DIRECT DETECTION OF PROTEIN KINASES ON ELECTROPHEROGRAMS

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1. Introduction

Since their discovery protein kinases have been usually tested by taking advantage of the solubility of ATP in deproteinizing agents like 10% trichloroacetic acid, in which protein bound phosphorylserine and phosphorylthreonine are quite stable. By such procedures at the end of incubation the excess of [^{32}P]ATP is easily removed from the ^{32}P-labelled protein either by centrifugation followed by several washings [1–3] or by applying the sample on chromatographic paper [4] or on filters [5] which are subsequently eluted by trichloroacetic acid solutions. The counting of the protein residue at the end of both these procedures gives a reasonably precise measure of the amount of ^{32}P transferred by the protein kinase from [^{32}P]ATP to the phosphoprotein.

In the last few years more and more attention has been devoted to protein kinases which have been found to differentiate both for their substrate specificity and for their sensitivity to cyclic nucleotides (see [6]). As expected, investigators make extensive use of analytical gel electrophoresis to characterize protein kinases, though the only known procedure to localize the enzyme activity is to cut the gel into slices and to test the protein kinase eluted from every single segment by one of the methods previously mentioned [5,7,8].

In the present note we shall describe a much less complicated and more rapid procedure which proved quite satisfactory for a direct evaluation of protein kinase activities on both polyacrylamide and acetylcellulose electropherograms. In principle such a method consists in laying down the gel, after electrophoresis, on a strip of chromatographic paper imbibed with a sample containing a buffer system, [^{32}P]ATP with high specific radioactivity, $MgCl_2$ (as activator), and a substrate phosphoprotein like phosvitin. After a short incubation the gel is removed and the paper exhaustively washed with a solution containing 1.0 mM unlabelled ATP in 10% trichloroacetic acid plus silicotungstic acid, in order to remove all the radioactivity due to the labelled ATP, while precipitating the phosphoprotein inside the paper. Finally the zones where the precipitated phosphoprotein had been labelled by protein kinase diffused from the gel are evidenced by a radiochromatoscanner or by autoradiography.

2. Experimental

Rat liver cytosol phosvitin kinase was purified as previously described [9], concentrated by ultrafiltration with Diaflo UM 10 membranes and dialyzed against 500 vol of 0.13 M Tris–HCl buffer pH 8.1 for two hr before electrophoresis.

Polyacrylamide gels at pH 8.1 were prepared according to Kish and Kleinsmith [5] except acrylamide concentration was made 5%. Before adding the enzyme the gels were prerun for 10–12 hr in the cold room at 2.0 mA per gel using 0.13 M Tris–HCl pH 8.1 in both upper and lower buffer chambers.

50 to 100 μl enzyme solution, containing about 2 μg protein, were applied at the top of each gel column and included in 20 mg of Sephadex G-25. A reference gel was also always run where the marker dye Bromophenol Blue was added to the enzyme solution. Electrophoresis was carried out at 4.0 mA per gel, at 4°C for 6 hr, while Bromophenol Blue reached the bottom of the gel after about 4 hr.

Phosvitin kinase activity was determined in the gels by the following two methods:

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A) The gel was cut into 3 mm slices which were eluted for one night in 0.5 ml of 0.1 M Tris–HCl buffer pH 7.0 at 2°C. 0.30 ml of each eluate were assayed for protein kinase activity by incubation at 37°C for 30 min in 1 ml of a reaction mixture containing: 100 mM Tris–HCl buffer pH 7.5; 6 mM $MgCl_2$; 10 μM [γ^{32}P] ATP having a specific radioactivity of about 0.5 μCi/nmol; and 1.0 mg phosvitin prepared according to Mecham and Olcott [10]. The reaction was stopped by addition of 4.0 ml of 10% trichloroacetic acid. Precipitated protein was recovered by centrifugation, washed 4 times with 10% trichloroacetic acid, dissolved in 10 ml of Instagel (Packard) and counted in a liquid scintillator.

B) The gel was put down horizontally in the middle of a 2 × 9 cm strip of Whatman 3 MM chromatographic paper laying on a glass plate and previously soaked with a reaction sample containing: 100 mM Tris–HCl buffer pH 7.5; 6 mM $MgCl_2$; 25 μM [γ^{32}P] ATP having a specific radioactivity of 1.0 μCi/nmole; and phosvitin (5 mg/ml). After 20 min at room temperature (20°–22°) the gel was removed and the reaction blocked by dipping the paper in 20 ml of an ice cold solution just prepared by mixing 10% trichloroacetic acid and silicotungstic acid solution [11] (9:1) and containing 1.0 mM unlabelled ATP. After 10 min stirring the paper was removed and then washed twice more following the same procedure. In order to eliminate any trace of contaminating [^{32}P] ATP it was found convenient to furtherly submit the washed strip to overnight descending chromatography from a small trough filled with trichloroacetic/silicotungstic acid solution. Finally the paper was dried in an oven at 100°C and its radioactivity localized by a Packard Radiochromatogram scanner (mod. 7201), using the 2.5 mm collimator opening.

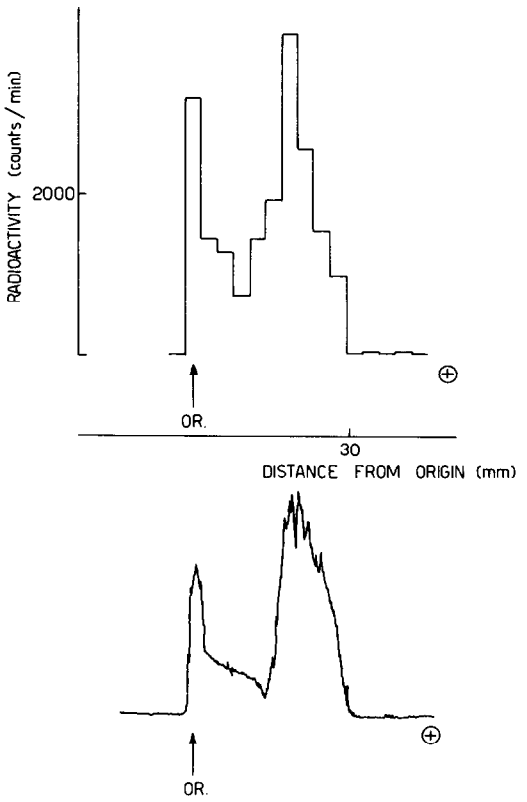

Fig.1. Detection of phosvitin kinase activity on polyacrylamide gel electropherograms. General conditions are described in the Experimental section. Phosvitin kinase activity was determined in two gel columns run together following the procedures A (top figure) and B (bottom figure) respectively.

3. Results and discussion

In fig.1 a typical experiment is described performed by submitting to 5% polyacrylamide gel electrophoresis two identical samples of rat liver cytosol phosvitin kinase [9] and determining the protein kinase acitivity either by slicing the gel, eluting the segments and applying to each sample the traditional procedure (top figure), or by the novel method here described (bottom figure). It can be seen that the two profiles are quite superimposable which demonstrates the reliability of the new method. It is worthy to underline that quite satisfactory results are obtained just by laying the whole gel column as such on the paper strip, the surface of contact between the slightly collapsing gel column and the paper being large enough to allow the diffusion of detectable amounts of enzyme into the reaction mixture wetting the paper. Of course the sensitivity is increased if the gel column is longitudinally sliced and both the resulting half cylinders are parallely layed with their flat surface on the paper. Any pressure on the gel during its contact with the paper must be avoided in order to prevent the shifting of the reacted areas from their original position, due to hydrodynamic effects.

Quite good results have been also obtained by

applying the described method to acetylcellulose electrophoresis (Cellogel RS, pH 8.2) and by replacing phosvitin with casein as substrate phosphoprotein. Moreover the presence of silicotungstic acid in the washing solution should make possible also the use of phosphorylable substrates like protamines which are otherwise soluble in 10% trichloroacetic acid.

In conclusion the procedure described in the present note makes possible the rapid and direct detection of protein kinase activities on gel electropherograms, avoiding the wearing and painstaking analysis of enzymic activity in a very large number of gel segments. Moreover the slicing of the gel along its axis and proper modifications of the reaction mixture will make possible the contemporaneous detection in the same electropherogram of distinct protein kinases, differing, e.g., for substrate specificity or for the requirement of activators.

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