Labeling and Isolation of Plasma Membranes from Corn Leaf Protoplasts

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ABSTRACT

A plasma membrane-enriched fraction has been isolated from corn leaf mesophyll protoplasts and its identity confirmed with the aid of an external label, diazotized 125Iiodosulfanilic acid. Gentle cell disruption enabled internal organelles to be maintained intact and thus facilitated separation from the plasma membrane. The plasma membrane-enriched fraction was devoid of chloroplast or mitochondrial markers, whereas markers for the endoplasmic reticulum and golgi indicated minimal contamination. The highly enriched plasma membrane fraction contained a Mg2+-dependent, K+ stimulated ATPase with a pH optimum near neutrality. The position of the membranes on sucrose density gradients indicates that the plasma membranes have characteristics similar to other plasma membrane fractions.

MATERIALS AND METHODS

Plant Material and Protoplast Isolation. Zea mays L., cv. Golden Bantam, was grown in a fertilized peat-vermiculite soil mixture at room temperature under continuous cool-white fluorescent lighting. Protoplasts were isolated from the youngest fully expanded leaves of 12- to 14-day old plants by the method described by Earle et al. (3). All conditions were identical except that the digestion enzyme was passed through a Sephadex G-50 column prior to use and digestion was carried out in the dark without shaking. Protoplasts were liberated by gently swirling the digested tissue. Following isolation, the protoplasts were washed five times in 0.6 M sorbitol, 50 mM K-phosphate buffer (pH 7.5), and 5 mg/ml BSA, by repeated low speed centrifugation (50g). The inclusion of BSA was necessary because released cellular debris, most notably chloroplasts, would adhere to the protoplast surface. BSA eliminated this problem and had the added advantage of promoting greater total yield of intact protoplasts. The washed protoplasts were further purified by aqueous two-phase polymer separation (11) and resuspended in 0.6 M sorbitol and 50 mM phosphate buffer (pH 7.5) and stored on ice.

Labeling. 125I Iodosulfanilic acid of high specific radioactivity (greater than 1,000 Ci/mmol) was converted to its diazonium salt by the sequential addition of equal quantities of NaNO3 and HCl, as described in the instructions accompanying the labeling kit supplied by New England Nuclear. Unlabeled diazotized sulfanilic acid was prepared in the same manner. The final concentration of diazotized sulfanilic acid was 50 μM, and 60–80 μCi of 125I iodine were used per experiment.

Washed protoplasts were incubated in the presence of label for 30 min at 4°C. Labeling was terminated by removal of the labeling medium and replacement with 0.6 M sorbitol, 50 mM phosphate buffer (pH 7.5), and 10 mg/ml BSA. The additional protein was needed to complex loosely adsorbed, unreacted label. The protoplasts were washed at least five times in the above medium.

Plasma Membrane Isolation. Labeled protoplasts were resuspended in 0.6 M sorbitol and 50 mM phosphate buffer (pH 7.9). An equal volume of 50 mM Tris-Mes (pH 7.9), 2 mM Na2EDTA, and 6 mM DTT was added to the suspension. The protoplasts were allowed to swell for 5 min causing them to become extremely fragile. Lysis was readily achieved by six gentle passes of a Teflon plunger in a glass Teflon hand-held homogenizer. This process resulted in a nearly complete lysis of intact protoplasts. The lysate was layered on a 35% sucrose pad and centrifuged at 10,000g for 10 min (Fig. 1). Dense organelles (e.g. chloroplasts and mitochondria) readily moved into the sucrose pad along with unbroken protoplasts and membrane aggregates. The supernatant fraction was then layered on a discontinuous sucrose gradient (31 and 38%

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Abbreviations: Na2EDTA: ethylenedinitrilotetraacetic acid, disodium salt.
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Table I. Distribution of the Diazotized \[^{125}I\]iodosulfanilic Acid Label in Different Subcellular Fractions

<table>
<thead>
<tr>
<th>Fraction*</th>
<th>Total Radioactivity</th>
<th>Total Protein</th>
<th>Specific Activity</th>
<th>Label Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm</td>
<td>mg</td>
<td>cpm/mg protein</td>
<td>%</td>
</tr>
<tr>
<td>A</td>
<td>33,500</td>
<td>32.1</td>
<td>1.1 × 10^3</td>
<td>49</td>
</tr>
<tr>
<td>B</td>
<td>17,520</td>
<td>11.8</td>
<td>1.48 × 10^3</td>
<td>37</td>
</tr>
<tr>
<td>C</td>
<td>17,980</td>
<td>17.1</td>
<td>1.05 × 10^3</td>
<td>37</td>
</tr>
<tr>
<td>D</td>
<td>12,960</td>
<td>0.56</td>
<td>23.1 × 10^3</td>
<td>37</td>
</tr>
<tr>
<td>E</td>
<td>5,020</td>
<td>16.3</td>
<td>0.32 × 10^3</td>
<td>37</td>
</tr>
</tbody>
</table>

* See Figure 1.

Table II. Activity of Major Organelle Enzyme Markers in the Various Subcellular Fractions Isolated

The value in parentheses represents the total activity of the fraction.

<table>
<thead>
<tr>
<th>Fraction*</th>
<th>Cyt c Oxidase</th>
<th>NADH Cyt c Reductase</th>
<th>NADPH Cyt c Reductase</th>
<th>Latent IDPase</th>
<th>K(^+)-Stimulated ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.68</td>
<td>(21.6)</td>
<td>(31.4)</td>
<td>(31.1)</td>
<td>(31.1)</td>
</tr>
<tr>
<td>B</td>
<td>1.5</td>
<td>(43.8)</td>
<td>(31.4)</td>
<td>(31.1)</td>
<td>(31.1)</td>
</tr>
<tr>
<td>C</td>
<td>0.04</td>
<td>(0.66)</td>
<td>(31.9)</td>
<td>(31.8)</td>
<td>(31.8)</td>
</tr>
<tr>
<td>D</td>
<td>0.20</td>
<td>(0.22)</td>
<td>(0.34)</td>
<td>(1.57)</td>
<td>(7.32)</td>
</tr>
<tr>
<td>E</td>
<td>0.00</td>
<td>(0.00)</td>
<td>(31.6)</td>
<td>(31.4)</td>
<td>(31.1)</td>
</tr>
</tbody>
</table>

* See Figure 1.

Table III. Distribution of Labeled Chloroplasts and Total Chlorophyll in Different Subcellular Fractions

Chloroplasts were isolated from lysed protoplasts and collected in 0.3 \( \text{M} \) sorbitol and 50 \( \text{mM} \) phosphate buffer (pH 7.5). Labeling was identical to the methods used for protoplast labeling.

<table>
<thead>
<tr>
<th>Fraction*</th>
<th>Chlorophyll</th>
<th>Total Radioactivity</th>
<th>Specific Radioactivity</th>
<th>Label Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.35</td>
<td>88,500</td>
<td>2,160</td>
<td>95.4</td>
</tr>
<tr>
<td>B</td>
<td>2.51</td>
<td>84,400</td>
<td>4,200</td>
<td>95.4</td>
</tr>
<tr>
<td>C</td>
<td>ND(^\text{b})</td>
<td>5,750</td>
<td>295</td>
<td>6.5</td>
</tr>
<tr>
<td>D</td>
<td>ND</td>
<td>120</td>
<td>600</td>
<td>0.14</td>
</tr>
<tr>
<td>E</td>
<td>ND</td>
<td>5,000</td>
<td>250</td>
<td>5.7</td>
</tr>
</tbody>
</table>

* See Figure 1.

b Not detectable.

RESULTS

A primary concern during the labeling process was to prevent modification of specific membrane properties. Diazotized \[^{125}I\]iodosulfanilic acid used at 50 \( \mu \text{M} \) did not significantly alter membrane-bound enzyme activity, particularly the K\(^+\)-stimulated ATPase (Fig. 2). Once labeled, the plasma membrane could be followed easily through the isolation procedure. Table I indicates the presence of label through the fractionation process and demonstrates that the fraction with highest specific radioactivity came from recovery of the final 80,000g pellet (fraction D). The specific radioactivity of this fraction was much greater than that of any other fraction recovered. Analysis of this fraction for major organelle contamination indicated that it was essentially devoid of markers for the ER, mitochondria (Table II), and chloroplasts (Table III). Golgi contamination was also demonstrated to be minimal, yet it contributed proportionately more to the fraction than the other organelles.

Fraction D contained the highest specific activity of K\(^+\)-stimu-

FIG. 1. Diagram illustrating how each major subcellular fraction was obtained. Fractions were designated as follows (see under "Materials and Methods"): (A) lysate; (B) 10,000g pellet; (C) 10,000g supernatant; (D) final 80,000g pellet; and (E) fraction above the 31 and 38% sucrose interface in the first 80,000g centrifugation.

FIG. 2. The effect of diazotized sulfanilic acid on membrane-bound ATPase activity.

sucrose) and centrifuged at 80,000g for 45 min. The interface (31/38) was removed with a pasteur pipette, diluted in 0.25 M sucrose and 10 mM Tris-Mes (pH 7.0), and pelleted at 80,000g. The pellet was resuspended and assayed.

Enzyme Assays. Protein was determined by a modified Lowry procedure (18). ATPase activity was determined by the method described by Ames (1). All reductase assays were performed according to Hodges and Leonard (10), and latent IDPase activity was determined by the method of Ray et al. (20).
lated ATPase activity (Table II). This enzyme activity has been associated with plasma membranes from root and coleoptile tissue (6, 8, 9, 13–15). Further characterization of this fraction revealed that the enzyme activity had a clear pH optimum at pH 6.5 (Fig. 3), was stimulated by Mg\(^{2+}\), and was further stimulated by K\(^+\) (Table IV).

Sucrose density centrifugation of fraction D showed that peak radioactivity corresponded with the peak of K\(^+\)-stimulated activity but did not coincide with markers for major organelles (Fig. 4). Since mitochondria and plasma membranes reach sedimentation equilibrium at approximately the same density it was important to show that these two membranes were separate. This was done by contaminating the gradient with isolated mitochondria and comparing the peak of radioactivity to the peak of Cyt c oxidase activity. Figure 4 clearly demonstrates this separation. Thus, labeling and marker enzyme studies would indicate that the final 80,000g pellet (fraction D) was enriched with plasma membranes.

In a further attempt to demonstrate the purity of the proposed plasma membrane fraction, we hypothesized that a small mitochondrial contamination might contribute significantly to Mg\(^{2+}\)-ATPase activity. In an effort to test this hypothesis, the ATPase activity was measured as a function of pH in the presence and absence of oligomycin (Fig. 5). Oligomycin has been shown to be a potent inhibitor of the mitochondrial coupling factor ATPase. The plasma membrane ATPase activity was unaffected by this inhibitor and the validity of this test was clearly demonstrated by contaminating one fraction with mitochondria and reversing the effect with oligomycin. Thus, mitochondrial contamination appears insignificant and certainly does not contribute to the ATPase activity observed.

One of the prime difficulties in using green tissue as a starting material for plasma membrane isolations is the problem of disrupting chloroplasts and producing small fragments that are difficult to separate from light membranes by conventional differential centrifugation. We have addressed this problem in two direct ways. One was to measure the presence of Chl throughout the isolation procedure and the other was to label chloroplasts (isolated from lysed protoplasts) in the same fashion as the protoplasts and follow the label as previously described. Table III indicates that all the Chl was recovered in the 10,000g fraction and this was substantiated by the lack of perceptible Chl contamination of the final 80,000g pellet. In addition, 95% of the label was recovered in the initial 10,000g pellet and 6% from the supernatant of the first 80,000g centrifugation (Table III), this latter 6% possibly being accounted for by light chloroplast envelope membranes or soluble protein.

![Fig. 3. A profile of Mg-ATPase activity as a function of pH in the presence and absence of added 50 mM KCl. KCl included (X), KCl excluded (O), and the difference between the two activities (O).](image)

![Fig. 4. Density gradient profile of the final 80,000g pellet (fraction D). Fractions enriched in the following markers: (A) NADH Cyt c reductase (O), latent IDPase (●), (B) radioactivity (●), Cyt c oxidase (O); (C) K\(^+\)-stimulated ATPase activity (●), per cent sucrose (O). Panels B and C were obtained by adding a portion of fraction B to fraction D and running the sample on a 15–45% linear sucrose gradient at 80,000g for 3 h. Panel A was obtained by pooling two 100,000g fraction E pellets and running the membranes on a separate linear sucrose gradient. The percent sucrose shown in panel C applies to panels B and C, but also closely approximates the positions shown in panel A.](image)
A plasma membrane-enriched fraction has been isolated from corn leaf mesophyll protoplasts. It has been positively identified by labeling with diazonized [125I]iodosulfanilic acid. External labeling of the plasma membrane is the most effective means of identifying this subcellular membrane when intrinsic markers are absent or in doubt. The basic requirements used to select an effective cell surface label have been outlined by Maddy (17). It should (a) be nonpermeant and small for accessibility; (b) react under physiological conditions of pH and temperature; (c) form stable, covalent linkages with membrane constituents; (d) be detectable in small amounts; and (e) cause little or no perturbation of membrane structure and function. Diazonized [125I]iodosulfanilic acid was chosen because it readily met all of the above criteria and has been shown to be an effective label in other systems (4).

The labeling of purified protoplasts with this external marker permitted rapid identification of the plasma membrane during fractionation. Table I demonstrates that label was detected in each fraction but the 80,000g pellet (fraction D) was markedly enriched in label. It is reasonable to suggest that this fraction was highly enriched with cell surface membranes.

A previous labeling study has demonstrated the isolation of a highly labeled plasma membrane-enriched fraction. However, those membranes displayed significant golgi contamination (5). In contrast, analysis of fraction D for major organelle contamination indicated that it was relatively free of mitochondria, ER, golgi (Table II), and chloroplasts (Table III). Thus, highly purified plasma membranes must be present in this fraction. Further analysis on sucrose density gradients indicated that the membranes were separate and distinct from major organelles (Fig. 4). In addition, the position of these membranes on the sucrose density gradient agrees with the cell surface labeling study of Galbraith and Northcote (5). This confirms other work in establishing the position of plasma membranes in sucrose density gradients (9, 10, 14-16).

Partial characterization of the plasma membrane ATPase indicated that the activity was Mg2+-dependent, K+-stimulated (Table IV), and showed a pH optimum near neutrality (Fig. 3). In addition, the enzyme is substrate specific, showing a distinct preference for ATP over other substrates (unpublished data). This enzyme activity is characteristic of root and coleoptile plasma membranes (6, 8, 9, 13-15) and has also been detected in leaf tissue (16). While a K+-stimulated ATPase was readily detected, Table II indicates that this enzyme is best utilized as a qualitative marker rather than a quantitative marker. This is due to the presence of nonspecific phosphatases which are present in large amounts. The K+-stimulated ATPase activity was reflective of the plasma membrane fraction isolated and did not represent contamination due to mitochondria since ATPase activity was unaffected by the presence of oligomycin (Fig. 5).

Protoplasts proved to be effective for cell surface isolation since internal organelles could be maintained intact and thereby be rapidly separated from lighter membranes. However, due to the gentle nature of the lysis process, plasma membrane yields tended to be less than optimal. This was readily seen by determining the total label recovered in the 80,000g pellet (fraction D), an amount which was always less than 50% (Table I). This loss was probably due to the predominant formation of large, dense membrane sheets during lysis as opposed to small, light vesicles. The large sheets were readily moved and pelleted under relatively low centrifugal fields (10,000g).

We conclude that a plasma membrane-enriched fraction may be isolated from corn leaf mesophyll protoplasts by gentle lysis, and differential and density gradient centrifugation. An external label, diazonized [125I]iodosulfanilic acid, was used to monitor the cell surface membranes and a correlation was established between label and enzyme activity associated with the label. Analysis for organelle contamination indicated that the plasma membranes were of high purity.

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