BENCHMARKS

Benchmarks are brief communications that describe helpful hints, shortcuts, techniques or substantive modifications of existing methods.

Low-Voltage Separation of Phosphoamino Acids by Silica Gel Thin-Layer Electrophoresis in a DNA Electrophoresis Cell

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A major objective in the characterization of protein kinase activity is to determine phosphoamino acid identity in substrate proteins. Several fundamentally different phosphoamino acid analysis (PAA) procedures are available to identify phosphorylated amino acids (2,3). Phosphoamino acids can be released from proteins by partial hydrolysis (4) and identified directly by one- or two-dimensional high-voltage thin-layer electrophoresis (HV-TLE) and/or thin-layer chromatography (TLC) (1,3,5). PAA by conventional HV-TLE (≥1000 V) is quick but requires the use of expensive power supplies and temperature-regulated equipment not readily available to most laboratories. PAA by TLC does not require elaborate equipment but is time-consuming, requiring 7–12 h for sample migration (3,5).

Low-voltage thin-layer electrophoresis (LV-TLE) (≤500 V) is relatively quick and eliminates the need for cooling the TLE unit and other expensive equipment. Although the separation of complex mixtures of amino acids was originally achieved by LV-TLE (6), it is generally not accepted that separation of phosphoamino acids can be achieved at lower voltages because detailed methods for PAA by LV-TLE are unavailable. Several simple units designed for LV-TLE have been described (6); their similarity to modern agar electrophoresis equipment prompted us to investigate whether this equipment could be used for PAA.

We have developed a simple one-dimensional LV-TLE procedure that will resolve the three most common eukaryotic phosphoamino acids. Our technique uses a standard method for partial acid hydrolysis of proteins (4) but includes significant modifications of existing HV-TLE protocols (1) that result in a simplified PAA technique that most laboratories are equipped to perform. Specifically, electrophoresis is performed on silica gel plates using a commonly used PAA buffer (2) in a DNA electrophoresis cell. The thickness and type of thin-layer sorbent used and the electrophoresis voltage differ from published procedures (1,2). The advantages of our technique over conventional HV-TLE and/or TLC are that it is economical, it utilizes standard laboratory equipment and it allows rapid separation of phosphoamino acids. We describe our LV-TLE protocol in detail and demonstrate that it can resolve phospho-serine (pSer), threonine (pThr) and tyrosine (pTyr) residues in protein hydrolysates as discrete spots.

We have purified a maize protein kinase, ZmMEK1, as a glutathione *S*-transferase (GST) fusion expressed in *E. coli*. During in vitro kinase reactions with [γ-^{32}P]ATP, ZmMEK1 is capable of autophosphorylation on the kinase portion of the fusion product (our unpublished observations) and phosphorylation of myelin basic protein (MBP) (Figure 1). These reaction products

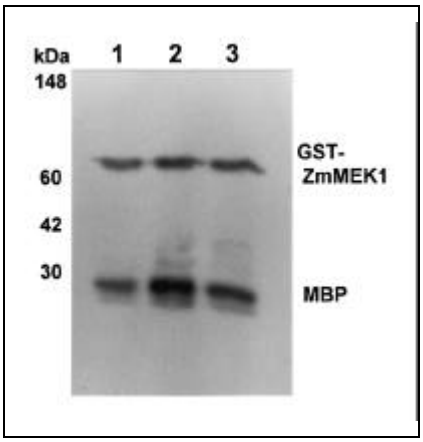

Figure 1. Autoradiogram of in vitro protein kinase assay products transferred to a PVDF membrane. Lanes 1–3 each contain the products of identical kinase reactions that included 1 µg of GST-ZmMEK1 kinase and 2 µg of MBP substrate that were incubated in 30 mM HEPES (pH 7.5), 5 mM Mg^{2+}, 5 mM Mn^{2+}, 10 mM ATP and 10 µCi [γ-^{32}P]ATP for 30 min at 30°C, fractionated on 10% polyacrylamide SDS gels and transferred to PVDF membranes for 60 min at 100 V. The air-dried membrane was exposed to film for 17 h. The numbers to the left of the figure indicate the size of protein standards in kDa. The positions of GST-ZmMEK1 and MBP are indicated; these proteins were cut from the membrane, separately acid-hydrolyzed in 5.8 *N* HCl, dried, resuspended in a phosphoamino acid standard solution and ca. 150 cpm (4 µL) of each sample spotted onto a silica gel TLE plate.

were separated on a 10% polyacrylamide sodium dodecyl sulfate (SDS) minigel and electrophoresed onto a polyvinylidene difluoride (PVDF) membrane (Immobilon®-P; Millipore, Bedford, MA, USA) in transfer buffer (15.6 mM Tris-HCl, pH 8.3, 120 mM glycine). The membrane was washed three times in water for 5 min each and air-dried for 45 min. After marking the membrane with fluorescent ink for accurate alignment with the film, autoradiography was performed at -70°C with an intensifying screen for 8–24 h.

Radio-labeled ZmMEK1 and MBP (Figure 1) were cut out of the PVDF membrane and placed into separate screw-cap microcentrifuge tubes. The protein-bound membrane pieces were wetted in 200 µL of 100% methanol for 30 s and rinsed twice in 200 µL of water for 30 s each, and the proteins were partially acid-hydrolyzed (4) by incubation in 100 µL of 5.8 *N* HCl at 110°C for 90 min. The supernatant was placed into a new tube, dried for 90 min in a SpeedVac® concentrator (Savant Instruments, Farmingdale, NY, USA), resuspended in 4 µL of phosphoamino acid standard solution (1 mg/mL of *O*-phospho-DL-serine, threonine and tyrosine [Sigma Chemical, St. Louis, MO, USA] dissolved in water) and counts per minute (cpm) determined.

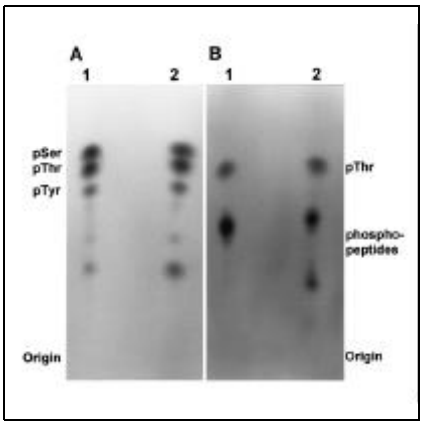

Figure 2. PAA by LV-TLE of acid-hydrolyzed in vitro kinase reaction products. (A) Photograph of the phosphoamino acid standards. After LV-TLE separation of the acid hydrolysates, the positions of pTyr, pThr and pSer were determined by spraying the silica gel TLE plate with ninhydrin reagent and heating for 15 min at 60°C. The position of each standard and the sample application origin are indicated. Lane 1 contained the ZmMEK1 autophosphorylation hydrolysate and lane 2 the MBP hydrolysate. (B) Autoradiogram of the TLE plate. The TLE plate was exposed to film for 3.5 days at -70°C with an intensifying screen. The positions of pThr, partially hydrolyzed phosphopeptides and the sample application origin are indicated. Lane 1 contained the ZmMEK1 autophosphorylation hydrolysate and lane 2 the MBP hydrolysate. By re-aligning the TLE plate and the autoradiogram, it was determined that both ZmMEK1 autophosphorylation and MBP phosphorylation occurred on threonine residues.

In preparation for LV-TLE, we cut a 6.25- × 11.5-cm TLE plate from a Whatman 250-µm silica gel (6 nm mean pore diameter), polyester-backed chromatography plate (Catalog No. 4410 221; Fisher Scientific, Pittsburgh, PA, USA). We applied 100–300 cpm (1–4 µL) of the resuspended acid hydrolysates as spots along an origin 2 cm from the base of the plate. Up to four samples were applied per plate and air-dried for 45 min. We prepared 250 mL of electrophoresis buffer (pH 3.5) (2) (i.e., 236.25 mL water: 12.5 mL glacial acetic acid: 1.25 mL pyridine) and poured 65 mL into both electrode chambers of a Mini Sub™ (Bio-Rad, Hercules, CA, USA) DNA cell. This solution did not harm the electrophoresis cell. Two 6.25- × 8.5-cm buffer wicks were cut from VWRbrand® 238 blotting paper (VWR Scientific, Bridgeport, NJ, USA) and folded in half so they were 4.25 cm long. They were folded again along a line approximately 1 cm from the folded edge. An 11.5- × 13-cm plate blotter was cut from blotting paper and folded in half so it was 6.5 cm wide. Round holes were cut into the plate blotter 2 cm from the base with a No. 6 cork borer (1.2 cm diameter) for each sample applied. The wicks and plate blotter were soaked in electrophoresis buffer. The plate blotter was partially dried by briefly laying it on a piece of filter paper, and the TLE plate was evenly wetted with electrophoresis buffer by placing the plate blotter on it with the holes over the sample application spots. The sample spots were wetted first by gently pushing buffer into the plate so that it concentrated into the center of the sample (1), and the rest of the plate was wetted by applying light pressure.

The TLE plate was placed onto the casting tray of the Mini Sub DNA cell and the wetted wicks placed onto the ends of the TLE plate so that the 1-cm fold was in good contact with the plate. The samples were electrophoresed at 250 V (17 V/cm, 14–20 mA) for 45 min toward the anode (positive terminal) at room temperature. After electrophoresis, the plates were air-dried in a fume hood for 90 min. The plate blotter and wicks were also dried and reused. To detect the phosphoamino acid standards, which appear as three purple spots against a white background (Figure 2A), the TLE plates were lightly sprayed with ninhydrin reagent (0.25% ninhydrin in acetone) and placed at 60°C for 15 min. The TLE plate was then marked with fluorescent ink to allow accurate alignment with the film, and autoradiography was performed at -70°C with an intensifying screen for 1–7 days. The identity of radioactive phosphoamino acids in the samples was determined by aligning the autoradiogram with the TLE plate and comparing their migration to the stained phosphoamino acid standards (Figure 2B).

Our rapid one-dimensional LV-TLE protocol significantly simplifies the determination of phosphoamino acid content in protein kinase substrates. We demonstrate its utility by showing that the ZmMEK1 protein kinase phosphorylates both itself and MBP on threonine residues (Figure 2B). This procedure is performed using commonly available equipment and should be widely applicable.

At pH 3.5, pTyr, pThr and pSer are all expected to have a charge of -1 and will therefore migrate towards the anode in an electrophoretic field. Separation of the phosphoamino acids occurs through differential adsorption to the silica gel sorbent and partitioning in the electrophoresis buffer because of differences in their hydrophobicities and molecular weights. Under the conditions we describe, average mobilities (n = 8) from the origin were 5.9 cm for pTyr, 6.6 cm for pThr and 7.2 cm for pSer. The average spot diameter (0.2–0.5 cm) differed with the amount of hydrolysate loaded. Partially hydrolyzed peptides were distributed between pTyr and the origin (Figure 2B). Free [^{32}P]phosphate migrated off the plate, so the anode wick and buffer should be treated as radioactive waste. The amount of separation achieved by our procedure between adjacent pairs of phosphoamino acids (ca. 0.6 cm) is as good as or better than

that achieved by TLC with numerous buffers (1,2,5) or by HV-TLE protocols (2) when multiple samples are analyzed. We have found that when 1 µL of a dye solution (1% orange G, 1% phenol red in pH 3.5 buffer) was electrophoresed in parallel with the phosphoamino acid standards, orange G migrated slightly ahead of pSer and phenol red migrated with pTyr, providing useful visible markers for electrophoretic progression in our system.

When proteins are labeled by in vitro kinase reactions, the only radioactive compounds in the hydrolysates will be phosphopeptides, phosphoamino acids and phosphate. Under these conditions, a one-dimensional separation by TLE at pH 3.5 is considered adequate (2). When proteins are labeled in vivo and only partially purified, a high resolution two-dimensional HV-TLE/TLC separation system is required. These hydrolysates frequently contain labeled compounds that migrate with the phosphoamino acids (2). The procedure we describe is therefore limited to PAA of purified proteins and substrates labeled in vitro.

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In Situ Detection Method for Glutaminyl Cyclase Activity in Polyacrylamide Gels

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Glutaminyl cyclase (QC; EC 2.3.2.5) is an enzyme associated with the posttranslational maturation of bioactive peptides in the neuroendocrine system. It acts on glutaminyl residues at the peptide amino terminus, cyclizing them to pyroglutamyl residues and producing ammonia in the process. The enzyme is widespread in mammalian tissues (2,5), and it has been suggested that multiple forms might be present. A method of detecting glutaminyl cyclase activity in polyacrylamide gels might, therefore, be useful in detecting tissue-specific variants.

Because QC produces ammonia in the course of catalysis, initial attempts were made to adapt detection methods published for other ammonia-producing enzymes, such as urease. However, indicator dyes, such as phenol red or the silver-staining method of de Llano et al. (3), proved unable to detect QC activity, probably because they depend on the rise in pH resulting from the production of ammonia, and the peptide substrates of QC might have buffered the system too much. However, an adaptation of our standard liquid-phase assay for QC activity (1), which links the production of ammonia to the conversion of nicotinamide-adenine dinucleotide [reduced] (NADH) to oxidized nicotinamide-adenine dinucleotide (NAD^+) with glutamate dehydrogenase to the filter paper overlay method of Nelson et al. (4), proved to be effective in detecting QC activity.

Bovine tissue samples were homogenized at a ratio of 0.3 g of tissue to 1 mL of 0.1 M Tris-HCl, pH 7.6. The extracts were subjected to two freeze/thaw cycles and clarified by centrifugation at 12 000× *g* for 15 min. They were then diluted 1:1 with a sample buffer (62.5 mM Tris-HCl, 20% glycerol, pH 6.8) that contained bromophenol blue as the running dye. Thirty microliters (ca. 90 µg) of each tissue sample were electrophoresed in the absence of detergent on a 10% NuPAGE™ gel (8 × 8 cm, 1-mm thick; Novex, San Diego, CA, USA) using the morpholino propanesulfonic acid (MOPS) buffer system (pH 7.7) at 20 V for 44 h at 4°C. Recombinant human QC (1.8 U) expressed as a fusion protein with glutathione *S*-transferase (GST) QC was treated in the same manner as a positive control (6). One unit of enzyme activity is defined as the amount of enzyme required to convert one nanomole of Gln-NH_2 (8 mM initial concentration) to pGlu-NH_2 in one minute (1).

Once electrophoresis was complete,

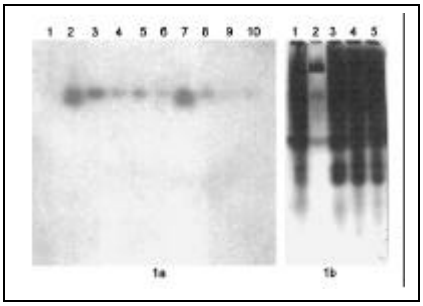

Figure 1. Filter paper overlay of glutaminyl cyclase native gel viewed under UV light. (a) Lane 1: mature bovine pituitary tissue extract; lane 2: recombinant human enzyme (positive control); lane 3: mature bovine hippocampus; lane 4: bovine calf hippocampus; lane 5: mature bovine striatum; lanes 6–10: duplicates of lanes 1–5. (b) Lanes are the same as 1–5 in Panel a except the filter paper has been removed and the gel stained for protein with Coomassie Blue.