BIOSYNTHESIS OF GRAMICIDIN S BY A CELL-FREE SYSTEM OF BACILLUS BREVIS*

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Several Studies on the biosynthesis of gramicidin S by Bacillus brevis have been reported in recent years from different laboratories, such as those by Barry and Ichihara (1958), Winnick et al. (1961) and Eikhom et al. (1963, 1964). However, most of these were concerned with experiments on intact cells of this organism. Kurahashi (1961) reported the formation of D-phenylalanyl-L-prolyl diketopiperazine as the starting peptide of gramicidin S synthesis by a cell-free system of B. brevis Nagano, and Tomino and Kurahashi (1964) obtained D-phenylalanyl-L-prolyl-L-valine with the same system.

In our laboratory, gramicidin S has been synthesized by a cell-free system of B. brevis Nagano and part of this work has already been presented by Yukioka et al. (1963, 1964). This communication is on some features of the enzymatic formation of gramicidin S.

MATERIALS AND METHODS

B. brevis strains Nagano and ATCC 9999 were used and essentially the same results were obtained with both strains.

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Bacterial cells, which were grown in peptone yeast extract medium with vigorous shaking, were collected in the late logarithmic phase of growth. They were washed 3 times with saline solution, and then disrupted by sonic oscillation at 10 KC, 100W, for 4 minutes in the presence of 0.01 M $MgCl_2$ and 0.01 M β-mercapto-ethanol. The homogenate was fractionated by successive differential centrifugation at 14,500 x g for 30 minutes, 41,500 x g for 60 minutes and 105,000 x g for 120 minutes. Each supernatant and precipitated fraction and recombined fractions were incubated with standard reaction mixture as shown in Table I. After 3 hours incubation at 37°C, the reaction was stopped by addition of 10 ml of ethanol. The mixture was centrifuged and the precipitate was extracted with 10 ml of ethanol. The ethanol fractions were combined and passed through Dowex 50 (H^+ form in ethanol) using a 0.9 x 10 cm column. The column was washed successively with 100 ml of ethanol, 100 ml of water and 400 ml of 4 N aqueous ammonia. Finally, the radioactive gramicidin S was quantitatively eluted from the column with 200 ml of ammonia saturated 95% methanol. The radioactivity of the gramicidin S was determined with a gas-flow counter on samples of infinite thinness. The gramicidin S formed was identified by scanning the radioactivity of paper chromatograms (Rf 0.92 with n-butanol, acetic acid, water (4:1:1 by volume) and 0.76 with pyridine, iso-amylalcohol, water (1.75:2:1 by volume)), and a highvoltage electropherogram (Mobility, 22 cm toward to the cathode with formic acid, acetic acid, acetone, water (1:6:3:10 by volume) medium and 60 volts/cm for 150 minutes). Also some samples were identified by bioautography using Micrococcus pyogenes var. aureus.

RESULTS AND DISCUSSION

As shown in Table I, the gramicidin forming activity found in

Table I. Gramicidin S Formation by Different Enzyme Fractions

Enzyme fraction	Radioactivity of gramicidin S formed
0 Time	295 cpm
14,500 x g supernatant	303,350
41,500 x g supernatant	297,940
" precipitate	790
" supernatant and precipitate	258,330
105,000 x g supernatant	267,300
" precipitate	810
" supernatant and precipitate	288,635
" supernatant, precipitate and 41,500 x g precipitate	276,715

The standard reaction mixture contained, enzyme (6 mg of protein), 15 μmoles of Tris buffer, 10 μmoles of KCl, 15 μmoles of $MgCl_2$, 1 μmole each of L-leucine, L-valine, L-proline and L-ornithine, 5 μmoles of ATP, 5 μmoles of phosphoenolpyruvate, 20 μg of pyruvatekinase and 0.1 μmole of C^{14}-L-phenylalanine (500,000 cpm) in a total volume of 1.5 ml. (pH 7.0). For a description of the procedure used for isolation of radioactive gramicidin S formed, see Materials and Methods in the text.

the supernatant fraction after centrifugation at 14,500 x g was almost quantitatively recovered in the supernatant after successive centrifugation at 41,500 x g and 105,000 x g, and addition of the precipitates to this material did not have any effect on the activity. The enzyme system was precipitated from the supernatant fraction with ammonium sulfate at between 35 and 45 per cent saturation. These results show that gramicidin formation requires only a soluble enzyme system and no particulate fraction is involved. For disruption of cells, the best results were obtained by 4 minutes sonication, prolonged sonication causing a marked decrease in the activity. The optimum concentration of Mg ions was found at 0.01 M for both sonic disruption of cells and enzyme incubation. Under the standard conditions, formation of the peptide was observed over a period of 3 hours. Omission of ATP and an ATP generating system from the complete

mixture caused a substantial decrease in gramicidin S formation. The optimal pH for its formation was around 7.0. Addition of chloramphenicol, puromycin, streptomycin or penicillin G had no inhibitory effect, as shown in Table II.

Table II. Effect of Chloramphenicol, Puromycin, Streptomycin and Penicillin G.

Inhibitor added	Radioactivity of gramicidin S synthesized
Complete system (0 hr)	150 cpm
Complete system (3 hrs)	260,585
Chloramphenicol	
5×10^{-5} M	242,360
5×10^{-4}	257,495
5×10^{-3}	260,585
Streptomycin	
5×10^{-5} M	267,825
5×10^{-4}	257,215
5×10^{-3}	271,580
Puromycin	
5×10^{-5} M	262,095
5×10^{-4}	247,500
5×10^{-3}	261,660
Penicillin G	
5×10^{-5} M	267,985
5×10^{-4}	251,650
5×10^{-3}	265,535

Table III shows the effect of pretreatment of the 105,000 x g supernatant fraction with bovine pancreatic RNase and DNase. Neither enzyme had any appreciable effect on peptide synthesis.

Table III. Effect of RNase and DNase

Pretreatment	Radioactivity of gramicidin S formed
Complete system (0 hr)	155 cpm
Complete system (3 hrs)	
withour pretreatment	290,750
with RNase (200 μg)	267,790
with DNase (200 μg)	258,400

From these results, it is most likely that the mechanism for synthesis of gramicidin S is not like that of protein synthesis contrary to the observations on the biosynthesis of gramicidins and tyrocidines by the Dubos strain of B. brevis reported by Uemura et al. (1963), Okuda et al. (1964 a, b) and Bodley et al. (1964).

Furthermore, several smaller peptides with amino acid sequences related to that of gramicidin S have been isolated from an incubation mixture in which a specified constituent amino acid(s) is omitted. Identification and studies on the nature of these peptides, and further purification of enzyme system are now in progress.

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