FRACTIONATION OF AN ENZYME SYSTEM RESPONSIBLE FOR GRAMICIDIN S BIOSYNTHESIS

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In previous papers, Yukioka et al. (1964. 1965) reported that an antibiotic polypeptide, gramicidin S, was synthesized by a soluble enzyme system of B. brevis. It was supposed that the mechanism of gramicidin S formation might be undertaken in a different way from that of usual protein synthesis, because there was no inhibitory effect by RNase, DNase, puromycin or chloramphenicol on this cyclic peptide synthesis. Similar observations were also reported by Berg et al. (1965). In addition, peptides having amino acid sequences closely related to gramicidin S were isolated from cell-free system of B. brevis; D-phenylalanyl-L-prolyl diketopiperazine by Kurahashi (1961), D-phenylalanyl-L-prolyl-L-valine tripeptide by Tomino and Kurahashi (1964), and D-phenylalanyl-L-prolyl-L-valyl-L-ornithine tetrapeptide by Tsuji (1966) and Holm et al. (1966). These results strongly suggest that gramicidin S synthesis would be initiated by D-phenylalanyl-L-proline dipeptide formation, and followed by successive addition of appropriate amino acids. This communication deals with the further fractionation of a soluble enzyme system for gramicidin S synthesis.

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MATERIALS AND METHODS

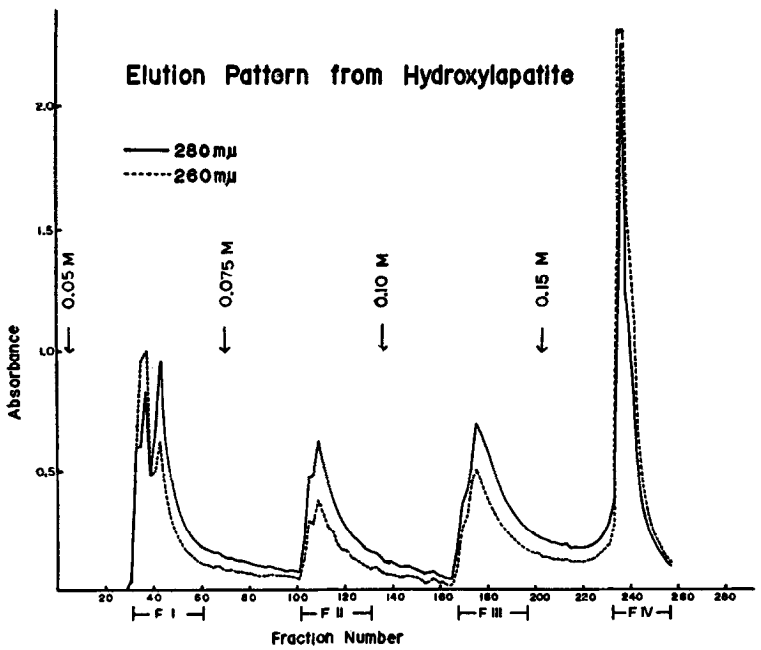
B. brevis (Nagano) was used throughout the experiments. The fraction precipitated from 105,000 x g supernatant of the sonicate of B. brevis with ammonium sulfate, between 36 and 45 per cent saturation, was prepared as described by Yukioka et al. (1965), and used as a starting enzyme preparation. Sephadex G-200, previously equilibrated with 0.01 M Tris:HCl buffer, pH 7.0, containing 0.01 M $MgCl_2$ and 0.01 M β-mercaptoethanol, was put into a 3 x 30 cm column. Hydroxylapatite was prepared according to the procedure of Tiselius et al. (1956), and was stored in 0.001 M phosphate buffer, pH 6.8. Density gradient centrifugation was performed with 4 ml of 0 to 50 per cent linear sucrose gradient per tube at 35,000 rpm for 12 hours at 0°C, using a Spinco SW39L rotor. The reaction system for gramicidin formation was the same as in the previous report. In this system ^{14}C-L-phenylalanine was used as a marker, and cold L-proline, L-valine, L-ornithine and L-leucine were added. For D-phenylalanyl-L-prolyl diketopiperazine formation, only L-proline was added instead of the four amino acids. After incubation for 90 minutes at 37°C, the reaction was stopped by heating in boiling water and the reaction products were extracted twice using ethanol. The ethanol extracts were passed through a Dowex-50 (H^+ form) 1 x 7 cm column and successively eluted with ethanol, water, 4 N aqueous ammonia and ammonia saturated 90 per cent methanol. Since the ammoniacal methanol eluate contained gramicidin S only, the amount of gramicidin S synthesized was determined by the radioactivity of this fraction. The fraction eluted by ethanol was passed through a Dowex-2 (OH^- form) 1 x 3 cm column and eluted with 100 ml of ethanol. The formation of D-phenylalanyl-L-prolyl

diketopiperazine was determined by the radioactivity of this eluate, because it contained only a single radioactive substance corresponding to the diketopiperazine reported by Kurahashi (1961). Radioactivity was determined with a gas-flow counter on samples of infinite thinness. Protein was measured by the method of Kalckar (1947).

RESULTS AND DISCUSSION

Neither chromatography using Sephadex G-200 nor the sucrose density gradient centrifugation could fractionate the crude enzyme system. However, the enzyme system was fractionated into four protein portions by use of the hydroxylapatite column chromatograph. The crude enzyme preparation was dissolved in 0.03 M potassium phosphate buffer, pH 6.8, and dialyzed against the same buffer for 60 minutes, and then was absorbed on the hydroxylapatite column (3 x 20 cm) pre-equilibrated with the same buffer. The elution was carried out by step wise increase of the buffer concentration: 300 ml of 0.05 M, 0.075 M, 0.10 M

Fig. I



and 0.15 M potassium phosphate buffers, pH 6.8, with a flow rate of 60 ml per hour. Five ml aliquots of the eluates were collected. The elution pattern is shown in Figure 1. To each protein fraction was added an equal volume of saturated ammonium sulfate solution. The resulting precipitates were dissolved in minimum amounts of Tris:HCl buffer, pH 7.0, and the activities of gramicidin S and of D-phenylalanyl-L-prolyl diketopiperazine were assayed on each separated and recombined fractions. The results are shown in Table I. Gramicidin formation was not found in any single fraction, but was almost completely restored when recombination of the separated enzyme fractions was carried out. As shown in Table I, although a small amount of diketopiperazine was formed by Fraction 4, the rate of formation markedly increased when Fractions 2 or 3 were added to Fraction 4. It is known that diketopiperazine may be formed by the non-enzymatic cyclization of D-phenylalanyl-L-proline dipeptide, when a following amino acid, L-valine, was omitted

Combination of fractions	Gramicidin formed	DKP formed
	cpm	cpm
1	0	0
2	0	0
3	0	0
4	0	4,290
1 + 2	0	160
1 + 3	0	740
1 + 4	530	6,125
2 + 3	50	390
2 + 4	10,990	13,070
3 + 4	22,195	13,495
1 + 2 + 3	0	480
1 + 2 + 4	32,520	16,165
1 + 3 + 4	42,235	13,600
2 + 3 + 4	33,620	13,020
1 + 2 + 3 + 4	37,620	12,715

Table I.

from the reaction mixture. Therefore, it is evident that the enzymes responsible for D-phenylalanyl-L-proline dipeptide synthesis were resolved in Fraction 2, 3 and 4. Fraction 1 seemed to contribute to the further elongation of the dipeptide, since it showed only a slight increasing effect on diketopiperazine formation, and it is seen that this fraction was essential for full gramicidin S synthesis. Phenylalanine found in the synthesized peptides was shown to be the D-form in spite of using the L-isomer as a starting material. Furthermore, replacing ^{14}C-L-phenylalanine by the D-isomer, the incorporation of radioactivity into the peptides was far less than when the L-isomer was used. From these results, it is expected that the conversion of L-phenylalanine to D-isomer occured during the dipeptide formation. ATP-dependent phenylalanine racemization was found in this organism by Yamada et al. (1964), but this activity was almost negligible in our enzyme preparations. Further purification of the enzymes is now in progress to elucidate the mechanism of each peptidation step, including conversion of L-phenylalanine to the D-isomer.

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