PURIFICATION BY AFFINITY CHROMATOGRAPHY OF AN ENZYME INVOLVED IN GENTAMICIN INACTIVATION

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1. Introduction

It is now well known that there are enzymes found in strains of bacteria carrying resistance (R) factors which can inactivate many of the aminoglycoside antibiotics [1].

One enzyme in particular adenylates gentamicin, kanamicin and tobramicin [2, 3] and has provided the basis for a rapid and sensitive assay for gentamicin in biological fluids [4, 5].

We believe that the technique reported here might lead to a faster, simpler and more efficient method of purification than the multistep chemical process used for this kind of enzyme and others which are able to inactivate aminoglycoside antibiotics.

An additional interest of this method is its probable application to the efficient isolation of several inactivating enzymes produced by the same bacteria and ribosomal units which are the targets of such drugs.

It is well established that the close proximity of small active molecules to the matrix leads to an adsorbent of low efficiency probably because of a reduction in the likelihood of complementary specific sites being brought into sufficiently close contact. A spacer is often necessary to overcome this difficulty.

In view of the ease of the process, however, and the thread-like shape of the aminoglycoside antibiotics, we studied the coupling of gentamicin C 1 and kanamicin A to indubiose 4 A (agarose beads). The efficiency of the column so obtained was tested in the purification of an acetyltransferase involved in gentamicin inactivation.

This enzyme had been first identified by Davies [6] in *Pseudomonas* AE *ruginosa* which inactivated gentamicin and sisomicin but not kanamicin A. The same resistant character was then found by Witchitz [7] in other strains which transfered this resistance to *E. coli*. We also found [8] the same gentamicin transmissible resistance in a *Klebsiella pneumoniae* and the recipient *E. coli* K_{12} so obtained was used to produce the enzyme.

2. Material and methods

Indubiose 4 A has been kindly supplied by l'Industrie Biologique Française, the gentamicin by les Laboratoires Unilabo and the Kanamicin by les Laboratoires Bristol. *E. coli* was K_{12} and had received the R-factor character by transfer from a *K. pneumoniae* strain resistant to gentamicin and sisomicin.

2.1. *Preparation of the columns*

Indubiose 4 A (5 g of dried adsorbent) in 100 ml water was treated at 20° with 5 g cyanogen bromide, the pH being kept at 11 by continual addition of sodium hydroxide 8 M. The adsorbent was then washed very quickly with a litre of sodium carbonate (8.2 g) and sodium bicarbonate (10.6 g) buffer and treated with either gentamicin C_1 (0.5 g) or kanamicin A (0.5 g) in 100 ml of the same buffer. The resulting suspension was gently rocked overnight at 0° (pH 9.5 to 10) then washed extensively with water [9, 10]. A 20 × 300 mm column was filled with the adsorbent and equilibrated with sodium chloride 0.05 M.

Microanalysis showed that indubiose had fixed about 0.015 mmole of antibiotic per gram of dried adsorbent.

2.2. *Preparation of the crude enzymatic extract*

The *E. coli* K. 12 carrying the R factor was grown in 100 ml yeast extract tryptone medium, harvested in the late logarithmic phase of growth by centrifugation, washed with 10 mM Tris, 50 mM NH_4Cl, pH 7.8 (at 4°). The pellets were resuspended in the same buffer and disrupted by sonication, the cell debris being removed by centrifugation at 18 000 rpm for 30 min. The supernatant fluid was collected and applied to the column.

2.3. *Purification of the enzyme*

The elution of the enzyme (25 ml/hr) was performed by means of a gradient of sodium chloride from 0.05 M to 2 M, the course of the chromatography being followed by continual determination of the absorption at 280 nm. (Solid line in the figure). The acetylating activity was monitored (dashed line) by using [^{14}C]acetyl coenzyme A([^{14}C]AcCoA) and gentamicin C_1 as previously described [11]. The reaction mixture contained 50 µl of eluted enzyme, 10 nmoles [^{14}C]AcCoA (specific activity 2.65 µCi/µmole), 4 nmole gentamicin C_1, 10 nmole of Tris (pH 6.6 at 25°), 10 nmole $MgCl_2 \cdot 6\,H_2O$ and 2 µmoles of 2-mercaptoethanol in a total volume of 65 µl. Incubation was at 30° for 20 min after which 20 µl was pipetted on to a 1 cm square of phosphocellulose paper (Whatman P81). The squares were immersed in hot, distilled water (70 to 80°) for 2 min to stop the reaction and remove any radioactivity that was not bound to the antibiotics. They were then washed several times with large volumes of distilled water, dried and counted in an Intertechnique SL 31 scintillation spectrometer. Control reactions for non specific binding of [^{14}C]AcCoA to the paper were run in the absence of either enzyme or antibiotic.

3. Results

It is clear from a study of the figure that, under the conditions used, indubiose alone is insufficient to purify the enzyme as the maximum of radioactivity measured is coincident with the maximum concentration of protein eluted. On the other hand when either the gentamicin or kanamicin bound indubiose column is used it is evident that an excellent purification is obtained as the maximum radioactive incorporation occurs with fractions that contain no detectable protein.

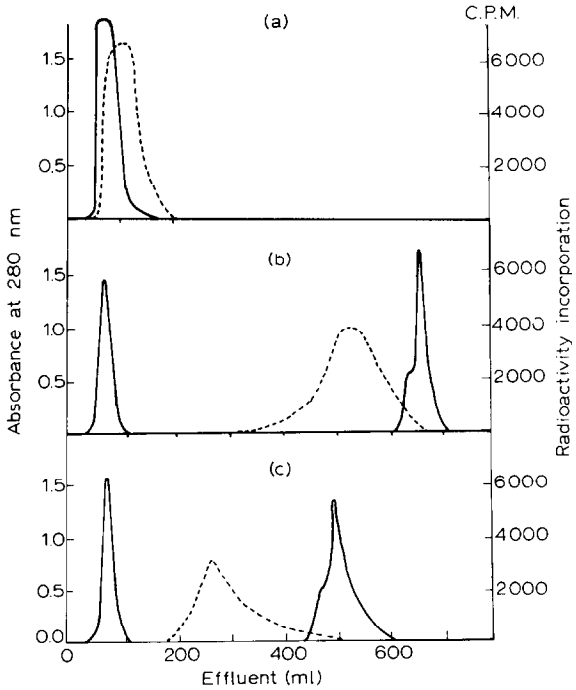

Fig. 1. Elution patterns of acetyltransferase on indubiose (A); indubiose–gentamicin (b); indubiose–kanamicin (c). Absorbance at 280 nm (——); enzymatic activity (– – –).

The figure also shows that, with the antibiotic treated columns, product is eluted after the enzyme. It is possible that this peak is due to a mixture of ribosomal fragments as it is not observed when indubiose alone is used.

4. Discussion

It is evident from the results that affinity chromatography is a simple and efficient method for purifying the enzyme involved in resistance phenomena to aminoglycoside antibiotics. Its use appears to be very broad as an aminosidic antibiotic like kanamicin A, which is neither a substrate nor an inhibitor of this enzymatic reaction, is apparently as efficient a substrate as gentamicin C_1. It is important to emphasize

that the conditions of elution are critical.

Experiments aimed at optimizing conditions and extending this procedure to the purification of other enzymes responsible for bacterial resistance are now under way.

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