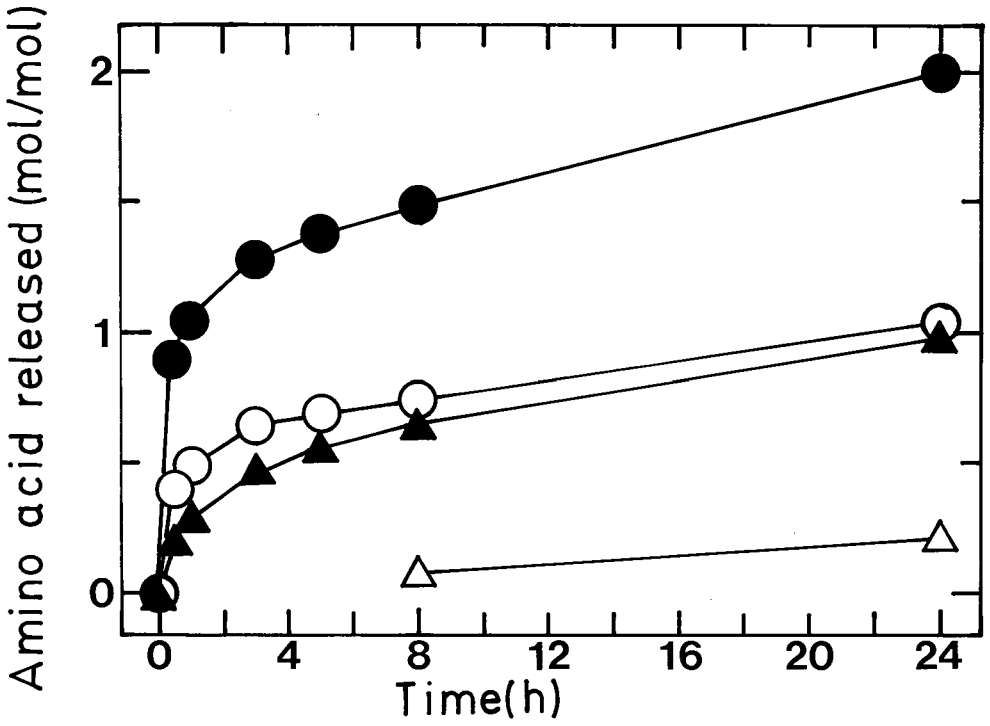

Fig. 15. Digestion of a performic acid-oxidized subtilosin A with carboxypeptidase A-DFP.

Amino acids released are expressed as follows: Open and closed circles, Gly and Ala, respectively; open and closed triangles, Glu and Ile, respectively. Digestion with carboxypeptidase Y instead of carboxypeptidase A gave the following result, amino acids released (mol/mol): In incubation for 5 h, Ala (1.62), Gly (0.83), Ile (0.65), Asp (0.16), Glu (0.07), Leu (0.06); for 18 h, Ala (1.96), Gly (1.17), Ile (1.16), Pro (0.50), Asp (0.48), Leu (0.23), Val (0.21), Glu (0.16). Both digestive conditions were described in "Materials and Methods".

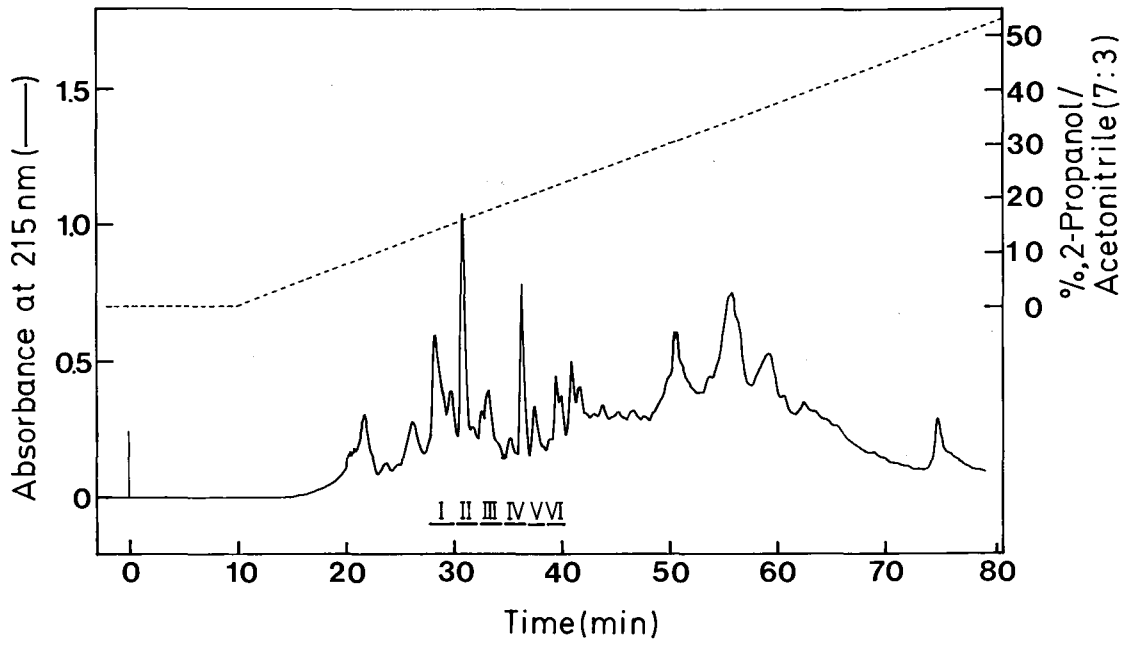

Fig. 2S. Reverse-phase HPLC of dilute acid hydrolyzates of RCM-subtilosin A.

Hydrolysis of the sample with a dilute acid was carried out as described in "Materials and Methods". Half of the hydrolyzates was applied to a reverse-phase column (Yanapak ODS-T). Elution was performed with a 80 ml linear gradient of a 7:3 mixture of 2-propanol and acetonitrile in 0.1% TFA from 0% to 60% at a flow rate of 1.0 ml/min, and monitored by changes of absorbance at 215 nm. Fractions pooled were marked by bars and numbered.

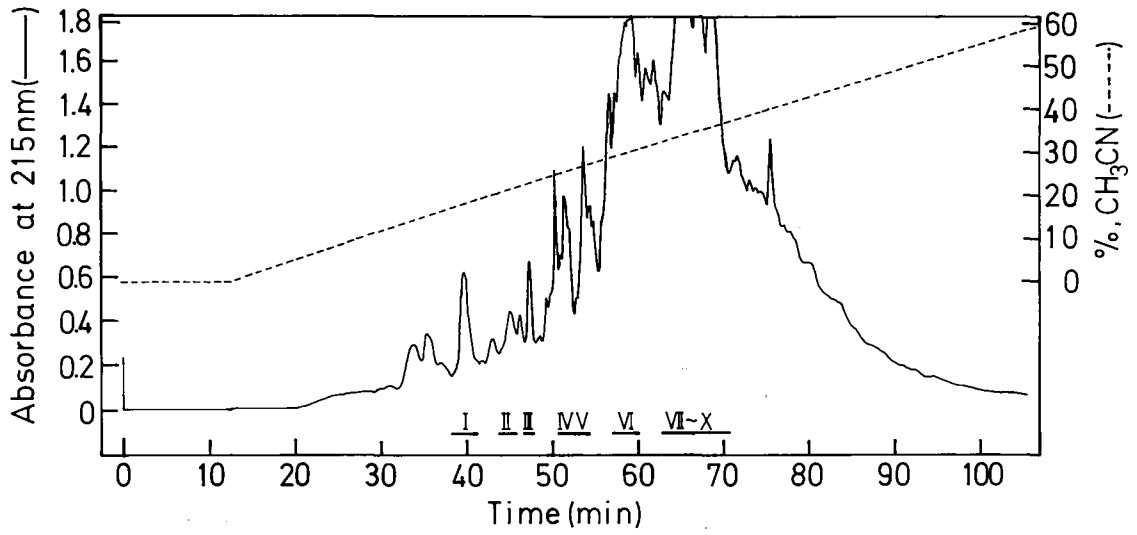

Fig. 3S. Reverse-phase HPLC of concentrated HCl hydrolyzates of intact subtilosin A.

The sample (3 mg) was hydrolysed as described in "Materials and Methods". The hydrolyzates were chromatographed by HPLC on a reverse-phase column (Yanapak ODS-T) with a 80 ml linear gradient of acetonitrile in 0.1% TFA from 0% to 60% at a flow rate of 0.8 ml/min. Eluates were monitored by changes of absorbance at 215 nm. Fractions pooled were marked by bars and numbered.

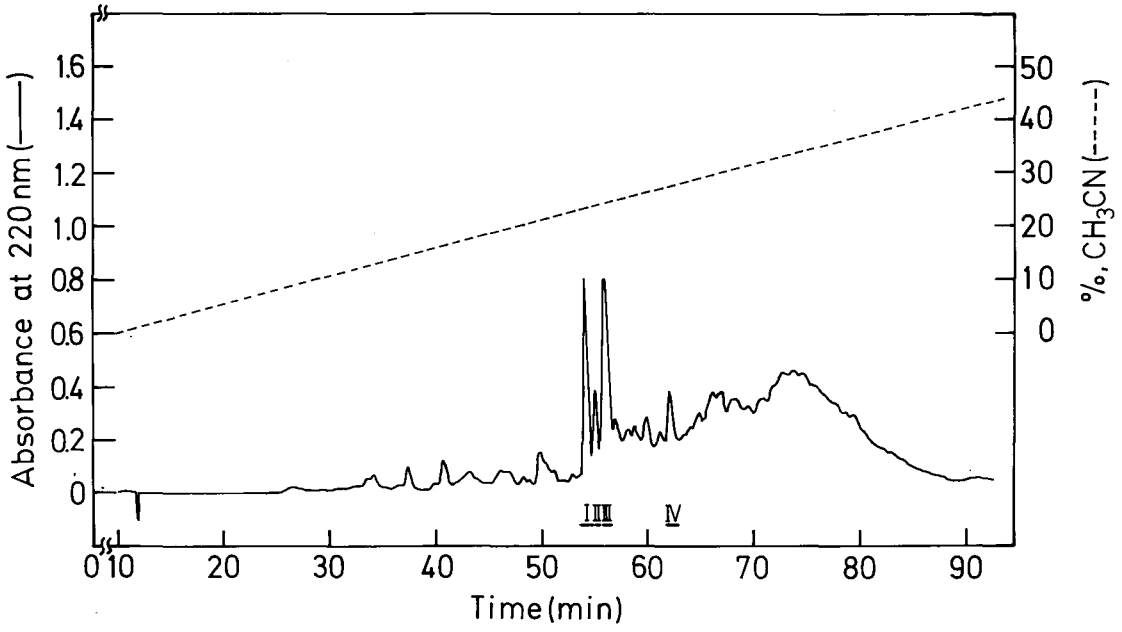

Fig. 4S. Reverse-phase HPLC of digests of performic acid-oxidized subtilosin A with Achromobacter protease I. (I)

The sample (1.4 mg) was digested with 28 µg of the protease in 0.14 ml of 0.1 M NH_4HCO_3 containing 10 mM $CaCl_2$ at 37°C for 2 h. The digests were subjected to reverse-phase HPLC on a Yanapak ODS-T column. Elution was carried out with a 80 ml linear gradient of acetonitrile in 0.1% TFA from 0% to 40% at a flow rate of 1.0 ml/min, and monitored at 220 nm. Fractions pooled were indicated by bars and numbered.

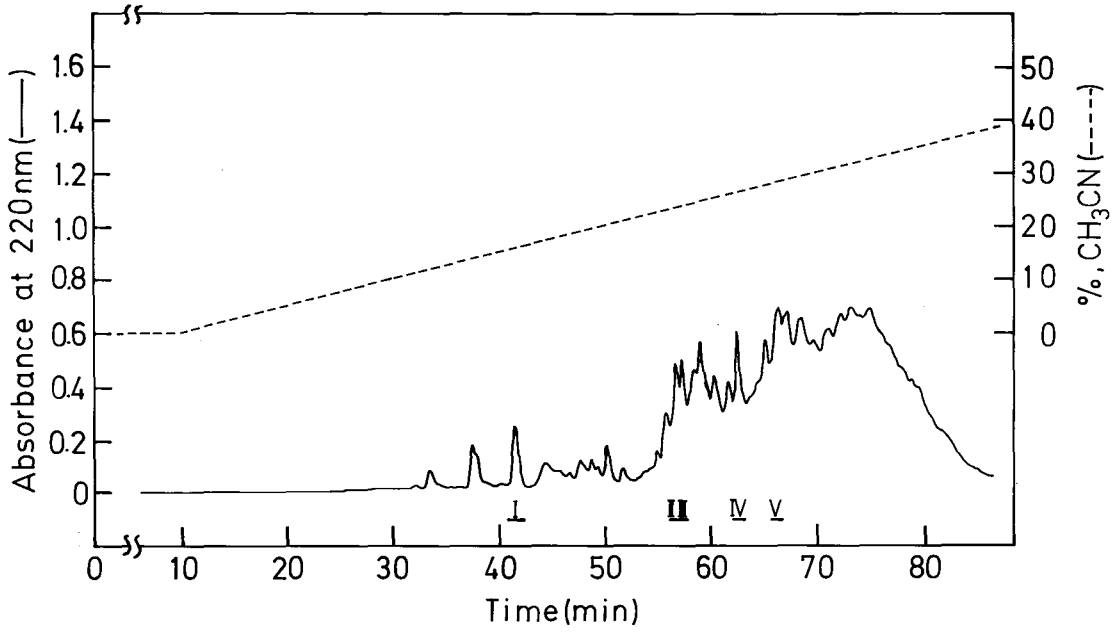

Fig. 5S. Reverse-phase HPLC of digests of performic acid-oxidized subtilosin A with Achromobacter protease I. (II)

All procedure except that the sample was digested with 3.5 μg of Achromobacter protease I at 37°C for 45 min was performed as described in the legend to Fig. 4S. Fractions indicated by bars were pooled and numbered.

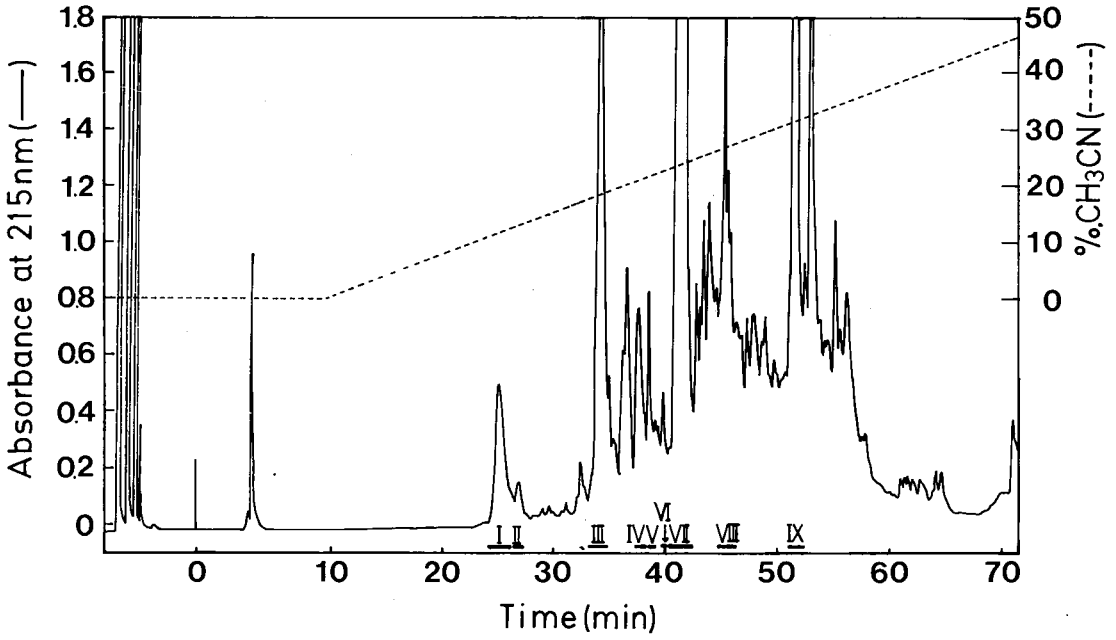

Fig. 6S. Reverse-phase HPLC of chymotryptic peptides of RCM-subtilosin A.

Digestion was carried out as described in "Materials and Methods". The digests were chromatographed on a reverse-phase HPLC column (Chemcopak $_7C_8$, 4.6 x 250 mm, 7 µm, C_8) with a 80 ml linear gradient in 0.1% TFA from 0% to 60% at a flow rate of 1.0 ml/min. Eluates were monitored by changes of absorbance at 215 nm. Fractions pooled were marked by bars and numbered.

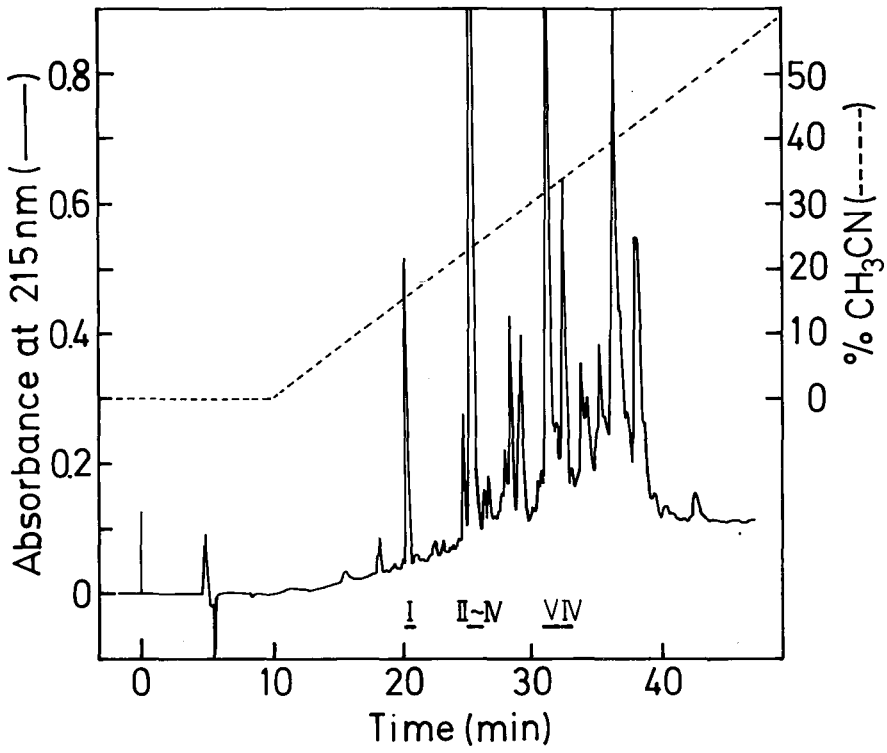

Fig. 7S. Reverse-phase HPLC of thermolysin peptides of RCM-subtilosin A. Digestion was performed as described in "Materials and Methods". Half of the digests was applied to a reverse-phase HPLC column (Chemcopak $_5C_{18}$, 4.6 x 250 mm), and eluted with a 40 ml linear gradient of acetonitrile in 0.1% TFA from 0% to 60%. The eluates were monitored by changes of absorbance at 215 nm. Fractions marked by bars and numbered were pooled.

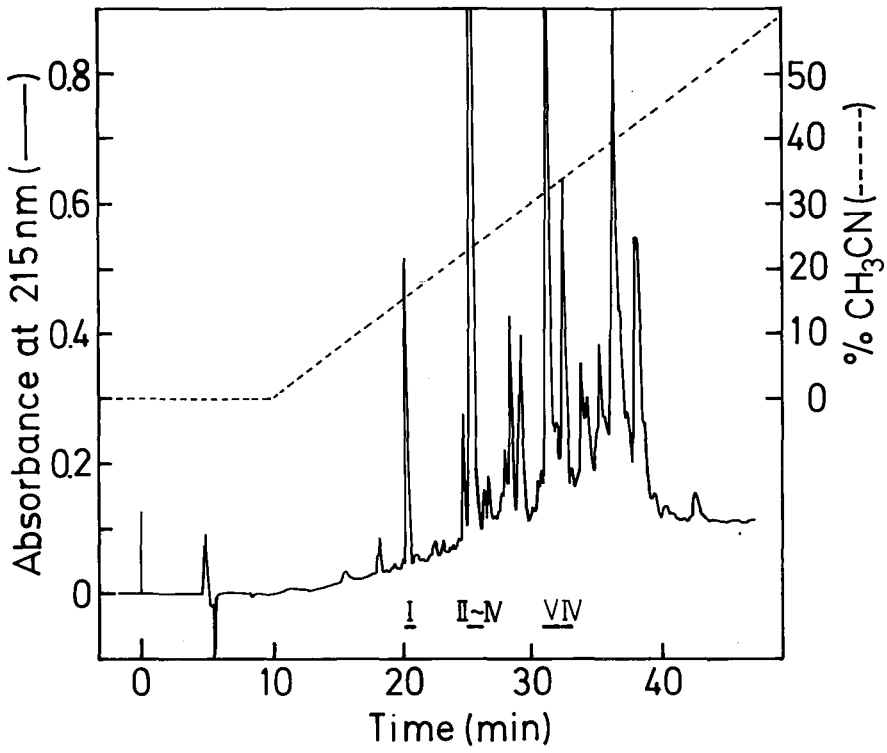

Fig. 7S. Reverse-phase HPLC of thermolysin peptides of RCM-subtilosin A. Digestion was performed as described in "Materials and Methods". Half of the digests was applied to a reverse-phase HPLC column (Chemcopak $_5C_{18}$, 4.6 x 250 mm), and eluted with a 40 ml linear gradient of acetonitrile in 0.1% TFA from 0% to 60%. The eluates were monitored by changes of absorbance at 215 nm. Fractions marked by bars and numbered were pooled.

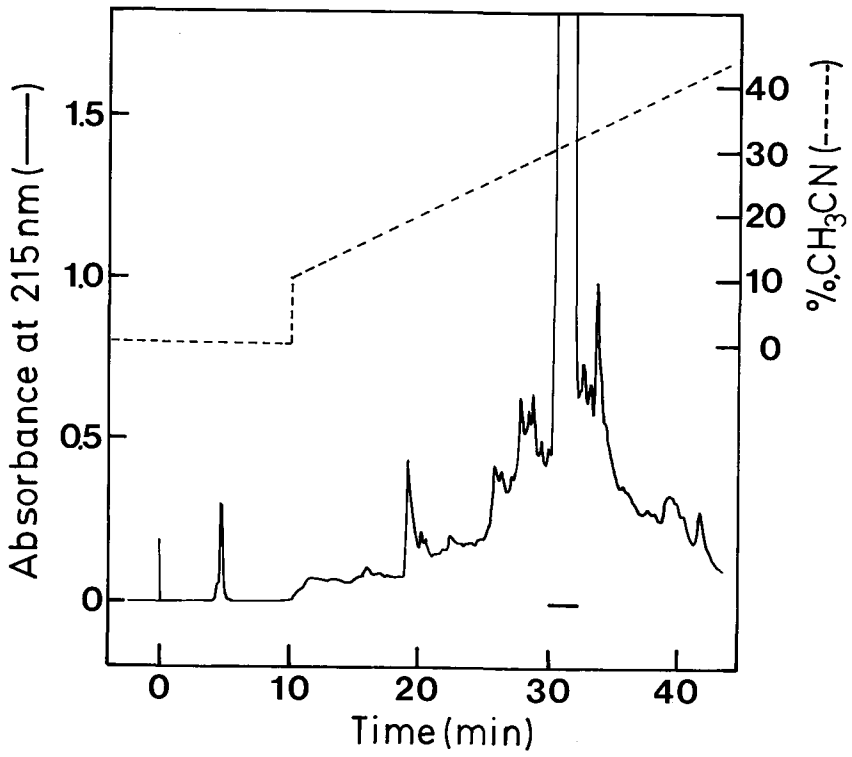

Fig. 8S. Isolation of amino-terminal frgaments from chymotryptic peptides of RCM-subtilosin A by reverse-phase HPLC.

RCM-subtilosin A (1.53 mg) was digested with 75 μg of α-chymotrypsin in 0.2 M N-ethylmorpholine acetate buffer, pH 8.5, at 37°C for 11 h. The digests was applied to a SP-Sephadex C-25 column (12 x 50 mm) equilibrated with 25 mM phosphoric acid, pH 2.0, and then washed with the same solvent thoroughly. The washings was chromatographed by reversed-phase HPLC on a Chemcopak $_5C_{18}$ column with a 40 ml linear gradient of acetonitrile in 0.1% TFA from 10% to 50%. Elution was monitored by absorbance at 215 nm. The fraction indicated by a bar was further purified by another reverse-phase HPLC to obtain the fragment designated as CH-1 (See Fig. 9).

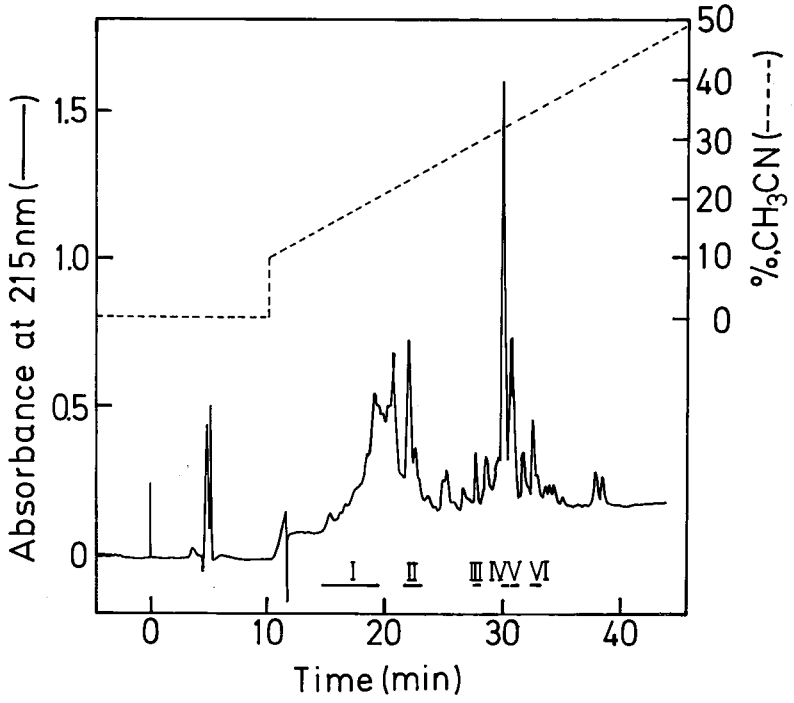

Fig. 9S. Isolation of amino-terminal fragments from thermolysin peptides of RCM-subtilosin A by reverse-phase HPLC.

RCM-sutilosin A (3 mg) was digested with 300 μg of thermolysin in 0.1 M NH_4HCO_3 containing 10 mM $CaCl_2$ at 60°C for 3 h. The digests were subjected to ion-exchange chromatography and reverse-phase HPLC in the same manner as described in the legend to Fig. 8S. Bars indicate the fractions pooled.

Fragment	Amino acid sequence
Table IIS, V:	C-L-V
Table IIIS, I:	G-P-I-P-B
III:	G-L-(W,G)
IV:	G-(A,L,G)
V:	G-(A,L,G)
Table IVS, I:	G-C-A-T-C-S-I-G-A-A-C-L-V-B-G-P-I-P-B
II:	G-C-A-T-C-S-I-G-A-A-C-L-V-B-G-P-I-P-B
IV:	Z-I-A-G-A-(G,L)
Table VS, IV:	Z-I-A-G-A-(G,L)
Table VIS, II:	V-D-G-P-I-P-(B)
V:	G-L-(w)
Table VIIS, VI:	L-(V,B,G,P,I,P,B,Z,I,A,G,A)
Table IXS, I:	X-G
II:	X-G
III:	X-G
V:	X-G
VI:	X-G

Fig. 10S. Amino acid sequences of fragments other than those shown in Fig. 9.

Amino acid sequence was determined by the dansyl-Edman degradation method, and expressed in one-letter notation of amino acids.