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AFFINITY CHROMATOGRAPHY OF β-LACTAMASES

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SUMMARY

β-Lactamase (penicillin amido-β-lactamhydrolase, EC 3.5.2.6) will bind affinitively to adsorbent-containing covalently-bound ampicilline. It can be released by a gradient of NaCl with a resultant 500-fold purification.

INTRODUCTION

As bacteria are becoming more and more resistant to antibiotics, the effectiveness of these substances is decreasing. Very often such bacteria are producing enzymes which chemically modify the antibiotics, so their knowledge is one of the fundamental aspects of antibiotherapy. β-Lactamases (penicillin amido-β-lactamhydrolase, EC 3.5.2.6) are the enzymes which are able to cleave one amide bound from penicillins and cephalosporins and make the penicilloic acids thus obtained completely inactive.

The general potential of affinity chromatography in enzyme isolation and purification is now well recognized[1–3] and has proved its efficiency in many circumstances.

This paper describes the preparation and the effectiveness of an affinity column in the purification of a β-lactamase involved in bacterial resistance to penicillins and cephalosporins.

EXPERIMENTAL

Materials and methods

Indubiose ACA-3/4 and polyacrylamide gel plates for electrophoresis are supplied by l'Industrie Biologique Française. *Escherichia coli* is K_{12} and has received the R factor character by transfers from another *E. coli* resistant to ampicillin, penicillin G and penicillin V. The titration of the enzyme is performed with a Mettler (DK serial) pH stat.

Preparation of the affinity column

Indubiose ACA-3/4 (2 g dried adsorbent) in 50 ml distilled water is treated

three times at 20 °C overnight with 10 ml of a 25% aqueous solution of glutaraldehyde, the pH being kept at 11 by continual addition of 1 M NaOH (ref. 4). The adsorbent is then extensively washed with water (4 l) to remove the excess of glutaraldehyde and resuspended in 100 ml of distilled water. This mixture is gently rocked with 0.4 g ampicillin trihydrate at 4 °C for 48 h, the pH being kept at 7. It is then well washed with water (8 l). A 200 mm × 8 mm column is filled with this adsorbent and equilibrated with an aqueous solution of 0.01 M NaCl.

Microanalysis shows that activated indubiose has fixed about 1.5 μmoles of antibiotic per gram of dried resin.

Preparation of the crude enzymatic extract

The recipient *E. coli* K_{12} are grown in 50 ml yeast extract, bactotryptone, glucose medium, harvested in the late logarithmic phase of growth by centrifugation and washed with 0.1 mM NaCl solution. After a second centrifugation the pellets are suspended in 0.1 M NaCl and disrupted by sonication, the cell debris being removed by an additional centrifugation at 20 000 rev./min for 30 min. The supernatant is collected and applied to the column.

Running of the column

The running of the column is performed as follows: 0.01 M NaCl (25-ml/h fractions of 6 ml) is first used as an irrigant for the elution of "nonspecificic binding proteins". 1 M NaCl eluates the enzyme afterwards as shown in Fig. 1. The course of the chromatography is followed by continual determination of the adsorption at

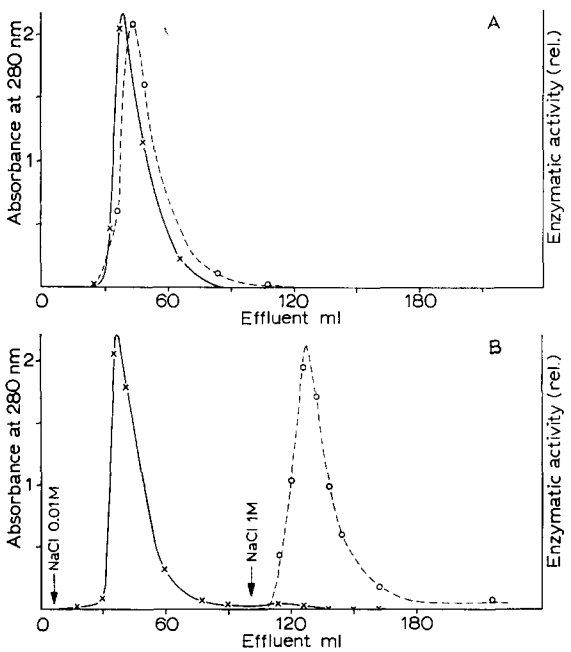

Fig. 1. Elution patterns of β-lactamase on indubiose (A) and indubiose ampicilline (B). ×—×, absorbance at 280 nm; ○— — —○, enzymatic activity.

280 nm (continuous line in the figure). The hydrolytic activity is monitored (dotted line) by the microacidimetric method which will be extensively described elsewhere[5].

In a thermostated cell at 37 °C, we introduce 9.5 ml of a water solution containing 5 g NaCl and 50 μg ampicillin per l. After the pH has been adjusted to 7.00 and 0.5 ml of the chromatographic fraction to be tested is added, the titration is automatically performed with a $5 \cdot 10^{-3}$ M NaOH solution in water.

Polyacrylamide gel electrophoresis

The chromatographic fraction where the maximum enzymatic activity is found, is concentrated by ultrafiltration and then examined by polyacrylamide gel electrophoresis. The sample is applied to a strip of polyacrylamide gel then electrophoresed at 10 mA/180V for 3 h at 4 °C. The buffer used is 0.01 M Tris–0.3 M glycine, pH 8.7. Proteins are stained with 0.12% Coumassie blue in 5% acetic acid and destained with the acetic anion-exchange resin Dowex 1-X1 in 5% acetic acid in water (with gentle rocking for 3 h).

RESULTS

Purification of the enzyme

It is clear from a study of Fig. 1 that, under the conditions used, indubiose alone is insufficient to purify the enzyme as the maximum hydrolytic activity measured is coincident with the maximum concentration of protein eluted. On the other hand, when the ampicilline-bound indubiose column is used it is evident that an excellent purification is obtained as the maximum enzymatic activity is found in fractions that contain no detectable protein.

The purification factor for this enzyme is about 500.

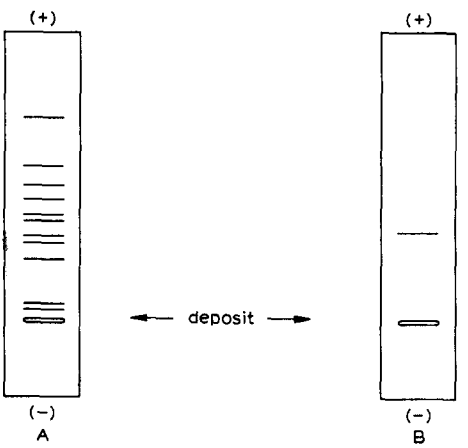

Fig. 2. Polyacrylamide gel plates electrophoresis of β-lactamase. A. Fraction of partially purified enzyme by chromatography on indubiose. B. Peak fractions of purified enzyme after chromatography on indubiose–ampicillin column.

Electrophoresis characterisation

Polyacrylamide electrophoresis reveals 1 band when the enzyme is purified by

affinity chromatography, whereas the material obtained after purification on the indubiose column is revealed as a complex mixture.

DISCUSSION

The ampicillin-bound indubiose column selectively adsorbs only penicillinase. The enzyme can be eluted in good yield by a 1 M NaCl solution. Our method leads to a complete separation of penicillinase as shown by electrophoresis and proves to be much more efficient and simpler than gel filtration on Sephadex G 100 (ref. 6) or the multi-step procedure of Richmond[7]. The purification factor is about 500 which has never been obtained before in a one-step procedure. This new technique might have of course other interesting implications: the β-lactamases produced by different bacteria and the exo-penicillinases mediated by chromosomal genes or plasmid genes in the same strain of bacteria are now easily available in a pure form which makes their comparison much more accurate.

An additional interest of this column is its probable use for the pinpointing of the receptor of β-lactamase antibiotics.

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