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Detection and purification of glucose 6-phosphate dehydrogenase, malic enzyme, and NADP-dependent isocitrate dehydrogenase by blue native polyacrylamide gel electrophoresis

We describe a blue native polyacrylamide gel electrophoretic technique that allows the facile detection, quantitation and purification of three NADPH-producing enzymes. Glucose 6-phosphate dehydrogenase, malic enzyme and NADP-dependent isocitrate dehydrogenase were detected simultaneously. Activity staining based on the formation of NADPH from the respective substrates and the subsequent precipitation of formazan enabled the relative quantitation of enzymatic activities, while Coomassie staining on one-dimensional or two-dimensional gels helped monitor the amount of protein associated with these enzymatic activities. This technique provides a simple and effective route to obtain homogeneous protein for further analyses and also enables the screening of these NADPH-producing enzymes in various cellular systems.

Keywords: Activity staining / Band quantification / Blue native polyacrylamide gel electrophoresis / NADPH-producing enzymes / Protein expression DOI 10.1002/elps.200500040

1 Introduction

NADPH-producing enzymes are essential to all living organisms as they play a key role in biosynthetic processes and in regulating the cellular redox status [1, 2]. Specifically, NADPH is pivotal in lipid synthesis, cellular replication and the functioning of such antioxidative enzymes as catalase and glutathione reductase [3–5]. Glucose 6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), NADP-dependent isocitrate dehydrogenase (NADP-ICDH; EC 1.1.1.42), malic enzyme (ME; EC 1.1.1.40) and phosphogluconate dehydrogenase (PGDH; EC 1.1.1.44) are the four main enzymes involved in NADPH production in most organisms. These enzymes contribute significantly to the intracellular NADPH redox pool, and are pivotal in maintaining a reducing environment in aerobic organisms. G6PDH is a critical component of the oxidative stage of the pentose phosphate pathway, a metabolic network known to produce NADPH and ribose 5-phosphate, two metabolites essential for DNA synthesis [4]. ME generates NADPH and pyruvate; its participation in diverse metabolic pathways ranging from photosynthesis to lipogenesis has been reported [6]. NADP-ICDH is emerging as an essential component of oxidative defence mechanisms due to its ability to generate both NADPH and 2-oxoglutarate, two strong antioxidants [7, 8].

Most NADPH-producing enzymes are routinely measured spectrophotometrically [7, 9]. However, this has to be done separately for each enzyme and this technique measures activity without quantifying actual protein expression. Furthermore, this analytical procedure is at a disadvantage due to low sensitivity and its requirement for large quantities of protein *per* sample. Other methods for measuring NADPH-producing enzymes, such as modified carbon paste electrodes for flow injection amperometric determination and double starter pH-metry, require electrodes that are both expensive and fragile [10, 11]. The use of electrodes has to be done separately for each enzyme and protein expression cannot be measured. The staining of enzymatic activities in gels has been utilised to monitor numerous soluble enzymes. However, only native PAGE protocols have been reported and the in-gel activity staining of these three NADPH-producing enzymes has not been shown on a same gel [12].

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Abbreviations: BN-PAGE, blue native polyacrylamide gel electrophoresis; **CFE**, cell-free extract; **G6PDH**, glucose 6-phosphate dehydrogenase; **INT**, iodonitrotetrazolium; **ME**, malic enzyme; **NADP-ICDH**, $NADP^+$-dependent isocitrate dehydrogenase; **PMS**, phenazine methosulphate

Here, we report the use of blue native polyacrylamide gel electrophoresis (BN-PAGE) to detect, quantify and purify three NADPH-generating enzymes. Although this electrophoretic method is routinely utilised in the study of mitochondrial proteins due to the ability of CBB G-250 dye to induce a negative charge on the protein complexes without destabilising them [13], it has not been exploited to monitor soluble enzymes. This is the first report demonstrating simultaneous in-gel activity staining of three NADPH-generating enzymes by BN-PAGE. This method appears to be better than regular native PAGE as it generates well-resolved bands and the enzymes are less labile, *i.e.*, in-gel activity can be maintained for longer periods.

2 Materials and methods

CBB G-250 was purchased from Eastman Kodak Co. (Rochester, NY, USA); ammonium persulphate, BioRad Protein Assay reagent and *N*,*N*'-methylenebisacrylamide were from BioRad Laboratories (Mississauga, ON, Canada); all other chemicals were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). The bacterial strain *Pseudomonas fluorescens* 13525 was obtained from the American Type Culture Collection (Rockville, MD, USA) and was maintained on a mineral medium containing citric acid. The medium was solidified by addition of 2% w/v agar. The sterile agar test tubes were maintained at 4°C. Control media represented those lacking the test metal. The media consisted of Na_2HPO_4 (6.0 g), KH_2PO_4 (3.0 g), NH_4Cl (0.8 g), $MgSO_4 \cdot 7H_2O$ (0.2 g) and citric acid monohydrate (4.0 g) in 1 L deionised, distilled water. Trace elements in concentrations as described in [14] were also included. The pH was adjusted to 6.8 with dilute NaOH. The media were dispensed in 200 mL amounts in 500 mL Erlenmeyer flasks, stoppered with foam plugs and autoclaved for 20 min at 121°C. Aluminium-citrate media were prepared in the same manner as the control medium with the following modifications: 4 g citric acid monohydrate and 3.621 g $AlCl_3 \cdot 6H_2O$ were first allowed to form a complex in approximately 50 mL deionised, 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 utilised. 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 cells were harvested at the desired time intervals.

P. fluorescens 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 cell storage buffer (50 mM Tris-HCl, 1 mM phenylmethylsulphonyl fluoride, 1 mM DTT, pH 7.4) and ruptured by sonication at 0°C using a Brunswick sonicator, power level 4, for four time intervals at 15 s *per* interval. The cell-free extract (CFE) was centrifuged at 180 000 × *g* for 60 min at 4°C to yield membrane and soluble components. The membrane fraction was discarded while the soluble CFE fraction was centrifuged again at 180 000 × *g* for 2 h to get a membrane-free preparation. Protein concentrations were determined by Bradford method using the BioRad Protein Assay reagent [15]. BN-PAGE was performed according to the method described by Schägger [13] with the following modifications. Lauryl-maltoside was unnecessary as there were no membranes to dissolve. For all gels, 1 mm spacers were used to make small gels (8 × 7 cm^2) 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 using a gradient former (BioRad). Soluble fractions from CFE obtained from *P. fluorescens* grown in citrate (control), Al-citrate and menadione-enriched media were analysed. Samples were prepared by diluting the soluble protein with 3× BN buffer and water to a final concentration of 4 mg/mL protein equivalent and 1× BN buffer (50 mM Bis-Tris, 500 mM ε-aminocaproic acid, pH 7.0 at 4°C) respectively. Samples were kept at −20°C until use. To each lane was added the desired concentration of protein and run under BN 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 Bis-Tris, 0.02% w/v CBB G-250; pH 7.0 at 4°C) was exchanged with a colourless one (identical buffer as blue cathode with the omission of CBB G-250). (Note: the chamber was not washed so that a small amount of CBB still remained.) During the run, the gel usually destains and protein bands may be easily visualised. Electrophoresis was stopped before the running front moved out of the gel. For 2-D BN-PAGE, the activity bands were excised. These pieces of gel, with the desired protein, were placed vertically in a second BN-gel and stacking gel was poured around them. Again, the gel was run as before. In the case of 3-D SDS-PAGE, gels from a 2-D 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 modified from Laemmli [17] (0.025 M Tris-HCl, 0.192 M glycine and 0.1% w/v SDS; pH 8.3), then dots corresponding to the desired protein

were excised and placed onto a 10% SDS gel (8 × 7 cm^2) before being overlaid with stacking gel. Electrophoresis was conducted at 200 V for 45 min at room temperature. Following BN-PAGE, the gels were allowed to equilibrate in 3–5 mL of reaction buffer (25 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4) for 15 min. This treatment helped optimise enzymatic activity associated within the gel. The gels were then placed in 3 mL of reaction buffer (1.5 mL/lane) with the appropriate substrates and cofactors, depending on the enzyme to be monitored. Enzyme activities in the gel were detected by the precipitation of formazan from iodonitrotetrazolium (INT). NADP-ICDH activity was tested by incubating BN gels with isocitrate (5 mM), $NADP^+$ (0.5 mM), 0.4 mg/mL INT and 0.2 mg/mL phenazine methosulphate (PMS) in activity buffer. For ME activity, malate (5 mM) was the substrate while for G6PDH, glucose 6-phosphate (5 mM) was included in the reaction mixture. In experiments where the three enzymes were detected simultaneously, the three substrates (isocitrate, glucose 6-phosphate and malate) were present in the reaction mixture (3 mL). To fix bands and remove excess CBB 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 utilised for activity measurement were stained with 0.05% w/v CBB R-250, 40% methanol and 7% acetic acid for 1 h and destained overnight. Band intensities were quantified using Scion Image for Windows (Scion Corporation, Frederick, MD, USA). To quantitate band intensities 'Analyzing Electrophoretic Gels' was followed from the Scion Image Manual [18].

3 Results and discussion

BN-PAGE has been exclusively utilised for assessing the activity and/or relative protein concentration of the mitochondrial complexes [13, 19]. Here, we demonstrate a modified BN-PAGE technique that can readily yield very important information about NADPH-generating enzymes. This BN-PAGE technique allows the visualisation of the migrating bands and maintains enzymes in their active form. All the three enzymes are identified by in-gel activity staining, an observation that is being reported for the first time. The activity intensity and the sharpness of bands were less marked when a regular native PAGE was performed (data not shown). The charge provided by the dye coupled with the enzymatic stability promoted by ε-aminocaproic acid *via* preferential hydration may contribute to the efficacy of this procedure. This procedure yielded more intense bands. The enzymes from the BN-PAGE experiments were even active after storing the gels overnight at 4°C. The interaction of CBB G-250 dye and the ε-aminocaproic acid with these enzymes may contribute to this increased stability. This feature is essential for resolving the activity of enzyme complexes in membrane components. In this instance, these compounds confer stability to these soluble enzymes. Compared to native PAGE, this electrophoretic method can easily reveal both the activity of an enzyme and its concentration. Here, the enzymes involved in the maintenance of cellular redox potential can all be simultaneously monitored. Following BN-PAGE of the soluble extract from *P. fluorescens* grown in a citrate medium, the gel was incubated with isocitrate, $NADP^+$, INT and PMS. A band due to formazan precipitation was evident in 5 min. When ME and G6PDH were tested with their respective substrates, malate and glucose 6-phosphate, bands with different mobility were detected. However, when the gel was incubated with the three different substrates, the bands that were observed individually were also evident, albeit at different times. The band corresponding to ICDH activity appeared fastest while that attributable to G6PDH was observed in 15 min. These bands were found to augment when increasing protein samples were loaded. Figure 1 shows the activity bands corresponding to G6PDH, ME and ICDH after BN-PAGE. Lanes 1, 2 and 3 contained 20, 40 and 60 μg of the soluble CFE protein, respectively. The molecular masses corresponding to these bands appeared to correlate with that reported in literature where G6PDH, ICDH and ME have decreasing molecular masses, respectively [20–22]. In another gel where soluble CFE protein was loaded in increasing concentrations, a corresponding increase in band intensities was observed when the proteins were visualised by staining the gel with CBB R-250 dye (Fig. 2). When activity

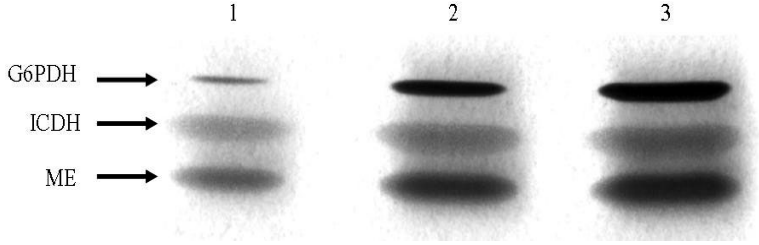

Figure 1. In-gel activity staining of NADP-ICDH, ME and G6PDH in *P. fluorescens* grown in a citrate medium. Lanes 1, 2 and 3 correspond to 20, 40 and 60 μg soluble CFE protein, respectively.

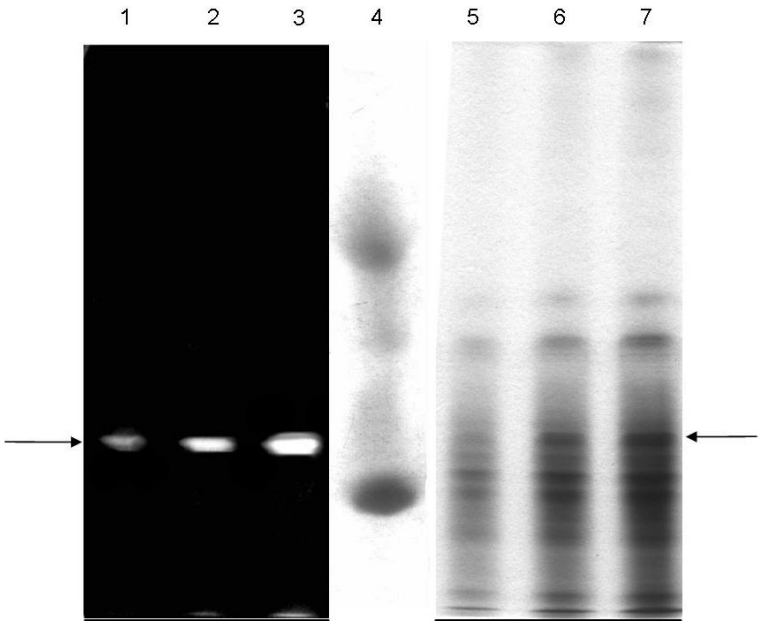

Figure 2. In-gel activity and CBB staining of NADP-ICDH in *P. fluorescens* isolated from a citrate medium. Lanes 1–3: activity staining (image has been digitally inverted to facilitate the visualisation of activity bands); lanes 4–7: CBB staining. Lanes 1 and 5, 2 and 6, and 3 and 7 correspond to 20, 40 and 60 μg soluble CFE protein, respectively. Lane 4: BSA molecular mass standard where top band corresponds to 133 kDa and bottom band corresponds to 66.5 kDa.

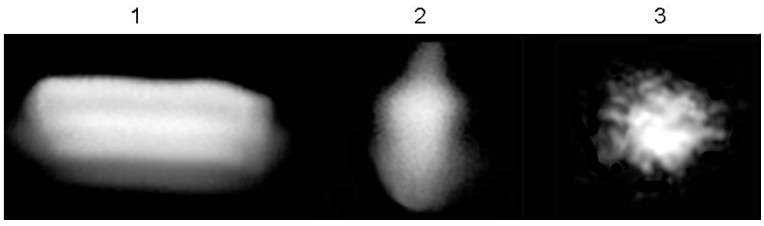

Figure 3. Purification of NADP-ICDH with BN-PAGE (image has been digitally inverted in order to facilitate the visualisation of bands). Lane 1: 1-D in-gel activity staining of soluble CFE; lane 2: 2-D in-gel activity staining of NADP-ICDH from soluble CFE; lane 3: CBB staining of NADP-ICDH from soluble CFE where 1-D and 2-D were BN-PAGE and 3-D was SDS-PAGE (CFE obtained from *P. fluorescens* in citrate medium).

bands of ICDH were compared to the protein bands observed with the CBB R-250 dye, a concomitant increase was readily discernable. Thus, it is evident that the higher activity observed was due to increased protein concentration. This simple procedure indeed obviates the need for expensive immunoblot experiments and enables the monitoring of both activity and expression of a given enzyme, two key biochemical parameters necessary to decipher cellular behaviour.

Furthermore, BN-PAGE can be a potent tool to generate homogeneous protein. Figure 3 shows a 1-D BN-PAGE of ICDH activity along with a 2-D BN-PAGE of ICDH activity and a 3-D SDS-PAGE of ICDH expression. This allowed for the resolution of a sharper activity band, as interfering proteins that were present during the 1-D BN-PAGE experiment were absent in this case. This represents a facile and more accurate strategy to determine the amount of protein expressed. The 3-D technique of combining BN- and SDS-PAGE also helped confirm the purity and the homomeric nature of this enzyme, and it is also a quick way of purifying an enzyme before subjecting it to more detailed analyses like mass spectrometric identification. A similar technique was used to identify the different subunits of the mitochondrial electron transport chain by MALDI-TOF-MS [16]. Only a 1-D BN-PAGE followed by a 2-D SDS-PAGE enabled further structural studies. However, in the present study further treatment of the gel *via* 3-D SDS-PAGE would help assure a relatively pure protein. The determination of activity of NADPH-producing enzymes is useful for evaluating the redox status of cellular systems under various stress conditions. Figure 4 demonstrates how ME can be studied in *P. fluorescens* subjected to superoxide stress. The more intense band in the menadione culture is a clear indication of the elevated activity of this enzyme. These bands can be readily quantified by programs such as Scion Image. In this instance, the stronger band intensities in menadione cultures were supported by values that were numerous folds higher to those obtained in the control cultures. Hence, BN-PAGE can help determine the redox status of organisms under various stress conditions. An additional benefit of this technique is the possible identification of different isozymes. Figure 5 shows the

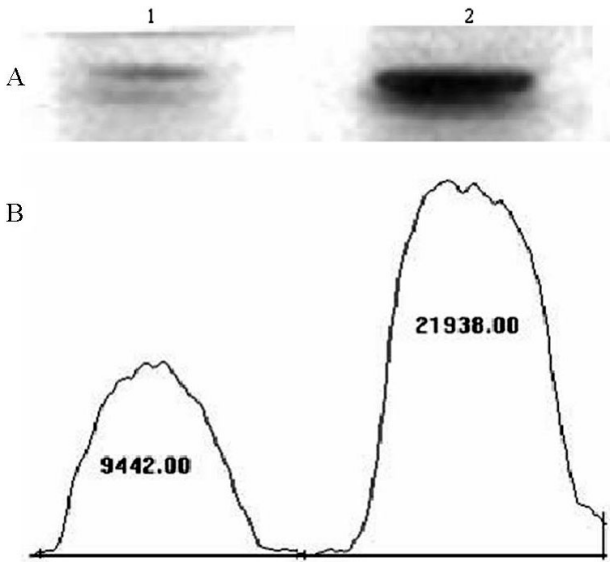

Figure 4. In-gel activity staining of ME from soluble CFE obtained under various conditions. Lane 1: citrate medium; lane 2: citrate medium enriched with menadione. (A) In-gel activity stain; (B) band intensity of in-gel activity stain as quantified by Scion Image (60 μg of protein sample was utilised).

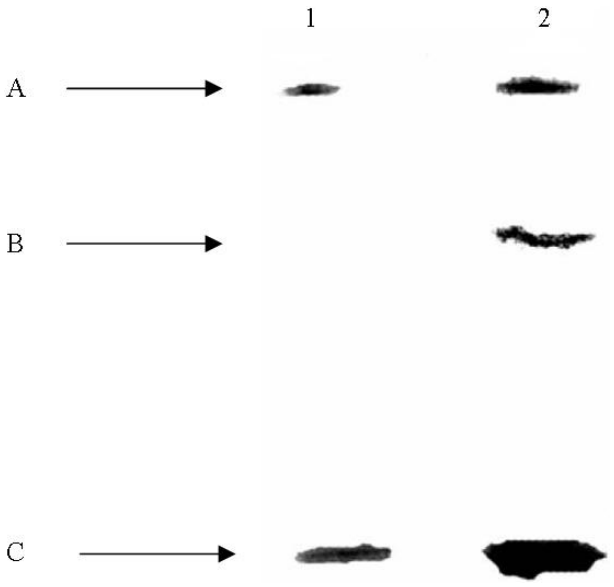

Figure 5. In-gel activity staining of G6PDH under aluminium stress. Lane 1: citrate medium soluble CFE; lane 2: Al-citrate medium soluble CFE. Note the appearance of a novel isozyme (B) in the Al-stressed CFE. These activities were further confirmed by incubating pieces of gel separately (60 μg of protein sample was utilised).

appearance of different isozymes of G6PDH in cells exposed to Al. Although these bands appeared at different times, incubating different segments of the gel confirmed the presence of three enzymes interacting with glucose 6-phosphate. Such an observation would never have been reported if the activity was only measured spectrophotometrically and/or by a regular native PAGE, where the bands were poorly resolved and the enzymatic activity labile.

4 Concluding remarks

In conclusion, the BN-PAGE technique described in this report provides an efficient tool for studying NADPH-producing enzymes. Activity measurement and enzymatic expression can be readily monitored. The enzymes can also be purified, excised and subsequently subjected to further analyses. Multiple forms of enzymes can be readily identified and the temporal variation and status of these enzymes under various stress conditions can be studied. Simultaneous detection of these NADPH-generating enzymes within a cellular system is also possible. Thus, BN-PAGE is a powerful analytical tool to monitor even soluble enzymes. The inclusion of CBB G-250 and ε-aminocaproic acid helps maintain in-gel activity. Hence, it appears to be superior to regular native PAGE as this technique gave poor resolution and was characterised by less intense activity bands. Furthermore, this is the first report of an in-gel activity staining of three NADPH-producing enzymes on the same gel.

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