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## **CHAPTER 8: Isolation of the inner and outer membranes of the chloroplast envelope from Angiosperms**

### **Author:**

Maryse A Block, Eric Maréchal

### **Corresponding author:**

Eric Maréchal, Laboratoire de Physiologie Cellulaire et Végétale, CNRS, CEA, INRAE, Univ. Grenoble Alpes, IRIG, CEA Grenoble, 17 Rue des Martyrs, 38000 Grenoble, France

email: [eric.marechal@cea.fr](mailto:eric.marechal@cea.fr)

Phone: +33 438784985

**Running Head:** Purification of chloroplast inner and outer envelope membranes

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## **Abstract**

The outer and the inner membrane of the chloroplast envelope, also called OEM and IEM, have distinct lipid and protein compositions. They host molecular systems involved in biogenesis of the organelle, its cellular function and its communication with other compartments. Here we describe a method for the isolation of these two membranes starting from intact chloroplast preparations, with two alternative procedures based on the starting material. One was developed from spinach leaves, the other one from pea leaves. The two procedures differ by the method used to isolate and rupture chloroplasts and separate each membrane.

**Key Words:** Chloroplast, chloroplast envelope, membrane, outer envelope membrane, inner envelope membrane, differential centrifugation.

## 1. Introduction

In plant cells, chloroplasts are bounded by an envelope composed of two membranes. Classically called the OEM and the IEM, the outer and inner membranes have distinct lipid and protein compositions. They host molecular systems involved in biogenesis of the organelle, its cellular function and its communication with other compartments. The isolation of the OEM and IEM was an initial breakthrough for their subsequent characterization. Here we describe two alternative procedures for their preparation from isolated intact chloroplasts. In both procedures, after isolation of intact chloroplasts in an isosmotic medium close to 0.3 M, chloroplasts are transferred to a hypertonic medium, which induces the export of water from the matrix and the rapid reduction of the stromal volume. This organelle shrinkage also induces a rapid reverse transport of small molecules present in the hypertonic medium. The IEM being less permeable to these small molecules, compared to the OEM [1], the interspace between the two envelope membranes increases. Each membrane of the chloroplast envelope can then be extracted and isolated by differential centrifugation. One procedure was developed from spinach leaves, the other one from pea leaves. Both membranes of the chloroplast envelope exhibit several contact sites between each other, and with other membranes of the cell, which are still poorly known. Since these two procedures differ in the way chloroplasts are isolated and broken, each protocol may result in slightly different partitioning of these membrane contact sites. The isolation of the OEM and IEM from spinach leaf chloroplasts is adapted from the method first described by [2]. Following their transfer into hypertonic medium, *Spinacia oleracea* chloroplasts are compressed to a low pressure and decompressed to atmospheric pressure. Moderate variation of pressure causes rupture of the membranes. The isolation of the OEM and IEM from pea leaf chloroplasts is adapted from the protocol described in [3]. *Pisum sativum* chloroplasts are broken by a freeze-thaw cycle and a chloroplast envelope fraction containing a mixture of the inner and outer membranes is first

recovered by flotation centrifugation, then the two membrane fractions of the chloroplast envelope are resolved by another round of centrifugation on sucrose gradient. Importantly, although total envelope fractions can be purified from lower amounts of leaves, such as from Arabidopsis (Chapter **XXI**), the starting material should be in sufficient amount to adapt the protocol detailed here. Alternatively, features identified in the spinach and /or pea models can be used to analyze proteomes from mixed envelope membranes from models such as Arabidopsis [4].

## **2. Materials**

### **2.1 Isolation of the OEM and IEM from spinach leaf chloroplasts**

1. Argon gas bottle, with a gas regulator holding an inlet pressure gauge (max 250 bars) and an outlet pressure gauge (max 10 bars) (see Note 1).
2. Yeda press connected to the bottle gas
3. Plant material: 1-1.5 kg of spinach (*Spinacia oleracea L.*) leaves are deribbed, washed in water and drained (see Note 2). They are then kept in the dark overnight at 4°C.
4. Grinding medium: 0.33 M mannitol, 1 g/L bovin serum albumin, 30 mM 3-(N-morpholino) propanesulfonic acid (MOPS) NaOH, pH 7.8. First prepare a stock solution of 1 M MOPS NaOH, pH 7.8. This solution can be stored at 4 °C several weeks. For grinding medium, prepare 2 L with 120 g of mannitol and 30 mL of 1 M MOPS. Adjust pH to 7.8 with a diluted NaOH solution. Adjust volume. Grinding medium is cooled to 4°C and 2 g of defatted bovin serum albumin is added to the medium just before leaf grinding.
5. Washing medium: 0.33 M mannitol, 20 mM MOPS NaOH, pH 7.8. Prepare 1 L of washing medium with 60 g of mannitol and 20 mL of 1 M MOPS NaOH, pH 7.8. Adjust pH with a diluted NaOH solution. Adjust volume.

6. Hypertonic medium: 0.7 M mannitol, 4 mM MgCl<sub>2</sub>, 1 mM phenylmethylsulfonyl fluoride, 1mM benzamidine, 0.5 mM ε-amino caproic acid, 20 mM MOPS NaOH, pH 7.8. First prepare stock solutions of 100 mM phenylmethylsulfonyl fluoride in isopropanol, 100 mM benzamidine, and 50 mM ε-amino caproic acid. Then prepare 200 mL of hypertonic medium with 25.2 g mannitol, 0.8 mL of 1M MgCl<sub>2</sub>, 2 ml of 100 mM phenylmethylsulfonyl fluoride solution in isopropanol, 2 mL of 100 mM benzamidine, 2 mL of 50 mM, ε-amino caproic acid, 4 mL of 1M MOPS NaOH, pH 7.8. Adjust pH and volume.
7. Dilution medium: 1 mM phenylmethylsulfonyl fluoride, 1mM benzamidine, 0.5 mM ε-amino caproic acid, 10 mM MOPS NaOH, pH 7.8. Prepare 500 mL of dilution medium with 5 ml of 100 mM phenylmethylsulfonyl fluoride solution in isopropanol, 5 mL of 100 mM benzamidine, 5 mL of 50 mM ε-amino caproic acid, 5 mL of 1M MOPS, NaOH pH 7.8. Adjust volume.
8. 40% Percoll: 40% (w/v) of Percoll, 0.33 M sucrose, 20 mM MOPS NaOH, pH 7.8. For preparation of 200 mL of Percoll 40%, solubilize 12 g of mannitol in 4 mL of 1M MOPS NaOH, pH 7.8, 80 mL of Percoll (GE Healthcare), and water. Adjust volume to 200 mL.
9. 80% Percoll: 80% (w/v) of Percoll, 0.33 M sucrose, 20 mM MOPS NaOH, pH 7.8. For preparation of 100 mL of Percoll 80%, solubilize 6 g of mannitol in 2 mL of 1M MOPS NaOH, pH 7.8, 80 mL of Percoll (GE Healthcare), and a very small volume of water. Adjust volume to 100 mL.
10. Percoll gradients: Four Percoll gradients are prepared in 120 mL HS4 centrifuge tubes with 25 mL of 80% Percoll and 50 mL of 40% Percoll. 50 mL of 40% Percoll are poured in each tube, then 25 mL of 80% Percoll are gently loaded under the 40% Percoll layer with a pipette.

11. 0.4 M sucrose: 0.4 M sucrose, 4 mM MgCl<sub>2</sub>, 1 mM phenylmethylsulfonyl fluoride, 1mM benzamidine, 0.5 mM ε-amino caproic acid, 20 mM MOPS NaOH, pH 7.8. For 200 mL of 0.4 M sucrose, weight 27.5 g of sucrose, add 4 mL of 1 M MOPS NaOH, pH 7.8 and 0.8 mL of 1 M MgCl<sub>2</sub>. Adjust pH to 7.8 with diluted NaOH. Add 2 mL of 100 mM phenylmethylsulfonyl fluoride solution in isopropanol, 2 mL of 100 mM benzamidine, 2 mL of 50 mM ε-amino caproic acid. Adjust volume.
12. 0.65 M sucrose: 0.65 M sucrose, 4 mM MgCl<sub>2</sub>, 1 mM phenylmethylsulfonyl fluoride, 1mM benzamidine, 0.5 mM ε-amino caproic acid, 20 mM MOPS NaOH, pH 7.8. For 200 mL of 0.65 M sucrose, weight 44.4 g of sucrose, add 4 mL of 1M MOPS NaOH, pH 7.8 and 0.8 mL of 1M MgCl<sub>2</sub>. Adjust pH to 7.8 with diluted NaOH. Add 1 mL of 100 mM phenylmethylsulfonyl fluoride solution in isopropanol, 1 mL of 100 mM benzamidine, 1 mL of 50 mM ε-amino caproic acid. Adjust volume.
13. 1 M sucrose: 1 M sucrose, 4 mM MgCl<sub>2</sub>, 1 mM phenylmethylsulfonyl fluoride, 1mM benzamidine, 0.5 mM ε-amino caproic acid, 20 mM MOPS NaOH, pH 7.8. For 200 mL of 1 M sucrose, weight 68.4 g of sucrose, add 4 mL of 1M MOPS, NaOH, pH 7.8 and 0.8 mL of 1M MgCl<sub>2</sub>. Adjust pH to 7.8 with diluted NaOH. Add 1 mL of 100 mM phenylmethylsulfonyl fluoride solution in isopropanol, 1 mL of 100 mM benzamidine, 1 mL of 50 mM ε-amino caproic acid. Adjust volume
14. Sucrose gradients: Six sucrose gradients are prepared in SW27 ultracentrifuge tubes with 8 mL of 1 M sucrose, 8 mL of 0.65 M sucrose and 8 mL of 0.4 M sucrose medium (see Note 3).

### **2.1 Isolation of the OEM and IEM from pea leaf chloroplasts**

1. Refractometer
2. Dilution medium: 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) KOH, pH 7.8. 200 mL of

dilution medium are prepared with 2 mL of 0.1 M EDTA, 2 mL of 1 M HEPES, KOH, pH 7.8 and water. Adjust pH to 7.8 with KOH.

3. 0.65 M sucrose: 0.65 M sucrose, 1 mM EDTA, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) KOH, pH 7.8. For 100 mL of 0.65 M sucrose, solubilize 22.2 g of sucrose with 1 mL of 0.1 M EDTA, 1 mL of 1 M mM HEPES KOH, pH 7.8. Adjust pH to 7.8 with KOH. Adjust volume to 100 mL.
4. 1.1 M sucrose: 1.1 M sucrose, 1 mM EDTA, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) KOH, pH 7.8. For 100 mL of 1.1 M sucrose, solubilize 37.6 g of sucrose with 1 mL of 0.1 M EDTA, 1 mL of 1 M mM HEPES, KOH, pH 7.8. Adjust pH to 7.8 with KOH. Adjust volume to 100 mL.

### **3. Methods**

#### **3.1. Isolation of the OEM and IEM from spinach leaf chloroplasts**

1. 1 to 1.5 kg of spinach leaves are ground in 2 L of medium (see Note 4). Leaves are ground in a large blender equipped with sharp blades and cooled to 4°C (see Note 5). Crushed leaves are filtered on 6 sheets of muslin and a 50 µM-aperture nylon cloth.
2. Filtered suspension is centrifuged at 3,000 x g for 15 min in 500 mL buckets. Supernatant is removed. Pellets are resuspended into 160 mL of washing medium (see Note 6).
3. 40 mL of the suspension is loaded on each Percoll gradient and centrifuged at 3,500 x g for 20 min (see Note 7). Intact chloroplasts are collected at the 80% - 40% Percoll interface after removing the upper fraction by aspiration.
4. Chloroplast suspension is diluted in at least 4 volumes of washing medium and centrifuged at 4,000 x g for 10 min into 2 pellets. To completely remove Percoll, chloroplasts are washed again by dilution in washing medium and centrifugation at 4,000 x g for 10 min.



5. Each chloroplast pellet is resuspended in hypertonic medium up to a final volume of 30 mL (see Note 8). Suspension is rapidly homogenized by filtration through a 50  $\mu$ M-aperture nylon cloth and incubated 10 minutes on ice before loading in a cold Yeda press. Gas pressure is set to 6 bars. Liquid is regularly and slowly released from the press (10 mL/min) and collected on ice (see Note 9).
6. Chloroplast suspension is centrifuged at 12,000 x g for 5 min. The supernatant is centrifuged twice again at 12,000 x g for 5 min to remove non ruptured chloroplasts into the pellet. The supernatant is diluted with medium up to 78 mL. 13 mL of the diluted supernatant are loaded on top of each sucrose gradient, composed of 8 mL of 1.1 M sucrose, 8 mL of 0.65 M sucrose and 8 mL of 0.4 mM sucrose and centrifuged at 80,000 x g for 1h30.
7. After centrifugation, the outer envelope fraction is collected as a layer at the 0.4 M - 0.65 M sucrose interface and the inner envelope fraction at the 1 M - 0.65 M sucrose interface. Both fractions are diluted in dilution medium at least 4 times and centrifuged at 100,000 x g for 2h (see Note 10). The membrane pellets are collected in a minimal volume of dilution medium leading to about 0.4 mL of OEM fraction at a 1 mg/mL protein concentration and 0.4 mL of IEM at a 3 mg/mL protein concentration. Each chloroplast subfraction is kept on ice when used straightway, otherwise stored at -80°C.

### **3.2. Isolation of the OEM and IEM from spinach leaf chloroplasts**

1. An envelope fraction of pea chloroplasts obtained as described in chapter 7 is first diluted to 0.45 M sucrose by addition of dilution medium (see Note 11). 15 mL of this dilution are then loaded on a sucrose discontinuous gradient composed of 12 mL of 1.1 M sucrose and 12 mL of 0.65 M sucrose. It is then centrifuged at 100,000 x g for 3 h.

2. The outer envelope membrane fraction is collected at the 0.45 M- 0.65 M sucrose interface, the inner envelope membrane fraction at the 1.1 M- 0.65 M sucrose interface. Both fractions are diluted at least 4 times in 10 mM HEPES KOH, pH 7.8 before centrifugation at 100,000 x g for 1h30 and collection in pellets (see Note 12).

#### 4. Notes

1. As an alternative, nitrogen gas can be used. Outlet pressure gauge should be accurate in the 0-10 bar range.
2. Commercial fresh leaves are generally used. Transfer to dark several hours prior chloroplast preparation reduces chloroplast starch content that causes chloroplast rupture during centrifugation.
3. Prepare sucrose gradients by smoothly pouring 8 mL of 0.65 M sucrose medium above an 8 mL layer of 1 M sucrose medium. Add on top 8 ml of 0.4 M sucrose. A peristaltic pump is convenient to delicately add layers and with this apparatus, layers can be added in reverse order, from the lightest to the heaviest. Sucrose gradients are best used shortly after preparation.
4. All operations are carried out at 0-5 °C, in the cold room or on an ice bucket, all media are cooled to 4 °C prior use and all centrifuges are refrigerated prior starting.
5. Grinding should result in consistent small leaf pieces of about 2 mm side.
6. Gentle mixing of pellets with a pencil can help to get a homogenous suspension and to preserve chloroplast integrity. 1 kg of spinach leaves can generally yield 60 to 90 mg of chlorophyll of purified intact chloroplasts. Chlorophyll concentration can be measured as follows: 10 µL of the fraction is diluted in 10 mL acetone / water, 80%, v/v. The mixture is centrifuged at 7,000 x g for 15 minutes. The optical density (OD) of the supernatant is measured at 652 nm. Chlorophyll content of the fraction (mg/ mL) is given by OD\*26 [5].

7. It is better to set low centrifuge acceleration rate and brake off in order to avoid abrupt disturbance of the gradients.
8. As an indication, a chlorophyll concentration of 1 to 1.5 mg/ mL is adapted for chloroplast rupture.
9. As an alternative, chloroplasts can be ruptured in a Potter- Elvehjem homogenizer [6]. It is recommended to test the homogenizer type and the number of sucker back and forth to have correct chloroplast opening.
10. Membrane pellets may be lost because they easily detach from the bottom of the tube. Thus it is recommended to remove the supernatant by aspiration.
11. All operations are carried out on an ice, all media are cooled to 4 °C prior use and all centrifuges are refrigerated prior starting. We start from the envelope fraction collected as a layer at the top of the sucrose gradient. It is possible to start from a mix of different frozen fractions isolated at different times. The sucrose concentration is controlled by refraction index measurement. A 0.45 M sucrose solution has a refraction index of 1.354.
12. For quality preservation, addition of protease inhibitors such as 100 mM phenylmethylsulfonyl fluoride, 100 mM benzamidine, and 50 mM  $\epsilon$ -amino caproic acid is recommended as well as storage at -80°C.

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