Basic protocol for the analysis of thylakoid membrane protein complexes by blue native gel electrophoresis

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SUMMARY
Basic protocol for the elucidation of plant thylakoid protein complex organization and composition with blue native polyacrylamide gel electrophoresis (BN-PAGE) and 2D-SDS-PAGE is described. The protocol is optimized for Arabidopsis thaliana, but with minor modifications can be used for other plant species.

ABSTRACT
Photosynthetic electron transfer chain (ETC) converts solar energy to chemical energy in the form of NADPH and ATP. Four large protein complexes embedded in the thylakoid membrane harvest solar energy to drive electrons from water to $NADP^+$ via two photosystems, and use the created proton gradient for production of ATP. Photosystem (PS)II, PSI, cytochrome b_6f (Cyt b_6f) and ATPase are all multiprotein complexes with distinct orientation and dynamics in the thylakoid membrane. Valuable information about the composition and interactions of the protein complexes in the thylakoid membrane can be obtained by solubilizing the complexes from the membrane integrity by mild detergents followed by native gel electrophoretic separation of the complexes. Blue native polyacrylamide gel electrophoresis (BN-PAGE) is a analytical method used for the separation of protein complexes in their native and functional form. The method can be used for protein complex purification for more detailed structural analysis, but it also provides a tool to dissect the dynamic interactions between the protein complexes. The method was developed for the analysis of mitochondrial respiratory protein complexes, but has since beenoptimized and improved for the dissection of the thylakoid protein complexes. Here we provide a detailed up-to-date protocol for analysis of labile photosynthetic protein complexes

and their interactions in Arabidopsis thaliana.

INTRODUCTION

Large multisubunit protein complexes photosystem (PS) I and PSII, Cyt b_6f and ATPase coordinate the production of NADPH and ATP in photosynthetic light reactions. In higher plant chloroplasts, the complexes are located in the thylakoid membrane, which is a structurally heterogeneous membrane structure, comprising appressed grana and non-appressed stroma thylakoids. Blue native polyacrylamide gel electrophoresis (BN-PAGE) is an extensively used method in the analysis of large multisubunit protein complexes in their native and biologically active form. The method was established for the dissection of mitochondrial membrane protein complexes [1], but been customized for the separation of thylakoid protein complexes [2,3]. The method is suitable (i) for the purification of individual thylakoid protein complexes for structural analysis, (ii) for determining native interactions between protein complexes and (iii) for the analysis of overall organization of the protein complexes upon changing environmental cues.

Prior to the separation, protein complexes are isolated from the membrane with carefully chosen nonionic detergents, which are generally mild and preserve the native structure of the protein complexes. Detergents contain hydrophobic and hydrophilic sites and form stable micelles above a certain concentration, called a critical micellar concentration (CMC). Increasing the detergent concentration above the CMC results in disruption of the lipid-lipid interactions and in the solubilization of protein complexes. The choice of detergent depends on the stability of the protein complex of interest and on the solubilization capacity of the detergent. Routinely used detergents include α/β-dodecyl-maltoside and digitonin. Following the solubilization of protein complexes in their native state, insoluble material is removed by centrifugation. In higher plants the thylakoid membrane is highly heterogenic in structure and some detergents (e.g. digitonin) selectively solubilize only a specific fraction of the membrane[3]. Therefore, to characterize the protein complex organization or the interactions between the protein complexes, it is crucial to always determine the solubilization capacity of the chosen detergent by determining the chlorophyll content and the chlorophyll a/b ratio of supernatant to assess the yield and the represented thylakoid (sub)domain, respectively, of the solubilized fraction. The chlorophyll a/b ratio in intact thylakoids of growth-light acclimated plants is typically around 3, whereas the chl a/b value of thylakoid fractions enriched either with grana or stroma thylakoids falls below (~2.5) or exceeds (~4.5) the value of the total thylakoids, respectively.

To provide negative charge to the protein complexes, Coomassie brilliant blue (CBB, G-250) dye is added to the solubilized sample.Due to the charge shift, protein complexes migrate towards the anode and are separated on acrylamide (AA) gradient according to their molecular mass and shape. Effective and high resolution separation is achieved by using a linear acrylamide concentration gradient. During the electrophoresis, the protein complexes migrate towards the anode until they reach their size-dependent pore-size limit. The pore-size of polyacrylamide gel depends on (i) the total acrylamide/bis-acrylamide concentration (T) and (ii) on the cross-linker bis-acrylamide monomer concentration (C) relative to the total monomers [4]. After the separation with BN-PAGE, the protein complexes can be further subdivided into their individual protein subunits by second-dimension (2D)-SDS-PAGE. Here we describe a detailed protocol for the analysis of thylakoid membrane protein complexes by BN-PAGE/2D-SDS-PAGE.

PROTOCOL

1. Preparing BN gel [1–3]

1. Set up the gel caster with 8 x10 cm plates (rectangular glass and notched alumina plate) according to manufacturer's instructions using 0. 75 mm spacers.

2. Place a gradient mixer on a stir plate and connect it with the peristaltic pump by a tubing. Attach a syringe needle to the other end of the tubing and place the needle between the glass and aluminum plate. Place magnetic stirrer to the "heavy" (H)-chamber.

3. Prepare the 3.5% (v/v) and 12.5% (v/v) acrylamide (AA) solutions in 15 ml conical centrifuge tubes for the separation gel gradient (see recipes in Table 1). To prevent untimely polymerization, keep the centrifuge tubes on ice while preparing the solutions.

CAUTION: Acrylamide is neurotoxic and carcinogenic, wear protective clothes and gloves.

4. Add 5% APS and TEMED right before pipeting the solutions to the gradient mixer. Pipet the 12.5% solution to the H-chamber. Remove air bubbles from the channel connecting the "light" (L) and H-chamber by opening the valve connecting the two chambers allowing solution to enter to the L-chamber. Close the valve and pipet the traces of solution back to H-chamber. Finally, pipet the 3.5% solution to the L- chamber.

5. Switch on the magnetic stirrer (the speed of the stir is not critical, but it should ensure proper mixing of the the heavy and light solutions), open the valves and switch on the peristaltic pump. Allow the gel solutions to flow between the glass and aluminum plate, the flowrate should be roughly 0.5 mL/min. The needle must be above the liquid all the time, it can be attached to the upper part of the glass plate with a tape.

6. When the H- and L-chambers have emptied, fill them with ultrapurewater and allow it to gently overlay the gel surface. The gel polymerization takes around 1-2 hours at RT.

7. Prepare the 3% acrylamide solution (see recipe in Table 1) for the stacking gel at RT. Pipet the stacking gel on top of the polymerized separation gel (before casting the stacking gel, remove the water overlaying the gel surface) and place a sample gel comb between the glass and aluminum plate avoiding air bubbles. Allow to polymerize 30-60 min at RT. Remove the comb gently under ultrapure water. Store the gel at +4 ˚C.

Pause point. The gel can be stored at +4 ˚C few days. The gel should be kept in moist condition, the gel surface should not dry.

3. Thylakoid solubilization [1–3]

Note: All steps should be performed under very dim light. Keep samples and buffers on ice.

1. Dilute isolated thylakoids with ice-cold 25BTH20G buffer to a final chlorophyll concentration of 1 mg/mL. For 2D-BN-SDS-PAGE analysis roughly 4-8 µg of chlorophyll/ sample is suitable.

   Note: The thylakoids used in the experiments must be isolated from fresh leaves (for protocol of thylakoid isolation, see [3])

2. Add an equal volume of detergent buffer, i.e. 2% β-DM (w/v) or 2% digitonin (w/v). Mix the detergent to the thylakoid sample gently with the pipet tip and avoid making air bubbles.The final concentration of the detergent is 1% and that of the thylakoids 0.5mg/mL. Solubilize the thylakoids for 2 min on ice (β-DM) or 10 minutes at RT with continuous gentle mixing on a rocker/shaker (digitonin).

   Note: Digitonin and β-DM are generally used for the solubilization of thylakoid protein complexes. If other non-ionic detergents are used, the detergent concentration and the solubilization time must be first optimized. Usually the detergent concentration range varies from 0.5%-5% (w/v).

   CAUTION: Digitonin is toxic, wear protective clothes and gloves

3. Remove the insolubilized material by centrifugation at 18 000 x g for 20 min, at +4 °C.

4. Transfer the supernatant to a new 1.5 mLtube and add 1/10 (v/v) of CBB buffer to the sample.

   Note: When the overall composition of the thylakoid membrane protein complexes is examined, determining the yield and the represented thylakoid domain of solubilized fraction is recommended. To determine the yield of the solubilized material, take 5 µL of the supernatant to a new tube (before adding CBB) and measure the Chl content and Chl a/b ratio according to [5].

4. BN-PAGE [1–3]

1. Assemble the gel to a vertical electrophoresis system (e.g. Hoefer SE 250). Pour blue cathode buffer (see recipe) to the upper buffer chamber and anode buffer to the lower buffer chamber.

2. Load thylakoid sample (e.g. 5 µg of chlorophyll) into the wells.

3. Start the electrophoresis and gradually increase the voltage: 75 V for 30 min, 100 V for 30 min, 125 V for 30 min, 150 V for 1 h and 175 V until the complexes have been separated completely. The gel running should be done at +4˚C either at cold room or adjusted with a cooling system.

   Note: Change the blue cathode buffer to a clear cathode buffer when the sample front has migrated about one third of the gel.

4. After the electrophoretic run, scan the gel for image archiving.

5. 2D-SDS-PAGE

1. Assemble vertical electrophoresis system (gel size 16 x 20 cm). Use 1 mm spacers.

2. Prepare standard SDS gel (12% acrylamide, 6M Urea, see recipe in Table 2) with a 2D-comb (single large well for the strip and one standard well for molecular weight marker).

3. Cut the lane from BN-gel and place it in a (5 mL) tube. Add 2 mL of Laemmli buffer (containing 5 % β-mercaptoethanol) and incubate the strip for 45 min with gentle shaking at RT.

4. Place the lane with e.g. a spacer on top of the gel avoiding air bubbles.

5. Pipet 5 µl of molecular weight marker on a narrow piece of filter paper and place the paper to the standard well.

6. To seal the BN-gel strip and the marker paper, pour 0.5% agarose (in running buffer) on top of the gel strip and allow to solidify.

7. Perform electrophoresis according to standard protocols. After the electrophoretic run, visualize the proteins with e.g. Sypro Ruby stain or silver staining according to [6].

REPRESENTATIVE RESULTS

The representative results of 2D-BN/SDS-PAGE in Figure 1 shows the detailed protein subunit composition of digitonin and β-DM-solubilized thylakoids. The protein complex pattern of digitonin solubilized thylakoids contain the PSII-LHCII-PSI megacomplex, two large PSII-LHCII supercomplexes (sc), PSI-LHCII supercomplex, PSI monomer (m), PSII m/Cyt b_6f, loosely bound (L)-LHCII trimer (Figure 1A). The slightly stronger detergent, β-DM, solubilizes the entire thylakoid membrane, but is unable to preserve weak interactions between protein complexes. Thylakoid solubilization with β-DM typically produces four PSII-LHCII supercomplexes (with different amount of LHCII antenna attached), PSII dimer (d) and PSI m, ATPase, PSII m and Cytb_6f, M-LHCII, L-LHCII and LHCII monomer (Figure 1B). Unlike β-DM, digitonin produces only minor amount of LHCII monomer. The protein complex pattern may differ upon different light conditions and in

different mutant lines, since the protein complex interactions are dynamic and dependent on i.e. protein phosphorylation.

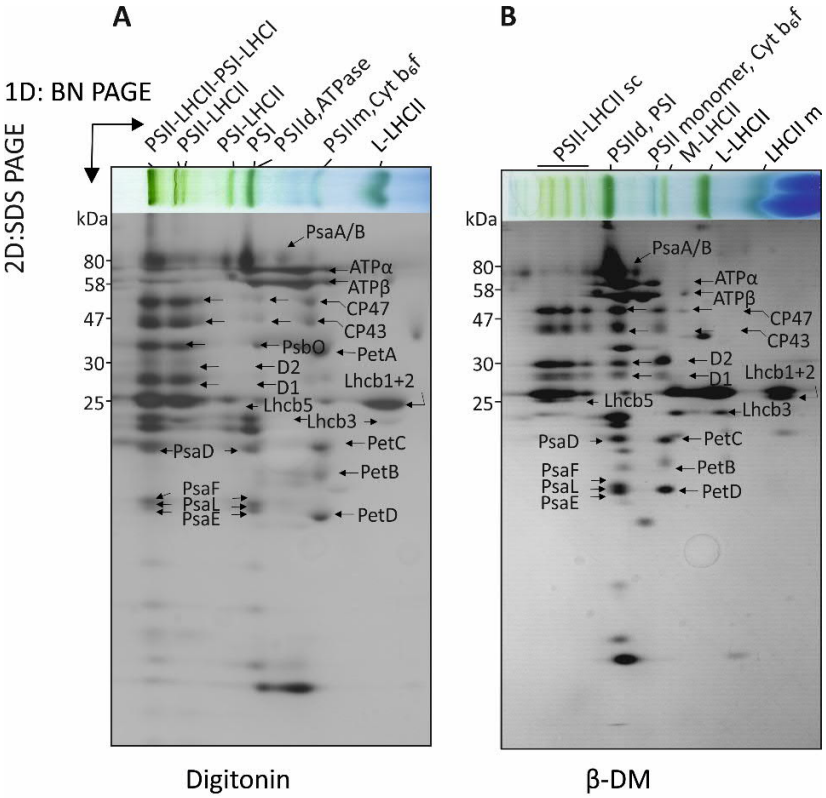

Figure 1. A two-dimensional BN-PAGE/SDS-PAGE of Arabidopsis thylakoid. Thylakoid protein complexes solubilized with (A) 1% digitonin and (B) 1% β-DM and separated first by 1D-BN-PAGE (the lanes on top) and subsequently on 2D-SDS-PAGE to demonstrate the individual protein composition of each complex. Due to the incubation of BN-strips with denaturing Laemmli buffer, the protein subunits of each complex (in the BN strip) dissociate and are separated in a vertical line during the 2D-SDS-PAGE. The protein identification is based on mass spectrometry analysis presented in references [7,8].

TABLES OF MATERIALS AND BUFFER RECIPES

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
6-aminocaproic acid (ACA)	Sigma-Aldrich	A2504	
BisTris	Sigma-Aldrich	B4429	
Sucrose	Sigma-Aldrich	S0389	
Acrylamide (AA)	Sigma-Aldrich	A9099	Caution: Neurotoxic!
n-dodecyl-β-D-maltoside	Sigma-Aldrich	D4641	
Tricine	Sigma-Aldrich	T0377	
Tris	Sigma-Aldrich	T1503	
SDS	VWR	442444H	
Urea	VWR	28877.292	
Glycerol	J.T. Baker	7044	
Sodium Fluoride (NaF)	J.T. Baker	3688	
EDTA disodium salt	J.T. Baker	1073	
Digitonin	Calbiochem	300410	Caution:Toxic!
Pefabloc SC	Roche	11585916001	
Serva Coomassie Blue G	Serva	35050	
β-mercaptoethanol	Bio-Rad	1610710	
APS (Ammonium persulfate)	Bio-Rad	161-0700	
TEMED (Tetramethylethylenediamine)	Bio-Rad	1610801	
(N,N'-Methylene)-Bis-Acrylamide	Omnipur	2610	
Glycine	Fisher	G0800	
Prestained Protein Marker, Broad Range (7-175 kDa)	New England Biolabs	P7708	
Falcon, Conical Centrifuge Tubes 15 ml	Corning	352093	
Dual gel caster with 10 x 8 cm plates	Hoefer	SE215	
Gradient maker SG5	Hoefer		
0.75 mm T-spacers	Hoefer	SE2119T-2-.75	
Sample gel comb, 0.75 mm	Hoefer	SE211A-10-.75	
Mighty Small SE250 vertical electrophoresis system	Hoefer	SE250	
IPC-pump	Ismatec		
Power supply, PowerPac HV	Bio-Rad	164-5097	
Centrifuge	Eppendorf	5424R	
Rocker-Shaker	Biosan	BS-010130-AAI	
PROTEAN II xi Cell	Bio-Rad	1651813	

Table 1. Buffers and solutions for native gel electrophoresis

Buffer	Content	Comments
3.5% (T) Acrylamide (AA) BN Separation gel	48% AA, 1.5% bis-AA: 148 µL 3xGel Buffer: 700 µL 75% (w/v) glycerol: 140 µL Ultrapure H_2O: 1092 µL 5% APS: 15 µL TEMED 3 µL	NOTE: Acrylamide is neurotoxic. Add APS and TEMED immediately before use. The recipe is sufficient for casting one small BN gel.
12.5% (T) Acrylamide (AA) BN Separation gel	48% AA, 1,5% bis-AA: 530 µL 3xGel Buffer: 700 µL 75% (w/v) glycerol: 560 µL Ultrapure H_2O: 290 µL 5% APS: 11 µL TEMED 2µL	NOTE: Acrylamide is neurotoxic. Add APS and TEMED immediately before use. The recipe is sufficient for casting one small BN gel.
3 % (T) Acrylamide (AA) BN Stacking gel	20% AA, 5% bis-AA: 180 µL 3xGel Buffer: 500 µL Ultrapure H_2O: 800 µL 5% APS: 30 µL TEMED 3 µL	NOTE: Acrylamide is neurotoxic. Add APS and TEMED immediately before use. The recipe is sufficient for one small BN gel.
3x Gel buffer	1.5M ACA 150mM BisTris/HCl (pH 7.0)	Store at + 4 ˚C.
CBB buffer	100 mM BisTris/HCl (pH 7.0) 0.5 M ACA 30% (w/v) sucrose 50 mg/ml Serva Blue G	Store at + 4 ˚C
Anode buffer	50 mM BisTris/HCl (pH 7.0)	Store at + 4 ˚C. Buffer can be prepared as 10x stock solution
Cathode buffer	50 mM Tricine 15 mM BisTris 0.01% Serva Blue G	Store at + 4 ˚C. Buffer can be prepared as 10x stock solution, add the dye to the 1x solution
25BTH20G	25 mM BisTris/HCl (pH 7.0) 20% (w/v) glycerol 0.25 mg/ml Pefabloc (add freshly) 10 mM NaF (add freshly)	Buffer can be prepared as 2X stock solution (store at + 4 ˚C) , but add Pefabloc and NaF freshly
Detergent buffer	2% β-dodecyl maltoside/Digitonin (w/v) 25 mM BisTris/HCl (pH 7.0) 20% (w/v) glycerol and 0.25 mg/ml Pefabloc (add freshly from the stock solution) 10 mM NaF (add freshly)	Detergents can be prepared as 5-10% stock solutions (in water). If other detergents are used, the final detergent concentration has to be optimized.

Table 2. Buffers for 2D-SDS-PAGE

Buffer	Content	Comments
Laemmli buffer	138 mM Tris/HCL pH 6.8 6M Urea 22.2 % (v/v) glycerol 4.4 % SDS	Reference: 9
12% Acrylamide, 6M Urea SDS Separation gel	50% AA, 1,33% bis-AA: 10.5 mL 20% SDS: 0.7 mL 1.5 M Tris-HCl (pH 8.8): 8.05mL Urea: 12.6 g MQ-H_2O: 6.16 mL 10% APS: 200 µL TEMED 28 µL	NOTE: Acrylamide is neurotoxic.The recipe is suitable for casting one big SDS-gel.
6% Acrylamide, 6M Urea SDS Stacking gel	50% AA, 1.33% bis-AA: 1.2 mL 20% SDS: 0.2 mL 0.5 M Tris-HCl (pH 6.8): 2.5 mL Urea: 3.6 g MQ-H_2O: 3.45 mL 10% APS: 100 µL TEMED 10 µL	NOTE: Acrylamide is neurotoxic.
SDS Running buffer	19 mM Tris 2.5 mM Glycine 0.01 % SDS	

DISCUSSION

The photosynthetic energy conversion machinery is composed of large multisubunit protein complexes, which are embedded in the thylakoid membrane. This protocol describes a basic method for analysis of the plant thylakoid protein complexes from Arabidopsis thaliana with BN-PAGE combined with 2D-SDS-PAGE. The protocol is also suitable for the analysis of thylakoid protein complexes from tobacco and spinach thylakoids, but might require small adjustments.

For the solubilization of membrane protein complexes, nonionic detergents are commonly used for their ability to preserve the complexes in their native form. Here, two commonly used detergents β-DM and digitonin were applied. Dodecyl maltoside solubilizes individual protein complexes, whereas digitonin can be used for the analysis of larger protein complex assemblies [10]. The bulky structured digitonin is unable to fit to the tightly appressed grana and therefore solubilizes only the non-appressed regions of the thylakoid membrane [3, 11]. It is therefore suited for the analysis of stroma thylakoids and grana margins. However, when digitonin is used together with aminocaproic acid (ACA), the combination solubilizes the entire thylakoid membrane, including also the appressed grana thylakoids [12]. Through an unknown mechanism, ACA allows digitonin to have an access to the partition gap between adjacent grana membrane layers. Importantly, digitonin preserves labile interactions between protein complexes and can therefore be used for the analysis of labile protein super and megacomplexes, which are most

abundant in the non-appressed thylakoid regions[8]. It must be noted that detergents always interfere with some of the labile interactions between the protein complexes and therefore it is not possible to isolate completely intact network of protein complexes. Some of the complexes are degradation products that have been disconnected from larger protein complexes during the solubilization and the electrophoresis. The quality of the BN-PAGE separation depends not only of the sample preparation (protein complex solubilization), but also on the quality of the thylakoid isolation, which must be done from fresh leaves. If special care is not taken, the PSII-LHCII supercomplexes typically degrade during β-DM solubilization.

BN-PAGE maintains the integrity of the solubilized protein complexes. The separation capacity of the BN gel depends on the acrylamide gradient, and the gradient should be optimized based on the protein complexes of interest. The pore-size of the polyacrylamide gel can be modified by changing the concentration gradient (total acrylamide concentration, T) or by adjusting the bis-acrylamide concentration (C) relative to the total amount of acrylamide monomers[4]. The BN-PA gel gradient used here is optimized and well suited for the analysis of large protein super- and megacomplexes[3]. After protein complex separation with BN-PAGE, the composition, structure or the spectroscopic properties of each individual protein complex band can be further analyzed. For analysis of the subunit composition of the protein complexes, a denaturing 2D-SDS-PAGE is described here. The subunits of each complex are separated in a vertical line and can be easily identified. It has to be noted that several proteins may be present in a single spot and the subunits in the same vertical line may belong to separate complexes co-migrating in BN-PAGE.

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DISCLOSURES

The authors have nothing to disclose.

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