An Improved Method for the Isolation of Spinach Chloroplast Envelope Membranes

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ABSTRACT

A three-phase, discontinuous sucrose gradient yielded two distinct fractions of envelope membranes from spinach (Spinacia oleracea L.) chloroplasts. Their buoyant densities were 1.08 g cm\(^{-2}\) and 1.11 g cm\(^{-3}\). Electron micrographs showed the lighter and heavier fractions to consist primarily of single and double membranes, respectively. The milligrams of lipid-milligrams of protein ratio for the complete envelope membrane (double membrane fraction) was 1.74. Thin layer chromatograms showed that the lipids of the complete envelope membranes were similar to those found in earlier preparations which consisted of single and double membranes. This isolation procedure is superior to earlier methods in that the percentage of complete envelope membranes is greater and the yield is almost three times as great. Enzymatic and chemical analyses and microscopic examination showed the complete envelope membranes were free of bacterial, fungal, microsomal, mitochondrial, and lamellar membrane contamination as well as stromal contamination. The specific activities of nonlatent Mg\(^{2+}\)-dependent ATPase (80 \(\mu\)moles of phosphate released hr\(^{-1}\) mg protein\(^{-1}\)) were about 10-fold higher than those values found with earlier preparations consisting of single and double membranes, indicating that the ATPase is largely lost in preparations containing single membranes. These higher values show that the ATPase is located in the double membrane and probably functions in the transport processes of the envelope membrane.

Methods for the isolation of envelope membranes from chloroplasts have been developed recently by Poincelot (14) and others (2, 7). Although these envelope membrane preparations are relatively free of lamellar and stromal contamination, they suffer from two disadvantages. First, they have low levels of ATPase activity (approximately 12 \(\mu\)moles Pi released hr\(^{-1}\) mg protein\(^{-1}\)), and second, they consist of a mixture of single and double membrane vesicles (2, 7, 14).

Inasmuch as the Mg\(^{2+}\)-dependent ATPase has been suggested as an enzymatic marker for chloroplast envelope membranes (2, 14), higher activities would be advantageous. It seems that this Mg\(^{2+}\)-dependent ATPase is localized between the two membranes comprising the envelope membrane (16), hence, the presence of single membranes may indicate a loss of ATPase. Preparations with greater percentages of double membrane vesicles may thus have higher ATPase activities and be better suited for biochemical studies of the envelope membrane. This paper details a procedure for isolating preparations of envelope membranes from spinach chloroplasts, which have more double membrane vesicles and higher ATPase activities than those prepared by former methods (2, 7, 14).

MATERIALS AND METHODS

Plant Material and Growing Conditions. Spinach (Spinacia oleracea L., var. Viroflay, Asgrow Seed Co.) was grown in the greenhouse in sand to which nutrient solution was added weekly.

Chloroplasts. Intact chloroplasts were prepared from 10-g batches of freshly harvested leaves by procedures already described (4, 12). Plants, when harvested, were 4 to 6 weeks old.

Removal and Isolation of Envelope Membranes. The method is summarized in Figure 1 and is based on the fact that envelope membranes are easily removed from intact chloroplasts by suspending the latter in hypotonic medium. Two pellets of intact chloroplasts (derived from 20 g leaves) were suspended in 6 ml of hypotonic medium (Tricine buffer, pH 7.6, 50 mM). These suspensions of 0.58 to 0.75 mg of Chl/ml were kept at 4 C for 20 min with occasional swirling, whereupon they were pooled and homogenized by making three complete passes in a TenBroeck homogenizer. The homogenized suspension was mixed with 2 ml of 48% sucrose (w/v) in 50 mM Tricine buffer (pH 7.6) to yield about 8 ml of a suspension containing 12% sucrose (w/v). The suspension was purified on a three-phase, discontinuous sucrose gradient in a Beckman Model L preparative centrifuge. The sucrose gradient, buffered as above, consisted from top to bottom of 2.5 ml of the osmotically shocked suspension of chloroplasts in 12% sucrose (w/v), 1.5 ml of 23% sucrose (w/v), and 1 ml of 30% sucrose (w/v). In all, three tubes of above sucrose gradient were centrifuged in a swinging bucket rotor (type SW 50L) at 4 C for 60 min at 27,000 rpm (78,000 g at R\(_{max}\)). Two pale yellow bands, visible as milky white bands with back lighting, were found at the two interfaces. Avoiding turbulence, we carefully removed each band (approximately 0.5 ml). The upper and lower bands contained the incomplete and complete envelope membranes, respectively.

Electron Microscopy. Envelope membrane suspensions were diluted with an equal volume of Tricine buffer and centrifuged at 78,000 g (R\(_{max}\)) for 60 min (4 C). Pellets of envelope membranes were fixed (4 C) with 2% glutaraldehyde in 100 mM, pH 7.4 cacodylate buffer for 30 min. After removing the glutaraldehyde, each pellet was immersed in three 5 ml changes (30 min, each change) of fresh cacodylate buffer and postfixed with 1% osmium tetroxide in cacodylate buffer for 30 min at 4 C. The osmium tetroxide was decanted and the pellet was washed in three changes of cacodylate buffer as
above. After dehydration in ethanol, pellets were embedded in plastic according to Spurr (17). Sections were stained with uranyl acetate and lead citrate and examined with a Zeiss EM9 S-2 electron microscope.

**Lipid Extraction and Thin Layer Chromatography.** Lipids were extracted successively with chloroform, methanol, and water, and their dry weight was determined as described previously (1, 14). Two-dimensional TLC was carried out in chloroform-methanol-acetic acid-water (170:25:25:6) and chloroform-methanol-7N NH4OH (65:30:4) and lipids were identified as before (14).

**Enzyme Assays.** Carbonic anhydrase and ribulose diphosphate carboxylase activities were assayed by the procedures of Rickli et al. (15) and Paulsen and Lane (11), respectively. The Mg2+-dependent ATPase activity was measured by the procedure of Nelson et al. (8) \( \text{NAD(P)}^+ \text{H:Cyt C oxidoreductase} \) activity was determined by the method of Douce et al. (3). Inorganic phosphorus was measured with molybdate (5), and protein with Folin reagent (6).

**RESULTS**

After centrifugation in the discontinuous sucrose gradient, two yellowish white bands were observed at the interfaces (Fig. 1). The upper and lower membrane bands had buoyant densities of 1.08 and 1.11 g cm\(^{-3}\), respectively. The upper layer consisted primarily of single membrane vesicles and the lower layer of double membrane vesicles as demonstrated by electron microscopy (Fig. 2, A and C, 2, B and D, respectively). The double membrane vesicles (complete envelope membranes) had a mg of lipid-mg of protein ratio of 1.74. From 80 g of spinach leaves, the total yield of complete envelope membranes was approximately 2.5 mg. Pelleted material at the bottom of the tube contained lamellar material.

That the upper layer resulted from damaged or separated envelope membranes was easily seen. When envelope membranes were deliberately allowed to remain at room temperature or were vigorously stirred before centrifugation, the color of the upper layer increased in intensity as did the protein, with a corresponding decrease in the lower layer. Under normal conditions of preparation, the quantity of protein in the upper layer (after washing-centrifugation to remove contaminating stromal protein) was less than 10% of that in the lower or complete envelope membrane layer.

In Figure 2B, many envelope membranes in the lower layer do not appear to be double. However, under higher magnification, these single-appearing envelope membranes were observed to have the 100-Å spacing (Fig. 2D) typical of the chloroplast double membrane structure (18). A few membranes had larger spacing between the two membranes, suggesting some separation during the isolation process. The upper layer (Fig. 2A) contains single-appearing membranes, which under higher magnification (Fig. 2C) were not resolved into double membranes with 100-Å spacing. Examination of many electron micrographs indicated that the upper layer had about 80% single membrane vesicles (incomplete envelope membranes) and the lower layer about 75% complete (double) envelope membranes. Those double-appearing membranes, found occasionally in the upper layer of incomplete envelope membranes, were substantially different from the complete envelope membranes in the lower layer. Sometimes sections of the former were missing or appeared as a diffuse mass, rather than a sharp line. Others were observed with as much as 400-Å spacing between the single membranes of a pair. These observations would be consistent with damaged membranes. In summary, the upper layer contained mostly incomplete envelope membranes and a few badly damaged complete ones, as contrasted with the lower layer, which consisted mostly of complete envelope membranes.

Chloroplast envelope membranes were examined for various contaminants. Bacterial and fungal contaminants were not observed with light or electron microscopy. Subsequent plating on nutrient media showed no contamination when aseptic techniques were used. Great care must be taken to control aphids on leaves before harvest, as their sticky carbohydrate excretions were found to harbor yeasts and could cause contamination if aphid populations are high.

Stromal contamination was measured by assaying for the stromal enzymes, carbonic anhydrase and ribulose diphosphate carboxylase (9, 13). Envelope membranes had no detectable carbonic anhydrase activity with as much as 0.12 mg of membrane protein, and levels of ribulose diphosphate carboxylase, with similar amounts of protein, indicated less than 1% contamination by stromal material.

Lamellar membrane contamination was ascertained from the protein-Chl ratio. Lamellar membranes have a \( \mu \text{g} \) of protein/\( \mu \text{g} \) of Chl ratio of 4.2 (10), whereas the complete envelope membranes which contain no Chl, had a \( \mu \text{g} \) of protein/\( \mu \text{g} \) of Chl ratio of 315. Therefore the complete envelope membranes were estimated to be at least 98% pure with respect to lamellar membrane contamination.

Contamination of the complete envelope membranes from microsomes or mitochondria was not evident, since no \( \text{NAD(P)}^+ \text{H:Cyt C oxidoreductase} \) activity was detected with up to 0.10 mg of envelope membrane protein. This enzyme is found on the inner and outer membranes of plant mitochondria and microsomes (3).

The complete envelope membranes described here contained a nonlatent. Mg2+-dependent ATPase activity of 80 \( \mu \text{moles} \) of Pi released hr\(^{-1}\) mg protein\(^{-1}\) on the average (Table I). Rates as high as 150 were observed. When envelope mem-
Lipids in the complete envelope membranes were not qualitatively different from those found in earlier preparations consisting of single and double membranes (14). Lipids were not quantitatively determined as previously, but the chromatogram spot intensities indicated that the major lipids were monogalactosyldiglyceride, digalactosyldiglyceride, and phosphatidylcholine. Minor lipids were phosphatidylycerol, phosphatidylinositol, sulfolipid, trigalactosyldiglyceride, acylated sterylglucoside, sterylglycoside, and neutral lipids. Trace amounts of Chl, carotenoid, lysophosphatidylcholine, diphasphatidyglycerol, cerebroside, phosphatidylethanolamine, and unidentified glycolipids were observed.

**DISCUSSION**

Methods for isolating chloroplast envelope membranes have been developed recently (2, 7, 14). As with any new method, confusion in terminology is apt to arise. The term "envelope membranes" is certainly correct, but does not allow us to differentiate between different types produced according to the isolation procedure used. In the present paper, we have chosen the form "complete envelope membranes" to mean those envelope membranes appearing as double membrane vesicles.

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**Table I. Nonlatent Mg2+-dependent ATPase Activity in Chloroplast Envelope Membranes**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Method of Isolation of Envelope Membranes</th>
<th>Previous ref. 14</th>
<th>Present</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>μmoles of Pi released hr-μg protein</td>
<td>6</td>
<td>146</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>3</td>
<td>65</td>
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<tr>
<td>2</td>
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<td>8</td>
<td>80</td>
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1 In Experiments 1 and 2, isolated chloroplasts were divided into two portions and envelope membranes were prepared simultaneously by the previous and present method.

2 Experiment 3 represents the average of four experiments carried out with separate preparations of chloroplasts for each method. Values of about 13 were obtained by Douce et al. (2) with their envelope membrane preparations.

Envelopes were prepared from the same batch of chloroplasts by both the old (14) and present method, the ATPase activities with the new method were consistently at least 10-fold higher (Table I).

Fig. 2. Representative fields of the incomplete (A and C) and complete (B and D) envelope membranes, which range in diameter from 0.2 μm to 2.0 μm. At high magnification, the incomplete envelope membranes were single-appearing, except for a few exceptions illustrated in C. A complete envelope membrane in the process of separating (C, a) shows approximately a 400-Å spacing. An envelope membrane which has a section washed out (appearing as a diffuse mass) is shown by C, b. Complete envelope membranes showing a spacing of 75 to 100 Å between the two membranes comprising the envelope can be seen in D, c.
whereas those with only single membranes are designated as “incomplete envelope membranes”.

The present method is clearly better than the previous one (14). The time required for preparation is reduced by the elimination of the washing-centrifugation steps, because the removal of contaminants is accomplished with an additional phase in the discontinuous gradient. The yield of complete envelope membranes is increased 2.5-fold, a conservative estimate, because the previous preparations consisted of only 50% complete envelope membranes, compared with 75% in the present method. The increased yield probably resulted from the elimination of the 2000g centrifugation in the preliminary purification step, where some envelope membranes were trapped and lost during the sedimentation of damaged chloroplasts. The new procedure also results in higher percentages of complete envelope membranes than those described by Douce et al. (2) and substantially purer complete envelope membranes than were obtained by Mackender and Leech (7), where mitochondrial and lamellar contamination were observed.

When percentages of complete envelope membranes were higher, levels of nonlatent Mg\(^{2+}\)-dependent ATPase were 10-fold higher (2, 14). Much ATPase activity was lost during the washing steps in the older procedure. The larger percentage of complete envelope membranes also precludes the loss of non-latent Mg\(^{2+}\)-dependent ATPase. Histochemical evidence indicates that this ATPase is localized between the two membranes comprising the complete envelope membrane (16). Although proposed as a marker enzyme for the complete envelope membrane, earlier levels of activity (2, 14) were too low for this purpose. However, the present levels (80 μmoles of Pi released hr\(^{-1}\) mg protein\(^{-1}\)) of activity found here make it an excellent marker enzyme, and suggest it functions in the transport processes of the envelope membrane.

In conclusion, complete chloroplast envelope membranes can be prepared free of microbial, mitochondrial, microsomal, lamellar, and stromal contamination. The procedure gives good yields of envelope membranes, of which 75% are complete, with a minimum of preparation time. A nonlatent Mg\(^{2+}\) dependent ATPase is present in levels high enough to be assayed as a marker enzyme. The lipid composition has been extensively characterized (14). These complete envelope membranes should enhance our understanding of the biochemistry, enzymology, and permeability of the chloroplast envelope membrane.

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LITERATURE CITED