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A procedure for the electrophoretic analysis of phosphoenolpyruvate carboxylase*

ROBERT C. KARN [a], PETER A. KIVIC [b] and GEORGE A. HUDOCK

[a] *Department of Medical Genetics, Indiana University Medical Center, Indianapolis, Ind. 46202 (U.S.A.) and* [b] *Electron Microscope Unit, The University of Sydney, Sydney (Australia)*

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SUMMARY

Phosphoenolpyruvate carboxylase from *Euglena gracilis* was analyzed electrophoretically using an agar suspension of the reaction mixture to detect the enzyme bands. Since the stain is specific for oxaloacetate produced by the enzyme, it should be possible to extend this technique to other enzymes concerned with oxaloacetate metabolism.

Starch gel electrophoresis[1] has been widely employed in studies of enzymes. A specific method of detection is required, however, for the analysis of isoenzymes catalyzing a particular reaction. Detection is usually accomplished by incubating the gel in a reaction mixture containing a dye which forms a colored complex in the presence of the enzyme product. Ideally, the colored complex becomes immobilized on the gel at the reaction site. This report concerns a detection technique which makes possible the study of several anaplerotic enzymes associated with the citric acid cycle.

Babson and co-workers[2] reported a simple method for detecting oxaloacetate utilizing the diazonium dye 6-benzamido-4-methoxy-*m*-toluidine diazonium chloride (referred to here as Fast Violet B). Fast Violet B changes from a faint yellow color to bright red when in solution with oxaloacetate but not with other organic acids common to the citric acid cycle. The dye remains soluble after changing color and the color fades after 1.5 to 2.5 h.

We have modified this method for the electrophoretic assay of phosphoenolpyruvate carboxylase (orthophosphate:oxaloacetate carboxy-lyase (phosphorylating)

*Contribution from the Department of Zoology, Indiana University, Bloomington, Ind. 47401, U.S.A.

EC 4.1.1.31), an enzyme previously not amenable to analysis by electrophoretic techniques

Phosphoenolpyruvate carboxylase[3] catalyzes the reaction:

$$\text{Phosphoenolpyruvate} + CO_2 \rightleftharpoons \text{oxaloacetate} + P_i$$

The solubility of the dye presented a problem since it must be immobilized at the site where it reacts with the enzyme product (oxaloacetate). This difficulty was overcome by suspending the reaction mixture, including the unreacted dye, in a dilute solution of agar which was then spread over the surface of the gel[4].

The enzyme preparation used here was a crude extract of *Euglena gracilis* strain Z prepared by freeze–thawing a thick slurry of cells suspended in 0.1 M Tris buffer, pH 7.5, and removing insoluble cell material by centrifugation at 10 000 x *g* for 30 min at 0 °C. The proteins in this preparation were separated by vertical starch gel electrophoresis[5].

The specific stain employed to detect phosphoenolpyruvate carboxylase activity on the starch gel sheet was a modification of a spectrophotometric oxaloacetate assay[2]. Two mixtures, given in Table I, were prepared shortly beforehand; Solution A was kept at 4 °C and Solution B at 50 °C. The two were rapidly mixed to form the staining solution, which was then immediately poured over the freshly cut gel surface, in as even a layer as possible, and allowed to solidify. The gel was incubated at room temperature until red bands could be seen through the agar layer (usually 30–60 min). The agar was then scraped from the gel surface and the zymogram photographed immediately.

TABLE I

COMPOSITION OF THE SOLUTIONS WHICH WERE COMBINED TO PRODUCE THE STAINING MIXTURE USED TO DETECT PHOSPHOENOLPYRUVATE CARBOXYLASE ON STARCH GEL

Solution A	
0.1 M Tris buffer (pH 7.5), Sigma Chem. Co.	12.5 ml
Fast Violet B, Sigma	62.5 mg
$MgSO_4$	30.8 mg
$KHCO_3$	62.5 mg
Phosphoenolpyruvate, Sigma	50.0 mg
This solution was kept at 4 °C until used	
Solution B	
0.1 M Tris buffer (pH 7.5)	12.5 ml
Agar, Difco	250.0 mg
This solution was heated at 100 °C until the agar dissolved, then kept at 50 °C until used	

A gel incubated with the complete stain mixture shows a single distinct red band (Fig. 1) which lasts for about 2 h. Detection of the enzyme was absolutely dependent on the presence of phosphoenolpyruvate, and greatly enhanced by the presence of $KHCO_3$. The faint pattern obtained in the absence of $KHCO_3$ is probably due to CO_3^{2-} present in the distilled water in which the reaction mixture was prepared.

The value of this new enzyme staining technique should extend beyond the detection of the one enzyme reported here. It can doubtlessly be modified for the analyses

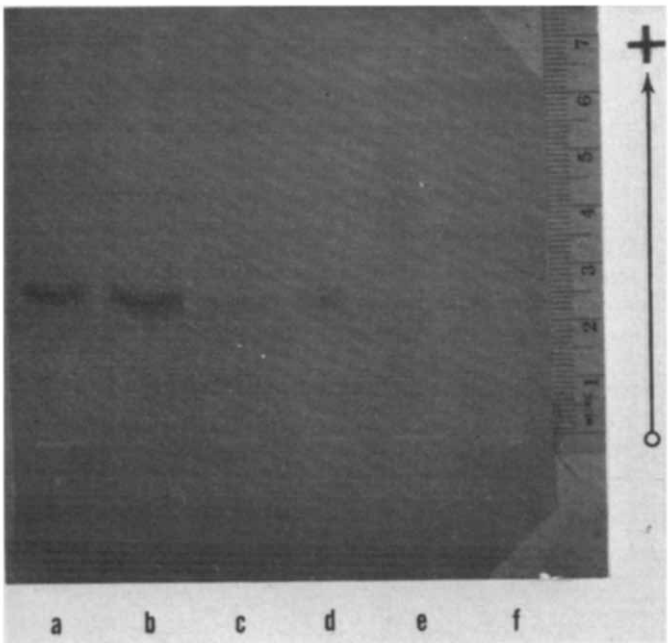

Fig. 1. An electrophoretic starch gel stained for phosphoenolpyruvate carboxylase. (a and b) The single band (red in the original) locates the single phosphoenolpyruvate carboxylase isozyme present in *Euglena gracilis*. (c–f) Two other portions of the same gel, stained with partial reaction mixtures lacking $KHCO_3$ (c and d) and phosphoenolpyruvate (e and f).

of other enzymes involving oxaloacetate, an important component of intermediary metabolism. Several anaplerotic enzymes associated with the citric acid cycle for instance, also produce oxaloacetate[6]. Such studies would help clarify the functional relationships between the citric acid cycle and the various anaplerotic reactions. One such enzyme, malic enzyme, has been shown to be regulated in *E. gracilis* by the balance of metabolites resulting from photomixotrophic growth[5]. It is likely that other anaplerotic pathways are similarly regulated in order to control metabolic balance.

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