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A Method for Activity Staining after Native Polyacrylamide Gel Electrophoresis Using a Coupled Enzyme Assay and Fluorescence Detection: Application to the Analysis of Several Glycolytic Enzymes[1]

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We describe a method for the detection of isoforms of several glycolytic enzymes by activity staining after native PAGE. The staining is based on coupled enzyme assays carried out on the gel after electrophoresis and is linked to the disappearance of NADH, which is visualized by fluorescence. This method offers reliable and sensitive detection for phospho*enol*pyruvate carboxylase, PPi-dependent phosphofructokinase, and pyruvate kinase from plant tissues. It can be applied to the detection of all enzymes which are normally detected spectrophotometrically using coupled enzyme assays consuming NAD(P)H. © 2001 Elsevier Science

Key Words: **native polyacrylamide gel electrophoresis; isozyme; activity stain; phospho*enol*pyruvate carboxylase; pyruvate kinase; PPi-dependent phosphofructokinase; coupled enzyme assay; fluorescence detection.**

Compared with nonplant glycolysis, the plant glycolytic pathway is structured as a complex network of reactions. The plant glycolytic pathway exists in both the cytosol and the plastid, with parallel reactions being catalyzed by distinct isozymes in each compartment (1). Different isozymes of pyruvate kinase (PK, EC 2.7.1.40), for example, are present in both compartments and are involved in distinct metabolic functions (1). For phospho*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31), another important glycolytic enzyme, different isoforms are present in C3 and C4 plant tissues (2). Different PEPC isoforms with contrasting structural and kinetic properties are also present in unicellular green algae (3). Several plant glycolytic enzymes are also expressed in a tissue- or developmental-stage-specific manner (1) or can be induced by environmental stresses such as anoxia or phosphate starvation (1, 4, 5). This often results in a complex situation when it comes to elucidating the quantitative relationships, the respective metabolic functions, and the regulatory properties of different isoforms. Enzyme activity staining after native PAGE is a widely used technique for isozyme analysis. It allows the detection and visualization of multiple isoforms of a specific enzyme present in a small amount of extract. The study of isoforms by native PAGE, however, is often limited by the availability of a specific and sensitive detection method. A number of glycolytic enzymes can be linked to a dehydrogenase and to $NAD(P)^+$ reduction (4). Using this approach, NAD(P)H generation can be visualized by reduction of electron acceptors phenazine methosulfate and nitroblue tetrazolium (which forms an insoluble purple precipitate). This type of activity stain is widely used. However, this approach cannot be used with some enzymes. This is the case for PEPC, which catalyzes an irreversible reaction. In spectrophotometric assays, PEPC activity is measured by a coupled assay linked to malate dehydrogenase (MDH, EC 1.1.1.37) in the oxidizing (NADH consuming) direction, precluding

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[3] Abbreviations used: PEP, phospho*enol*pyruvate; PEPC, phospho*enol*pyruvate carboxylase; PK, pyruvate kinase; PFP, PPi-dependent phosphofructokinase; DTT, dithiothreitol; MDH, malate dehydrogenase; LDH, lactate dehydrogenase.

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the use of nitroblue tetrazolium detection for staining after native PAGE. PEPC can be detected after native PAGE using an activity stain (6–8). This stain is based on the detection of PEPC reaction product oxaloacetate by reaction with Fast Violet B. However, this method has several drawbacks such as long incubation times and lack of sensitivity. For this reason, we found that it can only be used reliably with purified enzymes (7, 8).

We describe below a staining technique that allows the sensitive detection of PEPC. The method is very specific since it is based on the same reaction used for the spectrophotometric determination of the enzyme. We also describe a native PAGE stain for PK and PPi-dependent phosphofructokinase (PFP) based on the same principle. This serves to illustrate that the method can probably be generalized to assay all enzymes that can be linked to an NAD(P)H-consuming coupled enzyme assay.

MATERIALS AND METHODS

Chemicals and plant materials. All chemicals were of analytical grade from Sigma Chemical Co. (St. Louis, MO) or BDH Chemicals (Toronto, Ontario, Canada). Butyl Sepharose Fast Flow, Protein A–Sepharose CL-4B, prepacked Superose 6 HR 10/30, and Mono Q 5/5 columns were from Pharmacia (Baie d'Urfé, Québec, Canada). Fractogel EMD DEAE-650(S) was from VWR Canlab (Mississauga, Ontario, Canada). *Chlamydomonas reinhardtii, Selenastrum minutum,* and *Brassica napus* cells were grown, maintained, and harvested as described (9). Affinity-purified anti-(banana fruit PEPC) antiserum, anti-(developing castor seed endosperm plastidic PK) IgG, anti-(*B. napus* cytosolic PK) antiserum, and anti-(native potato tuber PFP) antisera were obtained as described, respectively, in Refs. (7, 10–12). Alkaline phosphatase-tagged goat anti-(rabbit IgG) was from Promega (Madison, WI). For the purification of recombinant tobacco PEPC, the PEPC-deficient strain of *Escherichia coli* PCR1 harboring the plasmid pTE6 containing a tobacco PEPC cDNA under the control of the *LacZ* promoter was used (13).

Enzyme extraction and spectrophotometric assays. Enzyme extractions were carried out at 4°C as described previously for *C. reinhardtii* PEPC (8), *S. minutum* PEPC (14), *B. napus* PK (11), and *B. napus* PPi-dependent phosphofructokinase (PFP, EC 2.7.1.90) (15). The extracts were kept on ice and used immediately for enzyme assay and native PAGE analysis. All enzymes were assayed spectrophotometrically on a Dynatech microplate reader. *C. reinhardtii, S. minutum,* and recombinant tobacco PEPC activities were assayed using a coupled enzyme assay with MDH, as described before (8). Cytosolic and plastidic PK isozymes (PK_c and PK_p, respectively) were assayed using a coupled enzyme assay with lactate dehydrogenase (LDH, EC 1.1.1.27) (11). PFP was assayed in the forward direction using a coupled enzyme assay with aldolase (EC 4.1.2.13), triose phosphate isomerase (EC 5.3.1.1), and glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) (15). One unit of enzyme activity (U) is defined as the amount of enzyme catalyzing the production of 1 μmol of product/min at 20°C. Protein was determined using the method of Bradford (16) with bovine γ-globulin as a standard.

Purification of recombinant tobacco PEPC. Recombinant tobacco PEPC was purified as described (8). The enzyme preparation was >95% homogenous as judged from SDS–PAGE analysis.

Partial purification of B. napus PK_c and PK_p. Partial purification of PK_c and PK_p from *B. napus* suspension culture was done using polyethylene glycol fractionation and chromatography on butyl Sepharose and DEAE Fractogel columns according to Ref. (11). PK_p and PK_c isozymes were resolved using the DEAE Fractogel column. Peak isozyme activity fractions were pooled separately, frozen in liquid N_2, and stored at −80°C. Final specific activities for PK_c and PK_p were 7 and 1 U/mg protein, respectively. Aliquots of the partially purified fractions were used for native PAGE analysis.

Native PAGE and activity stains using fluorescence detection. Native PAGE was performed on a Bio-Rad Mini Protean II system using a discontinuous system (17) with the modifications described in Ref. (7) except that gels were run for 4 h at 150 V and 4°C. Enzyme activity staining was carried out at room temperature.

For all the coupled enzyme assay/fluorescence detection methods, immediately after electrophoresis, gels were incubated at room temperature in the indicated equilibration buffer (10 ml) for 15 min. This solution was removed and replaced by the indicated development buffer. Staining developed over 5–10 min (actual incubation times are given in the figure legends) and was visualized on a UV transilluminator. Activity bands (sites of NADH oxidation in the gel) appeared as dark bands over a fluorescent background. In all cases, control experiments were carried out in order to verify that enzyme activity staining was substrate-dependent and therefore specific for the activity tested. The gels were routinely photographed using a Polaroid T-667 film (3000ISO/36DIN) and an exposure time of 3 s. Image analysis and quantification were done with ImageJ, a public domain program available from http://rsb.info.nih.gov/ij/. Unless otherwise stated, the solutions used in equilibration and development were as follows: PEPC equilibration buffer, 50 mM Bis–Tris propane (pH 8.4), 2.5 mM $KHCO_3$, 10 mM $MgCl_2$, 5 mM DTT, and 15% (v/v) glycerol; PEPC development buffer, PEPC equilibration buffer plus 1.5 mM PEP, 0.15 mM NADH, and 2 U/ml rabbit muscle MDH; PK equilibration buffer, 50 mM Mes–50 mM Bis–Tris pro-

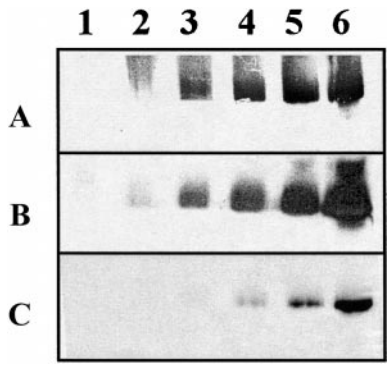

FIG. 1. Detection of recombinant tobacco PEPC after native PAGE by immunoblot and activity stain. The purified enzyme was analyzed by native PAGE and subjected to immunoblot followed by immunodetection with anti-(banana fruit PEPC) affinity-purified IgGs (A) (7), activity stain using the coupled enzyme assay/fluorescence detection method (B), and activity stain using the Fast Violet B method (C). The proteins migrated from the cathode (top) to the anode (bottom). Incubation in the development buffer was for 10 min (B) and 30 min (C). PEPC activity loading of the lanes was lane 1, 0.1 mU; lane 2, 0.25 mU; lane 3, 0.5 mU; lane 4, 1 mU; lane 5, 2.5 mU; and lane 6, 5 mU.

pane (pH 6.8), 1 mM DTT, 5% PEG (M_r 8000), 50 mM KCl, and 10 mM $MgCl_2$; PK development buffer, PK equilibration buffer plus 2 mM PEP, 1 mM ADP, 0.15 mM NADH, and 2 U/ml rabbit muscle LDH; PFP equilibration buffer, 50 mM Hepes–KOH (pH 6.8), 5 mM $MgCl_2$; PFP development buffer, PFP equilibration buffer plus 5 mM fructose 6-phosphate, 5 μM fructose 2,6-bisphosphate, 0.4 mM PPi, 0.15 mM NADH, 1 U/ml aldolase, 10 U/ml triose-phosphate isomerase, and 1 U/ml glycerol-3-phosphate dehydrogenase.

PEPC activity stain using the Fast Violet B method. Detection of PEPC by the Fast Violet B method was adapted from Ref. (6). Immediately after electrophoresis, the gel was soaked for 15 min in 10 ml of 50 mM Bis–Tris propane (pH 8.4), 10 mM $MgCl_2$, 5 mM DTT, and 15% (v/v) glycerol. This solution was then replaced by 10 ml of 50 mM Bis–Tris propane (pH 8.4), containing 2.5 mM $KHCO_3$, 1.5 mM PEP, 10 mM $MgCl_2$, 5 mM DTT, 15% (v/v) glycerol, and 3 mg/ml freshly prepared Fast Violet B. PEPC activity appeared as red bands. After 30 min staining, the reaction was stopped by soaking the gel in 50 ml of 5% (v/v) acetic acid.

Immunoblot analysis. Native polyacrylamide gels were routinely electroblotted onto PVDF membranes for 120 min at 125 V and 4°C. Immunodetection was carried out as described previously (8).

RESULTS AND DISCUSSION

Comparison of PEPC activity staining using the coupled enzyme assay/fluorescence detection and the Fast Violet B methods. Various amounts of purified recombinant tobacco PEPC (0.1–5 mU) were subjected to native PAGE. An immunoblot analysis of the gel was conducted to locate PEPC and verify loading (Fig. 1A). Gels were stained in parallel using the coupled enzyme assay/fluorescence detection method (Fig. 1B) and a modification of a previously published PEPC detection method based on oxaloacetate reaction with Fast Violet B (6) (Fig. 1C). Both methods were specific for PEPC activity as no band developed in the absence of the substrate PEP (not shown; see below). With fluorescence detection, the activity was faintly detectable in lane 2 (0.25 mU, corresponding approximately to 10 ng of PEPC protein) and a clear signal appeared with a loading of 0.5 mU (lane 3). With the Fast Violet B method, the detection limit was between 1 and 2.5 mU (lanes 4 and 5). In addition, the visualization of activity was faster with the fluorescence (10-min reaction time) than with the Fast Violet B (30-min reaction time). Therefore, the coupled enzyme assay/fluorescence method offered more than a fivefold improvement in sensitivity and three times faster reaction compared to the Fast Violet B method for PEPC analysis. The data of gels carried out in parallel to those presented in Fig. 1B were quantified using the ImageJ software to ascertain the linearity of the method (Fig. 2). The plot of PEPC fluorescent stain signal versus amount of PEPC loaded per lane generated a positive linear slope with a good correlation coefficient ($r^2 = 0.97$) over the tested range of activity. This linear relationship was verified for gels that were stained for less than 10-min periods and were loaded with less than 5 mU. We found that extending incubation times in the staining solution did not improve detection and that results obtained with 30–60 min incubation in development buffer deviated greatly from linearity. Hence, our coupled enzyme assay/fluorescence detection method offers a rapid, simple, quantitative, and sensitive alternative to the previously published method used for PEPC detection.

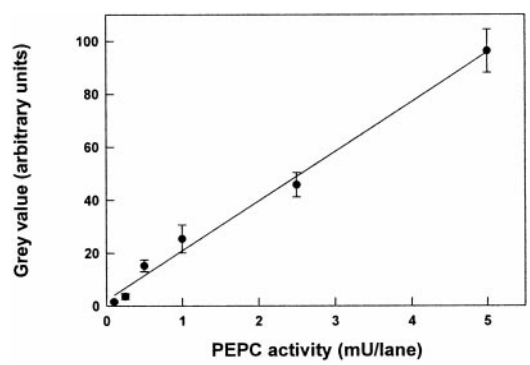

FIG. 2. Signal intensity as a function of PEPC activity. The PEPC activity bands were scanned from positive prints of gels carried out in parallel to Fig. 1B. The scans were quantified using ImageJ software. Grey values represent the integrated signal intensities of each activity band (measured as area under the curve). Data are means ± SD for the quantification of three gels.

This method could therefore potentially be used to visualize and quantify low amounts of PEPC activity such as can be found in clarified plant extracts (see below). These results also prompted us to explore the use of a similar native PAGE activity stain to visualize PK, another important glycolytic enzyme.

Development of the PK activity stain and visualization of partially purified B. napus PK_c and PK_p. A native stain exists for detecting PK activity (18). With this method, the PK reaction (PEP + ADP → pyruvate + ATP) is detected by coupling with hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49). ATP consumption is thus coupled to nitroblue tetrazolium reduction. However, we found that this stain can be difficult to use with certain clarified tissue extracts due to heavy interference by adenylate kinase or myokinase (EC 2.7.4.3), which consumes ADP (2 ADP → ATP + AMP). To detect PK activity, we therefore developed a similar approach to that of the coupled enzyme assay/fluorescence detection method used for PEPC. The spectrophotometric assay of PK uses LDH as a coupling enzyme and is linked to NADH oxidation. PK_c and PK_p partially purified from *B. napus* suspension cell cultures were analyzed by native PAGE followed by immunoblot and activity staining (Fig. 3). Immunoblot analysis was carried out using anti-(*B. napus* PK_c) affinity-purified IgGs and no cross-reaction was observed with the PK_p preparation (Fig. 3A). Conversely, immunoblot analysis carried out with the anti-(developing castor bean endosperm PK_p) affinity-purified IgGs shows no cross-reaction with the PK_c preparation (Fig. 3B). These data demonstrate that the two partially purified preparations are homogenous for their respective PK isoform. Both PK isoforms were detected under the conditions given under Materials and Methods (Fig. 3C). The staining intensity was proportional to loading and was specific for PK, since no bands were detected in the absence of ADP (Fig. 3D). These results thus offer an alternative to the existing activity stain for PK, a key regulatory enzyme in the glycolytic pathway. This method should facilitate future identification and characterization of the various isoforms of this enzyme, which are present in higher plant tissues and in unicellular organisms.

Application of the coupled enzyme assay/fluorescence detection to the characterization of PFP and isoforms of PEPC and PK present in clarified higher plant and algal cell extracts. To further demonstrate the general applicability of this staining method, we developed a stain to identify PFP activity in clarified *B. napus* extracts. PFP has been suggested to play an important role in the metabolic acclimation of plants to Pi deprivation (1) and O_2 deprivation (19). The enzyme has been purified and characterized from *Brassica nigra* suspension cells where it is present as a single isoform

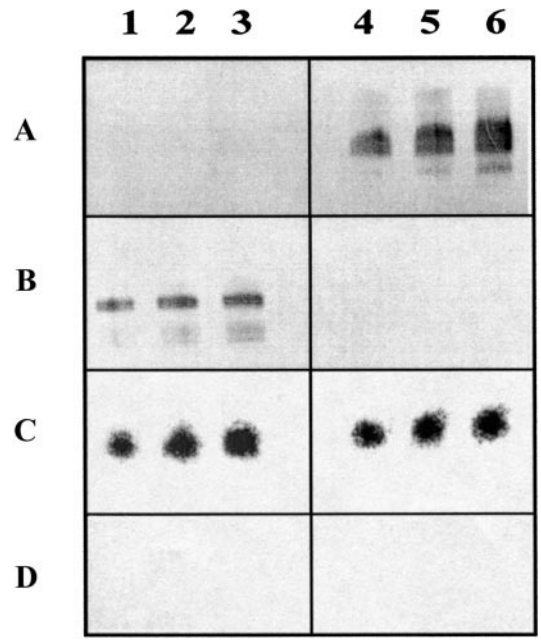

FIG. 3. Detection of partially purified *B. napus* PK_p (lanes 1–3) and PK_c (lanes 4–6) after native PAGE by immunoblot and activity stain. The enzyme preparations were analyzed by native PAGE and subjected to (A) immunoblot followed by immunodetection with anti-(*B. napus* PK_c) affinity-purified IgGs (11), (B) immunoblot followed by immunodetection with anti-(developing castor bean endosperm PK_p) affinity-purified IgGs (10), (C) activity stain using the complete PK development buffer, and (D) activity stain using PK development buffer lacking ADP. The proteins migrated from the cathode (top) to the anode (bottom). Incubation in the development buffer was for 10 min. Activity loading of the lanes was as follows: lanes 1 and 4, 1 mU; lanes 2 and 5, 2 mU; and lanes 3 and 6, 3 mU.

of 520 kDa (15). PFP activity can be detected on native gels in the "reverse" (gluconeogenic) direction using a published procedure (15). To detect the *B. napus* enzyme using the "forward" (glycolytic) reaction, we prepared clarified cell extracts and analyzed them by native PAGE (Fig. 4A). The gels were stained for PFP activity as described under Materials and Methods. A single activity band was resolved and staining was dependent on the presence of PPi in the incubation mixture (compare lanes 1 and 2). The activity band comigrated with a band recognized by anti-(native potato tuber PFP) immune serum (lane 3).

We wished to further demonstrate the high sensitivity of the PK and PEPC detection methods by resolving and visualizing these activities in clarified extracts. Green algal extracts were analyzed for the presence of PK and PEPC isoforms. Two PK isoforms have been purified from the unicellular green alga *S. minutum* (20), whereas *C. reinhardtii* contains only one PK isoform (21). Figure 4B shows that one PK activity band is present in *C. reinhardtii* (lane 3) and that two activity bands are present in the *S. minutum* extract (lane 4). These bands are specific since they were not detected

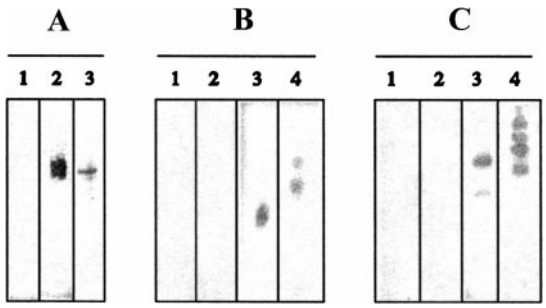

FIG. 4. Detection of PFP (A), PK (B), and PEPC (C) activities after native PAGE in clarified vascular plant and green algal cell extracts. Clarified extracts were prepared and immediately analyzed by native PAGE followed by activity staining. (A) *B. napus* PFP detection (3 mU/lane) in the absence (lane 1) or presence (lane 2) of the cosubstrate PPi in the development buffer. Lane 3 corresponds to an immunoblot detection of PFP carried out with an anti-(native potato tuber PFP) serum (12). (B) *C. reinhardtii* (lanes 1 and 3) and *S. minutum* (lanes 2 and 4) PK detection (1 mU/lane). Staining was done in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of the cosubstrate ADP. (C) *C. reinhardtii* (lanes 1 and 3) and *S. minutum* (lanes 2 and 4) PEPC detection (2 mU/lane). Staining was performed in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of PEP. Incubation in the development buffer was for 10 min (A) and 5 min (B, C). The proteins migrated from the cathode (top) to the anode (bottom).

in the absence of ADP (lanes 1 and 2). The data are also consistent with the known distribution of PK isoforms in these organisms. A similar experiment was run to detect green algal PEPC isoforms (Fig. 4C). Green algal PEPCs characterized so far fall into two classes. Class 1 PEPCs are homotetrameric, whereas Class 2 PEPCs correspond to large protein complexes (14). *C. reinhardtii* cells contain two PEPC isoforms, one representative of each class. *S. minutum* cells contain one tetrameric PEPC and three Class 2 PEPCs. The two classes have contrasting kinetic properties and metabolic functions (3). Analysis of clarified algal extracts allowed us to visualize these isoforms (Fig. 4C, lanes 3 and 4): two bands are apparent in *C. reinhardtii* and four in *S. minutum*. Staining was dependent on the presence of the substrate PEP in the development buffer (lanes 1 and 2). Moreover, relative staining intensity of the various isoforms followed the known relative distribution of Class 1 and Class 2 PEPCs in *C. reinhardtii* and *S. minutum* where the faster-moving (anodal) Class 1 PEPC represents 10–20% of total PEPC activity (3, 8).

During the different trials that led to the development of these procedures, we observed one possible limitation to their application. The detection of closely migrating bands could prove difficult because of the glowing aspect of the gel background and the somewhat diffuse aspect of activity bands under UV light during staining. Such was the case in initial trials of *S. minutum* PEPC isoform separation using clarified algal cell extracts. These difficulties were circumvented by (i) increasing electrophoretic migration times from 2 to 4 h and (ii) optimizing activity loaded on the gel to achieve the best resolution. However, the methods described above are easy to use and highly specific for each enzyme activity since they are based on the reactions used for spectrophotometric assays.

In conclusion, our stain methods can be used to follow and quantify the relative distribution of PFP, PEPC, and PK isoforms under the different physiological conditions known to affect these activities (1, 9, 22). They can also be used to identify and follow isoform developmental and tissue-specific expression patterns (1, 23) without extensive purification.

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