Optimized native gel systems for separation of thylakoid protein complexes: novel super- and mega-complexes

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Gel-based analysis of thylakoid membrane protein complexes represents a valuable tool to monitor the dynamics of the photosynthetic machinery. Native-PAGE preserves the components and often also the conformation of the protein complexes, thus enabling the analysis of their subunit composition. Nevertheless, the literature and practical experimentation in the field sometimes raise confusion owing to a great variety of native-PAGE and thylakoid-solubilization systems. In the present paper, we describe optimized methods for separation of higher plant thylakoid membrane protein complexes by native-PAGE addressing particularly: (i) the use of detergent; (ii) the use of solubilization buffer; and (iii) the gel electrophoresis method. Special attention is paid to separation of high-molecular-mass thylakoid membrane super- and mega-complexes from Arabidopsis thaliana leaves. Several novel super- and mega-complexes including PS (photosystem) I, PSII and LHCs (light-harvesting complexes) in various combinations are reported.

Key words: Arabidopsis, blue native gel electrophoresis, clear native gel electrophoresis, light-harvesting complex, photosystem, thylakoid megacomplex.

INTRODUCTION

Great diversity in the composition of the protein complexes is a distinct feature of the thylakoid membrane. PS (photosystem) II and PSI supercomplexes and subcomplexes are involved in (i) the linear and cyclic electron transfer, (ii) dynamics of light absorption, and (iii) the repair cycle of PSII. The high-molecular-mass supercomplexes composed of the PSII dimer and LHC (light-harvesting complex) II dominate the granal thylakoid membrane and represent the most active form of PSII [1]. Upon light-induced damage, PSII–LHCII supercomplexes monomerize, and damaged PSII cores migrate to unstacked stroma thylakoid membrane, where the PSII repair cycle takes place [2,3]. Hence the low-molecular-mass PSII subcomplexes in the stroma thylakoid membrane mainly represent intermediates of the repair cycle and/or the biogenesis of PSII [2]. Two high-molecular-mass PSI megacomplexes, PSI–LHCCI and PSI–NDH [NAD(P)H dehydrogenase], were identified from the stroma membrane [4–6] and were shown to represent the state transition and NDH-dependent cyclic electron transfer-specific megacomplexes respectively.

In order to understand the dynamics in the composition of the thylakoid membrane protein complexes, it is important to develop techniques allowing reliable separation of the protein complexes. Native-PAGE is an excellent tool for analyses of proteins and protein complexes in their native form. In early native-PAGE analyses, low concentrations of anionic detergents, such as SDS, DOC (sodium deoxycholate) or zwitterionic detergent disodium N-dodecyl-β-midomopropionate (Deriphat 160) have been applied to improve the resolution of proteins and/or protein complexes [7–9]. Since 1991, BN (blue native)-PAGE, where anionic Coomassie Brilliant Blue dye is used to introduce a negative charge and visual stain for the proteins and protein complexes [10], has been the prevalent method for native-PAGE separation. In BN-PAGE, the superior separation capacity of protein complexes is combined with the maintenance of the integrity of protein complexes. The first analysis of thylakoid membrane protein complexes using BN-PAGE was described in 1997 [11], and since then this method has been routinely used in plant science [2]. Prior to native-PAGE, the thylakoid membrane is solubilized with a low concentration of a mild non-ionic detergent, such as DM (dodecyl maltoside), TM (tridecyl maltoside), digitonin or Triton X-100. For two-dimensional separation, the protein complexes are denatured by incubating the native gel strips with urea, 2-mercaptoethanol and high concentrations of SDS, after which the distinct subunits of the protein complexes can be separated by SDS/PAGE.

In the present paper, we describe optimized methods for isolation and separation of thylakoid membrane protein complexes by CN (clear native), BN and lpBN (large pore BN)-PAGE. We have addressed the effects of (i) selected detergents, (ii) solubilization buffers, and (iii) the gel-electrophoresis method, and show that all of these steps have a pronounced effect on the composition of the thylakoid membrane protein complexes. Moreover, the methods were scaled to the mini-gel system, which saves biological material and reagents, and also enables faster separation of the protein complexes, thus maintaining the protein complexes as intact as possible. Using these optimized methods, we have identified several novel thylakoid membrane megacomplexes.

EXPERIMENTAL

Plant material

Arabidopsis thaliana ecotype Columbia (hereafter referred to as Arabidopsis) WT (wild-type) plants as well as the strn7 [12] and strn7/8 [13] mutant lines were grown under a photon flux density of 120 μmol of photons·m⁻²·s⁻¹ in an 8 h light/16 h dark regime at 23°C.
Preparation of native-PAGE gels

A Hoefer gel caster with 10 cm x 8 cm plates, a Hoefer SG5 gradient maker and the Hoefer SE 250 running system were used for native-PAGE. Native-PAGE gels were cast as described in [10] with minor modifications applied for the total concentration of both acrylamide and bisacrylamide monomers (T) and for the concentration of the cross-linker bisacrylamide relative to the total monomer concentration (C). Optimal separation of the thylakoid membrane protein complexes by BN-PAGE and CN-PAGE was obtained by using an acrylamide gradient of 5–12.5% (w/v) T and 3% (w/v) C in the separation gel, and 4% (w/v) T and 3% (w/v) C in the stacking gel. Optimal separation of the thylakoid membrane protein complexes by lpBN-PAGE, in turn, was obtained using an acrylamide gradient of 3.5–12.5% (w/v) T and 3% (w/v) C in the separation gel, and 3% (w/v) T and 20% (w/v) C in the stacking gel. Importantly, the comb of lpBN-PAGE was removed under water after 40 min of polymerization.

Sample preparation for CN, BN and lpBN gels

Thylakoid isolation was performed under very dim light at 4°C. Thylakoids were isolated from fresh leaves ground in ice-cold grinding buffer [50 mM Hepes/KOH (pH 7.5), 330 mM sorbitol, 2 mM EDTA, 1 mM MgCl₂, 5 mM ascorbate and 0.05% BSA with or without 10 mM sodium fluoride]. The suspension was filtered through two layers of Miracloth followed by centrifugation at 5000 g at 4°C for 4 min. The pellet was resuspended in a shock buffer [50 mM Hepes/KOH (pH 7.5), 5 mM sorbitol and 5 mM MgCl₂ with or without 10 mM sodium fluoride], followed by centrifugation at 5000 g at 4°C for 4 min. Remnants of the shock buffer were removed by suspending the pellet into storage buffer [50 mM Hepes/KOH (pH 7.5), 100 mM sorbitol and 10 mM MgCl₂ with or without 10 mM sodium fluoride] followed by centrifugation at 5000 g at 4°C for 4 min. Finally, the thylakoid pellet was suspended into a small aliquot of storage buffer. The subfractionation of thylakoid membrane with digitonin was performed essentially as described in [14], except that the samples were shaken for 8.5 min at 20°C after the addition of digitonin. The chlorophyll concentration of thylakoid preparations was determined as described in [15].

The thylakoid membrane (8 μg of chlorophyll, if not otherwise stated) was resuspended either into ice-cold 25 BTH20G [25 mM BisTris/HCl (pH 7.0), 20% (w/v) glycerol and 0.25 mg · mL⁻¹ Pefabloc] or ACA (amino-n-caproic acid) buffer [50 mM BisTris/HCl (pH 7.0), 750 mM ACA, 1 mM EDTA and 0.25 mg · mL⁻¹ Pefabloc] with or without 10 mM sodium fluoride, to a chlorophyll concentration of 1.0 mg · mL⁻¹. An equal volume of detergent solution (diluted in 25 BTH20G or ACA buffer) was added to a final concentration of 0.5–2.0% (w/v) for both DM (Sigma) and TM (Affymetrix) or 0.5–3.0% (w/v) for digitonin (Calbiochem). Thylakoid membrane was solubilized in darkness for 5 min either on ice when using DM (and TM) or at 20°C with continuous gentle mixing when using digitonin. Traces of insoluble material were removed by centrifugation at 18000 g at 4°C for 20 min. Prior to loading, the CN-PAGE samples were supplemented with DOC (Sigma, final concentration of 0–0.5%) and the BN-PAGE samples were supplemented with a one-tenth volume of Serva Blue G buffer [100 mM BisTris/HCl (pH 7.0), 0.5 M ACA, 30% (w/v) sucrose and 50 mg · mL⁻¹ Serva Blue G] to introduce a negative charge and to increase the solubility of the sample. For the lpBN gels, sample preparation was performed as described for BN-PAGE samples.

Separation of protein complexes by CN, BN and lpBN gels

Anode buffer [50 mM BisTris/HCl (pH 7.0)] was used for BN-PAGE, CN-PAGE and lpBN gels. For BN-PAGE and lpBN-PAGE, the cathode buffer contained Serva Blue G dye [50 mM Tricine, 15 mM BisTris/HCl (pH 7.0) and 0.01% Serva Blue G], and for CN-PAGE, the cathode buffer contained aliquots of DOC and DM [50 mM Tricine, 15 mM BisTris/HCl (pH 7.0), 0.05% DOC and 0.02% DM]. For certain experiments, the CN-PAGE cathode buffer was supplemented with 0.1% disodium N-dodecyl-β-mimidopropionate (Deriphat 160) (Henkel) or 0.03% DM. Electrophoresis was performed at 0°C with a gradual increase in the voltage as follows: 75 V for 30 min, 100 V for 30 min, 125 V for 30 min, 150 V for 1 h and 175 V for 30 min, followed by 200 V until the sample reached the end of the gel (total running time was approximately 4 h).

Separation of the subunits of the protein complexes in the second dimension

For two-dimensional separation, the strips from the first-dimension native-PAGE were excised and incubated with gentle shaking in Laemmli buffer [16] [138 mM Tris/HCl (pH 6.8), 6 M urea, 22.2% (v/v) glycerol, 4.3% (w/v) SDS and 5% (v/v) 2-mercaptoethanol] for 1 h at 20°C. After solubilization, the strips were transferred to the top of the SDS/PAGE gel [15% (w/v) polyacrylamide and 6 M urea] and sealed with 0.5% agarose in SDS/PAGE running buffer (25 mM Tris base, 190 mM glycine and 0.1% SDS) followed by separation of the protein subunits of the complexes. After electrophoresis, the proteins were visualized by silver [17] or Coomassie Blue staining.

RESULTS AND DISCUSSION

Typical patterns of the thylakoid membrane protein complexes, after solubilization with DM or digitonin and separation by BN-PAGE, are shown in Figure 1 (the identification of the protein complexes was based on [2,5]). With DM, the PSII and PSI complexes, together with various combinations of LHC, were resolved. LHClII also migrated as a trimer and monomer, without the Ps attached. Furthermore, the chloroplast NDH complex together with PSI, cytochrome b₆/f and the ATP synthase complexes were resolved. It is of note that when digitonin was used as a solubilization agent, a completely different pattern of thylakoid protein complexes was obtained. A distinct feature of digitonin-solubilized thylakoids was a PSI–LHClII megacomplex and a remarkable amount of thylakoid material, which either remained insoluble or did not penetrate into the separation gel, but instead stayed in the stacking gel. Moreover, an up-shift in the mobility of protein complexes was observed when the migration of digitonin-solubilized thylakoid membrane protein complexes was compared with those solubilized with DM.

Phosphorylation of the thylakoid membrane proteins

Since the phosphorylation status of the LHClII and PSII core proteins has been shown to affect the dynamics of the thylakoid membrane protein complexes [5,12,13], we applied a general protein phosphatase inhibitor, sodium fluoride, during both the thylakoid isolation and solubilization of the protein complexes. The snf7 kinase mutant, deficient in LHClII phosphorylation [12], and the snf7/snf8 double-kinase mutant, deficient in the PSII core and LHClII protein phosphorylation [13], were used as controls. Isolation and solubilization of the thylakoid membrane in the presence or absence of sodium fluoride had virtually no effect on the content of the most active form of PSII, the
Thylakoid membrane (8 μg of chlorophyll) solubilized with either 1 % (w/v) DM or 1 % (w/v) digitonin was subjected to BN-PAGE. PSI and PSII complexes, together with various combinations of LHClCs, as well as the chloroplast NDH, cytochrome (Cyt) b6f and the ATP synthase are resolved. Of these, PSI overlaps with the PSII dimer and cytochrome b6f overlaps with the PSI monomer. mc, megacomplex; sc, supercomplex.

PSII–LHCII supercomplex in native-PAGE (Figure 2a). In line with this result, the stn7/stn8 kinase mutant and the WT thylakoids revealed a similar content of the PSII–LHCII supercomplexes (Figure 2a). In contrast, the accumulation of the state-transition-specific PSI–LHCII megacomplex [5] in digitonin-solubilized thylakoid membrane was highly dependent on the presence of sodium fluoride in the thylakoid isolation buffers and was likewise absent from the stn7 kinase mutant (Figure 2b).

Impact of the detergent subtraction of the thylakoid membrane

Next, the impact of detergent subtraction of the thylakoid membrane on the integrity and distribution of the thylakoid protein complexes was tested. Thylakoid membrane was solubilized with digitonin and Triton X-100 followed by stepwise centrifugation steps to obtain the grana core, grana margins and stroma thylakoid membrane fractions as described previously [14]. The intact thylakoid membrane and the thylakoid membrane subtraction were then solubilized with DM and separated by two-dimensional BN/SDS/PAGE. As shown in Supplementary Figure S1 (at http://www.BiochemJ.org/bj/439/bj4390207add.htm), the PSII–LHCII supercomplexes, PSII dimers and the LHCII assembly complexes were concentrated in the grana core fraction, whereas the PSI–NDH megacomplex, PSI, ATP synthase, PSI monomer and the cytochrome b6f complex were strongly represented in the stroma thylakoid fraction. Grana margins, in turn, contained more of the PSI complex and PSII dimers and monomers, as well as the LHCII assembly complex, compared with the total thylakoid membrane (Supplementary Figure S1).

Optimal solubilization conditions of the thylakoid membrane depend on the purpose of research

The potential of three mild detergents, DM, TM and digitonin, to solubilize the thylakoid membrane was next tested. When DM or TM was used as a detergent, the PSII–LHCII supercomplexes, PSII dimer and monomer, as well as the PSI complex, ATP synthase, cytochrome b6f and PSI–NDH megacomplex were solubilized (Figure 3). When digitonin was used as a detergent, a low amount of the PSI complex and ATP synthase, as well as the PSII monomer and cytochrome b6f, were solubilized and separated from the PSI–LHCII megacomplex [5] in digitonin-solubilized thylakoid membrane (Figure 3). When digitonin was used as a detergent, a low amount of the PSI complex and ATP synthase, as well as the PSII monomer and cytochrome b6f, were solubilized and separated from the PSI–LHCII megacomplex [5] in digitonin-solubilized thylakoid membrane (Figure 3). In line with this, the proportion of the PSI–LHCII megacomplex was remarkably lower in digitonin-solubilized thylakoid membrane when using ACA buffer as compared with 25BTH20G buffer (Figure 3). Taken together, these results indicate that ACA buffer, in combination with each of the three non-ionic detergents, DM, TM or digitonin, enhanced the solubilization of the thylakoid membrane protein complexes at the expense of the integrity of supercomplexes as compared with 25BTH20G buffer (Figure 3). Therefore 25BTH20G buffer can be regarded to be superior to ACA buffer for preserving the integrity of the thylakoid membrane protein complexes.

To find out the best possible detergent concentration for solubilization of the thylakoid membrane for BN-PAGE, four different concentrations [0.5–2.0 % (w/v)] of DM or TM and five different concentrations [0.5–3.0 % (w/v)] of digitonin were tested in combination with 25BTH20G buffer. DM (or TM)
Thylakoid membranes (8 μg of chlorophyll) solubilized with 1% (w/v) TM, DM or digitonin in 25BTH20G or ACA buffer were subjected to BN-PAGE. Cytochrome; mc, megacomplex; sc, supercomplex.

at 0.5% was not sufficient to solubilize the thylakoid protein complexes, whereas 2% (w/v) DM (or TM) oversolubilized the thylakoid membrane, leading to a decrease in the amount of PSII–LHCII supercomplexes (Figure 4a). Instead, 1% (w/v) DM (or TM) resulted in efficient solubilization without disassembling the PSII–LHCII supercomplexes (Figure 4a). When the thylakoid membrane was solubilized either with 1% (w/v) DM or TM (in 25BTH20G buffer) and separated by two-dimensional BN/SDS/PAGE, no major difference in the solubilization pattern of the thylakoid membrane protein complexes was observed (Supplementary Figure S2 at http://www.BiochemJ.org/bj/439/bj4390207add.htm). TM has a longer aliphatic tail than DM, and in Chlamydomonas reinhardtii, TM has been observed to be more suitable for solubilizing the large thylakoid membrane protein complexes than was DM [19]. A slight up-shift in the migration of PSI and PSII complexes, as well as that of the LHCII trimer, was shown to be accompanied by a diminished amount of the LHCII assembly complex in BN-PAGE, when the thylakoid membrane solubilized with 1% (w/v) TM was compared with the thylakoid membrane solubilized with 1% (w/v) DM (Figure 4a). This suggests that TM either maintains more intact protein complexes compared with DM or, more probably, different amounts of lipids were attached to the complexes. It is important to note that both TM and DM solubilized the entire thylakoid membrane network (no insoluble material was recovered by centrifugation). This conclusion is strongly supported when the membrane protein complexes solubilized from the intact thylakoid membrane with DM (or TM) are compared with those individually solubilized from different thylakoid subfractions (grana core, grana margins or stroma thylakoids) (Supplementary Figures S1 and S2). Therefore both DM and TM allow the analysis of the protein complexes from the entire higher plant thylakoid membrane. Interestingly, the amount of the LHCII assembly complex (composed of Lhcb1, Lhc2, Lhc3 Lhc4.1, Lhc4.2 and Lhc6 [2]) and PSII–LHCII supercomplexes were directly and inversely proportional respectively to the amount of DM used for solubilization (Figure 4a). Thus the LHCII assembly complex, whose origin has not been studied previously, is likely to represent a dissociation product of the PSII–LHCII supercomplex generated during solubilization and/or the electrophoretic separation of protein complexes by native-PAGE.

With digitonin solubilization of the thylakoid membrane, it was found that 0.5% digitonin (in 25BTH20G buffer) was not sufficient to solubilize the membrane protein complexes, but 1–3% (w/v) digitonin efficiently solubilized the membrane protein complexes, with 1% (w/v) digitonin being optimal (Figure 4b).

A distinct feature of digitonin-solubilized thylakoids was a high quantity of PSI (Figure 4b). When the pellet of 1.0% (w/v) digitonin sample was further solubilized with 1.0% (w/v) DM before BN-PAGE, the various thylakoid membrane protein complexes were observed in high quantities, indicating that a large proportion of thylakoid membrane remained insoluble during digitonin treatment (Figure 4b, rightmost lane). The efficiency of digitonin for solubilizing preferably PSI has been demonstrated previously [20], and is likely to be due to the bulky structure of the digitonin molecules, which hinders the penetration of digitonin to the dense-packed grana stacks [21]. Taken together these results indicate that the solubilization of the thylakoid membrane with digitonin is extremely selective and mostly targets the complexes residing in the stroma thylakoids and grana margins. Therefore digitonin is highly suitable for analysis of the PSII–LHCII and other labile megacomplexes in stroma thylakoids or grana margins, but not appropriate for quantitative analysis of higher plant thylakoid protein complexes. This is in line with previous studies showing that digitonin is capable of maintaining weak protein–protein interactions [21,22].

**Specialties of CN-PAGE in separation of thylakoid membrane protein complexes**

Prior to BN-PAGE, CN-PAGE was used as a predominant gel-based separation method of protein complexes. The advantage of
CN-PAGE compared with BN-PAGE lies in its better suitability for in-gel fluorescence detection and catalytic activity assays [23]. However, compared with BN-PAGE, CN-PAGE has been postulated to suffer from aggregation and broadening of protein bands during electrophoresis. An improved CN-PAGE method has been introduced to increase the electrophoretic mobility of mitochondrial protein complexes by supplementing the cathode buffer with the mild anionic detergent DOC [23].

Since the solubilization pattern of the thylakoid membrane protein complexes was highly similar using either DM or TM as a detergent in BN-PAGE analysis, only two detergents, DM and digitonin, were optimized for CN-PAGE analysis. The capacity of DOC, and also of a zwitterionic detergent Deriphat 160 [9,24] to introduce a charge shift to the thylakoid membrane protein complexes was tested first. Either 0.1 % Deriphat 160 or 0.05 % DOC, together with 0.02 % DM, was added to the cathode buffer of CN-PAGE. While present in the cathode buffer of CN-PAGE, both DOC and Deriphat 160 improved the electrophoretic mobility of thylakoid membrane protein complexes (Figure 5a and Supplementary Figure S3 at http://www.BiochemJ.org/bj/439/bj4390207add.htm).

As a control, the cathode buffer was supplemented only with 0.03 % DM in order to decrease aggregation, but not introducing a charge shift to the protein complexes. The resolution of the CN-PAGE system without addition of external charge was inferior to that of the samples treated with DOC or Deriphat 160 (Figure 5a). Nevertheless, the two-dimensional separation of the thylakoid membrane protein complexes revealed that, in particular, the separation of ATP synthase and its subunits was markedly improved in the absence of agents introducing the charge shift (Supplementary Figure S3).

Moreover, addition of an external charge during native PAGE had a major impact on the heterogeneity of the PSII complexes. In the presence of Coomassie Blue dye, DOC or Deriphat 160, the PSII complexes separated as PSII–LHCII supercomplexes, PSII dimers and PSII monomers (Figure 1 and Supplementary Figure S3). However, in the absence of these substances, the PSII complexes were present mostly as monomers (Supplementary Figure S3). This result raised an interesting question concerning the relevance of high-molecular-mass PSII complexes in vivo. Indeed, a recent publication demonstrated that in cyanobacteria the monomeric PSII is converted into a dimeric form after treatment with 5 % DM, and it was speculated that in cyanobacteria the native form of PSII could be a monomer instead of a dimer [25]. In higher plants, however, the biophysical studies recording the activity of PSII in different subfractions of the thylakoid membrane isolated without detergents [1] strongly support the existing understanding of the higher oligomeric forms of PSII. In accordance with this, the accumulation of the high-molecular-mass PSII complexes was observed in the native-PAGE separation of grana thylakoid membrane protein complexes as compared with the total thylakoid membrane sample, in both the presence and the absence of external charge (E.-M. Aro, S. Järvi and M. Suorsa, unpublished work). Therefore we conclude that the presence of external charge is important for the integrity of PSII complexes during native-PAGE. To finally solve the formation of thylakoid membrane supercomplexes in vivo, the role of thylakoid lipids should be thoroughly investigated.

The effect of the two solubilization buffers on the integrity of protein complexes was next studied using the 25BTH20G and ACA buffers. Similar to BN-PAGE, in CN-PAGE also the 25BTH20G buffer appeared to be better for the integrity of the protein complexes as compared with the ACA buffer. The proportion of the PSII–LHCII supercomplexes was again remarkably higher, whereas the proportions of PSII monomer and LHCII assembly complex were remarkably lower when using 25BTH20G buffer compared with ACA buffer (Figure 5a).

In order to improve the mobility of the protein complexes, the thylakoid samples were supplemented with 0–0.5 % DOC prior to separation with CN-PAGE. When using DM as a detergent, the addition of 0.1–0.5 % DOC was found to increase the amount of PSII–LHCII supercomplexes, decrease the amount of PSII monomers and in general sharpen the separation of the protein complexes in CN-PAGE, as compared with conditions where DOC was present only in the cathode buffer (Figure 5b).

Instead, addition of DOC into digitonin-solubilized thylakoid sample prior to electrophoresis did not have any impact on the thylakoid membrane protein complexes, except that the LHCII trimer migrated faster with an increasing concentration of DOC (Figure 5c). This is in line with an early experiment showing that the presence of DOC shifted hydrophobic proteins anodally [8].
Figure 6 Comparison of two-dimensional CN and BN/SDS-PAGE analyses of Arabidopsis thylakoid membrane protein complexes

Thylakoid membrane (8 μg of chlorophyll) was solubilized either with 1 % (w/v) DM or 1 % (w/v) digitonin in 25BTH20G buffer prior to CN-PAGE and BN-PAGE. For CN-PAGE, the samples were supplemented with 0.3 % DOC and the cathode buffer with 0.02 % DM and 0.05 % DOC. Native-PAGE was followed by separation of protein complexes in the second dimension by SDS/PAGE and silver staining. The molecular mass in kDa is indicated. cyt, cytochrome; mc, megacomplex; sc, supercomplex.

Comparison of two-dimensional CN-PAGE and BN-PAGE in resolution of thylakoid membrane protein complexes

The resolution capacity of the protein complexes by two-dimensional CN-PAGE and BN-PAGE was compared after solubilization of the thylakoid membrane with DM and digitonin. Based on optimization, the thylakoid membrane was solubilized with 1 % (w/v) DM or digitonin in 25BTH20G buffer. Before loading, the CN-PAGE sample was supplemented with 0.3 % DOC. After the first-dimension electrophoresis, the strips were excised and incubated in Laemmli buffer containing 5 % (v/v) 2-mercaptoethanol for 1 h at 20°C. Subsequently, the strips were transferred to the top of the SDS/PAGE gel, with samples that were solubilized with DM and digitonin side by side, and the subunits of the protein complexes were separated in the second dimension and stained with silver nitrate. No major difference between the CN-PAGE and BN-PAGE separation of the thylakoid membrane protein complexes was recorded by this optimized method (Figure 6). Intriguingly, an unknown high-molecular-mass megacomplex was observed after digitonin solubilization.

Figure 7 lpBN-PAGE of Arabidopsis thylakoid membrane protein complexes

Thylakoid membrane (8 μg of chlorophyll) was solubilized with 1 % (w/v) DM or 1 % (w/v) digitonin in 25BTH20G buffer and the protein complexes were separated by lpBN-PAGE. The molecular mass in kDa is indicated. Cyt, cytochrome; mc, megacomplex; sc, supercomplex.

lpBN-PAGE is superior in separation of thylakoid membrane megacomplexes

In order to characterize the megacomplex described above (Figure 6, right-hand side), a method suitable for separation of high-molecular-mass megacomplexes from mitochondria [26] was optimized for separation of thylakoid membrane megacomplexes and named lpBN-PAGE. In the lpBN-PAGE method, the increased pore size of the stacking gel enabled superior migration of the high-molecular-mass protein complexes as compared with the normal BN-PAGE (Figures 1 and 7). Optimal separation of the thylakoid membrane protein complexes by lpBN-PAGE was obtained by using an acrylamide gradient of 3.5–12.5 % (w/v) T and 3 % (w/v) C in the separation gel, and 3 % (w/v) T and 20 % (w/v) C in the stacking gel (for explanations of T and C, see the Experimental section). Such lpBN-PAGE analysis of Arabidopsis thylakoid membrane protein complexes considerably improved the separation of the high-molecular-mass protein complexes, such as the PSI-NDH megacomplex, as compared with conventional BN-PAGE (Figures 1 and 7). Moreover, whereas a remarkable proportion of the 1 % (w/v) digitonin-solubilized thylakoid membrane proteins remained in the stacking gel of the conventional BN-PAGE (Figure 1), lpBN-PAGE allowed all of the material to enter the separation gel (Figure 7).

Intriguingly, not only one, but instead several super- and mega-complexes of ≥1000 kDa were resolved from thylakoid membrane when digitonin or DM was used for solubilization (Figure 7). In order to identify the novel high-molecular-mass thylakoid megacomplexes, the thylakoid membrane protein complexes were subjected to two-dimensional lpBN/SDS-PAGE.
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Figure 8 Two-dimensional lpBN-PAGE analysis of Arabidopsis thylakoid membrane protein complexes solubilized with 1% (w/v) DM

Thylakoid membrane (8 μg of chlorophyll) was solubilized with 1% (w/v) DM and the protein complexes were separated by lpBN-PAGE. Native-PAGE was followed by separation of protein complexes in the second dimension by SDS/PAGE and silver staining. Assignment of the distinct subunits of the thylakoid membrane protein complexes is based on [2]. The molecular mass in kDa is indicated. Cyt, cytochrome; mc, megacomplex; sc, supercomplex.

Based on analysis, Mc1b, Sc4b and Mc5b were of high abundance, whereas megacomplexes 1a, 2a, 3a, 2b and 3b were present in lower abundance (Figures 8 and 9). The analysis of the protein composition of the megacomplexes of 1% (w/v) DM-solubilized thylakoid membrane revealed at least three novel super- and mega-complexes in addition to the PSI–NDH megacomplex identified previously (Figure 8). On the basis of their subunit composition, two of these represented PSI megacomplexes (Mc1a and Mc3a) and one PSI–LHCII supercomplex (Sc2a) (Figure 8). This result implied that like cyanobacterial PSI [27], higher plant PSI (600 kDa) might also be present as a trimer (1.8 MDa) and a megacomplex (>2.4 MDa) in vivo. The high-molecular-mass PSII–LHCII supercomplex (Sc2a), in turn, probably represents the high-molecular-mass PSII–LHCII supercomplex observed previously by sucrose-density-gradient and single-particle analysis [28].

The most striking results were obtained by lpBN-PAGE when the thylakoid membrane was solubilized by 1% (w/v) digitonin, which is capable of maintaining weak interactions between protein complexes. Indeed, five novel super- and mega-complexes (Figure 9), in addition to the state-transition-specific PSI–LHCII [5], were resolved and these megacomplexes are apparently located in the stroma membrane and grana margins in vivo. Two of the novel complexes represent PSI–LHCII megacomplexes (Mc3b and Mc5b) and one is a PSII–LHCII supercomplex (Sc4b). Since an up-shift in the mobility of protein complexes was observed when migration of digitonin-solubilized thylakoid membrane protein complexes was compared with DM (Figure 1), it is likely that Sc4b represents a PSII–LHCII supercomplex localized to the grana margins and is thus prone to solubilization by digitonin. Most interestingly, the PSI and PSII complexes were for the first time shown to migrate together (in two different complexes, Mc1b and Mc2b), hence providing evidence that the PSI and PSII complexes probably interact with each other in grana margins. This observation supports the theories on state transitions and interaction of the PSII–LHCII–PSI complexes in grana margins of higher plant thylakoids [29,30].

Conclusions

Taken together, the solubilization of the thylakoid membrane with DM or TM gives information of the entire thylakoid membrane, whereas digitonin solubilization gives information only on protein complexes in the stroma lamellae and also in grana margins. Three gel-based methods for separation of higher plant thylakoid membrane protein complexes, BN-, CN- and lpBN-PAGE, were optimized (Table 1). In particular the lpBN-PAGE method, suitable for separation of novel high-molecular-mass megacomplexes, was shown to provide an invaluable tool to analyse the dynamics of the thylakoid membrane complexes upon changing environmental and metabolic cues.

AUTHOR CONTRIBUTION

Sari Järvi and Marianna Suorsa designed and performed experiments, analysed data and wrote the paper. Virpi Paakkarinen designed and performed experiments and
analysed data. Eva-Mari Aro designed and supervised experiments, and wrote the paper.

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REFERENCES


Table 1

<table>
<thead>
<tr>
<th>Thylakoid membrane protein complex</th>
<th>Detergent</th>
<th>Gel system</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSI</td>
<td>1 % (w/v) DM/TM</td>
<td>BN/CN + 0.3 % (w/v) DOC/lpBN</td>
</tr>
<tr>
<td>Cyt b6f</td>
<td>1 % (w/v)</td>
<td>BN/CN + 0.3 % (w/v) DOC/lpBN</td>
</tr>
<tr>
<td>LHCII assembly</td>
<td>1 % (w/v) DM/TM/digitonin</td>
<td>CN without an additional charge</td>
</tr>
<tr>
<td>LHCII trimer</td>
<td>1 % (w/v) DM/TM</td>
<td>BN/CN + 0.3 % (w/v) DOC/lpBN</td>
</tr>
<tr>
<td>LHCII monomer</td>
<td>1 % (w/v) DM/TM</td>
<td>BN/CN + 0.3 % (w/v) DOC/lpBN</td>
</tr>
<tr>
<td>PSI–NDH mc</td>
<td>1 % (w/v) DM/TM</td>
<td>lpgBN</td>
</tr>
<tr>
<td>PSI–LHCII mc</td>
<td>1 % (w/v) digitonin</td>
<td>lpgBN</td>
</tr>
<tr>
<td>Megacomplexes</td>
<td>1 % (w/v) DM/TM/digitonin</td>
<td>lpgBN</td>
</tr>
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</table>

Optimal detergent and gel system for thylakoid membrane protein complexes are shown. The optimal solubilization buffer in all cases was found to be 25BTH20G. Cyt, cytochrome; mc, megacomplex; NDH, NAD(P)H dehydrogenase.
SUPPLEMENTARY ONLINE DATA
Optimized native gel systems for separation of thylakoid protein complexes: novel super- and mega-complexes

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Department of Biochemistry and Food Chemistry, Molecular Plant Biology, University of Turku, FI-20014 Turku, Finland

Figure S1 Two-dimensional BN/SDS/PAGE analysis of protein complexes from Arabidopsis thylakoid membrane and thylakoid subfractions

Thylakoid membrane was fractionated into grana margins, grana core and stroma thylakoid membrane with digitonin and Triton X-100. Thylakoid fractions (20 μg of chlorophyll) were solubilized with 1% (w/v) DM in 25BTH20G buffer, and subjected to two-dimensional BN/SDS/PAGE followed by silver staining. The most abundant thylakoid membrane protein complexes in each fraction are marked in bold. The molecular mass in kDa is indicated. Cyt, cytochrome; mc, megacomplex; sc, supercomplex.

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Thylakoid membrane protein complexes (8 μg of chlorophyll) were subjected to two-dimensional BN/SDS/PAGE followed by silver staining. The molecular mass in kDa is indicated. Cyt, cytochrome; mc, megacomplex; sc, supercomplex.

Thylakoid membrane (8 μg of chlorophyll) was solubilized with 1 % (w/v) DM in 25BTH20G buffer. CN-PAGE cathode buffer was supplemented either with 0.03 % DM, 0.02 % DM together with 0.05 % DOC, or 0.1 % disodium N-dodecyl-β-iminodipropionate (Deriphat 160) (see also Figure 5a of the main text). CN-PAGE was followed by separation of protein complexes in the second dimension by SDS/PAGE and silver staining. The molecular mass in kDa is indicated. Cyt, cytochrome; sc, supercomplex.