A Systematic Strategy for Proteomic Analysis of Chloroplast Protein Complexes in Wheat

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Abstract

A systematic strategy was developed for the proteomic analysis of wheat chloroplast protein complexes. First, comprehensive centrifugation methods were utilized for the exhaustive isolation of thylakoid, envelope, and stromal fractions. Second, 1% n-dodecyl-β-D-maltoside was selected from a series of detergents as the optimal detergent to dissolve protein complexes effectively from membranes. Then, blue native polyacrylamide gel electrophoresis (BN–PAGE) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) were improved to separate and analyze the protein complexes. By this systematic strategy, envelopes, thylakoids, and stromata were enriched effectively from chloroplasts in the same process, and more than 18 complexes were obtained simultaneously by BN–PAGE. Finally, thylakoid protein complexes were further analyzed by BN/SDS–PAGE, and nine complex bands and 40 protein spots were observed on BN–PAGE and SDS–PAGE respectively. Our results indicate that this new strategy can be used efficiently to analyze the proteome of chloroplast protein complexes and can be applied conveniently to the analysis of other subcellular protein complexes.

Key words: chloroplast thylakoids; protein complexes; proteome; wheat; analysis strategy

Chloroplasts are plant cell organelles in which photosynthesis and other biosynthetic pathways take place. Protein complexes in thylakoid membranes, envelope membranes, and chloroplast stroma are relevant mainly to photosynthetic and biosynthetic functions.1) Chloroplasts capture light energy to conserve free energy in the form of ATP and reduce NADP to NADPH through a photosynthetic reaction catalyzed by integral membrane protein complexes in thylakoid membranes.2) There are four major protein complexes in thylakoid membrane, photosystem I (PSI), photosystem II (PSII), the cytochrome b6f complex, and ATP synthase.3) These complexes are composed of at least 70 different proteins that work together ultimately to create ATP and NADPH as products.4) The outer and inner envelope membranes of the chloroplasts also contain multisubunit protein complexes for the import of preproteins, which are encoded by nuclear genes, and was synthesized in the cytoplasm, into the chloroplasts.5) These protein complexes are termed Toc (located on the outer envelope membrane of chloroplasts) and Tic (located on the inner envelope of chloroplasts).6) In addition, there are other complexes in chloroplast stroma, such as the RuBisCO protein complex, the PRK/GAPDH/CP12 protein complex and the Cor15a protein complex.7–9) It is predicted that all plastid types contain 2,000 to 4,000 different proteins in Arabidopsis.10) Approximately 550 α-helical proteins are integral to thylakoid and envelope membranes, and an unknown number of peripherally associated proteins are also present in these membranes.11) Hence it is of great significance to research the proteome of chloroplasts in various physiological states.

Several approaches are available for chloroplast protein complex separation and proteomic analysis.12–15) Gel-based and MS-based techniques to determine quantitative differences between proteomes have greatly improved in recent years. With the development of proteomics and mass spectrometry, the systematic identification of proteins has become possible. Nevertheless, identification of chloroplast protein complexes is still a great challenge, because chloroplast protein complexes are highly hydrophobic and low in abundance as compared with cytoplasmic proteins. In addition, the presence of about 100 abundant photosynthetic proteins in thylakoids, which represent some 98% of the protein mass, provides an additional challenge in the quantification of low-abundance proteins. Methods of detergent fractionation and subcellular fractionation have been applied successfully to enrich hydrophobic proteins. Blue native polyacrylamide gel electrophoresis (BN–PAGE),16–18) which can separate protein complexes under native conditions, is also a powerful technique for the study of protein complexes.5,19,20

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Abbreviations: ACN, acetonitrile; BN–PAGE, blue native polyacrylamide gel electrophoresis; CBB, Coomassie Brilliant Blue G-250; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; CHCA, alpha-cyano-4-hydroxycinnamic acid; DDM, n-dodecyl-β-D-maltoside; EGTA, ethylene glycol tetracetic acid; MALDI-TOF MS/MS, matrix assisted laser desorption ionization time of flight tandem mass spectrometry; PMSF, phenylmethylsulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid
Here we present a systematic strategy for proteomic analysis of chloroplast protein complexes. Different subcellular fractions of wheat chloroplasts were prepared and collected by differential density gradient centrifugation methods. Then 1.0% DDM was used to dissolve protein complexes effectively from the membranes while also preventing disaggregation of protein complexes. The protein complexes were easily and independently analyzed by SDS–PAGE and BN–PAGE. Finally, two-dimensional electrophoretic separation (BN/SDS–PAGE) was performed to analyze the proteomes of the chloroplast protein complexes, and MALDI-TOF MS/MS was used to identify separated protein spots quickly. By this strategy, we analyzed the complex proteome of the chloroplast efficiently.

Materials and Methods

Plant materials. Wheat Taichung29 seeds (Triticum aestivum) were provided by Professor Shichang Xu (Institute of Plant Protection, Chinese Academy of Agricultural Sciences). After sterilization with 1% hydrogen peroxide and soaking in water for 2 d at 20°C, the seeds were planted and grown in a glass greenhouse under a 16-h photoperiod at a constant temperature of 20°C. The leaves of 7- to 14-d-old seedlings were harvested for proteomic analysis of chloroplast protein complexes.

Isolation of wheat chloroplasts. Leaves (15–20 g) were cut into short pieces 3–5 mm in length and were mixed with 100 mL of fresh homogenate buffer (50 mM Hepes-KOH pH 7.8, 330 mM sorbitol, 5 mM caproic acid, 20 mM NaCl, 2 mM EDTA, and 10% glycerol). After acid, and 1 mM PMSF). Then they were homogenized for 3 min at 1000 rpm (PRO Scientific D-Series, USA). The resulting homogenate was moved into a 12.5-mL ultracentrifugation tube and then centrifuged at 4°C for 15 min. Then it was frozen with 3 mL of homogenate buffer and laid on top of a 45–20% Percoll step gradient (Fig. 1). The intact wheat chloroplast fraction was collected by centrifugation at 10,000 g for 30 min at 4°C. The samples were incubated on ice for 30 min, and the insoluble materials were removed by ultracentrifugation at 100,000 g for 1 h (4°C). Supernatant protein concentrations were determined using a 2D Quant Kit (GE Healthcare, Piscataway, NJ). Then the effects of different levels of DDM (0.4%, 0.7%, 1.0%, 1.3%, and 1.6%) on protein complex extraction were compared by SDS–PAGE and BN–PAGE, as described below.

SDS–PAGE analysis. SDS–PAGE was based on the approach described by Wang, with slight modifications.22 In our experiment, a 40-µL aliquot of each protein complex extraction was treated by reduction-alkylation and then loaded onto SDS–PAGE gels through the following steps. First, 5 µL of 200 mM DTT in 50 mM NH₄HCO₃ was mixed with 40 µL of extraction solution (the final DTT concentration was about 20 mM) and kept at 25°C for 30 min. Then 5 µL of 500 mM iodoacetamide (IAA) in 25 mM NH₄HCO₃ was added (the final IAA concentration was 50 mM) and kept at room temperature for 30 min. All of the pre-prepared protein samples was mixed with 10 µL of 5 × SDS–PAGE loading buffer (250 mM Tris–HCl pH 6.8, 10% w/v SDS, 50% v/v glycerol, 5% v/v mercaptoethanol, 0.5% w/v and bromophenol blue) at room temperature for 30 min. The samples were then added to an SDS–PAGE gel (resolving gel, 12% w/v acrylamide/bis-acrylamide 30:1, 380 mM Tris–HCl pH 8.8, 1% w/v SDS, 130 mM × 100 mM × 1.0% gel, 1.0% agarose, 0.1% w/v acrylamide/bis-acrylamide 30:1, 126 mM Tris–HCl pH 6.8, 1% w/v SDS, 1% agarose), the gel was stained by CBB by the method of hand (Fig. 2A). The chloroplast fraction was used or stored at −80°C freezer for future use.

Chloroplast thylakoid and envelope separation. The separation of chloroplast thylakoids and envelope mainly followed the outline of procedure in Fig. 1. The intact wheat chloroplast fraction was resuspended in 10 mL of fresh hypotonic ice-cold buffer (1.6 M sucrose, 5 mM Tricine-KOH pH 7.8, 10 mM EDTA, 2 mM MgCl₂, and 1 mM PMSF), and was kept on ice for 15 min. Then it was frozen with liquid nitrogen. The solution was diluted by adding 3 volumes of ice-cold hypo-osmotic buffer (5 mM Tricine-KOH pH 7.8, 10 mM EDTA, 2 mM MgCl₂) and centrifuged at 3,000 g for 15 min (4°C). The sediment, which contained the bulk of the thylakoids, was used to extract thylakoid protein complexes at the next step, and the supernatant was moved into a 12.5-mL ultracentrifugation tube and then centrifuged at 40,000 g for 30 min (4°C). The resulting supernatant (Supernatant 2, Fig. 1), which contained stromal protein complexes, was enriched by ultrafiltration and then directly used in further gel electrophoretic analyses. The result centrifuged pellets, which contain envelope membranes, were resuspended with 2 mL of lysate buffer and laid on the top of a discontinuous sucrose density gradient, which was prepared by overlaying 3 mL each of 0.8 and 1.0 M sucrose solutions in lysate buffer (20 mM Bis-Tris pH 7.0, 500 mM L-arginine, 20 mM NaCl, 2 mM EDTA, and 10% glycerol). After centrifugation at 150,000 g for 12 h, the band in the 0.8/1.0 M interphase and the pellets were collected separately, washed 3 times with lysate buffer, and then ultracentrifuged at 100,000 g for 1 h. The precipitated pellets were used for the further extraction of envelope and thylakoid protein complexes.

Protein complex extraction. Envelope and thylakoid membrane pellets were independently resuspended in lysate buffer and diluted to 2 mg/mL of chlorophyll by the method of Porra et al.23 Then, 1.0% DDM, Triton X-114, NP-40, CHAPS, or Triton X-100 was added to the membrane sample solutions to release the proteins. The mixed samples were incubated on ice for 30 min, and the insoluble materials were removed by ultracentrifugation at 100,000 g for 1 h (4°C). Supernatant protein concentrations were determined using a 2D Quant Kit (GE Healthcare, Piscataway, NJ). Then the effects of different levels of DDM (0.4%, 0.7%, 1.0%, 1.3%, and 1.6%) on protein complex extraction were compared by SDS–PAGE and BN–PAGE, as described below.

BN–PAGE analysis. BN–PAGE was carried out using the Protean II XL cell (Bio-Rad) by published methods.24–26 First, 20 µL 0.5% w/v CBB the mother liquor was added to an 80-µL protein complex sample solution to a final concentration of about 0.1% w/v, and 50 µL of mixed sample was loaded into each sample well. Then the proteins were stacked in a 3.5% gel in the presence of 0.1% DDM at 100 V at 8°C and then separated in a 4–10% gradient gel in the presence of 0.1% DDM at 250 V at 8°C. The BN–PAGE operations were ended when the CBB ran out of the bottom of the gel. Finally the gel was further stained by CBB by the method of hand (Fig. 2A).

SDS–PAGE loading buffer (250 mM Tris–HCl pH 6.8, 10% w/v SDS, 50% v/v glycerol, 5% v/v mercaptoethanol, 0.5% w/v and bromophenol blue) at room temperature for 30 min. The samples were then added to an SDS–PAGE gel (resolving gel, 12% w/v acrylamide/bis-acrylamide 30:1, 380 mM Tris–HCl pH 8.8, 1% w/v SDS, 130 mM × 100 mM × 1.0% gel, 1.0% agarose, 0.1% w/v acrylamide/bis-acrylamide 30:1, 126 mM Tris–HCl pH 6.8, 1% w/v SDS, 1% agarose), the gel was stained by CBB by the method of hand (Fig. 2A). Then, 1.0% DDM solution was removed, and 10 µL of 40 mM NH₄HCO₃/0.5% w/v and 0.5% w/v 6 M urea, 4.0% w/v SDS, 25% v/v glycerol, 140 mM Tris–HCl pH 6.8, 5% v/v β-mercaptoethanol) at 37°C for 30 min with gentle agitation. The second dimension of SDS–PAGE was then performed by placing the strip on top of 4% stacking gel, and monomeric proteins of protein complexes were separated in a 12% resolving gel containing 1% urea for SDS–PAGE analysis as described above.

In-gel protein digestion. In-gel protein digestion was done by an optimized method following Hanrieder et al. and Kumarathasan et al. Each protein spot gel was cut into dices of about 1 mm and washed and destained with 50% ACN and 25 mM NH₄HCO₃ twice (50 µL, 30 min for each destaining) at room temperature. Next, the gel fragments were dehydrated with 50 µL acetonitrile for 10 min and dried with a rotary evaporator for 20 min. 20 µL of 45 mM dithiothreitol was then added and then incubation at 50°C for 30 min. 20 µL of 100 mM iodoacetamide was added and the mixtures were incubated in the dark at room temperature for 30 min. The treated gel fragments were washed and dehydrated and then dried again, as described above. 25 µL of 25 ng/µL trypsin was added to each dried gel fragment and this was incubated for 1 h at 4°C. Excess trypsin solution was removed, and 10 µL of 40 mM NH₄HCO₃/10% ACN was added to each of the fragments. The fragments were incubated at 37°C for 16 h. After digestion, the samples were centrifuged at 10,000 g for 5 min and supernatants were collected. Gel fragments were extracted step-by-step with 0.1% TFA, 30% ACN, 250 µM bis-Tris pH 7.0, 500 mM L-arginine, 20 mM NaCl, 2 mM EDTA, and 10% glycerol). After centrifugation at 150,000 g for 12 h, the band in the 0.8/1.0 M interphase and the pellets were collected separately, washed 3 times with lysate buffer, and then ultracentrifuged at 100,000 g for 1 h.
and then 0.1% TFA, 60% ACN at 30 °C for 30 min. The supernatant was obtained at each step. The supernatants were combined, and the digested peptides were dried with a rotary evaporator for subsequent MS analysis.

**MS/MS analysis.** MS/MS analyses were performed on a MALDI-TOF tandem mass spectrometry instrument (Autoflex III MALDI-TOF/TOF System, Bruker Daltonik, Germany). The dried digested peptides were dissolved in 2 μL of 50% ACN and 0.1% TFA, and 0.8 μL of the analyte peptide solution was spotted on an AnchorChip MALDI target plate (Bruker Daltonik) and dried at room temperature. Then 0.8 μL of a 30% saturated solution of CHCA dissolved in 70% ACN and 0.1% TFA was used to cover the peptides. MALDI-TOF/TOF spectra were recorded in reflection mode, and were calibrated externally by a standard peptide mixture (Bruker Daltonik). FlexAnalysis 3.0 software (Bruker Daltonik) was used for subsequent data processing and peak list generation. Protein identification was performed by on-line search through Mascot MS/MS Search (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=MIS), with the following parameters: Taxonomy, Viridiplantae; Database, NCBInr; Enzyme, Trypsin; global carbamidomethylation of cysteine; variable oxidation parameters: Taxonomy, Viridiplantae; Database, NCBInr; Enzyme, Trypsin; global carbamidomethylation of cysteine; variable oxidation

### Results and Discussion

**Combined methods for the separation of envelopes and thylakoids**

In the past, BN–PAGE was successfully used to investigate protein complexes and protein interactions in subcellular membranes and cytoplasm, especially the chloroplast protein complexes involved in photosynthesis. As a rule of thumb, the preparation of protein complex samples is very important in BN–PAGE analysis and protein complex identification. Chloroplasts contain several membrane systems, including chloroplast envelope and thylakoids, and the content of some protein complexes in membrane systems is too low so that they cannot easily be detected or separated directly. To improve the separation of protein complexes, a combined method modified from Kleffmann et al. and Shingles et al. was used in this study to collect different intact chloroplast fractions following the procedure shown in Fig. 1.39,40

After Percoll density gradient centrifugation, intact chloroplasts at the 45/20% interphase were collected for the next procedure (Fig. 2A). Using the Percoll gradient step, most broken chloroplasts and cell residues were separated from intact chloroplasts, and the interference of cytosolic proteins was reduced. Then a combination of freezing and hyper- and hypo-osmotic treatments were employed to lyse intact chloroplasts. The hyper-osmotic treatment caused shrinkage of the chloroplasts and resulted in the physical separation of the envelope to some extent. After the freezing treatment, chloroplast envelopes formed predominantly, and vesicles were separated during rapid warming and hypo-osmotic treatment. Through this protocol, the separation efficiency of different membrane systems was improved. After discontinuous sucrose density gradient centrifugation, envelope membranes were separated well from the 0.8/1.0 M interphase (Fig. 2B). By this systematic strategy, envelopes, thylakoids, and stromata were effectively enriched from wheat chloroplasts through the same process.

**Optimizing the method of chloroplast protein complex extraction**

Chloroplasts contain a large number of protein complexes that are composed mostly of hydrophobic and transmembrane proteins. Identifying intrinsic protein complexes from membranes remains a challenge. Many strategies have been developed for the solubilization and extraction of membrane proteins, including the use of organic solvents31,32) and of nonionic and zwitterionic detergents.33–36 but organic solvent treatments do not keep protein complexes in their native forms. Hence finding suitable detergents is a key issue in investigating membrane protein complexes. Since a suitable detergent must be mild to minimize the disruption of protein complexes while solubilization and extraction are performed,37 in this study, five mild detergents (DDM, Triton X-100, Triton X-114, NP-40, and CHAPS) were tested for the extraction of protein complexes from thylakoids. The efficiencies of extraction were determined by a quantitative method (2D Quant Kit) that is detergent compatible (Fig. 2C). As shown in Fig. 2C, DDM and Triton X-100 were more effective than the other detergents in releasing proteins at a concentration of 1%. The minimization of protein complex disruption and detergent compatibility with electrophoresis were further compared by BN–PAGE for DDM and Triton X-100 (Fig. 2E). In the presence of 1% Triton X-100, high-mass protein complexes were lost, and protein bands were not as well defined. During
1% DDM treatment, more protein complexes were extracted, and the protein bands were better defined. To determine the optimal DDM concentration, various levels of DDM were used to release membrane proteins from equal amounts of membrane fraction (Fig. 2D). Quantification showed that the quantity of extracted protein gradually increased with increasing DDM concentration, especially between 0.7% and 1.0%. This trend could also be observed through the band numbers and densities of different lanes on BN–PAGE (Fig. 2F) and SDS–PAGE (Fig. 2G). BN–PAGE indicated that supercomplexes were not released from membranes at a low level of DDM (0.4%). The higher level of DDM (1.6%) was used, the more proteins were extracted. But the protein bands could not be clearly observed on a BN–PAGE gel. By a comprehensive comparison (Fig. 2E–G), a DDM concentration of about 1.0% was determined to be necessary for the extraction of chloroplast protein complexes.

**Improvement of BN–PAGE and BN/SDS–PAGE**

Since the early use of blue native electrophoresis to investigate protein complexes, great improvements have been made in sample preparation and the compositions of electrophoresis buffer and electrophoretic medium. BN–PAGE is typically performed by adding 0.02% CBB to the cathode buffer, and sometimes to the sample buffer. To enhance protein complex solubilization and prevent aggregation and precipitation during migration towards the anode in our experiments, we added 0.1% DDM to the acrylamide gel and 0.1% anionic CBB to the protein samples before loading them into the gel wells. Since normal BN–PAGE can only be used for the isolation of protein complexes in a mass range of 10 kDa to 10 MDa and chloroplast membrane protein complexes also have a very wide mass range, a continuous gradient gel (4–10%) was used to improve electrophoretic resolution. After BN–PAGE separation of the protein samples in the presence of CBB G-250, several major bands were observed clearly. The gradient BN–PAGE gel containing DDM enhanced not only resolution but also the reliability of maintaining various protein complexes (Fig. 3A, C). On BN/SDS–PAGE, after completion of the first dimension by BN–PAGE, the gel strip was incubated and then placed at the top of an SDS–PAGE gel that contained 1 M urea. As is known, most thylakoid proteins are hydrophobic, and the addition of urea to the SDS–PAGE gel improves protein solubility and increases protein transfer efficiency.

**Electrophoresis and MS analysis of chloroplast protein complexes**

When carrying out the procedure shown in Fig. 1, the thylakoid, envelope, and stromal protein complexes of the chloroplasts were collected and enriched at the same processing step. During envelope fraction analysis, about 4 and 12 bands were separated by BN–PAGE and SDS–PAGE respectively from the same sample (Fig. 3B, C). This means that the bands in the BN–PAGE lane contained protein complexes. Analysis of chloroplast extracts showed that more than 18 protein complexes in native form BN–PAGE while many different protein complexes were observed on SDS–PAGE (Fig. 3B, C). To analyze the complexes from the BN–PAGE lane, SDS–PAGE was used as second dimension for the separation of the thylakoid fraction. A total of 40 individual protein spots were detected routinely by blue silver staining (Fig. 3D). Figure 3E shows the pattern of thylakoid protein complex proteins and subunits. This
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more clearly and accurately reveals the components of each protein complex. All the spots marked in Fig. 3E were cut from the gel, digested by trypsin, and analyzed by MALDI-TOF MS/MS. In this study, 20 proteins were identified from thylakoid protein complexes. First, we used a density gradient centrifugation process for exhaustive isolation of thylakoid membranes, envelope membranes and stromal protein fractions from the same sample. This resulted in enrichment of the envelope membranes and reduced the dissociation of protein complexes. Secondly, we found that 1.0% DDM effectively dissolved protein complexes from membranes and prevented the disaggregation of them. A total of 18 protein complexes were detected from the thylakoids, envelope, and stroma. By two-dimensional electrophoretic separation, BN–PAGE was used as the first dimension in the presence of 0.1% DDM, followed by SDS–PAGE as the second dimension in the presence of 1 M urea. We obtained nine protein complex bands and 40 protein spots from wheat thylakoids. Finally, we quickly identified 20 proteins from those spots by MALDI-TOF MS/MS. Our results indicate that this new strategy can be used efficiently for proteomic analysis of chloroplast protein complexes and can conveniently be applied to analyze other subcellular protein complexes.

Conclusions

In the present study, we developed a systematic strategy for the proteomic analysis of wheat chloroplast protein complexes. First, we used a density gradient centrifugation process for exhaustive isolation of thylakoid membranes, envelope membranes and stromal protein fractions from the same sample. This resulted in enrichment of the envelope membranes and reduced the dissociation of protein complexes. Secondly, we found that 1.0% DDM effectively dissolved protein complexes from membranes and prevented the disaggregation of them. A total of 18 protein complexes were detected from the thylakoids, envelope, and stroma. By two-dimensional electrophoretic separation, BN–PAGE was used as the first dimension in the presence of 0.1% DDM, followed by SDS–PAGE as the second dimension in the presence of 1 M urea. We obtained nine protein complex bands and 40 protein spots from wheat thylakoids. Finally, we quickly identified 20 proteins from those spots by MALDI-TOF MS/MS. Our results indicate that this new strategy can be used efficiently for proteomic analysis of chloroplast protein complexes and can conveniently be applied to analyze other subcellular protein complexes.

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References

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