Cell-Free Synthesis of Gramicidin S*

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ABSTRACT: Biosynthesis of gramicidin S is studied in a cell-free system from *Bacillus brevis* Nagano strain. The ammonium sulfate precipitate of the high-speed supernatant fraction of sonic extract catalyzes *de novo* synthesis of gramicidin S. The reaction requires adenosine triphosphate (ATP), $MgCl_2$, reducing agent, and component amino acids. Gel filtration of enzyme preparation provides evidence that two complementary fractions (fractions I and II) are involved in gramicidin S synthesis. Both of the fractions are also required for the synthesis of intermediary peptides as well as gramicidin S, while fraction II alone is able to form amide-like substance of D-phenylalanine when incubated in the presence of NH_4^+. The results suggest that fraction II possibly supplies activated D-phenylalanine which is later incorporated into peptides.

Recently, an increasing number of papers have become available which dealt with the biosyntheses of the antibiotic peptides, such as gramicidins and tyrocidines. Much of interest has been concerned to know whether these peptides could be synthesized along ribosomal templates or they might be synthesized by stepwise addition of amino acids. Evidence has been accumulating which showed that the biosyntheses of these peptides were catalyzed only with the soluble fractions obtained from *Bacillus brevis* strains and were not affected by the inhibitors of protein synthesis (Mach *et al.*, 1963; March, 1963; Yukioka *et al.*, 1965; Berg *et al.*, 1965; Fujikawa *et al.*, 1966). The concept in support of the ribosomal pathway for the synthesis of these peptides which had been contradictory to the above findings for past few years (Uemura *et al.*, 1963; Okuda *et al.*, 1964; Bodley *et al.*, 1964; Uemura *et al.*, 1965; Hall *et al.*, 1965) has recently been retracted by Bhagavan *et al.* (1966) of the same group, reporting that they have obtained a soluble enzyme system for the synthesis of gramicidin S different from their previous one.

We have also been engaged in the study of the biosynthesis of gramicidin S in a cell-free system from *B. brevis* Nagano strain and have obtained evidence that there is difference between the mechanism of gramicidin S biosynthesis and protein biosynthesis. In hope of the elucidation of further mechanism we have performed the characterization and purification of the enzyme system responsible for gramicidin S biosynthesis. In this communication we report that (1) gramicidin S synthesizing system is resolved into two complementary fractions by molecular sieving; (2) intermediary peptides are not accumulated when each fraction is individually incubated with adenosine triphosphate (ATP)[1] and the component amino acids; and (3) D-phenylalanine as well as L-phenylalanine serves as the precursor of D-phenylalanine residue of gramicidin S although L form is preferentially incorporated into gramicidin S when both forms of phenylalanine are simultaneously present.

Materials

Organism. B. brevis Nagano strain was supplied by Dr. S. Otani of Osaka City University Medical School.

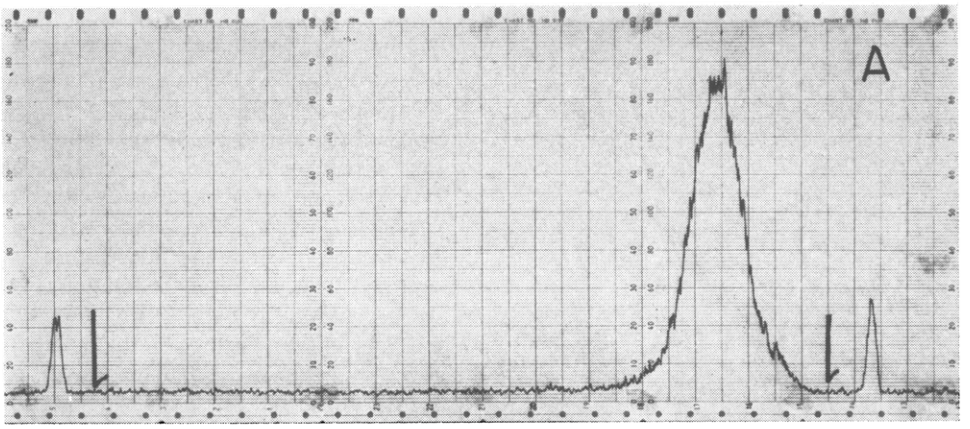
Medium. Nutrient broth was used throughout the experiments which contained 10 g of polypeptone (Kyokuto), 10 g of meat extract (Daigo Eiyo), 2.5 g of NaCl, and 1.5 ml of 20% solution of NaOH/l. of tap water. Nutrient agar contained 1% of agar in nutrient broth.

Enzymes. D-Amino acid oxidase was prepared from hog kidney following the method of Kubo *et al.* (1960). Snake venom (L-amino acid oxidase) was a gift of Dr. T. Suzuki of this institute. Creatine kinase was prepared from rabbit muscle according to the method of Noda *et al.* (1955).

Chemicals and Isotopes. Amino acids were purchased from Mann Research Laboratories Inc., New York, N. Y. ATP and phosphocreatine were obtained from Sigma Chemical Co., St. Louis. β-Mercaptoethanol was a product of California Corp. for Biochemical Research, Los Angeles. Streptomycin sulfate was kindly provided from Mr. T. Yano of Banyu Pharmaceutical Co., Okazaki. L-[U-^{14}C]Phenylalanine (sp act. 300 mc/mmole) was obtained from Daiichi Pure Chemicals Co., Tokyo, and purified by treatment with D-amino acid oxidase and subsequent chromatography on Dowex 50 (H^+). The final solution of L-[U-^{14}C]phenylalanine was made up to contain 50 μc/5 μmoles per ml (6770 cpm/mμmole) by the addition of an appropriate

* From the Institute for Protein Research, Osaka University, Osaka, Japan. *Received February 20, 1967.* This work was supported in part by a U. S. Public Health Service Grant AM-04600 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md.

[1] Abbreviation used: ATP, adenosine 5′-triphosphate.



Paper Chromatogram

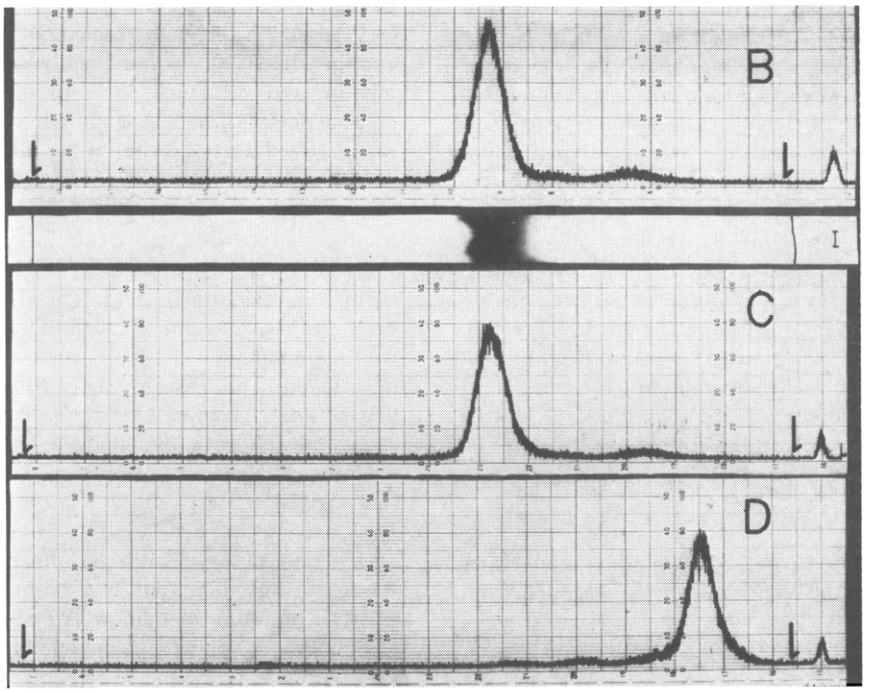


FIGURE 1: Radio paper chromatograms of the reaction product and its hydrolysates. Experimental conditions for the reaction were the same as described in Experimental Procedures except that the incubation mixture of 1ml containing 10 mg of enzyme protein was adopted. After incubation for 1 hr, the mixture was applied on to Dowex 50 (H^+) column (1 × 4 cm) and the column was washed successively with 25 ml of H_2O and 25 ml of 5 N NH_4OH. Subsequently, gramicidin S was eluted with 25 ml of 7.5 N NH_4OH in 50% methanol. Samples were applied onto Toyo No. 51 filter paper (2.5 × 60 cm) and developed descendingly with a solvent system of 1-butanol–acetic acid–water (4:1:1, v/v). After drying the chromatograms, radioactivities were located by scanning the chromatograms by an Aloka paper chromatogram scanner Model PCS-4. Gramicidin S was visualized by dipping the chromatogram into 1% solution of bromophenol blue and then washing out the unreacted dye. Phenylalanine was detected by a ninhydrin method. (A) Eluate from Dowex 50 (H^+) column with ammonia methanol plus 100 μg of gramicidin S; (B) hydrolysate of A; (C) hydrolysate, treated with L-amino acid oxidase; and (D) hydrolysate, treated with D-amino acid oxidase. Arrows in the figure represent origin and solvent front of chromatogram (left to right), respectively.

amount of cold L-phenylalanine. L-[3-^{14}C]Phenylalanine and D-[3-^{14}C]phenylalanine were prepared from DL-[3-^{14}C]phenylalanine (a product of Section Molécules Marquées Fabriqué par CEA-France, sp act. 10 mc/mmole) by treatment with D- and L-amino acid oxidase, respectively. Each isomer of [3-^{14}C]phenylalanine was purified by column chromatography on Dowex 50 (H^+) and the final solution was made up to contain 25

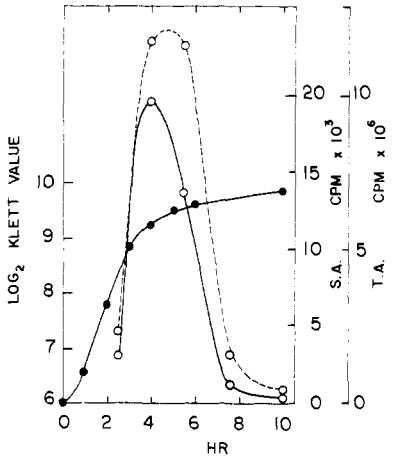


FIGURE 2: Relationship between enzyme activity and growth of bacteria. An aliquot of overnight culture was inoculated to a fresh medium and cells were grown by shaking at 37°. At various time intervals as indicated, cells were harvested by centrifugation and the cell-free extract was prepared as described in Experimental Procedures. Cell-free extract was centrifuged at 105,000*g* for 90 min and a 25-μl aliquot of the supernatant was determined for gramicidin S synthesis. Growth of cells was followed by measuring the turbidity of culture with a Klett-Summerson photometer with a no. 66 filter (●——●). Specific activity (S. A.) represents cpm of phenylalanine incorporated into gramicidin S/30 min per mg of 105,000*g* supernatant (O——O). Total activity (T. A.) represents the activities in cell-free extracts obtained from 1 l. of culture (O---O).

μc/2.5 μmoles per ml (sp act. 8400 cpm/mμmole). Other chemicals used in this experiment were obtained from commercial sources.

Experimental Procedures

Cultivation of Organism. An overnight nutrient slant culture of *B. brevis* Nagano strain was inoculated to 500 ml of nutrient broth in 2-l. erlenmeyer flask and grown overnight in a gyratory incubator shaker (New Brunswick) at 37°. Portions (40 ml) of overnight culture were transferred to twelve 5-l. erlenmeyer flasks, each containing 1 l. of fresh medium and shaken in a reciprocating shaker at 37° for 4 hr. Cells were harvested by a Sharples centrifuge and washed once with 0.01 M triethanolamine-HCl buffer (pH 7.4) containing 0.01 M $MgCl_2$ (buffer A). Usually, 50–60 g wet wt of cells was obtained from 12 l. of medium. Packed cells were kept frozen at −20° until use.

Preparation of Cell-Free Extract. All preparations were carried out in the cold. Frozen cells were thawed and suspended in four volumes of buffer A, and 50-ml portions of cell suspension were treated with a Raytheon 10-kcycle sonic oscillator at maximum power set for 4 min. Unbroken cells and cell debris were removed by centrifugation at 18,000*g* for 20 min.

Streptomycin Precipitation. Cell-free extract was centrifuged at 78,000*g* for 2 hr and the precipitate was discarded. To the supernatant fraction was added 10% streptomycin sulfate in buffer A to make the final concentration of streptomycin sulfate 1%. The mixture was stirred for 15 min and centrifuged at 18,000*g* for 15 min, and the precipitate was discarded.

Ammonium Sulfate Precipitation. To the streptomycin supernatant fraction was added an equal volume of saturated ammonium sulfate solution (pH 7.8) containing 10^{-3} M EDTA with stirring over a period of 20 min. Stirring was continued for another 20 min followed by centrifugation at 18,000*g* for 20 min. The precipitate was dissolved in a minimum volume of 0.02 M triethanolamine-HCl buffer (pH 7.4) containing 0.01 M $MgCl_2$ (buffer B). Ammonium sulfate was removed by passing the solution through a column of Sephadex G-50 (coarse) which had been equilibrated with buffer B and the effluent was frozen quickly in a Dry-Ice–acetone mixture and kept at −20°. For the chromatography on Sephadex G-200, the ammonium sulfate precipitate was dialyzed against buffer B for 2 hr.

Assay of Gramicidin S Synthesis. Incubation mixture contained the followings in a total volume of 0.11 ml: triethanolamine-HCl buffer (pH 7.4), 5 μmoles; $MgCl_2$, 1 μmole; ATP, 500 mμmoles; L-[U-^{14}C]phenylalanine (sp act. 10 μc/μmole), 5 mμmoles; 20 mμmoles each of L-proline, L-valine, L-ornithine, and L-leucine; β-mercaptoethanol, 1 μmole; and enzyme solution to be assayed. The mixture was incubated at 37° for a defined time. At the end of incubation, a 0.1-ml aliquot was removed from the mixture and applied onto a column of Dowex 50 (H^+, 1 × 1 cm). The column was washed successively with 5 ml of H_2O and three 5-ml portions of 5 N NH_4OH to elute amino acids and peptides. Gramicidin S, remaining adsorbed to the resin, was eluted with two 2.5-ml portions of 7.5 N NH_4OH in 50% methanol. An aliquot (0.5 ml) of the eluate was plated on an aluminum planchet and dried. Radioactivity on the planchet was measured in a Nuclear-Chicago gas-flow counter fitted with a micromil window.

Identification of Product. For the identification of the reaction product, the scale of the incubation mixture was increased tenfold (1 ml) and ammonia–methanol-elutable fraction was examined by paper chromatography. A typical chromatogram is shown in Figure 1A. The R_F value of the radioactivity is the same as that of an authentic sample of gramicidin S. In order to verify further, acid hydrolysis of the product was carried out. Fraction eluted with ammonia–methanol was evaporated to dryness and taken up with 2 ml of a 1:1 mixture of concentrated HCl and glacial acetic acid. Hydrolysis was performed with 100 μg of authentic unlabeled gramicidin S in a sealed tube for 18 hr at 110°. After evaporation, the residue was dissolved in 0.3 ml of water and divided into three portions. To tube 1 was added 20 μl of water. To tubes 2 and 3 was added 20 μl each of L-amino acid oxidase (40 mg/ml) and D-amino acid oxidase (10 mg/ml), respectively. The tubes were incubated at 37° for 30

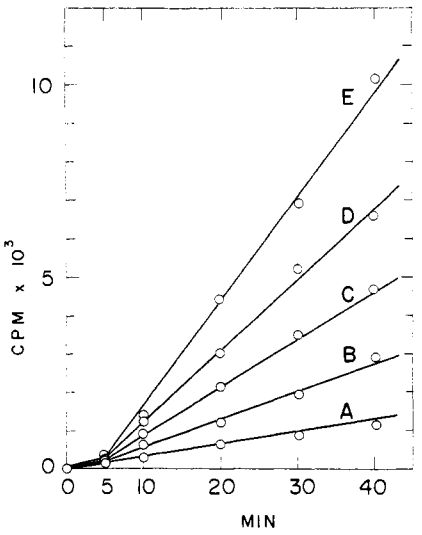


FIGURE 3: Rate of gramicidin S synthesis with various amount of enzyme protein. The experimental conditions were the same as those given in the Experimental Procedures, except that the volume of incubation mixture was 1.0 ml. At the various time intervals as indicated, 0.1-ml aliquots were withdrawn and gramicidin S synthesized was determined. The amounts of enzyme per milliliter of reaction mixture were 0.5 (A), 1.0 (B), 1.5 (C), 2.0 (D), and 2.5 mg (E).

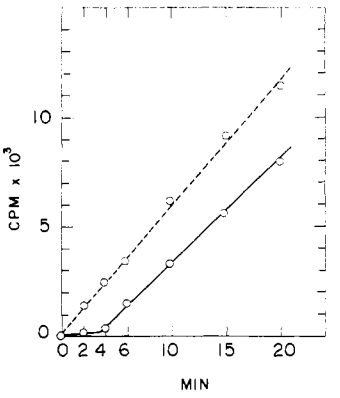


FIGURE 4: The effect of preincubation of enzyme with ATP on the rate of gramicidin S synthesis. Each 4 mg of enzyme protein was incubated for 5 min with water (O——O) or 2.5 μmoles of ATP (O----O) in 0.5 ml. At time 0, each mixture was made 1 ml by the addition of the remainders of reaction components, preequilibrated at 37° (another 2.5 μmoles of ATP was supplied to the fraction preincubated with ATP to make final concentration of ATP 5 μmoles/ml for each fraction). Time course of gramicidin S synthesis was followed as described under Figure 3.

min and the content of each tube was developed by paper chromatography. As shown in Figure 1B, only phenylalanine was recovered as a radioactive compound from tube 1. Phenylalanine in the hydrolysate was oxidized only with D-amino acid oxidase to produce phenylacetic acid (Figure 1C,D) indicating that the optical configuration of phenylalanine residue of the reaction product was D.

Assay of Protein. Protein was determined by Biuret method (Layne, 1957).

Results

Growth of B. brevis Nagano and the Gramicidin S Forming Activity. For the study of gramicidin S biosynthesis it was desired to obtain a cell-free extract with the highest activity. It has been reported by several workers that gramicidin S began to be produced in the cells at the late logarithmic phase of growth (Akiyama *et al.*, 1960; Okuda *et al.*, 1963; Eikhom *et al.*, 1963). However, these experiments were performed by determining the amount of gramicidin S in the cells of various growth phase and it was not clear when the activity of the gramicidin S synthesis was the highest. Thus, an experiment to determine the relationship between the enzyme activity of gramicidin S synthesis and the growth phase of the organism was undertaken. An aliquot of overnight culture was inoculated to a fresh medium and the growth of the cells was followed by determining the optical density. Cells were harvested at various time intervals and extracts for the assay of gramicidin S were prepared. In Figure 2, it is shown that the specific as well as total gramicidin S forming activity was low at the middle logarithmic phase of growth but it increased abruptly and reached the maximum at the late logarithmic phase of growth (4 hr after inoculation). The activity, then fell down within a few hours to a level of the middle logarithmic phase. We interpret these results to mean that the gramicidin S forming system is synthesized in the cells at the late logarithmic phase, then the enzyme(s) responsible for the synthesis of gramicidin S suffers degradation or inactivation. Hereafter, the cells harvested at the 4th hr of the cultivation which had the highest activity for gramicidin S formation were utilized for the preparation of cell-free extract.

Characteristics of Cell-Free Synthesis of Gramicidin S. Figure 3 shows the time course of gramicidin S synthesis with various amounts of enzyme preparation. The ammonium sulfate precipitate of high-speed supernatant fraction actively incorporated L-phenylalanine into gramicidin S. As is seen in the figure, the time course of gramicidin S synthesis proceeded linearly after short lag period. Under the experimental conditions the rate of gramicidin S synthesis was fairly proportional to the amount of enzyme preparation added. The lag period had originally been considered to be time required for the elongation of peptide chain leading to gramicidin S formation. However, this lag period was not observed when the enzyme preparation was preincubated with ATP for 5 min (Figure 4), while, preincubation of the enzyme with other reaction components, such as amino acids or β-mercapto-

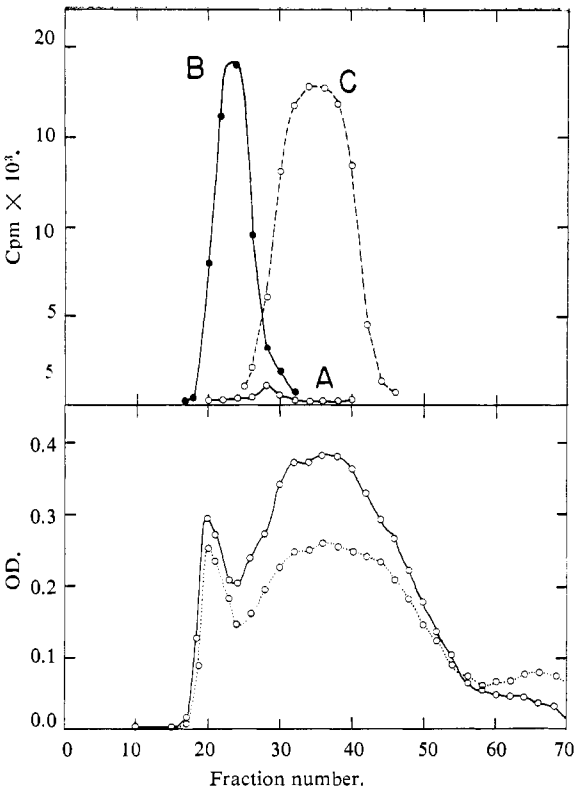


FIGURE 5: Chromatography of the ammonium sulfate fraction on Sephadex G-200. The dialyzed ammonium sulfate fraction (30 ml) (protein concentration 55 mg/ml) was applied to a column (5 × 50 cm) and eluted with buffer B as described in the text. (upper half) Gramicidin S synthesis with 25 μl of each fraction assayed individually (O——O) and in combination with 50 μl of fraction 34 (●——●) and 50 μl of fraction 22 (O----O). Incubation time was 30 min. (lower half) Protein distribution in elutate fractions. Each fraction was diluted tenfold with water and optical densities of 280 (O——O) and 260 mμ (O---O) were measured.

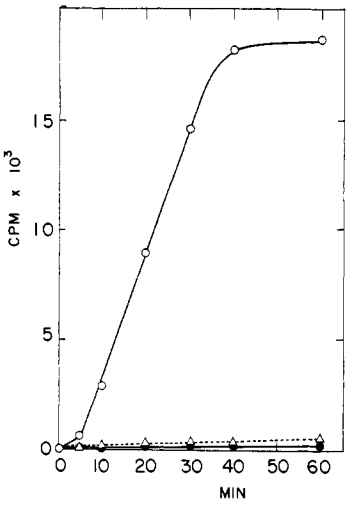


FIGURE 6: Rate of gramicidin S synthesis by the resolved fractions. The experimental conditions were the same as those given in the legend of Figure 3. The amounts and sources of enzyme per milliliter of the reaction mixture were 1.5 mg of fraction I (△----△), 1 mg of fraction II (●——●), and 1.5 mg of fraction I plus 1 mg of fraction II (O——O).

ethanol, showed no effect upon the lag period. This effect of ATP may be explained that activation of enzyme(s) by ATP is required before the formation of gramicidin S begins.

The incorporation of L-phenylalanine into gramicidin S was dependent on ATP, $MgCl_2$, amino acids (proline, valine, ornithine, and leucine), and β-mercaptoethanol as shown in Table I. When Sephadex-treated extract was used as an enzyme source, the omission of one amino acid caused a marked reduction in the incorporation of L-phenylalanine into gramicidin S, indicating that the *de novo* synthesis of gramicidin S occurred during the incubation. Addition of ATP-generating system (phosphocreatine and creatine kinase) slightly stimulated the reaction. β-Mercaptoethanol could be replaced by other reducing agents, such as cysteine, glutathione, or dithiothreitol (Cleland's reagent).

The formation of gramicidin S was not affected by the inhibitors of protein synthesis, such as puromycin,

TABLE I: Requirements for the Synthesis of Gramicidin S.[a]

Conditions	[^{14}C]Phenylalanine Incorp into Gramicidin S (cpm)
Omissions	
None (complete)	14,200
$MgCl_2$	2,550
ATP	780
β-Mercaptoethanol	1,080
Proline	920
Valine	3,350
Ornithine	800
Leucine	1,360
Additions	
KCl (0.1 M)	13,500
Phosphocreatine (5 mM) and creatine kinase (20 μg)	14,500

[a] Experimental conditions were as described under Experimental Procedures except for the omissions and the additions listed in the table. The amount of enzyme/0.11 ml of reaction mixture was 0.4 mg of ammonium sulfate fraction which had been eluted from Sephadex G-50 column with buffer B without containing $MgCl_2$. Incubation time was 30 min.

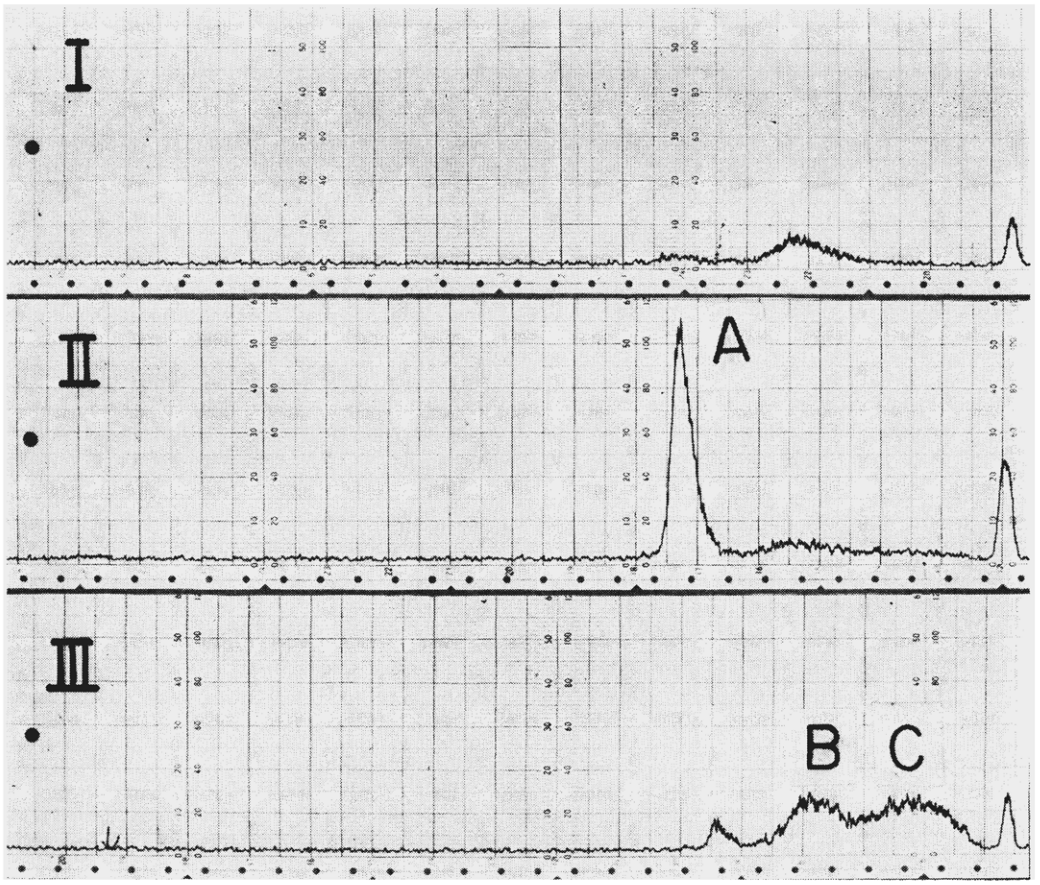


FIGURE 7: Radio paper chromatograms of the reaction products with complementary fractions. Incubation mixtures of 1 ml were adopted. After incubation for 1 hr at 37°, proteins were precipitated by 80% methanol and methanol was evaporated. The residue was applied on Dowex 50 (H^+, 1 × 4 cm) column and washed with 25 ml of water. Subsequently, the column was eluted with 5 N NH_4OH and the eluate was concentrated and applied on to Dowex 1 (OH^-, 1 × 4 cm) column. The column was then washed with 25 ml of 50% methanol and the effluent and washing were concentrated and developed by paper chromatography. The amounts and sources of enzyme were: 3 mg of fraction I (I), 2 mg of fraction II (II), and 3 mg of fraction I plus 2 mg of fraction II (III).

chloramphenicol, and RNase, nor by the complete removal of nucleic acid from enzyme preparation by chromatography on DEAE-cellulose (S. Tomino, unpublished data). These observations are well in agreement with the results presented by Yukioka *et al.* (1965), Berg *et al.* (1965), and Bhagavan *et al.* (1966). These facts are interpreted to support the concept that the nucleic acids, such as mRNA and tRNAs are not required for gramicidin S synthesis.

Partial Purification and Resolution of Gramicidin S Synthesizing System. As described in the previous section, partial purification of the gramicidin S synthesizing system was undertaken by the use of differential ultracentrifugation, streptomycin precipitation, ammonium sulfate fractionation, and DEAE-cellulose column chromatography. Some of these procedures were found to be useful for the partial purification. Further studies on this line revealed that molecular sieving of the ammonium sulfate precipitate successfully resolved the enzyme system into two distinctly separate fractions. The established method is as follows. Starting from 540 ml of crude extracts of *B. brevis*, fractionation of the enzyme system was carried out through the ammonium sulfate precipitation as described earlier. Dialyzed ammonium sulfate fraction (30 ml) (protein concentration, 55 mg/ml), which contained more than 90% of enzyme activity originally present in the crude extract, was layered onto the column of Sephadex G-200 (5 × 50 cm) which had been equilibrated with buffer B for 2 days. Elution was performed with buffer B and eluate was collected every 15 ml by a LKB fraction collector equipped with a Uvicord ultraviolet monitor.

The distribution of the proteins in the eluate is shown in Figure 5 (lower half). The gramicidin S synthesizing activity in the eluate was very low, when each fraction alone was assayed (upper half, curve A). However, when each fraction was assayed in the presence of fraction 22, a large peak of activity appeared over a range of fractions 28–42 (curve C), and another peak of activity was detected when assayed in the presence of fraction 34 (curve B).

This shows that the gramicidin S synthesizing system

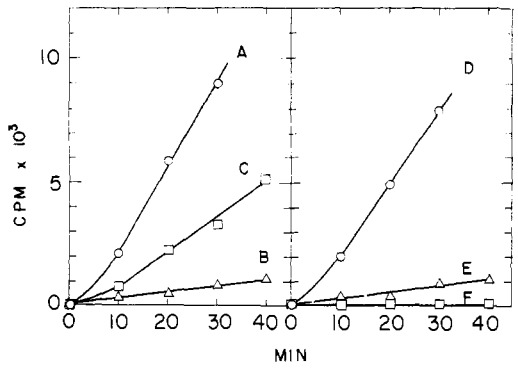


FIGURE 8: Rate of incorporation of L- and D-phenylalanine into gramicidin S. Experimental conditions were the same as those described under Figure 3, except that 4 mg of enzyme protein/ml was used. Phenylalanine to the reaction mixture is as follows: 50 mμmoles of L-[3-^{14}C]phenylalanine (A), 50 mμmoles of L-[3-^{14}C]phenylalanine plus 500 mμmoles of L-[^{12}C]phenylalanine (B), 50 mμmoles of L-[3-^{14}C]-phenylalanine plus 500 mμmoles of D-[^{12}C]phenylalanine (C), 50 mμmoles of D-[3-^{14}C]phenylalanine (D), 50 mμmoles of D-[3-^{14}C]phenylalanine plus 500 mμmoles of D-[^{12}C]phenylalanine (E) or 50 mμmoles of D-[3-^{14}C]phenylalanine, plus 500 mμmoles of L-[^{12}C]phenylalanine (F).

is resolved into two complementary fractions by molecular sieving. These fractions are referred to as fractions I and II, respectively, according to the order of the elution from column. Up to this step, purifications of 50-fold for fraction I and 16-fold for fraction II were achieved above crude extract with the recoveries of 45 and 70%, respectively. After separation, fraction I was unstable and more than 50% of the activity was lost when kept overnight at 0°, while fraction II was rather stable and no loss in activity was marked under the same condition. To protect against inactivation, each fraction was pooled and concentrated by ammonium sulfate precipitation and stored under 5% sucrose at −20°. The complementary nature of the two fractions are also shown in Figure 6.

Role of Fractions I and II in Gramicidin S Synthesis. As reported previously, the incubation of L-phenylalanine, L-proline, L-valine with enzyme, and ATP produced D-phenylalanyl-L-prolyl-L-valine (Tomino and Kurahashi, 1964) and D-phenylalanyl-L-prolyldiketopiperazine (Kurahashi, 1961). Recently, Holm *et al.* (1966) also isolated a conjugated phenylalanyl-prolyl-valyl-ornithine from an incubation mixture containing 11,000*g* supernatant fraction, ATP, $MgCl_2$, and the component amino acids of gramicidin S. These peptides possess a part of peptide sequence of gramicidin S and it was considered that they might be released as incomplete products from enzyme during gramicidin S synthesis.

In order to test whether these peptides might accumulate when each fraction alone was utilized as the enzyme source, an incubation of each fraction and the recombined fraction together with the necessary components for gramicidin S synthesis were carried out. After incubation, the respective mixtures were applied onto Dowex 50 (H^+) columns and fractions eluted with 5 N NH_4OH were again passed through the Dowex 1 (OH^-) columns. Compounds which remained adsorbed to Dowex 1 columns were eluted with 50% HCOOH. The eluates from Dowex 1 were revealed by paper chromatography to contain no significant radioactive peptide other than phenylalanine, indicating that no intermediary peptide with a free carboxyl group was accumulating during the incubation with either of fraction I or II or the recombined. However, as shown in Figure 7, in the fraction which passed through Dowex 1 column, a radioactive product (compound A) appeared when fraction II alone was incubated with ATP and component amino acids (Figure 7, II, peak A). In the presence of fractions I and II, accumulation of this compound was hardly observed and two other compounds (B and C) appeared (Figure 7, III, peaks B and C). Although further confirmation was necessary, A was likely to be D-phenylalanine amide from the following observations. (1) It was not formed when fraction II was freed of ammonium ion by Sephadex G-50 treatment. (2) Considering the behavior on Dowex 50 (H^+) and Dowex 1 (OH^-), A possessed amino group but no free carboxyl group. (3) Upon acid hydrolysis of A, D-phenylalanine was recovered. (4) Paper chromatographic nature of A was the same as that of authentic phenylalanine amide.

Acid hydrolysis of B gave rise to phenylalanine, proline, valine, and ornithine,[2] whereas, all amino acids present in gramicidin S were recovered from the hydrolysate of C. Considering the results of hydrolysis and the behavior on Dowex 1 (OH^-), these compounds are supposed to be the peptides of which carboxyl ends are substituted.

A possible interpretation on the accumulation of A with fraction II may be that fraction II forms activated D-phenylalanine from L-phenylalanine and ATP, and then it is utilized for the synthesis of gramicidin S and/or B and C as the side products of gramicidin S synthesis when fraction I is simultaneously present. In the absence of fraction I, activated D-phenylalanine combines with NH_4^+ which is present in the enzyme preparation resulting from incomplete dialysis to form D-phenylalanine amide.

Incorporation of L- and D-Phenylalanine into Gramicidin S. In the previous experiments described, L-phenylalanine was added to the reaction mixture, although phenylalanine present in the product, gramicidin S, had the D configuration. If L-phenylalanine added is converted to free D-phenylalanine by a racemase and subsequently incorporated into the peptide, the enzyme preparation should also incorporate D-phenylalanine.

[2] Compound B is likely to be Phe-Pro-Val-Orn-X which has been isolated by Holm *et al.* (1966).

In Figure 8, it is shown that D-phenylalanine is incorporated into gramicidin S, although the rate of the incorporation is slightly smaller than that of L-phenylalanine (Figure 8, curves A and D). When each form of [^{14}C]phenylalanine was diluted with ten times as much the homologous [^{12}C]phenylalanine, the incorporation of radioactivity was diminished to nearly the theoretical level (curves B and E). However, the effect of dilution of [^{14}C]phenylalanine with heterologous [^{12}C]phenylalanine was markedly different; the incorporation of L-[^{14}C]phenylalanine was inhibited only 50% by the presence of ten times as much cold D-phenylalanine (curve C), while the incorporation of D-[^{14}C]-phenylalanine was inhibited completely by the presence of ten times as much cold L-phenylalanine (curve F). These results indicate that while free D-phenylalanine itself can serve as a precursor of gramicidin S synthesis, L-phenylalanine is preferentially incorporated into gramicidin S when it is present together with D-phenylalanine and that the formation of free D-phenylalanine from L-phenylalanine is not obligatory prior to the incorporation into gramicidin S.

Discussions

The molecular sieve chromatography of enzyme solution on Sephadex G-200 clearly shows that the gramicidin S formation is catalyzed by at least two fractions. The resolution of the enzyme system had first been achieved by sucrose density gradient centrifugation but the introduction of Sephadex G-200 has been found to be preferable because of its larger capacity and better reproducibility. Recently, Otani *et al.* (1966) have also described the fractionation of gramicidin S forming system by a hydroxylapatite chromatography.

Preliminary results are indicating that ATP–PP_i-exchange activities dependent on L-leucine and L-valine are in both fractions I and II, whereas the activities of ATP-dependent racemization of phenylalanine (Yamada *et al.*, 1964) and ATP–PP_i-exchange reaction dependent on L-phenylalanine and D-phenylalanine are only in fraction II. It is not known at present whether these ATP–PP_i-exchange activities are essential for the gramicidin S synthesis or due to the contamination of amino acid activating enzymes for usual protein synthesis. However, the formation of D-phenylalanine amide with fraction II suggests that this fraction supplies the activated form of D-phenylalanine leading to peptide synthesis. Purifications of fractions I and II are now in progress. At present, further resolution of fraction I or II has been unsuccessful by any methods hitherto adopted, such as chromatography on DEAE-cellulose or hydroxylapatite gel and treatment with alumina Cγ gel. Some other method of fractionation may resolve each fraction further, but the failure of further resolution of enzyme system may suggest, on the other hand, that the enzymes other than the one(s) which is responsible for the formation of activated D-phenylalanine, are clustered in fraction I.

It is well known that many peptides with antibiotic activity produced by microbial organisms contain D-amino acid residues. During the course of the studies of biosynthesis of penicillin (Arnstein and Morris, 1960), actinomycin (Katz and Weissbach, 1963), and tyrocidines (K. Fujikawa, personal communication) it has been shown that the D-amino acid residues in the peptides were derived from L isomers of the amino acids and D isomers were hardly utilized for the synthesis of antibiotics. The results of dilution experiments presented in this communication also show that L-phenylalanine is more readily incorporated into gramicidin S than D isomer although the latter itself can serve as a substrate. We have found the activity of ATP-dependent racemization of phenylalanine in the extract of *B. brevis* Nagano strain. As described before, this activity has also been detected in fraction II together with the activity of D- and L-phenylalanine activation. Taking these observations into account, the incorporation of L- and D-phenylalanine into gramicidin S together with the result of the dilution experiment may be explained by assuming that the activation of each form of phenylalanine is catalyzed by a single enzyme which has higher affinity toward L form than D, and racemization of activated phenylalanine subsequently takes place followed by the specific utilization of activated D-phenylalanine for the peptidation. Glutamine synthetase is known to catalyze the activation of D- as well as L-glutamic acid although activated L form is specifically utilized for subsequent reaction (Krishnaswamy *et al.*, 1962). For further clarification, one has to await the elucidation of the mechanism of activation and racemization of phenylalanine by *B. brevis* extract.

Acknowledgment

We wish to express our gratitude to Dr. S. Sakakibara of this institute for kind by supplying the synthetic intermediary peptides of gramicidin S, prepared by the peptide center of this institute.

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