Gradient Flotation Centrifugation of Chloroplast Membranes

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[Abstract] Plastoglobules are lipoprotein particles physically attached to thylakoids in chloroplasts (Kessler *et al.*, 1999). They are mainly composed of polar lipid, neutral lipids, and proteins (Vidi *et al.*, 2006). Here we used simple sucrose gradient flotation centrifugation method to purify the plastoglobules from total chloroplast membranes (Vidi *et al.*, 2007, Shanmugabalaji *et al.*, 2013).

Materials and Reagents

1. Anti-PGL35 (Agrisera, catalog number: AS06 116)
2. Anti-TOC75 (Agrisera, catalog number: AS06 150)
3. Anti-LHCB2 (Agrisera, catalog number: AS01 003)
4. Na-ascorbate (Sigma-Aldrich, catalog number: 11140)
5. BSA fraction V (Sigma-Aldrich, catalog number: 05470)
6. PMSF/isopropanol (Sigma-Aldrich, catalog number: P7626)
7. Tricine-HCl (Sigma-Aldrich, catalog number: T0377)
8. DTT (Sigma-Aldrich, catalog number: 43819)
9. HB buffer (see Recipes)
10. TE buffer (see Recipes)

Equipment

1. Miracloth (pore size: 22-25 µm) (Merck KGaA, catalog number: 475855)
2. Centrifuge with JA-14 rotor
3. Potter homogenizer
4. Polycarbonate UltraClear SW28 tube
5. SW41Ti rotor (Beckman Coulter)
6. Spectrophotometer

Procedure

1. Harvest leaves from of 3-4 weeks old *Arabidopsis* or tobacco seedlings (better if seedlings were kept in dark for 12 h before, to minimize the starch) and collect them in chilled water. Let them in cold room for at least 30 minutes.
2. Grind leaves 3 times in 100 to 400 ml HB buffer, using a Waring blender homogeniser (1 time high, 5 sec; 3 times low, 3 sec).
3. Filter the homogenate immediately through two cheese cloth and one miracloth.
4. Centrifuge the filtrate 2 min at 2,200 rpm at 4 °C in JA-14 rotor.
5. Resuspend the pellet in the 3 ml of HB buffer and quantify the chlorophyll.
6. Adjust the volume to 50 ml with HB buffer and centrifuge at 4 °C at 3,600 rpm for 2 min.
7. Resuspend the chloroplast pellet from the chloroplast prep in 0.6 M sucrose in TE buffer to a concentration of 1-2 mg/ml chlorophyll.
   Note: Chlorophyll content is measured by diluting 5-10 µl of resuspended chloroplasts into 1 ml of 80% acetone. Mix well and spin for 2 min in the microfuge. Remove the supernatant and measure against a blank of 80% acetone in a quartz cuvette at 652 nm.
   Chlorophyll concentration in mg/ml = OD_{652} x dilution factor/36
8. Freeze the chloroplast suspension at -80 °C for 1-2 h.
9. Thaw the suspension and dilute with at least 3 volumes of TE buffer.
10. Homogenize for 20 strokes in a Potter homogenizer with a pestle.
11. Spin the lysed chloroplasts at 39,000 rpm for 1 hour at 4 °C. Remove the brownish supernatant (stroma) by pipetting and store it in frozen at -20 °C for 30 min.
12. Resuspend the pellet in 45% sucrose in TE buffer to a concentration of 2-3 mg chlorophyll/ml. Homogenize the pellet in a Potter homogenizer for 20 strokes as given above. The membranes may be stored at -20 °C at this point for later fractionation.
13. Pipette the resuspended membranes into a polycarbonate UltraClear SW28 tube.
14. Membranes were overlaid with a linear sucrose gradient created using 15 ml 5% sucrose and 15 ml of 45% sucrose in TE buffer and centrifuged for 17 h at 100,000 *x g* and 4 °C (SW41Ti rotor).
15. Collect 1 ml of fractions from the gradient. The fractions can be stored at -20 °C.
    Starting from the top of the gradient (fraction 1) and ending at the bottom (approximately 32 fractions) plastoglobules are present in the fractions 1-6; envelopes are in fractions 14-18, and thylakoid membranes in fractions 25-32. Nevertheless, the exact chloroplast membrane distribution could be checked by immunoblotting with anti-PGL35 (plastoglobules), anti-TOC75 (envelopes) and anti-LHCB2 (thylakoid membranes).

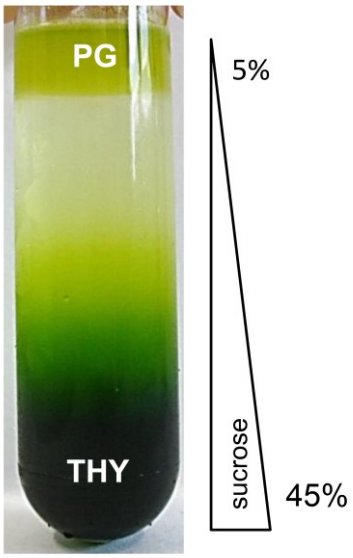

Figure 1. Purification of plastoglobules by flotation centrifugation. Total membranes from isolated chloroplasts were separated by flotation on a continuous sucrose gradient. Plastoglobules are visible as a yellowish green layer at the top of the gradient. THY, thylakoid membranes; PG, plastoglobules.

Recipes

1. HB buffer

Stock	Final concentration	For 400 ml	For 200 ml
Sorbitol (182.2 g/mol)	450 mM	32.8 g	16.4 g
1 M Tricine/KOH pH 8.4	20 mM	8 ml	4 ml
0.5 M EDTA pH 8.5	10 mM	8 ml	4 ml
0.5 M $NaHCO_3$	10 mM	8 ml	4 ml
1 M $MnCl_2$	1 mM	0.4 ml	0.2 ml
Add the day of use:			
Na-ascorbate (200 g/mol)	5 mM	0.4 g	0.2 g
BSA fraction V	0.05%	0.2 g	0.1 g
0.2 M PMSF/isopropanol	1 mM	2 ml	1 ml

2. TE buffer

Stock	Final concentration
Tricine-HCl pH 7.5	50 mM
EDTA	2 mM
DTT	2 mM

Acknowledgments

This protocol was adapted from Shanmugabalaji *et al.* 2013. The project was supported by the NRP59, the University of Neuchatel, NCCR ‘‘Plant Survival’’ (National Center of Competence in Research), SystemsX PGCE and the Swiss National Foundation.

References

1. Kessler, F., Schnell, D. and Blobel, G. (1999). Identification of proteins associated with plastoglobules isolated from pea (*Pisum sativum* L.) chloroplasts. *Planta* 208(1): 107-113.
2. Shanmugabalaji, V., Besagni, C., Piller, L. E., Douet, V., Ruf, S., Bock, R. and Kessler, F. (2013). Dual targeting of a mature plastoglobulin/fibrillin fusion protein to chloroplast plastoglobules and thylakoids in transplastomic tobacco plants. *Plant Mol Biol* 81(1-2): 13-25.
3. Vidi, P. A., Kanwischer, M., Baginsky, S., Austin, J. R., Csucs, G., Dormann, P., Kessler, F. and Brehelin, C. (2006). Tocopherol cyclase (VTE1) localization and vitamin E accumulation in chloroplast plastoglobule lipoprotein particles. *J Biol Chem* 281(16): 11225-11234.
4. Vidi, P. A., Kessler, F. and Brehelin, C. (2007). Plastoglobules: a new address for targeting recombinant proteins in the chloroplast. *BMC Biotechnol* 7: 4.