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Running title: Analysis of thylakoid membrane protein complexes by native gel electrophoresis.

Abstract

Gel-based analysis of thylakoid membrane protein complexes represents a valuable tool to monitor the dynamics of the photosynthetic machinery. The native polyacrylamide gel electrophoresis (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 due to a great variety of native-PAGE and thylakoid solubilization systems. Here, we describe optimized methods for separation of higher plant thylakoid membrane protein complexes by native-PAGE addressing particularly (*i*) the use of detergent and (*ii*) solubilization buffer as well as (*iii*) the gel electrophoresis method. Special attention is paid on separation of high molecular mass thylakoid membrane super- and megacomplexes from *Arabidopsis thaliana* leaves. Several novel super- and megacomplexes including the photosystem (PS)I, PSII and light harvesting complexes (LHC) in various combinations are reported.

Keywords: *Arabidopsis*, blue native gel electrophoresis, clear native gel electrophoresis, light harvesting complex, photosystem, thylakoid megacomplex

Abbreviations: ACA, amino-n-caproic acid; BN, blue native; C, concentration of the cross-linker *bis*-acrylamide relative to the total monomer concentration; CN, clear native; cyt, cytochrome; 2D, two-dimensional; Deriphat 160, disodium N-lauryl-β-iminodipropionate; DM, n-dodecyl β-D-maltoside; DOC, sodium deoxycholate; LHC, light harvesting complex; lpBN, large pore blue native; NaF, sodium fluoride; NDH, NAD(P)H dehydrogenase, PAGE, polyacrylamide gel electrophoresis; PS, photosystem; SDS, sodium dodecyl sulfate; T, total concentration of both acrylamide and *bis*-acrylamide monomers; TM, n-tridecyl-β-D-maltoside; WT, wild type

1. Introduction

Great diversity in the composition of the protein complexes is a distinct feature of the thylakoid membrane. Photosystem (PS)II and PSI supercomplexes and subcomplexes are involved in *(i)* the linear and cyclic electron transfer, *(ii)* dynamics of light absorption as well as *(iii)* the repair cycle of PSII. The high molecular mass supercomplexes composed of the PSII dimer and the light harvesting complex (LHC)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 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]. Recently, two high molecular mass PSI megacomplexes, PSI-LHCII and PSI-NAD(P)H dehydrogenase (NDH), 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 polyacrylamide gel electrophoresis (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 sodium dodecyl sulphate (SDS), sodium deoxycholate (DOC) or zwitterionic detergent disodium N-lauryl-β-iminodipropionate (Deriphat 160) have been applied to improve the resolution of proteins and/or protein complexes [7-9]. Since 1991, blue native gel electrophoresis (BN-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 to the maintenance of the integrity of protein complexes. First analysis of thylakoid membrane protein complexes using BN-PAGE was published in 1997 [11] and since then this method has been routinely used in plant science [2]. Prior to native-PAGE, thylakoid membrane is solubilized with a low concentration of a mild non-ionic detergent, such as dodecyl maltoside (DM), tridecyl maltoside (TM), digitonin or Triton X-100. For two-dimensional (2D) 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 this paper we describe optimized methods for isolation and separation of thylakoid membrane protein complexes by clear native (CN), BN and large pore blue native (lpBN)-PAGE. We have addressed the effects of (*i*) selected detergents, (*ii*) solubilization buffers and *(iii)* gel electrophoresis method, and show that all 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 retaining the protein complexes as intact as possible. By these optimized methods we have identified several novel thylakoid membrane megacomplexes.

2. Experimental

2.1. Plant material

Arabidopsis thaliana ecotype Columbia (hereafter *Arabidopsis*) wild type (WT) plants as well as the *stn7* [12] and *stn7/8* [13] mutant lines were grown under a photon flux density of 120 μmol photons $m^{-2}s^{-1}$ in 8h light regime at 23ºC.

2.2. Preparation of native-PAGE gels

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

2.3. 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 $MgCl_2$, 5 mM ascorbate, 0.05% (w/v) bovine serum albumin, ± 10 mM sodium fluoride (NaF)). Suspension was filtered through two layers of Miracloth followed by centrifugation at 5000 *g* at 4°C for 4 min. Pellet was resuspended in a shock buffer (50 mM Hepes-KOH, pH 7.5, 5 mM sorbitol, 5 mM $MgCl_2$, ± 10 mM NaF), 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, 10 mM $MgCl_2$, ± 10 mM NaF) 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 in [14], except that the samples were shaken for 8.5 min at 20°C after

adding of digitonin. 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 25BTH20G (25 mM BisTris-HCl, pH 7.0, 20% (w/v) glycerol and 0.25 mg ml^{-1} Pefabloc) or amino-n-caproic acid (ACA, Sigma A-7824) (50 mM BisTris-HCl, pH 7.0, 750 mM ACA, 1 mM EDTA, 0.25 mg ml^{-1} Pefabloc) buffer with or without 10 mM NaF, to a chlorophyll concentration of 1.0 mg/mL. An equal volume of detergent solution (diluted in 25BTH20G or ACA buffer) was added to the final concentration of 0.5 – 2.0% (w/v) for both n-dodecyl β-D-maltoside (DM) (Sigma D-4641) and n-tridecyl-β-D-maltoside (TM) (Affymetrix T323) or 0.5 – 3.0% (w/v) for digitonin (Calbiochem 300410). 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 sodium deoxycholate (DOC) (Sigma D5670, final concentration of 0 – 0.5% (w/v)) and the BN-PAGE samples were supplemented with 1/10 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 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.

2.4. 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 gel, the cathode buffer contained Serva Blue G dye (50 mM Tricine, 15 mM BisTris-HCl, pH 7.0, 0.01% (w/v) Serva Blue G) and for CN-PAGE, the cathode buffer contained aliquot of DOC and DM (50 mM Tricine, 15 mM BisTris-HCl, pH 7.0, 0.05% (w/v) DOC, 0.02% (w/v) DM). For certain experiments, the CN-PAGE cathode buffer was supplemented with 0.1% (w/v) disodium N-lauryl-β-iminodipropionate (Deriphat 160) (Henkel) or 0.03% (w/v) DM. Electrophoresis was performed at 0°C with gradually increasing the voltage as follows: 75 V for 30 min, 100 V for 30 min, 125 V for 30 min, 150 V for 1 h, 175 V for 30 min, followed by 200 V until the sample reached the end of the gel (total running time was approximately 4h).

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

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

complexes. After electrophoresis, the proteins were visualized by silver [17] or Coomassie staining.

3. 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. LHCII also migrated as a trimer and monomer, without the photosystems attached. Furthermore, the chloroplast NDH complex together with PSI, the cytochrome (cyt) *b6f* and the ATP synthase complexes were resolved. Noteworthy, when digitonin was used as a solubilization agent, completely different pattern of thylakoid protein complexes was obtained. A distinct feature of digitonin solubilized thylakoids was a PSI-LHCII 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 to those solubilized with DM.

3.1. Phosphorylation of the thylakoid membrane proteins

Since the phosphorylation status of the LHCII 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 NaF during both the thylakoid isolation and solubilization of the protein complexes. The *stn7* kinase mutant, deficient of LHCII phosphorylation [12] and the *stn7/stn8* double kinase mutant, deficient of the PSII core and the LHCII protein phosphorylation [13] were used as controls. Isolation and solubilization of the thylakoid membrane in the presence or absence of NaF had virtually no effect on the content of the most active form of PSII, the PSII-LHCII supercomplex in native-PAGE (Figure 2a). In line with this result, the *stn7/8* kinase mutant and the WT thylakoids revealed a similar content of the PSII-LHCII supercomplexes (Figure 2a). On the contrary, the accumulation of the state transition specific PSI-LHCII megacomplex [5] in digitonin solubilized thylakoid membrane was highly dependent on the presence of NaF in the thylakoid isolation buffers and was likewise absent from the *stn7* kinase mutant (Figure 2b).

3.2. Impact of the detergent subfractionation of the thylakoid membrane

Next, the impact of detergent subfractionation 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 centrifugations to obtain the grana core, grana margins and stroma thylakoid membrane fractions as described in [14]. The intact thylakoid membrane and the thylakoid membrane subfractions were then solubilized with DM and separated by

2D BN/SDS-PAGE. As shown in Supplementary Figure 1, 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, PSII monomer and the cyt *b6f* complex were strongly represented in the stroma thylakoid fraction. Grana margins, in turn, contained more of PSI complex, PSII dimers and monomers as well as the LHCII assembly complex compared to the total thylakoid membrane (Supplementary Figure 1).

3.3. 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 tested next. 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, cyt *b6f* and PSI-NDH megacomplex were solubilized (Figure 3). When digitonin was used as a detergent, low amount of the PSI complex and ATP synthase as well as the PSII monomer and cyt *b6f* were solubilized and separated (Figure 3). In addition, the PSI-LHCII megacomplex was identified from digitonin solubilized thylakoid membrane (Figure 3).

Next, the effect of the thylakoid solubilization buffer on the integrity of protein complexes in the BN-PAGE gel was tested. Two commonly used solubilization buffers, 25BTH20G [18] and ACA [10], in combination with all three detergents were tested. The use of the ACA buffer substantially decreased the proportion of the PSII-LHCII supercomplexes in DM (or TM) solubilized thylakoid membrane as compared to 25BTH20G buffer (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 to 25BTH20G buffer (Figure 3). Taking together, ACA buffer, in combination with 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 to 25BTH20G buffer (Figure 3). Therefore, 25BTH20G buffer can be regarded 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. 0.5% (w/v) DM (or TM) was not sufficient to solubilize the thylakoid protein complexes, while 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 2D BN/SDS-PAGE, no major difference in the solubilization pattern of the thylakoid membrane

protein complexes was observed (Supplementary Figure 2). TM has 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 DM [19]. A slight up-shift in migration of PSI and PSII complexes as well as of the LHCII trimer was shown to be accompanied with diminished amount of the LHCII assembly complex in BN-PAGE, when the thylakoid membrane solubilized with 1% (w/v) TM was compared to the thylakoid membrane solubilized with 1% (w/v) DM (Figure 4a). This suggests that TM either maintains protein complexes more intact compared to DM or, more probably, different amount 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, stroma thylakoids) (Supplementary Figure 1, 2). 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, Lhcb2, Lhcb3 Lhcb4.1, Lhcb4.2, and Lhcb6 [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 before, 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% (w/v) digitonin (in 25BTH20G buffer) was not sufficient but 1.0–3.0% (w/v) digitonin efficiently solubilized the membrane protein complexes, 1.0% (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% (w/v) digitonin sample was further solubilized with 1% (w/v) DM and the protein complexes were separated by 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, last lane). The efficiency of digitonin for solubilizing preferably PSI has been demonstrated previously [20], and is likely due to the bulky structure of the digitonin molecules, which hinders the penetration of digitonin to the dense-packed grana stacks [21]. Taking together, 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 PSI-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 earlier studies showing that digitonin is capable of maintaining weak protein-protein interactions [21,22].

3.4. Specialities 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 to BN-PAGE lays in its better suitability for in-gel fluorescence detection and catalytic activity assays [23]. However, compared to BN-PAGE, CN-PAGE has been postulated to suffer from aggregation and broadening of protein bands during electrophoresis. Recently, an improved CN-PAGE method was 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% (w/v) Deriphat 160 or 0.05% (w/v) DOC together with 0.02% (w/v) DM was added into 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, Supplementary Figure 3).

As a control, the cathode buffer was supplemented only with 0.03% (w/v) 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 2D separation of the thylakoid membrane protein complexes revealed that especially the separation of the ATP synthase and its subunits was markedly improved in the absence of agents introducing the charge shift (Supplementary Figure 3).

Moreover, addition of external charge during native-PAGE had a major impact on the heterogeneity of the PSII complexes. In the presence of Coomassie dye, DOC or Deriphat 160, the PSII complexes separated as PSII-LHCII supercomplexes, PSII dimers and PSII monomers (Figure 1, Supplementary Figure 3). However, in absence of these substances, the PSII complexes were present mostly as monomers (Supplementary Figure 3). 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 to 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 the grana thylakoid membrane protein complexes as compared to the total thylakoid membrane sample, both in the presence and absence of external charge (unpublished results). 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 studied next using the 25BTH20G and ACA buffers. Similarly to BN-PAGE, also in CN-PAGE the 25BTH20G buffer appeared to be better for the integrity of the protein complexes as compared to 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 to ACA buffer (Figure 5a).

In order to improve the mobility of the protein complexes, the thylakoid samples were supplemented with 0 – 0.5% (w/v) of DOC prior to separation with the CN-PAGE. When using DM as a detergent, the addition of 0.1 - 0.5% (w/v) DOC was found to increase the amount of PSII-LHCII supercomplexes, decrease the amount of PSII monomers and in general to sharpen the separation of the protein complexes in CN-PAGE, as compared to 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].

3.5. Comparison of 2D CN-PAGE and BN-PAGE in resolution of thylakoid membrane protein complexes

The resolution capacity of the protein complexes by 2D 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% (w/v) 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 on the top of the SDS-PAGE gel, samples solubilized with DM and digitonin side by side, and the subunits of the protein complexes were separated in 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 6, right panels).

3.6. lpBN-PAGE is superior in separation of thylakoid membrane megacomplexes

In order to characterize the megacomplex described above (Figure 6, right panels), a method suitable for separation of high molecular mass megacomplexes from mitochondria [26] was optimized for separation of thylakoid membrane megacomplexes and named as large pore blue native (lpBN)-PAGE. In lpBN-PAGE method, the increased pore size of the stacking gel enabled superior migration of the high molecular mass protein complexes as compared to the normal BN-PAGE (Figures 1 and 7). Optimal separation of the thylakoid membrane protein complexes by lpBN-PAGE was obtained by using acrylamide gradient of 3.5-12.5% (w/v) T, 3% (w/v) C in the separation gel and 3% (w/v) T, 20% (w/v) C in the stacking gel (for T and C, see experimental section). Such lpBN-PAGE analysis of *Arabidopsis* thylakoid membrane protein complexes considerably improved the separation of the high molecular mass protein complexes, like the PSI-NDH megacomplex, as compared to the conventional BN-PAGE (Figure 7, Figure 1). Moreover, whereas 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 material to enter to the separation gel (Figure 7).

Intriguingly, not only one but instead several super- and megacomplexes 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 2D lpBN/SDS-PAGE. Based on analysis, Mc1b, Sc4b and Mc5b were of high abundance (bold) 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 megacomplexes in addition to the PSI-NDH megacomplex identified previously (Figure 8). Based on their subunit composition, two of these represented PSI megacomplexes (Mc1a, 3a) and one PSII-LHCII supercomplex (Sc2a) (Figure 8). This result implicated that likewise cyanobacterial PSI [27] also higher plant PSI (600 KDa) might 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, likely represents high molecular mass PSII-LHCII supercomplex observed previously by sucrose density gradient and single particle analysis [28].

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 megacomplexes (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 megacomplex (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 to 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 recent theories on state transitions and interaction of the PSII-LHCII-PSI complexes in grana margins of higher plant thylakoids [29,30].

3.7. 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). Particularly the lpBN-PAGE method, suitable for separation of novel high molecular mass megacomplexes, was shown to provide an invaluable tool to analyze the dynamics of the thylakoid membrane complexes upon changing environmental and metabolic cues.

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Figures and tables

Table 1. Optimized conditions for analysis of different thylakoid membrane protein complexes in *Arabidopsis* by native gel electrophoresis systems.
Optimal detergent and gel system for thylakoid membrane protein complexes are shown. Optimal solublization buffer in all cases was found to be 25BTH20G. Blue native (BN), clear native (CN), cytochrome (cyt), dodecyl maltoside (DM), light harvesting complexes (LHC), large pore blue native (lpBN), megacomplex (mc), NAD(P)H dehydrogenase (NDH), Photosystem (PS), sodium deoxycholate (DOC).

Thylakoid membrane protein complex	Detergent	Gel system
PSII	1% (w/v) DM/TM	BN / CN+0.3% (w/v) DOC / lpBN
Cyt *b6f*	1% (w/v) DM/TM/digitonin	BN / CN+0.3% (w/v) DOC / lpBN
PSI	1% (w/v) DM/TM	BN / CN+0.3% (w/v) DOC / lpBN
ATPase	1% (w/v) DM/TM	CN without an additional charge
LHCII assembly	1% (w/v) DM/TM	BN / CN+0.3% (w/v) DOC / lpBN
LHCII trimer	1% (w/v) DM/TM	BN / CN+0.3% (w/v) DOC / lpBN
LHCII monomer	1% (w/v) DM/TM	BN / CN+0.3% (w/v) DOC / lpBN
PSI-NDH mc	1% (w/v) DM/TM	lpBN
PSI-LHCII mc	1% (w/v) digitonin	lpBN
Megacomplexes	1% (w/v) DM/TM/digitonin	lpBN

Figure 1. BN-PAGE analysis of *Arabidopsis* thylakoid membrane protein complexes.
Thylakoid membrane (8 µg Chl) solubilized with either 1% (w/v) dodecyl maltoside (DM) or 1% (w/v) digitonin was subjected to BN-PAGE. Photosystem (PS) II and I complexes together with various combinations of light harvesting complexes (LHC), as well as the chloroplast NAD(P)H dehydrogenase (NDH), cytochrome (cyt) *b6f* and the ATP synthase are resolved. Of these, PSI overlaps with PSII dimer and cyt *b6f* overlaps with PSII monomer. Megacomplex (mc), supercomplex (sc).

Figure 2. BN-PAGE analysis of *Arabidopsis* thylakoid membrane protein complexes in the presence or absence of sodium fluoride (NaF).
Thylakoid membrane (8 µg Chl) solubilized with either (a) 1% (w/v) dodecyl maltoside (DM) or (b) 1% (w/v) digitonin, was subjected to BN-PAGE separation. In addition to the thylakoid membrane protein composition of WT plants, also those of the *stn7* kinase mutant, deficient in LHCII protein phosphorylation and the *stn7/stn8* kinase mutant, deficient in both LHCII and PSII core protein phosphorylation were analyzed. Cytochrome (cyt), light harvesting complexes (LHC), megacomplex (mc), NAD(P)H dehydrogenase (NDH), Photosystem (PS), supercomplex (sc).

Figure 3. BN-PAGE analysis of *Arabidopsis* thylakoid membrane protein complexes solubilized in 25BTH20G or ACA buffer.
Thylakoid membranes (8 µg Chl) solubilized with 1% (w/v) tridecyl maltoside (TM), dodecyl maltoside (DM) or digitonin in 25BTH20G or ACA buffer were subjected to BN-PAGE. Cytochrome (cyt), light harvesting complexes (LHC), megacomplex (mc), NAD(P)H dehydrogenase (NDH), Photosystem (PS), supercomplex (sc).

Figure 4. BN-PAGE analysis of *Arabidopsis* thylakoid membrane protein complexes solubilized with different concentrations of dodecyl maltoside (DM), tridecyl maltoside (TM) and digitonin in 25BTH20G buffer.
(a) Thylakoid membrane (8 µg Chl) was solubilized with 0.5 – 2.0% (w/v) DM or TM. (b) Thylakoid membrane (8 µg Chl) was solubilized with 0.5 – 3.0% (w/v) digitonin (lanes 1-5). The pellet from 1.0% (w/v) digitonin sample was further solubilized with 1.0% (w/v) DM before BN-PAGE (b, lane 6). Cytochrome (cyt), light harvesting complexes (LHC), megacomplex (mc), NAD(P)H dehydrogenase (NDH), Photosystem (PS), supercomplex (sc).

Figure 5. CN-PAGE analysis of *Arabidopsis* thylakoid membrane protein complexes.
(a) Thylakoid membrane (8 µg Chl) was solubilized with 1% (w/v) dodecyl maltoside (DM) in 25BTH20G (BTH) or ACA buffer. Cathode buffer was supplemented either with 0.03% (w/v) DM, 0.02% (w/v) DM together with 0.05% (w/v) sodium deoxycholate (DOC) or 0.1% (w/v) disodium N-lauryl-β-iminodipropionate (Deriphat) and the protein complexes were separated by CN-PAGE. (b) Thylakoid membrane (8 µg Chl) was solubilized with 1% (w/v) DM in 25BTH20G buffer and samples were supplemented with 0 – 0.5% (w/v) DOC. Cathode buffer was supplemented with 0.02% (w/v) DM and 0.05% (w/v) DOC and the protein complexes were separated by CN-PAGE. (c) Thylakoid membrane (8 µg Chl) was solubilized with 1% (w/v) digitonin in 25BTH20G buffer and the samples were supplemented with 0 – 0.5% (w/v) DOC. Cathode buffer was supplemented with 0.02% (w/v) DM and 0.05% (w/v) DOC and the protein complexes were separated by CN-PAGE. Cytochrome (cyt), light harvesting

complexes (LHC), megacomplex (mc), NAD(P)H dehydrogenase (NDH), Photosystem (PS), supercomplex (sc).

Figure 6. Comparison of 2D CN and BN/SDS-PAGE analyses of *Arabidopsis* thylakoid membrane protein complexes.
Thylakoid membrane (8 µg Chl) was solubilized either with 1% (w/v) dodecyl maltoside (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% (w/v) sodium deoxycholate (DOC) and the cathode buffer with 0.02% (w/v) DM and 0.05% (w/v) DOC. Native-PAGE was followed by separation of protein complexes in the second dimension by SDS-PAGE and silver staining. Cytochrome (cyt), light harvesting complexes (LHC), NAD(P)H dehydrogenase (NDH), Photosystem (PS), supercomplex (sc).

Figure 7. Large pore blue native gel electrophoresis (lpBN-PAGE) of *Arabidopsis* thylakoid membrane protein complexes.
Thylakoid membrane (8 µg Chl) was solubilized with 1% (w/v) dodecyl maltoside (DM) or 1% (w/v) digitonin in 25BTH20G buffer and the protein complexes were separated by lpBN-PAGE. Cytochrome (cyt), light harvesting complexes (LHC), megacomplex (mc), NAD(P)H dehydrogenase (NDH), Photosystem (PS), supercomplex (sc).

Figure 8. 2D lpBN-PAGE analysis of *Arabidopsis* thylakoid membrane protein complexes solubilized with 1% (w/v) dodecyl maltoside (DM).
Thylakoid membrane (8 µg Chl) was solubilized with 1% (w/v) dodecyl maltoside (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]. Cytochrome (cyt), light harvesting complexes (LHC), megacomplex (mc), Photosystem (PS), supercomplex (sc).

Figure 9. 2D lpBN-PAGE analysis of *Arabidopsis* thylakoid membrane protein complexes solubilized with 1% (w/v) digitonin.
Thylakoid membrane (8 µg Chl) was solubilized with 1% (w/v) digitonin in 25BTH20G buffer 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 [2,5]. Cytochrome (cyt), light harvesting complexes (LHC), megacomplex (mc), Photosystem (PS), supercomplex (sc).

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Figure 1

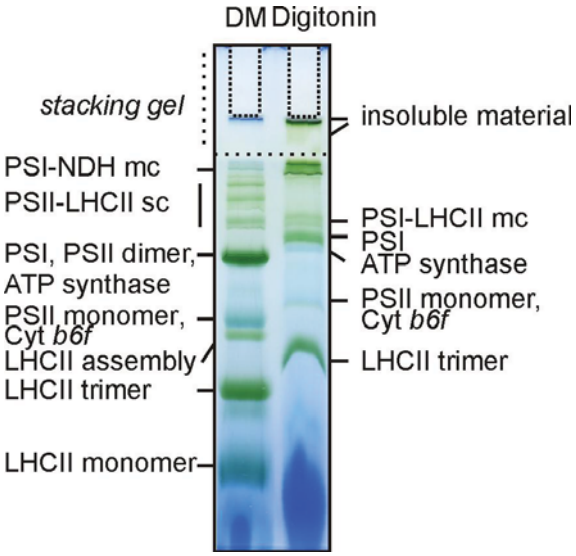

Figure 2

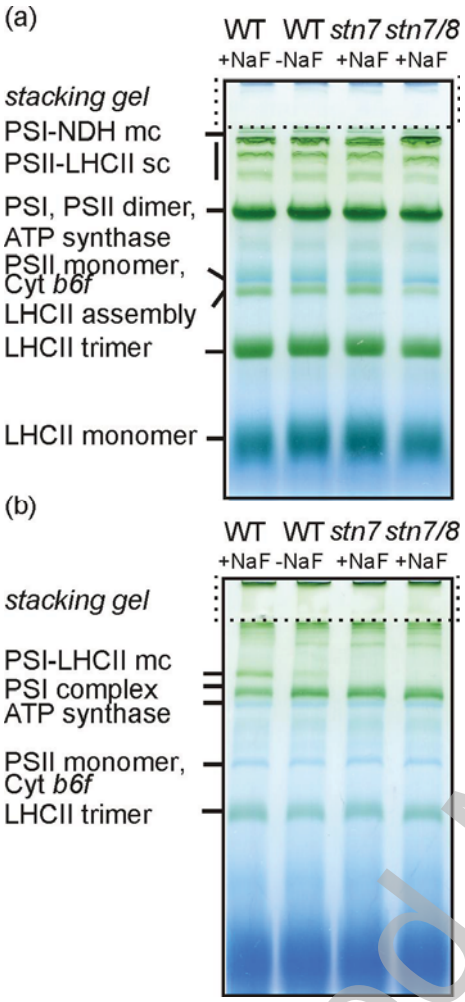

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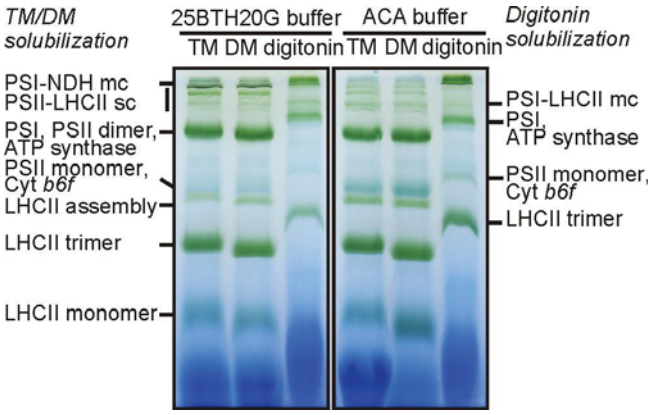
Figure 3



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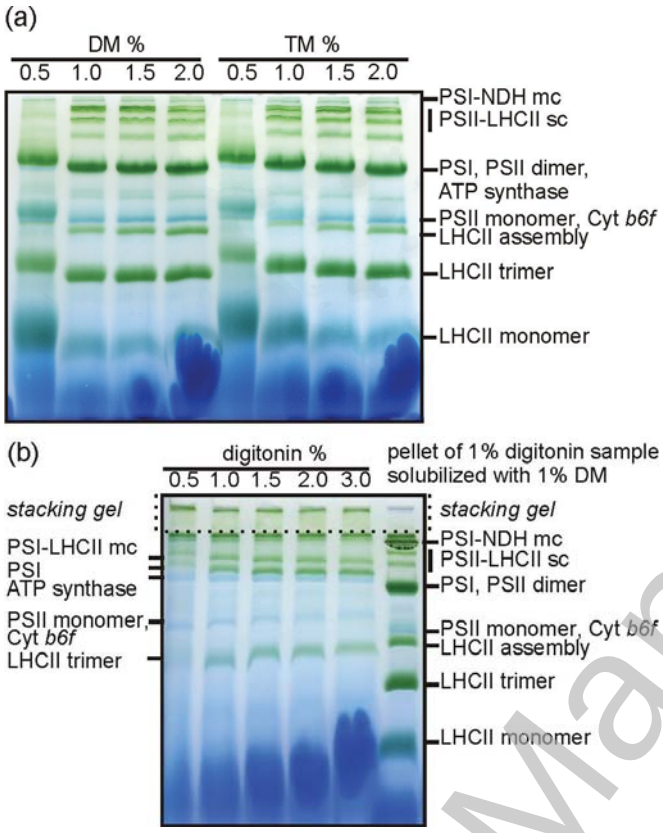

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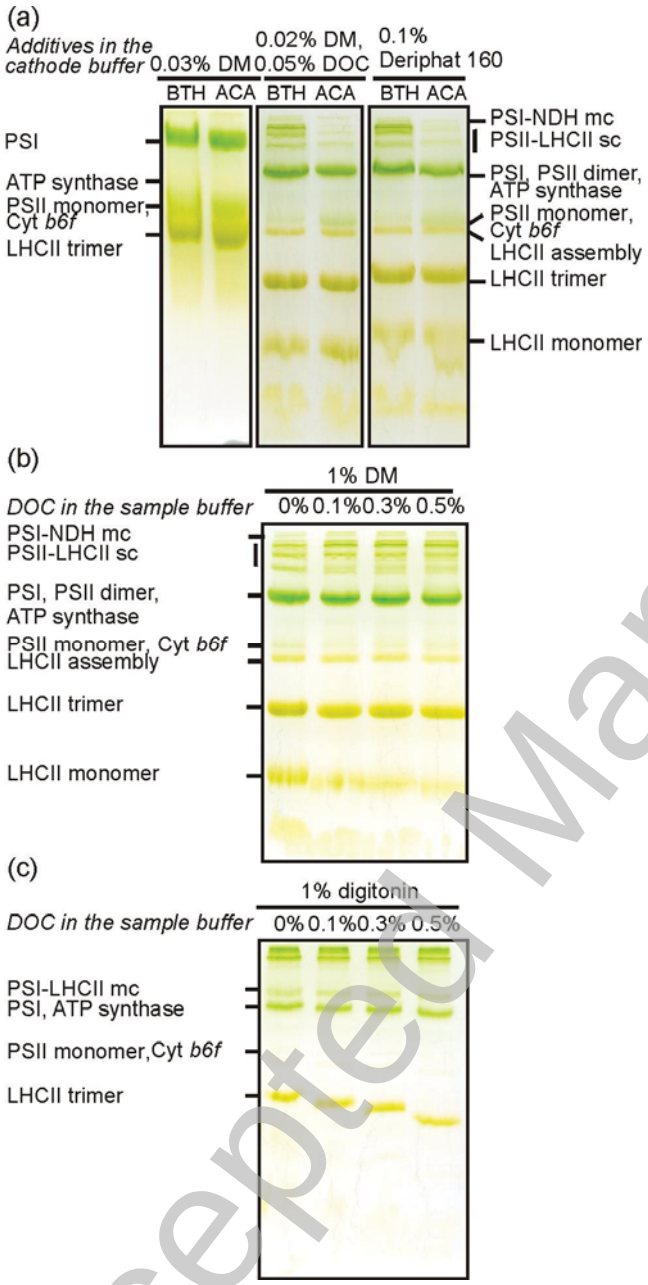

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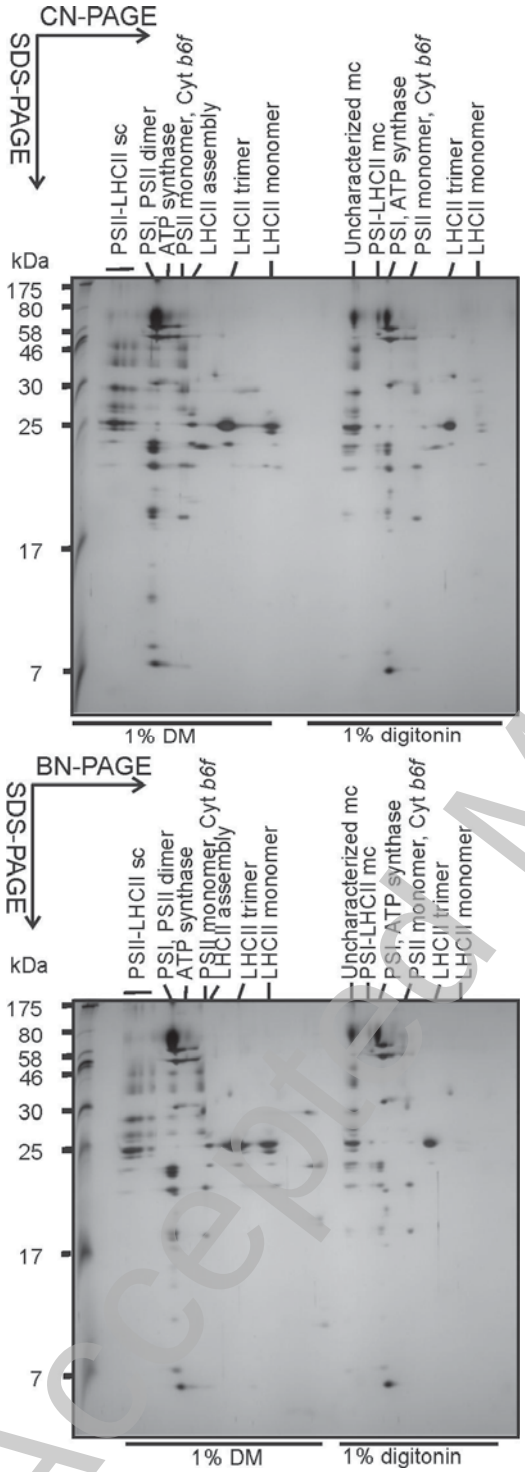

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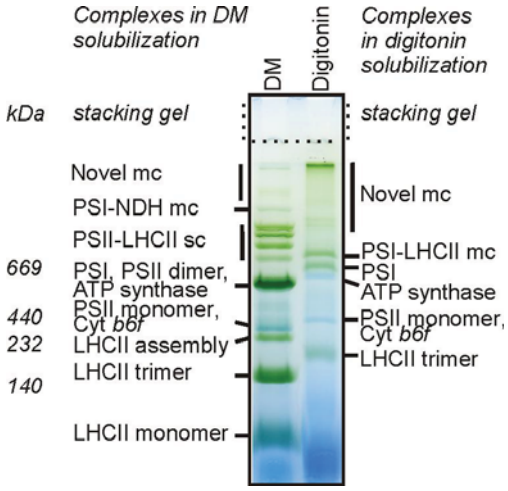

Figure 8

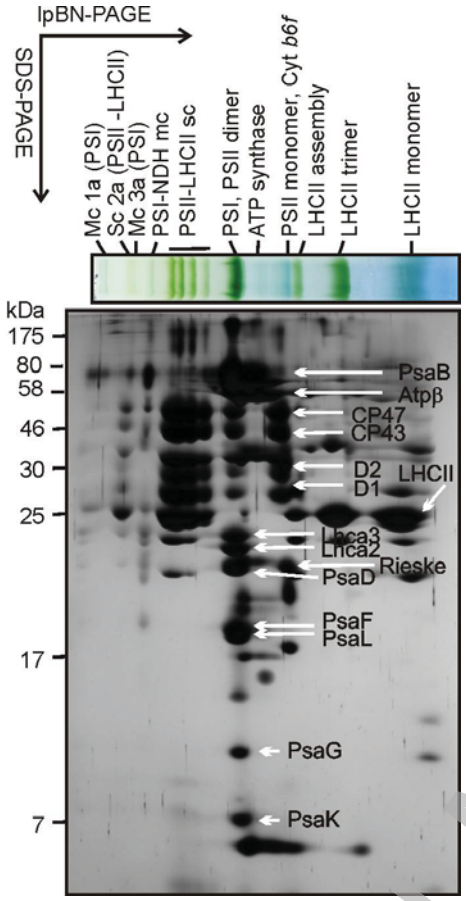

Figure 9