# THE PRESENCE OF 4′-PHOSPHOPANTETHEINE IN THE BACITRACIN SYNTHETASE

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## 1. Introduction

A soluble enzyme complex participates in the biosynthesis of bacitracin which is an antibiotic dodecapeptide produced by a strain of *Bacillus licheniformis* and the peptide is synthesized from amino acids bound to enzyme protein through thioester linkages [1,2]. These features of bacitracin synthetase resemble those in the biosynthesis of gramicidin S and tyrocidine [3]. Recently, it was reported that gramicidin S and tyrocidine synthetases contained one and two moles of 4′-phosphopantetheine per mole of synthetase, respectively [4,5], and that phosphopantetheine arm participated in peptide chain elongation [6]. Bacitracin synthetase has been separated into two complementary fractions (Peaks I and II) by column chromatography on hydroxyapatite [2]. The present work demonstrates that Peak II fraction was further separated into two components and that each of the three components responsible for bacitracin biosynthesis contains approximately one equivalent of 4′-phosphopantetheine.

## 2. Materials and methods

### 2.1. *Bacitracin synthetase*

The partially purified bacitracin synthetase was prepared from the crude extract of *B. licheniformis* ATCC 10716 as described previously [2], and the hydroxyapatite fractions (Peaks I and II) were used throughout the work.

### 2.2. *Assay of ATP–$^{32}PP_i$ exchange activity*

The incubating mixture and the procedure for assay of the exchange activity were as previously described except that bovine serum albumin (100 μg/ml) was added to the incubation mixture [2].

### 2.3. *Estimation of the molecular weight*

The molecular weights of the three components of bacitracin synthetase were estimated by the methods of Martin and Ames [7]. The marker enzymes used were catalase (mol. wt 250 000) and urease (mol. wt 480 000).

### 2.4. *Polyacrylamide disc gel electrophoresis*

Polyacrylamide gel electrophoresis was performed according to the methods of Davis [8]. 5% separation gels (0.5 × 8 cm, pH 8.9) and 2.5% stacking gels were prepared and electrophoresis was carried out in a cold room at 5°C with a constant current of 2 mA per tube for 2.5 hr. Three parallel experiments were performed. After electrophoresis, gel A was stained and scanned for protein absorbance using a densitometer. Gels B and C were sliced into uniform segments and used for assay of exchange activity and pantothenate content, respectively.

### 2.5. *Release of pantothenate from the enzyme protein*

Pantothenate was released from the enzyme protein according to the procedure of Pugh and Wakil [9]. The protein was hydrolyzed in 1 N NaOH at 100°C for 90–120 min. Then pH of the mixture was adjusted to about 10 with 1 N HCl. Glycine–NaCl–NaOH buffer (pH 9.6) and $MgCl_2$ were added to make final concentration 0.1 M and 1 mM, respectively. The mixture was incubated with alkaline phosphatase (10 μg/1.5 ml) at 37°C for 2–3 hr. Controls without the addition of alkaline phosphatase were treated identically.

2.6. *Assay of pantothenate*

Pantothenate was determined by microbiological assay with *Lactobacillus plantarum* ATCC 8014, using the medium based on USP description [10].

2.7. *Assay of β-alanine and taurine*

Enzyme protein was treated with 0.1 N NaOH at 70°C for 1 hr according to the method of Majerus et al. [11] for the liberation of 4'-phosphopantetheine. The solution was acidified with acetic acid and the resulting precipitate removed by centrifugation. The supernatant was concentrated under reduced pressure and submitted to oxidation with performic acid, as described by Hirs [12]. Oxidized sample was hydrolyzed in 6 N HCl in an evacuated sealed tube for 24 hr at 110°C. The hydrolysate was analyzed with a Hitachi Model KLA-3B amino acid analyzer. As a control experiment, CoA was treated in the same way as the enzyme protein. The recovery rate of β-alanine and taurine from CoA was used for the calculation of the ratio of these substances in the enzyme protein.

2.8. *Measurement of protein concentration*

Protein concentration was determined by the method of Lowry et al. with bovine serum albumin as a standard [13].

## 3. Results

Bacitracin synthetase was resolved into two complementary fractions by hydroxyapatite column chromatography as shown in fig.1. The mol. wt of Peak I was calculated to be about 200 000 from the profile of sucrose density gradient centrifugation (fig.2). Figs. 3 and 4 show that Peak II was further separated into two main components. The light fraction which activated L-lysine and L-ornithine has the mol. wt of about 210 000 and the heavy one, which activated L-isoleucine, L- and D-phenylalanine, L-histidine, L-aspartate and L-asparagine, has the mol. wt of about 380 000. The former fraction is now termed as Component II and the latter Component III, and then Peak I is termed as Component I.

In preliminary experiments, it was found that Peak II contained about two moles of pantothenate per mole of fraction. Pantothenate was then assayed for the fractions obtained by disc electrophoresis of

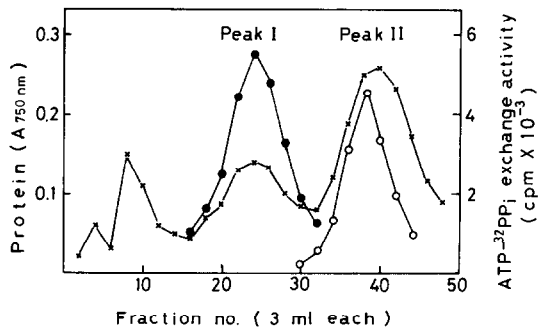


Fig.1. Resolution of bacitracin synthetase into two complementary fractions by hydroxyapatite column chromatography. The column (1.2 X 13 cm) was eluted with a linear gradient consisting of 75 ml each of 0.15 M and 0.30 M potassium phosphate buffer (pH 6.8). Protein (X–X–X); ATP–$^{32}PP_i$ exchange activities dependent on L-cysteine (●–●–●) and D-phenylalanine (○–○–○).

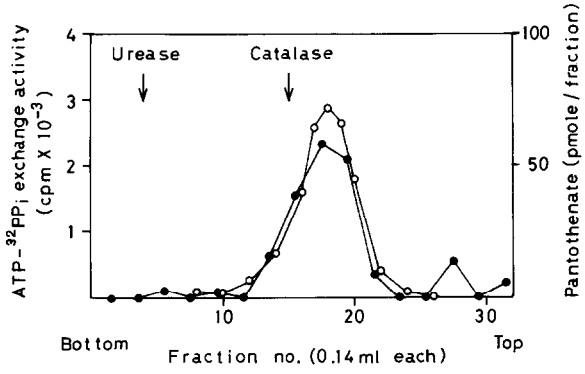


Fig.2. Sucrose density gradient centrifugation of Peak I. A 4 to 16% linear sucrose gradient (4.4 ml) containing 50 mM Tris–HCl (pH 7.4), 1 mM $MgCl_2$ and 5 mM dithiothreitol was used. Peak I (200 μg) and marker enzymes were layered and centrifuged at 100 000 *g* for 11 hr. In parallel experiment the sample solution without markers was centrifuged. Two fractions were combined and used for pantothenate determination. ATP–$^{32}PP_i$ exchange activity dependent on L-cysteine (○–○–○); Pantothenate content (●–●–●).

peak II where Components II and III were distinctly separated. Fig.4 shows that both Components contain pantothenate. In the case of Component I, pantothenate was assayed for the fractions obtained by sucrose density gradient centrifugation owing to a fairly low yield of pantothenate from the fractions obtained by disc electrophoresis. As shown in fig.2, it is clear that

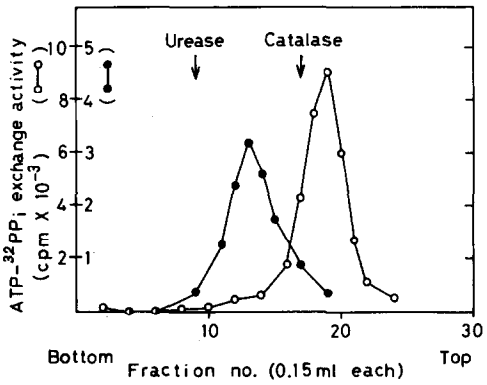


Fig. 3. Sucrose density gradient centrifugation of Peak II. A 4 to 16% linear sucrose gradient (4.4 ml) containing 0.3 M potassium phosphate buffer (pH 7.2) and 5 mM dithiothreitol was used. Peak II (90 μg) and marker enzymes were layered and centrifuged at 130 000 *g* for 7 hr. ATP–$^{32}$ $PP_i$ exchange activities dependent on a mixture of L-lysine and L-ornithine (o–o–o), and L-histidine (•–•–•).

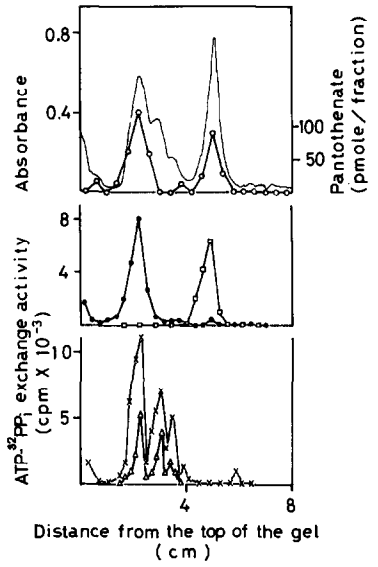


Fig.4. Polyacrylamide gel electrophoresis of Peak II. 170 μg fo Peak II was applied to each of four gels. For good separation of each of the protein components, electrophoresis was continued further for 2 hr after bromphenol blue marker emerged from gel at about 2.5 hr. Protein absorbance (——); Pantothenate content (o–o–o); ATP–$^{32}$ $PP_i$ exchange activities dependent on a mixture of L-histidine, L-aspartate and L-asparagine (•–•–•), a mixture of L-lysine and L-ornithine (□–□–□), L-isoleucine (X–X–X), and D-phenylalanine (△–△–△).

Component I also contains pantothenate. As summarized in table I, each Components contains about one mole of pantothenate per mole of Component.

Since pantothenate was not released from the enzyme protein without both alkali and alkaline phosphatase treatments and phosphopantothenate released by alkali treatment was very stable under alkaline conditions, it is suggested that pantothenate is present as 4′-phosphopantothenate [14]. By the amino acid analysis of performic acid-treated enzyme protein, it was shown that the ratio of taurine to β-alanine was 1.1:1 and 0.9:1 for Peaks I and II, respectively. This suggests that phosphopantothenate is present as phosphopantetheine. Following results may eliminate the possibility that phosphopantetheine was derived from CoA adventitiously bound to the enzyme protein; (1) previous thorough dialysis of enzyme solution against 8 M urea–1 mM dithiothreitol had no effect on the yield of pantothenate. (2) When CoA was subjected

Table 1
Phosphopantetheine content of three components of bacitracin synthetase

| Enzyme fraction | Amino acid activated | Mol. wt | Purity (%) | Phosphopantetheine (mol/mol fraction) |
|---|---|---|---|---|
| Component I | L-Ile, L-Cys L-Leu, L-Glu | 200 000 | 70 | 1.3 |
| Component II | L-Lys, L-Orn | 2[illegible]0 000 | 40 | 0.6 |
| Component III | L-Ile, L-and D-Phe, L-His L-Asp, L-Asn | 380 000 | 40 | 1.3 |

*Components II and III were derived from Peak II, therefore the purity of Peak II was about 80% as to bacitracin synthetase.

to alkali treatment in 1 N NaOH at 105°C, phosphopantothenate liberated from CoA increased linearly with time and about 30% of CoA was hydrolyzed in 4 hr. With enzyme protein, however, almost all phosphopantothenate was liberated within 1 hr under the same conditions.

## 4. Discussion

The present results revealed that bacitracin synthetase was composed of three components being dissociated under a mild condition, Frøyshov also reported the same result [15]. A minor protein band having only the L-phenylalanine- and L-isoleucine-activating activity was detected in disc electrophoresis as shown in fig.4, suggesting a possible separation of bacitracin synthetase complex into further smaller components.

Each of the three Components proved to possess about one equivalent of 4′-phosphopantetheine. However, the possibility that bacitracin synthetase contained originally one phosphopantetheine-containing protein and this protein associated at random with the three Components could be ruled out by the following results. When a Sephadex G-200 fraction, on the purification step previous to hydroxyapatite column chromatography [2] and this containing all of the three Components, was assayed for pantothenate, about three equivalents of pantothenate were detected, assuming a mol. wt of 800 000 for whole bacitracin synthetase.

From the results presented here as well as those in gramicidin S and tyrocidine systems [4,5], it may be generally said that in antibiotic polypeptide synthetase each of the enzyme complex, which has more than two active sites and which seems to dissociate from each other under physiological conditions, contains one equivalent of phosphopantetheine.

Present results show that the transpeptidation and transthiolation reactions mediated by enzyme-bound phosphopantetheine arm as demonstrated in gramicidin S and tyrocidine biosynthesis [6,16] probably operate also in bacitracin biosynthesis.

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