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Plastoglobule Lipid Droplet Isolation from Plant Leaf Tissue and Cyanobacteria

Kiran-Kumar Shivaiah[1,2], **Febri A. Susanto**[1,2], **Elsinraju Devadasu**[1,2], **Peter K. Lundquist**[1,2]

[1] Department of Biochemistry and Molecular Biology, Michigan State University [2] Plant Resilience Institute, Michigan State University

Corresponding Author

Peter K. Lundquist
pklundqu@msu.edu

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Abstract

Plastoglobule lipid droplets are a dynamic sub-compartment of plant chloroplasts and cyanobacteria. Found ubiquitously among photosynthetic species, they are believed to serve a central role in the adaptation and remodeling of the thylakoid membrane under rapidly changing environmental conditions. The capacity to isolate plastoglobules of high purity has greatly facilitated their study through proteomic, lipidomic, and other methodologies. With plastoglobules of high purity and yield, it is possible to investigate their lipid and protein composition, enzymatic activity, and protein topology, among other possible molecular characteristics. This article presents a rapid and effective protocol for the isolation of plastoglobules from chloroplasts of plant leaf tissue and presents methodological variations for the isolation of plastoglobules and related lipid droplet structures from maize leaves, the desiccated leaf tissue of the resurrection plant, *Eragrostis nindensis*, and the cyanobacterium, *Synechocystis* sp. PCC 6803. Isolation relies on the low density of these lipid-rich particles, which facilitates their purification by sucrose density flotation. This methodology will prove valuable in the study of plastoglobules from diverse species.

Introduction

The current understanding of plastoglobule composition and function has emerged through detailed proteomic and lipidomic studies[1,2,3,4,5]. Such studies have been greatly aided by a rapid and effective method of isolation that relies on their very low density for efficient separation using sucrose gradients. Initial methods of plastoglobule isolation were achieved from species such as the beech tree (*Fagus sylvatica*), scotch broom (S*arothamnus scoparius*), onion (*Allium cepa*), spinach (*Spinacia oleracea*), pansy (*Viola tricolor*), pepper (*Capsicum annuum*), and pea (*Pisum sativum*)[6,7,8,9,10,11,12,13]. An updated method to isolate chloroplast plastoglobules in a more efficient and better yielding manner was later presented by Ytterberg et al.[3,14]. While initially employed for the study of the plastoglobules of *Arabidopsis thaliana* leaf chloroplasts, we have successfully employed this updated method for the healthy leaf tissue of other plant species, both monocot and dicot, including maize (*Zea mays)*, tomato (*Solanum lycopersicum)*, lovegrass

(*Eragrostis nindensis)*, purple false brome (*Brachypodium distachyon)*, and wild tobacco (*Nicotiana benthamiana*; unpublished results). Furthermore, the isolation method has been successfully adapted to the plastoglobules of cyanobacteria, including *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120[15], and the desiccated leaf tissue of the resurrection plant, *E. nindensis*.

Chloroplast plastoglobules of healthy leaf tissue are physically connected to the thylakoid membranes[16]. Despite this physical continuity, the two chloroplast sub-compartments maintain distinct lipid and protein compositions, although the regulated exchange of lipid and protein between the two compartments has been proposed[2,4,17,18,19]. In fact, an interesting hemifusion model has recently been proposed for the trafficking of neutral lipids between chloroplasts and cytosol[19]. Due to the physical continuity of plastoglobules and thylakoids, the isolation method with healthy leaf tissue begins with the collection of a pelleted crude thylakoid preparation, which is subsequently sonicated to separate the plastoglobules from the thylakoids, which is in contrast to methods used for isolating cytosolic lipid droplets[20]. Ultracentrifugation on a sucrose cushion then floats the low-density plastoglobules up through the sucrose, effectively separating them from the thylakoids, nuclei, and other high-density material. In contrast, plastoglobules in cyanobacteria, as well as those of desiccated leaf tissue, evidently exist *in vivo* in a free-floating form. Hence, their isolation involves directly floating on a sucrose gradient. This article demonstrates the isolation method from healthy leaf tissue and further demonstrates two variations that can be used to isolate plastoglobules from desiccated leaf tissue or cyanobacterial cultures, greatly expanding the physiological breadth and evolutionary context in which plastoglobules can be studied.

Isolated plastoglobules can subsequently be used for any number of downstream analyses to investigate molecular characteristics. We have used the isolated plastoglobules from *A. thaliana* leaf tissue for extensive proteomic and lipidomic analysis under differing environmental conditions or genotypes, demonstrating the selective modification of protein and lipid composition in adaptation to stress[2,4,21,22]. In addition, *in vitro* kinase assays that demonstrate trans-phosphorylation activity associated with isolated plastoglobules have been performed[22], the oligomeric states of protein components has been investigated using native gel electrophoresis [21], and protease-shaving assays have been performed[23].

The primary benefit of this method is the relative speed of the procedure. In our experience, the protocols outlined below can be fully completed within approximately 4 h. An alternate method to isolate plastoglobules from leaf tissue has been described, which allows the simultaneous isolation of other chloroplast sub-compartments[24]. This alternative method offers some clear advantages when quantitative comparison to the other chloroplast sub-compartments is necessary or desired. However, this alternative method is also more tedious and will provide a significantly lower yield of isolated plastoglobules from comparable quantities of leaf tissue. When a focused study of plastoglobules is the aim, the methodology outlined here is the optimal choice. Nonetheless, total leaf and crude thylakoid aliquots can be collected during the sample preparation, and it is highly recommended to do so, to have reference samples for subsequent comparison.

Protocol

1. Crude plastoglobule isolation

1. Crude plastoglobule extraction from un-stressed maize leaf tissue
    1. Acquire six healthy maize seedlings approximately 3 weeks old and nearly at the V5 growth stage, weighing approximately 120 g.
    2. Clip off all the leaves at the base of the stem, rapidly dunk them in an ice bath, and transport to the cold room.
    3. Working under a green safety lamp, remove the maize leaves from the ice bath and snip them into smaller pieces (around 5 cm x 5 cm) using scissors.
    4. Gently but thoroughly grind half of the clipped leaf tissue in a commercial blender in 350 mL of grinding buffer (**Table 1**). Start-stop the blender 5x-6x to ensure that all the leaves are cut. Do not use higher than level 7 on the blender.
    5. Filter the homogenate through one layer of 25 µm nylon cloth on a large funnel into four 250 mL centrifuge bottles. Then, repeat step 1.1.4 with the second half of the clipped leaf tissue.
    6. Evenly divide the filtrate between the four bottles and centrifuge for 6 min at 1,840 x *g* at 4 °C. Remove an aliquot of the leaf filtrate and set it aside prior to centrifugation to be stored as a representative total leaf sample.
    7. Pour off the resulting supernatant and gently resuspend the pellet in 12 mL of medium R 0.2 containing 0.2 M sucrose (**Table 1**) by swirling and resuspending with gentle movements of the brush. After resuspending each pellet, carry the suspension over to the next bottle and repeat the resuspension. Pool the suspensions into one bottle.
    8. Distribute the pooled suspension between six 3 mL ultracentrifuge tubes, reaching a maximum volume of 2.5 mL in each tube.
    9. Sonicate each tube 4x, for 10 s each time, using a tip sonicator at an amplitude of 100%. Be careful to keep the sonicator horn submerged and away from the liquid surface to prevent frothing.
    10. Slowly move the sonicator horn up and down within the suspension during each round. Alternate between the four tubes, returning each tube to an ice bucket after each sonication to allow the sample to cool.
    11. Centrifuge the sonicated crude thylakoid suspension at 150,000 x *g* for 30 min at 4 °C. Harvest the resulting floating pad of crude plastoglobules from the surface of the sucrose cushion (readily seen as a yellow, oily pad) using a syringe and 22 G needle by skimming the surface of the cushion with the opening of the needle, recovering approximately 500 µL from each tube. Deposit into a 2.0 mL tube.
    12. Collect aliquots of the crude thylakoid prior to and after the sonication and release of plastoglobules. Continue to step 2.1. Alternatively, store the crude plastoglobules at −80 °C and purify at a later time.
2. Crude plastoglobule extraction from desiccated *E. nindensis* leaf tissue
    1. Acquire a pot (comprising of three individual plants per pot) of fully desiccated, 8-9-week-old *E. nindensis* (nearly 40 g of tissue).

2. Clip off all the leaf tissue from the base of the plant, just above the soil, using scissors and immediately dunk the tissue in an ice bath. Transport the tissue to the cold room.

3. Working under a green safety lamp, snip the *E. nindensis* leaves into smaller pieces (around 5 cm x 5 cm) using scissors.

4. Gently but thoroughly grind the leaves with 100 mL of grinding buffer (**Table 1**) in a commercial blender. Start-stop the blender several times to ensure that all leaves are being cut. Do not use higher than level 7 on the blender.

5. Filter the homogenate through one layer of 25 µm nylon cloth on a large funnel into a 250 mL conical flask. Remove an aliquot of the leaf filtrate and set it aside prior to centrifugation to be stored as a representative total leaf sample.

6. Add the appropriate amount of sucrose to bring to a final concentration of 0.5 M and distribute the solution into 250 mL centrifuge tubes.
   NOTE: A higher concentration of sucrose compared to the *Z. mays* preparation is used to ensure flotation of the cytosolic lipid droplets prior to the subsequent sonication/release of the thylakoid-bound plastoglobules.

7. Centrifuge the tubes at 45,000 x *g* for 30 min at 4 °C. A mixture of plastoglobules and cytosolic lipid droplets will be readily seen as a yellow, oily pad on or near the surface of the sucrose cushion (**Figure 1B**). Collect the floating material using a syringe with a 22 G needle by skimming the surface of the cushion with the opening of the needle, and deposit into a tube.

8. Discard the remaining supernatant after collecting the free-floating plastoglobule/lipid droplet mixture.

9. To isolate the plastoglobules connected to residual thylakoid, resuspend the pellet in 12 mL of medium R 0.2 by swirling and resuspending with gentle movements of the brush. Pool the suspensions into one bottle. Continue to step 1.1.8.

3. Crude plastoglobule extraction from cyanobacteria

   1. Grow a 50 mL culture of *Synechocystis* sp. PCC 6803 to the stationary phase (about 7-10 days) and adjust the cell density to an OD_{750} of 2.0 using a spectrophotometer. For culture conditions, use BG-11 media and grow in an incubator at a light intensity of 150 µmol photons/m^2/s, 2% CO_2, 32 °C, and with continuous shaking at 150 rpm.

   2. To remove the polysaccharides, wash the cells by centrifuging the 50 mL culture at 6,000 x *g* for 45 min at 4 °C and subsequently removing the supernatant. Continue by washing the cells twice in 50 mL of buffer A (**Table 1**). Remove an aliquot of the cell homogenate and set it aside prior to centrifugation to be stored as a representative total cell sample

   3. Resuspend the washed pellet in 25 mL of Buffer A and break the cells using a French pressure cell at 1,100 psi, repeating the process 3x (put the sample on ice between each cycle to avoid protein denaturation) until the lysed color changes from green to red-blue-green under white light. Use a pre-cooled cell and perform this step in the cold room.

   4. Distribute the resulting homogenate between eight 3 mL ultracentrifuge tubes filled to a maximum of 2.5

mL in each tube and then carefully overlay with 400 µL of medium R, producing a step gradient.

NOTE: Refer to Yang, et al. and Kelekar, et al. for detailed descriptions of sucrose gradient preparation[25,26].

5. Carefully balance the tubes by adding additional medium R, as necessary, and centrifuge for 30 min at 150,000 x *g* at 4 °C. The thylakoid and other heavier organelles (including any polyhydroxyalkanoate bodies) will pellet, while plastoglobules will be readily seen as a yellow, oily pad on or near the top of the sucrose gradient (**Figure 1C**).

6. Harvest the resulting floating pad of crude plastoglobules with a syringe and 22G needle and deposit into a 2 mL tube. Scrape plastoglobules off the side of the ultracentrifuge tube wall with the needle tip if necessary.

7. Continue to step 2.2. Alternatively, store crude plastoglobules at -80 °C and purify later.

2. Harvesting pure plastoglobules

1. Plant tissue processing

    1. Produce sucrose gradients in 2.5 mL ultracentrifuge tubes by first layering with 500 µL of crude plastoglobules from step 1.1 or step 1.2 mixed with 500 µL of medium R 0.7, to bring to a total volume of 1 mL, then overlay with 400 µL of medium R 0.2, followed by overlaying with 400 µL of medium R.

    NOTE: Refer to Yang et al. and Kelekar et al. for detailed descriptions of sucrose gradient preparation[25,26].

    2. Carefully balance the tubes by adding excess medium R to the top layer, as necessary. Then centrifuge at 150,000 x *g* for 1.5 h at 4 °C.

    3. Harvest the resulting floating pad of pure plastoglobules (**Figure 1A**) with a syringe and 22G needle and deposit into a 2 mL tube. Scrape plastoglobules off the top of the centrifuge tube wall with the needle tip if necessary.

    4. Aliquot the pure plastoglobules and flash freeze in liquid nitrogen. Store directly at -80 °C or lyophilize to a dry powder.

2. Cyanobacteria processing

    1. Produce a sucrose gradient in four 2.5 mL ultracentrifuge tubes layered first with 500 µL of the crude plastoglobules from step 1.3 mixed with 750 µL of medium R 0.7, then overlay with 750 µL of medium R 0.2.

    NOTE: Refer to Yang et al. and Kelekar et al. for detailed descriptions of sucrose gradient preparation[25,26].

    2. Centrifuge the sucrose gradient at 150,000 x *g* for 90 min at 4 °C. Collect the pure plastoglobules (**Figure 1C**) with a syringe and 22 G needle from the top phase of the sucrose gradient and transfer to a 1.5 mL tube.

    3. Aliquot the pure plastoglobules and flash-freeze in liquid nitrogen. Store directly at −80 °C or lyophilize to a dry powder.

Representative Results

Upon completion of step 1 of the protocol, one should be able to readily see a considerable amount of plastoglobule/ lipid droplet material floating on (or near) the top layer of

the sucrose cushion (**Figure 1B-C**). Other fractions could also be collected at this stage. For example, the thylakoids will be pelleted and can be re-suspended with medium R 0.2 for subsequent analyses. After subsequent centrifugation, purified plastoglobules will be obtained at or near the surface of the sucrose gradient, as shown in **Figure 1A**,**C**. It has been seen in certain circumstances (e.g., specific genotypic lines or environmental conditions) that the plastoglobules will isolate as two distinct sub-populations separating at different layers in the gradient: a low-density fraction on the gradient surface and a second, denser fraction that settles at the interface between the top two gradient layers. While a careful comparative analysis of the separating fractions has not previously been performed, it seems likely that these sub-populations represent differently sized plastoglobules, in which those with smaller diameter (and hence greater surface area to volume ratio and protein to lipid ratio) will settle lower in the gradient.

In an unsuccessful attempt, one will see little or no visible plastoglobules on the surface of the sucrose cushion after the first round of centrifugation. Failure to successfully isolate enough plastoglobules can depend on several factors that may need to be optimized. In particular, the sonication technique (when using healthy leaf tissue) can have a significant impact on the success. Additionally, the necessary amount of plant/cyanobacterial material will be dependent on the specific species and its stress condition.

After the successful isolation of plastoglobules, it is possible to validate the purity of the isolated plastoglobules using immunoblotting of the marker proteins for plastoglobules and thylakoids (the primary contaminating compartment). In **Figure 1D**, representative immunoblots are seen using antibodies raised against *A. thaliana* FBN1a, as a marker for plastoglobules[2], and against *A. thaliana* photosystem II subunit D1, as a marker of thylakoids[27]. The FBN homologs in *Synechocystis* sp. PCC 6803 associate primarily with thylakoids rather than plastoglobules.

One should also carefully consider the manner of storage, which may depend on the intended downstream studies. Especially for downstream analyses of prenyl-lipid pigment lipids, it is crucial to minimize exposure of the samples to direct light to avoid damage of the photo-labile compounds. Purified plastoglobules can be used for downstream experiments such as lipidomic- or proteomic-based experiments. Plastoglobules are characterized by a very low protein to lipid ratio, which manifests in their low density and capacity to float on the sucrose; thus, a low protein concentration of the plastoglobule samples is normal. For this reason, it is advisable to remove lipids prior to protein separation by SDS-PAGE using acetone protein precipitation or chloroform/methanol extraction.

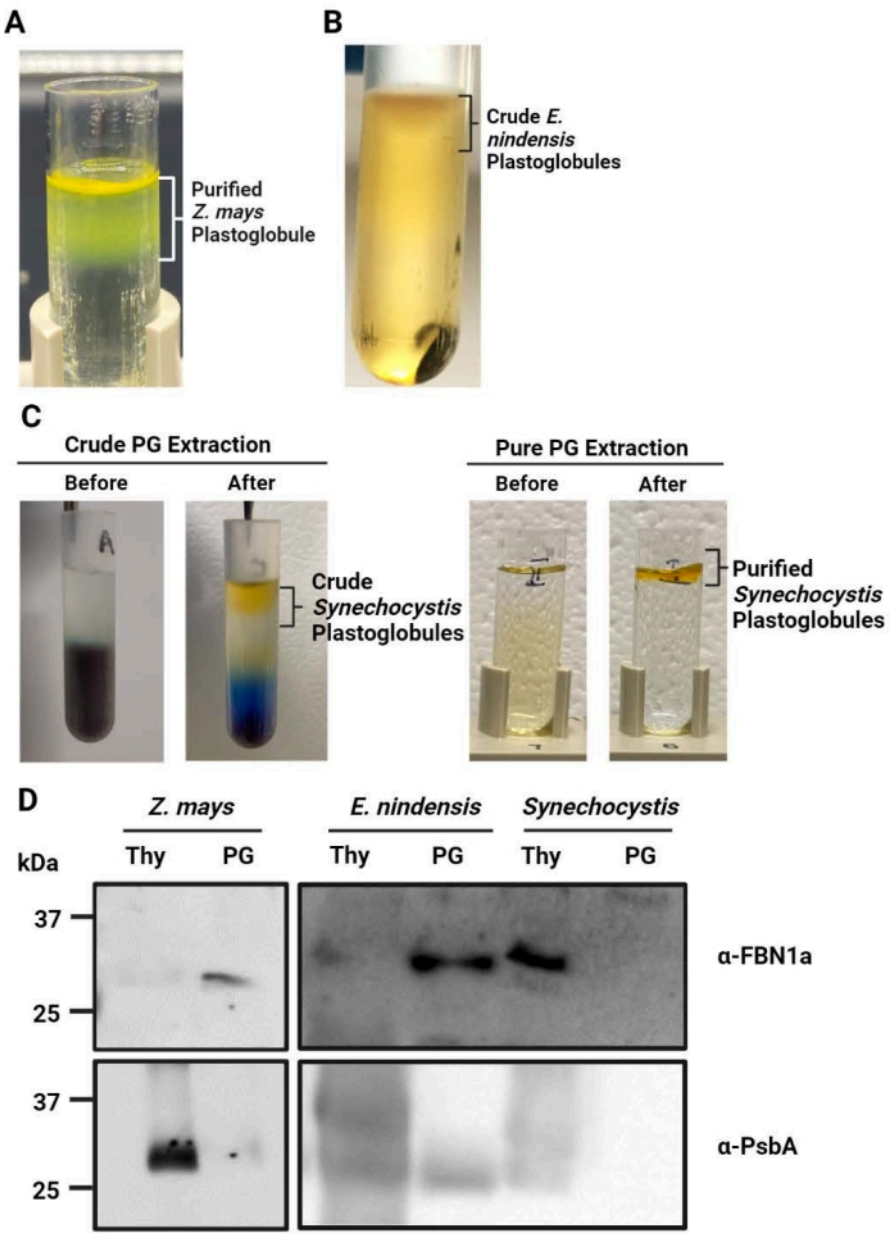

Figure 1: Isolation and immunoblotting of plastoglobules and thylakoids. (**A**) A representative purified plastoglobule sample from *Z. mays* prior to extraction from the sucrose gradient. (**B**) A representative crude plastoglobule sample from desiccated *E. nindensis* prior to extraction from the sucrose cushion. (**C**) A representative crude (left) and pure (right) plastoglobule sample from *Synechocystis* sp. PCC 6803, showing both before and after ultracentrifugation of the loaded sucrose cushion and gradient, respectively. (**D**) Anti-fibrillin1a antibody (α-FBN1a) was used to monitor the accumulation of fibrillin, a marker protein of plastoglobules in higher plants. The fibrillin ortholog accumulates predominantly in the isolated thylakoids of cyanobacteria (Synecho, *Syenchocystis* sp. PCC 6803). Anti-photosystem II subunit D1 (α-PsbA) was used to

validate the depletion of thylakoids from plastoglobule isolations. In each lane, 5 µg of thylakoids and 10 µg of plastoglobule protein were loaded. Abbreviations: PG = plastoglobules; Thy = thylakoids. Please click here to view a larger version of this figure.

Buffer compositions [a]	
Grinding Buffer	50 mM HEPES-KOH (pH 8.0)
	5 mM $MgCl_2$
	100 mM sorbitol
	5 mM ascorbic acid [b]
	5 mM reduced cysteine [b]
	0.05 % (w/v) BSA [b]
Medium R	50 mM HEPES-KOH (pH 8.0)
	5 mM $MgCl_2$
Medium R 0.2	50 mM HEPES-KOH (pH 8.0)
	5 mM $MgCl_2$
	0.2 M sucrose
Medium R 0.7	50 mM HEPES-KOH (pH 8.0)
	5 mM $MgCl_2$
	0.7 M sucrose
Buffer A	25 mM HEPES-KOH (pH 7.8)
	250 mM sucrose
[a] *Final concentrations of each buffer component are provided.*	
[b] *Must be added fresh on the day of the isolation. While buffers can be prepared the day before the isolation, these certain ingredients must be added fresh on the day of the isolation, as well as any phosphatase and protease inhibitors.*	

Table 1: Buffer recipes for plastoglobule isolation from plant leaf tissue or cyanobacteria.

Phosphatase Inhibitor Cocktail [b]	
Inhibitor	**Final Conc. (mM)**
Na-Fluoride	50
β-Glycerophosphate#2Na#5H_2O	25
Na-OrthoVanadate	1
Na-Pyrophosphate#10H_2O	10
[b] *Phosphatase inhibitors must be added fresh the day of the isolation.*	

Table 2: Phosphatase inhibitor cocktail.

Protease Inhibitor Cocktail [a]				
Inhibitor	**Stock (mg/mL)**	**Stock medium**	**Dilution factor**	**Final conc. (mg/mL)**
Antipain#2HCl	20	water	400x	50
Bestatin	1	0.15 M NaCl	25x	40
Chymostatin	20	DMSO	2000x	10
E-64	20	water	2000x	10
Leupeptin (hemisulfate)	20	water	4000x	5
P-ramidon#2Na	20	water	2000x	10
AEBSF	50	water	1000x	50
Aprotinin	10	water	5000x	2
[a] *The protease stock solutions must be stored at -20 °C in small aliquots for long term storage. Thaw and add fresh to appropriate buffer immediately prior to isolation.*				

Table 3: Protease inhibitor cocktail.

Discussion

To minimize physiological/biochemical changes to the material and protect certain photo- and thermo-labile prenyl-lipid pigments that are a rich component of plastoglobules, it is critical to perform the isolation at 4 °C and protected from light. As indicated above, the initial steps are performed in the cold room under a safety lamp using a green-emitting light bulb. The subsequent steps performed in the laboratory are under

dimmed lights and use ice or refrigerated centrifugation. For similar reasons, the inclusion of fresh protease inhibitors (and phosphatase inhibitors if there is an interest in studying the phospho-regulation of proteins) is critical (**Table 2** and **Table 3**).

While other methods (e.g., a Dounce homogenizer, freeze-thaw cycles) could, in principle, be employed to release plastoglobules from thylakoids in higher plant chloroplasts, sonication has been found to be far superior in providing the best yield and purity (unpublished results). Sonication may create artificial vesicles from thylakoid lamellae or endoplasmic reticulum; however, these vesicles would be very protein-rich and, hence, dense, precluding their flotation on the sucrose gradient.

When extracting the floating pad of pure plastoglobules from the sucrose gradient, it is beneficial to extract them in the smallest amount of medium R as possible. Acquiring concentrated plastoglobules in this manner facilitates downstream studies by minimizing the necessary volumes of sample. The use of a syringe and 22 G needle is the most effective strategy to extract the plastoglobules. Due to their hydrophobic, lipid-rich nature, they are extremely sticky, and the use of pipette tips or a spatula should be avoided at all costs. It has been found that the loss of plastoglobules due to sticking is best minimized with a syringe and needle, although it does not seem possible to completely prevent some loss during extraction.

If an easily visible, yellow-creamy layer of plastoglobules is not seen floating on or near the surface of the sucrose gradient, insufficient plant/cyanobacterial material may have been used for the isolation. It has been found that monocots give higher plastoglobule yields than dicots from comparable amounts of plant tissue (unpublished results). While proper amounts of starting biological material are indicated for the specific isolations exemplified in this article, the necessary amounts of tissue for efficient extraction must be determined empirically based on the species, tissue, and environmental conditions of the organism. In general, stressed (but not necrotic) leaf tissue or cyanobacterial cultures will give higher yields of plastoglobules. When using *A. thaliana* leaf tissue, two flats of *A. thaliana* plants (nearly 140 individual plants) from the mid-vegetative growth stage are typically sufficient for the isolation of plastoglobules, especially when the plant material is mildly stressed, for example, by a light stress treatment[2,4]. As an alternative explanation for poor yields, the initial homogenization of the leaf tissue with the blender may have been too vigorous and separated the plastoglobules from the thylakoids prematurely, or the sonication of the crude thylakoid material may have been insufficiently vigorous to effectively release the plastoglobules before flotation (these points are not an issue when extracting from cyanobacteria, which are free-floating *in situ*).

It is important to keep in mind that plastoglobule isolation will represent the bulk population of plastoglobules from a tissue sample. Hence, any possible heterogeneity in lipid, protein, or function would not be discernible from subsequent studies. Due to this limitation, it has not been possible to gauge how much heterogeneity may exist among individual plastoglobule lipid droplets. However, transmission electron micrographs of plant leaf tissue from multiple organisms reveal differential staining patterns amongst plastoglobules, even within the same chloroplast[28,29,30,31]. This strongly suggests heterogeneity in the lipid content, although the nature or purpose of this remains unclear. Significantly, electron micrographs of isolated *A. thaliana* plastoglobules

demonstrate that this method of isolation is not biased for the isolation of larger or smaller plastoglobules[2].

The isolation of pure plastoglobules represents the initial step toward their detailed molecular characterization. Numerous downstream applications can subsequently be carried out depending on the specific interests and purpose of the investigator. For example, to investigate the lipid or protein composition, the isolated sample is readily amenable to proteomic or lipidomic studies. The in-gel digestion approach is favored for bottom-up proteomics studies of plastoglobules samples. However, because lipids are in vast excess relative to protein, the lipid can interfere with the SDS-PAGE separation. The initial precipitation of protein using acetone, with subsequent resuspension in Laemmli solubilization buffer, eliminates most lipids, thereby allowing an efficient separation of protein in the SDS-PAGE separating gel. Initial resuspension of the protein pellet in 100 mM tris-HCl buffer and 2% SDS allows accurate determination of the protein content by the bicinchoninic acid method before the addition of reductant, dye, and glycerol. Likewise, lipid purification using liquid-liquid phase separation methods, such as the Bligh and Dyer method, is suitable for downstream lipidomic analyses[32].

Disclosures

No conflicts of interest to declare.

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