Vol. 116, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 31, 1983 Pages 751-758

PEPTIDE ANTIBIOTIC SUBTILIN IS SYNTHESIZED VIA PRECURSOR PROTEINS

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Received September 21, 1983

SUMMARY: Biogenesis of subtilin, an antimicrobial peptide produced by Bacillus subtilis ATCC 6633, was studied in growing cells. Pulse-chase labeling experiments with [^{35}S]cysteine revealed the presence of precursor proteins of subtilin. The synthesis of both precursor proteins and subtilin was inhibited by inhibitors of protein and RNA synthesis. When the precursor proteins were incubated with crude extracts of the organism in vitro, they were converted to subtilin. Pepstatin and phenylmethylsulfonyl fluoride in combination inhibited this conversion.

Subtilin is an antibiotic peptide produced by Bacillus subtilis ATCC 6633 and active against gram-positive bacteria (1). In the 1940's and 1950's, extensive studies of the production, purification and properties of subtilin were made (2), but the molecular structure remained ambiguous. In 1973, Gross et al. (3) elucidated its structure as shown in Fig. 1. It consists of 32 amino acid residues and contains some unusual amino acids, such as dehydroalanine, dehydrobutyrine, lanthionine and methyllanthionine.

In recent years the biosynthetic mechanism of small antibiotic peptides produced by bacteria, such as gramicidin S, tyrocidines, gramicidin A, bacitracins and polymyxin E, has been clarified. They are synthesized by the multienzyme thiotemplate mechanism without involvement of either nucleic acids or ribosomes (4). However, studies of the biosynthetic mechanism of larger antibiotic peptides, like subtilin and nisin which consists of 34 amino acid residues (3) and is produced by Streptococcus lactis (5), are scarce. On the basis of the results that inhibitors of protein synthesis inhibited nisin production by an S. lactis cell suspension, Hurst (6) and

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0006-291X/83 $1.50

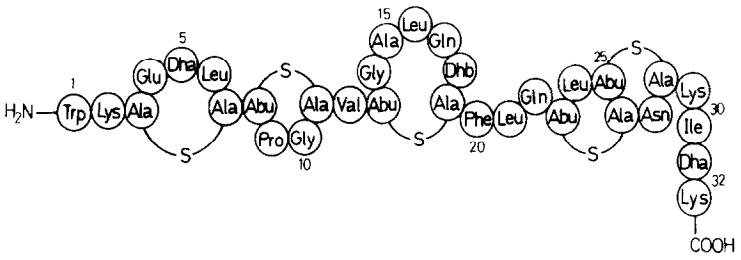

Fig. 1. Structure of subtilin (3). Abu, 2-aminobutyric acid; Dha, dehydroalanine; Dhb, dehydrobutyrine; Ala-S-Ala, lanthionine; Abu-S-Ala, β-methyllanthionine.

Ingram (7) reported that nisin may be synthesized by way of the protein synthetic mechanism. Hurst isolated basic peptides from a mutant strain of S. lactis which did not produce nisin, and converted them to a product, presumably nisin, by incubating the basic peptide preparations with the wild type cell extract (8). However, neither the nature of the precursors nor the mechanism of conversion were clarified.

In this report we describe the detection of precursor proteins of subtilin in vivo and their conversion to subtilin in vitro.

MATERIALS AND METHODS

Chemicals. The following chemicals were obtained commercially: L-[^{35}S]cysteine (1345 Ci/mmol), L-[^{35}S]methionine (1490 Ci/mmol), sodium [^{35}S]sulfate (42.8 mCi/mmol) and ^{14}C-labeled molecular weight marker proteins from the Radiochemical Center Amersham; pepstatin A from Protein Research Foundation; rifampicin and keyhole limpet hemocyanine from Calbiochem; puromycin dihydrochloride and actinomycin D from Makor Chemicals, Ltd; chloramphenicol from Sankyo Co., Ltd; PMSF[3] from Sigma Chemical Company; and Protein A-Sepharose CL-4B from Pharmacia.

Bacterial Strains and Growth Media. Bacillus subtilis ATCC 6633 was maintained on 1.5% agar slants of Medium A containing 100 g sucrose, 11.7 g citric acid, 4.0 g Na_2SO_4, 4.2 g $(NH_4)_2HPO_4$, 5.0 g yeast extract (Difco), 100 ml of a salt mixture (7.62 g KCl, 4.18 g $MgCl_2 \cdot 6H_2O$, 0.543 g $MnCl_2 \cdot 4H_2O$, 0.49 g $FeCl_3 \cdot 6H_2O$, and 0.208 g $ZnCl_2$ in 1000 ml of H_2O), and sufficient NH_4OH to bring the pH to 6.8-6.9 per liter (9). For labeling experiments Medium B was used, in which yeast extract was omitted and Na_2SO_4 reduced to 0.2 g.

Lactobacillus casei IFO 3425 used for bioassay of subtilin was maintained on 1.5% agar slants of Lactobacilli medium (Nissui Pharmaceutical Co., Ltd.) containing 5.5 g yeast extract, 12.5 g peptone, 11.0 g glucose, 0.25 g KH_2PO_4, 0.25 g K_2HPO_4, 10.0 g sodium acetate, 0.1 g $MgSO_4$, 5.0 mg $MnSO_4$, and 5.0 mg $FeSO_4$ per liter (Medium C).

Purification of Subtilin and Preparation of Rabbit Antiserum against Subtilin. B. subtilis ATCC 6633 cells were cultured for 24-48 hr in Medium

[3] Abbreviations: EGTA, ethyleneglycol-bis(β-aminoethylether)N,N'-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

A at 35°C with rotatory shaking. After acidification of the culture to pH 2 with HCl, subtilin was extracted with 1/2 volume of n-butanol, followed by precipitation with 3 volumes of acetone at 4°C. Crude subtilin was further purified by CM-Sephadex C-25 column chromatography and high-performance liquid chromatography as will be described elsewhere. Purified subtilin was conjugated with keyhole limpet hemocyanine (KLH) according to the methods of Reichlin and Schnure (10) and Baron and Baltimore (11). Rabbits were immunized by injecting KLH-subtilin complex in complete Freund's adjuvant weekly for 4 weeks. Antiserum obtained did not precipitate subtilin, but neutralized its antibiotic activity as shown in Fig. 2. The antibody-subtilin complexes were precipitated with Protein A-Sepharose.

Preparation of Radioactive Marker Subtilin. B. subtilis ATCC 6633 cells were cultured in 30 ml of Medium B with 96 μCi sodium [^{35}S]sulfate at 35°C for 22 hr. ^{35}S-Labeled subtilin was extracted with butanol and precipitated with acetone as described above. The dried material was dissolved in a pH 2.5 HCl solution. Subtilin was precipitated with 15% trichloroacetic acid and washed 3 times with 12.5% trichloroacetic acid. The precipitate was dried and washed with ether. This radioactive subtilin gave a single spot by thin-layer chromatography and polyacrylamide gel electrophoresis, and the migration coincided with a sample of subtilin purified by high performance liquid chromatography as described above.

Pulse-chase Labeling in Vivo. B. subtilis ATCC 6633 was grown in Medium B at 35°C with rotatory shaking until the cell density reached 300 to 400 at 660 nm in a Klett-Summerson colorimeter. To a 10-ml culture was added 45 μCi (22 μCi/nmol) of [^{35}S]cysteine/ml of culture and 1-ml samples were removed into 0.18 ml of 100% trichloroacetic acid at intervals. For chase experiments 171 μg of unlabeled cysteine per ml of culture was added after a 6 min pulse and 1-ml samples were removed thereafter as above.

Immunoprecipitation. Trichloroacetic acid precipitated cells were collected by centrifugation and washed with 12.5% trichloroacetic acid 5 times. Precipitates were solubilized by addition of 50 μl of 2% SDS solution and heating at 100°C for 2 min as described by Josefsson and Randall (12). After dilution with 1.1 ml of 10 mM Tris-HCl buffer (pH 7.4) contain-

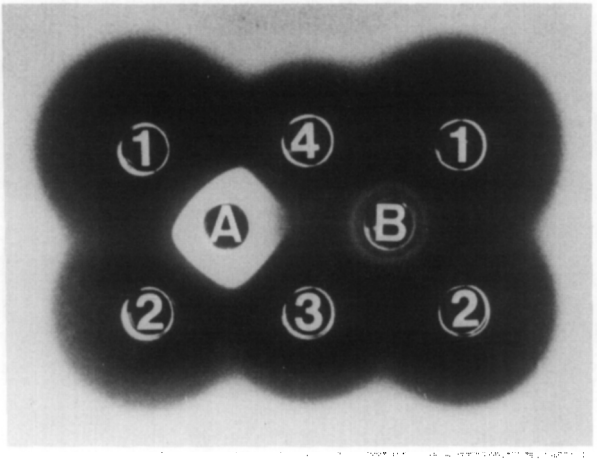

Fig. 2. Neutralization of antibiotic activity by antisubtilin rabbit serum. A plate was prepared by pouring 20 ml of Medium C containing 1% agar, 0.9% NaCl, 0.2% Brij 58 and seeds of L. casei. 7.5 μl each of antisubtilin serum and nonimmune serum were placed in Wells A and B, respectively. 0.4, 0.2, 0.1, and 0.05 μg of subtilin were placed in Wells 1, 2, 3 and 4, respectively. The plate was incubated at 37°C overnight.

ing 0.9% NaCl, 1% Triton X-100, 0.2% Brij 58, 1 mM PMSF and 10 mM EGTA, an aliquot (230 µl)was mixed with 20 µl of antisubtilin serum. The reaction mixture was incubated at 4°C overnight. Upon addition of 250 µl of Protein A-Sepharose (5.5 mg) the reaction was incubated at room temperature for 1 hr. Immune complexes were washed five times with 50 mM Tris-HCl buffer (pH 7.4) containing 0.9% NaCl, 0.2% Brij, 1 mM PMSF and 10 mM EGTA and the precipitated proteins were released into solution by addition of electrophoresis sample buffer (40 µl) of Laemmli(13) and heating at 100°C for 3 min.

SDS-polyacrylamide Gel Electrophoresis and Fluorography. SDS-polyacrylamide gel electrophoresis was carried out on a 1-mm thick slab gel by the method of Laemmli (13). The sample (5-8 µl) was loaded on a linear gradient of 10 to 20% polyacrylamide gel with a 3% stacking gel. After electrophoresis, gels were fixed with 12.5% trichloroacetic acid, 10% acetic acid, and 30% methanol at 4°C for 1 hr and then immersed in Enhance (New England Nuclear) for 1 hr and in cold water for 1 hr. The gels were dried on a sheet of filter paper, and exposed to X-ray film (Kodak X-Omat S) at -70°C for several days.

Preparation of Crude Enzyme for in Vitro Conversion of Subtilin Precursors to Subtilin. B. subtilis cells were grown in 25 ml of Medium A at 35°C until the cell density reached 540 at 660 nm in a Keltt-Summerson colorimeter. Cells were harvested and washed once with 30 ml of 50 mM Tris-HCl buffer, pH 7.0. Cells were suspended in 1.0 ml of the buffer and sonicated for 5 x 1 min in a Branson sonifier. The supernatant solution obtained by 10,000 x g centrifugation for 10 min was used as a converting enzyme source.

RESULTS

A culture of B. subtilis was labeled with [^{35}S]cysteine. At the times indicated in Fig. 3, cells were harvested by pipetting samples into trichloroacetic acid and subtilin solubilized with SDS was analyzed by immunoprecipitation and SDS polyacrylamide gel electrophoresis according to the method of Laemmli (13). Unexpectedly it was revealed that [^{35}S]cysteine was not incorporated into subtilin, but that compounds which migrated slower than subtilin in electrophoresis were labeled. Even after 4 min labeling no radioactive subtilin was formed, indicating that the synthesis of subtilin precursors with higer molecular weights was rapid, but that the processing to form subtilin was rather slow. Because Laemmli's system of gel electrophoresis used here does not give adequate resolution of low molecular weight polypeptides, it is not certain whether the band of precursors represents a single or multiple polypeptides. Lane I shows that no labeled products were precipitated by nonimmune serum.

Figure 4 shows the results of pulse-chase labeling experiments with [^{35}S]cysteine to study the in vivo events involved in subtilin synthesis.

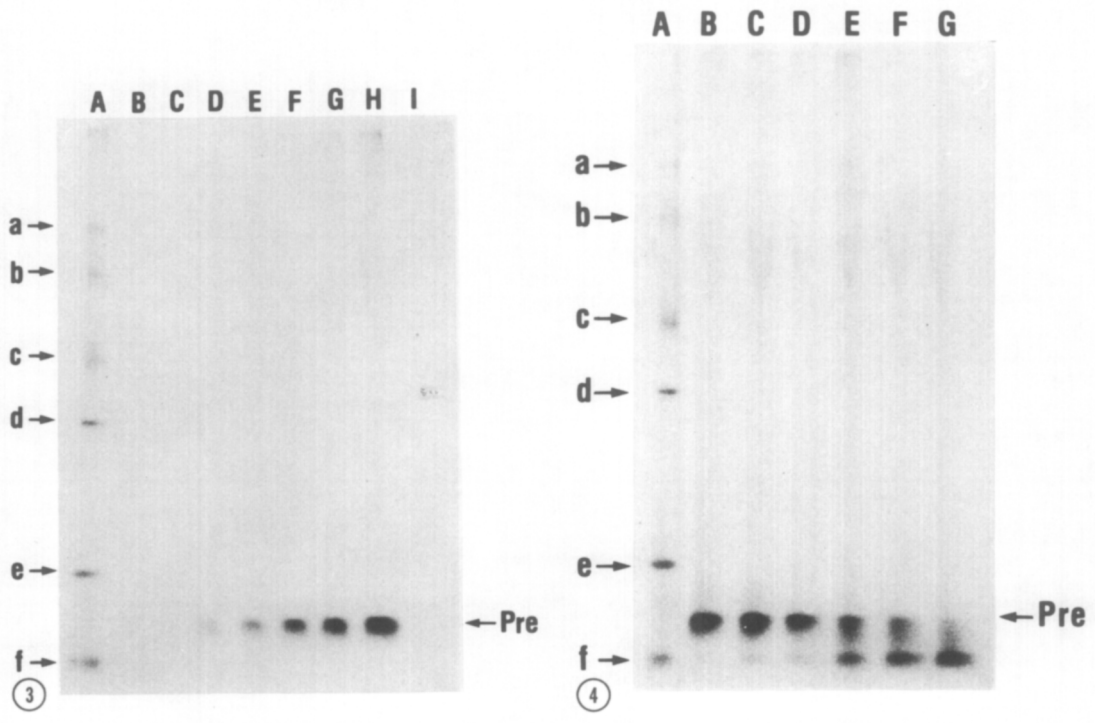

Fig. 3. Time course of incorporation of [^{35}S]cysteine into antisubtilin antibody precipitable materials in vivo. Labeling, immunoprecipitation, SDS polyacrylamide gel electrophoresis and fluorography were performed as described under "Materials and Methods." Lane A, ^{14}C-labeled molecular weight marker proteins (a, phosphorylase (92,500); b, bovine serum albumin (69,000); c, ovalbumin (46,000); d, carbonic anhydrase (30,000); e, lysozyme (14,300)) and ^{35}S-labeled subtilin (f). Lanes B through H represent samples labeled for 20 sec, 40 sec, 1 min, 1.5 min, 2 min, 3 min, and 4 min, respectively. Lane I, SDS extracts of 4 min pulse-labeled cells treated with nonimmune serum. Pre, subtilin precursors.

Fig. 4. Pulse-chase labeling with [^{35}S]cysteine of antisubtilin antibody precipitable materials in vivo. Experimental details are described under "Materials and Methods." Molecular weight marker proteins (Lane A) are the same as in Fig. 3. Lane B, 6 min pulse-labeled sample. Lanes C through G represent samples chased for 2, 4, 9, 14, and 24 min, respectively.

During a 6 min pulse-labeling radioactive precursors accumulated. By chasing with [^{32}S]cysteine, the radioactivity in the precursors decreased with concomitant appearance of the radioactivity in a compound which migrated similarly to a standard sample of labeled subtilin. During a 24 min chase nearly all the precursors were converted to subtilin. This conversion of the precursors to subtilin was confirmed by in vitro experiments. When the precursor proteins isolated by immunoprecipitation were incubated with crude extracts of B. subtilis cells, most of the precursors were converted

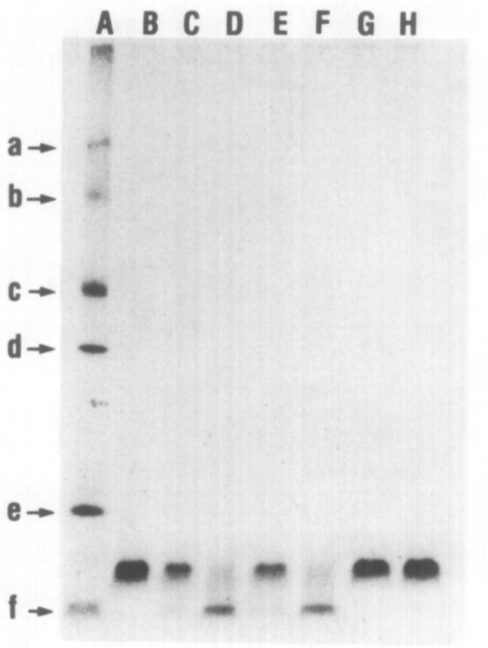

Fig. 5. Conversion of subtilin precursors to subtilin in vivo. B. subtilis cells were labeled with [^{35}S]cysteine for 4 min and SDS solubilized extracts were immunoprecipitated as described in "Materials and Methods." Radioactive immunocomplexes precipitated with protein A-Sepharose were extracted with 1 N acetic acid. The solution was lyophilized and dissolved in 100 µl of 50 mM Tris-HCl buffer, pH 7.0. The reaction mixture consisted of 12 µl of precursor proteins (22,000 cpm), 6 µl of 50 mM Tris-HCl buffer, pH 7.0, 12 µl of crude extracts of B. subtilis cells prepared as described under "Materials and Methods" and inhibitors as indicated below. Incubation was 1 and 3 hr at 37°C. 8 µl of each sample was analyzed by SDS polyacrylamide gel electrophoresis. Lane A, marker proteins as in Fig. 1; Lane B, no incubation without inhibitors; Lanes C and D, incubated without inhibitors for 1 and 3 hr, respectively; Lanes E and F, incubated with 10 mM EGTA for 1 and 3 hr, respectively; Lanes G and H, incubated with 10 mM EGTA, 2 mM pepstatin and 5 mM PMSF for 1 and 3 hr, respectively.

to subtilin in 3 hr as shown in Fig. 5. The product was also identified by thin-layer chromatography (data not shown). A mixture of pepstatin and PMSF inhibited the conversion, suggesting the participation of proteinases in the conversion. EGTA had no effect on the conversion. EGTA was shown to prevent the loss of antibiotic activity of crude subtilin on standing at room temperature (data not shown).

Figure 6 shows that inhibitors of protein and RNA synthesis inhibit the incorporation of [^{35}S]cysteine into antisubtilin serum immunoprecipitates of cell extracts. The synthesis of both subtilin and its precursors was inhibited completely by chloramphenicol and actinomycin D, but the

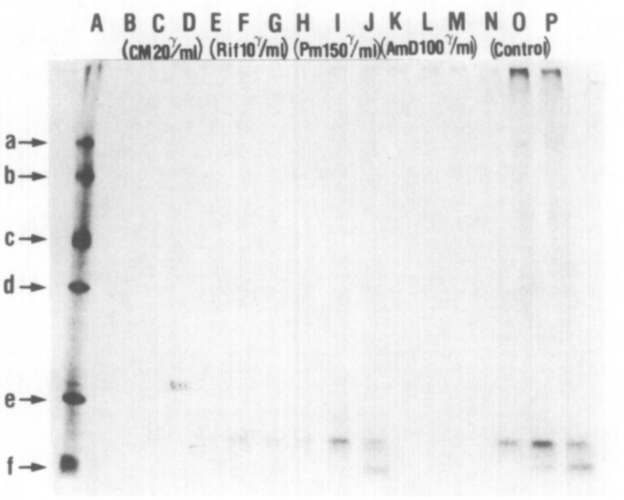

Fig. 6. Effect of inhibitors of protein and RNA synthesis on subtilin synthesis in vivo. 3.5-ml cultures of B. subtilis cells were preincubated at 35°C for 5 min with inhibitors as indicated. 62 μCi [^{35}S]cysteine (2.3 μCi/nmol) was added. Samples removed at intervals were precipitated with trichloroacetic acid and antisubtilin antibody precipitable materials were analyzed as described under "Materials and Methods". Lane A, molecular weight marker proteins as indicated in Fig. 1. Incubation times were 5 min for Lanes B, E, H, K and N; 15 min for Lanes C, F, I, L, and O; and 30 min for Lanes D, G, J, M, and P. CM, chloramphenicol; Rif, rifampicin; Pm, puromycin dihydrochloride; AmD, actinomycin D.

inhibition by puromycin was not pronounced. It was found that B. subtilis cells used were rather insensitive to this inhibitor. Erythromycin also inhibited subtilin and precursor synthesis completely at the level of 2 μg/ml culture.

DISCUSSION

Subtilin and nisin contain many residues of unusual amino acids, such as lanthionine, methyllanthionine, dehydroalanine and dehydrobutyrine. It may suggest that they are synthesized nonribosomally by the multienzyme thiotemplate mechanism like gramicidin S and tyrocidines (4). However, on the contrary the present work demonstrated that subtilin is made in forms of larger precursor proteins. Inhibitors of protein and RNA synthesis inhibited the synthesis of both subtilin and its precursors. Furthermore, [^{35}S]methionine which is not a constituent amino acid of subtilin was incorporated into the precursor proteins and during processing the methionine-containing fragments were removed (data not shown). These results

indicate that the synthesis of the precursors are carried out by ribosomes and RNA. Since SDS polyacrylamide gel electrophoresis with the buffer system of Laemmli (13) does not provide adequate separation of low molecular weight proteins, we cannot determine the number of precursor polypeptides during maturation of subtilin from the results presented, but Sephadex G-50 gel filtration of the immunoprecipitates with antiserum directed against subtilin revealed that at least three radioactive peptides with 8,000, 5,000, and 4,000 apparent molecular weight were present in the cell extracts (data not shown). Since subtilin contains several modified amino acid residues as seen in Fig. 1, the processing and modification of subtilin precursors must involve several steps. We are trying to isolate each intermediate peptide and identify its structure to understand the detailed mechanism of subtilin formation.

ACKNOWLEDGMENTS

This study was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan. We are indebted to Drs. M. Yamada, Y. Kuriki and Mr. K. Babasaki for helpful advice. We acknowledge the receipt of a subtilin sample purified by counter-current distribution from Dr. K. Folkers of the University of Texas at Austin.

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