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Characterization of Gramicidin S Synthetase Aggregation Substance: Control of Gramicidin S Synthesis by Its Product, Gramicidin S

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An aggregation substance of gramicidin S synthetases was found and purified by DEAE-cellulose chromatography and CM-chromatography from cell debris of *Bacillus brevis* Nagano. It specifically aggregated and inactivated gramicidin S synthetases 1 (GS1) and 2 (GS2). On the basis of amino acid composition analysis, reversed-phase HPLC, FAB mass spectrometry, amino acid sequence analysis, and antibacterial activity, this substance (GrS-aggregation substance) was identified as gramicidin S. A gramicidin S derivative bearing a lysine residue in place of one ornithine residue was also detected as a minor component of GrS-aggregation substance. The extent of the aggregation was dependent on the concentration and relative amount of gramicidin S. The inhibition of the enzyme activities was irreversible and the inhibition was proportional to the amount of gramicidin S, like the aggregation of the enzymes. The degree of GS2 inhibition in the amino acid-dependent ATP-PP_i exchange reaction varied with the amino acids of gramicidin S and increased in order of the amino acid sequence of gramicidin S. The degree of inhibition of the overall synthesis of gramicidin S was the same as that in the leucine-dependent exchange reaction.

Key words: *Bacillus brevis*, gramicidin S, gramicidin S synthetase, peptide antibiotic, peptide synthetase.

Bacillus brevis Nagano produces a cyclic decapeptide antibiotic, gramicidin S (D-Phe-L-Pro-L-Val-L-Orn-L-Leu)$_2$ as a secondary metabolite. Gramicidin S is synthesized nonribosomally by two complementary enzymes, gramicidin S synthetase 1 (GS1)/phenylalanine racemase [EC 5.1.1.11] and gramicidin S synthetase 2 (GS2), which have molecular weights of 120,000 and 510,000, respectively (*1-3*). Its formation begins with the activation, thioesterification, and racemization of phenylalanine by GS1 (*4-6*). GS2 catalyzes activation of the other constituent amino acids. Each activation step of the constituent amino acids is similar to that catalyzed by aminoacyl tRNA synthetase, as shown in the following scheme,

$$\text{Enz} + \text{Amino acid} + \text{ATP} \rightleftharpoons \text{Aminoacyl-AMP-Enz} + PP_i$$

$$\text{Aminoacyl-AMP-Enz} \rightleftharpoons \text{Aminoacyl-S-Enz} + \text{AMP}$$

Polymerization begins with the transfer of phenylalanine on GS1 to the 4′-phosphopantetheine residue of GS2 (*7, 8*) and proceeds through sequential thiolation and transpeptidation reaction *via* 4′-phosphopantetheine on GS2 (*8-12*), and then two identical pentapeptides cyclize head-to-tail *via* an unknown mechanism to form gramicidin S.

GS1 and GS2 are composed of repeated functional units of approximately 1,000 amino acid residues, which correspond to the activation units for specific amino acids. Each unit contains a highly conserved sequence of about 600 amino acids for aminoacyl adenylation and thioester formation (*13-17*).

Activities of the two soluble gramicidin S synthetases increase during the transition period between the log phase and the stationary phase, and then rapidly decrease. Some gramicidin S seems to be formed for long time after loss of the soluble activities of these enzymes. The involvement of membrane-bound GS2 in the synthesis of gramicidin S at the later period is suggested (*18, 19*). There may be some mechanism to locate the enzyme in the membrane. We tried to find a substance that locates GS2 in the membrane and found GrS-aggregation substance, which was extracted from cell debris of *B. brevis* by a membrane-solubilizing agent, Triton X-100. It specifically aggregated GS1 and GS2.

Here we report the isolation and purification of GrS-aggregation substance from cell debris of *B. brevis*, and its identification as gramicidin S. Gramicidin S aggregated GS1 and GS2, and irreversibly inhibited the activities of these enzymes. It may participate in the rapid disappearance of these soluble enzymes in the cell.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions—Gramicidin S-producing strain of *B. brevis* Nagano was cultivated in nutrient broth under agitation (250 rpm) at 37°C and harvested after 6.5 h (for enzyme preparation) or 8 h (for preparation of GrS-aggregation substance), as described previously (*3*). Cells were harvested by centrifugation at

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Abbreviations: *p*-amidino PMSF, (*p*-amidinophenyl)methane-sulfonyl fluoride; Orn, ornithine; TCA, trichloroacetic acid; FAB, fast atom bombardment; M_r, molecular weight.

room temperature and stored at −20°C.

Enzyme Preparation—GS1 and GS2 were purified from frozen cell paste of *B. brevis* as described previously (*2, 3, 20*).

Assay of Enzyme Activities—Activities of amino acid activation at individual activation sites were measured by amino acid-dependent ATP-$^{32}PP_i$ exchange reaction in the presence of each constituent amino acid as previously described (*3*) with some modifications. The incubation mixture contained 0.4 ml of 0.02 M Tris HCl buffer pH 8.0, 0.1 ml each of 15 mM DTT, 35 mM KF and 25 mM PP_i, 25 μl of 100 mM ATP, and 25 μl of one of the constituent amino acids, namely, 100 mM D-phenylalanine, L-proline, L-valine, L-ornithine, or L-leucine, 50,000 cpm of $^{32}PP_i$, and enzyme (GS1 or GS2) in a final volume of 1.0 ml. The mixture was incubated at 37°C for 15 min. The reaction was stopped by adding 3 ml of 12.5% trichloroacetic acid (TCA) (w/v) containing 50 mg of activated charcoal. ATP adsorbed on charcoal was eluted with 3 ml of 0.3 M ammonia in 50% ethanol after washing twice with 4 ml of distilled water. The radioactivity of a 1 ml aliquot of the eluate was measured with Cerenkov counting. Activity of gramicidin S synthesis was measured by the same procedure as used in the case of tyrocidine synthesis (*21*) with some modifications. A 0.1 ml aliquot of a mixture of GS1 and GS2 was added to 0.05 ml of amino acid mixture, which contained 1 mM each of L-proline, L-valine, L-ornithine, L-leucine, and [^{14}C]L-phenylalanine (20 μCi), 0.1 ml of ATP-buffer [50 mM ATP, 10 mM $MgCl_2$, 2.5 mM EDTA, 3 mM DTT, 50 mM Tris-HCl buffer (pH 8.0)], and 0.02 ml of pyrophosphatase. The mixture was then incubated at 37°C for 30 min. The reaction was stopped by the addition of 2.5 ml of cold 5% TCA and the whole was allowed to stand for 20 min at 0°C. The precipitate was collected on a Millipore filter (0.45 μm pore). The filter was washed with five 2-ml portions of cold 5% TCA, then dried and the radioactivity was measured. For the blank experiment, ATP and amino acids except [^{14}C]L-phenylalanine were omitted from the reaction mixture. For inhibition assay, the reaction was carried out after preincubation of GS2 or GS1 (or the mixture of GS1 and GS2) with an appropriate amount of GrS-aggregation substance or gramicidin S at 37°C for 20 min unless otherwise stated.

Detection of GrS-Aggregation Substance—Aggregation activity was measured in terms of the disappearance of GS2 in non-reducing PAGE on 5% acrylamide slab gel or rod gel after preincubation of GS2 with aggregation substance. About 10 μg (in 20 μl) of GS2 and 5 μl of sample were incubated for 15 min at 37°C and a 20 μl aliquot was electrophoresed and stained with 0.25% Coomassie Brilliant Blue G-250 as usual. About 100 μg (in 100 μl) of GS2 was used in the case of PAGE with a rod gel.

Preparation of GrS-Aggregation Substance—About 65 g of frozen cell paste was thawed and suspended in 4 volumes of buffer A: 50 mM potassium phosphate buffer, pH 7.5 containing 1 mM $MgCl_2$, 1 mM DTT, 1 mM *p*-amidino PMSF, 0.5 mM EDTA, and 10% glycerol. The suspension was sonically disrupted at 10 kHz, 100 W for 4 min at 0°C and centrifuged at 45,000 × *g* for 60 min. The precipitate, designated as cell debris, was then treated with 125 mg of lysozyme (1 mg per ml) for 15 min at 37°C and centrifuged similarly. The lysozyme was dissolved with 2 mg each of DNase and RNase in 20 mM Tris-HCl buffer pH 7.5 containing 0.25% Triton X-100, 1 mM DTT, 0.5 mM EDTA. GrS-aggregation substance was then extracted from the precipitate by incubating for 15 min at 37°C with 150 ml of 1% Triton X-100 buffer (20 mM Tris-HCl buffer pH 7.5, 1% Triton X-100, 1 mM DTT, and 0.5 mM EDTA). After centrifugation, the supernatant was dialyzed overnight against 0.2% Triton X-100 buffer which was the same buffer as above except for the Triton X-100 concentration.

DEAE-52 Cellulose Column Chromatography of GrS-Aggregation Substance—The dialyzed supernatant was applied to a DEAE-52 cellulose column (1.5 × 49 cm) equilibrated with 0.2% Triton X-100 buffer and 5 ml fractions were collected. GrS-aggregation activity was evaluated in terms of the aggregation of GS2 detected by polyacrylamide gel electrophoresis after preincubation of aliquots of the eluted fractions with GS2 at 37°C for 15 min. Active fractions were eluted in the void volume without adsorption. These fractions (fraction numbers 11 through 42) were combined, concentrated to about 70 ml using a Diaflo ultrafiltration membrane PM 10 (Amicon, Beverly, USA) and dialyzed against buffer B (10 mM potassium phosphate buffer pH 6.6, 1 mM DTT, and 1 mM EDTA) containing 0.2% Triton X-100.

CM-52 Cellulose Column Chromatography of GrS-Aggregation Substance—The dialyzed fraction after DEAE-52 cellulose chromatography was applied to a CM-52 cellulose column (1.5 × 45 cm) equilibrated with buffer B containing 0.2% Triton X-100. The column was extensively washed with about 500 ml of buffer B containing no Triton X-100 after the unadsorbed fractions were eluted. The active fractions were eluted at about 0.2 M KCl with a linear gradient of 0–0.4 M KCl in buffer B and 5 ml fractions were collected. The combined active fractions were concentrated using a Diaflo membrane to obtain a crystalline residue.

Reversed-Phase High-Performance Liquid Chromatography—HPLC was carried out using a Beckman System Gold consisting of a Programmable solvent module 126, a Programmable detector module 166, and an Autosampler 507. Separation was achieved using a Cosmosil $5C_{18}$-AR packed column (4.6 × 250 mm) (Nacalai Tesque) operating at a mobile-phase flow rate of 1 ml/min. The separation conditions were similar to those described by Thibault *et al.* (*22*). The initial mobile phase was 50:50 acetonitrile-water, programmed to 100% acetonitrile in 20 min; all solvents contained 0.1% trifluoroacetic acid. The detector was set to 214 nm. About 1 mg of GrS-aggregation substance or gramicidin S was dissolved in 1 ml of 10:20 acetonitrile-water and 20 μl of the resulting solution (200 μg) was injected into the chromatogram (preparative scale). Before loading on the HPLC column, crystals of GrS-aggregation substance were precipitated by centrifugation, dissolved in 0.2 ml of methanol, mixed with 1 ml of 0.25 potassium phosphate buffer, pH 7.5, then centrifuged. This procedure was repeated once and after washing with distilled water the precipitate was used for HPLC analysis.

Amino Acid Analysis—Samples were hydrolyzed in 6 N hydrochloric acid for 24 h, in sealed evacuated glass tubes at 110°C. Before the hydrolysis, crystals of GrS-aggregation substance were precipitated with methanol and phosphate buffer and washed with distilled water as described for of HPLC, then lyophilized after having been dialyzed against distilled water for 2 h under reduced pressure.

Hydrolysis of the crystal preparations was carried out at the concentration of 1.14 mg per ml (corresponding to 0.1 mM of gramicidin S). Each fraction from HPLC was hydrolyzed with 200 μl of 6 N HCl after drying *in vacuo*. Amino acid analysis was carried out using a Hitachi L-8500 automated amino acid analyzer, with ninhydrin detection.

Fast Atom Bombardment (FAB) Mass Spectrometry—A double beam focusing mass spectrometer, JEOL JMS-SX102A equipped with a JEOL JMA-DA7000 data system, was used for accurate mass determination. Experiments were typically carried out with a xenon atom beam source at 10 kV accelerating voltage. Mass assignment was made using Ultramark (Perfluoroalkylphosphasen). Approximately 1 μg of the HPLC fraction was loaded on a stainless steel plate and mixed with *m*-nitrobenzyl alcohol-glycerine (1:1) on the plate.

Terminal Amino Acid Sequence Analysis—The amino acid sequence of GrS-aggregation substance was analyzed with an Applied Biosystems sequencer system consisting of a Model 477A protein sequencer and a Model 120A PTH analyzer.

Antimicrobial Activity Assay—The amount of gramicidin S was determined microbiologically. *Staphylococcus aureus* 209P was used as a test organism. A sample was added to 5 ml of nutrient broth and 50 μl of overnight culture was inoculated into the broth. After cultivation for 20 h, antimicrobial activity was measured in terms of the turbidity at 600 nm.

Measurement of Protein Concentration—Protein concentration was estimated by using Coomassie protein assay reagent (Pierce, USA).

Biochemicals—$^{32}PP_i$ was purchased from the Radiochemical Centre (Amersham, UK). Gramicidin S of *B. brevis* Nagano and pyrophosphatase were purchased from Sigma Chemical (St. Louis, USA). HPLC-grade acetonitrile and distilled water for HPLC (Wako Chemicals, Osaka) were used in the preparation of mobile phases of HPLC. All other chemicals were standard commercial products.

RESULTS

Existence of GrS-Aggregation Substance in the Cell Debris of B. brevis—GrS-aggregation substance was found in the Triton X-100 extract of cell debris of *B. brevis*. Figure 1 shows the disappearance of GS2 in PAGE using rod gel after incubation with the Triton X-100 extract (Fig. 1A, lane 1). Aggregation of GS2 was observed at upper part of the separation gel and spacer gel, or within the spacer gel. A contaminant protein in the GS2 preparation, which was electrophoresed slightly more slowly than GS2, was not aggregated and remained in the gel. Although Triton X-100 itself also associated GS2 to some extent and formed high-molecular complexes (Fig. 1A, lane 2, in comparison with Fig. 1B, lane 1), its effect was small as compared with that of GrS-aggregation substance (Fig. 1A, lane 1). The purified preparation dissolved without Triton X-100 (described below) aggregated GS2 (Fig. 1B, lanes 2 to 4). The appearance of aggregation was dependent on the amount of GrS-aggregation substance. The aggregation complex increased in size (Fig. 1B, lanes 2 to 4) and finally precipitated as the amount increased.

Purification of GrS-Aggregation Substance—GrS-aggregation substance was extracted from cell debris of *B. brevis* with Tris-HCl buffer containing 1% Triton X-100, as described in "MATERIALS AND METHODS." This substance passed through a DEAE-52-cellulose column without adsorption. Aggregation activities of the fractions were detected by non-reducing PAGE using 5% slab gel. Figure 2 shows the aggregation profile of the non-adsorbed fraction from DEAE-52-cellulose chromatography. Aggregation activity was marked in fractions 11 through 39. These fractions contained large aggregation complexes, and the GS2 band nearly disappeared within the separation gel (Fig. 2A, lanes 3-5, 8-12; 2B, lanes 1-2). In the later fractions, the width of the GS2 band on PAGE gradually increased and slower-migrating bands appeared. They corresponded to the aggregation complex of GS2 with GrS-aggregation substance or Triton X-100 (Fig. 2B, lanes 3-5, 8-12). Contaminant protein in the GS2 preparation was seen close to GS2 through all lanes in the gel, indicating that GrS-aggregation substance specifically aggregated GS2. The elution profile could not be measured in terms of optical density due to the high absorption at 280 nm of Triton X-100 in the buffer. The pooled active fractions were concentrated and applied to a CM-52-cellulose column after dialysis. GrS-aggregation substance was adsorbed on the CM-52-cellulose column and eluted at about 0.2 M KCl with a linear gradient of 0 to 0.4 M KCl after extensive washing with buffer B containing no Triton X-100. Aggregation activity was clearly observed in fractions 62 through 83 (Fig. 3B, lanes 3-5, 8-12). Absorbance at 280 nm was small in the active fraction (maximum absorbance of about 0.05) and almost base line in the other fractions. The active

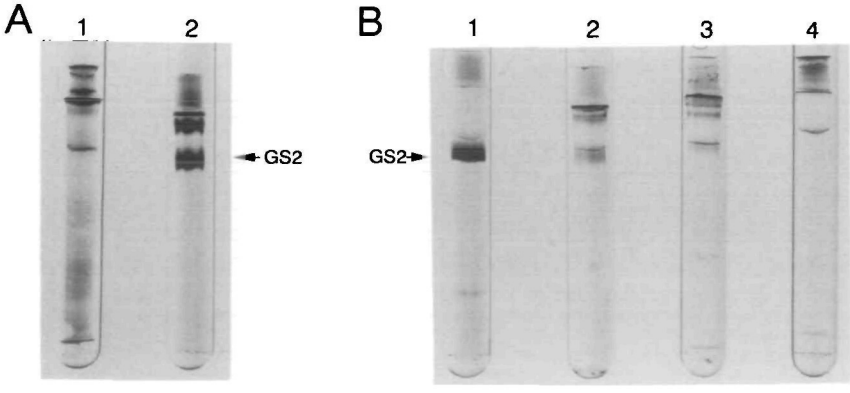


Fig. 1. **Existence of GrS-aggregation substance in the Triton X-100 extract from the cell debris of *B. brevis*.** Preparations of GrS-aggregation substance were preincubated with about 100 μg of GS2 in 100 μl of buffer A containing 0.1% Triton X-100 (A) or 5% ethanol (B) at 37°C for 15 min and the mixtures were electrophoresed in rod gels of 5% polyacrylamide and stained with Coomassie Brilliant Blue G-250. (A) Preincubation with 20 μl of Triton X-100 extract (lane 1) or enzyme only (lane 2). (B) Preincubation with the purified GrS-aggregation substance; 0 μg (lane 1), 12.5 μg (lane 2), 25 μg (lane 3), and 50 μg (lane 4).

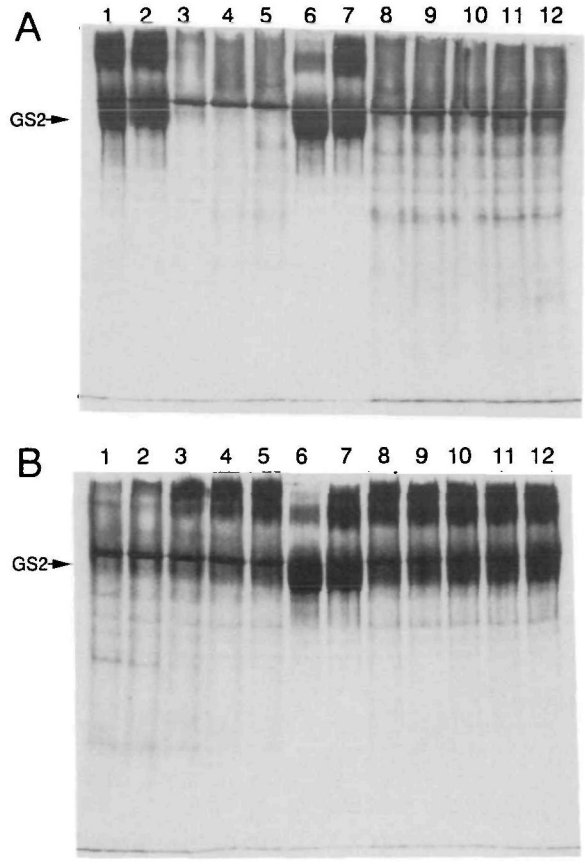


Fig. 2. **Detection of GrS-aggregation activity in the non-adsorbed fractions from the DEAE-52 cellulose column.** The Triton X-100 extract obtained from cell debris of *B. brevis* was applied to a DEAE-52 cellulose column and eluted with 0.2% Triton X-100 buffer as described in the text. Aliquots (5 μl) of the eluted fractions were preincubated with GS2 (10 μg in 15 μl of the buffer) at 37°C for 15 min, then mixtures of 5 μl of marker dye and 20 μl of each fraction were electrophoresed in a slab gel of 5% polyacrylamide and stained with Coomassie Brilliant Blue. The spacer gels were removed after electrophoresis. Lane 6, GS2 and 5 μl of buffer A; lane 7, GS2 and 5 μl of 0.2% Triton X-100 buffer. The fraction numbers of samples are; in gel A: 5, 9, 11, 14, and 17 (lanes 1-5); 20, 23, 27, 30, and 33 (lanes 8-12), respectively, in gel B: 36, 39, 42, 45, and 48 (lanes 1-5); 51, 54, 57, 60, and 65 (lanes 8-12), respectively.

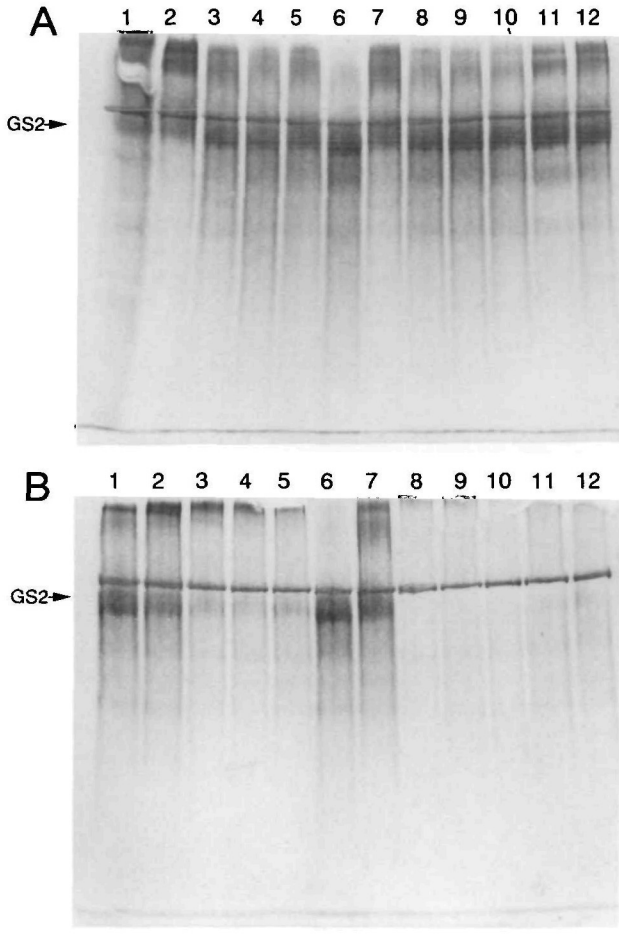


Fig. 3. **Detection of GrS-aggregation activity in the eluted fractions from CM-52 cellulose column chromatography.** The pooled active fractions from the DEAE-52 column chromatography were applied to a CM-52 cellulose column and eluted as described in the text. Fractions 1 to 30 were pass-through fractions. Elution with a linear gradient of 0-0.4 M KCl buffer was started at fraction 31 after extensive washing with buffer containing no Triton X-100. Electrophoresis was carried out as in Fig. 2. Lane 6, GS2 and 5 μl of buffer A; lane 7, GS2 and 5 μl of 0.2% Triton X-100. The fractions are; in gel A: lanes 1-5, 7, 12, 17, 22, and 27; lanes 8-12, 32, 37, 42, 47, and 52, respectively, in gel B: lanes 1-5, 56, 59, 62, 65, and 68; lanes 8-12, 71, 74, 77, 80, and 83, respectively.

fraction obtained from CM-52-cellulose column chromatography was concentrated to form needle-like crystals (Fig. 4). Table I shows a summary of the purification. Aggregation activities are indicated in terms of the amounts of gramicidin S. Five micrograms of gramicidin S was the minimum amount of the aggregation substance which completely aggregated 20 μg of GS2. Specific activity at the final step was about 1.0 indicating that the preparation was purified as gramicidin S.

HPLC Chromatograms of Gramicidin S and GrS-Aggregation Substance—Figure 5 shows reversed-phase HPLC of the purified GrS-aggregation substance (Fig. 5A), gramicidin S (Fig. 5B), which was prepared by the method of Otani *et al.* (*23*) under the same culture conditions as in the isolation of GrS-aggregation substance, together with commercial gramicidin S (Fig. 5C). A single major peak with five minor peaks was observed in the HPLC profile of GrS-aggregation substance. The elution position of the major peak was in good agreement with those of both gramicidin-S preparations which were located at 8.56 or 8.57 min (Fig. 5, B and C), corresponding to gramicidin S (*22*). The elution pattern of the commercial gramicidin-S preparation showed a similar profile to that previously described (*22, 24*), but the separation pattern of the preparation obtained by the method of Otani *et al.* slightly differed from that of commercial gramicidin S. There were one or two poorly resolved shoulders on the individual peaks eluting at 6.84, 7.70, and 8.52 min, respectively. The minor component of GrS-aggregation substance resembled that of the latter preparation and somewhat differed from the commercial preparation. This difference may be due to the culture conditions of the bacteria. Furthermore, the amount of minor components in GrS-aggregation substance was very small compared to that in the gramicidin-S preparations.

Amino Acid Composition of GrS-Aggregation Substance—Table II shows the amino acid composition of GrS-aggregation substance, gramicidin S from different sources and HPLC fractions from these preparations. GrS-aggregation substance mainly consisted of about equimolar quantities of phenylalanine, proline, valine, leucine, with about three-quarters mole of ornithine and a quarter mole of lysine. These amino acids except lysine are constituent amino acids of gramicidin S. The amino acid composition was similar to that of gramicidin-S preparation obtained by the method of Otani *et al.* (*23*), but somewhat differed from that of the commercial product (Table II). The former preparations contained significant amounts of lysine and the commercial preparation contained only a very small amount of lysine; further the content of leucine was larger in the former preparation than in the latter. This may be owing to culture conditions which produce different compositions of gramicidin-S analogues.

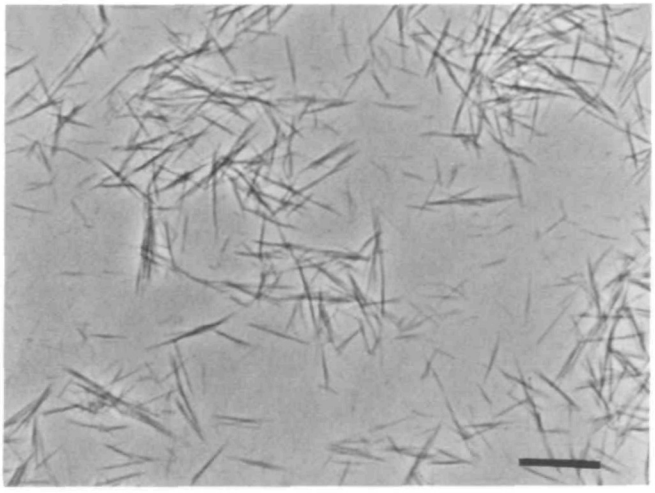


Fig. 4. **Photomicrograph of the crystals of GrS-aggregation substance.** Bar indicates 20 μm.

TABLE I. **Purification of GrS-aggregation substance from cell debris of *B. brevis*.** Activity of GrS-aggregation substance was measured in terms of the aggregation of GS2. The assay procedure is described in "MATERIALS AND METHODS."

Purification step	Total activity[a]	Total protein (mg)	Specific activity
1. Triton X-100 extraction	250	870	0.29
2. DEAE-52 cellulose column chromatography	178	302	0.59
3. CM-52 cellulose column chromatography	128	125	1.02
4. Crystallization	76	75	1.01

[a]Calculated as the weight (mg) of gramicidin S which has equivalent activity of aggregation.

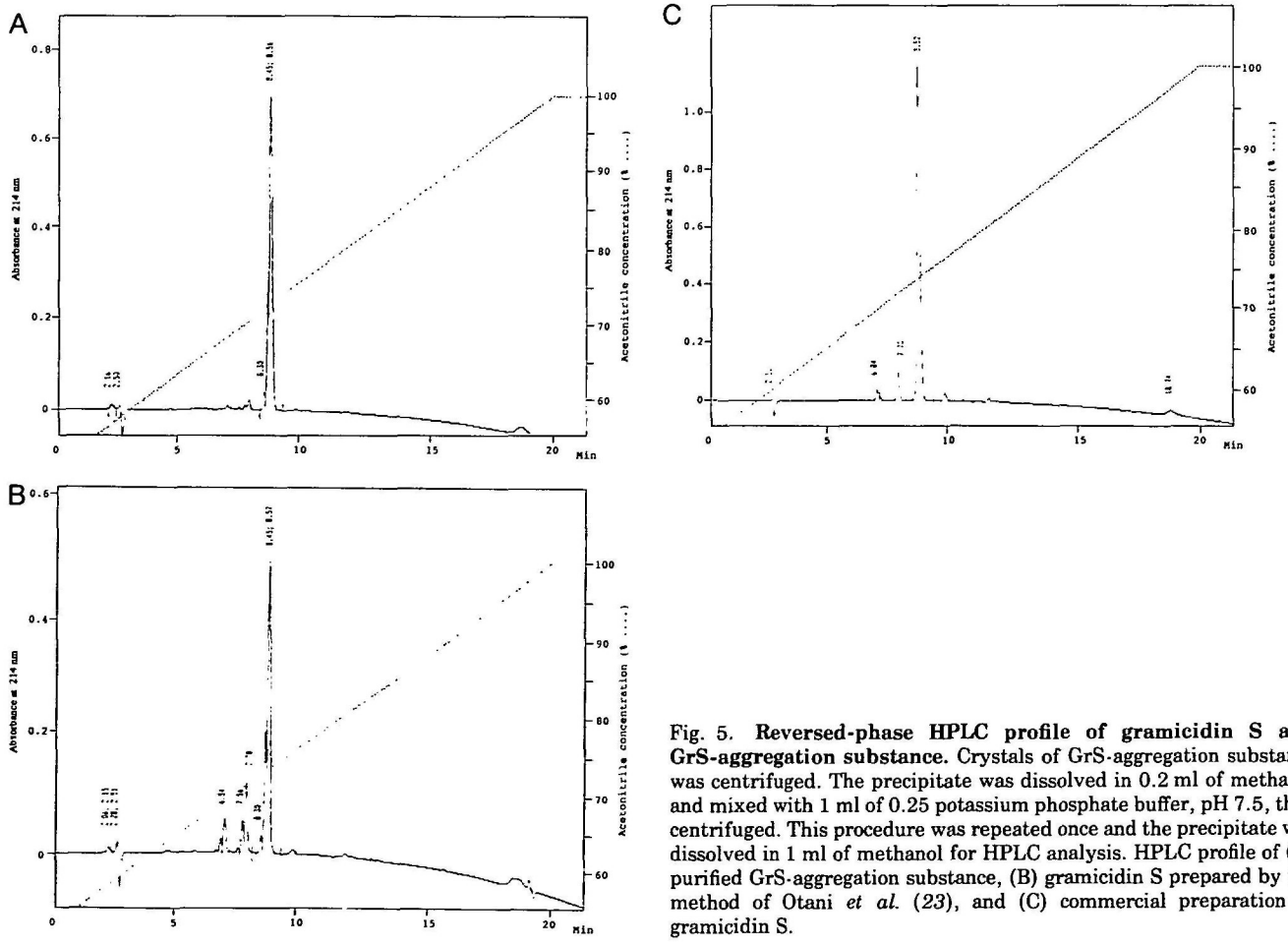
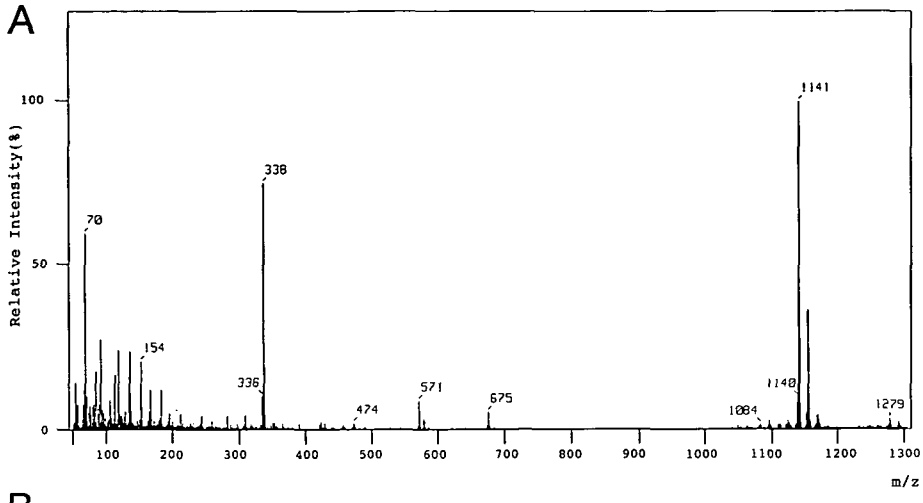


Fig. 5. **Reversed-phase HPLC profile of gramicidin S and GrS-aggregation substance.** Crystals of GrS-aggregation substance was centrifuged. The precipitate was dissolved in 0.2 ml of methanol and mixed with 1 ml of 0.25 potassium phosphate buffer, pH 7.5, then centrifuged. This procedure was repeated once and the precipitate was dissolved in 1 ml of methanol for HPLC analysis. HPLC profile of (A) purified GrS-aggregation substance, (B) gramicidin S prepared by the method of Otani *et al.* (*23*), and (C) commercial preparation of gramicidin S.



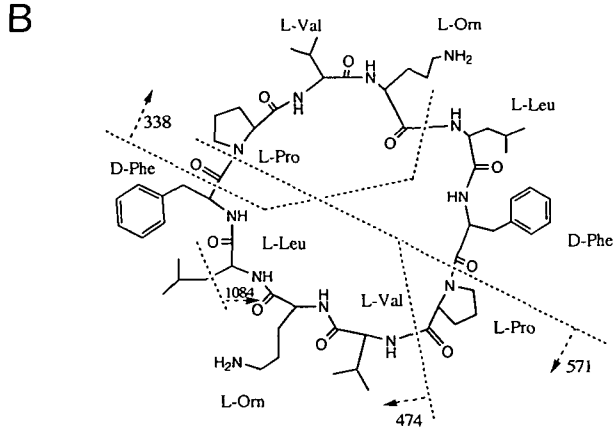


Fig. 6. **FAB mass spectra (positive ions) of GrS-aggregation substance (A) and proposed fragmentation pattern of gramicidin S (B).** The purified GrS-aggregation substance from the reversed-phase HPLC fraction (8.5 min) was analyzed.

TABLE II. **Amino acid analysis of GrS-aggregation substance and gramicidin S.** For details, see "MATERIALS AND METHODS." The values are given as nmol of residues in 2.6 nmol equivalent (crystal preparations) or 2.0 nmol equivalent (HPLC fractions) of gramicidin S. The values of HPLC fractions are measured using the peak fraction (eluted at 8.53–8.73 min).

	Crystal preparation			Reversed phase HPLC		
	GrS2-aggregation substance	Gramicidin S		GrS2-aggregation substance	Gramicidin S	
		I[a]	II[b]		I[a]	II[b]
Phe	4.79	4.84	5.10	3.88	3.34	3.04
Pro	4.54	4.73	5.26	3.73	3.95	3.03
Val	4.29	4.24	4.05	3.63	3.03	2.43
Orn	3.97	4.73	4.02	2.98	3.76	2.93
Leu	5.20	4.85	5.29	4.15	3.95	3.77
Lys	1.08	0.12	1.04	0.65	0.10	0.29

[a]Gramicidin S commercial product. [b]Gramicidin S prepared by the method of Otani *et al.* (*23*), under the same culture conditions as used to obtain GrS-aggregation substance. Values of other amino acids are omitted since they were not significant (below 0.1).

Amino acid compositions of these preparations in the HPLC fraction were analyzed. Table II compares the amino acid composition of the fraction at the main peak (8.53 to 8.73 min). Amino acid components of GrS-aggregation substance were similar to those of gramicidin S prepared under the same culture conditions, but slightly differed from those of the commercial product. The presence of lysine was noted in the former gramicidin S and GrS-aggregation substance, but very little lysine was detected in the commercial product. The presence of lysine in the gramicidin-S peak suggests that the gramicidin-S homolog containing lysine instead of ornithine has almost the same mobility as gramicidin S under these HPLC conditions. This was supported by more precise analysis of the amino acid composition at the main peak. The early-eluted portion of the main peak contained more lysine than the later-eluted portion. This result is consistent with the presence of a shoulder at the main peak in the HPLC of these preparations. A higher lysine content in the early-eluted fractions was also detected in the chromatogram of the commercial product, though it was not prominent (data not shown). These results indicate that the gramicidin S analogue containing lysine is eluted slightly earlier than gramicidin S.

The fractions of the main peak in the HPLC of GrS-aggregation substance aggregated GS2 and the degree of the aggregation was proportional to the absorbance value at 214 nm of each fraction. Similar results were obtained with gramicidin S preparations (data not shown). None of the isolated minor peaks from GS2-aggregation substance and gramicidin S aggregated GS2. It is not clear whether these analogues cannot aggregate gramicidin S synthetase or whether the aggregation did not occur because the amounts of these analogues were too small.

Analysis of N-Terminal Amino Acid Sequence—The reversed-phase HPLC fraction (8.5 min) was subjected to protein sequencing. No amino acid residue was detected during fifteen cycles of Edman degradation. This result indicates that the N-terminal residue of GrS-aggregation substance is blocked.

FAB Mass Spectrum of the Purified GrS-Aggregation Substance—Figure 6A shows the FAB mass spectrum of GrS-aggregation substance obtained from the reversed-phase HPLC chromatography (8.5 min). FAB mass values of the observed signals were well correlated with those from gramicidin S and its analogue. Two peaks at *m/z* 1,141 and 1,155 (accurate mass measurements; *m/z* 1,141.71 and 1,155.73, data not shown) corresponded to protonated gramicidin S (M_r of 1,140) and a gramicidin S analogue in which one ornithine residue of gramicidin S is substituted by lysine (M_r of 1,154). This result was consis-

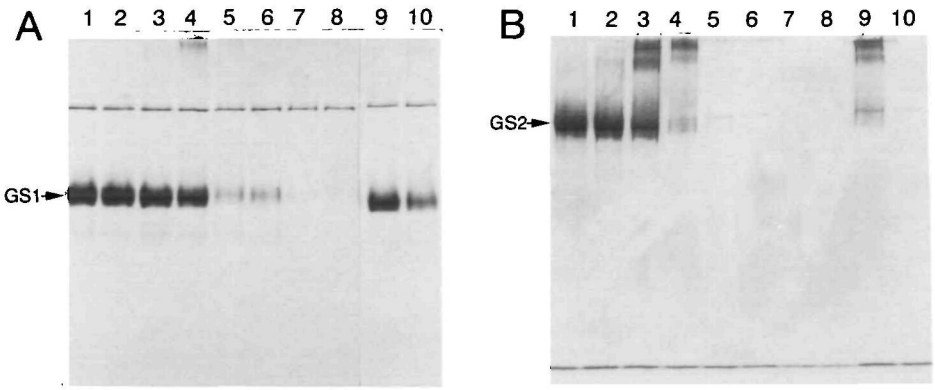


Fig. 7. **Aggregation of GS1 or GS2 by Gramicidin S.** About 21.7 μg of GS1 (A) or 11.3 μg of GS2 (B) was preincubated with various amounts of gramicidin S or GrS-aggregation substance in a volume of 20 μl at 37°C for 15 min and electrophoresed in 7.5% (GS1) or 5% (GS2) PAGE. The gel was stained with Coomassie Brilliant Blue G-250 as in Fig. 2. Lanes 1-8, amounts of gramicidin S are 0, 0.63, 1.25, 2.5, 5, 12.5, 25, and 50 μg, respectively. Lanes 9 and 10 are the aggregations by 2.5 and 5 μg of GrS aggregation substance (which was identified as gramicidin S in this paper).

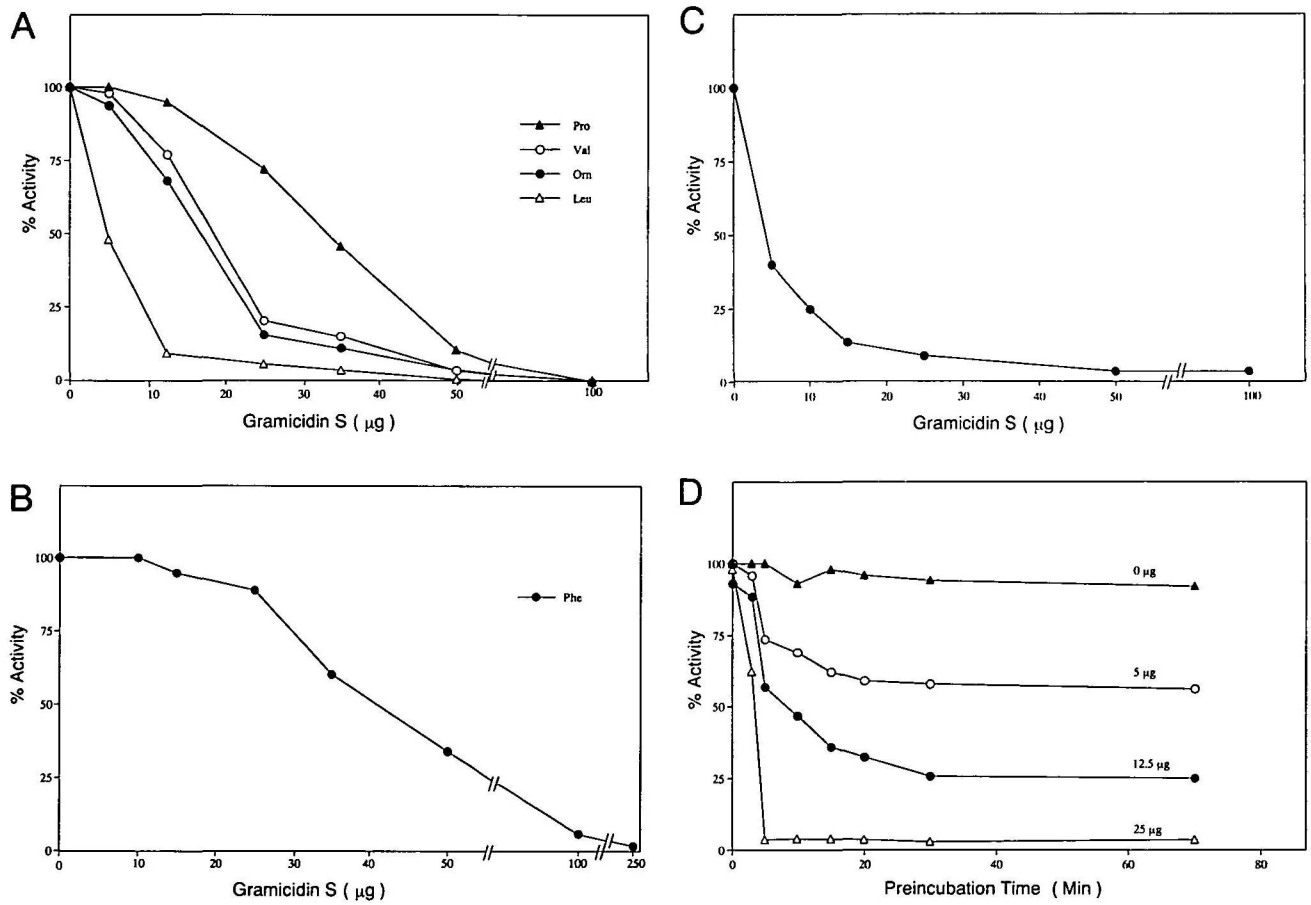


Fig. 8. **Inhibition of the activities of GS1 or GS2 by gramicidin S.** GS1 (about 40 μg; 45 milliunit) and/or GS2 (about 80 μg; 45 milliunit) was preincubated with various amounts of gramicidin S at 37°C for 20 min (A-C) or for various times (D). The preincubated enzymes were then added to the reaction mixture and individual activities were measured as described in "MATERIALS AND METHODS." One enzyme unit is defined as the amount of GS1 or GS2 which catalyzes phenylalanine- or ornithine-dependent ATP-$^{32}PP_i$ exchange of 1 μmol per min, respectively. (A) Amino acid-dependent ATP-$^{32}PP_i$ exchange activities of GS2. (B) Amino acid-dependent ATP-$^{32}PP_i$ exchange activities of GS1. (C) Gramicidin S synthesis by GS1 and GS2. (D) Effect of preincubation time on the inactivation of Orn-dependent ATP-$^{32}PP_i$ exchange activities of GS2.

tent with the analysis of amino acid composition. Since the peak at m/z 571 has a mass value of half that of protonated gramicidin S, it is considered to represent a pentapeptide fragment of gramicidin S, probably Pro-Val-Orn-Leu-Phe as judged from the fragment peak at m/z 474 (mentioned below) (Fig. 6, A and B). Mass values of m/z 338 and 675 match the MH^+ ion of O^+≡Pro-Val-Orn and that of the dimer, respectively. A peak at m/z 474 corresponds in mass value to the fragment lacking the proline residue from the above pentapeptide (M_r 570) (Fig. 6, A and B). The mass value of m/z 1,084 matches the molecular weight of the gramicidin S derivative lacking the $(CH_3)_3C$ radical of leucine (Fig. 6, A and B).

These results indicate that GrS-aggregation substance is

gramicidin S.

Antimicrobial Assay—GrS-aggregation substance showed antimicrobial activity against *Staphylococcus aureus* 209P. The minimum inhibitory concentration of GrS-aggregation substance was about the same as that of gramicidin S (2 μg/ml).

Effect of Gramicidin S Concentration on Aggregation of Gramicidin S Synthetases—Gramicidin S specifically aggregated GS1 in addition to GS2. The extent of aggregation was dependent on the concentration of gramicidin S (Fig. 7) and on the amount of the enzymes in the preincubation mixture. About 25 μg of gramicidin S completely aggregated about 100 μg of GS2 or 20 μg of GS1. The molecular ratio of the enzyme and gramicidin S in the aggregation mixture was calculated as about one to hundred for both enzymes. Commercial gramicidin S aggregated GS1 and GS2 more strongly than the crystalline preparation of GrS-aggregation substance. This may be due to the presence of a small amount of membrane lipids or Triton X-100 in the crystalline preparation, since it aggregated the enzymes to the same degree as the commercial product after the crystals had been washed with 80% ethanol solution containing 0.1 M phosphate buffer, pH 7.5. Nonspecific aggregation occurred when a large amount of gramicidin S was present in the incubation mixture. However, it did not occur unless GS1 or GS2 was completely aggregated. Figure 7A indicates the presence in the preparation of GS1 of a contaminant protein which is not aggregated by gramicidin S.

Aggregation was not absolutely irreversible since GS1 or GS2 aggregated by gramicidin S had the same mobility as the enzymes without gramicidin S in SDS PAGE (data not shown). This result indicates that the aggregation does not involve covalent bond formation other than disulfide bond by cysteine residues.

Inhibitory Effect of Gramicidin S on Activities of GS1 and GS2—Gramicidin S as well as GrS-aggregation substance inhibited the activities of GS1 and GS2. The inhibitory effects of gramicidin S on individual activation sites of constituent amino acids were measured by examining the corresponding amino acid-dependent ATP-$^{32}PP_i$ exchange reactions as shown in Fig. 8. Gramicidin S inhibited all the exchange activities of GS1 and GS2. This result indicates that all the activation sites were inhibited by gramicidin S. The degree of the inhibition differed among the exchange reactions. It increased in the order of proline-, valine-, ornithine-, and leucine-dependent exchange reactions on GS2 (Fig. 8A). This order is the same as the amino acid sequence of gramicidin S, except for phenylalanine. This is interesting, since the activation sites of the amino acids on GS2 are also arranged in the same order (*15-17*). The inhibition of D-phenylalanine-dependent exchange activity on GS1 was slightly weaker than that of the proline-dependent exchange activity when GS1 having about the same exchange activity as that of GS2 was used (Fig. 8B), though comparison is not strictly valid because GS1 is a different molecule and has about one-fourth the molecular size of GS2. Inhibition of gramicidin S formation was similar in degree to that of leucine-dependent exchange activity (Fig. 8C), indicating that inhibition of leucine-dependent exchange activity limits activity of the total synthesis.

The degree of inhibition was non-linear with respect to the preincubation time (Fig. 8D). Gramicidin S did not strongly inhibit these activities severely in the absence of preincubation. Without preincubation, about 55% of ornithine-dependent exchange activity still remained when the amount of gramicidin S in the reaction mixture was increased to 250 μg (data not shown). Furthermore, the presence of the substrate (ATP or constituent amino acids) prevented the inhibition (data not shown). As the conditions of the preincubation were the same as those of aggregation of these enzymes, the inhibition of the enzyme activities may correlate with the aggregation. Preliminary data indicated that these substrates protected the enzymes from aggregation by gramicidin S.

DISCUSSION

In this study, GrS-aggregation substance was identified as gramicidin S. There were some differences in the minor components of gramicidin S preparations from different sources, probably due to different culture conditions of the bacteria. Since substrate specificities for constituent amino acids of gramicidin S are not strict (*25, 26*), different analogues could be formed depending on the substrate analogues present in the cells.

Gramicidin S synthetases are formed at the end of exponential growth but the greater part of their activities rapidly disappear soon after their specific activities have reached the maximum level (*18*). Little is known about the mechanisms of regulation of the enzyme formation or of rapid disappearance of the enzyme activities.

Oxygen may inactivate gramicidin S synthetases during fermentation. The L-ornithine- and D-phenylalanine-activating activities of the gramicidin S synthetases were lost during aeration in the frozen-thawed cells and crude extract (*27*). The reducing agent DTT prevented the oxygen-dependent inactivation of the synthetases in the cell-free extract and nitrogen preserved the activity in both cells and extracts. Oxidation of thiol groups on the active sites was suggested to result in the loss of the activities. Vandamme *et al.* (*18*) reported that a high aeration rate resulted in a very rapid fermentation with little gramicidin S formation. Stabilization of the activities by nitrogen during fermentation was also reported by Friebel and Demain (*28*). However, this would not explain why even in low aeration conditions, the soluble synthetase levels drop drastically after they reach the peak.

In the present study, we isolated the aggregation substance of gramicidin S synthetases from the cell debris by Triton X-100 extraction. This substance was proved to be gramicidin S. It specifically aggregated these synthetases and inhibited the enzyme activities. A large quantity of gramicidin S is formed during the late logarithmic phase of growth, though the degree of the production varies depending on the fermentation conditions (*18*). Since gramicidin S is scarcely excreted outside of the cell, rapid increase of gramicidin S in the cell may occur with increase of the enzyme activities and result in rapid inactivation of the enzymes at their peak levels.

Gramicidin S inhibited individual ATP-PP_i exchange reactions of the synthetases, but the degrees of inhibition differed. The leucine-dependent exchange reaction was the most sensitive to gramicidin S. The degree of inhibition increased in the order of the amino acid sequence of gramicidin S in GS2. This result is probably related to the

fact that the activation sites of the specific amino acids on GS2 are arranged in the same order (*15-17*). Since leucine activation is the last step for the formation of the pentapeptide, this site is most likely near the cycling site. Therefore, gramicidin S may easily bind at or near the leucine site to block the leucine-activating reaction and has a lesser inhibitory effect on proline activation.

It remains unclear how gramicidin S aggregates the synthetases. As the concentration of gramicidin S synthetase is usually high *in vitro*, dimerization or polymerization of the enzyme with association of gramicidin S molecules may occur to form the aggregation complex. It is not known whether gramicidin S synthetases exist as the aggregated form with gramicidin S in the host cells.

Vandamme *et al.* reported the existence of a particle-bound form of GS2 which was obtained by detergent (Triton X-100) treatment of the cell pellets after extraction of the soluble enzyme (*18*). Gramicidin S was also extracted from cell debris with the detergent in this study. Gramicidin S forms a rigid ring structure of β-pleated sheet containing two type II′ β-turns with two cationic ornithine residues on one side of the molecular plane and hydrophobic residues on the other side (*29-31*). Gramicidin S may thus associate with membrane phospholipids *via* the hydrophobic residues.

The localization of gramicidin S and the detoxication mechanism in producing cells are still unknown.

It is also of interest to know why the membrane-bound form of GS2 maintains the enzymatic activities, escaping inhibition by gramicidin S. We are now studying the participation of membrane lipid in the protection of the synthetase activities and the localization of gramicidin S in the cell.

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