Purification of Intact Chloroplasts from *Arabidopsis* and Spinach Leaves by Isopycnic Centrifugation

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ABSTRACT

Chloroplasts are plant-specific organelles. They are the site of photosynthesis but also of many other essential metabolic pathways, such as syntheses of amino acids, vitamins, lipids, and pigments. This unit describes the isolation and purification of chloroplasts from *Arabidopsis* and spinach leaves. Differential centrifugation is first used to obtain a suspension enriched in chloroplasts (crude chloroplasts extract). In a second step, Percoll density gradient centrifugation is used to recover pure and intact chloroplasts. The Basic Protocol describes the purification of chloroplasts from *Arabidopsis* leaves. This small flowering plant is now widely used as a model organism in plant biology as it offers important advantages for basic research in genetics and molecular biology. The Alternate Protocol describes the purification of chloroplasts from spinach leaves. Spinach, easily available all through the year, remains a model of choice for the large-scale preparation of pure chloroplasts with a high degree of intactness. *Curr. Protoc. Cell Biol.* 40:3.30.1-3.30.14. © 2008 by John Wiley & Sons, Inc.

Keywords: chloroplast • *Arabidopsis* • spinach • purification • leaves

INTRODUCTION

Plastids are semiautonomous organelles found in plants and some protists. In plant leaves, plastids are photosynthetically active and named chloroplasts. They are also the site of essential metabolic pathways, such as syntheses of amino acids, vitamins, lipids, and pigments (Wise and Hoober, 2006). Biochemical, physiological, and proteomic analyses of the chloroplast can only be achieved using pure and intact chloroplasts that have conserved their metabolic activities and functional surrounding envelope membranes (Block et al., 2007). Localized at the interface between the plastid stroma and the cytosol, the envelope membranes have to remain intact to allow, for instance, physiological characterization of the various transport systems regulating plastid metabolism. Therefore, the isolation of pure and intact organelles becomes essential when characterization of plastid functions is expected. The procedure described here couples differential centrifugation to obtain a crude extract of chloroplasts with isopycnic centrifugation (Percoll-based density gradients) to recover pure and intact chloroplasts.

The Basic Protocol describes the step-by-step purification procedure to isolate chloroplasts from *Arabidopsis* leaves. Since the complete sequencing of its genome (The AGI, 2000), *Arabidopsis thaliana*, a small flowering plant, has become a widely used model organism in plant biology. The generation of large collections of insertion mutants, together with growing numbers of gene expression databases, offer important advantages for basic research in genetics and molecular biology. This Basic Protocol, to isolate chloroplasts from *Arabidopsis thaliana*, is provided with some tricks to optimize yield and purity. Conditions to grow *Arabidopsis* plants suitable for chloroplast isolation are also described in the Support Protocol.

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The Alternate Protocol describes the purification of chloroplasts from spinach (*Spinacia oleracea* L.) leaves. Due to its almost permanent availability in markets, this plant remains a traditional model plant for physiologists and biochemists. Spinach also contains low levels of phenolics and other inhibitory compounds (prevalent in numerous cultivated species), and thus is a good choice to easily obtain fully competent CO_2-fixing chloroplasts. Finally, the Alternate Protocol allows isolating large amounts of pure and intact chloroplasts.

BASIC PROTOCOL

ISOLATION OF PURE AND INTACT CHLOROPLASTS FROM *ARABIDOPSIS* LEAVES

The chloroplast is, in general, an extremely fragile structure. Thus, skill and expertise are required in order to avoid large-scale rupture of the two envelope membranes during chloroplast isolation. The media used throughout the procedure must contain an osmoticum (0.4 M sorbitol for *Arabidopsis* chloroplast) to avoid osmotic shock and thus, disruption of the organelles. The homogenization of the tissue is another critical step. The grinding process must be as short as possible. Longer blending, while apparently strongly improving the yield of recovered chlorophyll, rapidly increases the proportion of broken chloroplasts in the crude cell extract and strongly affects further purification steps. Filtration of the homogenate then allows limiting contamination with cellular debris and unbroken cells. A first centrifugation allows obtaining a crude chloroplast suspension. Intact chloroplasts are then purified by isopycnic centrifugation on preformed continuous Percoll gradients. The outline of the strategy is described in Figure 3.30.1.

Materials

Percoll gradients solution (see recipe)
Four to six boxes containing 3- to 4-week-old *Arabidopsis* plantlets (400 to 500 g of rosette material, see Support Protocol)
Grinding medium (see recipe)
Washing medium (see recipe)
80% (v/v) acetone

50-ml polypropylene tubes
Superspeed refrigerated centrifuge (e.g., Sorvall RC5), with the following rotors and corresponding tubes: fixed angle rotors GS-3 with six 500-ml plastic bottles and SS34 with eight 50-ml polypropylene tubes; swinging bucket rotor HB-6 with six 50-ml polycarbonate tubes or equivalent
1000-ml and 5-liter beakers
Ice and ice buckets
Motor-driven blender, 3 speeds, 1 gallon (3.785 liter; e.g., Waring blender)
Muslin or cheesecloth, 80-cm
Nylon blutex (50-μm aperture; Tripette et Renaud)
Pasteur pipet
Vacuum aspirator with a flask as a liquid trap
Curved plastic spatula
1- and 10-ml pipets
1-ml microcentrifuge tube
Vortex
1-ml spectrophotometer glass cuvette
UV spectrophotometer

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout.

NOTE: All operations are carried out at 0° to 5°C either by keeping samples on ice or by working in a cold room.

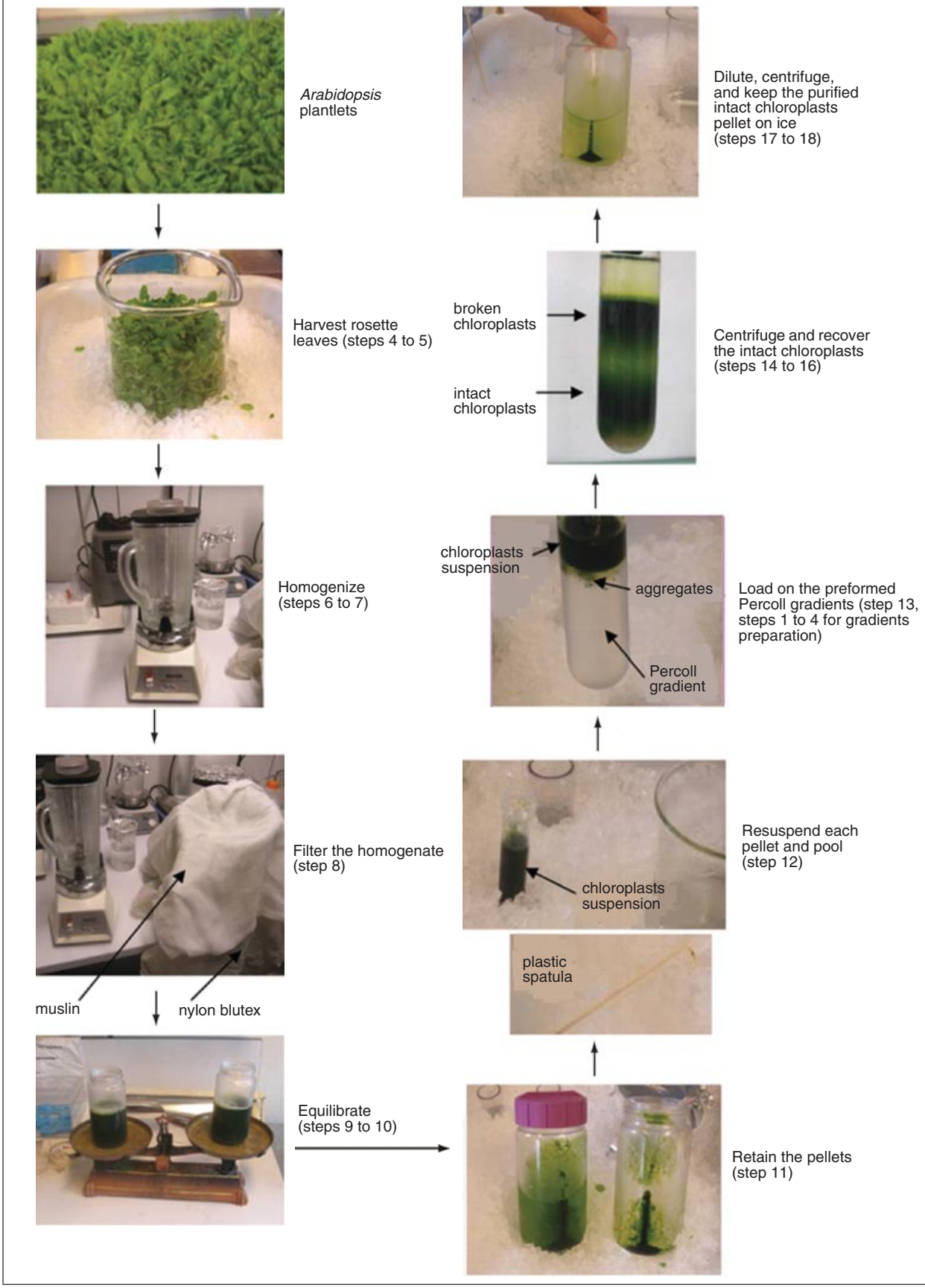

Figure 3.30.1 Outline of the strategy used to purify intact chloroplasts from *Arabidopsis* leaves. For color version of this figure see *http://www.currentprotocols.com*.

Prepare preformed continuous Percoll gradient

1. Prior to the experiment, prepare six 50-ml polypropylene tubes containing 30 ml Percoll gradients solution.

2. Preform Percoll gradients for chloroplast purification by centrifuging 55 min at 38,700 × *g* (Sorvall SS-34 rotor), 4°C.

   Vertical rotors can easily be used to obtain preformed Percoll gradients and subsequently purify chloroplasts (Douce and Joyard, 1982). It is recommended that the brake be disconnected or that the automatic rate controller (if available) of the centrifuge be used to prevent mixing of the gradients at the critical stage of deceleration.

3. Store the tubes containing preformed continuous Percoll gradients in the cold room until use.

   Percoll gradient can be prepared 1 or 2 days before the experiment.

Prepare Arabidopsis leaves and homogenize the tissue

4. Harvest 400 to 500 g of rosette leaves from boxes containing 3- to 4-week-old *Arabidopsis* plantlets.

   It is tempting to start from large amounts of material (huge rosettes with large leaves). It however appears that staring from younger leaves (3- to 4-week-old) improves yield, purity, and integrity of the purified organelles.

   The number of starch granules present in chloroplasts is critical for the preparation of intact chloroplasts: chloroplasts containing large starch grains will usually be broken during centrifugation (Douce and Joyard, 1982). Therefore, prior to the experiment, the plants should be kept for several hours in a dark and cold room (4°C) to reduce the amount of starch. A good way to proceed is to place the plants under such conditions the day before the extraction (we usually perform this at the beginning of the afternoon prior to the day of the experiment).

5. Preweigh a 1000-ml beaker, cool the beaker on ice, then place the rosette leaves inside the beaker. Reweigh the beaker and record the tissue weight. Transfer the beaker to a cold room for the next steps.

6. Put the leaf material and enough leaf grinding buffer (with BSA) to cover the leaves (usually ~2 liters of grinding medium for 400 to 500 g leaves) in the blender cup.

   The buffer to tissue ratio is 4 ml/g.

   A minimum of 60 g of leaves is required to obtain intact and purified chloroplasts.

7. Homogenize the leaf material three times, each time 2 sec in a Waring blender at high speed (see Video 1 at *http://www.currentprotocols.com*).

   The grinding process should be as short as possible. Longer blending improves the yield of recovered chlorophyll, but increases the proportion of broken chloroplasts in the homogenate, and then affects further purification steps.

8. Rapidly filter the homogenate through 4 to 5 layers of muslin and one layer of nylon blutex that line the inside of a precooled funnel placed over a 5-liter beaker. Collect the filtered homogenate in the beaker below the funnel. Gently squeeze the homogenate leaves inside the muslin/nylon blutex bag (see Video 1 at *http://www.currentprotocols.com*), to extract all the liquid.

   The filtration process should also be rapidly performed. After the grinding process, organelles are released in a crude extract consisting of broken cells, having released some very aggressive compounds (e.g., proteases, lipases, and phenolic compounds). Storing the organelles for too long in such a homogenate strongly affects their integrity and functioning. A short delay between grinding (step 7) and concentration (step 10) is thus expected to improve yield of intact chloroplasts.

9. Put the remaining tissue in the blender cup with enough grinding buffer to cover the tissue, and repeat steps 7 and 8. Collect the filtered homogenate in the same beaker.

Centrifuge the homogenate

10. Equally distribute the filtered suspension into six 500-ml bottles for centrifugation and centrifuge them 2 min at 2070 × *g* (Sorvall GS-3 rotor), 4°C.

    Do not forget to equilibrate the pairs of bottles on a balance prior to centrifugation.

11. Carefully discard the supernatant from each tube, retaining the pellets (pour out the liquid holding the tube with the pellet on the top of the tube; see Video 1 at *http://www.currentprotocols.com*). Remove the remaining supernatant with a Pasteur pipet connected to a vacuum aspirator with a flask as a liquid trap. Keep the pellets on ice.

    These pellets contain crude chloroplast fractions.

Purify the chloroplasts

12. Carefully resuspend each pellet, representing crude chloroplast fraction, by adding a minimal volume of washing medium using a curved plastic spatula (see Video 1 at *http://www.currentprotocols.com*).

    Use a 10-ml pipet to add 2 to 3 ml of washing medium in each tube. Carefully resuspend each pellet and gently aspirate the resuspended chloroplasts with a 10-ml pipet (do not use pipets with very fine tips, to limit mechanical breaking of the organelles).

    In order to homogenize chloroplast aggregates found in the bottom of the tube, carefully mix the suspension.

13. Pool the resuspended chloroplasts in one tube. Rinse each tube with additional washing medium.

    The final volume should not exceed 36 ml.

14. Load the crude chloroplast suspension on the top of the six preformed Percoll gradients (6 ml per tube).

    For this step, carefully tilt the Percoll gradient tube and load the chloroplast suspension very slowly, trying not to mix the chloroplast suspension with the upper Percoll layer. Again, do not use pipets with too fine tips.

15. Centrifuge the gradients 10 min at 13,300 × *g* (Sorvall swinging HB-6 rotor), 4°C. Carefully remove the upper part of the gradient containing the broken chloroplasts (Fig. 3.30.1) by aspirating with a Pasteur pipet connected to a vacuum aspirator with a flask as a water trap (see Video 1 at *http://www.currentprotocols.com*).

    It is recommended the brake be disconnected or that the automatic rate controller (if available) be used to prevent mixing of the gradients at the critical stage of deceleration.

16. Recover intact chloroplasts (a broad dark green band in the lower part of the gradient) with a 10-ml pipet. Gently pool the intact chloroplasts in a 500-ml plastic bottle.

    Broken chloroplasts are present in the upper part as a broad band. A small pellet containing cell pieces and large debris is found at the bottom of the tube. To limit cross-contamination with other cell fractions, do not aspirate this debris with the intact chloroplasts.

17. Dilute the chloroplast suspension 3- to 4-fold (up to a final volume of 500 ml) with 200 to 300 ml washing medium. Centrifuge the suspension 2 min at 2070 × *g* (Sorvall GS-3 rotor), 4°C.

18. Discard the supernatant and keep the purified intact chloroplasts pellet on ice.

    At this stage, the yield of intact chloroplasts is 50 to 60 mg protein.

19. Before use, add a minimal volume (~1 ml) of washing medium, or any other buffer containing an osmoticum, to resuspend the purified chloroplasts. Keep the purified chloroplast as a concentrated suspension on ice.

The excellent purity of the Arabidopsis chloroplasts prepared by the Percoll purification step was confirmed using immunological detection of marker enzymes from other subcellular compartments (Seigneurin-Berny et al., 2006). It is worth mentioning that mitochondrial and plasma membrane markers were not detected in the Percoll-purified Arabidopsis chloroplasts. This is also in good agreement with proteomic analyses, which demonstrated that only 5% (6 out of 112) of the Arabidopsis proteins identified in purified chloroplast envelope membranes (Ferro et al., 2003) may correspond to nonplastid proteins. Among them, only one protein appeared to correspond to a previously characterized major plasma membrane component; four proteins may indicate contamination by major tonoplast proteins, and one from glyoxysomes. Considering the high sensitivity of present mass spectrometers it is not surprising to detect minute amounts of these few extra-plastidial contaminants, which are major proteins in their respective subcellular compartments.

Quantify chlorophyll content

20. Quantify the chlorophyll content according to Bruinsma (1961). Add 10 µl of the purified chloroplasts to 1 ml 80% (v/v) acetone in a 1-ml microcentrifuge tube.

21. Vortex the mixture, incubate on ice and in the dark for 15 min, and centrifuge 15 sec at 16000 × *g*, 4°C.

22. Pour in a 1-ml spectrophotometer glass cuvette. Measure the absorbance at 652 nm against a tube containing 80% (v/v) acetone for the zero.

A ratio of $OD_{652}/36 = 1$ corresponds to 1 mg chlorophyll ml^{-1}.

23. Determine integrity of the organelle preparation.

In addition to morphological analysis by microscopy, the integrity of the purified chloroplasts can be confirmed using an oxygen electrode and performing the ferricyanide test for intactness (Walker, 1990). When lacking microscopy or oxygen electrode facilities, a simple way to estimate the integrity of the purified chloroplasts relies on the use of an SDS-PAGE analysis. This method relies on the demonstration that soluble proteins from the stroma are not lost during the purification procedure (Fig. 3.30.2). A protein profile obtained from pure and intact chloroplast preparations reveals that the amount of major soluble protein (RbcL or RubisCO) from the stroma is higher than the amount of the major thylakoid membrane proteins (LHCPs or Light Harvesting Complex Proteins). When broken chloroplasts are analyzed, and due to the loss of soluble proteins resulting from the breakage of the limiting envelope membranes, the ratio of RbcL/LHCPs is strongly reduced. This is illustrated in the Fig. 3.30.2, using broken or intact chloroplasts as obtained, respectively, from upper and lower layers of the Percoll-density gradient.

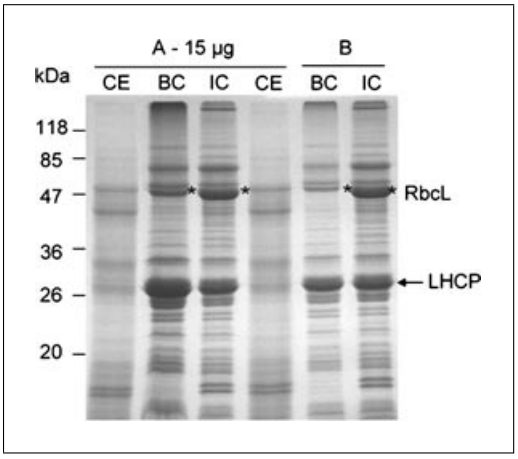

Figure 3.30.2 SDS-PAGE analysis of Percoll-purified chloroplasts from *Arabidopsis*. Fractions (15 µg protein) were analyzed on a 12% SDS-PAGE followed by Coomassie-blue staining. In lanes B, the amount of protein was calculated in order to load the same amount of LHCP in the two lanes of the gel. The star and the arrow respectively indicate the major soluble protein from the stroma, RbcL, and the major membrane proteins from the thylakoids, the LHCPs. Note that the ratio of RbcL/LHCPs is strongly reduced in broken chloroplasts, as a consequence of breakage of the limiting envelope membranes and thus of loss of soluble proteins from the stroma. Abbreviations: CE: crude extract, BC: broken chloroplasts, IC: intact chloroplasts.

ISOLATION OF PURE AND INTACT CHLOROPLASTS FROM SPINACH LEAVES

ALTERNATE PROTOCOL

Spinach leaves remain a tissue of choice for the preparation of chloroplasts with a high degree of intactness. They can be obtained in large amount from markets all through the year, and thus do not require plant growth facilities. Furthermore, chloroplasts isolated from spinach leaves are less fragile than chloroplasts purified from *Arabidopsis* leaves, and due to the unlimited availability of starting material, large amounts of chloroplasts can be obtained daily. Several important differences exist between the protocols used to purify spinach or *Arabidopsis* (Basic Protocol) chloroplasts. First, as starting material can be obtained in large amount, volumes of buffers or numbers of bottles and tubes need to be adapted. Second, the composition of buffers varies (the osmoticum used is sucrose instead of sorbitol). Finally, and as another difference between the two protocols described here, spinach chloroplasts are purified on a discontinuous Percoll gradient (Douce and Joyard, 1982) instead of preformed continuous Percoll gradient.

Materials

3 kg of spinach leaves from the market
Spinach leaf grinding medium (see recipe)
Ice
Spinach washing medium (see recipe)
40% (v/v) Percoll and 80% (v/v) Percoll solutions (see recipes)

Motor-driven blender, 3 speeds, 1 gallon (3.785 liter; Waring blender)
Muslin or cheesecloth, 80-cm
Nylon blutex (50-μm aperture; Tripette et Renaud)
5-liter beakers
Superspeed refrigerated centrifuge (Sorvall RC5), with the following rotors and corresponding tubes: fixed angle rotors GS-3 with six 500-ml plastic bottles and SS34 with eight 50-ml polypropylene tubes; swinging bucket rotor HS-4 with four 150-ml polycarbonate tubes or equivalent
Pasteur pipet
Vacuum aspirator with a flask as a liquid trap
Ice buckets
Curved plastic spatula
1- and 10-ml pipets
250-ml cylinder

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout.

NOTE: All operations are carried out at 0° to 5°C either by keeping samples on ice or by working in a cold room.

Prepare spinach leaves and homogenize the tissue

1. Wash the spinach leaves and then remove large veins from the leaves. Strain the leaves and store them in the dark and in a cold room (4°C) overnight.

   The overnight storage in the dark reduces the amount of starch granules in the spinach leaves (as for Arabidopsis leaves).

2. Put the leaf material and enough spinach leaf grinding buffer (with BSA) to cover the leaves (usually ~3 liters of spinach leaf grinding medium for 3 kg leaves) in the blender cup.

3. Homogenize the leaf material two times, each time 2 to 3 sec in a Waring blender at low speed and a third time (2 to 3 sec) at high speed.

   The grinding process should be as short as possible (see Basic Protocol, step 7).

4. Rapidly filter the homogenate through 4 to 5 layers of muslin and one layer of nylon blutex as described in Basic Protocol, step 8. Collect the filtered homogenate in a 5-liter beaker.

   Proceed rapidly.

Centrifuge the homogenate

5. Equally distribute the filtered suspension into 500-ml bottles for centrifugation and centrifuge them 10 min at 1200 × *g* (Sorvall GS-3 rotor), 4°C.

   Do not forget to equilibrate the pairs of bottles on a balance.

6. Carefully discard the supernatant from each bottle, retaining the pellets. Remove the remaining supernatant with a Pasteur pipet connected to a vacuum aspirator with a flask as a liquid trap. Keep the pellets on ice.

   These pellets contain crude chloroplast fraction.

Purify the chloroplasts by isopycnic centrifugation

7. Carefully resuspend each pellet, containing a crude chloroplast fraction, by adding 10 ml spinach washing medium using a curved plastic spatula.

   Carefully resuspend each pellet and gently aspirate the resuspended chloroplasts with a 10-ml pipet (do not use pipets with very fine tips).

8. Pool the chloroplast suspension in one tube. Rinse all tubes with additional spinach washing medium.

   The final volume should not exceed 200 ml.

9. Filter the crude chloroplast suspension through one layer of nylon blutex in order to separate the aggregates, and adjust the final volume to 200 ml with spinach washing buffer using a 250-ml cylinder.

10. Prepare the discontinuous Percoll gradients by successively layering 20 ml of 80% and 50 ml of 40% Percoll solutions in four separate 150-ml polycarbonate tubes.

11. Load the crude chloroplast suspension on the top of the discontinuous Percoll gradients (50 ml per tube).

    Carefully layer the chloroplast suspension on the top of the gradients trying not to mix the chloroplast suspension with the upper Percoll layer. Again, do not use pipets with too fine tips.

12. Centrifuge the gradients 20 min at 3000 × *g* (Sorvall swinging-bucket HS-4 rotor), 4°C.

    Intact chloroplasts are recovered at the bottom interface (80%/40% Percoll) and broken chloroplasts + extrachloroplastic membrane systems form a band at the upper interface (sample/40% Percoll; Fig. 3.30.3).

    It is recommended that the brake be disconnected or that the automatic rate controller (if available) be used to prevent mixing of the gradients at the critical stage of deceleration.

13. Carefully remove the upper part of the gradient containing the broken chloroplasts by aspirating with a Pasteur pipet connected to a vacuum aspirator with a flask as a liquid trap.

14. Recover intact chloroplasts (at the interface 80%/40% Percoll) with a 10-ml pipet. Gently pool the intact chloroplasts in a 500-ml plastic bottle.

15. Dilute the chloroplast suspension 3- to 4-fold (up to a final volume of 500 ml) with the spinach washing medium (500 ml final). Centrifuge the suspension 5 min at 4000 × *g* (Sorvall GS-3 rotor), 4°C.

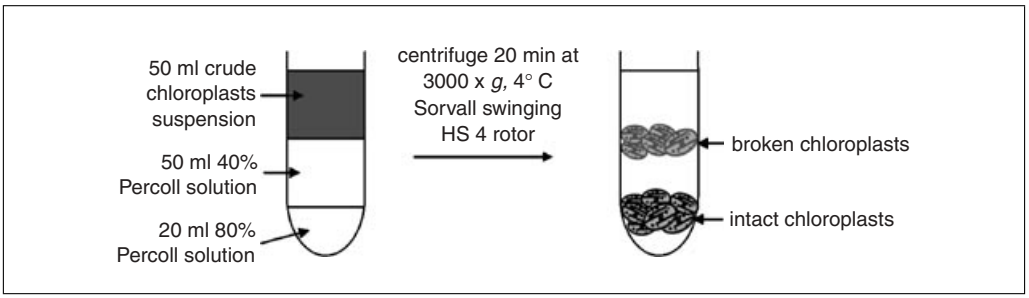

Figure 3.30.3 Schematic representation of the discontinuous Percoll-gradient fractionation of the crude spinach chloroplasts preparation to yield intact and broken chloroplasts.

16. Carefully discard the supernatant and repeat the wash step. Gently resuspend the purified pellet with 150 ml of spinach washing medium and equally distribute the suspension in 50-ml polypropylene tubes.
17. Centrifuge 5 min at 3000 × *g* (Sorvall SS34 rotor), 4°C.
18. Discard the supernatant and keep the pellet (purified intact chloroplasts) on ice.

At this stage, the yield of intact spinach chloroplasts is 150 to 200 mg chlorophyll or 2.5 to 4 g proteins.

After this purification step, the chloroplast preparation is devoid of enzymatic activities such as NADH:cytochrome c oxidoreductase, fumarase, catalase, glycolate oxidase, or nitrate reductase, indicating that it is essentially free of contamination by endoplasmic reticulum, mitochondria, peroxisomes, or cytoplasmic proteins.

Integrity of the chloroplast obtained is determined by phase contrast microscopy (Douce and Joyard, 1982), using an oxygen electrode and the ferricyanide test for intactness (Walker, 1990), or by SDS-PAGE analysis (Fig. 3.30.2).

GROWING OF *ARABIDOPSIS* PLANTLETS

SUPPORT PROTOCOL

Rosette leaves from 3- to 4-week-old *Arabidopsis thaliana* plantlets are required for isolation of the chloroplasts.

Materials

Arabidopsis thaliana seeds
Compost

Large plastic cases (30-cm × 45-cm)
Growth rooms

1. Fill large (30-cm × 45-cm) plastic cases with compost and water.
2. Sow seeds onto the surface of the compost by scattering them carefully at a high density (~30 mg of seeds for a whole box).
3. Grow *A. thaliana* plantlets in growth rooms with a 12-hr light cycle at 23°C (day)/18°C (night) with a light intensity of 150 μmol.m^{-2}sec^{-1}.

Four to six boxes containing 3- to 4-week-old Arabidopsis plantlets are expected to provide 400 to 500 g of rosette material.

4. Harvest the rosette leaves when they are ~1 to 2 cm (~3 to 4-week-old).

The chloroplasts purification procedure described in this unit was applied efficiently to both Columbia and Wassilewskija ecotypes of Arabidopsis.

REAGENTS AND SOLUTIONS

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

EDTA, pH 8

104.05 g EDTA (0.5 M final)
Adjust pH to 8 using NaOH
Add H_2O to 500 ml
Store up to 6 months at 4°C

Grinding medium

291.5 g sorbitol (0.4 M final)
80 ml 1 M Tricine buffer, pH 8.4 (20 mM final; see recipe)
80 ml 0.5 M EDTA, pH 8 (10 mM final; see recipe)
3.36 g $NaHCO_3$ (10 mM final)
Adjust to pH 8.4 using NaOH
Add H_2O to 4 liters
Store up to 1 to 2 days at 4°C
Add 4 g BSA (0.1% w/v) just before use

MOPS buffer, pH 7.8

41.85 g MOPS (1 M final)
Adjust to pH 7.8 using NaOH
Add H_2O to 200 ml
Store up to 6 months at 4°C

Percoll gradients solution

Mix 1 vol of Percoll with 1 vol of 2× washing medium stock solution (see recipe) to obtain a 50% (v/v) Percoll/0.4 M sorbitol solution.

Percoll solution, 40% (v/v)

20.6 g sucrose (0.33 M final)
80 ml Percoll (Pharmacia; 40% v/v final)
2 ml 1 M MOPS (10 mM final; see recipe)
Adjust to pH 7.8 using NaOH
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C

Percoll solution, 80% (v/v)

10.3 g sucrose (0.33 M final)
80 ml Percoll (80% v/v final)
1 ml 1 M MOPS (10 mM final; see recipe)
Adjust to pH 7.8 using NaOH
Add H_2O to 100 ml
Store up to 1 to 2 days at 4°C

Spinach leaf grinding medium

451 g sucrose (0.33 M final)
30 g pyrophosphate (30 mM final)
Adjust to pH 7.8 using NaOH
Add H_2O to 4 liters
Store up to 1 to 2 days at 4°C
Add 4 g BSA (0.1% w/v final) just before use

Spinach washing medium

112 g sucrose (0.33 M final)
20 ml 1 M MOPS (20 mM final; see recipe)
Adjust to pH 7.8 using NaOH
Add H_2O to 1 liter
Store up to 1 to 2 days at 4°C

Tricine buffer, pH 7.6

89.6 g Tricine (1 M final)
Adjust to pH 7.6 with KOH
Add H_2O to 500 ml
Store up to 6 months at 4°C

Tricine buffer pH 8.4

89.6 g Tricine (1 M final)
Adjust to pH 8.4 with KOH
Add H_2O to 500 ml
Store up to 6 months at 4°C

Washing medium

2× stock solution:
73 g sorbitol (0.8 M final)
20 ml 1 M Tricine buffer, pH 7.6 (40 mM final; see recipe)
5 ml 1 M $MgCl_2$ (5 mM final)
5 ml 0.5 M EDTA, pH 8 (2.5 mM final; see recipe)
Adjust to pH 7.6 using NaOH
Add H_2O to 500 ml
Store up to 1 to 2 days at 4°C

1× working solution:
Add 100 ml of 2× washing medium stock (see recipe) solution to 100 ml distilled water.

The final concentrations in the working solution are 0.4 M sorbitol, 20 mM Tricine, 2.5 mM $MgCl_2$, and 1.25 mM EDTA.

COMMENTARY

Background Information

The development of methods for the isolation of functional chloroplasts from plant tissue has been a long process, starting from the late 1930s when Robert Hill prepared chloroplast fractions shown to evolve oxygen in presence of an artificial oxidant, but not in presence of CO_2 (Hill, 1937, 1939). For a long time, chloroplast preparation was restricted to the isolation of green particles capable of supporting the Hill reaction. Arnon and coworkers (for instance Arnon et al., 1954; Allen et al., 1955) demonstrated CO_2-dependent O_2 evolution by chloroplast suspension, but the rates were considerably lower than those of intact leaves. It is only when the importance of maintaining chloroplast integrity throughout the preparation process was fully recognized that preparations showing high rates of CO_2-dependent O_2 evolution were obtained (Walker, 1964; Kalberer et al., 1967; Walker and Hill, 1967; Walker, 1971). Indeed, the use of sugar as an osmoticum (as suggested initially by Hill) together with a very brief homogenization and rapid separation allowed the isolation of chloroplasts with an intact envelope (Walker, 1964). Furthermore, Jensen and Bassham (1966) significantly improved the medium, especially the buffer, used for chloroplast isolation. Thus, in the late 1960s, methods to prepare physiologically active chloroplasts by differential centrifugation were widely available. Indeed, such chloroplast preparations paved the way for an extensive characterization of metabolite transport and mechanisms across the envelope membranes, studies of protein,

and lipid synthesis. For instance, Heber and Santarius (1970) demonstrated the exchange of ATP and ADP across the chloroplast envelope, whereas Heldt and Sauer (1971) identified the inner chloroplast envelope membrane as the site of specific metabolite transport. One should also mention the parallel development of non-aqueous methods to prepare chloroplast (for example, see Stocking, 1959), with the aim of analyzing the intracellular distribution of water-soluble molecules between plastids and the cytosol. Despite their interest, the development of such procedures remained limited.

Although physiologically and structurally intact, chloroplast fractions prepared by differential centrifugation were actually rather crude since they contained pieces derived from other cell compartments (e.g., nuclei and mitochondria) and even a few intact cells. The next step was therefore to remove these contaminants and prepare chloroplasts as pure as possible. Sucrose was first used for preparing gradients to separate chloroplasts (Leech, 1964), and then various media like colloidal silica-derived compounds (such as Ludox) were developed (for example, see Morgenthaler and Price, 1974). Unfortunately, chloroplasts purified on sucrose gradients were unable to perform CO_2-dependent oxygen evolution, mostly because the envelope became leaky during the course of the centrifugation at a high sucrose concentration. Actually, the development of Percoll (review by Pertoft, 2000) was the major breakthrough that allowed (around 1980) the development of procedures to prepare intact and almost pure chloroplasts from a wide variety of tissues (for example, see Takabe et al., 1979; Grant and Wright, 1980; Ortiz et al., 1980; Mourioux and Douce, 1981). Percoll-purified chloroplasts were able to achieve CO_2-dependent oxygen evolution almost identical (on a chlorophyll basis) to that of the leaves (Mourioux and Douce, 1981). Percoll has several key characteristics for the purification of physiologically active organelles: (1) Percoll is made of silica beads of various size and is highly suitable to the formation of density gradients that can be self generated just by high-speed centrifugation; (2) Percoll is an inert compound, its low osmolarity does not change that of the assay medium, even at a high concentration, thus maintaining the integrity of the organelle structure during density gradient centrifugation; (3) in Percoll, silica beads are coated with polyvinylpyrrolidone, which helps remove harmful phenolic compounds from the medium. In the early 1990s, a large number of publications were produced with the term "Percoll" as a keyword (i.e., 50 papers in 1990, 241 in 1991, and 224 in 1992). Screening the literature with the term "Percoll" now identifies >5000 published papers, thus demonstrating the importance of a powerful method—density gradient centrifugation—applicable to any cells or organelles in suspension for which differences in size or buoyant density exist (Pertoft, 2000).

Chloroplast purification was an essential step to prepare and characterize envelope membranes (Douce and Joyard, 1979). Since in chloroplasts, envelope membranes only represents a minor fraction (1% to 2% of the total chloroplast proteins), any contamination of the initial chloroplast fraction is expected to represent, at the end, a major proportion of the fraction enriched in envelope membranes. Indeed, Douce et al. (1973) used sucrose-purified chloroplasts to prepare and characterize envelope membranes from spinach chloroplasts, whereas Percoll-purified chloroplasts were used by Cline et al. (1981) and Block et al. (1983a,b) to prepare and characterize inner and outer envelope membranes from pea and spinach, respectively.

The Alternate Protocol described here to perform Percoll purification of pure and intact chloroplasts from spinach leaves essentially relies on a method published by Douce and Joyard (1982). More recently, and since the complete sequencing of the *Arabidopsis* genome (The AGI, 2000), *Arabidopsis thaliana* has become a widely used model organism, supplanting spinach and pea as model plants. Somerville et al. (1981) had already described the isolation of photosynthetically active protoplasts and chloroplasts from *Arabidopsis thaliana*. However, the method did not rely on the use of Percoll-gradients to isolate the organelles. More recently, Kuntz (1998) and Ferro et al. (2003) published alternative methods for the Percoll-based purification of chloroplasts from *Arabidopsis*. The Basic Protocol presented here, to isolate chloroplasts from *Arabidopsis thaliana*, is adapted from these previously published protocols, and provided with some tricks to optimize yield and purity.

Critical Parameters and Troubleshooting

There are several essential points to take into consideration for successful purification of chloroplasts, e.g., the leaf material, medium composition, and critical steps of the protocol.

The best results are obtained when starting from 3- to 4-week-old *Arabidopsis* rosette leaves. Leaves that are too old are enriched in phenolic compounds that are known to affect integrity of the chloroplasts (Walker, 1990). The quantity of leaves is also critical. No intact chloroplasts can be recovered when the leaf starting material is <50 g. The day before the extraction, put the plants (*Arabidopsis* or spinach) overnight at 4°C in the dark to reduce the amount of starch in leaves. Chloroplasts containing large starch grains will generally be broken during centrifugation (Douce and Joyard, 1982).

For *Arabidopsis* chloroplast purification, we found that the optimal osmoticum concentration was 0.4 M sorbitol instead of 0.33 M (Kuntz, 1998). Addition of EDTA in the grinding and washing media is necessary to recover intact *Arabidopsis* chloroplasts as EDTA has a protective effect (Sommerville et al., 1981).

When purification is performed with less material than the amount described in the protocols described here, the number of Percoll gradient tubes should also be reduced (e.g., use only one preformed Percoll gradient when starting from 60 g of *Arabidopsis* leaves). The tissue must remain cold throughout the protocol to help reduce endogenous protease activities. The grinding process must be as short as possible. Longer blending improves the yield of recovered chlorophyll, but increases the proportion of broken chloroplasts.

The crude chloroplast suspension obtained after homogenization of leaves that are too young is mainly composed of broken chloroplasts. Once the leaf material has been homogenized, rapidly filter and centrifuge the homogenate to protect chloroplasts from activities of proteases released after disruption of the cells. Addition of BSA immediately before use of the grinding medium helps to limit the activity of proteases.

Anticipated Results

Using the Basic Protocol for *Arabidopsis* chloroplast purification, an average of 54 mg chloroplast proteins can be purified from 500 g of 3- to 4-week-old *Arabidopsis* leaves. Excellent purity of these Percoll-purified *Arabidopsis* chloroplasts was confirmed through immunological detection of markers enzymes (Seigneurin-Berny et al., 2006) and proteomic analysis (Ferro et al., 2003). Only 5% (6 out of 112) of the identified *Arabidopsis* proteins may correspond to non-plastid proteins. On a chlorophyll basis, we determined that the yield (~2% to 3%) of Percoll-purified and intact chloroplasts is equivalent in *Arabidopsis* and spinach (Douce and Joyard, 1982), the major limit for *Arabidopsis* being the availability of large amounts of starting material. The yield of intact spinach chloroplasts obtained from 3 kg of leaves is ~150 to 200 mg chlorophyll or 2.5 to 4 g proteins. A classical preparation of *Arabidopsis* chloroplasts starts from 500 g of leaves. On average, the yield of intact *Arabidopsis* chloroplasts obtained from 3 kg of leaves would be ~45 to 55 mg chlorophyll or 0.18 to 0.32 g proteins.

Time Considerations

An efficient chloroplast preparation should be completed within 1 to 1.5 hr. Preformed continuous Percoll gradients can be prepared 1 or 2 days before. Concentrated purified chloroplasts can be stored on ice for a few hours before loss of their structural and metabolic integrity. *Arabidopsis* chloroplasts are more fragile than spinach chloroplasts and can be stored intact for 3 to 4 hr only. Intact chloroplasts do not survive freezing. Buffers with osmoticum must be used to keep the chloroplasts intact.

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