Isolation of Glyoxysomes from Pumpkin Cotyledons

The isolation of organelles is essential for a variety of biochemical procedures. The Basic Protocol in this unit delineates the efficient isolation and purification of glyoxysomes from pumpkin cotyledons. It has been used, with minor modifications, to isolate peroxisomes from multiple plant tissues and species. In addition, effective means to quantify and determine the integrity of the isolated organelles are discussed. A Support Protocol for growing pumpkin seedlings for use in the Basic Protocol is also provided.

ISOLATION OF GLYOXYSOMES

BASIC PROTOCOL

Glyoxysomes are found in germinating seedlings and contain the enzymes of the glyoxylate cycle. They are isolated by differential and density gradient centrifugation.

Materials

5- to 7-day, dark-grown pumpkin seedlings (Support Protocol)
1× grinding buffer working solution (see recipe)
2 M sucrose
28% (v/v) Percoll/resuspension buffer solution (see recipe)
1× resuspension buffer working solution (see recipe)

250-ml beakers
Balance accurate to 0.1 g
Waring blender (2-speed) and 500-ml blender cup, precooled to 4°C
Miracloth (Calbiochem)
Glass funnel, precooled to 4°C
50-ml round-bottom polypropylene centrifuge tubes
Sorvall RC5C centrifuge with HB-6 swinging-bucket rotor (or equivalent refrigerated centrifuge and rotor)
15-ml Corex tubes, cooled to 4°C
Small, soft paintbrush (natural hair)
UV spectrophotometer

NOTE: Keep all equipment and all solutions cold (4°C) for the duration of the procedure.

Prepare cotyledons

1. Record the age and growth conditions of the pumpkin seedlings.
2. Harvest the pumpkin cotyledons (Fig. 3.19.1) in dim room light by manually separating the cotyledons from the hypocotyls. For very small seedlings, remove the seed coat and radicle as well.
3. Preweigh a 250-ml beaker to the nearest 0.1 g, cool the beaker on ice, then place the cotyledons inside the beaker. Reweigh the beaker, and record the tissue weight to the nearest 0.1 g.

   Be sure to keep the tissue cold. Avoid exposure to bright light, as this will cause the cotyledons to become green. Normal room lighting can be resumed during the first centrifugation step.

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Figure 3.19.1 Five-day-old dark-grown pumpkin seedlings**.** 1, seed coat; 2, cotyledons; 3, hypocotyl; 4, radicle.

Homogenize the tissues

4. Put the tissue and enough 1× grinding buffer working solution (with BSA) to cover the tissue (usually about 150 to 170 ml) in the blender cup. Homogenize the tissue with the Waring Blender, using three short bursts of ∼3 sec each, on low speed. Do not overgrind.

5. Filter the homogenate through a piece of Miracloth that has been folded in half and used to line the inside of a precooled funnel placed over a 250-ml beaker on ice. Collect the filtered homogenate in the beaker below the funnel. Squeeze the macerated tissue inside the Miracloth bag gently, to extract all the liquid. Discard the remaining tissue and used Miracloth.

6. Distribute the filtrate evenly between four 50-ml round-bottom polypropylene centrifuge tubes and centrifuge in a swinging-bucket rotor 10 min at 3000 × *g*, 4°C.

7. Remove the thick lipid layer floating on top of each supernatant using a Kimwipe wrapped around the wide end of a 1000-µl plastic pipet tip.

8. Decant the supernatant into a second set of four clean 50-ml round-bottom polypropylene centrifuge tubes.

9. Centrifuge this supernatant in a swinging-bucket rotor 20 min at 10,500 × *g*, 4°C. Carefully decant and discard the supernatant from each tube, retaining the pellets (keep on ice).

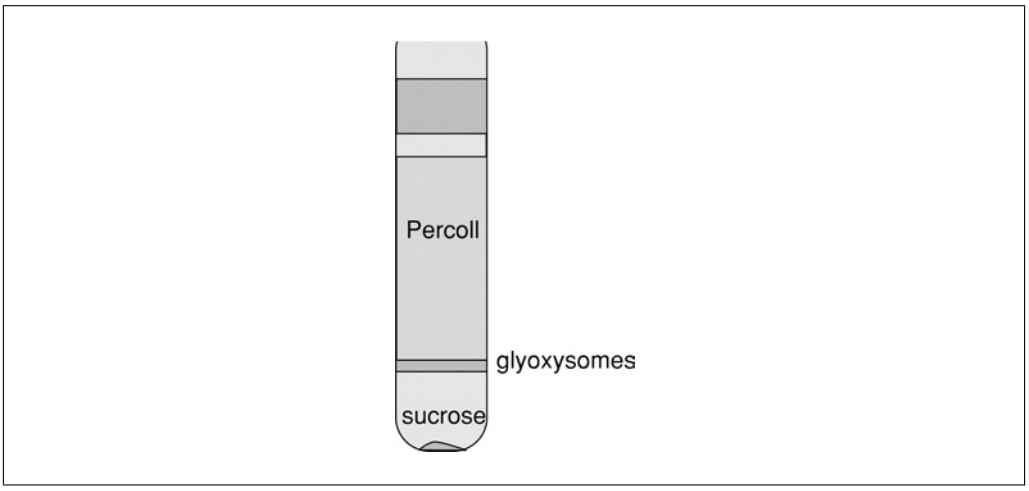

Figure 3.19.2 Percoll gradient separation of glyoxysomes.

Centrifuge the homogenate

10. Prepare a Percoll gradient by placing 1.5 ml of 2 M sucrose solution in a chilled 15-ml Corex tube. Carefully overlay with 10 ml 28% Percoll/1× resuspension buffer solution, being very careful not to mix the sucrose and Percoll layers.

11. Using a small paintbrush, gently resuspend each pellet (from step 9) in 250 µl of 1× resuspension buffer working solution. Pool the resulting suspension in one tube and swirl gently to mix.

12. Carefully overlay the pooled suspension on to the top of the prepared Percoll gradient.

13. Centrifuge the supernatant in a swinging-bucket rotor 30 min at 18,000 × *g*, 4°C, without the brake.

    Using the brake on this step will disturb the gradient and greatly diminish the final glyoxysome yield.

Purify the glyoxysomes

14. Carefully remove the upper layers of lipid and broken organelles in the gradient with a Pasteur pipet (Fig. 3.19.2). Finally, carefully collect and transfer the glyoxysomes (the visible yellowish band at the Percoll/sucrose interface) with a clean pipet to a 15-ml Corex tube.

    Removing too much Percoll or sucrose cushion will reduce the purity of the glyoxysomes, while removing too little will decrease the overall yield.

15. Dilute the glyoxysomes 3- to 5-fold with 1× resuspension buffer (usually ~8 ml is required).

16. Centrifuge glyoxysomes in a swinging-bucket rotor 16 to 20 min at 7000 × *g*, 4°C, with the brake on.

    A shorter (16-min) centrifugation will result in a soft organelle pellet, making it harder to remove all of the supernatant without losing part of the pellet. A longer (20-min) centrifugation will yield a tighter pellet.

17. Carefully remove and discard the supernatant. Gently resuspend the pellet of purified glyoxysomes in 100 µl of 1× resuspension buffer.

18. To approximate the yield, take an aliquot of the glyoxysome suspension, dilute it 1:500 with 1× resuspension buffer, and measure the absorbance at A_{280} and A_{235}. Use these values in the equations below to obtain the approximate concentration and yield.

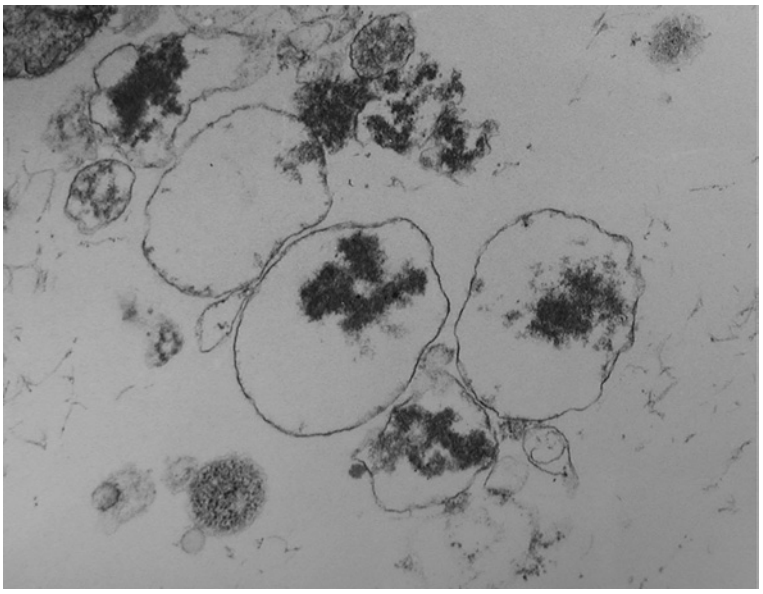

Figure 3.19.3 Electron micrograph of purified pumpkin glyoxysomes. Magnification, 10,000× (micrograph by Mary Alice Webb, Purdue University).

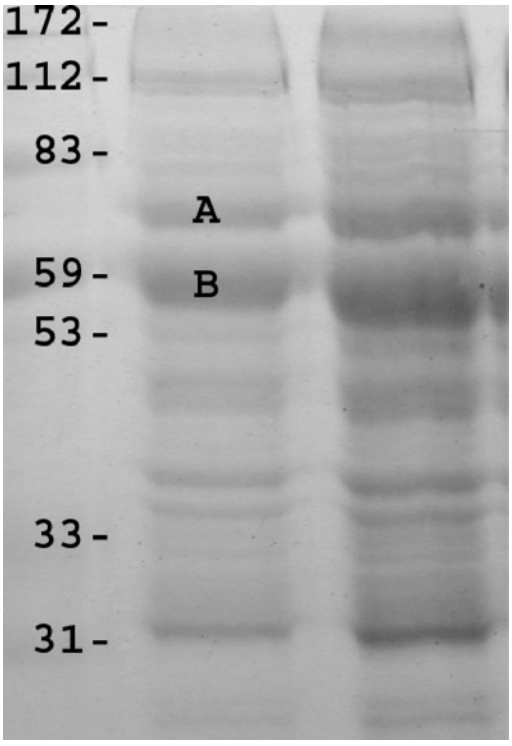

Figure 3.19.4 Coomassie blue–stained gel of proteins from purified pumpkin glyoxysomes. Proteins from freeze-thawed, lysed glyoxysomes were separated by 10% SDS-PAGE: left lane, 25 μg; right lane 50 μg. The major glyoxysomal protein bands indicated in the figure are: (**A**) isocitrate lyase (64 kDa) and (**B**) catalase (55 kDa).

Approx. conc. (mg protein/ml) $= [(A_{235} - A_{280})/2.51] \times 500$

Approx. yield (mg total protein) = (mg protein/ml)
× (final resuspension volume in ml).

These numbers are only approximate, but they are quick and easy to obtain and are consistent between organelle preparations. The authors use this calculation to standardize the amount of glyoxysomes used in each experiment. If more accurate protein concentrations are needed, one can do a biochemical protein assay, such as Pierce's BCA assay or any other protein assay of choice (APPENDIX 3H).

19. Determine integrity of the organelle preparation.

In addition to morphological analysis by electron microscopy (Fig. 3.19.3), there are many enzymatic assays that can be used to verify the presence of intact glyoxysomes, including catalase assays (Cooper and Beevers, 1969; Aebi, 1984; also see UNIT 3.4) and isocitrate lyase assays (Cooper and Beevers, 1969). Mitochondrial contamination can be assessed by performing fumarase assays (Cooper and Beevers, 1969; Hatch, 1978). The intactness of the organelles can be determined by performing the enzyme assays in the presence (to disrupt the membrane) and absence of 0.5% (v/v) Triton X-100. Though substantially enriched for glyoxysomes, the final organelle pellet usually contains some mitochondrial contamination. The proteins can be analyzed by SDS-PAGE and Coomassie blue staining (Fig. 3.19.4).

GROWING SEEDLINGS FOR GLYOXYSOME ISOLATION

SUPPORT PROTOCOL

Seedlings are required for isolation of the glyoxysomes.

Materials

Pumpkin seeds (or other plant seeds depending on desired organelle source)
Medium-coarse vermiculite (available from gardening supply stores)
Flat for germination (available from gardening supply stores)
Plastic wrap

1. Place ~75 ml pumpkin seeds on about 2 cm of medium-coarse vermiculite in a germination flat and sprinkle with a thin layer of vermiculite just sufficient to cover the seeds. Water the seeds liberally and cover the flat loosely with plastic wrap to maintain high humidity for germination.

The amount of seeds may need to be adjusted for other plant types to generate 25 to 35 g of tissue.

2. Germinate pumpkin seeds (or desired organelle source) in the dark at 22° to 25°C.

Optimize the temperature for the particular tissue source.

Germination and growth routinely takes 5 to 7 days, depending on the time of year (shorter during the summer months).

3. Water the seedlings 3 to 4 days after planting to promote germination.

4. Harvest the seedlings when they are ~5 to 8 cm tall.

Seeds that have just begun to germinate can also be harvested, as long as the seed coat is removed prior to grinding.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Grinding buffer

2× stock solution:
17.844 g tetrasodium pyrophosphate (40 mM final)
0.744 g EDTA (2 mM final)
109.32 g D-mannitol (0.6 M final)
Add 950 ml H_2O
Adjust pH to 7.5 with glacial acetic acid
Add H_2O to 1 liter final volume
Sterilize through 0.45-μm filter
Store up to 6 months at 4°C
1× working solution (170 ml): Add 85 ml of 2× grinding buffer stock solution to 85 ml water. Add 170 mg BSA (1 mg/ml) to the buffer and mix well. Make fresh working solution for each glyoxysome preparation.

The final concentrations in the working solution are 20 mM tetrasodium pyrophosphate, 1 mM EDTA, and 0.3 M D-mannitol.

Percoll, 28% (v/v)/resuspension buffer, 1×

28 ml Percoll
50 ml 2× resuspension buffer stock solution (see recipe)
22 ml H_2O
Store up to 6 months at 4°C

Resuspension buffer

2× stock solution:
4.776 g HEPES (20 mM final)
109.32 g D-mannitol (0.6 M final)
Add 950 ml H_2O
Adjust pH to 7.2 with KOH
Adjust volume to 1 liter with H_2O
Sterilize through 45-μm filter
Store up to 6 months at 4°C
1× working solution (20 ml): Add 10 ml of 2× resuspension buffer stock solution to 10 ml distilled water. Make fresh working solution for each glyoxysome preparation.

The final concentrations in the working solution are 10 mM HEPES and 0.3 M D-mannitol.

Sucrose, 2 M

Dissolve 85.575 g sucrose in water. Bring final volume to 125 ml. Filter the solution through a 45-μm filter. Store up to 6 months at 4°C.

Filtration can be tricky because of the high viscosity of the solution. A vacuum or syringe filter may be used. Filtering may be easier if the solution is warm.

COMMENTARY

Background Information

In the late 1960s, cell biologists determined that the spherical particles observed in plant cell electron micrographs were in fact classes of a distinct and vital organelle, the peroxisome (Tolbert, 1971). Peroxisomes derive their name from their critical function in the metabolism of hydrogen peroxide. For example, the degradation of hydrogen peroxide is catalyzed by the matrix protein catalase, arguably the most abundant peroxisomal enzyme. Catalase is also a major constituent of the distinctive peroxisomal core or crystalline inclusion that is sometimes observed in

plant peroxisomes (Heinze et al., 2000). Peroxisomes are small organelles that are bound by a single phospholipid membrane. They can range in size from 0.5 to 1.5 μm. Although they have been observed in a variety of shapes, they are most commonly spherical. While all plant peroxisomes have some enzymes in common (e.g., catalase, thiolase), they are typically divided into several classes based upon their physiological roles, as defined by spatial and temporal parameters (Olsen and Harada, 1995; Beevers, 2002). For example, cotyledons of oilseeds, such as pumpkins, contain a special class of peroxisomes, called glyoxysomes. These sequester all of the enzymes required for the glyoxylate cycle, to supply the growing seedling with energy. In contrast, peroxisomes found later in leaf development have a partially different enzyme complement because of their function in other pathways, including photorespiration (Olsen, 1998).

An interesting feature of peroxisomes is their lack of an organellar genome. This means that nuclear genes encode all constituent proteins. Peroxisomal proteins are synthesized in the cytoplasm and are post-translationally translocated into peroxisomes (Brickner et al., 1997). The biogenesis of peroxisomes most likely involves the growth and division of preexisting peroxisomes (Olsen, 1998; Purdue and Lazarow, 2001). Intense study has elucidated the molecular machinery (PEX proteins) required for the interrelated processes of peroxisome biogenesis and protein import. Import of peroxisomal matrix proteins occurs through two receptor-mediated import pathways. Each pathway is defined by one of two peroxisome targeting signals (PTS1, PTS2) on the cargo proteins that the receptors recognize and transport. Thus, there are two cytosolic receptors (Pex5p, Pex7p) that recognize the targeting signals and bind to the cargo proteins (Subramani, 1996; Johnson and Olsen, 2001; Brown and Baker, 2003). In vitro protein import assays have been invaluable in studies of the dynamics of peroxisomal protein import, addressing questions about the interactions between the receptors and cargo proteins, the energy requirements, the roles of protein chaperones, and the molecular mechanisms of translocation (Mori and Nishimura, 1989; Behari and Baker, 1993; Brickner et al., 1997; Brickner and Olsen, 1998; Crookes and Olsen, 1998; Johnson and Olsen, 2003). The success of these assays relies on the reproducible purification and manipulation of intact peroxisomes and the presence of the relevant protein cargo, receptors, and other biochemical constituents.

In addition to questions of organelle biogenesis, peroxisome biochemistry and function has received recent attention. Many previously uncharacterized enzymes, including alanine aminotransferase, alanine:glyoxylate aminotransferase, and sarcosine oxidase, have been localized to plant peroxisomes (Liepman and Olsen, 2001, 2003; Goyer et al., 2004). A role for peroxisomal β-oxidation in auxin metabolism has been extensively studied (e.g., Zolman et al., 2000, 2001; Zolman and Bartel, 2004). An isozyme of 12-oxophytodienoate reductase, OPR3, which catalyzes the final step of jasmonate biosynthesis, also possesses a peroxisomal targeting signal, suggesting a role for peroxisomes in jasmonic acid signaling (Sanders et al., 2000; Stintzi and Browse, 2000). Thus, peroxisomes have many critical physiological functions throughout the life cycle of plants.

Critical Parameters and Troubleshooting

There are several critical points for consideration during the glyoxysome purification procedure. Care must be taken to ensure that plants are grown in the dark, that the seedlings are ~5 to 8 cm tall, and that exposure to light during harvesting is minimized to get a good yield of glyoxysomes. This is because, when seedlings begin greening and producing the first true leaves, some glyoxysomes transition into leaf peroxisomes while others are probably degraded (there are fewer peroxisomes present in leaves than there are glyoxysomes present in cotyledons). The tissue must remain cold throughout the protocol to help reduce endogenous protease activities that can cause the degradation of glyoxysomes and glyoxysomal peripheral membrane proteins. The tissue should not be overground during the homogenization step. Instead subject the tissue to just enough homogenization needed to break up the majority of the tissue. When homogenization is done correctly, there should be no large chunks of intact tissue evident after homogenization, though smaller pieces of ground tissue should be present.

Anticipated Results

A preparation of 25 g of pumpkin cotyledons should yield 100 μl of glyoxysomes with an approximate protein concentration of 100 mg/ml. The yield largely depends on the successful removal of the glyoxysome layer from the Percoll gradient.

Time Considerations

An efficient glyoxysome preparation should be completed within 2 to 2.5 hr. Purified glyoxysomes can be stored on ice, but will lose structural and metabolic integrity within hours. Intact glyoxysomes do not survive freezing, though many glyoxysomal enzymes will continue to have measurable activity.

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