PROTOCOL 1.1 Isolation Glyoxysomes from Soybean Cotyledons

This protocol should be used for isolation of glyoxysomes from soybean cotyledons. We usually use soybean seeds (*Glycine max* cv. Bansei Shirodaizu). Figure 1 shows the steps to isolate glyoxysomes by Percoll density gradient centrifugation. Glyoxysomes are isolated from etiolated soybean cotyledons germinated at 22 ºC in the dark for 7 days. All procedures should be carried out at 4 ºC.

<Equipments and reagents>

- Grinding buffer [20 mM pyrophosphate-HCl, pH 7.5, 1 mM EDTA, 0.3M mannitol]
- Isolation buffer [10 mM HEPES-KOH, pH7.2, 1 mM EDTM, 0.3 M mannitol]
- 50% (v/v) Percoll solutions [50% (v/v) Percoll, 10 mM HEPES-KOH, pH 7.2, 1mM EDTA, 0.3 M raffinose]
- 28% (v/v) Percoll solutions [28% (v/v) Percoll, 10 mM HEPES-KOH, pH 7.2, 1mM EDTA, 0.3 M raffinose]
- Homogenizer (SMT COMPANY, Japan)
- Fractionator (SJ-1211; ATTO, Japan)
- Centrifuge and centrifuge tubes

1. Prepare gradients that consist of 5 ml of 50% (v/v) and 30 ml of 28% (v/v) Percoll solutions.
2. Harvest the soybean cotyledons (100 g fresh weight), and homogenize with 200 ml of grinding buffer in a chilled homogenizer twice for 3 seconds.
3. Squeeze the homogenate through four layers of cheesecloth, and homogenize the residue with another 200 ml of grinding buffer.
4. Combine the filtrates and centrifuge at 1,500×g for 10 min to remove plastids and cell debris.
5. Centrifuge the supernatant at 10,000×g for 20 min, resuspend the pellet in 150 ml of grinding buffer, and centrifuge at 1,500×g for 10 min to remove remains of plastids and cell debris.
6. Centrifuge the supernatant at 10,000×g for 20 min, and then resuspend the pellet in 4 ml of isolation buffer.
7. Put the suspended solution on the top of the Percoll solutions, and centrifuge at 40,000×g for 40 min with slow acceleration and deceleration.
8. After centrifugation, fractionate samples into each 1.0 ml by the fractionator.
9. Measure catalase activity as a maker of glyoxysomes in each fraction.

PROTOCOL 1.2 Purification of Glyoxysomes

Figure 2 shows the steps to purify glyoxysomes by iodixanol density gradient centrifugation. All procedures should be carried out at 4 ºC.

<Equipments and reagents>

- Isolation buffer [10 mM HEPES-KOH, pH7.2, 1 mM EDTM, 0.3 M mannitol]
- 50% (w/v) Iodixanol solution [50% (w/v) Iodixanol, 25 mM sucrose, 2.5 mM MOPS-HCl pH 7.2, 0.5 mM EDTA, 0.05% ethanol]
- 15.5% (w/v) Iodixanol solution [15.5% (w/v) Iodixanol, 440 mM sucrose, 6 mM MOPS-HCl pH 7.2, 1.2 mM EDTA, 0.12% ethanol]
- 36% (w/v) Iodixanol solution [36% (w/v) Iodixanol, 170 mM sucrose, 4 mM MOPS-HCl pH 7.2, 0.8 mM EDTA, 0.08% ethanol]
- Ultracentrifuge, swinging rotor (SW 28.1; Beckman Coulter, San Diego, CA, USA), and tubes
- Centrifuge and centrifuge tubes

1. Prepare gradients that consist of 2 ml of 50% (w/v) iodixanol solution and 13.6 ml of a linear iodixanol density gradient from 36% (w/v) to 15.5% solution. The gradients should be kept in 4 ºC for O/N.
2. Combine glyoxysomal fractions obtained from Percoll gradients, and dilute 5-fold with isolation buffer, followed by centrifugation at 4,800×g for 10 min.
3. Resuspend the pellet in 2 ml of isolation buffer.
4. Put the solution carefully onto the iodixanol density gradient.
5. Centrifuge at 100,000×g for 2.5 h in the SW28.1 swinging rotor.
6. Collect glyoxysomal fractions obtained from the iodixanol density gradient.

The fractions should be analyzed by immunoblotting to confirm that the peroxisomal fraction is not contaminated with proteins of other organelles.

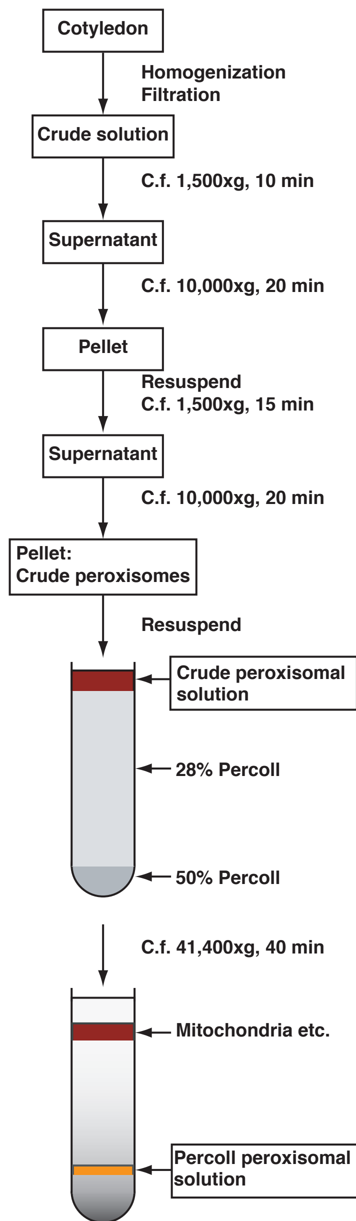

Figure 1. Isolation Glyoxysomes from Soybean Cotyledons

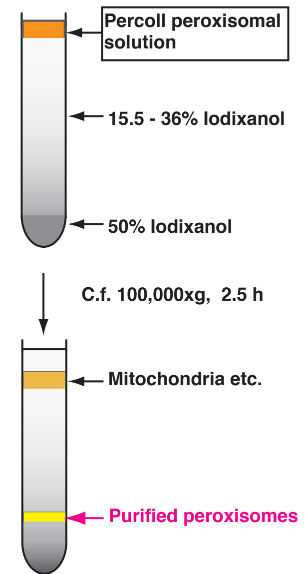

Figure 2. Purification of Glyoxysomes