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Simultaneous Monitoring of Activities of Numerous Tricarboxylic Acid Cycle Enzymes by Blue Native Polyacrylamide Gel Electrophoresis

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Abstract: Numerous soluble and membrane enzymes that mediate the tricarboxylic acid (TCA) cycle were simultaneously monitored for their activity and expression by Blue Native Polyacrylamide Electrophoresis (BN-PAGE). These enzymes were characterized by the specific staining obtained following the precipitation of formazan upon incubation of the gel with their respective substrates. NAD^+-dependent isocitrate dehydrogenase (NAD-ICDH), α-ketoglurarate dehydrogenase (KGDH), malate dehydrogenase (MDH) and succinate dehydrogenase (SDH) were identified on the same gel. Fumarase (FUM) and aconitase (ACN) were detected through the formation of their products malate and isocitrate, respectively. Purple bands were observed when the gels were incubated with either MDH and iodonitrotetrazolium (INT) or ICDH and INT. Two dimensional (2D) BN-PAGE and/or 2D SDS-PAGE enabled the quantitation and purification of these enzymes. These results demonstrate that BN-PAGE can be adapted to measure the activity and relative expression of various TCA cycle enzymes and can also be utilized to study the fate of these enzymes in various biological systems.

Key words: Metabolic enzymes, activity, expression, electrophoresis

Introduction

Tricarboxylic acid (TCA) cycle is a pivotal metabolic network in all oxidative organisms. It provides reducing factors such as NADH and $FADH_2$ that drive the generation of ATP and also acts as a pool of precursors essential in various anabolic reactions (Fernie *et al*., 2004). This metabolic circuit is mediated by 8 enzymes that essentially convert acetyl-CoA into CO_2 and reducing equivalents. The formation of citrate from acetyl-CoA and oxaloacetate, a process aided by citrate synthase (CS), charges up the TCA cycle engine. ACN and NAD-ICDH help in the formation of α-ketoglurarate and NADH. The decarboxylation of α-ketoglurarate to succinyl-CoA is catalyzed by KGDH (Shi *et al*., 2005). This succinate derivative is subsequently oxidized to oxaloacetate via a series of enzymes such as succinyl-CoA synthetase, SDH, FUM and MDH (Dunn, 1998).

Owing to its central role in cellular metabolism and its contribution in ATP formation, a variety of spectrophotometric assays have been widely used to monitor the TCA cycle enzymes in cell free extracts (CFE) (Hamel and Appanna, 2001; Kulkarni *et al*., 2004). The measurement of NADH production, to the quantitation of side-products formed as a result of sometimes complex reactions

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involving dinitrophenylhydrazones, dithionitrobenzoate or 4-nitrosoanilines, have often been utilized (Williams *et al*., 1998; Campbell *et al*., 2002). However, these techniques suffer from three limitations. First, it is prone to interference from non-specific reactions in the CFE. Second, it cannot quantify the amounts of protein associated with the enzymatic activity and third, the experimental design necessitates adequate CFE. Other techniques of measuring enzymatic activities such as nuclear magnetic resonance spectroscopy and mass spectrometry involve the use of expensive and elaborate equipments that are not readily accessible. To overcome these shortcomings, we applied BN-PAGE to monitor the activity and expression of these TCA cycle enzymes. Furthermore, this technique can also be utilized to purify these enzymes for subsequent analyses. BN-PAGE was originally developed by Schagger and von Jagow (1991) for the separation of enzymatically active heteromultimeric proteins localized in the inner mitochondrial membrane. Coomassie dye (G-250) that is included in the sample cathode buffer induces a negative charge shift on the proteins and the 6-amino hexanoic acid helps stabilize the proteins. These features enable the migration of the proteins without impeding their activities (Schagger *et al*., 1994).

We have utilized this technique to simultaneously detect SDH, ICDH, KGDH and MDH. The production of the reducing factor was detected by the formation of a band at the site of the enzyme due to the precipitation of INT as formazan. The formation of malate by FUM and isocitrate by ACN was detected by coupling these products with the enzymes MDH and ICDH, respectively. Coomassie staining on one dimensional (1D) BN-PAGE, 2D BN-PAGE and SDS-PAGE allowed for the relative quantitation of protein using densitometry. This is the first report demonstrating simultaneous in-gel activity staining of several TCA cycle enzymes by BN-PAGE. Thus, BN-PAGE provides a facile and inexpensive procedure to quantitate, purify and monitor numerous TCA cycle enzymes.

Materials and Methods

Chemicals

Ammonium per sulphate, Bio-Rad Protein Assay, Bio-Rad Silver Stain Kit and N, N'-methylene-bis-acrylamide were purchased from Bio-Rad Laboratories (Mississauga, ON, Canada); Coomassie Brilliant Blue G-250 from Eastman Kodak Co. (Rochester N.Y., USA); all other chemicals were from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).

Bacterial Growth Conditions

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 as the sole carbon source. The medium was solidified by the addition of 2% (w/v) agar. The sterile agar test tubes were maintained at 4°C. Control media represented media without the test metal. The media consisted of Na_2HPO_4 (6.0 g), KH_2PO_4 (3.0 g), NH_4Cl (0.8 g), $MgSO_4 \bullet 7H_2O$ (0.2 g) and citric acid monohydrate (4.0 g) in one liter of deionized, distilled water. Trace elements were also included in concentrations as described in (Anderson *et al*., 1992). 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.

Aluminum-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 $AlCl_3 \bullet 6H_2O$ 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. Media were inoculated with 1 mL of stationary phase *P. fluorescens*, grown in control media and aerated on a gyrotory water bath shaker, model 76 (New Brunswick Scientific) at 26°C. The cells were harvested at the desired time intervals.

Bacterial Isolation, Sample Preparation and Electrophoresis Conditions

P. fluorescens were collected by centrifugation at 16,500 x g for 10 min at 4°C. The bacterial pellet was washed in 0.85% (w/v) NaCl and centrifuged again for 10 min at 16,500 x g. The cells were resuspended in cell storage buffer (50 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, pH 7.4) and ruptured by sonication at 0°C using a Brunswick sonicator, power level 4, for four intervals at 15 sec per interval. The CFE was centrifuged at 180,000 x g for 60 min at 4°C to yield membrane and soluble components. The membrane CFE fraction was resuspended in cell storage buffer while the soluble CFE fraction was centrifuged again at 180,000 x g for 2 h to afford a membrane-free preparation. Protein concentrations were determined by the Bradford method (Bradford, 1976).

BN-PAGE was performed according to the method described in (Schagger and von Jagow, 1991) with the following modifications. For all gels, 1 mm spacers were used to make 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 using a gradient former (Bio-Rad). Membrane and soluble fractions from CFE obtained from *P. fluorescens* grown in citrate (control) and Al-citrate media were analysed. Membrane samples were prepared by diluting the soluble protein with 3X Blue Native (BN) buffer, 10% (w/v) lauryl-maltoside and water to a final concentration of 4 mg mL^{-1} protein equivalent, 1% (w/v) lauryl-maltoside and 1X BN buffer (50 mM BisTris, 500 mM 6-amino hexanoic acid, pH 7.0 at 4°C), respectively. Preparation of soluble protein samples was identical except that lauryl-maltoside was omitted. Samples were kept at -20°C until further use. To each lane was added the desired concentration of protein and ran under blue native conditions. Eighty volt 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 with the omission of 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 easily visualized. Electrophoresis was stopped before the running front moved out of the gel.

For 2D SDS-PAGE, gels from a 1D BN-PAGE were rinsed twice for 5 min then soaked for two hours in a solution of 1% (w/v) SDS and 1 % (v/v) 2-mercaptoethanol (Devreese *et al*., 2002). Gels were then rinsed twice for 5 sec with 1X SDS-PAGE electrophoresis buffer (0.025 M Tris-HCl, 0.192 M glycine and 0.1% (w/v) SDS; pH 8.3) (Laemmli, 1970), then bands corresponding to the desired protein were excised and placed onto a SDS gel before being overlaid with stacking gel. During 2D BN-PAGE, the excised bands from the 1D BN-PAGE were run under the same conditions as aforementioned. Electrophoresis was conducted at 200 V for 45 min at room temperature. In the case of colorless native PAGE (CN-PAGE) one cathode buffer was utilized and it contained 0.001% (w/v) Ponceau S instead of Coomassie G 250 (Schagger *et al*., 1994). This protocol obviated the need for a colorless cathode buffer.

In-Gel Activity Staining and Silver Staining

Following BN-PAGE, the gels were allowed to equilibrate in a reaction buffer (25 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4) for 15 min. The gels were then placed in the reaction buffer with the appropriate substrates and cofactors, depending on the enzymes to be monitored. Enzyme activities

in the gel were detected by the precipitation of formazan from INT (Beriault *et al*., 2005). ACN activity was tested by incubating BN gels with citrate (10 mM), 40 U mL^{-1} NADP-ICDH from porcine heart, NADP (0.5 mM), 0.4 mg mL^{-1} INT and 0.2 mg mL^{-1} phenazine methosulfate (PMS) in activity buffer. NAD-ICDH activity was tested by incubating BN gels with isocitrate (5 mM), NAD (0.5 mM), 0.4 mg mL^{-1} INT and 0.2 mg mL^{-1} PMS in activity buffer. For KGDH activity α-ketoglutarate (5 mM), NAD (0.5 mM) and Coenzyme A (0.1 mM) were included in the reaction mixture. For SDH, succinate (20 mM), 0.4 mg mL^{-1} INT and 0.2 mg mL^{-1} PMS were incubated in activity buffer enriched with cyanide (25 mM Tris-HCl, 5 mM $MgCl_2$, 5 mM KCN, pH 7.4). FUM activity was measured by including fumarate (5 mM), malate dehydrogenase from porcine heart (5 U mL^{-1}) and NAD (0.5 mM) in activity buffer while for MDH, malate (5 mM) and NAD (0.5 mM) were included in the reaction mixture. Following the identification of the activity band associated individually and in sequence, the four enzymes were detected simultaneously. In experiments where the four enzymes were detected simultaneously, the four substrates (isocitrate (5 mM), α-ketoglutarate (5 mM), succinate (20 mM) and malate (5 mM), Coenzyme A (0.1 mM) and NAD^+ (0.5 mM)) were present in the reaction mixture. To fix the 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 Coomassie R-250 or the Bio-Rad Silver Stain Kit and destained overnight. Band intensities were quantified using Scion Image for Windows (Scion Corporation, Frederick, Maryland, USA). To quantitate band intensities Analyzing Electrophoretic Gels was followed from the Scion Image Manual. The apparent molecular masses were estimated by using molecular mass markers: bovine serum albumin dimer (133 kDa) and bovine serum albumin monomer (66 kDa). Further confirmation of the nature of the enzymes was determined by the appropriate, specific inhibitors and/or antibodies. ACN polyclonal antibody was provided by Dr. R.S. Eisenstein, University of Wisconsin, Madison, Wisconsin.

Results

To analyze the activity and expression of TCA cycle enzymes, CFE were obtained from *Pseudomonas fluorescens* grown in citrate and Al-citrate media. The membrane CFE was solubilized by the non-ionic detergent lauryl-maltoside and applied on a non-denaturing gradient polyacrylamide gel. The resolution of the various membrane proteins and discrete patterns were distinctly evident (data not shown). In an effort to identify the various enzymes operative in the TCA cycle, the gel was incubated individually with different substrates in the presence of PMS and INT. The production of NADH or $FADH_2$ at the enzyme site was identified by the precipitation of formazan. The substrate specific for each enzyme was included in the reaction mixture and the whole gel strip was incubated for 10 to 40 min depending on the enzyme to be screened. A band corresponding to MDH activity appeared the fastest, while that attributable to NAD-ICDH was evident in 40 min. This experiment was subsequently performed in sequence. Following the appearance of an activity band, the gel was placed in the equilibration buffer and incubated with other substrates. As the activity bands had disparate Rf values (data not shown), the four substrates, namely isocitrate, malate, α-ketoglutarate and succinate and the respective cofactors were all subsequently included in the reaction mixture. A similar banding pattern compared to when the substrate was individually present in the reaction mixture was observed (Fig. 1). There was no significant variation in the Rf values nor in band intensities whether the enzymes were detected either individually or simultaneously. To investigate the detection limit of this in-gel activity staining technique, various amounts of protein were loaded.

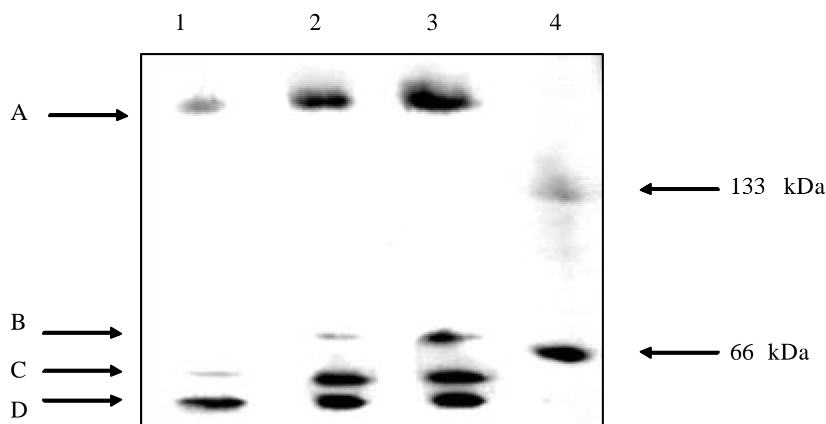

Fig. 1: In-gel activity staining of TCA cycle enzymes.A: SDH; B: KGDH; C: ICDH; D: MDH. Lane 1: 60 µg protein; lane 2: 90 µg protein; lane 3: 120 µg protein; lane 4: Coomassie staining of a BSA standard. (membrane CFE from *P. fluorescens* were utilized and the substrates were succinate (A), α-ketoglutarate (B), isocitrate (C) and (D) malate.

It appeared that the detection limit of MDH was the lowest. However, 90 µg of membrane CFE gave bands that were easily quantified by densitometry (data not shown). The nature of these enzymes were further confirmed by their apparent molecular masses that were within the range reported in the literature (Pettit *et al*., 1973; Evans and Ratledge, 1985; Unden and Kroger, 1986; Tayeh and Madigan, 1987) and by the use of inhibitors such as malonate for SDH, oxaloacetate for MDH and 1,2,3, propanetricarboxylic acid for ICDH. The omission of any cofactor from any of these enzymes also resulted in no activity band. KGDH was also probed with polyclonal antibodies (data not shown).

To quantitate the amount of protein associated with the enzymatic activities, the activity bands from 1D BN-PAGE were excised and 2D BN-PAGE and/or 2D SDS-PAGE was performed. This technique enabled the monitoring of the numerous relative quantities of proteins responsible for the activity. This obviates the need for antibodies that may be not readily available. It is important to take into consideration the cross-reaction with other proteins that may decrease the effectiveness of assays involving antibodies. The specificity of enzymes for a particular substrate affords a degree of selectivity that is not usually evident in assays involving polyclonal antibodies. Furthermore, BN-PAGE enables the monitoring of both activity and expression, features essential for the proper explanation of biochemical phenomena. In Fig. 2 and 3, the activities associated with ICDH and KGDH are shown. The amount of proteins that may be contributing to these activities did correspond to the data obtained by staining the 1D and 2D gels with Coomassie R 250 dye, respectively. The proteins appeared to be relatively homogeneous and can be readily subjected to further structural analyses. Figure 2 III shows how this technique can be readily invoked to study the status of an enzyme in control and stressed conditions. Only a faint band, indicative of diminished NAD-ICDH activity in stressed cultures, was evident.

The other TCA cycle enzymes studied during this investigation were visualized with the aid of the coupling enzymes ICDH and MDH. The former was utilized to detect ACN while the latter aided in the identification of FUM. ACN, a protein that acts both as an enzyme and a transcription factor, is relatively labile (Middaugh *et al*., 2005). The tricarboxylic acid, 1,2,3-propanetricarboxylic acid, was utilized as a stabilizer. Sixty microgram of soluble CFE did give a band corresponding to ACN activity.

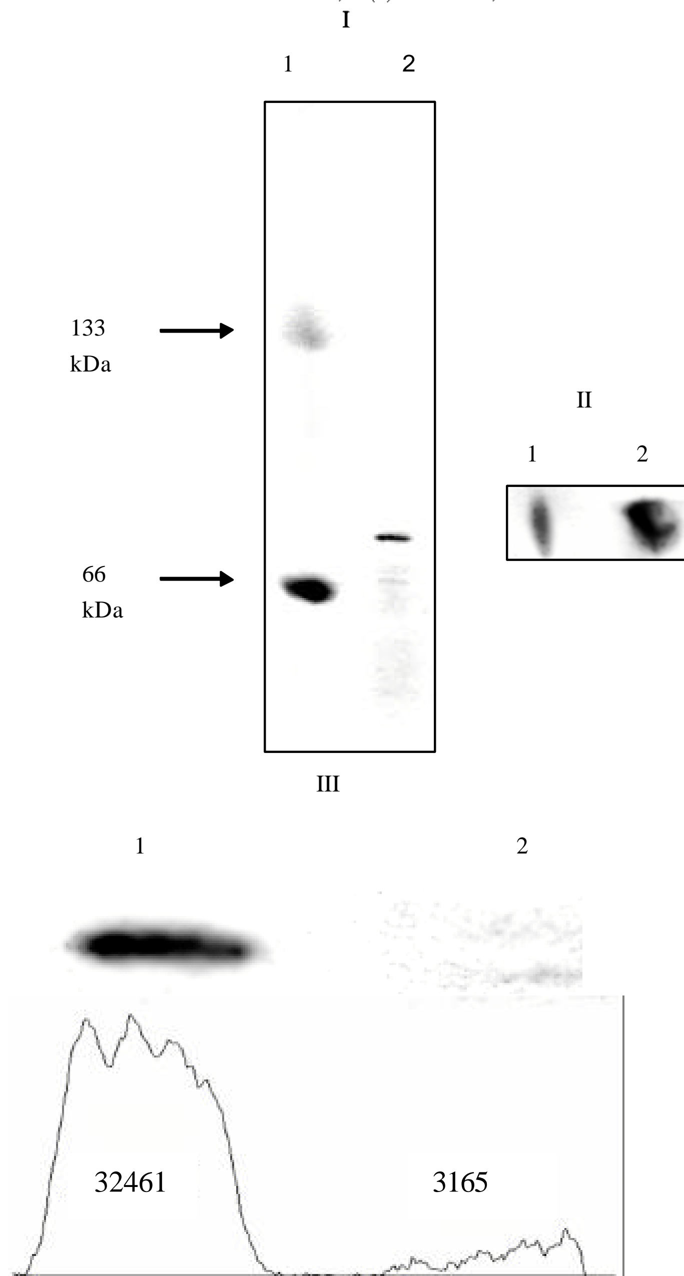

Fig. 2 I: In-gel activity staining and protein quantitation of NAD-ICDH by BN-PAGE. Lane 1: BSA standard; lane 2: in-gel activity stain of NAD-ICDH from citrate membrane CFE with BN-PAGE. II. 2D PAGE analysis of NAD-ICDH in the membrane CFE. Lane 1: 2D PAGE in-gel activity staining (1D BN-PAGE and 2D CN-PAGE). Lane 2: silver stain of 1D BN-PAGE followed by 2D SDS-PAGE. Slabs of gel from the 1D were soaked in 1% SDS and 1% 2-mercaptoethanol for 2 h before running the 2D. III. In-gel activity and quantitation of NAD-ICDH. Lane 1: citrate membrane CFE; 2: Al-citrate membrane CFE; Band intensity was quantified with Scion Image

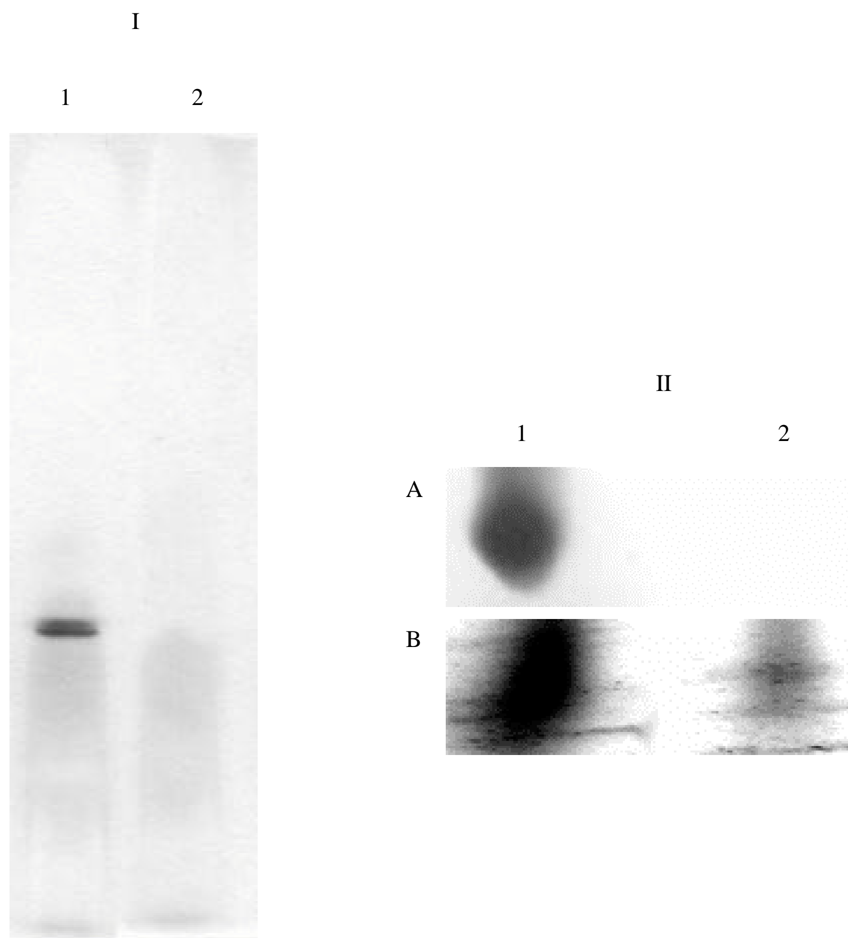

Fig. 3: Activity staining and protein expression of KGDH by BN-PAGE. I. 1D BN-PAGE in-gel activity staining of KGDH. Lane 1: membrane CFE from citrate medium; 2: membrane CFE from Al-citrate medium. II. Panel A: 1D and 2D BN-PAGE activity stain. Panel B: silver stain where 1D and 2D were BN-PAGE. Lane 1: membrane CFE from citrate medium; 2: membrane CFE from Al-citrate medium

However, 80 µg of soluble CFE appeared to yield optimal band and was routinely utilized to study this enzyme (Fig. 4I). It is interesting to note that the intensity of the band may be enhanced by incubating the gel in the reaction for a longer time. This technique was pivotal to monitor the changes associated with this enzyme in cells subjected to Al-stress. A Western blot analysis would have demonstrated similar ACN expressed in the control and stressed medium. The BN-PAGE helped establish that the decrease in ACN activity in the Al-stressed *P. fluorescens* was due not to protein concentration but to some intrinsic property associated with the enzyme (Fig 4. A II). When FUM was probed, two bands were observed in the membrane CFE. In this instance, fumarate, NAD, INT, PMS and MDH were included in the reaction mixture. FUMA, an enzyme that has an Fe-S cluster, usually has a molecular mass of 120 kDa while FUMC, an enzyme devoid of a Fe-S cluster has a molecular mass of 200 kDa (Weaver *et al*., 1998). The latter is not sensitive to oxidative stress while the former is. Susceptibility to H_2O_2 and O_2^- and molecular mass markers helped identify the upper

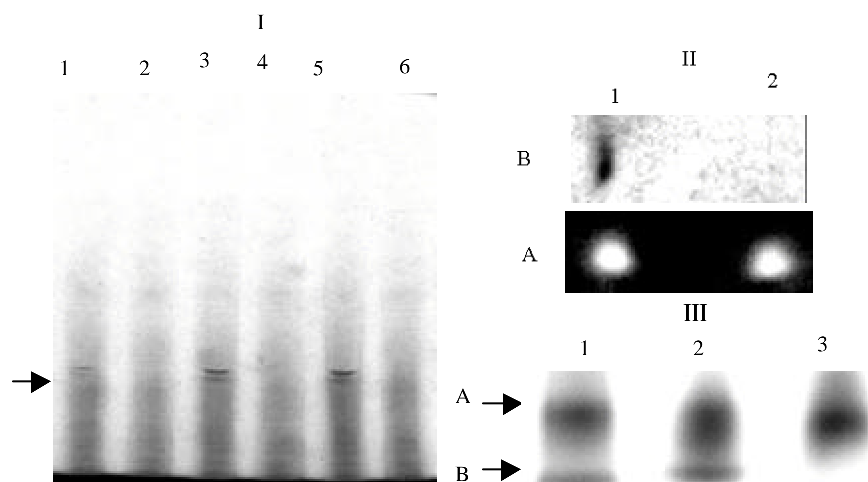

Fig. 4 I and II: In-gel activity staining of two Fe-S containing enzymes of the TCA cycle by BN-PAGE. I. In-gel activity staining of ACN in increasing protein concentrations. Lanes 1 and 2, 3 and 4 and 5 and 6 contain 60, 70 and 80 μg soluble CFE. Odd-numbered lanes are citrate soluble CFE while even-numbered lanes contain Al-citrate soluble CFE (reaction time: 15 min). II. 2D in-gel activity staining and 2D BN/SDS-PAGE/Western blot of ACN. Panel A: 2D BN-PAGE of ACN activity in soluble CFE. Panel B: 1D BN-PAGE, 2D SDS-PAGE (Western blot). Lane 1: soluble CFE from citrate medium; 2: soluble CFE from Al-citrate medium. III. BN-PAGE activity stain of FUM in Al-stressed CFE membranes. Lanes 1, 2 and 3: citrate media enriched with 5, 10 and 15 mM Al, respectively. (A) corresponds to FUMC while (B) corresponds to FUMA. Note the disappearance of FUMA (Fe-S protein) as the concentration of Al was increased

band as FUMC and the lower band as FUMA. Figure 4 III depicts the variation in two bands in cultures grown in 5, 10 and 15 mM Al media, respectively. The enzyme FUMA that does possess an Fe-S cluster was completely absent in cultures with 15 mM Al. The lower band attributable to FUMA was also absent in the CFE treated with either H_2O_2 or O_2^- (data not shown). Hence, this technique is a powerful tool to decipher both the activity, expression and the nature of enzymes in organisms subjected to various stresses.

Discussion

The electrophoretic procedure described in this report provides a facile means of studying six TCA cycle enzymes. Compared to spectrophotometric protocols, two key advantages are inherent in this BN-PAGE methodology. First, activities of these enzymes can be monitored free of interference from other enzymes as the enzymes are separated before in-gel activity studies are performed. In spectrophotometric procedures, inhibitors are usually utilized to selectively arrest the activities of competing enzymes. However, incomplete inhibition and cross-inhibition of non-specific reactions may introduce errors in measurement and tend to provide inaccurate data. Second, relatively small quantities of proteins can be utilized to evaluate numerous enzymes on the same gel, an important consideration in studies with cell lines where obtaining sufficient biomass may be very fastidious. This

technique also allows for the simultaneous determination of the amount of proteins expressed and obviates the need to use expensive antibodies that are often handicapped by lack of specificity. Antibodies have to be stripped before additional Western analyses can be performed. Hence, this procedure is inordinately time-consuming compared to BN-PAGE and enzymatic activity cannot be monitored. However, this technique is superior to native PAGE as the membrane proteins migrated discretely and the enzymatic activity is maintained in the gel. For instance, no activity for KGDH and SDH were detected by PAGE (data not shown). However, since the BN-PAGE method measures relative quantity and activity of the TCA cycle enzymes, it is essential that experimental and control samples are prepared and processed in an identical manner. It is also critical that the proteins be run on the same gel and stained and developed under similar conditions if the results are to be compared.

In summary, our study clearly demonstrates that BN-PAGE is a very effective analytical tool to study the status of six TCA cycle enzymes in various cellular systems. Numerous enzymes can be evaluated simultaneously and both relative activity and protein expression can be readily quantified in an inexpensive manner.

Conclusions

This is the first report on the effectiveness of BN-PAGE to monitor simultaneously numerous TCA cycle enzymes. The relative quantities of proteins associated with the enzymatic activities of ICDH, MDH, SDH and KGDH can be obtained on the same gel and the status of the pivotal metabolic network can readily assessed. This procedure also has an added benefit compared to classical spectrophotometric method as only small quantities of proteins are required. The band corresponding to the enzymatic activities can be readily excised and electrophoresed in the second dimension to afford relatively pure proteins that may be quantified and further characterized. Isoenzymes may also be identified as in the case of FUM since the native confirmation of the enzyme with disparate molecular masses is maintained. In this instance, the BN-PAGE is very useful to detect the status of the two key Fe-dependent enzymes of the TCA cycle. Compared to native PAGE, the migration of the protein bands are more discrete. Furthermore, enzymatic activity is less labile and thus resolution is better. Hence, detection of proteins is more sensitive.

Simplified Description of the Method and its Applications

This BN-PAGE technique provides a facile and effective means to measure the activities and expression of numerous enzymes involved in the TCA cycle. It can be readily utilized to study this crucial metabolic network in a variety of organisms exposed to different environments.

Acknowledgments

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List of Abbreviations

Blue native polyacrylamide gel electrophoresis (BN-PAGE), colourless native polyacrylamide gel electrophoresis (CN-PAGE), NAD^+-dependent isocitrate dehydrogenase (NAD-ICDH), α-ketoglurarate dehydrogenase (KGDH), malate dehydrogenase (MDH) and succinate dehydrogenase (SDH), fumarase (FUM), fumarase A (FUMA), fumarase C (FUMC), aconitase (ACN), iodonitrotetrazolium (INT), tricarboxylic acid (TCA) cycle

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