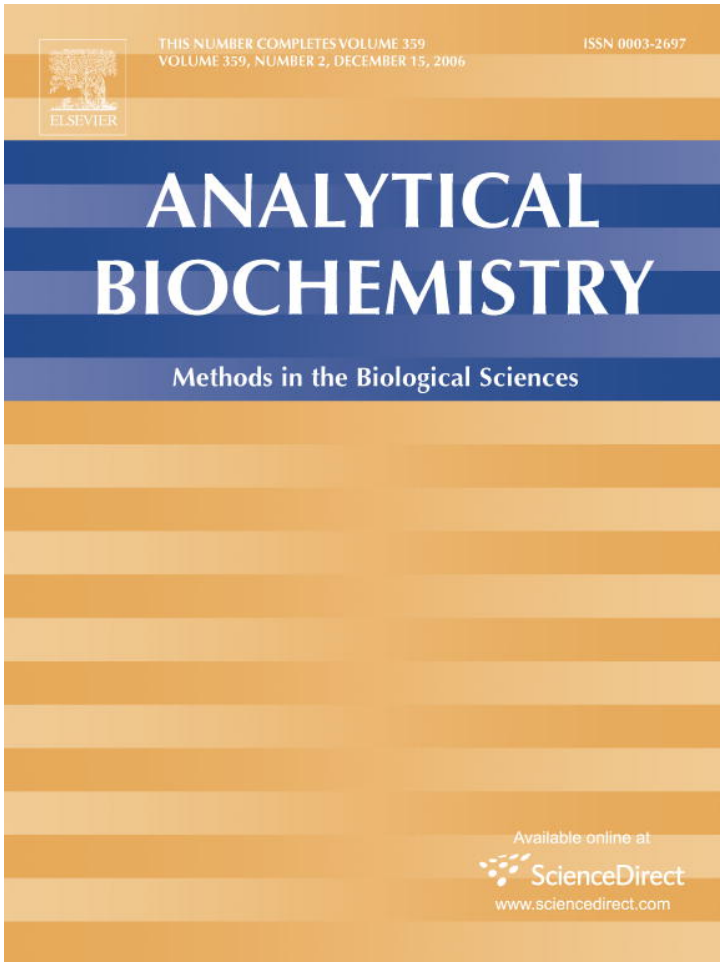

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In-gel activity staining of oxidized nicotinamide adenine dinucleotide kinase by blue native polyacrylamide gel electrophoresis

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Abstract

Oxidized nicotinamide adenine dinucleotide (NAD^+) kinase (NADK, E.C. 2.7.1.23) plays an instrumental role in cellular metabolism. Here we report on a blue native polyacrylamide gel electrophoretic technique that allows the facile detection of this enzyme. The product, oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$), formed following the reaction of NADK with NAD^+ and adenosine 5′-triphosphate was detected with the aid of glucose-6-phosphate dehydrogenase or $NADP^+$–isocitrate dehydrogenase, iodonitrotetrazolium chloride, and phenazine methosulfate. The bands at the respective activity sites were excised and subjected to native and denaturing two-dimensional electrophoresis for the determination of protein levels. Hence this novel electrophoretic method allows the easy detection of NADK, a critical enzyme involved in pyridine homeostasis. Furthermore, this technique allowed the monitoring of the activity and expression of this kinase in various biological systems.

Keywords: NAD^+ kinase; BN–PAGE; Activity bands; NAD^+; $NADP^+$

Aerobic organisms are subjected to constant bombardment of reactive oxygen species (ROS)[1] as a consequence of their regular metabolism [1]. These ROS are highly oxidative moieties that need to be detoxified if the cell is to survive. Although catalase, superoxide dismutase, and glutathione peroxidase play pivotal roles in this process, their effectiveness is dependent on the reductive environment generated by the steady supply of NADPH [2]. Enzymes such as ICDH, malic enzyme, and G6PDH have been shown to participate in the production of NADPH [3–6]. However, these NADPH-generating enzymes require $NADP^+$ to maintain their function. NADK is the key enzyme which catalyzes the phosphorylation of NAD^+ to $NADP^+$ [7]. Thus, the regulation of the cellular concentrations of $NADP^+$ mediated by NADK may play a critical role in the generation of a reductive environment and hence the detoxification of ROS [8]. NADK has been regarded the sole participant in the de novo synthesis of $NADP^+$. The maintenance of $NADP^+$ levels is crucial for anabolism, photosynthesis, and cell signaling [9]. NADK has been found in both prokaryotes and eukaryotes, suggesting that this enzyme is required for nicotinamide homeostasis in a number of organisms [8,10,11].

This enzyme is usually monitored by spectrophotometric techniques that involve enzyme-coupled assays [12]. In these methods, an enzyme such as G6PDH is employed to quantitate the formation of $NADP^+$. Interfering reactions, especially in the cell-free (CFEs), extracts can hinder the accuracy of the assay. In addition high quantities of protein are normally required and the sensitivity is relatively low [3,13–16]. To quantitate the expression of the NADK, antibodies that are expensive and/or not readily available have to be used. The procedure described here allows the

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[1] *Abbreviations used:* BN–PAGE, blue native polyacrylamide gel electrophoresis; G6PDH, glucose-6-phosphate dehydrogenase; INT, iodonitrotetrazolium chloride; $NADP^+$-ICDH, $NADP^+$-dependent isocitrate dehydrogenase; PMS, phenazine methosulfate; NADK, nicotinamide adenine dinucleotide kinase; ROS, reactive oxygen species; CSB, cell storage buffer; CFE, cell-free extract; MEM, minimal essential medium; 2D, two-dimensional.

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monitoring of both the enzyme activity and the relative protein expression without necessitating the use of antibodies. BN–PAGE provides a facile and inexpensive method to analyze NADK. Although BN–PAGE has been widely utilized to study mitochondrial membrane enzymes, we have recently adapted this technique to monitor a wide variety of soluble and membrane enzymes [17,18]. In this report we describe the in-gel detection and monitoring of NADK activity with the aid of either G6PDH or $NADP^+$–ICDH. This readily accessible procedure enables the quick identification and quantitation of this nicotinamide-sensitive kinase in various biological systems.

Materials and methods

Cell culture and subcellular fractionation

The strain of *Pseudomonas fluorescens* (ATCC 13525) was grown in a mineral medium containing Na_2HPO_4 (6.0 g), KH_2PO_4 (3.0 g), $MgSO_4$ (0.2 g), NH_4Cl (0.8 g), and citric acid (4.0 g) per liter of distilled and deionized water. Trace elements were present as previously described [19]. The pH was adjusted to 6.8 with dilute NaOH. The medium was dispensed in aliquots of 200 ml in 500 ml Erlenmeyer flasks and autoclaved for 20 min. Menadione experiments were performed by the addition of the menadione (100 μM) to the sterilized citrate media. The medium was inoculated with 1 ml of stationary-phase cells grown in a medium unamended with menadione and aerated on a gyratory water bath shaker (Model 76; New Brunswick Scientific) at 26 °C at 140 rpm. The Bradford protein assay was performed in triplicate to determine the amount of cells in the culture by measuring the solubilized cellular proteins [20]. The cells were treated with 1 M NaOH to solubilize the protein (cells obtained from 10 ml culture fluid were heated for 5 min in 1 ml NaOH). The bacterial cells were harvested at various growth intervals and suspended in a cell storage buffer (CSB; 50 mM Tris–HCl pH 7.3, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). The cells were homogenized by sonication and centrifuged at 3000*g* for 30 min at 4 °C to remove intact bacteria. The cell homogenate was then centrifuged at 180,000*g* for 1 h to afford a soluble CFE and a membrane CFE. The soluble fraction was further centrifuged at 180,000*g* for 2 h to ensure the removal of any residual membrane fraction. The CFEs were kept at 4 °C for up to 5 days.

HepG2 cells were a gift from Dr. D. Templeton (University of Toronto) and were maintained in α-MEM supplemented with 5% fetal bovine serum (v/v) and 1% antibiotics (v/v). Cells were routinely seeded at 1×10^5 cells/ml, cultured in 175 cm^2 flasks with loosened caps, and incubated with 5% CO_2 (v/v) in a humidified atmosphere at 37 °C. Upon reaching 70% confluency the medium was removed, replaced with serum-free α-MEM containing citrate (2.5 mM) or aluminum (Al)–citrate (0.5 mM Al:2.5 mM citrate) and exposed for a 24-h period. Cell viability was monitored using the Trypan blue exclusion assay [21]. Following the exposure, the cell monolayer was trypsinized and the cells were pelleted by centrifugation at 250*g* for 10 min at 4 °C. The cellular pellet was washed twice in phosphate-buffered saline, resuspended in mammalian CSB (mCSB), and stored at −86 °C until needed. The CSB used for the HepG2 cells contained 250 mM sucrose, 0.6 mM $MnCl_2$, 1 mg/ml pepstatin, and 0.1 mg/ml leupeptin. Cells were thawed and centrifuged at 250*g* for 10 min at 4 °C and resuspended in a minimal amount of ice-cold CSB. The cell suspension was ultrasonically disrupted on ice using a Brunswick sonicator for 20 s operating at 2-s intervals. The cell homogenate was then centrifuged at 12,000*g* for 30 min at 4 °C to afford a clean cytoplasmic fraction devoid of whole cells, mitochondria, and nuclei [22, 23]. The purity of the cytoplasm was confirmed by immunoblot using antibodies directed toward voltage-dependent anion channel, histone 2 A, and F-actin.

Blue native PAGE and two-dimensional (2D) electrophoresis

BN–PAGE was performed as described [17] with the following modifications. *P. fluorescens* and HepG2 proteins were prepared in a blue native buffer (50 mM Bis–Tris, 500 mM six-amino hexanoic acid (pH 7.0)) at final concentrations of 4 and 2 mg/ml, respectively. Proteins (20–80 μg) from bacterial and HepG2 cell free extract were loaded into each lane and electrophoresed under native conditions; 80 V was used for the stacking and this was increased to 200 V when the proteins reached the separating gel. The blue cathode buffer (50 mM Tricine, 15 mM Bis–Tris, 0.02% (w/v) Coomassie G-250 (pH 7.0) at 4 °C) was changed to colorless cathode buffer (50 mM Tricine, 15 mM Bis–Tris, (pH 7.0) at 4 °C) when the running front was half way through the separating gel. Electrophoresis was subsequently stopped once the running front had reached the bottom of the gel. The gel slab was removed from the chamber and equilibrated for 15 min in a gel equilibration buffer (25 mM Tris, 5 mM $MgCl_2$ (pH 7.4)). Immediately following the incubation the gel slab was placed in a reaction mixture consisting of equilibration buffer, NAD^+ (0.5 mM), ATP (3 mM), 5 mM glucose-6-phosphate, 5 units of G6PDH, INT (0.4 mg/ml), and PMS (0.2 mg/ml). Controls consisted of the reaction mixture without the substrate (ATP or NADH) or coupling enzyme or in the presence of the inhibitor iodoacetate (2 mM) and the nucleotide CTP (3 mM). A similar reaction mixture was utilized in the detection of NADK from HepG2 cells except that 5 mM isocitrate and 10 units of $NADP^+$–ICDH substituted for G6PDH and glucose-6-phosphate. HepG2 control experiments were also performed as described above. The activity band was excised and subjected to analysis by 2D BN–PAGE as described above. 2D SDS–PAGE using a discontinuous buffer system was performed according to the method of Laemmli [24]. The activity gel was equilibrated in the electrophoresis buffer (24 mM Tris, 191 mM glycine,

1% SDS (w/v)) containing 1% mercaptoethanol (v/v) for 30 min at room temperature. The band was then excised and electrophoresed at a constant 200 V. Gel slabs were fixed and destained according to standard procedures. Protein levels were ascertained by silver stain. Band intensity was determined using Scion Imaging for Windows (Scion Corp., Frederick, MD).

SDS–PAGE and immunoblot

SDS–PAGE was performed as described above. Samples were solubilized in 62.5 mM Tris–HCl (pH 6.8), 2% SDS (w/v), and 2% β-mercaptoethanol (v/v) at 100 °C for 5 min. The proteins were transferred to a Hybond-P polyvinylidene difluoride membrane electrophoretically for immunoblotting. Nonspecific binding sites were blocked by incubating the membrane in 5% non fat skim milk in TTBS (20 mM Tris–HCl (pH 7.6), 0.8% NaCl (w/v), 1% Tween 20 (w/v)) for 1 h. The antibody raised against NADK from rabbits was generously supplied by Dr. Snedden (Queen's university, Kingston, ON, Canada). The secondary antibody consisted of a horseradish-peroxidase-conjugated antirabbit (Santa Cruz). Detection of the desired antigen was achieved using Chemiglow (Alpha Innotech). The immunoblots were subsequently documented using the ChemiDoc XRS system (BioRad Imaging Systems). Band intensity was quantified using Alpha Innotech Software (Alpha Innotech Corp.).

Metabolite analysis

NADK activity was monitored by the consumption and production of NAD^+ and $NADP^+$, respectively. In a phosphate reaction buffer (10 mM Na_2HPO_4, 5 mM $MgCl_2$ (pH 7.3)) consisting of 3 mM ATP and 0.5 mM NAD^+, 2 mg of soluble CFE was added and allowed to react for 30 min. Reaction mixtures without ATP were utilized as controls. The reactions were stopped with cold 0.5% perchlorate and the resulting precipitate was removed by centrifugation at 12,000*g* for 10 min. HPLC analysis was carried out on a Waters Alliance HPLC equipped with a Waters model 2487 UV–vis dual wavelength detector and a C_{18} reverse-phase column (3.5 μm, amide cap, 4.6 × 150-mm inside diameter, Symmetry Column, Phenomenex, Torrance, CA). A mobile phase consisting of 20 mM KH_2PO_4 (dissolved in millipore water) was used operating at a flow rate of 0.7 ml/min at ambient temperature. The eluants were measured by UV absorption at 254 nm.

Results and discussion

NADK is a pivotal metabolic enzyme that allows living organisms to modulate the intracellular concentrations of NAD^+ and $NADP^+$. An increase in the former favors energy production while an increment in the latter promotes the synthesis of NADPH, an anabolic reductant. Owing to its instrumental role in metabolism, it is important to identify analytical tools that will allow the facile monitoring of this enzyme. Here, we show a BN–PAGE technique that allows the evaluation of the activity and expression of NADK. This procedure maintains the enzyme in its active state and allows the visualization of the migrating bands. The charge provided by the dye coupled with the enzymatic stability provided by the 6-aminohexanoic acid via preferential hydration may be a contributing factor that leads to the effectiveness of this method. Bands are more intense and sharper than those provided by regular native PAGE (data not shown). The dye, Coomassie blue G-250, also appears to contribute to the stability of the enzymes [25]. The ability of NADK to produce $NADP^+$ in the presence of ATP was employed to obtain an activity band. With the aid of the appropriate substrate and $NADP^+$-dependent enzyme (ICDH or G6PDH), the resulting NADPH interacts with INT to generate a purple precipitate at the site where the enzyme is immobilized. As shown in Fig. 1(I), an activity band was observed only when ATP was present in the reaction mixture. The two different coupling enzymes, G6PDH and ICDH, were utilized in an effort to ascertain the suitability and effectiveness of these two enzymes to detect $NADP^+$ formation as a consequence of NAD^+ kinase activity. Since G6PDH is a predominantly cytoplasmic enzyme, it may be more suitable to monitor NAD^+ kinase activity in membrane fractions. Interfering bands due to ICDH and G6PDH activities in the CFE were observed when higher concentrations of substrates were utilized and when the reaction was kept for prolonged periods. The activity band was excised and analyzed by 2D BN–PAGE. The activity band was more pronounced following 2D electrophoresis

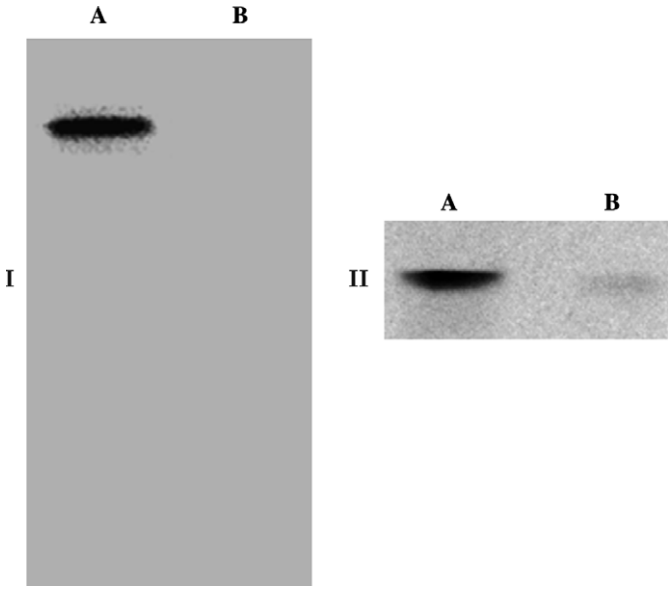

Fig. 1. In-gel detection of NADK activity. Soluble CFE (60 μg protein equivalent) obtained from *P. fluorescens* grown in citrate (control) medium was utilized. (I) NADK activity. (II) 2D BN–PAGE NADK activity (activity bands from (I) were excised and loaded). Lanes A, NAD^+ (0.5 mM), ATP (3 mM), isocitrate (2 mM) $NADP^+$–ICDH (5 U) (reaction mixture); lane B, reaction mixture–ATP.

(Figs. 1 and 2). Fig. 2 depicts the dependence of activity band intensity on protein concentration. A faint band was discernable when 40 μg protein equivalent of CFE was utilized. However, the intensity increased very significantly when 80 μg protein equivalent to CFE was loaded. These bands can be quantified by using appropriate computer software [4,6]. It is important to note that up to 10 μg of protein can be analyzed. However, the reaction time to observe the activity band was longer and interfering bands due to the activities ICDH and G6PDH in the CFE were also evident. Fig. 3 shows the influence of CTP and iodoacetate on the activity of NADK. The enzyme appeared to have a higher affinity for ATP than for CTP. It was also severely inhibited by iodoacetate. Indeed, it has been reported that the activity of the enzyme decreases in the following nucleotide order: ATP > CTP > UTP > GTP [26]. As the enzyme does possess an essential sulfydryl moiety, the presence of iodoacetate in the reaction mixture did have a negative effect on the activity of NADK. In Fig. 4(I), the 2D BN–PAGE of NADK in control and menadione cultures is depicted. In the cells isolated from the menadione cultures, the activity was more intense than in the control. When the 2D SDS–PAGE was performed, a prominent band at ~47 kDa was evident. No such band was observed with the cells from control cultures (Fig. 4(II)). However a faint band was observed when the gel was stained with silver staining solution instead of Coomassie blue. The ability of NADK to produce $NADP^+$ from NAD^+ and ATP was further confirmed by HPLC. As shown in Fig. 5, NADK was much more active in the cells grown in the presence of menadione as revealed by the intense $NADP^+$ peak. In comparison, the signal corresponding to NAD^+, the reactant, was far more intense in control cultures. The identities of these peaks (NAD^+, $NADP^+$) were confirmed by running commercial standards individually and simultaneously.

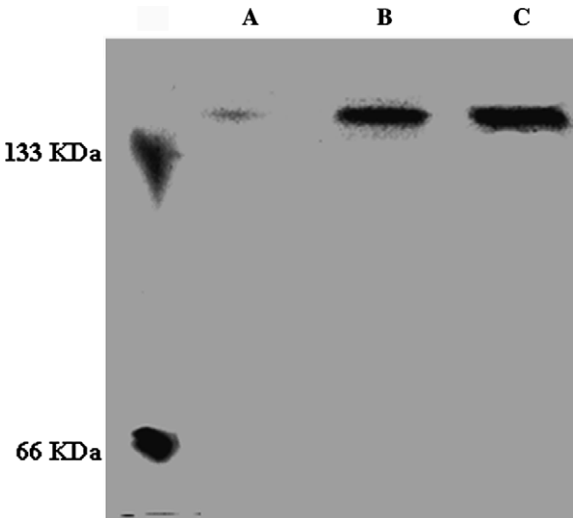

Fig. 2. In-gel detection of NADK activity and dependence on protein concentration. Lanes A, B, and C correspond to 40, 60, and 80 μg, respectively, of soluble CFE obtained from *P. fluorescens* grown in a citrate medium.

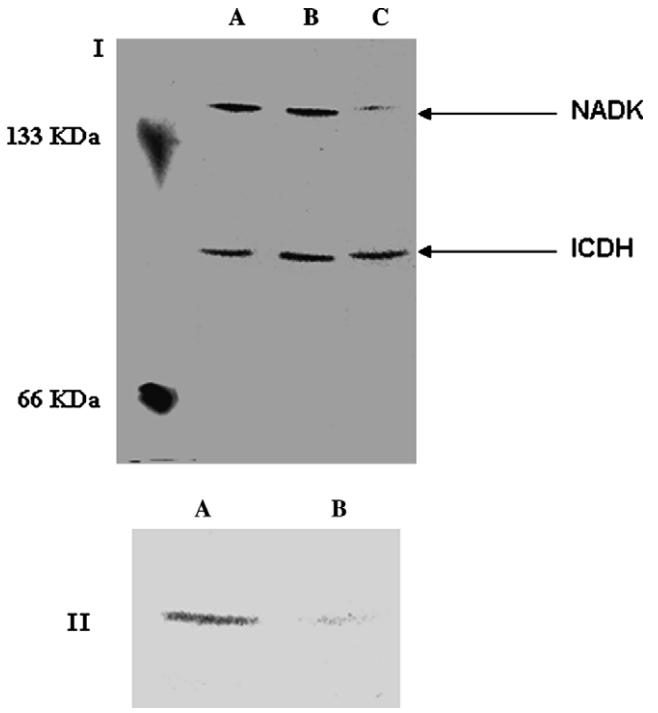

Fig. 3. Influence of various nucleotides and inhibitors on NADK activity. Soluble CFE from *P. fluorescens* (60 μg) was utilized. (I) Lane A, reaction with ATP; lane B, Reaction with CTP; lane C, reaction without ATP (note the absence of NADK activity). (II) Lane A, reaction mixture; lane B, reaction mixture + iodoacetate (2 mM).

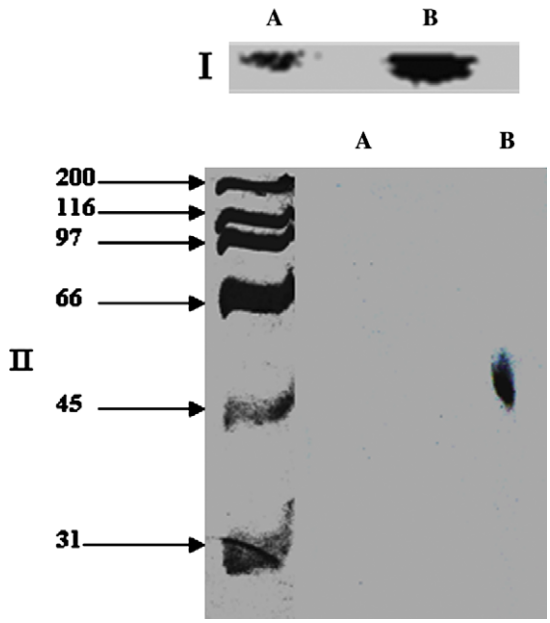

Fig. 4. Purification of NADK. (I) 2D BN–PAGE of NADK activity. Lane A, soluble CFE (control); lane B, soluble CFE (menadione culture). (II) 2D SDS–PAGE. Lane A, soluble CFE (control); lane B, soluble CFE (menadione culture). Coomassie staining was utilized to detect the protein.

The reaction mixtures were also spiked to confirm the presence of these nucleotides (data not shown). These data confirmed the presence of NADK in *P. fluorescens*.

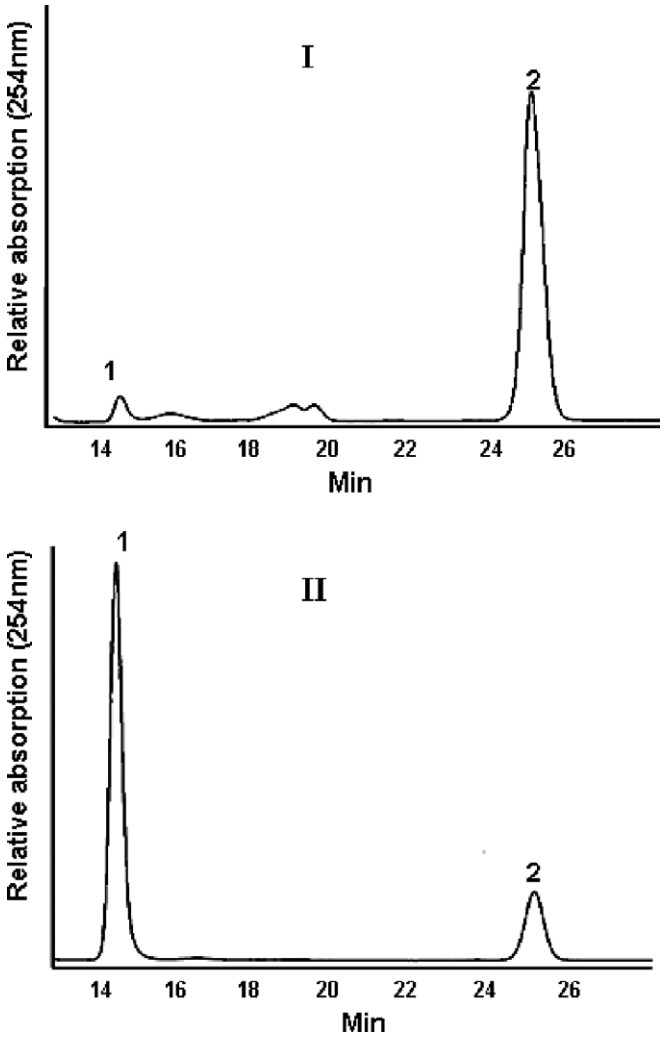

Fig. 5. HPLC analysis of NADK activity in *P. fluorescens*. (I) Soluble CFE (control). (II) Soluble CFE (menadione culture). Peak 1 corresponds to the $NADP^+$ retention time and peak 2 corresponds to the NAD^+ retention time.

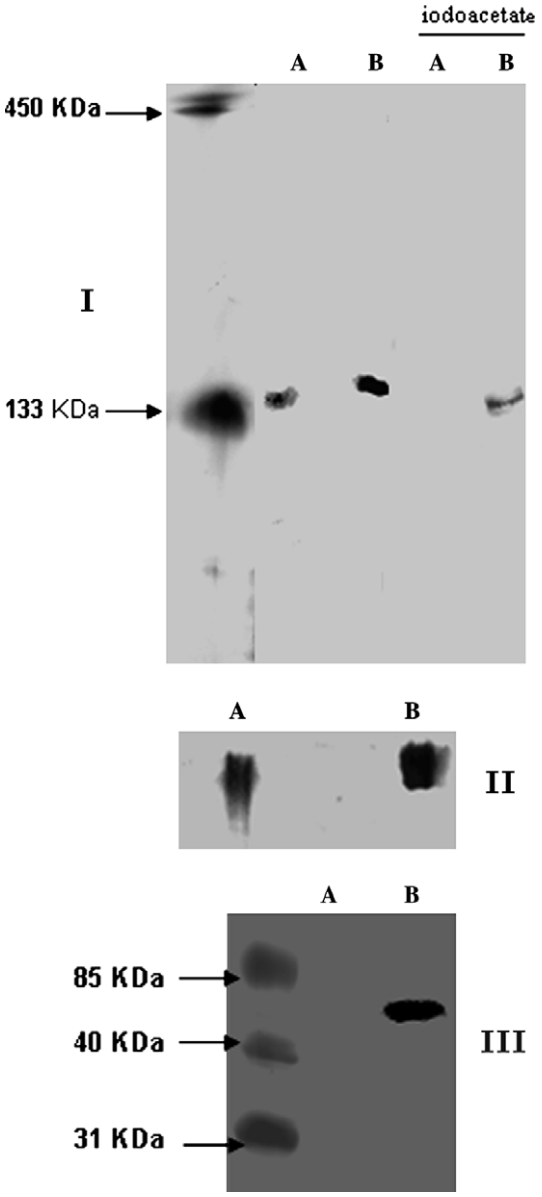

Fig. 6. In-gel detection of the activity and expression of NADK. HepG2 cells were grown to 70% confluency and exposed to α-MEM supplemented with lane A, citrate and lane B, Al–citrate for 24 h and soluble CFE was analyzed. (I) In-gel activity stain. (II) Analysis of protein levels by 2 D BN–PAGE. The activity band was electrophoresed and protein levels were detected by silver staining. (III) detection of NADK protein levels by immunoblot.

The activity and expression of NADK in the human liver cell line, HepG2, was also detected using the same technique. The cytoplasmic fraction of the HepG2 exposed to Al had higher levels of NADK activity than the control (Fig. 6(I)). In addition, the presence of iodoacetate in the reaction mixture had an inhibitory influence on NADK activity, thus confirming the significance of sulfydryl moieties in this enzyme. Following the activity stain, the bands were excised and subjected to 2D BN–PAGE and silver stain. Higher levels of NADK were detected in the HepG2 cells exposed to Al-stressed conditions (Fig. 6(II)). The protein levels of NADK were further confirmed by immunoblot analysis. Antibodies directed against NADK were able to reveal the higher expression of NADK in the Al-stressed cultures (Fig. 6(III)). However, HepG2 cells exposed to control did not exhibit any detectable levels of NADK. This discrepancy between the silver stain and the immunoblot may be due to the increased sensitivity of the silver stain. Accordingly, the higher activity and expression of NADK in the Al-stressed HepG2 cells suggests the importance of this enzyme in maintaining high levels of $NADP^+$. Indeed, Al is known to trigger oxidative stress and this enzyme may play a key role in promoting a reductive environment. Hence the BN–PAGE technique coupled with 2D electrophoresis is a very useful tool to monitor activity and expression of NADK in different cellular systems.

In conclusion, the BN–PAGE technique proved to be invaluable in the detection of NADK activity and expression. The versatility and sensitivity of this assay allowed the selective characterization of this enzyme in two separate organisms. Finally, this novel approach in detecting

NADK will allow the monitoring of this pivotal enzyme in various biological systems.

Acknowledgments

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