Isolation and Characterization of the Protein Body Membrane of Castor Beans

Irvin J. Mettler and Harry Beevers
Thimann Laboratories, University of California, Santa Cruz, California 95064

ABSTRACT

Intact protein bodies were isolated from dry castor bean seeds (Ricinus communis L.) after homogenization in nonaqueous medium. After repeated washing with glycerol to remove trapped lipid globules, the soluble protein respresented was removed by the addition of aqueous buffer. The membrane remained attached to the insoluble protein crystalloids and was subsequently released by sonication. Purification of the membraneous vesicles in diffferent gradients produced a single band at a density of 1.21 grams per cubic centimeter. Treatment with 6 molar urea, 1 molar KCl, or 0.25 molar galactose had no effect on the equilibrium density of the membrane. Electron microscopy revealed a highly pure and uniform collection of membrane vesicles. No enzyme activity was specifically associated with the membrane. Sodium dodecyl sulfate gel electrophoresis of the protein body fractions showed that the membrane contained unique proteins, two of which were glycosylated. The membrane contained 153 nanomoles of phospholipid per milligram of protein. The composition of the phosphoglycerides was 51% ethanolamine, 41% choline, 8% inositol, and a trace of serine.

The storage proteins of seeds were localized primarily in the organelles known as protein bodies. As summarized by Pernollet (19) three common structural types are recognized, those containing only amorphous protein, those which contain phytin globoids, and those with both phytin globoids and proteinaceous crystalloids. The protein bodies of the castor bean endosperm are of the best type (19, 30). All protein bodies are bounded by a single limiting membrane which is not always clearly visible in electron micrographs (but see Fig. 1).

The protein body membrane of developing cereal seeds such as maize appears to be closely related to the RER and may even have attached polyribosomes which are actively engaged in storage protein synthesis (10). Very little research has been reported on the characteristics of the protein body membrane from the dry seed. Youle and Huang (34) briefly described the separation of a crude membrane fraction from castor bean protein bodies isolated with hexane. The protein body membrane from such seeds is of particular interest since during germination the hydrated protein bodies fuse and form the vacuole (2, 29). Presumably, the membrane gives rise to the tonoplast or vacuolar membrane. The isolation of the protein body membrane from castor beans allows the characterization of a precursor to the tonoplast as well as contributing to our understanding of storage protein utilization.

MATERIALS AND METHODS

Protein Body Membrane Isolation and Purification. Intact protein bodies were isolated from dry seeds by the glycerol method (30, 33). One hundred dehulled castor beans (Ricinus communis L. cv. Hale) were homogenized in 80 ml glycerol in a blender for 2 min at low speed. Protein bodies were collected by centrifugation at 8,000g for 15 min and purified by suspension and recentrifugation three times in 40 ml glycerol. The matrix protein was dissolved upon addition of 20 ml cold aqueous buffer (0.1 m sucrose, 5 mM EDTA, 10 mM Tris-Mes [pH 6.5]). The insoluble crystalloids, with the protein body membrane still attached, were separated from the soluble protein by centrifugation at 250g for 5 min and were then resuspended in 20 ml of the same buffer solution. The membranes were released from the crystalloids by sonication for 90 s in a Wave Energy System (WW201 probe type sonicator set at 30). The crystalloids were removed by centrifugation at 250g for 5 min and the membrane fraction collected by centrifuging the supernatant solution at 100,000g for 1 h. The membrane pellet was resuspended in 1 ml of buffer and layered on top of a linear sucrose gradient (15-60% [w/w] sucrose in 1 mM Tris-Mes [pH 6.5]) and centrifuged at 80,000g in a Beckman SW 27.1 rotor for 15 to 17 h. Sequential 1.2-ml samples were collected and the sucrose concentrations determined refractometrically.

Electron Microscopy. Sections of dry seeds and protein bodies in glycerol were fixed sequentially in: 95% glycerol, 2.5% glutaraldehyde, 2.5% H2O2, 90% glycerol, 2% glutaraldehyde, 100 mM K-phosphate (pH 7.2); 40% glycerol, 2% glutaraldehyde, 100 mM K-phosphate (pH 7.2); and finally in 0.25 m sucrose, 2% glutaraldehyde, 100 mM K-phosphate. Aqueous samples were fixed in 0.25 m sucrose, 2% glutaraldehyde, 100 mM K-phosphate and were then treated with 2% glutaraldehyde for 2 to 4 h. While in the 2% glutaraldehyde the membrane fractions were centrifuged at 80,000g and the pellet subsequently treated in the same way as for the other aqueous samples. After the glutaraldehyde fixation, aqueous and nonaqueous samples were treated identically. The samples were washed with 0.1 m K-phosphate (pH 7.2), and postfixed with 1% OsO4 in 0.1 m K-phosphate overnight. Samples were then dehydrated with a graded acetone series and embedded in Spurr's resin (25). Ultrathin sections were cut with a diamond knife and poststained with uranyl-magnesium acetate and Reynolds's lead citrate. Sections were viewed with a Jeol 100B electron microscope.

Enzyme Assays. Phosphatases were assayed at 38 C in a 1-ml reaction volume containing 3 mM substrate, 30 mM Tris-Mes (at desired pH), 3 mM MgS04, and 50 mM KCl. With ATP as substrate, the sodium salt was first converted to the Tris salt (8). Pi release was determined by the Fiske and Subbarow procedure (7). Phytase was determined by the release of Pi from 10 mM Na-phytate in 0.1 M K-malate (pH 5.0).

NADH-Cyt c reductase (8), Cyt c oxidase (11), malate dehydrogenase (28), catalase (13), and fumarase (22) were determined spectrophotometrically as described. Acid lipase was determined fluorimetrically (17).

The various proteolytic assays were conducted after the methods of Tully and Beevers (31).

GEI ELECTROPHORESIS. Polyacrylamide gels (0.5 x 9.5 cm) con-
taining 5.6% acrylamide and 1% SDS were prepared according to Fairbanks et al. (6). Samples containing 50 to 100 μg protein were prepared for electrophoresis in 1% SDS, 10% sucrose, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), and 10 μg/ml pyronin Y. Forty μM DTT was included in some samples which were incubated at 37°C for 20 to 60 min, to reduce disulfide bonds. Electrophoresis was performed at 8 to 10 mamp/gel in a Bio-Rad model 150 gel electrophoresis cell. Gels were stained for protein with Coomassie blue or for carbohydrate using the PAS3 procedure as described (6). For mol wt approximations, the SDS gels were calibrated with the following known proteins: BSA, 68,000; ovalbumin, 44,000; aldolase, 40,000; glyceraldehyde-3-P dehydrogenase, 35,000; chymotrypsin, 25,700; myoglobin, 17,200; and Cyt c, 12,400.

Phospholipids. Lipids were extracted from membrane preparations according to Bligh and Dyer (3). For each 1-ml membrane fraction, 3 ml of methanol-chloroform (2:1, v/v) was added, mixed, and placed on ice for 5 min. One ml chloroform and 1 ml H2O were then added and the chloroform phase was washed twice with 1.5 ml water and then concentrated under N2. The extracts were applied to TLC plates (E. Merck silica gel 60) which had been prerun in chloroform-methanol (2:1) overnight and activated at 100°C for 1 h. Chromatography in chloroform-methanol-10% NH4OH (65:35:4.5) gave a good separation of the phospholipids (32). Phospholipids or neutral lipids were visualized by I2 vapor, a phospholipid spray (1) or ninhydrin (1) and identified by cochromatography with a series of phospholipid standards. For phospholipid analysis, regions of the silica gel were scraped off and transferred to a test tube. Percoll acid was added directly to the silica gel and the tube heated to facilitate digestion. The phosphorus content was assayed by a modification of the Bartlett method (4).

RESULTS

Membrane Isolation. Figure 1A shows the protein body in a section of endosperm tissue from the dry seed and the membrane is clearly seen in the enlargement of Figure 1B. Isolation and purification of protein body membranes was accomplished in four basic steps.

a. Protein bodies were isolated and purified by the nonaqueous procedure originally introduced by Yatsu and Jacks (33). This method, using glycerol, was preferred to alternatives in which acetone (24) or hexane (34) are used to avoid possible solvent effects on the membrane. Repeated washing of the protein body pellet in glycerol was necessary to remove the adhering lipid globules.

b. The purified protein bodies (Fig. 1C) were treated with aqueous buffer (see under “Materials and Methods”) to remove the soluble protein matrix. Surprisingly, as shown in Figure 1D, the membrane remained associated with the insoluble protein (crystallloids) which was recovered in the pellet after centrifugation at 250g. No significant amount of membrane was detected in the supernatant fraction when this was examined by electron microscopy and phospholipid analysis.

c. The membrane was separated from the crystalloids by sonication (see under “Materials and Methods”) and the pellet then recovered after centrifugation at 250g was principally composed of crystalloids without the membrane (Fig. 1E).

d. The protein body membrane was collected by ultracentrifugation of the remaining supernatant solution and purified by equilibrium centrifugation in a linear sucrose gradient. The membrane floated to a distinct band in the gradient at a density of 1.21 g/cm3 (Figs. 2A and 3).

A variety of contaminating material remained at the top of the sucrose gradient (Fig. 2B) but the membrane fraction appeared quite uniform and pure as judged by electron microscopy (Fig. 2, C and D). The purified membrane fraction was composed primarily of closed membrane vesicles 250 to 350 nm in diameter which under high magnification present the usual tri-partite image characteristic of biological membranes. Since the density of the membrane could possibly have been affected by contamination with the soluble lectins or by association with remnants of the insoluble crystalloid protein, membrane preparations were treated with 0.25 M galactose (to release lectin binding [18]) or with 1 M KCl, or 6 M urea (to solubilize crystalloid protein [30]). None of these treatments had a significant effect on the equilibrium density of the membrane. As shown in Table I the protein recovered in the purified membrane fraction represents less than 0.1% of that in the protein bodies. Even if only 50% of the protein body membrane was recovered in the purified fraction (a conservative estimate) it is clear that the contribution of the membrane to the total protein (and to the total enzyme activities described below) is exceedingly small.

Enzyme Activities. Although most of the hydrolytic enzyme activities in the endosperm develop after germination has begun, the dry seed has low amounts of peptidases, partly associated with the protein bodies (31). Assays of these and other enzymes were carried out on the three fractions obtained from the protein bodies to determine if any were specifically associated with the membrane and to assess the degree of contamination by other cell constituents. As shown in Table I, no enzyme marker for the membrane was found; by far the largest percentage of total activities measured were present in the soluble and crystalloid fractions. The enzymes with the highest activities were acid phosphatase and malate dehydrogenase. These enzymes show very high activities in crude extracts and the activities observed in the membrane fraction probably represent a trace of soluble contaminants trapped in the membrane vesicles (Fig. 3). No acid lipase was present in the membrane fraction showing that it was uncontaminated by spherosome membranes and the lack of NADH-Cyt c reductase shows that no membrane vesicles derived from the ER were present. The lack of catalase and fumarase shows that there was no mitochondrial or glyoxyosomal contamination in the protein body preparation.

Gel Electrophoresis. Typical SDS electropherograms of membrane proteins are shown in Figure 4. For comparison, those of soluble and crystalloid proteins are also shown and these display the characteristic patterns described by previous workers (30, 35). The major bands at mol wt ~125,000 and ~56,000 of the soluble fraction are, respectively, RCA1 and ricin D (30). Both stain with PAS and in the presence of DTT are converted to proteins of lower mol wt (Fig. 4). The two bands at mol wt ~12,000 appear to be the protein allergens described by Youle and Huang (36). The crystalloid fraction shows one major complex protein band (mol wt ~43,000) which is resolved into three components of lower mol wt by DTT. None of the crystalloid proteins stain with PAS (Fig. 4).

The pattern observed with the membrane proteins is clearly very different from that obtained with either soluble or crystalloid proteins, and shows at once that these latter proteins are not present as contaminants of the membrane preparation. Two major bands of protein, one of mol wt ~98,000 and a composite band around mol wt ~20,000, are seen. The mobility of these distinctive membrane proteins was not affected by treatment with DTT. PAS staining shows that the major band with mol wt of 98,000 and also a minor protein of mol wt ~26,000 are glycosylated. None of these constituents was observed in the soluble or crystalloid fractions.

Phospholipids. When chloroform-methanol extracts of the protein body fractions were subjected to TLC, three major lipid classes were detected by the phospholipid reagent in the purified membrane fraction, but were below the level of detectability in

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2 Abbreviation: PAS: periodic acid-Schiff reagent.
the other fractions and elsewhere in the sucrose gradient. Phosphatidylethanolamine and phosphatidylcholine were most prominent. The presence of phosphatidylinositol was also readily detected. A trace of phosphatidylserine was detected with ninhydrin and a small amount of neutral lipid was detected with $I_2$ at the solvent front.

Phosphorus analysis of the TLC spots gave the information on the levels of phospholipids shown in Table II. Phosphatidylethanolamine and phosphatidylcholine accounted for 90% of the total phospholipid. Phosphatidylinositol made up the remainder, with only a trace of phosphatidylserine. The proportions of the three phospholipids are similar to those reported for membranes of other organelles from this tissue (5) except that the protein body membrane has a noticeably higher content of phosphatidylethanolamine. The membrane of the protein body has a considerably higher protein to phospholipid ratio than the other membranes that have been studied (5).

**DISCUSSION**

The existence of a single limiting membrane surrounding plant protein bodies has long been established (19). The possibility of a membrane surrounding the phytin globoid inclusions of the protein body has not been completely resolved. Some authors have reported the existence of a membrane (21, 26), others have shown a boundary layer which does not have the ultrastructure of a typical biological membrane (9, 23, 27, 30), or no evidence for any discrete boundary between the globoid and matrix protein (12). With KMnO$_4$ fixation, a boundary “membrane” can be seen in castor bean protein bodies (30), but it does not have the fine structure of a phospholipid membrane and may represent some sort of protein interface.

Since the isolation of intact protein bodies from dry seeds of castor bean and other oil seeds requires the use of a nonaqueous solvent to prevent solvation of the matrix and subsequent disi-
Fig. 2. Sucrose gradient purification of protein body membrane. A: appearance of sucrose gradient after 16-h centrifugation of membrane fraction. B: electron micrograph of contaminating material located at top of sucrose gradient. C: electron micrograph of membrane fraction. D: high magnification of membrane fraction, note trilamellar ultrastructure (arrows). Bars represent 0.5 μm in C (also for B) and 0.1 μm in D.

Table I. Protein Content and Various Enzyme Activities of Soluble Protein, Insoluble Crystalloid Protein, and Purified Membrane from Castor Bean Protein Bodies

<table>
<thead>
<tr>
<th></th>
<th>Soluble</th>
<th>Crystalloid</th>
<th>Membrane</th>
</tr>
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<tbody>
<tr>
<td>Protein (mg)</td>
<td>660 (28)*</td>
<td>1700 (72)</td>
<td>1.9 (0.08)</td>
</tr>
<tr>
<td>Acid phosphataseb</td>
<td>8.2 (71)</td>
<td>1.3 (29)</td>
<td>1.2 (0.03)</td>
</tr>
<tr>
<td>ATP hydrolysib</td>
<td>2.5 (75)</td>
<td>0.33 (25)</td>
<td>0.23 (0.02)</td>
</tr>
<tr>
<td>Phosphodiesteraseb</td>
<td>1.07 (73)</td>
<td>0.15 (27)</td>
<td>0.93 (0.18)</td>
</tr>
<tr>
<td>Phytase</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>121 (95)</td>
<td>2.3 (5)</td>
<td>6.8 (0.02)</td>
</tr>
<tr>
<td>Amino peptidasee Leucyl-Prolyl</td>
<td>4.0 (57)</td>
<td>1.2 (43)</td>
<td>7.9 (0.3)</td>
</tr>
<tr>
<td>Acid protease</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Carboxypeptidase</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Acid lipase d</td>
<td>3 (42)</td>
<td>1.6 (58)</td>
<td>ND</td>
</tr>
<tr>
<td>Cyt c oxidased e</td>
<td>0.05 (49)</td>
<td>0.02 (51)</td>
<td>ND</td>
</tr>
<tr>
<td>Catalase</td>
<td>Trace</td>
<td>Trace</td>
<td>ND</td>
</tr>
<tr>
<td>NADH-Cyt c reductase</td>
<td>Trace</td>
<td>Trace</td>
<td>ND</td>
</tr>
<tr>
<td>Fumarase</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
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* Numbers in parentheses represent per cent of total activity recovered.
b Micromoles product/mg protein-h.
ND, not detected.
e Nanomoles product/mg protein-min.
f Nanomoles product/mg protein-h.

tegration, the glycerol method was used (16). The purified protein bodies were then used for the isolation of the outer membrane.

After purification in sucrose gradients, the membrane was recovered as uniform vesicles ~300 nm in diameter, with a mean density of 1.21 g/cm³. The protein bodies themselves have only low amounts of particular enzymes and none of these was specifically associated with the membrane. Only trace amounts of
The results suggest that the transformation of the protein body membrane to the tonoplast is not a simple one and may involve an increase in lipids and/or a loss of protein constituents.

**LITERATURE CITED**

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36. Youle RJ, AHC Huang 1978 Evidence that the castor bean allergens are the albumin storage proteins in the protein bodies of castor bean. Plant Physiol 61: 1040-1042