Soybean Protein Bodies: Phospholipids and Phospholipase D Activity[1]

W.L. Boatright* and **H.E. Snyder**
Department of Food Science, University of Arkansas, Fayetteville, Arkansas 72703

Protein bodies from mature soybeans were isolated by differential centrifugatioin to examine their composition and relationship with phospholipase D. Densities were adjusted by varying mixtures of soybean oil and carbon tetrachloride. The purity of the final isolate was confirmed by electron microscopy. Approximately 4.5% by weight of the protein bodies was lipid and 2.0% by weight phospholipid. Thin-layer chromatography revealed only a trace of phosphatidic acid. Treatment with 60% ethanol/40% sodium acetate buffer (pH 5.6) (vol/vol) resulted in phosphatidyl transferase activity with conversion of both phosphatidylcholine and phosphatidylethanolamine into phosphatidylethanol. It is proposed that protein bodies of soybeans contain phospholipase D.

KEY WORDS: Phosphatidyl transferase activity, phospholipase D, phospholipids, protein bodies, soybean, transmission electron microscopy.

The so-called "nonhydratable" phospholipids (NHP) in crude soybean oil are not readily removed during the typical commercial degumming process and require special, more expensive processing steps to facilitate their removal. These NHP consist largely of the calcium and magnesium salts of phosphatidic acid (PA) and phosphatidylethanolamine (PE) (1). The level of PA in healthy, undamaged soybeans is normally low and increases during germination (2). Other investigations have demonstrated that storage conditions (3) or the processes commonly used to extract the oil (4,5) can result in an increase of PA in soybeans. While formation of PA from the catalytic action of phospholipase D [phosphatidylcholine (PC) phosphatidohydrolase, EC 3.1.4.4] on PC and PE is well documented (6–8), the physiological function and subcellular location of this degradative enzyme in mature soybeans is not well understood.

Protein bodies from mung beans are the primary site of phospholipase D activity as well as a number of other hydrolytic enzymes, including acid phosphatase (9). Bair and Snyder (10) found soybean acid phosphatase activity associated exclusively with protein bodies. Yatsu and Jacks (11), working with cottonseed, obtained similar results earlier. This class of enzyme is often considered to be a marker for the storage site of hydrolytic enzymes. Scherer and Morré (12) found acid phosphatase (phosphatidic acid phosphatase) activity associated with soybean membrane fractions that also contain phospholipase D.

Jelsema *et al.* (13) isolated lipid bodies and protein bodies from aleurone layers of wheat. They demonstrated that activity from acid phosphatase, acid lipase and phospholipase D is associated with protein bodies rather than lipid bodies. This investigation also found that, upon incubation of barley aleurone system with the hormone gibberellic acid (GA^3), activity from the above-mentioned enzymes disappeared from protein bodies.

Fernandez and Staehelin (14) demonstrated that in barley aleurone cells the lipase activity, which is primarily associated with protein bodies, is transferred to lipid bodies after treatment with GA^3. Using freeze fracture electron microscopy, they showed that the monolayer membrane of the lipid body can become continuous with the outer leaflet of the protein bodies' bilayer membrane. From these structural data, they proposed that enzymes may be transferred between the two organelles by lateral diffusion.

In the present investigation, protein bodies from mature soybeans were examined to learn more about their composition and the subcellular location of the degradative enzyme phospholipase D.

MATERIALS AND METHODS

A single batch of Forrest variety soybeans, grown in Arkansas during 1990, was used throughout this study. Dehulling was done with a blender and the hulls were aspirated. Full-fat flour was produced with a UDY Cyclone Sample Mill (UDY Corp., Fort Collins, CO) without an outlet screen. Sieving was accomplished with an Alpine Air Jet Sieve (Alpine American Corp., Natick, MA) through a 200-mesh screen. Moisture and oil contents of soybean flour were determined according to the American Oil Chemists' Society (AOCS) Official Method Ac 3-44 (15). Nitrogen contents were determined by micro-kjeldahl, and values were multiplied by 5.8 to calculate protein.

Isolation of protein bodies. Protein bodies were isolated by using a modification of the differential centrifugation method of Deickert *et al.* (16). Approximately 2 g of ⩽200-mesh, full-fat flour was homogenized in 25 mL of refined, bleached and deodorized soybean salad oil in an Omni mixer-homogenizer (Omni International Inc., Waterbury, CT). The homogenate was centrifuged at 2,600 × *g* for 15 min. The resulting pellet was fractionated by resuspension and centrifugation in mixtures of soybean oil and carbon tetrachloride with specific gravities shown in Figure 1. The protein body isolate was washed several times with hexane to remove residual soybean oil. Hexane was removed with a stream of dry nitrogen followed by vacuum. The second isolation procedure was essentially a duplicate of the above except for the density scheme shown in Figure 2.

Analysis of isolated protein bodies. Moisture and nitrogen content determinations were similar to the methods used for soybean flour. Lipids were extracted from protein body isolates by two procedures. The first was a modification of the chloroform/methanol procedure of Folch *et al.* (17). Approximately 50 mg of isolate was extracted three times with 3 mL of $CHCl_3/CH_3OH$ (2:1, vol/vol) in the micro attachment of the Omni mixer-homogenizer for 60 s at setting 3. The homogenate was centrifuged at 500 × *g* for 5 min. Aqueous 1% NaCl was added to the combined supernatants at a ratio of 4 parts supernatant to 1 part NaCl solution, and the lower chloroform phase was obtained after overnight storage at −16°C

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*To whom correspondence should be addressed at University of Arkansas, Department of Food Science, 272 Young Avenue, Fayetteville, AR 72703.

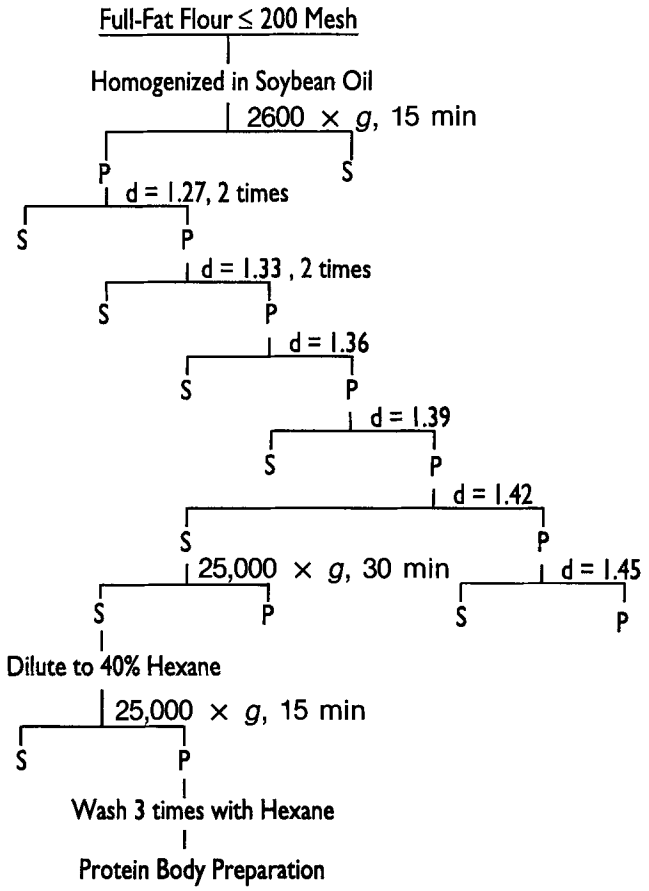

FIG. 1. Initial soybean oil/carbon tetrachloride scheme for isolation of protein bodies. Each step was centrifuged after adjusting density. Unless noted, centrifugations were 15 min at 2,600 × *g*. S = supernatant, P = precipitate, d = density.

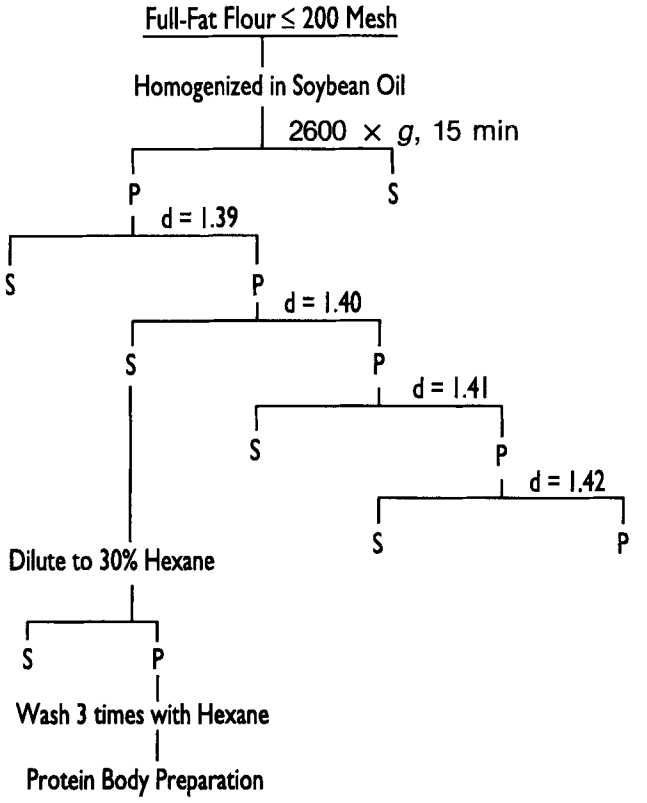

FIG. 2. Final soybean oil/carbon tetrachloride scheme for isolation of protein bodies. Each step was centrifuged after adjusting density. Unless noted, centrifugations were 20 min at 25,000 × *g*. S = supernatant, P = precipitate, d = density.

and analyzed. Results were adjusted for temperature-related changes in volume (18).

The second procedure was a modification of the method of Bligh and Dyer (19), based on chloroform/methanol/water/hydrochloric acid. Approximately 50 mg of isolate was extracted with 3 mL of $CHCl_3/CH_3OH/H_2O$ (50:100:40, vol/vol/vol) on the micro attachment of the Omni mixer-homogenizer for 60 s at setting 3. The homogenate was centrifuged at 500 × *g* for 5 min, and the residue (pellet) was extracted two more times with $CHCl_3/CH_3OH/H_2O$/concentrated-HCl (50:100:40:0.25, by vol). Immediately after each acidic extraction, 5 μL of concentrated NH_4OH was added to the combined supernatants to neutralize the acid. The combined supernatant was made biphasic by adding both 1% aqueous NaCl and $CHCl_3$ at ratios of 5 parts to 19 parts of the original supernatant, and the lower phase was formed and analyzed as before.

Total lipid from the protein body isolates was determined as the weight of total solids in the chloroform phase. Phospholipid contents were determined from the phosphorus content (20) of the chloroform phase multiplied by 25.

Thin-layer chromatography (TLC). Separation of phospholipids (PL) by TLC on activated silica gel G (Analtech Inc., Newark, DE) was accomplished according to AOCS Official Method Ja 7-86 (15). Each plate was loaded with approximately 10 μg of PL phosphorus. Resulting PL spots were identified with molybdenum blue spray reagent (21), primary amine-containing PL with ninhydrin spray reagent (22) (both from Sigma Chemical Co., St. Louis, MO), and all lipids were visualized with 4.2 M H_2SO_4 followed by charring at 130°C. Identification of PL spots was further confirmed by comparison of R_f values with those of known standards (Avanti Polar Lipids, Inc., Birmingham, AL).

Phospholipase D assay. Phospholipase D activity in the protein body isolates was determined from phosphatidyl transferase activity of this enzyme (23,24). Ethanol was the receptor. Approximately 50 mg of protein body isolate was added to 4.5 mL of 60% ethanol/40% 62.5 mM Na acetate/50 mM $CaCl_2$ (pH 5.6). After 45 min, 1 mL of 1% NaCl and 4.5 mL chloroform were added, and the lower phase was analyzed after overnight storage at −16°C. For a control, the protein body isolate was added to 4.5 mL of the above 60% ethanol solution and held at 90°C for 10 min followed by cooling. A biphasic solution was formed and handled as before.

Phosphatidylethanol (PEtOH) standard was prepared by a slight modification of the method used by Roughan *et al.* (25) to prepare phosphatidylmethanol. PC (100 μmole) in 5 mL of 95% ethanol was added to 40 mL of

TABLE 1

Proximate Analyses of Full-Fat Flour[a]

Moisture	6.40 (0.01)[b]
Oil (dry basis)	24.46 (0.01)
Protein (dry basis)	39.35 (0.31)

[a]Results from three analyses and expressed as percent by weight.
[b]Results in parentheses are mean standard errors.

62.5 mM Na acetate (pH 5.5) with 50 mM $CaCl_2$. After 1.5 mL of phospholipase D (2.25–2.5 mg protein) was added, the final volume was taken to 50 mL with water, and the mixture was incubated at 20–22°C for 90 min. Both PC (egg) and phospholipase D (cabbage) were purchased from Serdary Research Laboratories, London, Ontario, Canada. The solution was made biphasic by adding 15 mL of 95% ethanol and 35 mL chloroform. After 1.5 h at room temperature, the lower phase was washed with 63 mL of 27% aqueous ethanol (vol/vol) containing 2.5 mM HCl. The phases completed separation in the freezer overnight. The lower phase was concentrated by evaporation under N_2 and resuspended in 0.5 mL chloroform. The sample was separated on activated silica gel H with $CHCl_3/CH_3OH$/7.0 N NH_4OH (75:25:4), followed by visualization with iodine (I_2) vapors. The band that did not correspond to either PA or PC and that migrated near the solvent front was recovered, and PEtOH was eluted twice with 10 mL of $CHCl_3/CH_3OH/H_2O$ (50:45:5) and once with 10 mL CH_3OH (26). This solution was made biphasic by adding 10 mL chloroform and 15 mL H_2O. Separation was completed overnight in a freezer. The resulting lower phase was recovered, concentrated and stored in the freezer.

Microscopy. Samples were fixed for 24 h in 1% osmium tetroxide (OsO_4) buffered with 50 mM citrate (pH 5) followed by prestaining overnight in 0.5% uranyl acetate (0 to 4°C). Dehydration was accomplished in graded ethanol followed by 100% propylene oxide. The samples were embedded in Spurr's epoxy resin (27). Thick sections (0.5–2.0 μm), cut with a glass knife, were stained with 1% borax containing 1% toluidine blue and 1% Azure II and examined with phase-contrast microscopy. For electron microscopy, silver-gold sections (*ca.* 800 Å) were cut with a diamond knife, stained with uranyl acetate and lead citrate and examined with a Jeol 100 CX electron microscope (Tokyo, Japan).

RESULTS AND DISCUSSION

Proximate analyses of the full-fat flour used throughout this study (see Table 1) revealed values for oil and protein content within typical ranges for soybeans (28). After each centrifugation step of the first isolation procedure, the supernatant was collected and observed under a phase-contrast microscope. The protein bodies, spherical organelles 1–20 μm in diameter, were found almost exclusively in the supernatants with densities of 1.42 and 1.45. Only the supernatant with density 1.42 was examined further, as the supernatant with density 1.45 contained a large amount of cell-wall fragments and intact cells. These densities differed from the densities collected by Saio and Watanabe (29), who found the protein bodies to be predominately in supernatants with densities of 1.36 and 1.39. This difference is probably due to the different moisture content of the soybeans used. Another investigation, in which the same isolation procedure was used for green soybeans in the pods, found the protein bodies to predominate at lower densities (between 1.34 and 1.36) (30). The results from analyses performed on the current isolate are presented in Table 2 together with the corresponding results from Saio and Watanabe. The respective values for percent protein and total phosphorus from both investigations were similar.

Two different extraction techniques to analyze for total lipids and PL phosphorus were used for the protein body isolate from this study: A chloroform/methanol procedure similar to other investigations and a chloroform/methanol/water/hydrochloric acid method. Previous investigators found that a solvent system containing water and a mineral acid improves the extraction of PLs, particularly phosphatidylinositol (PI) (31,32). We found the same trend, with approximately 10% more PLs being extracted with the acidic solvent. Total lipids extracted remained about the same, indicating that the chloroform/methanol solvent must have been a more effective solvent for another class of lipid. For the PL extraction procedure in this investigation we employed freezer temperatures to complete phase separation (a modification to previously published procedures). This was evident when the final extract (separate lower phase) stored at freezer temperatures did not redevelop an upper phase. Furthermore, during subsequent concentration of the sample for TLC, the extract did not become cloudy when nearly dry. Both of

TABLE 2

Analyses of Soybean Oil/Carbon Tetrachloride-Isolated Protein Bodies

	Saio and Watanabe (Ref. 29)	$CHCl_3/CH_3OH$ (density 1.39–1.42)	$CHCl_3/CH_3OH/H_2O/HCl$ (density 1.39–1.42)
Moisture	9.5	5.48 (0.01)[a]	5.48 (0.01)[b]
Protein[c]	64.7	62.94 (0.21)[b]	62.94 (0.21)[b]
Total lipids[c,d]	—	2.97 (0.05)a[e]	2.94 (0.02)a
Total phospholipids[c,d]	—	1.79 (0.04)a	1.96 (0.03)b
Total phosphorus[c]	0.94	0.86 (<0.01)[b]	0.86 (<0.01)[b]
Phospholipids phosphorus[c,d]	—	0.072 (0.002)a	0.078 (0.001)b

[a]Figures in parenthesis are mean standard errors.
[b]Results from three analyses and expressed as percent by weight.
[c]Dry basis.
[d]Results from three analyses on each of two extractions and expressed as percent by weight.
[e]Means in horizontal rows followed by the same letter (base level) indicate no statistically significant difference at $P \geq 0.05$.

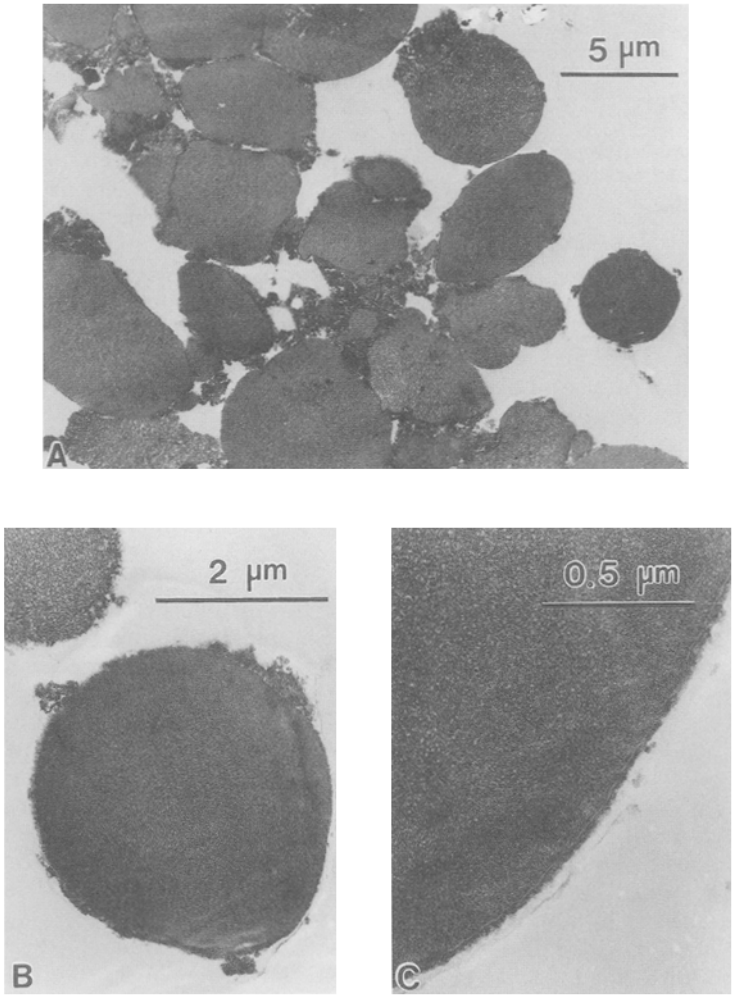

FIG. 3. Transmission electron micrograph of soybean protein bodies isolated by soybean oil/carbon tetrachloride differential centrifugation (density 1.39–1.40).

these problems occurred when the phase separation was carried out at room temperature.

After preparing this isolate (density 1.39–1.42), examination under both the phase-contrast microscope and the electron microscope confirmed that it was composed predominately of protein bodies. However, there also appeared to be some contamination from cell-wall fragments and other cytoplasmic components within partially intact cells. This same type of isolation procedure was repeated with emphasis on further fractionating the material between densities 1.39 and 1.42. Observations of these isolates under a phase-contrast microscope revealed that the supernatant from the solution with a density of 1.42 contained large amounts of cell-wall material. Thus, subsequent analyses focused on the supernatants from solutions with densities of 1.41 and 1.40.

Observations made on these two isolates after fixation and preparing thick sections revealed that the supernatant from density 1.41 contained a small amount of cell-wall fragments. The lighter density isolate, however, was virtually free from cell-wall fragments and cytoplasmic components. This isolate (density 1.39–1.40), as observed with the electron microscope (Fig. 3A and 3B), clearly showed predominantly intact protein bodies devoid of any lipid bodies or cell-wall fragments. Viewing the same isolate at a high magnification (Fig. 3C), the intact membrane surrounding the protein bodies (trilaminar structure) could be clearly seen. In Figure 3, some intact protein bodies appear to having adhering proteinaceous material. This nonmembrane-bound protein appears to be

TABLE 3

Analyses of Soybean Oil/Carbon Tetrachloride-Isolated Protein Bodies (density 1.39–1.40)

Moisture[a]	5.34 (0.07)[b]
Protein[a,c]	66.00 (1.52)
Total lipids[c,d]	4.47 (0.09)
Phospholipids[c,d]	2.08 (0.14)
Total phosphorus[a,c]	0.75 (0.01)
Phospholipids Phosphorus[c,d]	0.083 (0.005)

[a]Results from two analyses and expressed as percent by weight.
[b]Figures in parentheses are mean standard errors.
[c]Dry basis.
[d]Results from two analyses on each of two extractions with $CHCl_3/CH_3OH/H_2O/HCl$ and expressed as percent by weight.

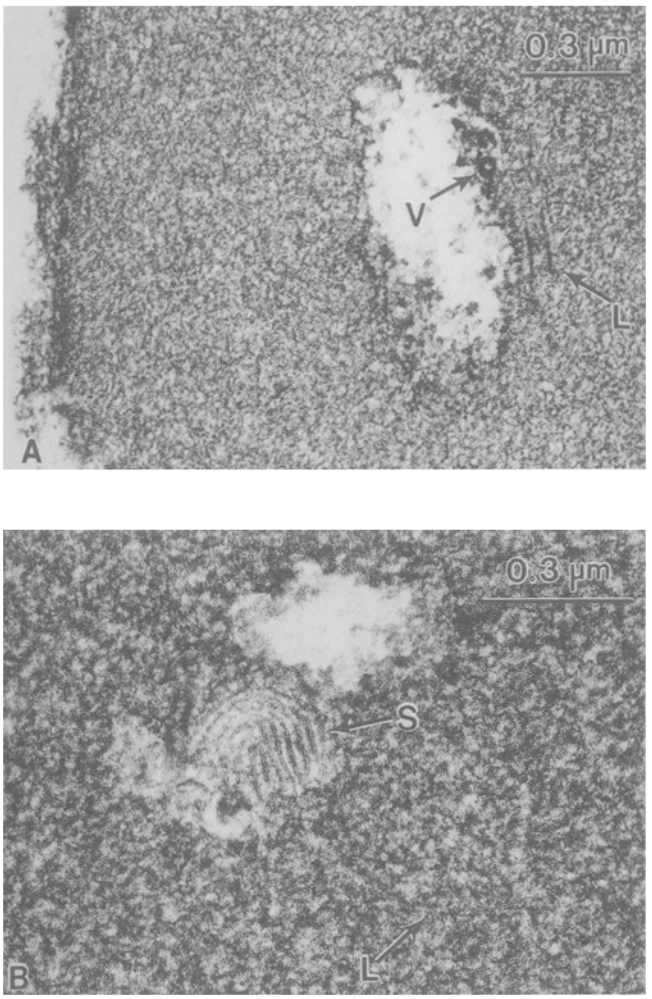

FIG. 4. Transmission electron micrograph of internal matrix of soybean protein bodies isolated by soybean oil/carbon tetrachloride differential centrifugation (density 1.39–1.40). V = membrane-bound vesicle; L = linear structures; S = stacks of membranous sacks.

the result of some protein bodies rupturing during the initial phase of fixation for electron microscopy.

Results from analysis of this isolate are presented in Table 3. The higher protein content in this isolate, compared to the isolate with a density of 1.39–1.42, confirms the increase in its purity. It was unexpected, however, to see the drop in percent total phosphorus in this isolate. Phytate, which contributes most of the phosphorus in soybeans, has been reported to occur primarily in association with protein bodies (28). This lower total phosphorus may relate to the lower density of these protein bodies.

A 2% by weight PL content for protein bodies is considerably higher than the expected contribution from the limiting membrane alone (33). Observations into the internal matrix of the protein bodies (Fig. 4A) revealed numerous membrane-bound vesicles in close association with what appeared to be internal cavities or regions not stained during preparation for electron microscopy. Unidentified linear structures can also be seen. Further observations (Fig. 4B) revealed stacks of membranous sacks also in close proximity to the internal cavities. This view also contains a similar linear structure. While the function of these internal membranous structures are yet unknown, their presence contributes to the overall PL content of the protein bodies.

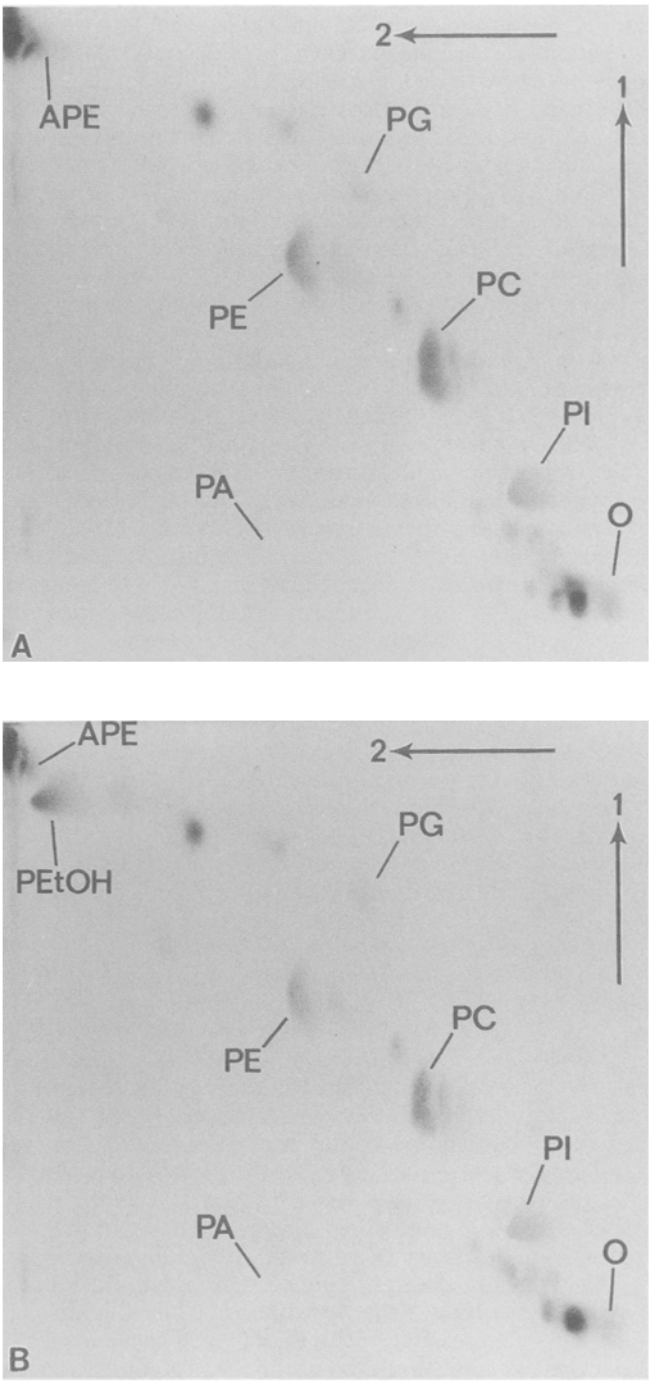

FIG. 5. Two-dimensional thin-layer chromatography of lipids extracted from soybean oil/carbon tetrachloride isolated protein bodies (density 1.39–1.40). A: Lipids extracted with $CHCl_3/CH_3OH/H_2O/HCl$; B: Lipids extracted after treatment with ethanol. 1 = direction of first dimension plate development; 2 = direction of second dimension plate development; O = origin; PA = phosphatidic acid; PI = phosphatidylinositol; PC = phosphatidycholine; PE = phosphatidylethanolamine; PG = phosphatidylglycerol; APE = N-acyl-phosphatidylethanolamine; PEtOH = phosphatidylethanol.

Separation of the protein bodies' lipid extract by TLC (see Fig. 5A) revealed similar types of PL that are reported in previous investigations as the overall content of PL in soybeans (2,34). The PA spot was faint, indicating that little, if any, phospholipase D activity occurred in the protein bodies during the isolation or extraction procedure. Little of the nonphosphorus-containing lipids (spots not labeled on TLC) appeared to be triglycerides. Based on the results from another investigation (35) and the characteristic blue to deep brown-purple color formed after charring with sulfuric acied (36), these lipids were assumed to be primarily glycolipids, sterols and/or derivatives of sterols.

The lipids extracted from the protein body isolate after treatment with ethanol contained a high content of an atypical PL (see Fig. 5B). This PL migrated near the solvent front in both directions. The control sample treated with hot ethanol solution contained only the typical PL of soybeans, similar to the $CHCl_3/CH_3OH/H_2O/HCl$ extract. Identification of the atypical PL as PEtOH was confirmed by using prepared PEtOH as an internal standard. Comparison of the PL from this extract with those from the untreated protein body extract exhibited (besides the emergences of PEtOH a reduction in the contents of PE and PC. Conversely, the level of PI appeared to remain fairly constant. By using the procedure with the ethanol pretreatment, PL totaling 1.94 ± 0.04% (dry basis) of the protein bodies were extracted. This was approximately 93% of the total, as determined by the $CHCl_3/CH_3OH/H_2O/HCl$ extraction method on the same isolate. Phosphatidyl transferase activity has been attributed to the activity of phospholipase D (23,24) and has also been used to assay for phospholipase D *in situ* (37). However, the possibility of an unknown enzyme possessing similar activity, although unlikely, cannot be entirely ruled out.

We put considerable effort into preparing a purified protein body isolate while maintaining the integrity of the membrane and without activating phospholipae D during the isolation. Extensive observations of the final prepared isolate by both light microscopy and electron microscopy, along with the low PA content of the isolate, confirmed that the procedure was effective. Therefore, based upon the observed purity of the protein body isolate used in this assay and the phosphatidyl transferase activity demonstrated, it is apparent that the protein bodies of soybeans act as a storage site for phospholipase D. This, however, does not rule out the possibility of the enzyme occurring at other locations (*e.g.*, lipid bodies or plasma membranes) in healthy, undamaged soybeans. Further investigation should be performed to address these other possible locations. This would determine if protein bodies of soybeans possess a "lysosomal nature," contributing a degradative enzyme to the membranes of other organelles.

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