A RAPID METHOD FOR THE PREPARATION OF THE THREE ENZYMES OF BACITRACIN SYNTHETASE ESSENTIALLY FREE FROM OTHER PROTEINS

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1. Introduction

Previous work by Frøyshov [1] and by Ishihara et al. [2] has shown that bacitracin synthetase from *Bacillus licheniformis* consists of three complementary enzymes, A, B and C. Enzyme A activates isoleucine, cysteine, leucine and glutamic acid, enzyme B activates lysine and ornithine and enzyme C activates phenylalanine, histidine, aspartic acid and asparagine. The present work describes a convenient procedure for preparing each of the three enzymes almost free from other proteins. The present procedure avoids time-consuming procedures and methods of limited capacity such as sucrose gradient centrifugation and polyacrylamide gel electrophoresis. When using the 43–53% $(NH_4)_2SO_4$-fraction of the cell-free extract, the subsequent procedure involves fractionation on an Ultrogel ACA 34 column which separates bacitracin synthetase into two fractions containing, respectively, A + C and B, followed by fractionation of each of the two fractions on hydroxyapatite.

2. Materials and methods

2.1. *Growth of* B. licheniformis

This was carried out as described [3].

2.2. *Preparation of bacitracin synthetase*

The cells were lysed with the aid of lysozyme and deoxyribonuclease [4]. 0.5 mM Paramethylsulphonylfluoride was added to prevent the action of proteolytic enzymes. An ammonium sulphate fraction (43–53% saturation) was prepared as described [4].

2.3. *Fractionation on Ultrogel ACA 34*

The 43–53% $(NH_4)_2SO_4$ precipitate (125 mg from 8 litre culture) was dissolved in 5 ml buffer A (pH 7.4, which contains 50 mM potassium phosphate, 0.2 mM $MgCl_2$ and 0.1 mM DTT) and put on a column (3 × 100 cm) equilibrated with buffer A containing 10% glycerol, and eluted at a rate of 35 ml/h (see fig.1).

2.4. *Fractionation on hydroxyapatite*

The columns used (1.5 × 7 cm) were equilibrated with buffer B (pH 6.8, which contains 100 mM potassium phosphate, 0.2 mM $MgCl_2$ and 0.1 mM DTT). The rate of elution was 25 ml/h.

2.4.1. Fractionation and purification of enzymes A and C

Fractions 69–79 (45 ml) from the Ultrogel column (fig.1) were put directly on the hydroxyapatite column. The column was eluted with 100 ml buffer B followed by a 350 ml linear phosphate gradient from 100 (buffer B) –300 mM.

2.4.2. Purification of enzyme B

Fractions 82–94 (50 ml) from the Ultrogel column (fig.1) were put directly on the hydroxyapatite column. The column was eluted with 100 ml 150 mM phosphate buffer followed by a 150 ml linear phosphate gradient from 150–300 mM.

2.5. *Concentration of fractions*

Pooled fractions of enzymes A, B and C were concentrated by ultrafiltration against buffer A at 0°C using a Sartorius membrane filter.

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2.6. *Estimation of ATP–[^{32}P]PP_i exchange reaction and bacitracin synthesis*

This was carried out as described [4].

2.7. *Polyacrylamide gel electrophoresis*

Gels containing 4% acrylamide, 0.28% *N,N'*-methylene-bisacrylamide, 0.04 mM sodium phosphate buffer (pH 7.0) polymerized with Temed and persulphate were used. Gels were run in the above buffer at 2 mA/gel for 15 min, then at 5 mA/gel for about 4 h, stained in 2.0% Coomassie brilliant blue 250 at 65°C for 30 min and destained in methanol/acetic acid/H_2O (2 : 1 : 7).

All gels were scanned at 600 nm with a Gilford instrument.

2.8. *Protein estimation*

Protein was estimated by the method of Schaffner and Weissman [5]. Bovine serum albumin was used as a standard.

2.9. *Estimation of bacitracin synthesis*

This was carried out as described [3].

2.10. *Labelled substances and measurement of radioactivity*

L-[U-^{14}C]Isoleucine and $Na_4{}^{32}P_2O_7$ were obtained from New England Nuclear Corp., Boston, and from the Radiochemical Centre, Amersham. For counting a Packard Tri-Carb Liquid Scintillation counter or a Frieseke and Hoepfner gasflow counter was used.

2.11. *Other materials*

Ultrogel ACA 34 was obtained from LKB-Produkter AB, Bromma, Sweden. Hydroxyapatite Bio-Gel HTP was obtained from BIO-Rad Lab., Richmond, CA.

3. Results and discussion

The gel filtration of the 43–53% $(NH_4)_2SO_4$ fraction on an Ultrogel ACA 34 column is shown in fig.1. By pooling fractions as indicated, it is possible to achieve considerable separation of enzymes A + C from B. These two fractions are separated from aminoacyl tRNA synthetases which appear further in the elution. The separation of enzymes A and C on a

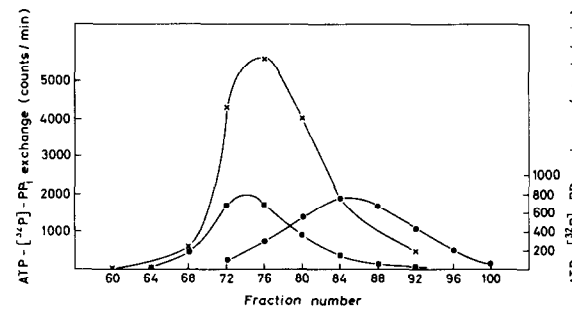

Fig.1. Fractionation of the 43–53% $(NH_4)_2SO_4$ fraction (125 mg) on Ultrogel ACA 34 in buffer A containing 10% glycerol. For details see Materials and methods. Each fraction was monitored for the presence of enzyme A, B and C using the [^{32}P]PP_i–ATP exchange reaction in the presence of cysteine (X ——X) enzyme A, ornithine (•——•) enzyme B and histidine (▪——▪) enzyme C, respectively. Fractions 69–79 were pooled to give enzyme A + C. Fractions 82–94 were pooled to give enzyme B.

hydroxyapatite column is seen in fig.2. It appears that this column permits separation of enzymes A and C. The elution diagram of enzyme B on a similar column is shown in fig.3. The purity of the three enzymes was examined by polyacrylamide gel electrophoresis. The gels obtained were scanned and the diagrams are shown in figs 4A, 4B and 4C. It is seen from fig.4A that enzyme A is almost free from other proteins. The

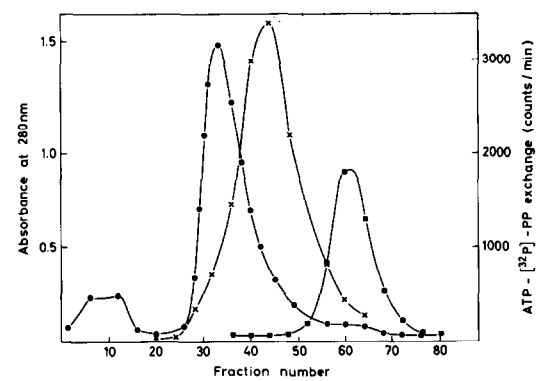

Fig.2. Fractionation of enzymes A + C from the Ultrogel column using a phosphate gradient on hydroxyapatite. For details see Materials and methods. Each fraction (4.5 ml) was monitored for enzyme A (X ——X) and C (▪——▪) as described in fig.1. Fractions 38–52 were pooled to give enzyme A and fractions 56–67 to give enzyme C. A_{280} nm (•——•) was recorded.

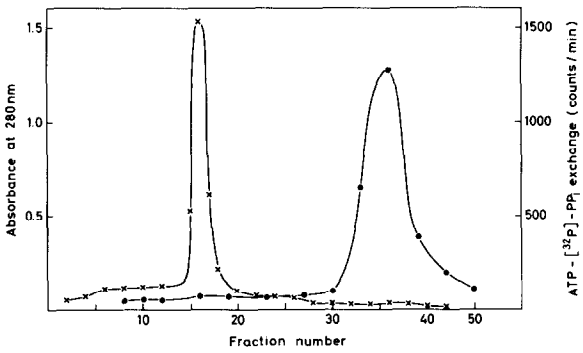

Fig.3. Fractionation of enzyme B (5.5 mg) from the Ultrogel column using a phosphate gradient on hydroxyapatite. For details see Materials and methods. Each fraction (4.5 ml) was monitored for enzyme B (•——•) as described in fig.1. Fractions 32–39 were combined. A_{280} nm (X ——X) was recorded and the phosphate gradient is indicated.

small slower moving peak is probably a dimer of enzyme A. Enzyme B (fig.4B) contains small amounts of enzyme A and C and of an unidentified component. Enzyme C contains small amounts of enzyme A. The degree of cross contamination can easily be reduced by pooling fewer fractions after Ultrogel fractionation and by hydroxyapatite chromatography. The yield of enzyme A, B and C from 8 litre culture using the procedure described in figs 1, 2 and 3 was 2.4 mg, 0.3 mg and 1.3 mg, respectively.

In our hands the present procedure, which only takes about 3 days, has proved to be very reproducible.

It is seen from fig.2 that the ultraviolet absorption at 280 nm in enzyme A peak is high compared to the amount of protein present. It was found that enzyme A contained non-dialyzable material having maximum absorption at 260 nm. It does not show up on polyacrylamide gel after staining. The material is most likely traces of nucleic acids. The content of this material could be reduced considerably by pooling only fractions 45–48 (see fig.2).

Each of the three enzymes of bacitracin synthetase contains a number of catalytic activities all required for the synthesis of bacitracin. It is therefore difficult to achieve significant purification and to retatin synthesizing activity. If only one of the many activities is destroyed, synthesis is abolished. In the present case the three enzymes obtained will not make bacitracin but have retained their ability to activate all the appropriate amino acids (results not shown) and should be useful for the study of subunits' physical chemical properties.

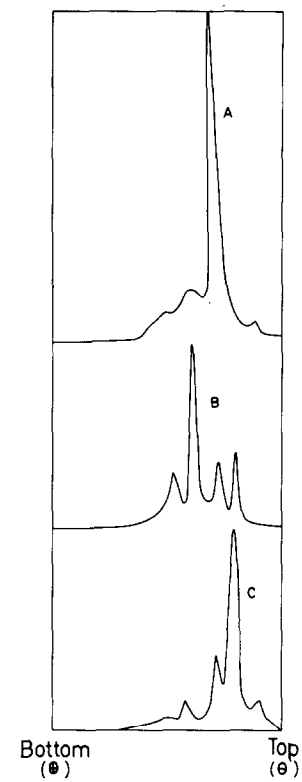

Fig.4. Scan of polyacrylamide gel electrophoresis in phosphate buffer, pH 7.0, of enzyme A, B and C.

Acknowledgement

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References

[1] Frøyshov, Ø. (1974) FEBS Lett. 44, 75–78.
[2] Ishihara, H., Endo, Y., Abe, S. and Shimura, K. (1975) FEBS Lett. 50, 43–46.
[3] Frøyshov, Ø. (1977) FEBS Lett. 81, 315–318.
[4] Frøyshov, Ø. and Laland, S. G. (1974) Eur. J. Biochem. 46, 235–242.
[5] Schaffner, W. and Weissman, C. (1973) Anal. Biochem. 56, 502–514.