AN IMMUNOCHEMICAL METHOD FOR THE CHARACTERIZATION OF POLYNUCLEOTIDE PHOSPHORYLASE FROM E. COLI

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1. Introduction

Enzyme-antibody complexes resolved by gel immunodiffusion have proved useful as an analytical tool for the characterization of enzymes in biological mixtures [1,2]. This paper describes the demonstration of polynucleotide phosphorylases (PNPase) by combining in the same experience the immunochemical assay and the enzymatic reaction.

The principle of the method is the following: after development in agarose of the antigen-antibody precipitates, the immuno-plates are incubated in an appropriate medium containing a radio-labeled nucleotide. The polynucleotide synthesized under the catalytic action of the enzyme remains attached to the PNPase–antibody complexes and can subsequently be visualized by autoradiographic techniques and/or specific staining.

2. Material

2.1. *Crude extracts of E. coli B*

These were prepared by grinding the cells with alumina (1:1, v/v) and centrifuging at 30,000 *g*. The supernatant was dialysed overnight against 0.02 M Tris, pH 8 and stored at −20°C.

2.2. *Purified PNPase*

The preparation used is the side fractions eluted on Sephadex G-200 according to the method described by Williams and Grunberg-Manago [3]. The preparation was stored at −60°C.

2.3. *Anti-coli B antiserum*

Rabbits were inoculated intradermically in the hind foot pad with 0.5 ml of crude extract (14 mg/ml) emulsified in 0.5 ml of complete Freund adjuvant, per animal. The immunization was continued by intramuscular (IM) or intravenous (IV) routes in the following manner: 15th (IM), 16th (IM and 17th (IV) days, 0.5 ml of crude extract; 24th (IM), 25th (IM), 26th (IV), 33rd (IM), 34th (IM) and 35th (IV) days, 0.5 ml of purified PNPase (1.5 mg/ml).

The animals were bled by canulating the internal carotid artery six days after the last intravenous injection.

3. Immunoelectrophoresis

Immunoelectrophoresis was carried out according to the method of Grabar and Williams [4] as modified by Scheidegger [5]. Agarose gels were prepared in veronal buffer 0.025 M, pH 8.2. After development of antigen-antibody precipitates (24 to 36 hr) the plates were washed in buffered saline for several days in the cold room and then dried under filter paper at room temperature [6].

4. Characterization of the antigen-antibody complex

The enzyme-antibody complex was identified by the synthesis of polynucleotide in situ. The incubation mixture contained in millimolar concentration:

Tris, pH 8, 100; $MgCl_2$, 5; EDTA, 0.5; (^{14}C) ADP (specific activity 0.1 $\mu c/\mu mole$), 10. The immunoplates were incubated (approximately 5 ml mixture per plate) for two hours at room temperature. The plates were then removed and washed for 4 to 6 hr in several baths of a phosphate buffer (0.05 M, pH 7) containing 1% of streptomycin.

Proteins were revealed with amido-black, as previously described [6]. The polynucleotide synthesized in situ was revealed by staining with 0.1% solution of toluidine blue and washing in 2% acetic acid. An alternative method consists of staining the plate with acridine orange, as described elsewhere [7]. After destaining, all plates are left to dry.

A further proof to demonstrate polynucleotide synthesis by polynucleotide phosphorylase was obtained by autoradiographic technique. The dried, destained plates were put in contact with Kodirex films (Kodak) during 2 to 3 weeks, depending on the concentration of the sample studied, and/or their initial activity. The films were then developed in the usual way.

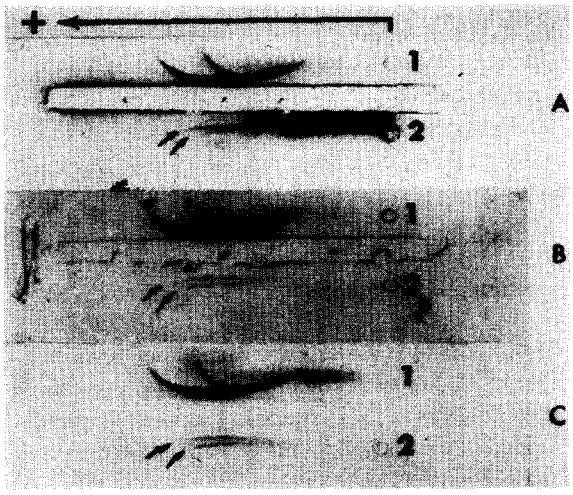

Fig. 1. Demonstration of polynucleotide phosphorylase (PNPase) activity in a purified preparation of PNPase (1) and in crude extract of *E. coli* B (2) after immunoelectrophoresis in agarose gels. Protein stain (A), autoradiography of plate A after three weeks exposure (B) and selective stain of polynucleotides with toluidine blue (C). Two precipitin lines immunologically distinct show enzymatic activity in the purified PNPase preparation. The two small arrows show the localization of PNPase activity among the 10 to 12 antigens revealed by the anti-Coli antiserum on crude extract of *Coli* B (sample 2, in plate A).

A typical assay with *E. coli* crude extract and a purified preparation of polynucleotide phosphorylase is illustrated in fig. 1. Two antigen-antibody precipitates, immunologically distinct, show polynucleotide phosphorylase activity, characterized by the formation of poly A with ADP as substrate. The position of the polymers formed on the immunoplates, revealed either by autoradiogram or by specific staining, can be perfectly superimposed on the position of the proteins.

The behavior of polynucleotide phosphorylase in enzyme-antibody precipitates obtained by gel diffusion is similar to that of many other enzymes [1]: a) the precipitates retain a catalytic activity significant enough to allow their demonstration in complex mixtures, and b) the enzymatic activity seems to be protected from denaturation when the enzyme is complexed with its homologous antibody [2]. Since, under the experimental conditions of the method (drying, standing at room temperature for several days) free solutions of polynucleotide phosphorylase would entirely loose their catalytic properties.

The significance of the presence of two polynucleotide phosphorylase-antiserum complexes and the cross-experiments with heterologous enzymes and antibodies will be discussed in a further communication.

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