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Two dimensional blue native / blue native polyacrylamide gel electrophoresis for the characterization of mitochondrial protein complexes and supercomplexes

Stephanie Sunderhaus, Holger Eubel and Hans-Peter Braun

Prof. Dr. Hans-Peter Braun
Abteilung Angewandte Genetik
Naturwissenschaftliche Fakultät
Universität Hannover
Herrenhäuser Str. 2
30419 Hannover, Germany

Tel: +49 5117622674
Fax: +49 5117623608
braun@genetik.uni-hannover.de

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Abstract

Blue-native polyacrylamide gel electrophoresis (BN PAGE) employs the blue wool dye Coomassie for labeling of proteins and protein complexes under native conditions. Electrophoresis under native conditions subsequently allows resolving proteins and protein complexes according to their molecular mass. BN-PAGE can be combined with second gel dimensions for extended analyses. Best known is the 2D BN / SDS PAGE system, which allows resolution of subunits of protein complexes. Recently, a 2D BN / BN PAGE system was suggested, which proved to be useful for investigating the substructure of protein complexes and protein supercomplexes. Basis of this 2D system is a variation in the conditions used for the two BN gel dimensions. Here we present a basic protocol for the analysis of mitochondrial fractions by 2D BN / BN PAGE. The 2D BN / BN system is compatible with in gel enzyme activity stainings, because both gel dimensions are carried out under native conditions.

Introduction

Blue-native polyacrylamide gel electrophoresis (BN-PAGE) is based on incubation of proteins with Coomassie-blue. Coomassie belongs to the trimethylmethane dye family and was used as a wool dye since the late 19th century. Due to its efficient and specific binding to proteins it was introduced in biochemistry to visualize proteins on gels after electrophoresis and later to photometrically determine protein concentrations [1,2]. Coomassie is a negatively-charged compound which carefully introduces charges into proteins and protein complexes. Because of this property it was suggested to be used for protein labeling prior to electrophoretic separations [3]. Coomassie does not denature proteins. In combination with non-ionic detergents, BN-PAGE proved to be an ideal tool for the analysis of membrane bound protein complexes, especially for those of mitochondria [3]. Even supramolecular assemblies of membrane bound mitochondrial protein complexes were shown to be stable during BN-PAGE, if the membrane solubilization step is carried out under very mild conditions [4]. Classically, a first Blue-native gel dimension is combined with SDS-PAGE as a second gel dimension, which allows to separate subunits of protein complexes. On the resulting gels, subunits of protein complexes form vertical rows. Detailed protocols for 2D BN / SDS PAGE were published recently [5-7].

Five years ago, a novel two-dimensional gel electrophoresis system was suggested which is based on BN-PAGE for both gel dimensions [4]. If the two gel dimensions are carried out under identical conditions, protein complexes form a diagonal line on the resulting 2D gels. However, if conditions of the second dimension BN-PAGE are slightly less gentle than conditions of the first dimension BN-PAGE, protein complexes are dissected into subcomplexes, which are visible underneath this diagonal line. 2D BN / BN PAGE proved to be a very powerful tool for the investigation of the protein complex composition of supercomplexes or the subcomplex composition of protein complexes. It successfully was used to characterize the supramolecular structure of the protein complexes of the oxidative phosphorylation (OXPHOS) system in mitochondria [4, 6, 8-14] and of the photosynthetic electron transport system in chloroplasts [15]. Conditions to be varied between the two BN gel dimensions can refer to detergent type (e.g. digitonin / dodecylmaltoside), detergent concentration (e.g. 0.5 / 2.0 % dodecylmaltoside), temperature (e.g. 4° / 20°C) or presence of chaotropic compounds (absence / presence of urea). 2D BN / BN PAGE is compatible with in gel enzyme stainings, allowing to determine the activity of supramolecular assemblies of protein complexes as well

as the activities of subcomplexes of protein complexes (Fig. 1). In this chapter, we present a basic protocol for the analysis of mitochondrial protein complexes by BN / BN PAGE, which is based on detergent variation between the two BN gel dimensions. The presented protocol also is suitable for the analysis of other organell fractions or bacteria.

2. Materials

2.1. Preparation of BN gels for first and second gel dimensions

1. Acrylamide solution: 49.5 %, acryl / bisacryl = 32 / 1 (AppliChem, Darmstadt, Germany)
2. Gel buffer BN (6x): 1.5 M amino caproic acid, 150 mM BisTris, pH 7.0 (adjust at 4°C)

2.2. Sample preparation

1. Digitonin solubilization solution: 5.0 % digitonin (e.g. Fluka, Buchs, Switzerland), 30 mM HEPES, 150 mM potassium acetate, 10 % (v/v) glycerol, pH 7.4 (adjust at 4°C). This buffer should be freshly prepared and shortly heated to 98°C to dissolve the detergent. Add PMSF directly before use (final concentration: 2 mM; stock solution: 200 mM PMSF [w/v] in EtOH)
2. Coomassie-blue solution: 5 % Coomassie G 250 (e.g. Merck, Darmstadt, Germany), 750 mM amino caproic acid

2.3. First dimension BN-PAGE

1. Cathode buffer BN (5x): 250 mM Tricine, 75 mM BisTris, 0.1 % (w/v) Coomassie G 250 (e.g. Merck, Darmstadt, Germany), pH 7.0 (adust at 4°C)
2. Anode buffer BN (6x): 300 mM BisTris, pH 7.0 (adjust at 4°C)

2.4. Transfer of gel stripes of first gel dimensions onto second gel dimensions

1. 1x Cathode buffer BN + dodecylmaltoside: 50 mM Tricine, 15 mM BisTris, 0.03% dodecylmaltoside, 0.02 (w/v) Coomassie G 250 (e.g. Merck, Darmstadt, Germany), pH 7.0 (adjust at 4°C)

2.5. Second dimension BN-PAGE

1. Agarose solution: 1.5 % (w/v) Agarose
2. Cathode buffer BN + dodecylmaltoside: (see 2.4.)
3. Anode buffer BN (6x): (see 2.3.)

2.6. Enzyme activity staining procedures of 2D BN / BN gels

1. Phosphate buffer stock solution: 1M phosphate, pH 7.4
2. DAB stock solution: 0.1 M 3,3'-diamino benzidine tetrahydrochloride dihydrate
3. KCN stock solution: 1 M KCN
4. EDTA stock solution: 0.1 M EDTA
5. Tris stock solution: 2 M Tris-HCl, pH 7.4
6. Cytochrome c oxidase staining solution: 10 mM Phosphate buffer, pH 7.4, 0.1 % (w/v) DAB, 7.5 % (w/v) sucrose, 19 U/ml catalase and 16 mM cytochrome c (Sigma-Aldrich, St. Louis, MO, USA)
7. Succinate dehydrogenase staining solution: 50 mM Phosphate buffer, pH 7.4, 84 mM succinic acid, 0.2 mM N-Methylphenazonium methyl sulfate (Fluka, Buchs, Switzerland), 0.2 % Nitro tetrazolium blue (Fluka, Buchs, Switzerland), 4.5 mM EDTA, 10 mM KCN
8. NADH dehydrogenase staining solution: 0.1 M Tris-HCl, pH 7.4, 0.14 mM NADH, 0.1 % Nitro tetrazolium blue (Fluka, Buchs, Switzerland)
9. Fixing solution: 40 % (v/v) methanol, 10 % (w/v) acetic acid

3. Methods

Digitonin currently is considered to be the mildest detergent for mitochondrial research, which better stabilizes most supramolecular structures than Triton X100, dodecylmaltoside or other non-ionic detergents. In the protocol given below, first dimension BN-PAGE is therefore carried out after membrane solubilization using digitonin and second dimension BN-PAGE after incubation of the gel stripe of the first gel dimension with dodecylmaltoside. However, numerous variations of this experimental design are possible (*see* Note 1)

3.1. Preparation of BN gels for first and second gel dimensions

Best resolution capacity of BN gels is achieved if the electrophoretic separation distance is > 12 cm. The following instructions refer to the Protean II electrophoresis unit (BioRad, Richmond, Ca, USA; dimensions 0.1 x 16 x 20 cm for the gel of the first dimension, 0.15 x 16 x 20 cm for the gel of the second dimension). However, units from other manufacturers are of comparable suitability for BN-PAGE, e.g. the Hoefer SE-400 or SE-600 gel systems (Amersham Biosciences, Uppsala, Sweden). Usage of gradient gels is recommended, because molecular masses of protein complexes can vary between 50 kDa and several thousand kDa. (*see* Note 2)

First dimension gel (thickness of the gel: 1.0 mm)

1. Prepare a 4.5 % separation gel solution by mixing 1.2 ml Acrylamide solution with 2.2 ml Gel buffer BN and 10.0 ml ddH_2O
2. Prepare a 16 % separation gel solution by mixing 4.5 ml Acrylamide solution with 2.2 ml Gel buffer BN, 4.0 ml ddH_2O and 2.7 ml glycerol
3. Transfer the two gel solutions into the two chambers of a gradient former and connect the gradient former via a hose and a needle with the space in-between two glass plates, which are pre-assembled in a gel casting stand. Gradient gels can either be pored from the top (16 % gel solution has to enter the gel sandwich first) or from the bottom (4.5 % gel solution has to enter first). Poring gradient gels at 4°C is recommended to avoid premature polymerization.
4. Add APS and TEMED to the two gel solutions (60 µl 10 % APS / 6 µl TEMED to the 4.5 % gel solution, 40 µl APS / 4 µl TEMED to the 16 % gel solution)
5. Pour the gradient gel, leaving space for the stacking gel, and overlay with ddH_2O. The gel should polymerize in about 60 minutes
6. Pour off the ddH_2O
7. Prepare the stacking gel solution by mixing 1.2 ml Acrylamide solution, 2.5 ml Gel buffer BN and 11.3 ml ddH_2O
8. Add 65 µl APS and 6.5 µl TEMED and pour the stacking gel around an inserted the comb. The stacking gel should polymerize within 30 minutes.

Second dimension gel (thickness of the gel: 1.5 mm)

1. Prepare a 5.0 % separation gel solution by mixing 2.0 ml Acrylamide solution with 3.3 ml Gel buffer BN and 14.7 ml ddH_2O
2. Prepare a 20 % separation gel solution by mixing 8.0 ml Acrylamide solution with 3.3 ml Gel buffer BN, 4.7 ml ddH_2O and 4.0 ml glycerol
3. Transfer the two gel solutions into the two chambers of a gradient former as described for the first dimension gel
4. Add APS and TEMED to the two gel solutions (90 µl 10 % APS / 9 µl TEMED to the 5 % gel solution, 50 µl APS / 5 µl TEMED to the 20 % gel solution)
5. Pour the gradient gel, leaving space for the stacking gel, and overlay with ddH_2O. The gel should polymerize in about 60 minutes
6. Pour off the ddH_2O
7. Prepare the stacking gel solution by mixing 1.2 ml Acrylamide solution, 2.5 ml Gel buffer BN, 1.7 ml glycerol and 9.6 ml ddH_2O
8. Add 65 µl APS and 6.5 µl TEMED and cast the stacking gel until 1 cm below the upper edge of the inner glass plate (do not insert a comb). Overlay with ddH_2O. The stacking gel should polymerize within 30 minutes. Finally remove the overlaying water.

Gels for both gel dimensions should be prepared one day before usage and should be stored at 4°C.

3.2. Sample preparation

All steps of the sample preparation should be carried out at 4°C.

1. Prepare mitochondria of interest (*see* Note 3)
2. Determine the protein concentration, e.g. according to Lowry [16].
3. Adjust the protein concentration to 10 µg / µl
4. Centrifuge 50 µl fractions (about 0.5 mg protein) for 10 minutes at 15 000 xg to sediment organelles
5. Resuspended pellets in 50 µl Digitonin solubilization solution
6. Incubate the fractions for 20 minutes on ice

7. Centrifuge the fractions for 20 minutes at 20 000 xg to remove insoluble material
8. Supplement the fractions with 2.5 µl Coomassie-blue solution
9. Load 30 - 50 µl of the supernatants (corresponding to about 0.3 – 0.5 mg mitochondrial protein) directly into the wells of a BN gel (protein amounts are adjusted to allow staining of gels by Coomassie; if silver staining shall be applied, protein amounts can be reduced by factor 10).

3.3. First dimension BN-PAGE

1. Prepare 1xAnode and 1xCathode buffers BN by diluting the corresponding stock solutions
2. Carefully remove the comb of the first dimension BN gel
3. Assemble the gel electrophoresis unit, add 1xCathode and 1xAnode buffers BN to the upper and lower chambers of the gel unit. Cool down the unit to 4°C
4. Load Coomassie-blue pre-treated protein samples (see 3.2.) into the gel wells
5. Connect the gel unit to a power supply. Start electrophoresis at constant voltage (100V for 45 minutes) and continue at constant current (15 mA for about 5 hours, *see* Note 4). Electrophoresis should be carried out at 4°C. Blue gel bands should already be visible during the electrophoresis run.

3.4. Transfer of gel stripes of first gel dimensions onto second gel dimensions

1. Cut out a lane of the first dimension BN gel
2. Incubate the gel stripe for 10 minutes in Cathode buffer BN + dodecylmaltoside at 4°C

3.5. Second dimension BN-PAGE

1. Assemble the gel electrophoresis unit and transfer the gel stripe of the first gel dimension onto a second dimension gel. Make sure it is placed centrally and that is has close contact to the second dimension gel. Fix the gel stripe with 1.5 % agarose solution (boil the solution before use and allow it to cool down to approximately 45°C). (*see* Note 5)
2. Prepare 1xAnode buffer BN by diluting the corresponding stock solution
3. Add 1x Cathode buffer BN + dodecylmaltoside and 1xAnode buffer BN to the upper and lower chambers of the gel unit.

4. Connect the gel unit to a power supply. Start electrophoresis at constant voltage (100V for 45 minutes) and continue at constant current (15 mA for 6 - 12 hours, *see* Note 6). Electrophoresis should be carried out at 4°C. Blue gel spots should already be visible during the electrophoresis run.

3.6. Enzyme activity staining procedures of 2D BN / BN gels

After completion of the electrophoresis run, 2D BN / BN gels can be stained using Coomassie colloidal [17, 18] or silver [19] (*see* Notes 7 and 8). However, since both gel dimensions are carried out under native conditions, 2D BN / BN PAGE also is compatible with in gel enzyme activity stainings [20, 21]. Three classical in gel staining procedures for enzymes of the respiratory chain are given below (activity staining procedures for several other enzymes can be found in the literature).

1. incubate the gel with ddH_2O for two times 10 minutes
2. incubate the gel with 100 ml freshly prepared staining solution (Cytochrome c oxidase staining solution, or Succinate dehydrogenase staing solution, or NADH dehydrogenase staining solution) Staining takes minutes until hours depending on the abundance of the stained enzyme.
3. Stop the reaction by transferring the gel into Fixing solution (*see* Notes 9 and 10).

4. Notes

1. Conditions to be varied in the two gel dimensions of BN / BN PAGE can refer to detergent type, detergent concentration, temperature, presence of chaotropic compounds and others. Conditions to monitor dissection of supercomplexes into protein complexes and / or dissection of protein complexes into subcomplexes should be optimized for the mitochondria of interest.
2. If very large protein complexes (>3 MDa) have to be resolved, the acrylamide gradient gel of the BN gel dimension can be substituted by a 2.5 % agarose gel prepared in Gel buffer BN [22].

3. Alternatively, mitochondrial subfractions can be used as starting material for BN / BN PAGE, e.g. an inner membrane or a matrix fraction.
4. Electrophoresis should not be carried out for more than 5 hours, because protein complexes might get stuck in the pores of the gradient polyacrylamide gel, which might prevent migration of the complexes into the second gel dimension. In general, 2/3 completion of the electrophoresis run is sufficient for protein complex resolution on the first gel dimension.
5. Transfer of a lane of a BN gel onto a second gel dimension is proposed to be carried out by fixing a lane of a first dimension BN gel onto a pre-poured BN gel for second gel dimension. By using this procedure, time between first and second gel dimension is minimized, which is advantageous for activity stainings of 2D BN / BN gels. However, physical contact between the lane of the first dimension gel and the second dimension gel might be better if the lane of the second gel dimension is embedded into the stacking gel of the second gel dimension (see [5-7] for corresponding protocols). On the other hand, TEMED and APS of the gel solution for the second gel dimension can diffuse into the gel stripe of the first gel dimension, which usually greatly reduces enzymatic activities. Therefore, this procedure should only be applied for 2D BN / BN gels in combination with Coomassie and silver staining.
6. Electrophoresis of the second gel dimension should be carried out for 6-12 hours or longer. Often, sharpness of protein spots is best after long electrophoresis, because protein complexes get stuck into the pores of the gradient gel of the second gel dimension at defined polyacrylamide concentrations.
7. Protein complexes resolved on 2D BN / BN gels also can be blotted onto membranes. Short pre-blots should be carried out to electrophoretically de-stain gels from excess of Coomassie-blue [23]. Alternatively, the Cathode-buffer BN can be replaced by a Cathode buffer BN without Coomassie blue after 50 % completion of the electrophoresis run of the second BN gel dimension. Furthermore, protein complexes excised from 2D BN / BN gels also can be separated on a third gel dimension, which is carried out in the presence of SDS [4].
8. Protein complexes resolved by 2D BN / BN PAGE also can be cut out and prepared for analysis by mass spectrometry. Usually the subunits of protein complexes are first fragmented by Trypsin. Afterwards, peptides are best analyzed by coupled liquid choromatography and electrospray tamdem mass spectrometry (LC-MS/MS).

9. Alternatively, in gel activity staining also can be stopped by adding inhibitors of the monitored enzymes
10. If 2D BN / BN gels shall be Coomassie or silver stained after activity staining, wash the gels several times with ddH_2O before fixation to remove proteins of the activity staining solutions. This will reduce background staining of the gels.

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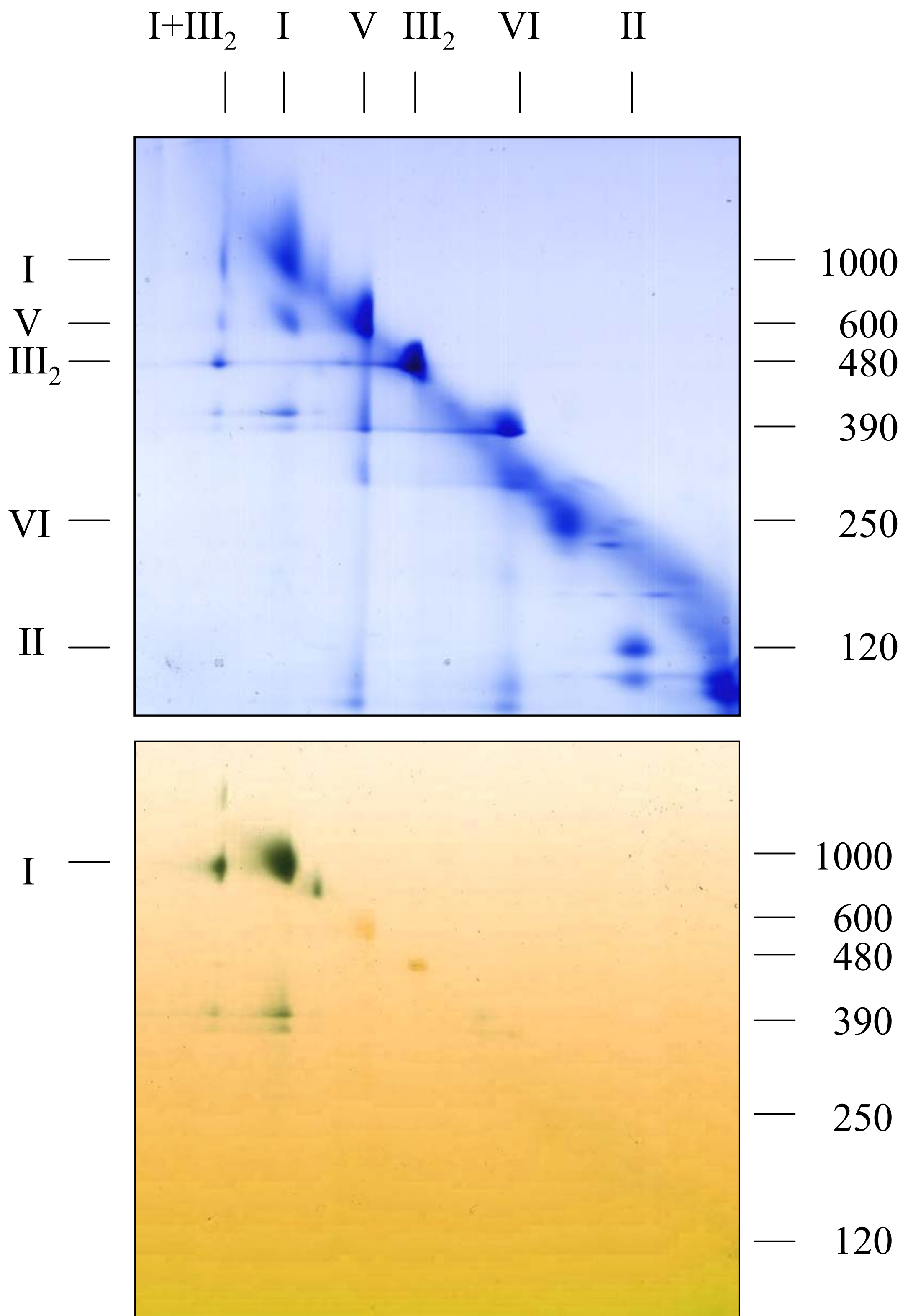
Figure legend:

Resolution of mitochondrial protein complexes from Arabidopsis by two-dimensional BN / BN PAGE. Proteins were solubilized by Digitonin solubilization solution. A: 2D BN / BN gel after Coomassie-staining, B: 2D BN / BN gel after activity staining for NADH dehydrogenase (complex I). The numbers to the right refer to the molecular masses of standard proteins. $I+III_2$: supercomplex composed of complexes I and III_2; I: NADH dehydrogenase (complex I); V: ATP synthase (complex V); III_2: dimeric cytochrome c reductase (complex III); IV: cytochrome c oxidase (complex IV), II: succinate dehydrogenase (complex II). Note: activity staining allows to visualize the singular and the supercomplex-bound form of complex I. Furthermore, complex I is partially dissected into fragments of 600 kDa (hydrophobic arm) and 400 kDa (matrix exposed arm). The 400 kDa arm includes the NADH oxidizing domain and therefore is also labeled by activity staining, whereas the 600 kDa arm is not.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