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BIOSYNTHESIS OF POLYMYXIN E

III. TOTAL SYNTHESIS OF POLYMYXIN E BY A CELL-FREE ENZYME SYSTEM

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SUMMARY

Polymyxin E, an antimicrobial branched cyclic decapeptide, was synthesized by an enzyme fraction partially purified from crude extracts of the producing organism, Aerobacillus polyaerogenes. For the synthesis, three constituent amino acids (L-2,4-diaminobutyric acid, L-leucine, and L-threonine), ATP, Mg^{2+} and an acylating system consisting of octanoyl CoA and an ammonium sulfate fraction of cell extracts are required.

Polymyxin E (colistin) produced by Aerobacillus polyaerogenes or Bacillus colistinus Koyama is one of the polymyxin series of antibiotics effective against gram-negative bacteria (1, 2). The amino terminal residue, L-2,4-diaminobutyric acid (DAB), is acylated with either 6-methyloctanoic acid, isooctanoic acid, or octanoic acid (OA) as shown in Diagram 1 (1, 3).

Studies of the biosynthesis of antibiotic peptides produced by bacteria of the genus Bacillus, such as gramicidin S, tyrocidines, bacitracins and gramicidin A, have revealed that they are synthesized by the multienzyme thiotemplate mechanism without the involvement of nucleic acids or ribosomes (4-10). A number of studies using whole cells showed that inhibitors of protein synthesis have no effect on polymyxin production (11-13), suggesting that its biosynthesis is also carried out according to the multienzyme thiotemplate mechanism. Ito et al. (14) reported that crude extracts of B. colistinus Koyama catalyzed the synthesis of colistin (polymyxin E), but the details of the biosynthetic mechanism have remained unclarified.

Abbreviations: DAB, 2,4-diaminobutyric acid; OA, octanoic acid; OA-DAB, N^2-octanoyl-L-2,4-diaminobutyric acid; OA-CoA, octanoyl coenzyme A.

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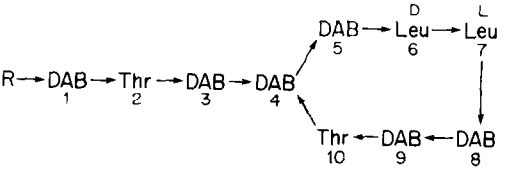

Polymyxin E_1 : R=6-methyloctanoic acid (MOA)
Polymyxin E_2 : R=isooctanoic acid (IOA)
Polymyxin E_3 : R=octanoic acid (OA)
DAB : 2,4-diaminobutyric acid

Diagram 1. Structure of Polymyxin E

We reported previously that a partially purified enzyme of A. polyaerogenes could activate and bind DAB, L-threonine and L-leucine through thioester bonds (2). We further proved that OA was transferred from octanoyl coenzyme A (OA-CoA) to a part of enzyme-bound DAB, when an ammonium sulfate fraction of the crude extract was added as a source of acyltransferase to the reaction for the amino acid binding experiments (15). In this communication we present evidence that the same enzyme system is able to synthesize polymyxin E when three constituent amino acids of polymyxin E are present in the reaction mixture.

MATERIALS AND METHODS

Chemicals and enzymes. The following chemicals were obtained commercially: L-DAB, OA-CoA and phosphoenolpyruvate from Sigma Chemical Co., and L-[U-^{14}C]-leucine (240 mCi/mmole) from Daiichi Pure Chemical Co. Inorganic pyrophosphatase [EC 3.6.1.1] and pyruvate kinase [EC 2.7.1.40] were purchased from Boehringer Mannheim GmbH. Polymyxin E sulfate and deacylpolymyxin E were the generous gift from Banyu Pharmaceutical Co. Ltd. and Dr. Y. Kimura, respectively.

Enzyme preparation. Preparation procedures of the DAB activating enzyme (Sepharose 4B fraction) and an ammonium sulfate fraction were described in earlier papers (2, 15).

Polymyxin E biosynthesis. The reaction mixture contained, in a final volume of 0.2 ml, 10 μmoles of Tris-HCl buffer (pH 7.7), 0.8 μmole of ATP, 2 μmoles of $MgCl_2$, 1 μmole of dithiothreitol, 0.8 μmole of phosphoenolpyruvate, 0.2 μmole of L-DAB, 0.2 μmole of L-threonine, 0.16 μmole of OA-CoA, 4 nmoles of L-[U-^{14}C]leucine (372,000 cpm), 0.4 μg of pyruvate kinase, 1 μg of inorganic pyrophosphatase, 206 μg of the ammonium sulfate fraction and 312 μg of the enzyme (Sepharose 4B fraction). The mixture was incubated at 30°C for 40 minutes. After incubation, the reaction was stopped by addition of 10 μl of trichloroacetic acid to give a final concentration of 4.8 %. Fifty micrograms of polymyxin E sulfate and 1 μmole of L-leucine were then added as carriers. After standing at 0°C for 1 hour, the precipitated protein was removed by centrifugation. The pH of the supernatant solution was adjusted to 7 by addition of 1 N KOH. The methods of isolation and analysis of the products are described in the legends to Fig. 1 and 3.

Paper chromatography. Paper chromatography was carried out descendingly on Toyo No. 50 filter papers with the following solvent systems.

Solvent A: n-butanol : acetic acid : pyridine : H_2O (15 : 3 : 10 : 12)

Solvent B: n-butanol : acetic acid : H_2O (4 : 1 : 2)

RESULTS

Incorporation of L-[^{14}C]leucine into polymyxin E. The enzyme fraction was incubated with L-[^{14}C]leucine, DAB, L-threonine together with ATP and the DAB acylating system as described in MATERIALS AND METHODS. After incubation the reaction was stopped and the supernatant solution was applied to a column of Bio-Gel P-2. Fig. 1 shows a gel-filtration pattern of the radioactive products. The radioactive peak preceding the large free [^{14}C]leucine peak coincided with the position where polymyxin E was eluted. Fractions 8 through 10 were combined, concentrated and subjected to paper chromatography with the two different solvent systems as shown in Fig. 2. The radioactive compound migrated as same as an authentic sample of polymyxin E. Polymyxin E_1, E_2 and E_3 had the same Rfs on the paper

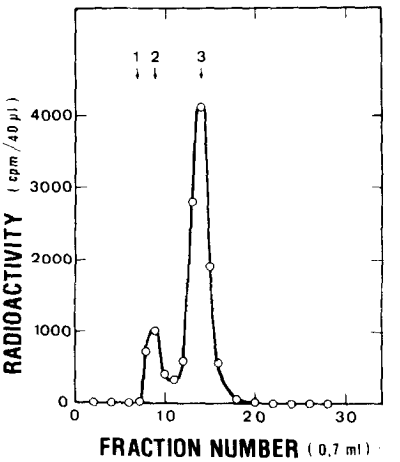

Fig. 1. Gel filtration of the reaction products on a Bio-gel P-2 column. The supernatant solution of the reaction mixture was obtained as described in MATERIALS AND METHODS and applied to a Bio-gel P-2 column (1 x 20 cm), previously equilibrated with 0.2 N acetic acid. The column was eluted with 0.2 N acetic acid. Forty microliters of each fraction were applied to a Whatman GF/C glass fiber paper. The paper was dried, placed in 5 ml of a scintillation fluid and the radioactivity was determined with a Beckman Model LS-250 liquid scintillation counter. Arrows 1, 2 and 3 indicate the elution position of blue dextran 2000, polymyxin E and L-leucine, respectively.

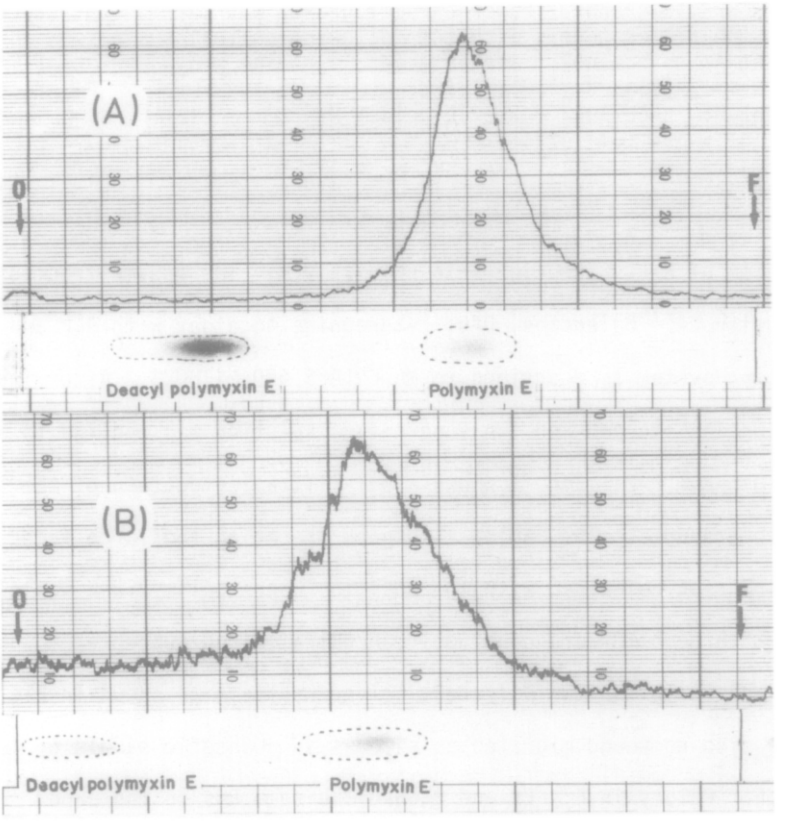

Fig. 2. Paper chromatography of the products eluted from a Bio-gel P-2 column. Paper chromatography was carried out as described in MATERIALS AND METHODS. (A) Solvent A; (B) Solvent B. The radioactivity was scanned with a Packard radiochromatogram scanner Model 7201. The markers, polymyxin E and deacylpolymyxin E, were visualized by ninhydrin spray.

chromatograms. [^{14}C]Leucine incorporated into polymyxin E accounted for 9.8 % of the total input in the reaction.

Requirement for the total synthesis of polymyxin E. Polymyxin E is a strongly basic peptide because it possesses six residues of DAB. It adsorbs to a Dowex 50 (NH_4^+-form) column very tightly and can be eluted only with a very basic solvent such as 2 N piperidine (Hayashi, K., personal communication). A reaction for polymyxin E synthesis was carried out as described in MATERIALS AND METHODS and the radioactive products were chromatographed on a Dowex 50W-X4 column. Fig. 3 shows the elution pattern of the radioactive products. In the control experiments

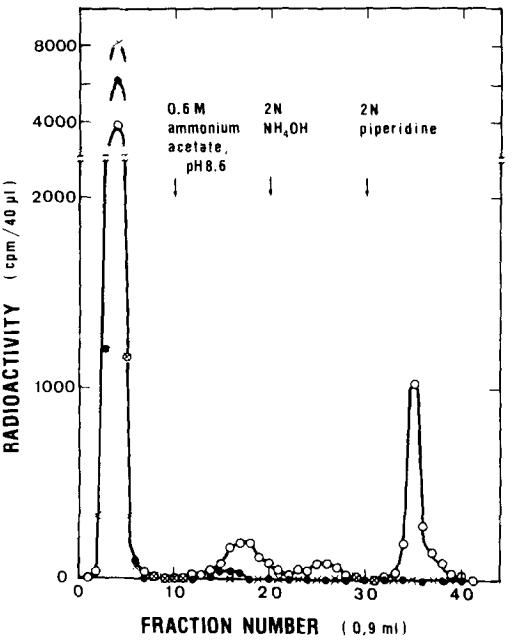

Fig. 3. Dowex 50W-X4 column chromatography of the reaction products. The supernatant solution of the reaction mixture was obtained as described in MATERIALS AND METHODS and applied to a Dowex 50W-X4 (NH^4-form), column (1 x 2 cm), previously equilibrated with 0.4 M ammonium acetate, pH 5.2. The column was eluted stepwise with 8 ml of the following solution: (1) 0.4 M ammonium acetate, pH 5.2, (2) 0.6 M ammonium acetate, pH 8.6, (3) 2 N NH_4OH, and (4) 2 N piperidine. Forty microliters of each fraction were applied to a Whatman GF/C glass fiber paper, and the radioactivity was determined with a liquid scintillation counter. - o -, Complete reaction mixture; - ● -, L-DAB, L-threonine and OA-CoA were omitted; - x -, no incubation.

without incubation or with the omission of OA-CoA, DAB and threonine, no radioactive product, which was eluted with 2 N piperidine, was formed. The radioactive product eluted with 2 N piperidine was verified to be polymyxin E by paper chromatography (data not shown).

Table I shows the requirement for the incorporation of L-[^{14}C]leucine into polymyxin E. Each of Sepharose 4B fraction, DAB, threonine, OA-CoA and an ammonium sulfate fraction is essential for the total synthesis of polymyxin E by the enzyme system. However, in the absence of threonine or an ammonium sulfate fraction, there was still an appreciable amount of incorporation of [^{14}C]leucine; we think that it resulted from the presence of endogenous threonine in the enzyme preparations and from the presence of acyltransferase contaminating in Sepharose 4B fraction, respectively.

Table 1. Requirement for the incorporation of L-[^{14}C]leucine into polymyxin E.

Conditions	Radioactivity incorporated into polymyxin E (cpm)
Complete	7,873
- DAB	113
- threonine	1,573
- octanoyl coenzyme A	193
- ammonium sulfate fraction	2,240

The reaction and isolation of the supernatant solution were carried out as described in MATERIALS AND METHODS. The supernatant solution was applied to a Dowex 50W-X4 (NH_4^+-form) column (1 x 1 cm), and then the column was washed as described in the legend to Fig. 3. The eluate with 2 N piperidine was evaporated to dryness and subjected to paper chromatography with Solvent B. The radioactivity in the area corresponding to the marker of polymyxin E was determined with a Beckman liquid scintillation counter.

DISCUSSION

The enzyme protein which catalyzed the total synthesis of polymyxin E had an approximate molecular weight of 300,000 (15). It activates and binds DAB, L-leucine and L-threonine as thioesters. About one tenth of DAB thioesterified to the enzyme is acylated by acyltransferase and OA-CoA (15). The addition of the acylating system is essential for the total synthesis of polymyxin E (Table 1). When DAB and [^{14}C]threonine were incubated under the conditions of the binding experiments without the acylating system, no intermediary peptide bound to the enzyme was formed (unpublished results). These results suggest that the synthesis of polymyxin E takes place with the initial formation of the enzyme-bound OA-DAB, followed by peptidation to a decapeptide stage and cyclization. It was reported that the initiation of gramicidin A biosynthesis was the formation of formylvaline thioesterified to the synthetase (16-18). Lipmann and his group reported that the large enzyme of tyrocidine synthetase with a molecular weight of 440,000 consists of six 70,000 dalton-subunits which activate one each of six constituent amino acids and a pantetheine-carrying subunit with a molecular weight of 20,000 (19, 20). It is rather unexpected that

polymyxin synthetase with a molecular weight of 300,000 can activate, thioesterify and conjugate ten constituent amino acids. No attempt to demonstrate enzyme-bound phosphopantetheine has been made. We are trying to purify further and characterize the synthetase.

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