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## Resolving mitochondrial protein complexes using non-gradient blue native polyacrylamide gel electrophoresis

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### Abstract

Blue native polyacrylamide gel electrophoresis (BN-PAGE) is a powerful technique for separation and proteomic analysis of high molecular weight protein complexes. It is often performed on gradient gels and is widely used for studying mitochondrial membrane complexes involved in electron transportation and oxidative phosphorylation. In this paper, we present an alternative BN-PAGE method that uses highly porous, non-gradient polyacrylamide gels for separation of rat brain mitochondrial protein complexes. Results demonstrate that this method not only resolves mitochondrial complexes I-V, allowing subsequent analysis by in-gel activity staining and mass spectrometry peptide sequencing, but also identifies Hsp60 polymers and dihydrolipoamide dehydrogenase (DLDH). Moreover, with this new method, it is shown for the first time that complex I and DLDH can be simultaneously detected on a single gel strip by in-gel activity staining. Overall, the method provides a simplified, non-gradient gel electrophoretic approach that should be useful in functional proteomics studies.

### Keywords

Blue native polyacrylamide gel electrophoresis; dihydrolipoamide dehydrogenase; mitochondria; protein complexes

### Introduction

Blue native polyacrylamide gel electrophoresis (BN-PAGE) is a powerful technique for isolation, separation and detection of high molecular weight protein complexes [1]. Two key features of BN-PAGE facilitate the separation of protein complexes in their native state, without loss of enzymatic activity. The first one is the introduction of Coomassie brilliant blue (CBB) G-250 into the protein samples, a procedure which produces a surface negative charge shift that does not inactivate the proteins, yet allows the proteins and their complexes to resolve in the gel according to their native molecular masses, their net surface charges, and their molecular shapes [2,3]. The second feature is the use of aminocaproic acid in sample preparation and as a gel buffer component, which improves the solubilization of membrane proteins [1]. Since its initial application for the separation of mitochondrial membrane complexes in the early 1990's [1], BN-PAGE has gained popularity and has been successfully used for separation and analysis of mitochondrial and non-mitochondrial protein complexes [4], including nuclear protein complexes [5], water soluble proteins [6,7], and whole cellular

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lysates [8]. Moreover, BN-PAGE has been successfully applied in clinical settings for diagnosing mitochondrial defects that are associated with human diseases [9–12].

Conventionally, gradient BN-PAGE has been performed for the separation of mitochondrial oxidative phosphorylation complexes [13,14]. While the gradient gel method is excellent for separation and detection of the five mitochondrial complexes that are involved in oxidative phosphorylation, other mitochondrial protein complexes are not detected under these conditions. For example, both complex I and dihydrolipoamide dehydrogenase (DLDH) can be stained using NADH and nitro blue tetrazolium (NBT) [15], yet the two have never been detected simultaneously on the same gel strip. Gradient BN-PAGE used to isolate complex I usually fails to resolve a DLDH homodimer band; conversely, the non-gradient BN-PAGE method reported recently for detection of DLDH, does not isolate complex I [15]. In the present study, we provide an alternative BN-PAGE approach that involves non-gradient gel electrophoresis on highly porous polyacrylamide gels. This technique yields, on the same gel strip, DLDH, Hsp60, and a separation of mitochondrial complexes I to V that are amenable to further analysis by in-gel activity measurements and mass spectrometry peptide sequencing.

## Materials and Methods

### Animals and chemicals

Tissues from adult Sprague-Dawley rats, obtained from Harlan (Indianapolis, Indiana), were used to isolate mitochondria. These experiments were conducted in adherence with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the University of North Texas Health Science Center Animal Care and Use Committee. The reagents and chemicals used for in-gel enzyme activity staining were purchased from Sigma (St. Louis, MO) unless otherwise stated. Acrylamide, bis-acrylamide (Bis), N,N-diallyltartardiamide (DATD), ammonium persulfate and Coomassie brilliant blue (CBB) G-250 were purchased from Bio-Rad laboratories (Richmond, CA). Tricine and  $\epsilon$ -amino-N-caproic acid were purchased from MP Biochemicals Inc. Bis-Tris was purchased from Calbiochem (La Jolla, CA). Serva Blue G-250 was from Serva (Heidelberg, Germany). Protease inhibitor cocktail tablets were purchased from Roche (Germany). Prestained SDS-PAGE markers were from Fermentas Life Sciences (Hanover, MD).

### Isolation of mitochondria and preparation of mitochondrial extracts for blue native PAGE

Mitochondria were isolated from whole rat brain using Percoll gradient centrifugation [15]. Preparation of total mitochondrial extracts for BN-PAGE analysis was performed as previously described [16] with modifications. Basically, mitochondrial pellet (either fresh or frozen) was resuspended at a protein concentration around 1 mg/ml in a solubilization buffer containing 50 mM Bis-Tris (pH 7.0), 1% n-dodecyl- $\beta$ -D-maltoside (v/v) and 750 mM  $\epsilon$ -amino-N-caproic acid. The suspension was kept on ice for 1 hr with occasional vortexing and was then clarified by centrifugation at  $20,000 \times g$  for 30 min. Following the centrifugation, 0.9 ml of the resulting supernatant, containing both membrane and water-soluble proteins, was mixed with 0.1 ml of concentrated BN-PAGE loading buffer (10 x) containing 0.75 M  $\epsilon$ -amino-N-caproic acid and 3% Serva Blue G-250 (w/v) [15]. The samples were then stored at  $-20^{\circ}\text{C}$  until analysis. All protein concentrations were determined by bicinchoninic acid protein assay [17] using BSA as the standard.

### Non-gradient blue native polyacrylamide gel electrophoresis (BN-PAGE)

An acrylamide/Bis solution that would yield highly porous gels upon polymerization was prepared as previously described [18,19] with modifications. Essentially, a stock solution containing 50% (w/v) acrylamide and 0.5% (w/v) Bis (acrylamide: Bis = 100:1, w/w) was prepared in deionized distilled water and used for both stacking and resolving gels. The final

concentration of the stacking gel was 4%, and that of the resolving gel was 8% except where specified. Non-gradient BN-PAGE was performed at room temperature using Bio-Rad Mini-PROTEAN III (Richmond, CA) as previously described [15]. Gel buffer was comprised of 500 mM aminocaproic acid and 50 mM Bis-Tris, pH 7.0. Cathode buffer contained 50 mM Tricine, 15 mM Bis-Tris, pH 7.0, 0.02% Serva blue G-250 (w/v); and anode buffer contained 50 mM Bis-Tris pH 7.0. Sample buffer was 75 mM aminocaproic acid (final concentration) containing 0.3% Serva blue G-250 (w/v, final concentration). Following sample loading (20 – 30  $\mu$ g proteins), the gel was run at 150 V until the front line had entered into one-third of the gel, whereupon the cathode buffer was replaced by the one that did not have Serva blue G-250 (50 mM Tricine, 15 mM Bis-Tris, pH 7.0). Gel running was then continued at 200 V until complete. The stacking gel was then carefully removed using a blade prior to in-gel activity staining. Where needed, gels were stained by CBB G-250 [20] followed by destaining in a solution containing 10% methanol and 8% acetic acid (all v/v). For BN-PAGE gel strips that were further processed by second dimensional SDS-PAGE, the gel strips, without fixing and staining, were equilibrated for 20 min in a solution containing 5% 2-mercaptoethanol (v/v), 62.5 mM Tris-HCl (pH 6.8), 2.3% SDS (w/v, and 10 mM glycerol [21], and placed onto SDS-PAGE for electrophoresis. Where indicated, clear native PAGE [22] was run under the same conditions except that the initial cathode buffer did not have Serva blue G-250. All gel images were documented using an EPSON PERFECTION 1670 scanner.

### In-gel enzyme activity staining

In-gel enzyme activity assays were performed for mitochondrial complexes I-V and dihydrolipoamide dehydrogenase (DLDH). All steps were conducted at room temperature with reactions being stopped at various time points by fixing the gel for 30 min in a solution containing 50% methanol (v/v) and 10% acetic acid (v/v). This was followed by a long-term preservation of the gel in a solution containing 10% methanol and 8% acetic acid at 4°C.

For complex I and DLDH activity staining, the gel strip was incubated in 50 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 0.2 mg/ml NBT and 0.1 mg/ml NADH [15]. For complex II staining, the gel strip was incubated in 20 ml of 5 mM Tris-HCl (pH 7.4) containing 0.5 M sodium succinate, 215 mM phenazine methosulfate (stock solution prepared in DMSO) and 20 mg NBT [23]. For complexes III and IV staining, the gel strip was incubated in a 50 ml solution containing 50 mM sodium phosphate, pH 7.2, 20 mg 3,3'-diaminobenzidine tetrachloride (DAB) and 50 mg cytochrome c [23]. Under our gel electrophoretic conditions, we found that complex IV, but not complex III, could also be stained by incubating the gel strip in the above solution that did not contain cytochrome c, though it usually took more than 6 hours for the color to develop. Finally, for complex V staining, the gel strip was incubated in a 50 ml solution containing 35 mM Tris, 270 mM glycine (pH 8.3), 14 mM MgCl<sub>2</sub>, 0.2% Pb(NO<sub>3</sub>)<sub>2</sub> and 8 mM ATP [24]. This method of complex V activity staining is based on the fact that the inorganic phosphate, originated from ATP hydrolysis catalyzed by complex V, reacts with lead nitrate to form lead phosphate that then accumulates on the enzyme's band [24].

### SDS-PAGE and Western blots

Second dimensional SDS-PAGE was performed according to Laemmli [25] using Bio-Rad Mini-PROTEAN III electrophoresis cell. Both the stacking and resolving gels (4% and 10%, respectively) were made from a 30% acrylamide/Bis (29:1, w/w) solution. Usually, two gels were run simultaneously, one for protein staining and the other for Western blot detection. After SDS-PAGE, gels were transferred to Hybond-C membranes with a Mini-Trans-Blot electrophoretic transfer cell (Bio-Rad, Richmond, CA) according to Towbin et al. [26] with some modifications [27]. Western blots were performed according to the procedure described previously [21].

## Protein identification by mass spectrometry peptide sequencing

Protein identification was performed at ProtTech (Norristown, PA) by using the NanoLC-MS/MS peptide sequencing technology. Briefly, a given blue native gel band was destained, cleaned, and in-gel digested with sequencing grade trypsin. The resulting peptide mixture was analyzed by an LC-MS/MS system, in which a high pressure liquid chromatography with a reverse phase C18 column (inner diameter: 75  $\mu$ m) was coupled on-line with an ion trap mass spectrometer. The collected mass spectrometric data were used to search the most recent non-redundant protein database using ProtTech's proprietary software suite. In contrast to MALDI-TOF based peptide mapping, the results from LC-MS/MS (tandem MS) are based on independent peptide sequencing. In these studies, only those proteins that were confirmed by two or more peptides sequenced (>99.9% certainty of identification) are reported. Hence, all proteins identified in the gel bands represent confirmed proteins rather than candidates. Moreover, the number of peptides sequenced by LC-MS/MS from each protein can be used as an indicator of their relative abundance in a mixture [28–30].

## Results

### Resolution of mitochondrial protein complexes

To establish the conditions of non-gradient BN-PAGE for resolving mitochondrial protein complexes, we tested a series of gel concentrations ranging from 7.5% to 12%. Fig. 1 shows protein band patterns at each acrylamide concentration. A comparison of the patterns with those resolved by gradient BN-PAGE [1,23,24] indicates that mitochondrial complexes I and V, the two biggest complexes involved in oxidative phosphorylation, were always recognizable after gel electrophoresis under our experimental conditions (Fig. 1, as indicated on the 7.5% gel). For a clear visualization of other well-resolved protein bands, it was necessary to further stain the gels with CBB G-250 followed by destaining. Upon destaining and storage, gels usually showed swelling that exhibited approximately a 20% increase in gel area.

As shown in Fig. 1, between 7.5% and 9% gels, complexes I and V were all well separated from each other, as were smaller protein complexes. Gel concentrations equal to or greater than 10%, however, yielded a progressively poorer separation of either complex I, complex V, or both. For example, on the 10% gel, complex I did not show a good separation whereas complex V did, while on the 12% gel, neither complex I nor complex V exhibited a clear-cut separation (Fig. 1). In general, on a 7.5% gel, native proteins with molecular weight as low as 140 kDa could be separated, while on a 12% gel, native proteins with molecular weight as low as 60 kDa could be resolved. It should be noted that on a 7.5% gel, complex II (~130 kDa) could run out of the gel if the running was not stopped at the time when the front line (CBB G-250) reached the bottom of the gel. Taken together, these results indicated that 8–9% gels would be appropriate for resolving mitochondrial membrane complexes I-V.

### Simultaneous staining of complex I and DLDH activity

Fig. 2A shows in-gel activity staining for both complex I and DLDH on an 8% blue native gel. Under our experimental conditions, complex I activity staining usually developed within 2 min of incubation in the presence of NADH and NBT, while DLDH activity staining developed at a much later time that was usually more than 15 min into the incubation. Additionally, a DLDH band was usually not visible by CBB staining and could only be visualized and localized upon activity staining. Taken together, these results indicate that, in the mitochondria, complex I content is much greater than that of DLDH. We also tested the resolution of complex I and DLDH using clear native PAGE (CN-PAGE) [23] wherein Serve blue G-250 was not included in the initial cathode running buffer, though the loading buffer did contain Serve blue G-250. The result in Fig. 2B shows that DLDH ran much slower on a clear native gel than on a blue native gel and, in addition, the DLDH activity band was more diffuse on the clear native gel.

These results suggest that BN-PAGE is better than CN-PAGE for DLDH separation and detection.

### In-gel activity staining of complexes II to V

We next tested the feasibility of the non-gradient electrophoretic method for in-gel activity detection of mitochondrial complexes II through V. Fig. 3A shows complex II activity staining using sodium succinate as the substrate and NBT as the electron acceptor in the presence of phenazine methosulfate [23]. Complex II is the smallest (~130 kDa) among the five mitochondrial oxidative phosphorylation complexes [31]. Hence, on an 8% gel, complex II was at the bottom of the gel and appeared diffuse (Fig. 3A, the left panel). On a 12% gel, however, a well-defined activity band of complex II could be visualized (Fig. 3A, the right panel). Under these experimental conditions, the activity staining usually took about 30 min. It should be noted that for the 8% gel, it was necessary to remove the front-line Coomassie blue by destaining so that the color of NBT formazan could be highlighted.

Under our experimental conditions, both complexes III and IV (along with an upper band indicated as supercomplex III), were stained by DAB/cytochrome c (Fig. 3B, panel 2). The color usually developed within 40 min of incubation. When the gel strip was incubated with DAB for a prolonged period (6–12 hrs) in the absence of cytochrome c, only complex IV could be stained (Fig. 3B, panel 1), and the activity band appeared less diffuse when compared with that stained by DAB/cytochrome c (Fig. 3B, panel 2). The rationale for naming the upper band as supercomplex III was based on the following three observations: (1) The band ran slower than did the usual complex III band (Fig. 3B, panel 2); (2) The band was stained by DAB/cytochrome c that could also stain the authentic complex III band (immediately below complex V in Fig. 3B, panel 2); (3) When analyzed by NanoLC MS/MS peptide sequencing, the band was found to contain comparatively abundant complex III subunits including ubiquinol cytochrome c reductase core proteins 1 and 2 (supplemental Table S1, numbers 3 and 6). It should be noted that the presence of a trace amount of three complex IV subunits in this band (supplemental Table S1, numbers 7, 13 and 14) would seem to be unrelated to its complex III activity staining, as the true complex III band did not contain any detectable complex IV subunits (supplemental Table S2), yet was stained by DAB/cytochrome c.

The staining of complex V by lead phosphate took approximately 60 min and resulted in two bands showing complex V activity (Fig. 3C). As previously reported [23,24], and as indicated in Fig. 3C, these two bands represented, respectively, the ATP hydrolysis activity of holocomplex V and ATP hydrolysis activity of the F<sub>1</sub> subcomplex. Therefore, the pattern of complex V resolved by non-gradient BN-PAGE was similar to that resolved by gradient BN-PAGE [23].

### Analysis of DLDH associated protein components

In connection with our studies of DLDH oxidative modifications [15,32], we were interested in investigating what proteins might associate with DLDH. For this purpose, the band exhibiting DLDH activity, such as the one shown in Fig. 2 (the left panel), was excised and analyzed by NanoLC-MS/MS peptide sequencing. Results in Table I show that a total of 24 proteins, including DLDH, were found to be contained in the DLDH band. Based on the establishment that the number of peptides sequenced from each protein usually reflects abundance of the protein in a sample [28–30], aconitase (number 2 in Table I), an enzyme in the Krebs cycle, was the major protein that was associated with DLDH. Furthermore, other Krebs cycle enzymes such as malate dehydrogenase (number 3), the complex II enzyme succinate dehydrogenase (number 8), and citrate synthase (number 9) were also found to be comparatively abundant in the DLDH band. These findings should not be surprising given the fact that DLDH is the E3 component of the  $\alpha$ -ketoglutarate dehydrogenase complex that



operates in the Krebs cycle. Additionally, the data are also suggestive that DLDH is associated with mitochondrial membrane proteins including ADP/TP translocase 1 (number 6) and complex V (number 16). Indeed, the pyruvate dehydrogenase complex, of which DLDH is also the E3 component, is known to be associated with mitochondrial membranes [33–35]. Interestingly, none of the complex I subunits could be detected in the DLDH-containing band, suggesting that complex I is not associated with DLDH. Likewise, when the complex I band was analyzed by mass spectrometry peptide sequencing, DLDH was not among the proteins that were identified (supplemental Table S3). Moreover, when a whole blue native gel strip was analyzed by second dimensional SDS-PAGE in conjunction with Western blots probed with anti-DLDH antibodies, no DLDH signal was detectable at the locations where complex I subunits were expected to resolve (Fig. 4). In any case, these results indicate that DLDH is not associated with complex I, which may explain why DLDH is not susceptible to oxidative attacks by complex I-generated reactive oxygen species (this laboratory, unpublished work).

### Overview of mitochondrial protein complexes resolved by non-gradient BN-PAGE

Based on the results presented in Figs. 2 and 3 and the related NanoLC MS/MS peptide sequencing results as described immediately below, the identity of each band on an 8% non-gradient BN-PAGE is given in Fig. 5. The very top two bands were labeled, respectively, as pseudo-complexes A and B, as each band was found to contain many different proteins that appeared to migrate together under these particular electrophoretic conditions (supplemental Tables S4 and S5). Band p, which also appeared to contain miscellaneous co-migrating proteins (supplemental Table S6) was labeled as pseudo-complex C. On the other hand, MS peptide sequencing results indicate that the predominant proteins contained in band b comprise Hsp60 (supplemental Table S7), as previously identified using gradient BN-PAGE [36] or gradient CN-PAGE [22]. Taken together, results of this study demonstrate that our non-gradient BN-PAGE approach is capable of resolving mitochondrial complexes I to V, Hsp60, and DLDH.

### Discussion

In the present report, we have described a non-gradient BN-PAGE method for separation and analysis of mitochondrial protein complexes. The establishment of the method was mainly achieved by increasing the ratio of acrylamide to Bis (100:1, w/w) that consequently increased the pore size of the gel [18,19]. The results presented demonstrate that this non-gradient blue native PAGE resolves not only all the five known mitochondrial protein complexes involved in oxidative phosphorylation, but also other protein complexes such as DLDH and Hsp60, as well as three gel bands that contain miscellaneous proteins migrating together under these gel electrophoretic conditions.

While Bis was used as the cross-linker in the present study for the method development, the effect of another cross-linker, N,N-diallyltartardiamide (DATD), on resolving mitochondrial protein complexes was also evaluated during the studies. For a given ratio of acrylamide to each cross-linker, DATD is known to generate a gel with a bigger pore size than does Bis [37,38]. Indeed, when DATD was used as the cross-linker, the gel concentrations could be moderately increased to achieve a similar and comparable protein resolution. For example, protein band patterns resolved by a 10% gel made from an acrylamide/DATD (100:1, w/w, 50%) solution were similar to those resolved by an 8.0% gel made from the acrylamide/Bis (100:1, w/w) solution (data not shown). Additionally, in agreement with previous findings [39,40], DATD-crosslinked gels were found to be elastic, sticky to glass, and to show swelling upon storage. Under our experimental conditions, the swelling of a 10% DATD-gel could eventually exhibit approximately a 130% increase in gel area in destaining solution. Nevertheless, no band distortions occurred (data not shown). One caveat associated with use of DATD for gel electrophoresis is that the 4% stacking gel has to be made from the 50%

acrylamide/Bis (100:1, w/w) solution such that firmly-formed loading wells can be established. This is because a 4% acrylamide/DATD (100:1, w/w) solution did not polymerize under our experimental conditions, probably due to the combining effect of the low DATD concentration in the 4% solution and the presence of ambient oxygen that could inhibit the gel polymerization. With due consideration of the above caveats, DATD-crosslinked, non-gradient blue native gels (10% resolving) also work well for the analysis of mitochondrial protein complexes.

Recently, we reported a BN-PAGE method for DLDH isolation and detection [15]. However, neither the gel concentration (9%) nor the pore size of the gel (acrylamide: Bis = 29:1, w/w) used in that study is conducive to complex I separation. We have found that complex I can be separated on a 6% blue native gel (acrylamide:Bis = 29:1, w/w), but DLDH fails to be detected on such a gel (data not shown), which is also too fragile for handling. Hence, non-gradient BN-PAGE made from a 29:1 (w/w) acrylamide/Bis solution [15] cannot accommodate complex I and DLDH simultaneously. By contrast, through increasing the pore size of the gels as described in the current study (acrylamide:Bis = 100:1, w/w), we show for the first time that complex I and DLDH can indeed be detected simultaneously on the same gel strip.

The results of the current study, together with previous observations [15,<sup>32</sup>], indicate that the successful separation of DLDH by blue native PAGE can be attributed to at least two factors. First, an active form of DLDH is a stable homodimer [32,<sup>41</sup>]. The DLDH homodimer is, in fact, so stable that the presence of 2 M urea in the gel failed to disrupt the DLDH homodimeric state (data not shown). Second, the presence of Serva blue G-250 in the loading sample and in the initial cathode running buffer greatly facilitates the separation of DLDH. Indeed, when mitochondrial protein extracts were analyzed by clear native PAGE, where Serva blue G-250 was omitted, a poor separation of DLDH from complex I occurred (Fig. 2B), demonstrating that Serva blue G-250 acts as a driving force for DLDH separation during blue native gel electrophoresis. It should be noted that with the present non-gradient gel approach, DLDH can only be located and identified by in-gel activity staining, while with our previously reported method [15], DLDH can be visualized by either Coomassie blue staining or by in-gel activity staining. The reason for this discrepancy remains unclear, but gel porosity and the respective detergents (n-dodecyl- $\beta$ -D-maltoside vs. Triton X-100) used in membrane protein solubilization may be partly responsible.

In addition to in-gel detection of complex I and DLDH, we further present evidence that other oxidative phosphorylation complexes can also be histochemically detected. In agreement with previous reports [23], we found that complex IV could be readily stained by DAB/cytochrome c. Interestingly, the same conditions (DAB/cytochrome c) also led to a discernable and relatively rapid staining of complex III. Mass spectrometry peptide sequencing of the complex III band failed to indicate the presence of any complex IV subunits (supplemental Table S2), and thus the reason that complex III enzymatic activity could be detected by DAB/cytochrome c under our experimental conditions remains unclear. These results may indicate that, under certain blue native gel electrophoretic conditions, it is possible to detect complex III activity using the DAB/cytochrome c staining system.

DAB itself, however, could not stain complex III activity under our experimental conditions. This is in disagreement with previous studies [23] wherein complex III separated by CN-PAGE was detectable using DAB. The reason for this disagreement is likely due to the use of Serva blue G-250 in our system that could interfere with DAB staining of complex III activity [23]. On the other hand, in agreement with previous studies [24], complex IV activity staining by either DAB alone (Fig. 3B, panel 1) or DAB/cytochrome c (Fig. 3B, panel 2) was found to be catalase-independent (data not shown), as was complex III activity staining by DAB/cytochrome c (Fig. 3B, panel 2). Hence, our results also ruled out the involvement of a basal level production of H<sub>2</sub>O<sub>2</sub> that would otherwise induce DAB polymerization and precipitation

[42]. For in-gel complex V activity detection, our results are similar to those reported previously, as we also found that complex V was resolved into two bands, both of which possessed ATP hydrolysis activity [23,<sup>24</sup>] (Fig. 3C).

Finally, as expected, our method of non-gradient BN-PAGE is compatible with mass spectrometry analyses of proteins (Table 1 and supplemental Tables S1–S7) that are of interest. Additionally, the gel strips generated by this method can also be further analyzed by second dimensional SDS-PAGE for Western blot probing of target proteins (Fig. 4). Moreover, with this new method, the isolation of much smaller native proteins or protein complexes (<100 kDa), including those of cytosolic and nuclear proteins, should be technically possible by increasing the acrylamide concentration of a non-gradient blue native gel (e.g., Fig. 1, the 12% gel).

In summary, a non-gradient BN-PAGE method for separation and histochemical staining of mitochondrial complexes is presented. This method can be used as an alternative simplified technique for isolating mitochondrial protein complexes and other cellular protein complexes for functional proteomics studies.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

BIS	bis-acrylamide
BN-PAGE	blue native polyacrylamide gel electrophoresis
CBB	Coomassie brilliant blue
DAB	3,3-diaminobenzidine tetrachloride
DATD	N,N-diallyltartardiamide
DLDH	dihydrolipoamide dehydrogenase
NBT	nitro blue tetrazolium

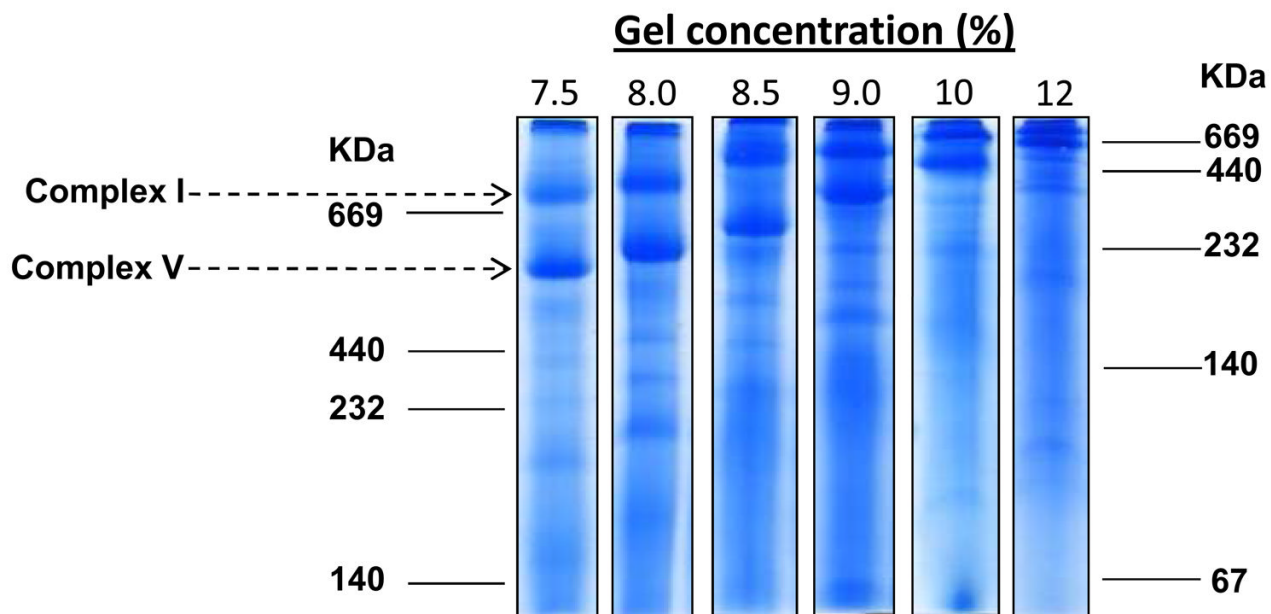
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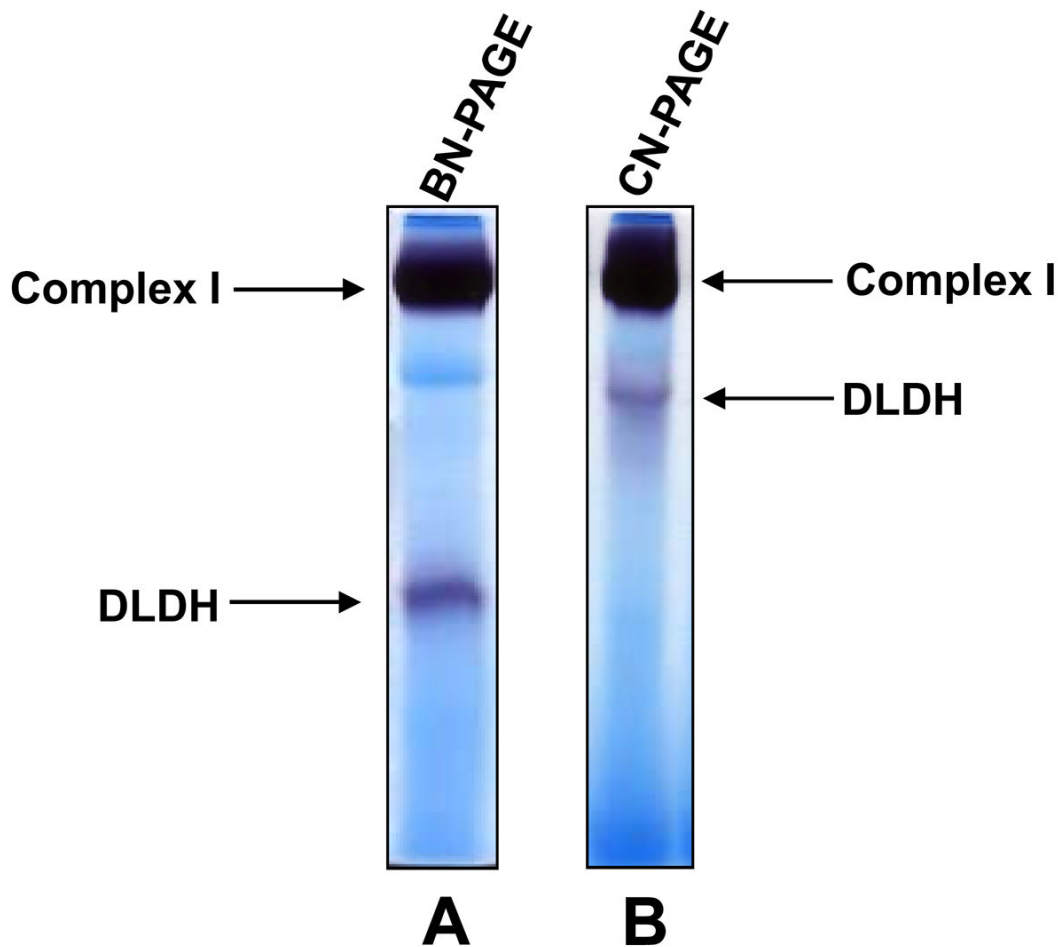
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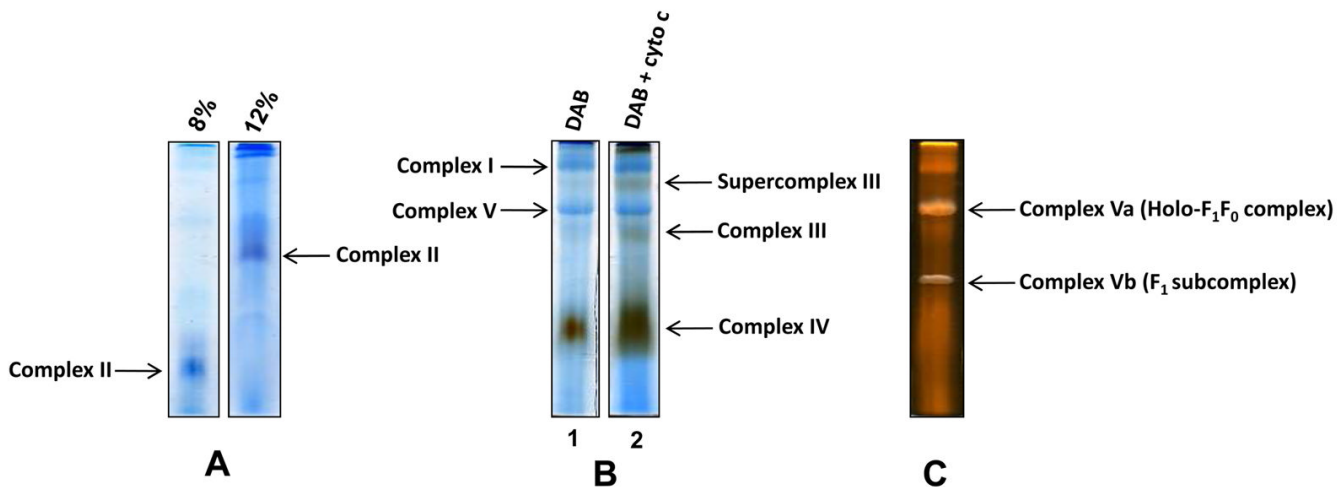
**Figure 1.**

Non-gradient BN-PAGE resolution of total mitochondrial extracts. Shown are gels ranging from 7.5% to 12% stained with Coomassie blue following gel electrophoresis. Twenty five micrograms of protein was loaded in each lane. Mitochondrial extracts were prepared as described in the text in a BN-PAGE sample buffer containing 50 mM Bis-Tris (pH 7.0), 1% n-dodecyl- $\beta$ -D-maltoside (v/v) and 750 mM  $\epsilon$ -amino-N-caproic acid. Complexes I, V and III are indicated, respectively, by arrows on the 7.5% gel. Native gel protein markers used in this figure as well as in other figures where indicated are: thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; lactate dehydrogenase, 140 kDa; BSA, 67 kDa.



**Figure 2.**

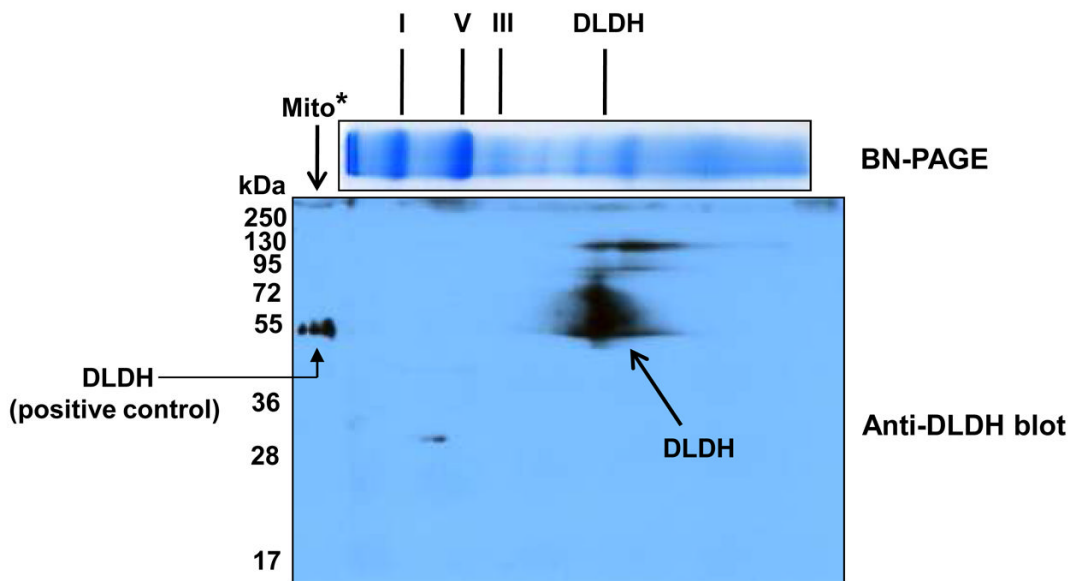
Simultaneous in-gel activity staining of complex I and DLDH on a single gel strip. Following gel electrophoresis (8% resolving gel for both A and B), each gel strip was incubated in 50 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 0.2 mg/ml NBT and 0.1 mg/ml NADH [15]. Shown is the resolution of complex I and DLDH by non-gradient blue native (A) and clear native (B) PAGE, respectively.



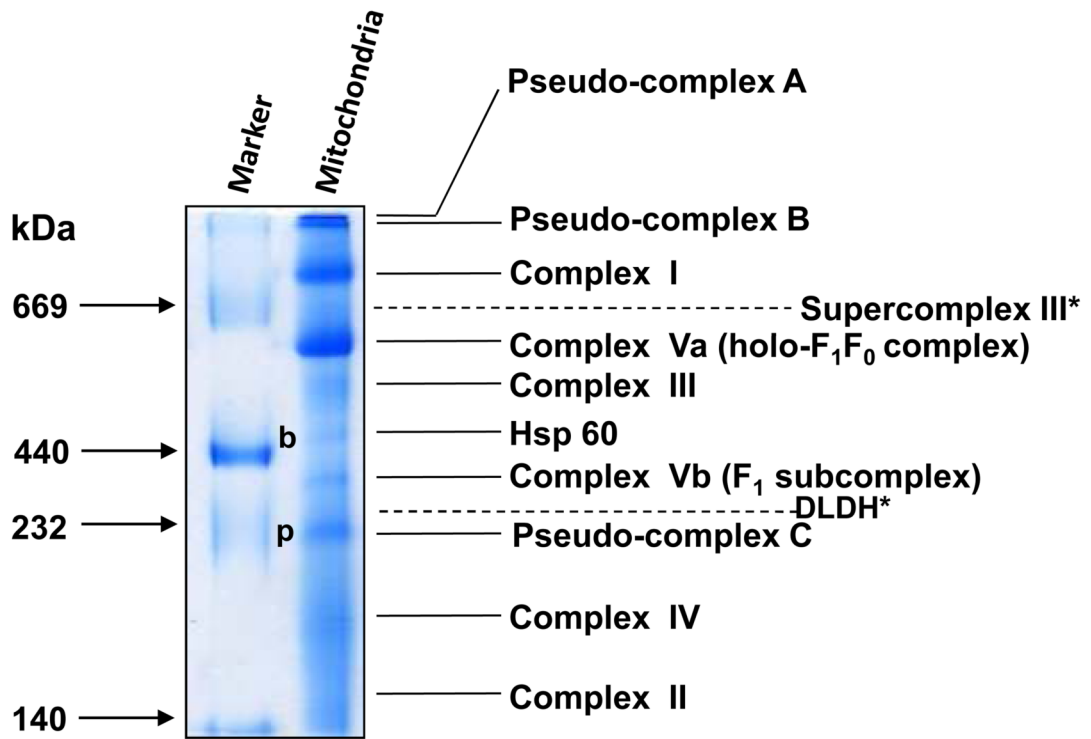
**Figure 3.**

In-gel activity staining of complexes II, III, IV, and V. Except where indicated, all gels were 8%. **(A)** Complex II activity staining on an 8% (the left panel) and a 12% (the right panel) blue native gel strip. Note that Coomassie blue at the bottom of the 8% gel had to be removed by destaining of the gel following activity staining so that a better complex II activity staining signal (NBT formazan) could be observed. **(B)** Activity staining of complexes III and IV. Under our gel electrophoresis conditions, DAB only stained complex IV (panel 1), while DAB and cytochrome c stained both complexes III and IV, and along with an upper band indicated as supercomplex III (panel 2). **(C)** Activity staining of complex V. Note that for a better visual effect, the color of the gel image was inverted to highlight complex V activity staining.





**Figure 4.** Two-dimensional Western blot probed with anti-DLDH antibodies. A BN-PAGE gel strip (the upper panel) was equilibrated for 20 min in a solution containing 5% 2-mercaptoethanol (v/v), 62.5 mM Tris-HCl (pH 6.8), 2.3% SDS (w/v), and 10 mM glycerol [21]. The gel strip was then placed on the top of a 10% polyacrylamide gel for SDS-PAGE that was followed by standard Western blot (the lower panel) procedures as described in the text. \*indicates the loading of mitochondrial extracts as a positive control for DLDH protein.



**Figure 5.** A representative non-gradient blue native gel image showing the resolution of rat brain mitochondrial protein complexes. In addition to the five complexes involved in oxidative phosphorylation, the band for Hsp60, the location of DLDH, and the bands containing miscellaneous proteins that co-migrated, are also indicated (pseudo-complexes A, B and C). Shown is the result of an 8% gel with 25µg mitochondrial proteins that were loaded. \*indicates labeling based on activity staining.

Proteins identified in the DLDH activity-associated gel band resolved by porous, non gradient BN-PAGE. Protein identification was carried out using NanoLC MS/MS peptide sequencing technique as described in the text.

**Table 1**

	<b>Protein name</b>	<b>MW (Da)</b>	<b>Access number (NCBI)</b>	<b>Number of peptides sequenced</b>
1	Dihydropyrimidinase dehydrogenase	54574.33	40786469	15
2	Aconitase	86121.31	40538860	13
3	Malate dehydrogenase	36116.98	42476181	7
4	Glutamate oxaloacetate transaminase 2	47683.27	6980972	6
5	4-aminobutyrate aminotransferase	57159.82	13591900	6
6	ADP/ATP translocase 1	33196.32	32189355	5
7	Hexokinase 1	103539.66	6981022	5
8	Succinate dehydrogenase complex, subunit A, flavoprotein	72596.11	18426858	5
9	Citrate synthase	52175.60	18543177	4
10	Aldolase A	39783.44	6978487	4
11	Tubulin, alpha 1B	50803.87	34740335	4
12	Pyruvate carboxylase	130349.04	929988	4
13	Neuron-specific class III beta tubulin	53599.25	20799322	4
14	Aldehyde dehydrogenase family 6 subfamily A1	58223.79	145651820	3
15	Predicted: similar to histone H2A type 1	18550.45	109505989	3
16	ATP synthase, H <sup>+</sup> transporting, mitochondrial F1 complex, alpha subunit	59830.73	40538742	3
17	Acetyl-coenzyme A acetyltransferase 1	45022.61	8392836	3
18	Pyruvate dehydrogenase	39299.18	56090293	3
19	Nicotinamide nucleotide transdehydrogenase	114537.59	61557127	3
20	Amphiphysin 1	74946.18	11560002	2
21	Aldolase C	39658.33	6978489	2
22	Dynammin 1	96209.41	18093102	2
23	Predicted: similar to succinate dehydrogenase Ip subunit	32607.22	109475694	2
24	Enoyl coenzyme A hydratase, short chain, 1	31895.33	17530977	2