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CHAPTER 7: Purification of chloroplast envelope, thylakoids and stroma from Angiosperm leaves

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Running Head: Purification of chloroplast subfractions

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Abstract:

Plant cell chloroplasts are bounded by a two-membrane envelope. Their photosynthetic function is based on the development of an operational large internal membrane network, called the thylakoids, and on enzymatic processes present in the chloroplast matrix, called the stroma. Thylakoid membranes are distinct from the chloroplast envelope and their biogenesis is dependent on biosynthetic and transport activities specific of the chloroplast envelope. Starting with the isolation of intact chloroplasts, the method presents the separation by differential centrifugation of the three compartments. Protocol is detailed for leaves of spinach, Arabidopsis or pea.

Key Words:

Chloroplast, stroma, envelope, thylakoids, membrane, differential centrifugation, chloroplast fractionation

1. Introduction

Chloroplasts are specific organelles of plant cells dedicated to photosynthesis and bounded by a two-membrane envelope. Their photosynthetic function is based on the development of an operational large internal membrane network, called the thylakoids and on enzymatic processes present in the chloroplast matrix, called the stroma. Thylakoids form membrane sacs, which can be piled in so-called grana, and are clearly different from the envelope. Their biogenesis is dependent on biosynthetic and transport activities located in the chloroplast envelope, but the precise mechanism is still elusive, as described in chapter 1. This chapter describes procedures to isolate intact chloroplasts and their three main compartments: the stroma, thylakoids and the bounding envelope. The method is based on differential centrifugation of ground leaf tissues. Three steps are involved: first isolation of intact chloroplasts, then organelle breakage and eventually sub-fraction separation. Protocol is detailed with variations adapted for leaves of spinach, Arabidopsis or pea. The protocol based on spinach leaves is adapted from the method first described by (1), then modified by (2). After intact chloroplast isolation, chloroplasts are broken due to transfer to a hypotonic medium. In this medium, water enters the chloroplast stroma, chloroplasts swell and tension imposed to the chloroplast envelope membranes leads to their rupture. The two envelope membranes are very close to each other. The protocol based on Arabidopsis leaves is adapted from (3). The method differs essentially regarding intact chloroplast isolation, because Arabidopsis chloroplasts have a less developed thylakoid network and are more susceptible to rupture. Eventually, the protocol developed for pea leaves is adapted from (4). By contrast with spinach and Arabidopsis protocols, after intact chloroplast isolation, chloroplasts are transferred to a hypertonic medium. Water exits from the stroma, which rapidly reduces its volume. The envelope membranes are thus separated from the organelle. A freeze-thaw cycle is then applied to break each chloroplast envelope membrane. A mix of inner and outer envelope membranes is recovered by flotation after several hours of

centrifugation in a sucrose gradient. Each membrane of the chloroplast envelope can be further isolated (see chapter 8).

2. Materials

2.1. Isolation of chloroplast subfractions from spinach leaves

1. Plant material: 3 kg of spinach (*Spinacia oleracea L.*) leaves are de-ribbed, washed in water and drained. Commercial fresh leaves are generally used. Transfer to dark several hours prior preparation reduces chloroplast starch content that causes organelle rupture during the first centrifugation. They are then kept in the dark overnight at 4°C.
2. Grinding medium: 0.33 M sucrose, 1 g/L bovine serum albumin, 30 mM tetrasodium pyrophosphate, pH 7.8 (as an alternative 50 mM N-Tris (hydroxymethyl) methyl]glycine (Tricine) NaOH can be used). For grinding medium, prepare 4 L with 451 g of sucrose and 31 g of tetrasodium pyrophosphate. Adjust pH to 7.8 with a diluted NaOH solution. Adjust volume. Grinding medium is cooled to 4°C and 4 g of defatted bovine serum albumin is added just before leaf grinding.
3. Washing medium: 0.33 M sucrose, 20 mM 3-(N-morpholino) propanesulfonic acid (MOPS) NaOH, pH 7.8. First prepare a stock solution of 1 MOPS NaOH, pH 7.8. This solution can be stored at 4 °C several weeks. Then prepare 1 L of washing medium with 112 g of sucrose and 20 mL of 1 M MOPS NaOH, pH 7.8. Adjust pH with a diluted NaOH solution. Adjust volume.
4. Swelling medium: 4 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 1mM benzamidine, 0.5 mM ε-amino caproic acid, 10 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 100 mL of swelling medium with 0.4 mL of 1M MgCl₂, 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, 1 mL of 1M MOPS NaOH, pH 7.8. Adjust volume.

5. 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.
6. 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 20.6 g of sucrose in 4 mL of 1M MOPS NaOH, pH 7.8, 80 mL of Percoll, and water. Adjust volume to 200 mL.
7. 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 10.3 g of sucrose in 2 mL of 1M MOPS NaOH, pH 7.8, 80 mL of Percoll, and a very small volume of water. Adjust volume to 100 mL.
8. Percoll gradient: 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.
9. 0.6 M sucrose: 0.6 M sucrose, 4 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1mM benzamidine, 0.5 mM ϵ -amino caproic acid, 10 mM MOPS NaOH, pH 7.8. For 100 mL of 0.6 M sucrose, weight 20.5 g of sucrose, add 1 mL of 1 M MOPS NaOH, pH 7.8 and 0.4 mL of 1 M $MgCl_2$. 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.

10. 0.93 M sucrose: 0.93 M sucrose, 4 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 1mM benzamidine, 0.5 mM ε-amino caproic acid, 10 mM MOPS NaOH, pH 7.8. For 100 mL of 0.93 M sucrose, weight 31.8 g of sucrose, add 1 mL of 1M MOPS NaOH, pH 7.8 and 0.4 mL of 1M MgCl₂. 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.
11. Sucrose gradient: Six sucrose gradients are prepared in ultracentrifuge tubes with 12 mL of 0.93 M sucrose and 10 mL of 0.6M sucrose medium. Prepare sucrose gradients by smoothly pouring 12 mL of 0.93 M sucrose medium underneath a 10 mL layer of 0.6 M sucrose medium. Sucrose gradients are best used shortly after preparation

2.2 Isolation of chloroplasts and chloroplast subfractions from Arabidopsis leaves

1. Plant material: Sow approximately 180 mg of *Arabidopsis thaliana* seeds on soil and synchronize germination by incubation 2 d in the dark at 4°C. Grow plants for 4 weeks at 22°C with a 10-h white light photoperiod. Culture is transferred to dark 16 h before chloroplast isolation. Rosette leaves are harvested with scissors and cooled on ice (see Note 1).
2. Grinding medium: 0.4 M sorbitol, 10 mM ethylenediaminetetraacetic acid (EDTA), 10 mM NaHCO₃, 40 mM N-Tris (hydroxymethyl) methyl glycine (Tricine) KOH, pH 8.4, 1 g/L defatted bovine serum albumin. First prepare a stock solution of 1 M N-Tris (hydroxymethyl) methyl glycine (Tricine) KOH, pH 8.4. This solution can be stored at 4 °C several weeks. For 2 L of grinding medium, weight 146 g of sorbitol, add 40 mL of 0.5 M EDTA, 20 mL of 1M NaHCO₃ and 80 mL of 1M Tricine KOH, pH 8.4. Adjust pH to 8.4

with a diluted solution of KOH, adjust volume to 2 L. Cool to 4°C and add 2 g of BSA just before leaf grinding

- 3 Washing medium: 0.4 M sorbitol, 2.5 mM EDTA, 5 mM MgCl₂, 20 mM MOPS NaOH, pH 7.6. First prepare a stock solution of 1 M MOPS NaOH, pH 7.6. This solution can be stored at 4 °C several weeks. For 1 L of washing medium, weight 73 g of sorbitol, add 5 mL of 0.5 M EDTA and 20 mL of 1M MOPS NaOH, pH 7.6. Adjust pH to 7.6 with a diluted solution of NaOH, adjust volume to 1 L.
- 4 50 % Percoll: 50 % (w/v) Percoll, 0.4 M sorbitol, 5 mM MgCl₂, 2.5 mM EDTA, 20 mM MOPS NaOH, pH 7.6. For 200 mL of 50 % Percoll, weight 14 g of sorbitol, add 4 mL of MOPS 1M, 1 mL of 1M MgCl₂, 1 mL of 0.5 M EDTA and some water. Adjust pH to 7.6 with diluted NaOH, add 100 mL of Percoll and adjust volume.
- 5 Prepare 6 Percoll gradients with 30 mL of 50 % Percoll. Centrifuge at 38,000 x g for 1 h (see Note 2).
- 6 Sucrose gradients are identical to those used for preparation of the spinach chloroplast envelope but with a reduced volume in smaller tubes. Six sucrose gradients are prepared in SW41 ultracentrifuge tubes with 4 mL of 0.93 M sucrose and 4 mL layer of 0.6 M sucrose medium. For sucrose gradient preparation, smoothly pour 4 mL of 0.93 M sucrose medium underneath a 4 mL layer of 0.6M sucrose medium Sucrose gradients are best used shortly after preparation.
- 7 Swelling and dilution media are the same as the ones used for spinach.

2.3 Isolation of chloroplasts and chloroplast subfractions from pea leaves

1. Refractometer

2. Plant material: 1 L of pea (*Pisum sativum*) seeds are soaked in slightly running water overnight before culture in wet vermiculite for 7 days at 24°C with a 12 H, photoperiod (white light). 1 kg of leaves are harvested at the start of the light period.
3. Grinding medium: 0.33 M mannitol, 2 mM EDTA, 1 g/L bovine serum albumin, 30 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) KOH, pH 7.5. Prepare 4 L of grinding medium with 240 g of mannitol, 80 mL of 0.1 M EDTA and 120 mL of 1M HEPES KOH. Adjust pH to 7.5 with a diluted KOH solution. Adjust volume. Grinding medium is cooled to 4°C and 4 g of defatted bovine serum albumin is added just before leaf grinding.
4. Washing medium: 0.33 M mannitol, 1 mM EDTA, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) KOH, pH 7.5. Prepare 1 L with 60.1 g of mannitol, 10 mL of 0.1 M EDTA and 10 mL of 1 M HEPES KOH, pH 7.5. Adjust pH with a diluted KOH solution. Adjust volume. Cool to 4°C.
5. 20 % Percoll: 20 % (w/v) of Percoll, 0.33 M mannitol, 2 mM EDTA, 30 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) KOH, pH 7.5. For 200 mL of 20 % of Percoll solution, mix 40 mL of Percoll with 160 mL of grinding medium deprived of bovine serum albumin. Cool to 4°C.
6. 40 % Percoll: 40 % (w/v) of Percoll, 0.33 M mannitol, 2 mM EDTA, 30 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) KOH, pH 7.5. For 100 mL of 40 % of Percoll solution, mix 40 mL of Percoll with 60 mL of grinding medium deprived of bovine serum albumin. Cool to 4°C.
7. Percoll gradient: Six Percoll gradients are prepared in ultracentrifuge tubes with 15 mL of 20 % Percoll and 5 mL of 40 % Percoll. 15 mL of 20 % Percoll are poured in each tube, then 5 mL of 40 % Percoll are gently loaded under the 20 % Percoll layer with a pipette. Gradients are prepared just before use.

8. 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 37.7 g of sucrose in 1 mM EDTA, 10 mM HEPES KOH, pH 7.8. Adjust pH to 7.8 with KOH. Adjust volume to 100 mL.
9. 2 M sucrose: 2 M sucrose, 1 mM EDTA, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) KOH, pH 7.8. For 100 mL of 2 M sucrose, solubilize 68.5 g of sucrose in 1 mM EDTA, 10 mM HEPES KOH, pH 7.8. Adjust pH to 7.8 with KOH. Adjust volume to 100 mL.
10. 1.3 M sucrose, 1 mM EDTA, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) KOH, pH 7.8. For 100 mL of 1.3 M sucrose, solubilize 44.5 g of sucrose in 1 mM EDTA, 10 mM HEPES KOH, pH 7.8. Adjust pH to 7.8 with KOH. Adjust volume to 100 mL.
11. 0.4 M sucrose, 1 mM EDTA, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) KOH, pH 7.8. For 100 mL of 0.4 M sucrose, solubilize 13.7 g of sucrose in 1 mM EDTA, 10 mM HEPES KOH, pH 7.8. Adjust pH to 7.8 with KOH. Adjust volume to 100 mL

3. Methods

3.1 Isolation of chloroplasts and chloroplast subfractions from spinach leaves

1. 1 to 1.5 kg of spinach leaves are ground in 2 L of medium (see Note 3). Leaves are shortly ground in a large blender equipped with sharp blades and readably cooled to 4°C (see Note 4).

2. Crushed leaves are filtered on 6 sheets of muslin and a 50 μM -aperture nylon cloth. Filtered suspension is centrifuged at 2,100 x g for 15 min in 500 mL buckets. Supernatant is removed. Green dense pellets are kept on ice until collection from another set of leaves.
3. Pellets are resuspended into 160 mL of washing medium (see Note 5).
4. 40 mL of suspension is loaded on each Percoll gradient and centrifuged at 3,500 x g for 20 min (50 mL swinging bucket rotor) (see Note 6).
5. Intact chloroplasts are collected at the 80 % - 40 % Percoll interface after removing the above fraction by aspiration.
6. Chloroplast suspension is diluted in at least 4 volumes of washing medium and centrifuged at 3,400 x g for 10 min. Chloroplast pellets are gently resuspended with a pencil into 200 mL of washing medium. They are then centrifuged in at 6,000 x g for 5 min. Supernatant is removed for isolation of intact chloroplast pellets and complete removal of Percoll (see Note 7).
7. Intact chloroplast pellets are resuspended in swelling medium up to a final volume of 78 mL. The swelling causes the chloroplasts to break into different parts. Suspension is greatly homogenized by active pipetting and final filtration through a 50 μM -aperture nylon cloth. 13 mL is then gently loaded on each sucrose gradient. Gradients are centrifuged at 72,000 x g for 1h.
8. After centrifugation, about 70 ml of stromal fraction at a 10 mg/mL protein concentration is collected from the top fraction of the gradients. It can be easily recognized with an amber color. The chloroplast envelope fraction shows as a yellow band at the 0.6M-0.93M sucrose interface. It is collected after removal of the remaining 0.6 M sucrose top layer, diluted to 210 mL with dilution medium, then centrifuged at 100,000 x g for 1 h.
9. The membrane pellets are collected in a minimal volume of dilution medium leading to about 1 mL of chloroplast envelope fraction at a 5 to 10 mg/mL protein concentration. An

even homogenate is obtained by 2 to 3 strokes in a Potter-Elvehjem homogenizer. The thylakoid fraction is very dense and sticks at the bottom of the sucrose gradient tubes. In order to reduce reliable cross-contamination by chloroplast envelope, the thylakoid pellets are diluted 10 times in dilution medium, before collection by a light centrifugation at 6,000 x g for 5 min.

10. Each chloroplast subfraction is kept on ice when used straightway, otherwise stored at -80°C.

3.2 Isolation of chloroplasts and chloroplast subfractions from Arabidopsis leaves

1. 0.5 kg of Arabidopsis leaves are ground in 2 L of grinding medium (see Note 8). Leaves are very shortly ground in a blender fitted with sharp blades and readably cooled to 4°C (see Note 9). Crushed leaves are filtered on 4 sheets of muslin and a 50 µM-aperture nylon cloth.
2. Filtered suspension is centrifuged at 2,100 x g for 10 min in 500 mL. Supernatant is removed by aspiration. Green pellets are resuspended into 48 mL of washing medium (see Note 10).
3. 6 mL of chloroplast suspension are loaded on top of each Percoll gradient. Gradients are centrifuged at 13,300 x g for 10 min (see Note 11). Intact chloroplasts are present in a layer at the bottom of the Percoll gradient which is collected by aspiration (see Note 12).
4. Chloroplast suspension is smoothly diluted in 4 volumes of washing medium and centrifuged for 5 min at 2,000 x g. The pellets are again smoothly diluted in washing medium and centrifuged at 2,000 x g for 3 min. As for spinach chloroplast fractionation, intact chloroplast pellets are resuspended in swelling medium.
5. A final volume of 21 mL is suitable for loading of 6 sucrose gradients (see Note 13). 3.5 mL of chloroplast suspension is gently loaded on each sucrose gradient. Gradients are centrifuged at 72,000 x g for 1h (swinging bucket rotor).

6. After centrifugation, 3 mL of stromal fraction is collected from the top of each gradient. The chloroplast envelope fraction is a yellow band at the 0.6M-0.93M sucrose interface. It is collected after removal of the remaining top layer, diluted at least 4 times with dilution medium, and then centrifuged at 100,000 x g for 1 h (swinging bucket rotor).
7. The envelope membrane pellets are collected in a minimal volume of dilution medium. The thylakoid fraction is at the bottom of the sucrose gradient tubes. Each chloroplast subfraction is kept on ice when used straightway, otherwise stored at -80°C..

3.3 Isolation of chloroplasts and chloroplast subfractions from pea leaves

1. 3 L of medium is required to grind 1 kg of pea leaves (see Note 14). Leaves are shortly ground in a blender fitted with sharp blades and readably cooled to 4°C (see Note 15).
2. Ground leaves are filtered on 4 sheets of muslin and a 50 µM-aperture nylon cloth. Filtered suspension is centrifuged at 4,200 x g for 10 min in 500 mL buckets. Supernatant is removed. Green pellets are kept on ice until collection and resuspension into 108 mL of grinding medium deprived of bovine serum albumin (see Note 16).
3. Suspension is filtered on a nylon cloth before loading of 18 mL on each Percoll gradient. Gradients are centrifuged at 72,000 x g for 30 min (swinging bucket rotor). Intact chloroplasts are present in the bottom Percoll layer which is collected by aspiration.
4. Chloroplast suspension is diluted in at least 8 volumes of grinding medium deprived of bovine serum albumin and centrifuged at 6,000 for 5 min x g (see Note 17). The pellets are again diluted in grinding medium deprived of bovine serum albumin and centrifuged at 6,000 x g for 5 min to pool them in a single pellet.
5. The chloroplast pellet is diluted into 35 mL of 1.1 M sucrose medium and frozen in liquid nitrogen after 10 min on ice (see Note 18). The suspension can be stored at -80°C.

6. For further envelope isolation, chloroplast suspension is thawed (see Note 19). A 2 M sucrose solution is added progressively until refraction index reaches 1.396 (see Note 20). 15 mL of chloroplast suspension is poured in a centrifugation tube, on which 12 mL of 1.1 M sucrose medium is added, before addition of 12 mL of 0.4 M sucrose on the top. The sucrose gradients are centrifuged at 100,000 x g for 5 h (swinging bucket rotor). The chloroplast envelope fraction is present in the top layer of the gradient (see Note).

4. Notes

1. 6 trays of 30 cm x 45 cm x 7 cm are used for culture. Transfer to dark several hours prior chloroplast preparation reduces chloroplast starch content that causes chloroplast rupture during centrifugation. 400 to 500 g of leaves are expected for chloroplast envelope preparation.
2. Centrifugation is necessary for formation of these Percoll gradients. They can be prepared the day before experiment and stored at 4°C.
3. 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.
4. Grinding should result in consistent small leaf pieces of about 2 mm side.
5. Gentle mixing with a pencil can help to get a homogenous suspension and to preserve chloroplast integrity.
6. It is better to set low centrifuge acceleration rate and brake off in order to avoid abrupt disturbance of the gradients.
7. 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 M acetone / water 80 % v/v. The mixture is centrifuged at 7,000 x g for 15

minutes. OD of the supernatant is measured at 652 nm. Chlorophyll content of the fraction (mg/ mL) is given by $OD \times 26$ (5).

8. 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.
9. Grinding should be soft to avoid chloroplast rupture however it should result in regular small leaf pieces of about 1 mm side.
10. Gentle mixing of the pellet with a pencil is used to get a homogenous suspension and to preserve chloroplast integrity.
11. It is better to set low centrifuge acceleration rate and brake off in order to avoid abrupt disturbance of the gradients.
12. To reduce contamination, it is better to collect the intact chloroplast layer after removal of the upper part of the gradient.
13. 20 to 30 mg of chlorophyll are expected from 500 g of leaves (see 2.3 Notes, Note 16). Depending on the amount of intact chloroplasts that was recovered, it may be best to load less than 6 sucrose gradients.
14. 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.
15. Grinding should result in consistent small leaf pieces of about 1 to 2 mm side.
16. Gentle mixing with a pencil is used to get a homogenous suspension and to preserve chloroplast integrity.
17. 70 to 90 mg of chlorophyll are expected from 1 kg of leaves (see 2.3 Notes, Note 16).
18. Transition to the 1.1 M sucrose chloroplast hyperosmotic concentration induces shrinkage of chloroplasts and disconnection of the envelope membranes from the other parts of the chloroplast. Freezing and thawing break the membranes.

19. The frozen mixture is set at room temperature but as soon as the suspension liquefies, it is returned onto ice.
20. A 1.3 M sucrose concentration has a refraction index of 1.396.
21. The chloroplast envelope fraction collected in the top layer of the sucrose gradient can be used like that or concentrated by centrifugation at 100,000 x g for 1h (ultracentrifuge, swinging bucket rotor) after dilution in an appropriate low density buffer (see spinach chloroplast envelope preparation) or further loaded on another sucrose gradient to separate inner and outer envelope membranes (see chapter 8). For quality preservation, addition of protease inhibitors such as 100 mM phenylmethylsulfonyl fluoride, 100 mM benzamidine, and 50 mM ϵ -amino caproic acid is recommended as well as storage at -80°C.

5. References

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