Phospholipid Composition of a Plasma Membrane-Enriched Fraction from Developing Soybean Roots

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ABSTRACT

Phospholipid polar head group and fatty acid composition were determined for plasma membrane enriched fractions from developing soybean root (Glycine max [L.] Merr. cult. Wells II). Plasma membrane vesicles were isolated from meristematic and mature sections of four-day-old dark grown soybean roots at pH 7.8 and in the presence of 5 millimolar ethylenediaminetetraacetate, 5 millimolar ethyleneglycol-bis (β-aminoethyl ether)N,N tetracetic acid, and 10 millimolar NaF. Lipid extracts analyzed for phospholipid composition revealed two major phospholipid components: phosphatidylcholine and phosphatidylethanolamine. Minor phospholipid components identified were phosphatidylserine, phosphatidyldserine, phosphatidylglycerol, and diphosphatidylglycerol. Lipid degradation by endogenous phospholipase D during membrane isolation at pH 6.5 and in the absence of chelating agents and NaF resulted in the recovery of large amounts of phosphatic acid. Phosphatidylethanolamine was the principal substrate for phospholipase D.

Fatty acid composition was determined for plasma membrane phosphatidylethanolamine and phosphatidylethanolamine from meristematic and mature root tissue. The fatty acids identified were 16:0, 18:0, 18:1, 18:2, and 18:3. Fatty acid composition varied with both phospholipid class and the developmental stage of the root. Results suggest that differences in the composition of the major phospholipids of plasma membrane from meristematic and mature root sections occur in the fatty acids and not in the polar head groups. These differences and those found in the composition of the polar head groups of the minor phospholipid components, e.g., phosphatidylglycerol, may be significant for structure-function relationships within the membrane.

The chemical composition of a membrane influences its functional properties including fluidity, permeability, protein activity, susceptibility to antibiotics and the structural configuration that the lipids assume under thermal and ionic conditions (22). Plant researchers have studied chloroplast and mitochondrial membranes extensively (12, 18, 21), but comparatively little work has been done to elucidate the composition of plasma membrane from higher plants. Plasma membranes from plants as well as other organisms are dynamic, their composition varying with environment and development. Studies on the plasma membrane of soybean root have demonstrated changes in sterol:phospholipid and sterol:protein ratios (28), sterol (27), and protein composition (5) with development. These changes may reflect alterations in membrane composition associated with differentiation of the average cell in the representative root segment and/or aging effects which may be common to all cells. The inability to prepare highly enriched fractions of plasma membranes from plants in quantities sufficient for analysis has hindered efforts to characterize plasma membrane composition. In a previous communication (28) we reported the isolation of a highly enriched plasma membrane fraction. Comparisons of isolated vesicles stained with the photophotographic acid-chromic acid procedure, specific for the plasma membrane, with vesicles stained with the general membrane stain uranyl acetate-lead citrate indicated these preparations were approximately 75% pure (2). Additional electron microscope studies with concanavalin A-ferritin stained vesicles suggested that the purity of the plasma membrane fraction may exceed 80% (27).

In this study, we have characterized the phospholipid composition of that enriched plasma membrane fraction from developing soybean root with respect to both polar head group and fatty acid composition. The enriched plasma membrane fractions showed minor changes in phospholipid polar head group composition with maturation. Changes in the acyl composition of the phospholipids during development were significant. These structural changes may be indicative of functional differentiation in the membrane with development.

MATERIALS AND METHODS

Plant Tissue. Soybean seedlings (Glycine max [L.] Merr. cult. Wells II) were germinated in darkness in plastic dishes containing moist Vermiculite at 30°C. Four-day-old roots, excised below the region of lateral root development, were separated into meristematic tissue (terminal 3–4 mm) and mature tissue (section 1.5–4 cm behind meristematic zone) (29). All tissue was excised into ice-cold aerated deionized H2O.

Isolation of Plasma Membrane Vesicles. Plasma membrane vesicles were prepared by differential and sucrose density gradient centrifugation as described previously (28). All media were modified from published isolation procedures to include 5 mM EDTA, 5 mM EGTA, and 10 mM NaF, and titrated to pH 7.8 in order to reduce endogenous phospholipase activity (10). Briefly, 80 g of mature or 2 to 4 g of meristematic root tissue were homogenized in a mortar and pestle with three volumes of grinding medium (50 mM Tris-Hepes [pH 7.8], 5 mM EGTA, 5 mM EDTA, 10 mM NaF, 2.5 mM DT, 250 mM sucrose, and 100 μg/ml BHT). The crude homogenate was filtered through Miracloth and centrifuged for 15 min at 15,000 g. The resultant supernatant was centrifuged at 80,000 g for 35 min (Spinco SW 27 rotor), the pellet resuspended in fresh grinding medium, and centrifuged again at 80,000 g (Spinco T 65 rotor) for further purification. This microsomal pellet was suspended in 1 ml resuspension buffer (1 mM Tris-Hepes [pH 7.8], 5 mM EDTA, 5 mM EGTA, 10 mM NaF, 20% sucrose, w/w) and layered on a discontinuous sucrose gradient consisting of 8 ml of 34% sucrose

1 Abbreviations: BHT, butylated hydroxytoluene; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatic acid; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol.

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(w/w) (containing 0.55 mM Tris-Hepes [pH 7.8], 5 mM EDTA, 5 mM EGTA, and 10 mM NaF) layered over 28 ml of 45% sucrose in the same buffer system. The gradients were centrifuged for 135 min at 80,000g (Spinco SW 27 rotor). The plasma membrane-enriched fractions recovered from the sucrose gradient interface were diluted with resuspension buffer, pelleted at 80,000g for 35 min (Spinco T 65 rotor), and resuspended in 0.5 ml resuspension buffer.

Phospholipid Analyses. Plasma membrane lipids were extracted by the method of Folch et al. (9). To inactivate membrane associated phospholipases, the plasma membrane suspension was first added to 5 ml of boiling isopropanol. After 5 min, 20 volumes of chloroform:methanol (2:1, v/v) were added to the mixture and lipids were extracted for 30 min. Extracts were washed twice with 1% (w/v) NaCl, evaporated to dryness under N2, redissolved in 100 µl chloroform:methanol (9:1, v/v), and stored overnight under N2 at −20°C.

For TLC, Silica Gel H plates (0.250 mm Redicoat H, Supelco, Inc.) were prewashed overnight in chloroform:methanol (2:1, v/v), dried at room temperature, and activated for 1 h at 100°C. Phospholipids were separated by two-dimensional TLC (25). Plates were developed in the first dimension with chloroform:methanol:28% aqueous ammonia in the proportions 65:25:5 and dried 20 min under vacuum. The second solvent was chloroform:acetone:methanol:acetic acid:water in the proportions 30:40:10:10:5. For some determinations, the proportions of the solvents were modified to improve the separation of PS and PI (65:35:5 and 50:20:10:10:5, respectively). Lipids were identified by co-chromatography with phospholipid standards and the use of the following detection reagents: molybdenum blue reagent for phosphorus, Dragendorff reagent for choline, ninhydrin for free amino groups and periodate-Schiff reagent for vicinal diol groups. For quantitative analyses, phospholipids were visualized with I2 vapor, scraped from the plates, and transferred to tubes for direct digestions. Lipid phosphorus was analyzed by the Bartlett method as described by Fattmer and Wels (8). Silica gel was removed from the samples by filtration through glass microfiber filters prior to reading the absorbance.

Fatty Acid Analysis. All organic solvents used contained 0.05% (w/v) BHT to prevent autoxidation. After separating the phospholipids by two-dimensional TLC, lipid spots corresponding to PC and PE were each vacuumed into Pasteur pipettes containing glass wool plugs. The silica gel was transferred to a screw cap tube with a Teflon-lined cap and the lipids were transterified directly on the silica gel. To the silica gel 0.5 ml of 2.5% H2SO4 in methanol:benzene (9:1, v/v) was added. The tubes were incubated for 20 min at 75°C. The methyl esters were extracted in benzene, dried under N2, and redissolved in 50 µl of benzene.

The resultant fatty acid methyl esters were separated and quantified on a Varian model 3700 gas chromatograph equipped with a flame ionization detector and an automatic data analysis system. Separations were done on a 1-m glass column packed with 10% SP-2340 on 100/120 Chromosorb W AW (Supelco, Inc.). Identification of the fatty acid methyl esters was made by comparison to known standards. All fatty acid data are presented as relative per cent of the five components.

Phospholipase D Assay. Cell-free extracts, microsome, and plasma membrane-enriched fractions were prepared from meristematic and mature root tissue by differential and sucrose density centrifugation (28). The procedure of Heller et al. (13) was used to assay phospholipase D in the crude enzyme preparations. Each assay contained 5 µmol phosphatidylcholine, 0.2 µCi 1-α-dipalmitylcholesterol-choline-methyl-3H] (New England Nuclear), 50 mM acetate buffer (pH 5.3) or 50 mM Tris-Hepes (pH 7.8), 2.5 mM SDS, 50 mM CaCl2, and 50 µl of crude extract (35–200 µg protein) in a volume of 1 ml. The reaction was performed at 30°C for 30 min with constant shaking and terminated by the addition of 4 ml chloroform:methanol (2:1, v/v). After centrifugation at 500g for 10 min, an 0.5 ml aliquot of the aqueous-methanol upper phase was added to 10 ml of Aquasol II (New England Nuclear). Radioactivity was determined with a Beckman LS 7000 scintillation counter. Protein was measured by a modification of the Lowry procedure (17).

RESULTS

Phospholipid Composition of Plasma Membrane Vesicles. Phospholipid composition of plasma membrane-enriched fractions from meristematic and mature soybean root tissue is shown in Table I. In preliminary analyses of plasma membrane vesicles isolated at pH 6.5, large amounts of PA were recovered: 11.8% (on a molar basis) of total membrane phospholipids in meristematic tissue and 26.6% in mature tissue. PA is thought not to occur in large quantities in membranes and its recovery is indicative of endogenous phospholipase D activity. To prevent this degradation, isolation media were modified to inhibit lipase activity (10). The high pH media did inhibit lipase activity but to a greater extent in the meristematic tissue than in the mature tissue. PC was the principal substrate of the enzyme (Table I).

PC and PE were the major phospholipid components in both meristematic and mature soybean root plasma membrane preparations (Table I). If PA recovered in membrane lipid is considered a degradation product due to the hydrolysis of PC, then PC and PE constitute 80% of membrane phospholipids. Differences in the level of these major phospholipids with respect to development were small: PC + PA remained constant and PE increased slightly with development.

Minor phospholipid components recovered from plasma membrane enriched fractions were PS, PI, PG, and DPG. There were small differences in the percentages of the first three components with development. Most notable was the decrease in PG as the root tissue matured. Differences in DPG were nonsignificant. Lyso-derivatives of the phospholipids were not detected in measurable quantities.

Fatty Acid Composition of PC and PE. The fatty acids recovered from PC and PE were palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3) (Table II). Relative percentages of palmitic and linoleic acids in PC from meristematic and mature plasma membrane preparations were similar; however, both stearic and oleic acids in PC declined with development. These decreases in relative percentage were concomitant with an increase in linolenic acid in mature tissue. The ratio of total saturated:total unsaturated fatty acids did not change as the tissue matured; however, the double bond index increased from 140.4 in meristematic root to 171.7 in mature root.

The fatty acid composition of PE maintained the same trends with development as noted in PC except that stearic acid did not decrease significantly with maturation. Comparison of the fatty acid composition of PC and PE reveals that the largest difference is in the per cent composition of palmitic acid in the two phospholipid classes.

Phospholipase D. Phospholipase D activities in the subcellular fractions from meristematic and mature root tissue are compared in Table III. Protein and total activity expressed per 10 g fresh root weight differ markedly for the two developmental stages. Recovery of protein and enzyme activity is clearly greater in meristematic tissue than in mature tissue. Comparisons of phospholipase D activity in meristematic and mature root tissue revealed no differences in the specific activity of the enzyme in the two tissue types.

The specific activity of phospholipase D was about the same for the crude homogenate, 15,000g supernatant, and 80,000g supernatant (Table III). It was somewhat reduced in the 15,000g
pellet and was substantially reduced in both the 80,000g pellet and plasma membrane-enriched fraction. The activity was heat labile in all fractions and increasing the pH of the enzyme assay from 5.3 to 7.8 resulted in 90% inhibition of activity (data not shown).

The 15,000g and 80,000g supernatants from mature tissue contained 87 and 73%, respectively, of the total enzyme activity recovered in the crude homogenate. This indicates that the majority of the enzyme present in these tissues is soluble. Less than 1% of total enzyme activity appeared in the microsome pellet. The reduced specific activities of the particulate fractions suggest that this phospholipase D is membrane bound.

**DISCUSSION**

There is general agreement among published lipid compositional analyses of higher plant plasma membranes that PC and PE are the major phospholipids (1, 7, 11, 18, 32). Reports differ, however, as to which of these phospholipid species constitutes the majority of lipids in the membrane. In the present system, PC comprised approximately 50% of membrane phospholipid in both meristematic and mature plasma membrane. In potato tuber (18) and oat root (15, 16) PE has been reported to be the major phospholipid component of the plasma membrane. This basic difference in membrane composition may reflect genotypic variation or may be the result of endogenous lipases. High activities of endogenous lipolytic enzymes such as phospholipase D and phosphatidic acid phospholipase (26), may significantly alter the composition of recoverable phospholipids, as in the present study. The persistently high levels of PA reported here for mature plasma membrane vesicles isolated at pH 7.8 were probably the result of rapid degradation of PC by phospholipase D during homogenization (24, 26). However, it is possible that some PA occurs naturally in this membrane.

PI, PS, PG, and DPG were minor phospholipid constituents in soybean root plasma membrane. Both PI and PG have been

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**Table I. Phospholipid Composition of Soybean Root Plasma Membrane Vesicles**

<table>
<thead>
<tr>
<th></th>
<th>pH 6.5</th>
<th>pH 7.8, EDTA, EGTA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Meristematic</td>
<td>Mature</td>
</tr>
<tr>
<td>PA</td>
<td>11.8 ± 1.6*</td>
<td>26.6 ± 1.7</td>
</tr>
<tr>
<td>PC</td>
<td>42.5 ± 2.0</td>
<td>29.3 ± 2.3</td>
</tr>
<tr>
<td>PA + PC</td>
<td>54.3 ± 0.8</td>
<td>55.9 ± 1.6</td>
</tr>
<tr>
<td>PE</td>
<td>28.6 ± 1.4</td>
<td>27.2 ± 2.2</td>
</tr>
<tr>
<td>PS</td>
<td>2.2 ± 0.3</td>
<td>5.7 ± 0.2</td>
</tr>
<tr>
<td>PI</td>
<td>9.0 ± 1.1</td>
<td>6.2 ± 0.2</td>
</tr>
<tr>
<td>PG</td>
<td>4.5 ± 0.5</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>DPG</td>
<td>0.9 ± 0.1</td>
<td>0.5 ± 0.2</td>
</tr>
</tbody>
</table>

* Values are means of 3 replicates ± SE.

**Table II. Fatty Acid Composition of PC and PE**

<table>
<thead>
<tr>
<th></th>
<th>Phosphatidylcholine</th>
<th>Phosphatidylethanolamine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Meristematic</td>
<td>Mature</td>
</tr>
<tr>
<td></td>
<td>mol %</td>
<td>mol %</td>
</tr>
<tr>
<td>16:0</td>
<td>22.4 ± 0.7a</td>
<td>23.8 ± 0.6a</td>
</tr>
<tr>
<td>18:0</td>
<td>11.6 ± 0.5a</td>
<td>6.9 ± 0.8b</td>
</tr>
<tr>
<td>18:1</td>
<td>12.3 ± 1.6a</td>
<td>3.2 ± 0.6b</td>
</tr>
<tr>
<td>18:2</td>
<td>32.6 ± 2.0a</td>
<td>29.9 ± 0.3b</td>
</tr>
<tr>
<td>18:3</td>
<td>21.0 ± 1.3a</td>
<td>36.2 ± 1.9b</td>
</tr>
</tbody>
</table>
| DBI
| 140.4 ± 8.4ac | 171.7 ± 8.8b | 134.8 ± 3.3c | 152.2 ± 13.0bc |

* Values are means of 3 replicates ± SE. Means in a horizontal line followed by the same letter do not differ significantly at the 5% level of probability.

**Table III. A Comparison to Phospholipase D Activity in Subcellular Fractions from Meristematic and Mature Soybean Root Tissue**

Activity was assayed with 0.10 mg protein at pH 5.3. For comparative purposes protein and total activities are expressed on the basis of 10 g fresh root weight.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>Total Activity</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Meristematic</td>
<td>Mature</td>
<td>Meristematic</td>
</tr>
<tr>
<td>Crude homogenate</td>
<td>539*</td>
<td>92.2</td>
<td>117</td>
</tr>
<tr>
<td>15,000g supernatant</td>
<td>375</td>
<td>69.2</td>
<td>93.4</td>
</tr>
<tr>
<td>15,000g pellet</td>
<td>88.0</td>
<td>13.3</td>
<td>12.8</td>
</tr>
<tr>
<td>80,000g supernatant</td>
<td>360</td>
<td>58.1</td>
<td>93.5</td>
</tr>
<tr>
<td>Microsome</td>
<td>17.9</td>
<td>1.5</td>
<td>1.1</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>1.0</td>
<td>0.1</td>
<td>Trace</td>
</tr>
</tbody>
</table>

* Values are means of three determinations.
PLASMA MEMBRANE PHOSPHOLIPIDS

reported in higher plant plasma membrane fractions (1, 7, 11, 14, 32). PS has also been detected but with less frequency (11, 32). There are no reports of DPG in higher plant plasma membrane fractions and its appearance is probably indicative of contamination by inner mitochondrial membranes (4). Based on reported values for DPG in inner mitochondrial membranes (19), contamination of the plasma membrane-enriched fraction by that membrane was less than 1%.

The fatty acids in PC and PE from both the meristematic and mature plasma membrane fractions were enriched in palmitic, linoleic, and linolenic acids, whereas stearic and oleic acids appeared in lesser amounts. These results are in general agreement with those of Rivera and Penner (23) who quantified total phospholipid fatty acids of plasma membrane isolated from whole soybean roots grown under various temperature regimes. The major fatty acids in plasma membrane-enriched fractions from oat root (14, 16), potato tuber (7, 18), corn root (11), and orchard grass crown (32) are palmitic and linoleic. In addition to soybean root, linoleic acid occurs in appreciable quantities in plasma membrane from cauliflower florets (18) and orchard grass crown (32). Palmotileic acid was not detected, although it has been observed in plasma membrane fractions from other systems (11, 14, 32). As in our study, unsaturated fatty acids accounted for 60 to 70% of plasma membrane fatty acids in the majority of these reports.

RER, inner mitochondrial membranes, and Golgi are known contaminants of plasma membrane-enriched fractions. Together they may constitute up to 25% of the membrane fraction. Based on rRNA concentration in these fractions, Berkowitz and Travis (3) have estimated that RER may represent 8% of membrane protein. They have also estimated mitochondrial contamination to be on the order of 2% (2). There is not currently a good estimate of Golgi contamination. The question arises as to how these contaminating membrane fractions affect the values reported here for the phospholipid composition of soybean plasma membrane. In both RER (20) and inner mitochondrial-enriched membrane fractions (19), phospholipid to protein weight ratios are less than that of soybean plasma membrane (28). As a result, contamination of the plasma membrane by phospholipids in these membranes will be proportionately reduced. Comparisons of cell membranes from plant organs devoid of chloroplasts reveal uniformity of phospholipid composition in minor variations and similar fatty acid compositions (18). This suggests that although contamination of the plasma membrane fractions by other organelles will affect the values reported, the actual composition of the plasma membrane is probably within our experimental error. Using the phosphotungstic acid chromic acid procedure, Berkowitz and Travis (2) have established that the levels of purity are comparable for plasma membrane vesicles isolated from meristematic and mature root tissue.

The effects of root development on the phospholipid composition of plasma membrane were relatively minor. Analysis of the polar head groups showed that the major phospholipid classes, PC and PE, changed little with development. Minor phospholipid components exhibited small differences in composition as the root tissue matured. These differences may be the result of minor changes in the levels of the various contaminating membranes with developmental stage of the tissue. Changes in the acyl composition of PC and PE with the developmental stage of the tissue were notable. Although most of the changes were small, the decrease in oleic acid and concomitant increase in linolenic acid were significant and indicated a trend toward increasing desaturation of the plasma membrane with maturation. Travis and Berkowitz (27) reported similar results on the sterols present in developing soybean root plasma membrane. In their study, sitosterol decreased during maturation with a corresponding increase in stigmasterol, stigmasteryl differing from sitosterol by the presence of a double bond at the C₂₅-C₂₆ position.

The reasons for these developmental changes in membrane lipid composition are not obvious. The major phospholipid in the membrane, PC, probably stabilizes the bulk of the membranes in a bilayer (6). The role of the minor phospholipid classes is less well understood. Some of these lipids may provide special environments for membrane proteins, e.g. the phospholipid anulus of Cyt oxidase, but this does not appear to be a general phenomenon (22). Recent studies indicate that the variety of phospholipids in biological membranes may be present to reduce the permeability of the membranes at the protein-lipid boundaries (30). In this case, the phospholipid composition of the membrane would change during cell differentiation with the protein complement (5) in order to maintain membrane integrity. Minor phospholipid components may also be important during nonbayer membrane processes such as fusion events (6). The increasing desaturation of acyl chains within the membrane noted here may be associated with development-related increases in membrane fluidity.

In addition to the destructive effects of phospholipase D, the apparent differences in hydrolyase activity in meristematic and mature tissue (Table I) suggested a role of the enzyme in lipid turnover. The product of phospholipase D activity, PA, is a precursor of all the phospholipid constituents within the membrane, in addition to di- and triglycerides. Comparisons of specific activities in the subcellular fractions from meristematic and mature tissue yielded no differences. The localization studies presented here demonstrated both soluble and membrane-bound forms of the enzyme. Similar results have been reported elsewhere (24, 26, 31). However, the enzyme was not enriched in the plasma membrane and therefore did not seem specifically involved in lipid turnover on the plasma membrane.

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