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A blue native polyacrylamide gel electrophoretic technology to probe the functional proteomics mediating nitrogen homeostasis in *Pseudomonas fluorescens*

Sungwon Han [a], Christopher Auger [a], Varun P. Appanna [a], Joseph Lemire [a], Zachary Castonguay [a], Elchin Akbarov [b], Vasu D. Appanna [a,*]

[a] Department of Chemistry and Biochemistry, Laurentian University, Sudbury, Ontario, Canada, P3E 2C6
[b] Department of Biological Sciences, Khazar University, Baku, Azerbaijan

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ABSTRACT

As glutamate and ammonia play a pivotal role in nitrogen homeostasis, their production is mediated by various enzymes that are widespread in living organisms. Here, we report on an effective electrophoretic method to monitor these enzymes. The in gel activity visualization is based on the interaction of the products, glutamate and ammonia, with glutamate dehydrogenase (GDH, EC: 1.4.1.2) in the presence of either phenazine methosulfate (PMS) or 2,6-dichloroindophenol (DCIP) and iodonitrotetrazolium (INT). The intensity of the activity bands was dependent on the amount of proteins loaded, the incubation time and the concentration of the respective substrates. The following enzymes were readily identified: glutaminase (EC: 3.5.1.2), alanine transaminase (EC: 2.6.1.2), aspartate transaminase (EC: 2.6.1.1), glycine transaminase (EC: 2.6.1.4), ornithine oxoacid aminotransferase (EC: 2.6.1.13), and carbamoyl phosphate synthase I (EC: 6.3.4.16). The specificity of the activity band was confirmed by high pressure liquid chromatography (HPLC) following incubation of the excised band with the corresponding substrates. These bands are amenable to further molecular characterization by a variety of analytical methods. This electrophoretic technology provides a powerful tool to screen these enzymes that contribute to nitrogen homeostasis in *Pseudomonas fluorescens* and possibly in other microbial systems.

1. Introduction

In all biological systems, the presence of nitrogen or its derivatives is necessary for proliferation and survival. The synthesis of cellular proteins and nucleic acids is two such examples where this moiety is essential (Painter, 1970; Sonenshein, 2008). However, at high intracellular concentration, nitrogen containing biomolecules are detrimental to the cell due to their influence on pH and inhibition of a variety of biological processes. Therefore, it is important for organisms to maintain and regulate their intracellular nitrogen content. Glutamate is one of the metabolites that plays a central role in nitrogen metabolism. The enzyme glutamate dehydrogenase (GDH) is the key in the homeostasis of glutamate and consequently nitrogen (Brosnan and Brosnan, 2009; Miller and Magasanik, 1990; Stitt et al., 2002). It fixes ammonia (NH_3) into glutamate that is subsequently utilized to generate essential nitrogen containing biomolecules or eliminate excess nitrogen as NH_3 by the oxidative deamination of glutamate (Prusiner et al., 1976). The by-product of the latter reaction yields α-ketoglutarate (αKG), which can then be used in the tricarboxylic acid (TCA) cycle for intracellular energy production. Therefore, this unique reaction is critical to many nitrogen-dependent metabolic networks throughout the biological systems.

Several enzymes, such as alanine transaminase (ALT) and carbamoyl phosphate synthase (CPS), either generate glutamate or NH_3 and can be coupled to GDH in order to monitor these enzymatic activities. The activity of ALT is commonly detected spectrophotometrically via the measurement of the NADH oxidation produced from the lactate dehydrogenase (LDH)-linked reaction upon the formation of pyruvate following the amination of αKG by alanine (Tarao et al., 1999; Vozarova et al., 2002). The measurement of CPS activity is performed by either tracking radiolabeled substrates or chemically converting carbamoyl phosphate into other metabolites for further analysis (Ahuja et al., 2001; Coleman et al., 1977; Duane, 1980; Mally et al., 1981). Numerous other glutamate, αKG or NH_3 producing enzymes are monitored spectrophotometrically or colorimetrically (Funck et al., 2008; Kim et al., 1994; Panakanti et al., 2010; Taylor and Jenkins, 1966; Wang et al., 2010; Yamaguchi et al., 2003). However, these methods tend to lack sensitivity and specificity in detection especially when measuring crude enzyme samples due to interferences caused by various endogenous materials existing in the cell-free extracts (Auger et al., 2012). More precise techniques such as radioisotope labeling assays are also available, but the complexity in procedures and high expense are some of the drawbacks associated with this type of analysis (Wu et al., 1997).

* Corresponding author. Tel.: +1 705 675 1151x2112; fax: +1 705 675 4844.
E-mail address: vappanna@laurentian.ca (V.D. Appanna).

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In this report, we demonstrate a simple alternative method for monitoring glutamate or NH_3 generating enzymes using blue native polyacrylamide gel electrophoresis (BN-PAGE). GDH is used as an auxiliary enzyme to react with the glutamate or NH_3 produced by a variety of enzymes immobilized in the gel. The conversion of glutamate into αKG reduces $NAD(P)^+$, and the production of NAD(P)H can be monitored using phenazine methosulfate (PMS) and iodonitrotetrazolium chloride (INT). The NH_3 reacts with αKG to generate glutamate in the presence of NAD(P)H and GDH. The oxidation of NAD(P)H is visualized with 2,6-dichloroindophenol (DCIP) and INT. This electrophoretic technique can be utilized to detect a variety of glutamate and NH_3 generating enzymes.

2. Material and methods

2.1. Cell culturing and fractionation

The bacterial strain of *Pseudomonas fluorescens* 13525 was obtained from American Type Culture Collection (ATCC). The culture was grown in a phosphate mineral medium containing Na_2HPO_4 (6 g), KH_2PO_4 (3 g), $MgSO_4$ (0.2 g), NH_4Cl (0.8 g) and citrate (4 g) or glucose (1.35 g) solubilized in 1 L of distilled and deionized H_2O (ddH_2O). The pH of the culture medium was adjusted to 6.8 with 2 M NaOH and trace elements were added to a final concentration of 1% v/v (Lemire et al., 2010). The autoclaved 200 mL aliquot of the media was inoculated with 1 mL of stationary phase cells grown in their respective cultures and aerated on a gyratory water bath shaker (Model 76; New Brunswick Scientific). Cells were isolated at 24 h growth period and pelleted. Cellular pellets were washed with 0.8% w/v NaCl and resuspended in cell storage buffer (CSB; 50 mM Tris–HCl pH 7.3, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonylfluoride, and 1 mM DTT). The cells were homogenized by sonication on ice and centrifuged to remove remaining intact cells. The homogenate was then centrifuged again to obtain soluble and membrane cell-free extracts (CFE) (Auger et al., 2011). The amount of protein was determined by the Bradford assay with bovine serum albumin (BSA) used as a standard (Bradford, 1976).

2.2. Blue native-polyacrylamide gel electrophoresis (BN-PAGE) and in-gel activity assays

BN-PAGE was performed as described in Mailloux et al. (2008) and Schagger and von Jagow (1991). A gradient gel (4–16%) was used for these assays. Protein (4 μg/μL) was prepared in blue native buffer (400 mM 6-amino hexanoic acid, 50 mM Bis–Tris [pH 7.0]). A final concentration of 1% dodecyl-β-maltoside was added to membrane fractions to solubilize membrane bound proteins for optimal protein separation. Each well of the native gel was loaded with 10–60 μg of prepared protein samples. Electrophoresis was performed at 4 °C under native conditions at 80 V and 15 mA for proper stacking, followed by 150 V and 25 mA in resolving gel until the migration of the protein reached half way through the gel. At the halfway point, blue cathode buffer (50 mM Tricine, 15 mM Bis–Tris, 0.02% w/v Coomassie G-250, pH 7 at 4 °C) was changed to a colorless cathode buffer (50 mM Tricine, 15 mM Bis–Tris, pH 7 at 4 °C). The electrophoresis was performed at 300 V and 25 mA beyond this point.

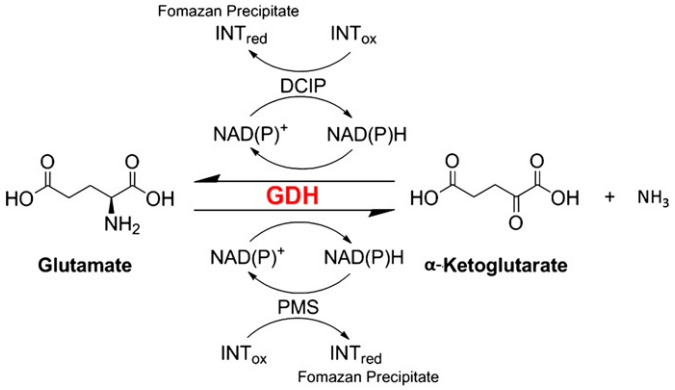

Fig. 1. A schematic representation of the electrophoretic method to detect GDH-linked enzymes. The formation of formazan precipitate is localized at the position of the enzyme in the gel. GDH = glutamate dehydrogenase.

The gel was incubated in reaction buffer (25 mM Tris–HCl, 5 mM $MgCl_2$ [pH 7.4]) for 15 min after completion of electrophoresis. In-gel activity assay was achieved by using a reaction mixture containing equilibrium buffer, 5 mM substrate (Table 1), 0.5 mM cofactors, 5 units of GDH, 0.2 mg/mL PMS or DCIP, and 0.5 μg/mL INT in a total volume of 3 mL. Destaining solution (40% methanol and 10% glacial acetic acid) was used to terminate the reactions. To ensure the equal protein loading, Coomassie staining was used. The specificity in detections was confirmed by performing in-gel reaction in the absence of a substrate or addition of inhibitor. To identify optimal conditions for these reactions, the protein, substrate concentrations and incubation time were varied.

2.3. HPLC analysis

Alliance HPLC with C18 reverse-phase column (Synergi Hydro-RP; 4 μm; 250 × 4.6 mm, Phenomenex) and Waters dual absorbance detector were used to confirm the specificity of the electrophoretic technique. The activity bands were excised from the gel and placed in a reaction mixture containing 2 mM substrates. After 30 min of incubation, 100 μL of the sample was collected and diluted with milli-Q water for HPLC analysis. A mobile phase consisting of 20 mM KH_2PO_4 (pH 2.9) was used at a rate of 0.7 mL/min at ambient temperature to separate the substrates and products, which were measured at 210 nm. Metabolites were identified by spiking biological samples using known standards, and peaks were quantified using the Empower software (Waters Corporation). This semi-quantitative procedure helped further confirm the specificity of enzymes probed in-gel. HPLC analyses were performed immediately after the reactions in order to minimize substrate and product degradation.

2.4. 2D SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described (Singh et al., 2005). The

Table 1
A list of enzymes detected via the GDH-linked in-gel assay.

Enzymes	Substance	Products
Alanine transaminase (EC: 2.6.1.2)[a]	L-alanine + α-ketoglutarate	Pyruvate + L-glutamate
Aspartate transaminase (EC: 2.6.1.1)[a,b]	L-asparate + α-ketoglutarate	Glyoxylate + L-glutamate
Ornithine aminotransferase (EC: 2.6.1.13)[a]	L-ornithine + α-ketoglutarate	L-Δ^1-pyrroline-5-carboxylate + L-glutamate
Glutaminase (EC: 3.5.1.2)[b]	L-glutamine	L-glutamate
Carbamoyl-phosphate synthase (ammonia. EC: 6.3.4.16)[a]	2 ADP + phospate + carbamoyl phospate	2 ATP + NH_3 + CO_2 + H_2O

[a] Enzyme location: soluble cell-free extract.
[b] Enzyme location: membrane cell-free extract (all in-gel reactions were carried out at ambient temperature).

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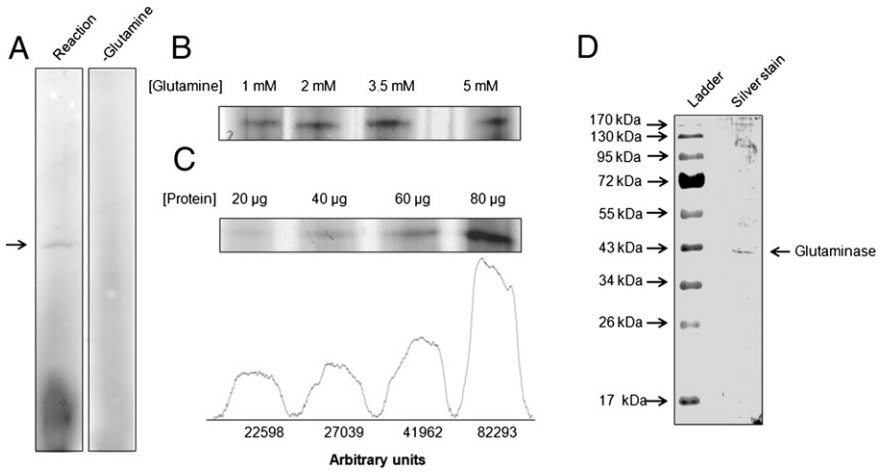

Fig. 2. BN-PAGE analysis of glutaminase via the GDH-linked enzyme assay. Panel A: Glutaminase activity was measured in the membrane CFE. The negative control was performed without glutamine in the reaction mixture. Panel B: A representative gel demonstrating the substrate dose dependence of activity band intensity for glutaminase. Panel C: A representative gel demonstrating the protein concentration dependence for glutaminase activity (densitometric analysis was performed with ImageJ for Windows). Panel D: The activity band for glutaminase was excised and subjected to a 2D SDS-PAGE followed by silver staining.

activity bands from the 1D BN-PAGE were cut from the gel, incubated in denaturing buffer (1% β-mercaptoethanol, 5% SDS) for 30 min and then loaded into a 10% isocratic gel. Electrophoresis was performed at 80 V and 15 mA for stacking, followed by 200 V and 25 mA for the remainder of the procedure. Upon completion of electrophoresis, the gel was subjected to silver stain described in Bio-Rad silver stain procedure (Gottlieb and Chavko, 1987). All experiments were performed independently at least twice and in duplicate. All chemicals were obtained from Sigma-Aldrich unless otherwise indicated.

3. Results and discussion

The primary role of GDH in living systems is to catalyze the reversible oxidative deamination of glutamate to produce α-ketoglutarate, utilizing either NAD^+ or $NADP^+$ as an electron acceptor. As such, this enzyme bridges amino acid catabolism to energy metabolism within the cell. GDH, in conjunction with glutamine synthase, also plays a key role in nitrogen flow and ammonia detoxification, as glutamate is capable of both receiving and donating a nitrogen group (Amon et al., 2009). Here, we demonstrate a simple BN-PAGE technique that exploits the reversibility of this enzyme in order to monitor the activities of enzymes that generate glutamate or NH_3. The reduction of $NAD(P)^+$ or oxidation of NAD(P)H is coupled to PMS or DCIP respectively, which readily reduces INT to generate a visible formazan precipitate that is quantifiable via densitometry (Fig. 1). Furthermore, the in-gel activity band lends itself to detailed molecular studies via a variety of analytical tools.

The ability of this assay to monitor glutaminase, a key enzyme in nitrogen homeostasis, was evaluated. The latter catalyzes the simple conversion of glutamine to glutamate, with the release of ammonia. After BN-PAGE, the gel slabs were incubated in reaction mixtures containing varying concentrations of glutamine. NAD (0.5 mM) was

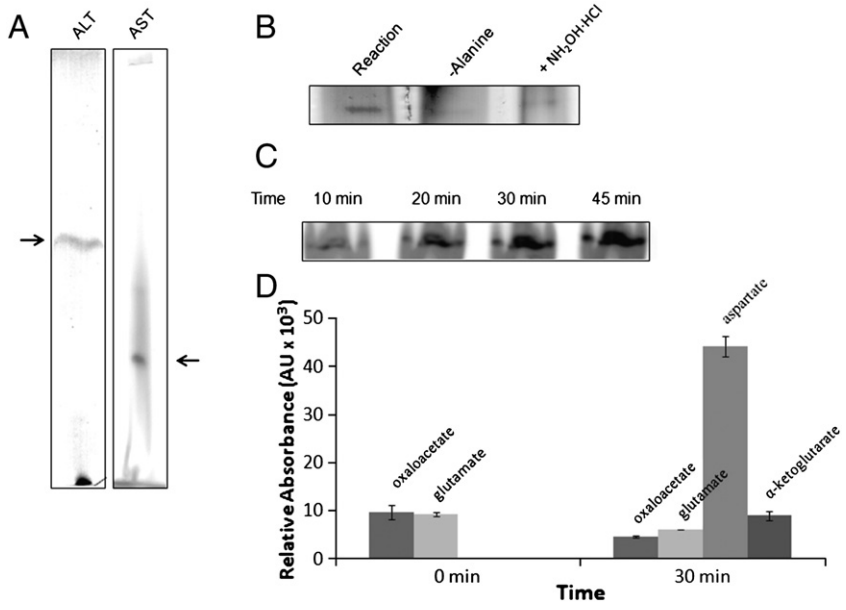

Fig. 3. Transaminase activity analysis via the GDH-linked assay. Panel A: Soluble and membrane CFE were probed for the activity of ALT and AST, respectively. Panel B: ALT was probed in-gel with and without the substrate alanine. The reaction was also performed with 2 mM hydroxylamine hydrochloride in the reaction mixture. Panel C: A representative gel demonstrating the time dependence of formazan precipitation for AST. Panel D: The activity band from panel A was excised and incubated in the reaction mixture for AST for 30 min then subjected to HPLC analysis ($n = 3 \pm$ standard deviation). (Note: When the reaction mixture without the activity band was incubated for 30 min, a slight decrease in oxaloacetate was observed).

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added to the mixture so that the glutamate formed could be coupled to exogenous GDH. To confirm the enzyme in question was indeed glutaminase, negative controls were performed without glutamine. When the substrate was removed in the BN-PAGE experiments, a band indicative of glutaminase was absent, thus confirming the specificity of this enzyme (Fig. 2A). The amount of formazan precipitate formed at the site of the enzyme increased in correlation with the amount of glutamine (Fig. 2B). To show that the increase in band intensity is related to the amount of enzyme in the sample, protein concentrations ranging between 20 and 80 μg were loaded into the gel. Indeed, the band signifying glutaminase activity appeared darker in lanes with higher protein (after 30 min of incubation with 5 mM glutamine, Fig. 2C). The activity bands in the presence of glutamine were excised and loaded into a SDS gel for expression analysis. This enzyme was detected at approximately 40 kDa after SDS-PAGE and silver staining (Abe et al., 1974). Although other bands were present, due to their migration to a similar location on the native gel, glutaminase expression was indeed more pronounced (Fig. 2D).

Transaminases are utilized by living systems to interconvert amino acids and α-keto acids via the removal and subsequent addition of amine groups. Hence, they play important roles in amino acid synthesis and the generation of intermediate metabolites. We next applied our technique to transaminases that function with α-ketoglutarate and glutamate in order to gauge its effectiveness. The enzymes alanine transaminase (ALT) and aspartate transaminase (AST) were probed in-gel following BN-PAGE and a formazan precipitate was visible after 30 min of incubation (Fig. 3A). Again, 0.5 mM NAD was added to the reaction mixture in order to couple the formation of glutamate to GDH. In order to ascertain the identity of ALT, a reaction was performed minus the substrate alanine (Fig. 3B). Additionally, a reaction mixture was created with all the required substrates plus a known inhibitor to hinder enzymatic activity. Indeed, with the addition of 2 mM hydroxylamine hydrochloride (Lain-Guelbenzu et al., 1991), band intensity was greatly reduced (Fig. 3B). To illustrate the time dependence of formazan precipitation, the lane containing 60 μg of soluble cell-free extract was incubated in a reaction mixture containing 5 mM aspartate and αKG for a period of 1 h. Maximum band intensity was achieved within 45 min (Fig. 3C). Confirmation of AST was accomplished using HPLC. The activity band from Fig. 3A was excised and incubated in a reaction buffer containing oxaloacetate and L-glutamate for various time intervals. After 30 min, the peaks attributable to the substrates were diminished, and the concentration of αKG and aspartate was clearly increased (Fig. 3D).

Perhaps the most intriguing application of this technique is the measurement of ammonia-generating enzymes, such as CPS. The latter is a key regulator of the urea cycle, playing a central role in the excretion and detoxification of ammonia in mammals. In bacteria, it is involved in the synthesis of nucleic acids (Koo et al., 2011). The activity of CPS is typically detected by coupling this enzyme to ornithine transcarbamoylase (OTC), followed by the colorimetric measurement of the citrulline formed. Unless the enzyme is purified prior to the assay, this method is subjected to interference by endogenous compounds present in the biological sample [9]. By coupling the formation of ammonia to the activity of exogenous GDH, our BN-PAGE technique permits the in-gel visualization of CPS activity (Fig. 4A). A full list of enzymes that were detected by this assay, including the aminotransferases that act on glycine and ornithine, is shown in Table 1. This technique is also ideal for comparing the enzymatic activity in biological systems growing in different environmental conditions. In *P. fluorescens* subjected to H_2O_2 as a stressor, the activity of glutaminase was increased versus the untreated control (personal observation, Fig. 4B). This up-regulation is not surprising, as the role of glutaminase in antioxidant defense has been reported (Hu et al., 2010). In order to ensure equal loading, protein from both samples were run in an SDS-PAGE and subjected to staining with Coomassie R-250 dye (Fig. 4B). Formazan precipitates more rapidly in the sample with higher biological activity of the enzyme being probed.

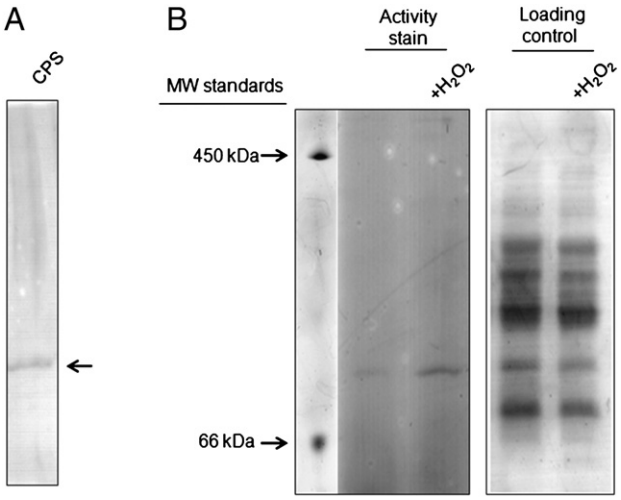

Fig. 4. The application the GDH-linked assay for CPS activity and qualitative analyses. Panel A: CPS was probed in-gel by the coupling of NH_3 formation to exogenous GDH. Panel B: Sixty micrograms (protein equivalent) of cell-free extract from control and 500 μM H_2O_2-treated cultures was loaded in a BN-PAGE gel. Once complete, the gel was incubated with the reaction mixture for glutaminase for 30 min (Note: the increased band intensity is indicative of higher enzymatic activity in that sample; the molecular weight ladder was stained separately with Coomassie Brilliant Blue R-250).

In summary, the BN-PAGE method presented here allows for the cost-efficient and rapid measurement of enzymes that generate glutamate or ammonia. Despite of the simplicity of classical spectrophotometric methods, they are prone to interference by competing enzymes and metabolites present in the biological cell-free systems. By singling out the enzyme of interest in gel, our method eliminates this hindrance. Additionally, the in-gel activity assay illustrated in this report generates a visible band which can be excised and utilized for further studies such as HPLC analysis or 2D electrophoresis (the latter can be followed up by silver staining, Western blotting or by further molecular characterization). Although this technique is being demonstrated using *P. fluorescens* as a model system, it is quite likely that these enzymatic assays can be applied to other microbial systems. For instance, the application of BN-PAGE technology in *Escherichia coli* and *Enterococcus faecalis* has been reported (Maddalo et al., 2011; Reinoso et al., 2012). This method can be employed to compare these enzymes under disparate conditions, making it ideal to probe the modulation of these enzymes in living systems. Hence, it is an important addition to the analytical repertoire aimed at deciphering functional proteomics dedicated to the homeostasis of the pivotal ingredient, nitrogen.

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