

A NEW RAPID AND POWERFUL TECHNIQUE TO OBTAIN PURIFIED RIBOSOMES

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1. Introduction

We recently developed a technique based on affinity chromatography [1–3] for the pinpointing of enzymes involved in the inactivation of gentamicin and other analogous antibiotics.

After elution of the enzyme we isolated a fraction with a high U.V. absorption at 280 nm. The present paper deals with the characterization of this fraction.

2. Materials and methods

Indubiose A 4 has been kindly provided by l'Industrie Biologique Française, gentamicin by the laboratoires Unilabo, streptomycin by the laboratoires Roger Bellon. Poly-uridilic Acid and tRNA were from Calbiochem and ³H-labelled phenylalanine from the Centre d'Energie Nucléaire de Saclay. The strains used are an *E. Coli* K 12, CP 78, Thy (–), Leu (–), His (–), Thr (–), Arg (–), SM R and an *E. Coli* MRE 600 deficient in ribonuclease I provided by Dr Springer Mathias (Institut de Biochimie physico-chimique PARIS).

2.1. Preparation of the columns

Indubiose 4 A (5 g of dried adsorbent) in 100 ml water is treated at 20°C with 5 g cyanogen bromide, the pH being kept at 11 by continuous addition of sodium hydroxide 8 M. The activated adsorbent is then washed very quickly with a litre of sodium carbonate (10.6 g)–sodium bicarbonate (8.2 g) buffer and treated with 0.5 g streptomycin or gentamicin

sulphate in 100 ml of the same buffer. The resulting suspension is gently rocked overnight at 0°C (pH 9.5 to 10) then washed extensively with water. A 20 × 300 mm column is filled with the adsorbent which is extensively washed with water then equilibrated by 10 mM Tris–HCl, 10 mM MgCl₂, 60 mM NH₄Cl, 6 mM β-mercaptoethanol pH 7.4 (buffer A).

2.2. Preparation of the ribosomal extract

2.2.1. *E. Coli* MRE 600

E. Coli MRE 600 are grown in 3 l of trypticase Soy Broth medium at 37°C, harvested in the late logarithmic phase of growth by centrifugation, washed at 4°C with Tris–HCl, 10 mM magnesium acetate, 40 mM NH₄Cl pH 7.4, then centrifuged at 23 000 g for 20 min. Ten g of the frozen cells are disrupted in the usual way by alumina grinding then extracted with 40 ml of buffer A. Cell debris and the alumina are removed by centrifugation at 30 000 g for 20 min. An aliquot (15 ml) of the supernatant fluid thus obtained is used for the chromatography which will be described later; the other part of the supernatant is separated by ultracentrifugation at 4°C for 3 hr (IEC B-60 centrifuge: 110 100 g). The upper part two thirds of the supernatant solution (S 100) are collected and divided into 1 ml fractions which are frozen and stored at –20°C. The residual pellet (crude ribosomes) is suspended in 5 ml buffer A and centrifuged at 30 000 g for 30 min to remove some residual cell debris. The corresponding supernatant is centrifuged at 100 000 g for 3 hr, the ribosomes are collected, suspended in 1 ml of buffer A and frozen at –20°C.

Ribosome concentration is calculated from O.D. 260 nm assuming 60 $\mu\text{g/ml/O.D. unit}$.

2.2.2. *E. Coli* K 12

E. Coli K 12 CP 78 are grown as described for *E. Coli* MRE 600. After the cells have been harvested by centrifugation they are disrupted as follows: 12 g of the cells are suspended in 100 ml 10 mM Tris-HCl, 10 mM NaCl pH 7.5 and treated at 0°C for 45 min with 10 ml of a 4 mg/ml solution of lysozyme in 0.1 mM EDTA pH 7.0. The resulting mixture is frozen at -20°C and then diluted with 10 ml distilled water. The cell debris is removed by centrifugation at 20 000 *g* for 30 min and the supernatant is used in the subsequent experiments.

2.3. Affinity chromatography of the crude ribosomal fractions

Affinity chromatography is performed in the cold (+ 4°C) and followed by continuous determination of the U.V. absorption at 206 and 254 nm (LKB Instrument Uvicord III). The speed of the elution is 60 ml per hour and the fractions are of 10 ml. After equilibration with buffer A, the crude ribosomal extracts (MRE 600, CP 78), as prepared in section 2.2.1. and 2.2.2. are applied to the columns. Elution is performed with buffer A until no further protein absorption at 280 nm is observed. A gradient of NH_4Cl from 0.06 to 1.3 M in buffer A is then applied. The ribosomal fractions (detectable U.V. absorption at 254 nm) are collected by ultrafiltration (Amicon MX 20 membrane) and frozen at -20°C. Protein and nucleic acid concentration is determined as described in section 2.2.1.

2.4. SDS polyacrylamide gel electrophoresis

Electrophoresis is conducted as described by Traut [4]. The ribosomal fraction (50 μl : O.D.₂₆₀ = 30) is treated for 2 min at 100°C in 100 μl of a 0.1% SDS, 1% β -mercaptoethanol, 0.01 M Na phosphate buffer (pH 7.1). The samples thus obtained are applied to the gels (10% acrylamide, 0.3% bisacrylamide, 0.1% SDS in 0.1 M Na phosphate solution (pH 7.1) and the electrophoresis is performed in 0.1% SDS, 0.1 M Na phosphate buffer (pH 7.1). The gels are stained with Coomassie blue and destained according to the procedure of Weber and Osborn [5].

2.5. *In vitro* polyphenylalanine synthesis

One mM Tris-HCl, 0.32 mM MgCl_2 , 2 mM NH_4Cl , 0.02 mM ATP, 0.006 mM GTP, 0.07 mM phosphoenol pyruvate, 25 mg t RNA of *E. Coli* B are dissolved in 10 ml distilled water (pH 7.4).

100 μl of the former solution, 10 μg of pyruvate kinase, 40 μl S 100 fraction of section 2.2.1. and limiting amounts of ribosomes are preincubated at 37°C for 30 min then 50 μg poly U, 10 nmoles ^3H -labelled phenylalanine (specific activity: 50 $\mu\text{Ci/ml}$) are added. Incubation is carried out at 37°C for 30 min and the reaction is stopped by addition of 1 ml 1 M NaCl and 1 ml 10% trichloroacetic acid (TCA). The resulting mixture is heated at 100°C for 15 min and filtered on GF/C (glass filter paper Whatman) filters. Each filter is well washed with 3% TCA and dried. Polymerisation is estimated by determination of bound radioactivity using an Inter-technique S.L. 30 scintillation spectrometer. The scintillation mixture is composed of 5 g of 2,5-diphenyloxazole and 0.1 g 1,4 bis (4-methyl 5-phenyl oxazolyl)-benzene per litre of toluene. At least two blanks, with either ribosomal fractions or poly U omitted, are carried through the entire experimental procedure.

3. Results

During the purification by affinity chromatography of enzymes involved in the inactivation of aminoglycoside antibiotics, a fraction with high affinity for the column was isolated [1-3]. This fraction was assumed to be ribosomal as the target for these drugs is the ribosome. Using a more sophisticated method for detection, we now obtain four profiles from the two ribosomal extracts (MRE 600 and CP 78 strains) when the purification is performed on either insolubilized streptomycin or insolubilized gentamicin (figs. 1 and 2).

Since electrophoresis shows that the MRE 600 ribosomal fractions isolated either by chromatography or by ultracentrifugation are closely similar, we compared their ability to induce the polymerisation of ^3H -labelled phenylalanine in the presence of poly U as template.

Fig. 4 shows that the fraction purified by chromatography is much more efficient in inducing polymerisa-

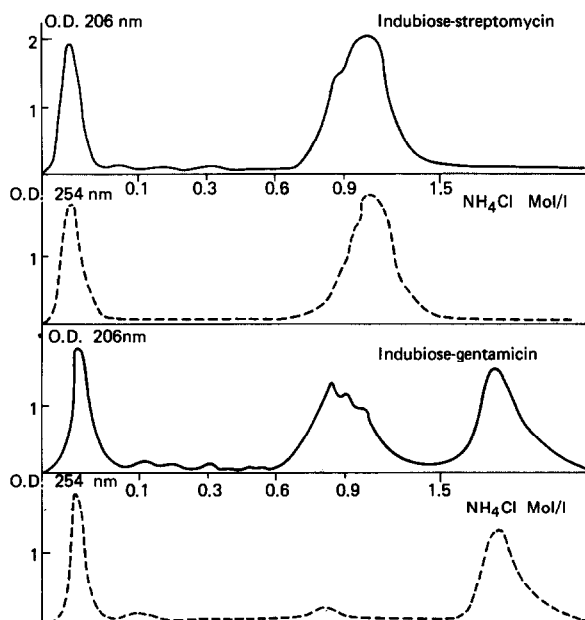


Fig. 1. Elution pattern of MRE 600 extract on indubiose streptomycin (A); indubiose gentamicin (B). Absorbance at 206 nm (—); absorbance at 254 nm (---).

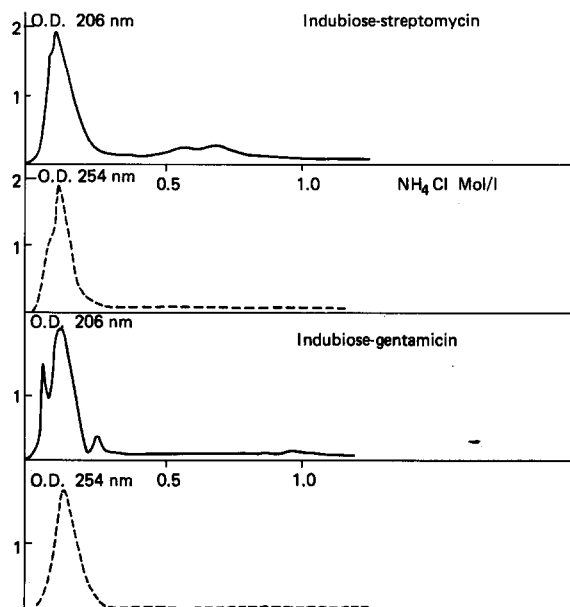


Fig. 2. Elution pattern of CP 78 extract on indubiose streptomycin (A); indubiose gentamicin (B). Absorbance at 206 nm (—); absorbance at 254 nm (---).

tion of ^3H -labelled phenylalanine when compared with ribosomes prepared by centrifugation. This result thus confirms that the column fraction indeed consists of ribosomes.

4. Discussion

We have demonstrated that the technique of affinity chromatography provides a simple and efficient means for purifying functional ribosomes from crude bacterial extracts. An advantage of the method is that the purification, starting from bacteria, can be completed in one day.

Fig. 1 shows clearly that the elution of ribosomes obtained from strain MRE 600 is retarded when either insolubilized streptomycin or gentamicin is used for the chromatography. The relative efficiency

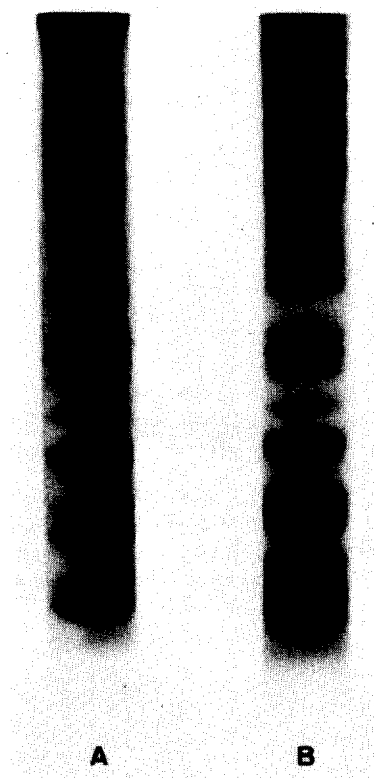


Fig. 3. Electrophoresis pattern of the ribosomal fractions purified by ultracentrifugation (A) or affinity chromatography (B). The electrophoresis is performed applying 10 mA per gel (5 mm \times 60 mm).

of these two resins is quite different, for, with the indubiose bound gentamicin, the ribosomal fraction is eluted with 1.3 M NH_4Cl whereas with the streptomycin bound agarose ribosomes are eluted with 0.6 M NH_4Cl . It is possible that the strength of the binding forces involved in the complex 'insolubilized antibiotic-ribosome' are different, but this is difficult to evaluate for we do not know at all how these antibiotics are bound to agarose beds.

The chromatographic situation is quite different when the crude extract of CP 78 strain is purified on gentamicin or streptomycin resins. It is well known [6] that in such mutants the aminoglycoside antibiotics cannot form a complex with the ribosome, thus there should be no retention by the columns. Our results completely confirm this expectation.

As shown in fig. 3, disc gel electrophoresis patterns of the two ribosomal fractions purified either by

chromatography or by ultracentrifugation are quite similar in the number of proteins. There is however an appreciable difference in their relative proportions. This fact may possibly explain the different efficiencies of these two preparations which are compared in fig. 4.

These results raise some questions.

Is there some explanation for the efficiency of ribosomes isolated by affinity chromatography? Is it possible to apply this new method to the study of the ribosome itself?

Experiments along these lines are now underway in the laboratory.

Acknowledgement

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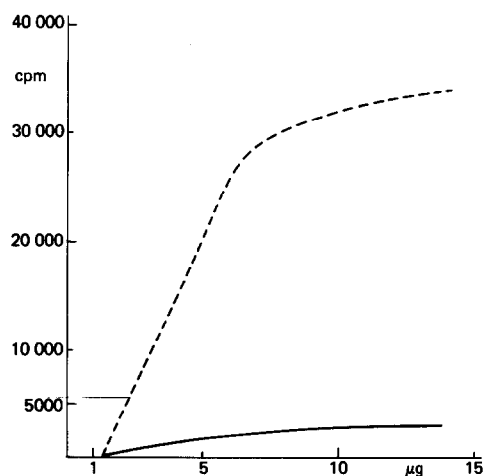


Fig. 4. Poly U directed ^3H -labelled phenylalanine polymerisation. (---) radioactivity incorporation with ribosomes obtained by affinity chromatography; (—) radioactivity incorporation with ribosomes isolated by ultracentrifugation.