Blue native polyacrylamide gel electrophoresis and the monitoring of malate- and oxaloacetate-producing enzymes

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Abstract

We demonstrate a facile blue native polyacrylamide gel electrophoresis (BN-PAGE) technique to detect two malate-generating enzymes, namely fumarase (FUM), malate synthase (MS) and four oxaloacetate-forming enzymes, namely pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK), citrate lyase (CL) and aspartate aminotransferase (AST). Malate dehydrogenase (MDH) was utilized as a coupling enzyme to detect either malate or oxaloacetate in the presence of their respective substrates and cofactors. The latter four oxaloacetate-forming enzymes were identified by 2,6-dichloroindophenol (DCIP) and p-iodonitrotetrazolium (INT) while the former two malate-producing enzymes were visualized by INT and phenazine methosulfate (PMS) in the reaction mixtures, respectively. The band formed at the site of enzymatic activity was easily quantified, while Coomassie staining provided information on the protein concentration. Hence, the expression and the activity of these enzymes can be readily evaluated. A two-dimensional (2D) BN-PAGE or SDS-PAGE enabled the rapid purification of the enzyme of interest. This technique also

provides a quick and inexpensive means of quantifying these enzymatic activities in normal and stressed biological systems.

1. Introduction

Enzymes involved in the synthesis of malate and oxaloacetate form a pivotal aspect of cellular metabolism in all organisms [1]. They help generate essential precursors that are involved in the production of energy, in gluconeogensis, in lipogenesis, and in the metabolism of amino acids. PC [E.C. 6.4.1.1], that mediates the formation of oxaloacetate from ATP, HCO_3^- and pyruvate, is a key enzyme that funnels metabolites towards the production of glucose [2]. CL is a lipogenic enzyme as it enables the transport of acetyl-CoA into the cytoplasm for its interaction with acetyl-CoA carboxylase. The formation of malonyl-CoA from acetyl-CoA, ATP and HCO_3^- is a critical step in the formation of fatty acid in most organisms [3]. The oxaloacetate generated by this cleavage is also essential in the production of NADPH, an essential ingredient for lipogenesis. AST [E.C. 2.6.1.1] is an enzyme that generates oxaloacetate via the transamination of aspartate and plays an important role in cellular nitrogen homeostasis [4]. PEPCK [E.C. 4.1.1.49], critical in linking the TCA cycle to gluconeogenesis, mediates the formation of phosphoenolpyruvate from oxaloacetate. This enzyme is crucial in carbohydrate metabolism [5]. FUM [E.C. 4.2.1.2], on the other hand, is part of the tricarboxylic acid (TCA) cycle and is involved in the reversible hydration of fumarate to malate. The malate is subsequently oxidized to generate NADH that eventually propels the synthesis of ATP in the electron transport chain [6]. Together with isocitrate lyase, MS [E.C. 2.3.3.9] forms the glyoxylate shunt. This metabolic network contributes to the survival of organisms exposed to nutrient and/or metal stresses by preventing the loss of carbon via the TCA cycle and in aiding the synthesis of oxalic acid [7–9].

Hence, it is very important to devise simple and inexpensive techniques to study these enzymes that cover a broad range of metabolic functions. Although spectrophotometric methods have been routinely utilized to monitor activities of these enzymes, interfering reactions hinder the accuracy of these assays. Sensitivity is relatively low and high quantities of proteins are required [4,10]. To quantitate the expression of these enzymes, antibodies that are expensive and/or not readily available have to be used. The procedure described here allows the monitoring of both enzymatic activity and relative protein expression without necessitating the use of antibodies.

Although BN-PAGE has been widely reported in the study of mitochondrial membrane proteins [11], we have adapted this technique to monitor both soluble and membrane-bound enzymes involved in the production of either malate or oxaloacetate. MDH is utilized as the coupling enzyme while INT is the detecting chromophore. In this report, we describe how BN-PAGE can detect, quantitate and purify six enzymes involved in malate

and oxaloacetate metabolism. This procedure is facile and inexpensive and allows the monitoring of these enzymes in various disparate cells.

2. Materials and methods

2.1. Chemicals

Ammonium persulfate, Bio-Rad Protein Assay reagent, Bio-Rad Silver Staining Kit and N, N'-methylene-bis-acrylamide were purchased from Bio-Rad Laboratories (Mississauga, ON, Canada) while Coomassie Brilliant Blue G-250 was obtained from Eastman Kodak Co. (Rochester N.Y., USA). Fetal bovine serum and trypsin-EDTA were from Invitrogen Canada Inc. (Burlington, Ontario, Canada) and tissue culture hardware was purchased from SARSTEDT INC. (Montréal, Québec, Canada). All other chemicals were from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).

2.2. Bacterium and cell culture conditions

Pseudomonas fluorescens (ATCC 13525) was obtained from the American Type Culture Collection (Rockville, MD, USA) and was maintained on a defined mineral medium containing citric acid. The medium was solidified by the addition of 2% (w/v) agar. The sterile agar test tubes were maintained at 4 °C after being autoclaved for 20 min at 121 °C. Control media, lacking the test metal, consisted of Na_2HPO_4 (6.0 g), KH_2PO_4 (3.0 g), NH_4Cl (0.8 g), $MgSO_4\cdot7H_2O$ (0.2 g) and citric acid monohydrate (4.0 g) in 1 L of deionized, distilled water. Trace elements in concentrations as described in Ref. [12] were also added. The pH was adjusted to 6.8 with dilute NaOH. The media were dispensed in 200 mL amounts to 500 mL Erlenmeyer flasks, stoppered with foam plugs and autoclaved as aforementioned.

HepG2 cells were seeded at 1×10^5 cells/mL and grown in α-modified Eagle's medium (α-MEM) supplemented with 5% fetal bovine serum, 1% penicillin and 1% streptomycin. Cells were routinely cultured in 175 cm^2 collagen-coated flasks with loosened caps and incubated with 5% CO_2 in humidified atmosphere at 37 °C.

2.3. Stress conditions

Bacterial Al–citrate media were prepared in the same manner as the control medium with the following modifications: 4 g citric acid monohydrate and 3.62 g $AlCl_3\cdot6H_2O$ were first allowed to complex in approximately 50 mL deionized, distilled water for approximately 30 min prior to being added to the remainder of the media. A final concentration of Al^{3+} and citric acid of 15 and 19 mM respectively was utilized. For oxidative stress experiments, the control media were enriched with 100 μM menadione prior to bacterial inoculations. Media were inoculated with 1 mL of stationary phase *P. fluorescens*, grown in control media and aerated on a gyrotory waterbath shaker, model 76 (New Brunswick Scientific) at 26 °C. The HepG2 cells were cultured to 70% confluency and were subsequently subjected to serum-free

media containing 2.5 mM citrate or Al–citrate (2.5 mM citrate : 0.5 mM AlCl$_3 \cdot$6H$_2$O) for up to 24 h.

2.4. Cell isolation and fractionation

At the desired time intervals, *P. fluorescens* cells were collected by centrifugation at 16,500 ×*g* for 10 min at 4 °C. The bacterial pellet was washed in 0.85% (w/v) NaCl and centrifuged again for 10 min. The cells were resuspended in bacterial cell storage buffer (bCSB; 50 mM Tris–HCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, pH 7.4), centrifuged once again for 10 min at 16,500 ×*g* at 4 °C, resuspended again in bCSB and ruptured by sonication at 0 °C using a Brunswick sonicator, power level 4, for four intervals at 15 s per interval. The cell free extracts (CFE) were centrifuged at 180,000 ×*g* for 60 min at 4 °C to yield membrane and soluble components. The membrane fraction was resuspended in bCSB while the soluble CFE fraction was further spun at 180,000 ×*g* for 2 h to afford a membrane-free preparation.

HepG2 cells were isolated at the desired exposure times. Cells were trypsinized and isolated by centrifugation (350 ×*g*) for 10 min at 4 °C, followed by two washings with phosphate buffered saline. The pellet was then resuspended in HepG2 cell storage buffer (HCSB; 50 mM Tris–HCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 250 mM sucrose, 2 mM citrate) and stored at −86 °C until needed. Protein concentrations were determined by the Bradford method using the Bio-Rad Protein Assay reagent [13].

2.5. BN-PAGE

BN-PAGE of bacterial protein was performed according to the method described by Schägger and von Jagow [14] with the following modifications. Lauryl-maltoside was added only to the membrane CFE. One millimeter spacers were used to obtain small gels (8 × 7 cm) for the Biorad MiniProtean™ 2 system. The final volume of the resolving gels was 5.8 mL, therefore 2.9 mL 4% acrylamide and 2.9 mL 16% acrylamide per gel were used to create linear gradients with the aid of Biorad Gradient Former model 385™. For experiments involving the enzyme PC, a 7.5% non-gradient gel was utilized.

Both membrane and soluble CFE obtained from *P. fluorescens* grown in citrate (control), Al–citrate and menadione-enriched media were analyzed. Samples were prepared by diluting the membrane protein with 3× Blue Native (BN) buffer, lauryl-maltoside and water to a final concentration of 4 mg/mL protein equivalent, 1 % (w/v) lauryl-maltoside and 1× BN buffer (50 mM BisTris, 500 mM 6-aminohexanoic acid, pH 7.0 at 4 °C). Soluble CFE protein was prepared identically with the omission of lauryl-maltoside. Samples were kept at −20 °C until use. To each lane of stacking gel was added the desired concentration of protein and electrophoresis was performed under blue native conditions. Eighty volts was used for running of the gel until the proteins reached the separating gel where the voltage was increased to 200 V or a constant current of 15 mA. Once the running-front was at the middle of the separating gel, the blue cathode buffer (50 mM Tricine, 15 mM BisTris, 0.02% (w/v) Coomassie blue G-250; pH 7.0 at 4 °C) was exchanged with a colorless one (identical buffer as blue cathode without Coomassie G-250) (note: the chamber was not washed so that a small amount of Coomassie still

remained). During the run, the gel usually destains and protein bands may be visualized. Electrophoresis was stopped before the running front moved out of the gel. The HepG2 cells were thawed and pelleted at 350 $\times g$ for 10 min at 4 °C and resuspended in HCSB with the addition of 1 mg/mL pepstatin A and 0.1 mg/mL of leupeptin. The cell suspension was homogenized at 0 °C using a Brunswick sonicator for 20 s in 2 s bursts. Immediately after homogenization, mitochondrial and soluble fractions were isolated by differential centrifugation [15]. The whole cells were removed by centrifugation at 600 $\times g$ for 10 min at 4 °C. The pellet was discarded and the supernatant was spun at 6700 $\times g$ for 30 min at 4 °C to pellet the mitochondria. The mitochondrial pellet was then resuspended in a minimal amount of HCSB. The samples collected by differential centrifugation were then subjected to several preparatory stages as described in Ref. [14].

The cytoplasmic fraction was solubilized by the addition of 4 mg of soluble protein and 83.3 μL of 3× Blue Native Buffer. The volume was adjusted to 250 μL with deionized, distilled water. Post preparation, the samples were stored at −20 °C until needed. To properly separate soluble proteins, 60 μg of protein was applied to a 4–10% Blue Native gel gently overlaid with BN cathode buffer, and subjected to electrophoresis.

2.6. 2D BN-PAGE and 2D SDS-PAGE

For 2D BN-PAGE, the activity bands from the 1D BN-PAGE were excised. The pieces of gel with the desired protein were placed vertically into the wells of a second BN-gel. Again, the gel was run as before. In the case of 2D SDS-PAGE, gels from a 1D BN-PAGE were soaked for 2 h in a solution of 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol [16]. Gels were then rinsed twice for 5 s with 1× SDS-PAGE electrophoresis buffer (25 mM Tris–HCl, 192 mM glycine and 0.1% (w/v) SDS; pH 8.3) [17], then bands corresponding to the desired protein were excised and placed into the wells of a 10 % SDS gel (8 × 7 cm). Electrophoresis was conducted at 200 V for 45 min at room temperature. Upon completion of the SDS-PAGE, gels were stained with the Bio-Rad Silver Staining Kit.

2.7. In-gel enzyme assays and protein expression

Following BN-PAGE, the gels were allowed to equilibrate for approximately 15 min in 1.5–2 mL of reaction buffer (25 mM Tris–HCl, 5 mM MgCl$_2$, pH 7.4) per lane of loaded protein. This treatment helped optimize enzymatic activity. Gels were then placed in reaction buffer (1.5 mL/lane) with the appropriate substrates and cofactors, depending on the enzyme to be monitored. Enzyme activities were detected in the gel by the reduction and precipitation of INT as formazan.

MS activity was tested in the soluble CFE by incubating BN gels with glyoxylate (5 mM), acetyl-CoA (0.5 mM), MDH (13 U/mL), NAD$^+$ (0.5 mM), 0.4 mg/mL INT and 0.2 mg/mL PMS in activity buffer. In the membrane CFE, FUM activity was detected by adding fumarate (5 mM), MDH (13 U/mL), NAD$^+$ (0.5 m), INT (0.4 mg/mL) and PMS (0.2 mg/mL) to the reaction mixture. For the four oxaloacetate-producing enzymes PMS was replaced with DCIP as the electron coupler [18]. In-gel PC activity was detected in the membrane CFE by adding pyruvate (5 mM), HCO$_3^-$ (10 mM), ATP (1 mM), NADH (0.5 mM), MDH (13 U/mL), INT (0.4 mg/mL) and DCIP (16.7 μg/mL) in activity buffer. For

PEPCK activity, phosphoenolpyruvate (10 mM), HCO_3^- (10 mM), ADP (1 mM), NADH (0.5 mM), MDH (13 U/mL), INT (0.4 mg/mL) and DCIP (16.7 μg/mL) were added to activity buffer; PEPCK was detected in the soluble CFE.

CL activity was tested by adding citrate (20 mM), coenzyme A (0.75 mM), ATP (0.37 mM), NADH (1.5 mM), MDH (13 U/mL), INT (0.4 mg/mL) and DCIP (16.7 μg/mL) in the reaction buffer to the soluble CFE. The detection of AST was possible by incubating the gel with aspartate (10 mM), α-ketoglutarate (10 mM), NADH (0.5 mM), MDH (13 U/mL), INT (0.4 mg/mL) and DCIP (16.7 μg/mL). Assays for bacterial samples were done at room temperature while those for the HepG2 cells were performed at 37 °C. The nature of these enzymes was further confirmed by using specific inhibitors and/or antibodies.

To fix activity bands and remove excess Coomassie G-250, gels were soaked overnight in destaining solution (40% methanol and 7% acetic acid). To monitor the amounts of protein expressed, lanes loaded with identical protein corresponding to those utilized for activity measurement were stained with 0.05% (w/v) Coomassie R-250, 40% methanol and 7% acetic acid for 1 h and destained overnight.

3. Results and discussion

3.1. Malate synthase and expression

MS, an enzyme that condenses glyoxylate and acetyl-CoA, was readily monitored by BN-PAGE. Fig. 1 depicts the activity band observed when various protein concentrations of the soluble CFE from *P. fluorescens* grown in a menadione medium were subjected to BN-PAGE analyses. A faint band indicative of MS activity was evident when 40 μg of protein was utilized. An increase in band intensity was recorded as the amount of protein loaded was augmented. It is important to note that even though the activity band was less intense at lower protein concentration, this could be enhanced if the gel was incubated in the reaction mixture longer. The molecular mass markers, the omission of one of the substrates and/or the inclusion of inhibitors enabled the confirmation of this band as that of MS activity (data not shown). The malate produced during this enzymatic reaction was detected by the presence of MDH and NAD^+ in the reaction mixture. The formation of NADH helped generate the formazan precipitate at the site of the immobilized MS in the gel via the transfer of electrons to INT via PMS. The protein concentration responsible for MS activity was detected by staining the gel with Coomassie. Densitometric analyses with Scion Image software enabled the quantitation of the protein. Fig. 1II depicts the absence of a MS activity band in control cells, and a sharp increase in the cells subjected to menadione stress.

3.2. Detection and expression of oxaloacetate-producing enzymes

PC, PEPCK, AST and CL were also readily detected by this technique. The oxaloacetate generated in the presence of the respective substrates and cofactors was subsequently identified by MDH, NADH, DCIP and INT in the reaction mixture. PC, a homotetramer with a molecular mass of 300 kDa in bacteria [19,20], was resolved in a
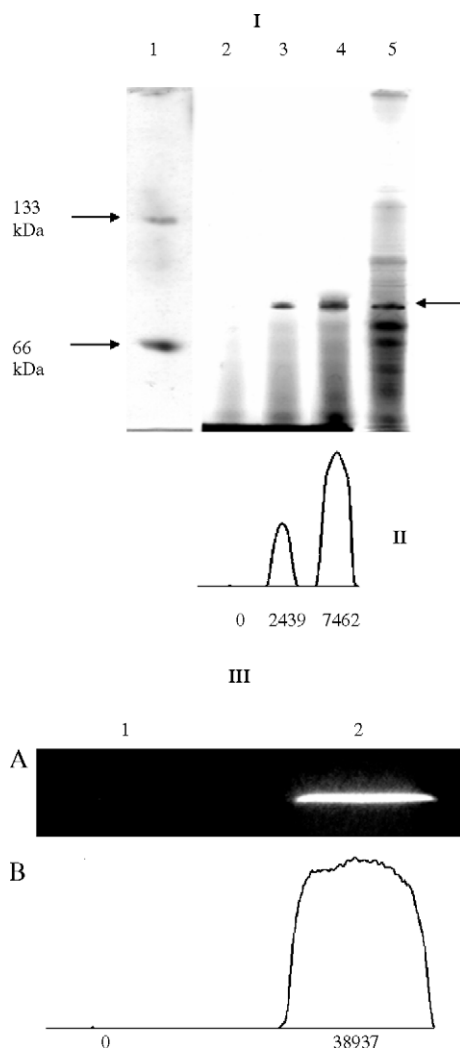
Fig. 1. I. In-gel activity staining of MS. Lane 1: BSA molecular mass marker. Lanes 2, 3 and 4: 20, 40 and 60 μg protein equivalents of soluble CFE obtained from menadione-stressed cells (activity staining); 5: soluble CFE from menadione-stressed cells (60 μg protein equivalent) stained by Coomassie R-250 dye. II. Relative band intensity from lanes 2,3 and 4 representing 20, 40 and 60 μg protein equivalent per lane respectively was quantified with Scion Image. III. In-gel activity staining (A) and relative quantification (B) of MS activity in *P. fluorescens*. Lane 1: soluble CFE from citrate cells; 2: soluble CFE from menadione-stressed cells. Relative band intensity was quantified with Scion Image.

7.5% acrylamide gel and it appeared that the presence of Coomassie G-250 dye and 6-aminohexanoic acid helped stabilize this enzyme, as no band was observed when a Native PAGE was performed (data not shown). 2D SDS-PAGE confirmed the purity of the enzyme as only a single band with a molecular mass of 66 kDa was detected. Furthermore, studies with avidin-conjugate and the omission of the cofactors provided

further evidence that enabled the attribution of this activity to PC (Fig. 2). The activity and expression of this protein were markedly increased in *P. fluorescens* subjected to oxidative stress.

3.3. PEPCK, AST and CL detection

Similarly, PEPCK and AST were detected by BN-PAGE (Fig. 3I). Attempts were also made to adapt this technique to CFE obtained from mammalian systems. CL activity in HepG2 cells was studied and it was evident that the activity of this enzyme was enhanced in cells exposed to Al. This increase in enzymatic activity was concomitant with the increase in enzyme concentration (Fig. 3II).

3.4. FUM activity and expression

FUM reversibly hydrates fumarate to malate, a product that reacts with malate in the presence of NAD^+ to produce NADH. This BN-PAGE technique was instrumental in detecting two isoenzymes associated with this activity. It is important to note that the lower band, that has been attributed to FumA was absent in the *P. fluorescens* subjected to Al (Fig. 4I). This clearly demonstrates a direct linkage between Al toxicity and dysfunctional Fe metabolism and/or ROS stress. FumA is dependent on a Fe–S cluster for its activity, while FumC is devoid of any requirement for Fe [21]. To confirm the purity of the enzyme the activity band was excised and loaded on a 2D SDS-PAGE or 2D BN-PAGE with different acrylamide concentrations (data not shown). This procedure allowed for the verification and/or the further purification of FUM. The discrete spot obtained appeared
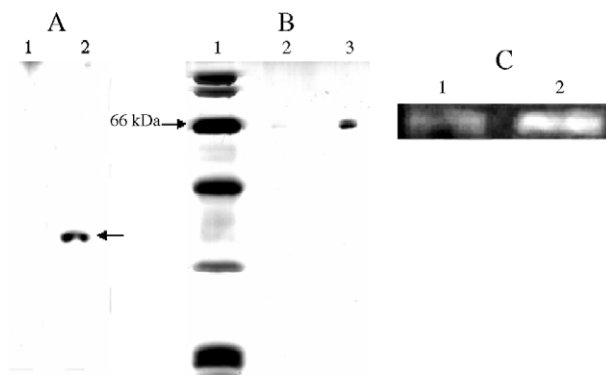

Fig. 2. PC activity and protein expression. Panel A: in-gel activity staining of PC in the membrane CFE. Lane 1: citrate cells; 2: menadione-stressed cells. Panel B: 2D SDS-PAGE of PC in a 7.5% acrylamide gel where activity bands from 1D BN-PAGE were utilized. Lane 1: SDS-PAGE molecular mass markers from Bio-Rad Laboratories (molecular masses ranging from 116 kDa (top band) to 21.5 kDa (bottom band)); 2: membrane CFE from control cells; 3: membrane CFE from menadione-stressed cells. Note: PC spot is only detected in lane 3, corresponding to 66 kDa when compared to molecular mass standards. Panel C: Western blot of PC as detected by avidin-conjugated horseradish peroxidase. Lane 1: membrane CFE from citrate cells; 2: membrane CFE from menadione-stressed cells.
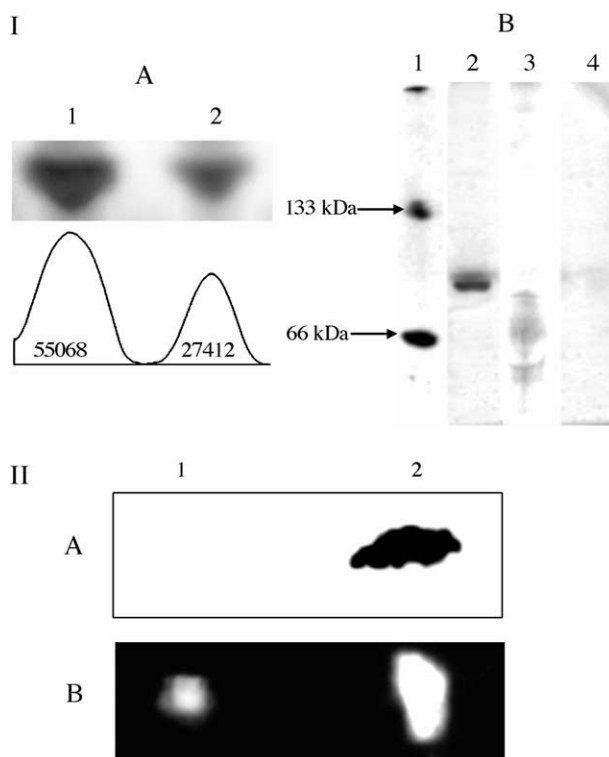
Fig. 3. I. In-gel activity staining of PEPCK and AST in soluble CFE. Panel A: in-gel activity staining of PEPCK. Lane 1: soluble CFE from citrate cultures, 2: soluble CFE from menadione-supplemented cultures. Relative band intensity was quantified with Scion Image (in vitro data revealed a specific activity of 2.1 and 1.5 mM min^{-1} mg^{-1} for CFE from control and menadione-supplemented cultures, respectively). Panel B: in-gel activity staining of AST. Lane 1: BSA molecular mass marker, 2: soluble CFE from control cells (reaction mixture), 3: soluble CFE from control cells (reaction mixture — aspartate), 4: soluble CFE from control cells (reaction mixture — α-ketoglutarate). II. CL in-gel activity stain and protein expression. HepG2 cells were grown to 70% confluency and treated with α-MEM (devoid of FBS) supplemented with 1 : 2.5 mM citrate and 2 : 2.5 mM citrate : 0.5 mM Al for 24 h. Panel A: 1D BN-PAGE in-gel activity stain; panel B: 2D BN-PAGE silver stain.

relatively uncontaminated with other proteins and can be subjected to further structural analyses. Thus, this method provides a simple technique that yields important biochemical details about an enzyme. The 2D SDS-PAGE was instrumental in quantitating the amount of enzyme expressed under these stress conditions. It was obvious that FumA was almost absent in the Al-stressed cells (Fig. 4II).

3.5. Conclusions

The utilization of MDH as a coupling enzyme has permitted the detection of six different enzymes from the TCA cycle (FUM), the glyoxylate shunt (MS) to gluconeogenesis (PC, PEPCK), fatty acid metabolism (CL) and amino acid metabolism (AST). Once the target enzyme has been identified in an in-gel enzyme assay, it can be
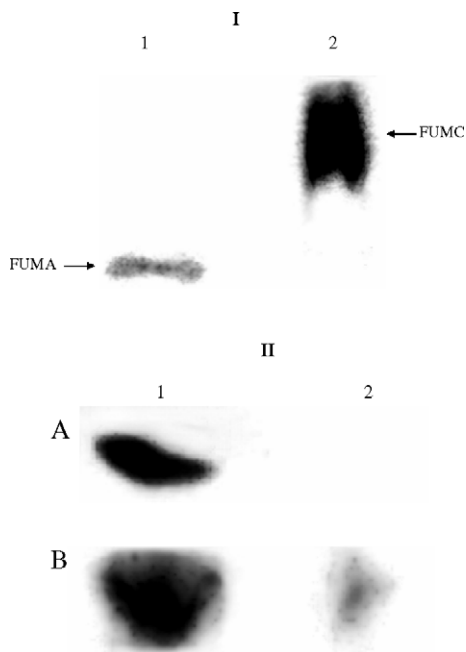
Fig. 4. I. BN-PAGE activity stain of FUM in CFE membranes. Lane 1: citrate medium; 2: Al–citrate medium. The formation of fumarate was detected with MDH, NAD$^+$, PMS and INT. II. 2D BN-PAGE analysis of FUMA in the membrane CFE. Panel A: 2D BN-PAGE activity stain. Panel B: silver stain after BN/SDS-PAGE. Slabs of gel from the 1D corresponding to FUMA activity were soaked in 1% SDS and 1% 2-mercaptoethanol for 2 h before running the 2D. Lane 1: citrate medium; 2: Al–citrate medium.

purified by 2D SDS-PAGE and subjected to further detailed chemical analyses. This technique is inexpensive and is readily accessible in most laboratories and obviates the need for time-consuming Western blot analysis that is ineffective for activity measurement.

4. Simplified description of the method and its applications

The BN-PAGE procedure coupled with the MDH-mediated detection of enzymatic activities yields very important biochemical information about enzymes involved in the metabolism of malate and oxaloacetate. This inexpensive technology can be extended to study a variety of biological systems where the homeostases of these four-carbon metabolites are critical for survival and proliferation.

Acknowledgements

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