

Localization and Topology of Thylakoid Membrane Proteins in Land Plants

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[Abstract] Thylakoids are a formation of flattened membrane vesicles and protein complexes found in cyanobacteria, algae and plants. In the chloroplasts of land plants the thylakoid membrane systems form a network of densely packed stacks called grana lamellae, which are connected by unstacked stroma lamellae. Photosystem II is mainly localized in the appressed grana region, while photosystem I and the ATP synthase complexes are enriched in the stroma lamellae. The cytochrome *b*₆/*f* complex is distributed laterally throughout both stacked and unstacked membrane regions. The photosynthetic complexes consist of integral and peripheral proteins. The first part of this protocol (A) shows how to fractionate thylakoids into grana and stroma lamellae. The second part of this protocol (B) shows how to distinguish between strong hydrophobic integral membrane associations and weak electrostatic membrane and/or membrane complex associations. As it is necessary to specifically detect the protein of interest in the fractions, a specific antibody raised against the protein of interest or a complemented null mutant of a structural component expressing a tagged fusion protein would be of great advantage. The last part of this protocol (C) shows, how to investigate the topology of integral and peripheral proteins. This method requires a specific antibody for the protein of interest. For integral membrane proteins peptide-specific antibodies or epitope-tagged versions are required. The protocol is suitable for the investigation of low molecular weight proteins (LMW) below 5 kDa (Torabi *et al.*, 2014).

Material and Reagents

1. Freshly isolated thylakoids
2. Digitonin (water soluble) (SERVA Electrophoresis GmbH, catalog number: 19551.02)
3. Thermolysin from *Bacillus thermoproteolyticus* (Calbiochem®, catalog number: 58656)
4. NaBr (Sigma-Aldrich, catalog number: S-9756)
5. NaSCN (Merck KGaA, catalog number: 6627)
6. Na₂CO₃ (Merck KGaA, catalog number: 6392)
7. NaOH (Roth North America, catalog number: 9097.2)
8. Sucrose (Roth North America, catalog number: 9097.2)
9. EDTA (SERVA Electrophoresis GmbH, catalog number: 11280)

10. HEPES (Roth North America, catalog number: 9105.3)
11. Na₂HPO₄ (Roth North America, catalog number: 4984.3)
12. NaH₂PO₄ (Roth North America, catalog number: K300.2)
13. 0.1 M sodium phosphate buffer (see Recipes)
14. Fractionation buffer (see Recipes)
15. HS buffer (see Recipes)
16. 0.4% digitonin solution (see Recipes)
17. Thermolysin stock solution (40 mg/ml) in HS buffer (see Recipes)
18. Salt containing HS buffers (see Recipes)

Equipment

1. Centrifuges (Beckmann Coulter, model: Avanti J-25; Eppendorf, model: 5430 R)
2. Ultracentrifuge (Beckmann Coulter, model: Optima LE-80K)
3. Sonifier (Branson, model: B-12)
4. Photometer (Amersham biosciences, model: Ultraspe™ 3100 pro)

Procedure

A. Fractionation of thylakoid membranes

1. To solubilize the thylakoids at the grana margins mix 5 ml of freshly isolated thylakoids (0.8 mg chlorophyll/ml) in fractionation buffer with 5 ml of the 0.4% digitonin solution and incubate 2 min at RT. Prevent sedimentation of the thylakoid solution by slightly agitating.
2. Stop the solubilization with the addition of 90 ml ice cold fractionation buffer.
3. Centrifuge the solution for 15 min (10,000 \times g, 4 °C). Carefully keep the supernatant for the next step and try to avoid contaminations from the pellet. Dissolve the pellet (grana fraction) in 2 ml fractionation buffer and store on ice.
4. Centrifuge the supernatant of step A3 for 30 min (40,000 \times g, 4 °C). Carefully remove the upper part of the supernatant for step A5 and leave about 3 cm of the solution to avoid contamination of the stroma lamellae fraction. (Optional) dissolve the pellet in the remaining supernatant and keep the intermittent fraction on ice.

Pellet stroma lamellae by ultracentrifugation of the supernatant of step A4 for 60 min (100,000 \times g, 4 °C). Carefully remove the supernatant and dissolve the pellet in fractionation buffer. Try to keep the concentration as high as possible to avoid additional ultracentrifugation steps when adjusting the fractions to the desired chlorophyll concentrations. For storage of native thylakoid fractions (-20 °C or -80 °C) high chlorophyll concentrations around 2-4 mg/ml are recommended. For SDS-PAGE analysis

final concentrations from 0.25 to 1 μg Chl/ μl are used depending on the protein of interest. As a general rule use a lower final protein concentration for bigger membrane proteins with many cysteins to reduce (e.g. CP47, PsaA). Higher protein concentrations are suitable for low molecular weight proteins to reduce the sample volume and to avoid an expanded loading buffer front which could interfere with the migration of very small proteins.

5. Measure the chlorophyll contents of the fractions in 80% acetone and adjust to equal amounts of μg chlorophyll/ μl .
6. Analyze the fractionation by SDS-PAGE (Figure 1).

B. Salt treatment of thylakoid membranes

1. Dissolve freshly isolated thylakoid membranes in HS buffer (0.5 mg chlorophyll/ml) and in the salt containing HS buffers.
2. Incubate the samples for 30 min on ice.
3. Dilute the samples with two volumes of HS buffer.
4. Separate into pellet and soluble fraction by 10 min centrifugation (20,000 $\times g$, 4°C).
5. Analyze the fractions by SDS-PAGE.

C. Thermolysin treatment of thylakoid membranes

1. Dissolve freshly isolated thylakoid membranes in HS buffer (0.5 mg chlorophyll/ml).
2. To produce 50% inside-out and 50% right-side out vesicles apply ultrasonic pulses (10-30 sec, 10-30 times) to the solution on ice. Wait about 20 sec in between the pulses for cooling.
3. Add the protease thermolysin to a final concentration of 100 $\mu\text{g}/\text{ml}$ to the untreated thylakoid membrane solution and the inside- and right-side out vesicles.
4. Take probes at different time points (for example 0, 1, 2, 5 min) and immediately stop the digestion by adding EDTA to a final concentration of 20 mM.
5. Wash probes in HS buffer containing 20 mM EDTA.
6. Compare the digestion of thylakoids and inside- and right-side out vesicles by SDS-PAGE.

Representative data

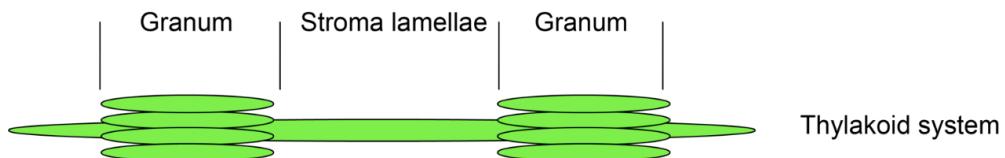
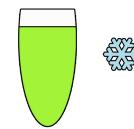


Figure 1. Thylakoid system

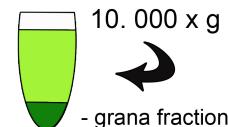
1. Solubilize 5 ml thylakoids (0.8 mg/ml) by addition of 5 ml digitonin solution (0.4%) for 2 min at RT



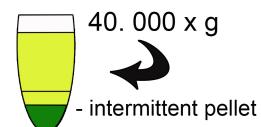
2. Stop the solubilization by addition of 90 ml ice cold fractionation-buffer



3. Pellet grana fraction (15 min, 10.000 x g, 4°C). Keep the supernatant for step 4. Dissolve grana pellet in fractionation-buffer and store on ice.



4. Pellet intermittent fraction (30 min, 40.000 x g, 4°C). Keep the upper part of the supernatant for step 5 and leave about 3 cm of the solution. Dissolve the pellet in the remaining supernatant and keep on ice.



5. Pellet stroma lamellae (60 min., 100.000 x g, 4°C). Remove the supernatant and dissolve the pellet in fractionation-buffer

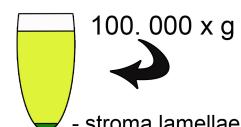


Figure 2. Scheme of the fractionation procedure (A)

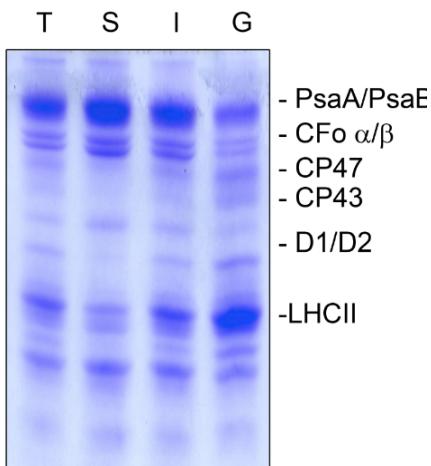


Figure 3. Coomassie staining of the thylakoid fractionation (A). Thylakoid membranes (T) were fractionated into stroma lamellae (S), intermittent fraction (I), and grana lamellae (G), and the separated proteins were subsequently stained with Coomassie to judge the purity of the fractions. PSI proteins PsaA and PsaB as well as the ATP synthase subunits CFo α/β were highly enriched in the stroma lamellae and only small amounts were present in the grana membranes. The PSII proteins CP47, CP43, D1, D2 and the antenna proteins of the light harvesting complex of PSII (LHCII) were predominantly present in grana fractions but almost lacking in the stroma lamellae fraction. The intermittent fraction contained proteins of both photosystems. An equal amount of chlorophyll (5 μ g) was loaded.

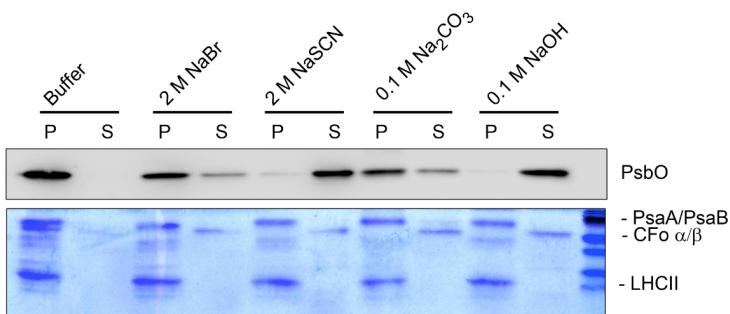


Figure 4. Washing experiment (B). Thylakoid membranes treated with different salt-containing buffers were fractionated into pellet (P) and supernatant (S) and separated by SDS-PAGE. The separated proteins were analyzed by Coomassie staining and immunoblot analysis. The dissociation of the hydrophilic ATP synthase subunits CFo α/β from the membrane can be identified by Coomassie staining under all salt conditions used. In contrast the hydrophobic light harvesting complex of PSII (LHCII) and the PSI core subunits PsaA/B could not be released from the pellet fraction. Specific antisera were used to identify the peripheral luminal PsbO protein, which is associated with PSII. The PsbO protein could be released completely from the membrane only under stringent salt conditions (2 M NaSCN and 0.1 M NaOH).

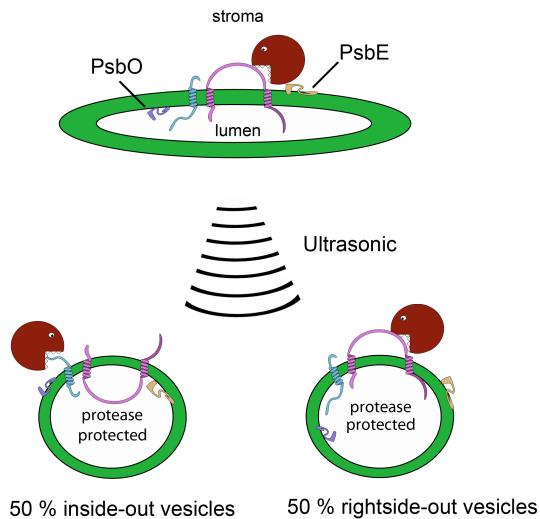


Figure 5. Scheme of the protease treatment (C)

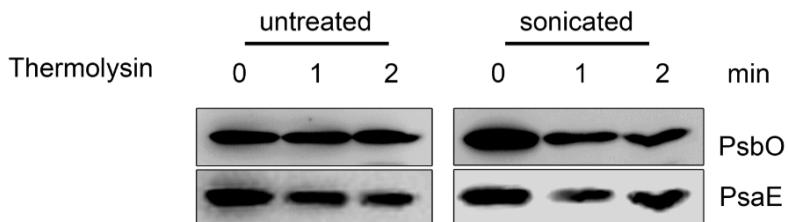


Figure 6. Topology studies (C). Untreated and sonicated thylakoids were incubated with thermolysin and subjected to immunodecoration using PsbO and PsaE antisera. In the untreated thylakoids the luminal PsbO is protected from thermolysin treatment, while the stromal exposed PsaE is degraded. In the sonicated thylakoids, which form about 50% inside-out vesicles, PsbO is partially degraded, while PsaE is partially protected.

Notes

1. To dissolve a large thylakoid pellet as fast and gentle as possible use a fine paint brush.
2. For the separation of thylakoid membrane proteins by SDS-PAGE and especially proteins ≤ 10 kDa Tricine-SDS-PAGE is highly recommended for reference (Schägger, 2006).
3. For immuno-blot analysis of low molecular weight thylakoid membrane proteins use 0.2 μ M PVDF or 0.1 μ M nitrocellulose membrane and shorten the transfer time.

Recipes

1. 0.1 M sodium phosphate buffer (pH 7.4)
1 M HEPES-NaOH (pH 8.0)

0.5 M EDTA (pH 8)

2. Fractionation buffer

100 mM sucrose

10 mM sodium-phosphate buffer (pH 7.4)

5 mM MgCl₂

5 mM NaCl

3. HS buffer

0.1 M sucrose

10 mM HEPES-NaOH (pH 8.0)

4. 0.4% digitonin solution

Dissolve 40 mg digitonin in 10 ml H₂O by mixing and heating

Solution can be stored at -20 °C

5. Thermolysin stock solution (40 mg/ml) in HS buffer

Stock can be stored at -20 °C

6. Salt containing HS buffers

2 M NaBr in HS buffer

2 M NaSCN in HS buffer

0.1 M Na₂CO₃ in HS buffer

0.1 mM NaOH in HS buffer A

Acknowledgments

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