Gradient Flotation Centrifugation of Chloroplast Membranes

Venkatasalam Shanmugabalaji[1*] and Felix Kessler[2]

[1]Department of Plant Biology, University of Geneva, Geneva, Switzerland; [2]Laboratoire de Physiologie Végétale, Université de Neuchâtel, Neuchâtel, Switzerland
*For correspondence: shanmugabalaji.venkatasalam@unige.ch

[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).

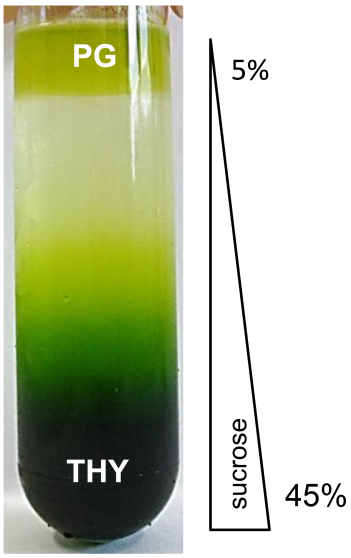

Figure 1. Purification of plastoglobules by flotation centrifugation. Total membranes from isolated chloroplasts were separated by flotation on a continuous sucrose gradient. Plastoglobules are visible as a yellowish green layer at the top of the gradient. THY, thylakoid membranes; PG, plastoglobules.

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

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