Electron Microscopy of Soybean Lipid Bodies

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ABSTRACT

Soybean lipid bodies in situ are small (0.2-0.5 μm in diameter) and have an affinity for plasmalemma, protein bodies, endoplasmic reticulum and other cell organelles but not for mitochondria or nuclei. Isolated lipid bodies contain 15% protein after extensive washing and have densities ranging from less than 1.0066 to 1.0788. We concluded that lipid bodies are surrounded by a specific delimiting membrane.

INTRODUCTION

Compared to the substantial economic importance of soybean oil, there is surprisingly little research that has been reported on the lipid-containing structures of soybeans. Bils and Howell (1) described lipid granules in electron micrographs of developing soybean cotyledons. Saio and Watanabe (2) and Wolf and Baker (3,4) also have made electron microscopic studies of soybeans and noted the lipid-containing organelles. To our knowledge, Kahn et al. (5) have published the only report on isolation and analysis of the lipid-containing organelles from soybeans.

There is considerable literature on lipid-containing organelles from other seeds and from other plant tissues (6-11), and a controversy exists about nomenclature of the lipid-containing organelles. The most common name is spherosome, but this term also has been associated with enzymatically active organelles such as glyoxysomes and lysosomes. Although the spherosome name has been used for lipid-containing organelles in soybeans (2-4), because of the controversy about spherosomes in other tissues and because of the lack of descriptive content (spherosome = round body), we prefer the term "lipid body" for the oil-rich organelles and will refer to them as lipid bodies in this paper.

MATERIALS AND METHODS

Amsoy 71 seed-grade soybeans were the experimental material for all micrographs and isolations of lipid bodies.

Sample Preparation for Transmission Electron Microscopy (TEM) and for Scanning Electron Microscopy (SEM)

For TEM, samples were cut into small pieces and fixed overnight in 3% glutaraldehyde-1.5% paraformaldehyde in 0.1 M phosphate buffer at pH 7.1 and 4C. The pieces were then rinsed for 3 20-min periods in phosphate buffer and postfixed for 2 hr in 2% osmium tetroxide (same buffer and pH) at room temperature. Fixation was followed by 3 20-min phosphate buffer rinses, dehydration through an acetone series, infiltration with propylene oxide and final infiltration and embedding in Spurr's resin (12). Sections having silver to gold interference colors were cut with glass knives using an LKB Ultratome III. Sections were picked up on copper grids and stained with methanolic uranyl acetate (13) and lead citrate (14). Samples were examined on a Hitachi HU-11C transmission electron microscope operated at an accelerating voltage of 50 kV.

For SEM the samples, after acetone dehydration, were frozen in parafilm pillows filled with absolute acetone and cryofractured in liquid nitrogen using a razor blade held in a hemostat. Fractured pieces were dropped into acetone to thaw, infiltrated with Freon TF (113) and critical-point dried using liquid carbon dioxide. Samples were mounted on brass discs with silver conductive paint, lightly coated with carbon and ca. 10 nm of gold in a Varian VE-30 vacuum evaporator. Samples were examined on a JEOL JSM-35 scanning electron microscope operated at 15 kV with a beam current of 80 μA.

Isolated lipid bodies were embedded in 2% agar and small cubes (ca. 1 mm^3) were taken through the fixation, dehydration and embedding procedures already outlined for TEM and SEM.

Isolation of Lipid Bodies

The methods of Jacks et al. (11), Kahn et al. (5) and Yatsu and Jacks (15) were used to separate a floating "fat pad" from the other soy constituents. After preliminary experiments, the Yatsu and Jacks method (15) was chosen for all subsequent isolations.

The floating layers were dialyzed for 2 days against distilled water at 4 C to remove salt or sugar before analysis. Samples for analysis were freeze-dried after dialysis.

Protein Analysis

The AOAC (16) micro-Kjeldahl procedure was used for digestion except that 0.2 g cupric selenite served as catalyst in place of mercuric oxide. A Lab Con Co micro-distillation unit was used to recover ammonia, and total nitrogen was multiplied by 6.25 to convert to protein.

Lipid Analysis

Crude lipid was extracted with hexane in a Goldfisch apparatus by using method (30-20 of AACC (17). For both protein and lipid analyses, duplicate samples were run and the results reported are the averages.

Density Gradient Centrifugation

The isolated lipid bodies were analyzed by both continuous and discontinuous sucrose density gradients. The lipid body isolate was suspended in distilled water and carefully layered on top of the gradients. The discontinuous gradient consisted of zones of 0.05 M, 0.1 M, 0.2 M, 0.4 M and 0.6 M sucrose. The continuous gradient ranged from 0.1 M to 0.6 M sucrose. The centrifugations were done at 4 C for 7 hr at 100,000 × g in a Beckman model L3-50 ultracentrifuge with a SW 41 rotor.

Tryptic Hydrolysis

Agar-embedded lipid bodies were placed in vials containing 5 ml of a trypsin solution (5 mg of trypsin in 0.001 N HCl) and incubated at 37 C for 12 hr.

Acid Phosphatase

Localization of acid phosphatase activity in isolated lipid bodies was done using a modification of the Barka and Anderson procedure (18). Aldehyde-fixed tissues were

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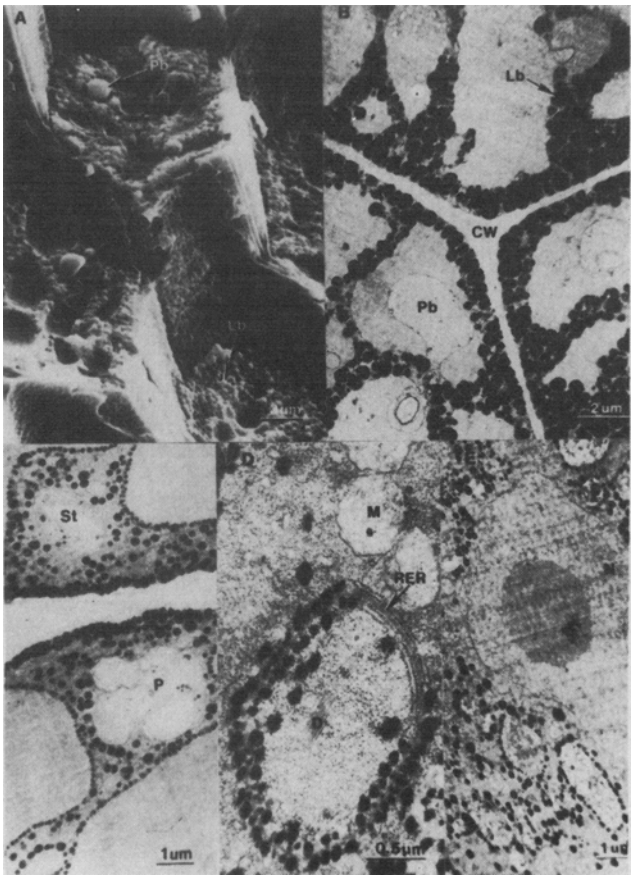

FIG. 1. Electron micrographs of lipid bodies in situ. A: SEM micrograph of freeze-fractured soybean cotyledon; B: TEM micrograph of soybean cotyledon showing lipid bodies densely packed; C: TEM micrograph showing lipid bodies concentrated at protein body and cell membranes but not at amyloplast membrane; D: TEM micrograph of soybean hypocotyl showing lipid bodies concentrated at rough endoplasmic reticulum but not at mitochondrial membrane; E: TEM micrograph of soybean hypocotyl showing lipid bodies concentrated around unidentified organelles but not around the nucleus. Labels are: Pb = protein body, Lb = lipid body, CW = cell wall, St = starch granule, P = amyloplast, M = mitochondrion, RER = rough endoplasmic reticulum and N = nucleus.

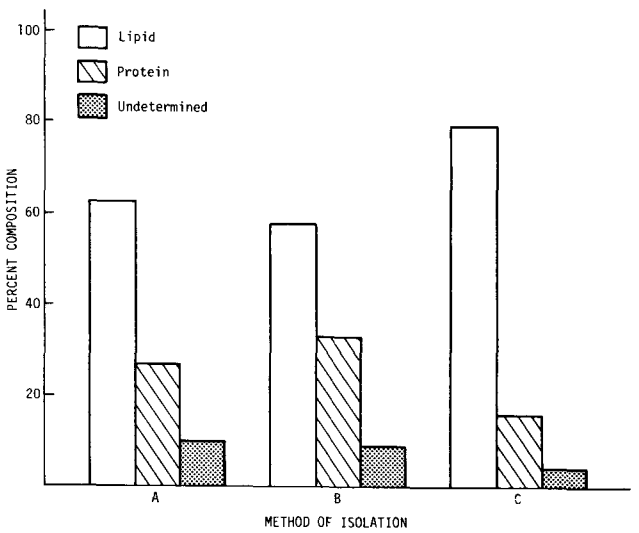

FIG. 2. Effect of the isolation method on the composition of the isolated lipid bodies. A is the Jacks et al. procedure (11), B is the Kahn et al. procedure (5) and C is the Yatsu and Jacks procedure (15).

placed in 5 to 10 ml of the reaction mixture (previously adjusted to pH 5.5) and incubated at 37 C for 30, 60, 90 and 120 min. These controls were used: (a) tissues incubated in the reaction mixture with no substrate (sodium *beta*-glycerol phosphate), (b) tissues incubated in complete reaction mixture plus 0.01 M sodium fluoride, and (c) tissues boiled for 15 min in water before incubating in the reaction mixture.

RESULTS AND DISCUSSION

Figure 1 shows lipid bodies in situ. When viewed by SEM (Fig. 1A), the lipid bodies were enmeshed in what has been referred to as a cytoplasmic network (4) presumably composed of protein. The spaces between protein bodies in cotyledon cells were filled with the lipid body network.

When viewed by TEM (Fig. 1B), lipid bodies also filled the spaces between protein bodies in some cotyledon cells and were discrete particles, ca. 0.2-0.5 μm in diameter, with no cytoplasmic network. In other cotyledon cells, lipid bodies were less concentrated (Fig. 1C) and definite patterns of concentration were discerned. Lipid bodies showed an affinity for protein bodies and for the cell membrane (cell wall) but not for amyloplasts or mitochondria. Figures 1D and 1E are from soybean hypocotyl and show further distinctions in affinities of lipid bodies for other cell organelles. In Figure 1D, lipid bodies are clustered about rough endoplasmic reticulum, and in Figure 1E, the lipid bodies show an affinity for unidentified organelles (possibly glyoxysomes) but no affinity for the nucleus.

Webster and Leopold (19) noted that lipid bodies from soybeans had affinity for protein bodies and for cell membrane (plasmalemma), and Jelsema et al. (10) observed that lipid bodies from wheat had an affinity for aleurone grains. The lack of affinity between lipid bodies and amyloplasts, mitochondria or nuclei suggested that lipid bodies adhered to those cell organelles having a single-unit membrane but did not adhere to organelles with a double-unit membrane.

Yatsu and Jacks (15) showed that the membrane surrounding peanut lipid bodies was a half-unit membrane and, when isolated, could recombine to form a unit membrane. Our observations show some differences between unit membranes on the basis of affinity for lipid bodies.

To further our understanding of the strucure and composition of soybean lipid bodies, we isolated them from mature cotyledons. Three isolation procedures were used (5,11,15), and the compositions of the fat pads isolated by these procedures are shown in Figure 2. We found more protein (32% vs 10%) and less lipid (58% vs 89%) than did Kahn et al. (5) who made the only other analysis of soybean lipid bodies of which we are aware. A possible explanation for the differences in analyses is that they isolated lipid bodies in 0.5 M sucrose and corrected dry weights for estimated sucrose content. Their lipid extraction was done with ethanol/ether/chloroform (2:2:1), which would have extracted sucrose and may have caused an overestimation of lipid, because they took the weight of the solvent-free extract to be lipid.

Jacks et al. (11) found 98.1% lipid in their isolated lipid body fraction from peanuts. The average diameter of peanut lipid bodies is greater than 1 μm. Since lipid bodies from peanuts are larger than those from soybeans, the larger size may have contributed to a cleaner centrifugal separation and a higher lipid content. Using the Jacks et al. procedure (11) on soybeans, we found 63% lipid and 27% protein.

The increased protein in our preparations may be a direct consequence of the small size of the lipid bodies and the corresponding increase in surface area. For example,

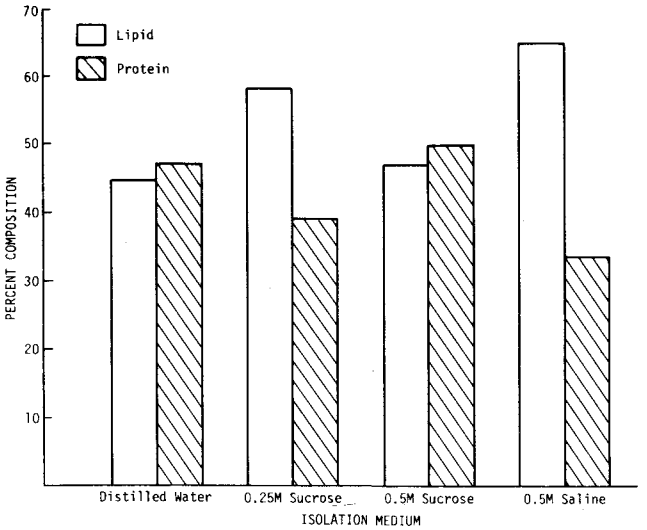

FIG. 3. Effect of the isolation medium on the composition of the isolated lipid bodies. The Jacks et al. method (11) was used for all isolations.

lipid bodies with a diameter of 0.2 μm compared to a diameter of 2 μm would have a 10-fold increase in surface area and presumably a 10-fold increase in protein content/unit vol of lipid.

To investigate the influence of the isolation medium on the composition of lipid bodies, we used water, 0.25 M sucrose, 0.5 M sucrose and 0.5 M saline to isolate lipid bodies, but all isolations were done by the Jacks et al. (1) procedure. The results in Figure 3 show that the most lipid and the least protein were obtained with 0.5 M saline. Because 0.5 M saline was the isolation medium used by Yatsu and Jacks (15), we did the remaining isolations of lipid bodies using their procedure.

Our analyses showed that lipid bodies contained considerable protein, yet TEM micrographs showed a clean-looking preparation (Fig. 4A) very similar to those for peanut lipid bodies (11,15). The protein must adhere tightly to the lipid bodies because it survives extensive (9X) washing.

The amount of floating fat pad recovered was dependent on the ratio of soybeans to isolating medium. With a ratio (by weight) of 1:3 or 1:4, an appreciable fat pad was separated, but if the ratio was 1:10, no floating fat pad was evident. One possible reason for the difference was that at soybean-to-isolating-medium ratios of 1:3 or 1:4, the protein bodies might remain intact, and less protein would be available to interact with lipid bodies. At a ratio of 1:10, protein bodies disintegrate and the soluble protein might adhere to lipid bodies, thereby increasing their density and decreasing the floating fat pad.

We observed by SEM that the cytoplasmic network evident in Figure 1A is greatly decreased in lipid body isolates (Fig. 4B), but some adhering material is visible at higher magnification (Fig. 4C).

When isolated lipid bodies were extracted with hexane (Fig. 4D), we could observe membranes that were about half the thickness of a unit membrane. This observation is the same as that made by Yatsu and Jacks (15) for isolated peanut lipid bodies.

To learn more about the distribution of densities in lipid body isolates, we centrifuged them in continuous and discontinuous sucrose density gradients. In continuous gradients, an even distribution was found. In discontinuous gradients, lipid bodies were found floating at all boundaries. Thus, the isolated lipid bodies have a wide range (<1.0066-1.0788) of evenly distributed densities.

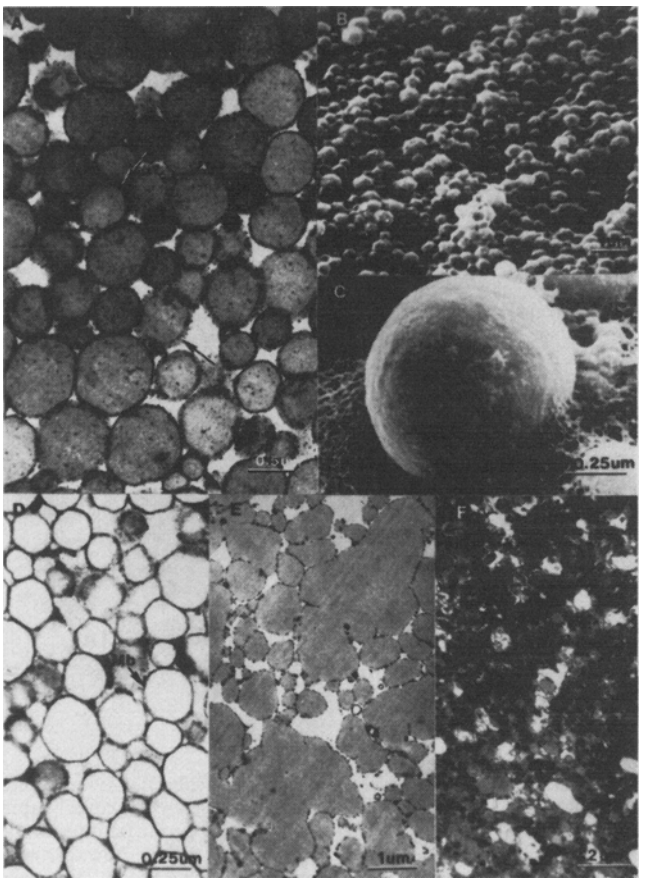

FIG. 4. Electron micrographs of isolated lipid bodies. A: TEM micrograph of isolated lipid bodies; B: SEM micrograph of isolated lipid bodies showing little cytoplasmic network; C: SEM micrograph of isolated lipid body at high magnification; D: TEM micrograph of isolated lipid bodies after extraction with hexane; E: TEM micrograph of isolated lipid bodies after treatment with trypsin; F: TEM micrograph of centrifugal pellet obtained during isolation of lipid bodies. Label: Mb = membrane.

Even when a floating fat pad was evident, we found considerable lipid in the supernatant fluid and in the centrifugal pellet during isolation in 0.5 M NaCl (D_{20}^{20} = 1.0205). The lipid bodies present in the centrifugal pellet (Fig. 4F) were considerably smaller than normal (most were 0.1 μm or less in diameter). Smaller lipid bodies, if coated with protein, would be of higher density than larger lipid bodies and might be expected to sediment in a centrifugal field.

Evidence for a protein component essential to the integrity of the lipid body membrane came from experiments on tryptic hydrolysis of lipid bodies. Figure 4E shows the results of subjecting lipid bodies to tryptic hydrolysis. Some breakdown of membranes and coalescence of lipid bodies were evident. Still the membranes did not break down entirely to give spherical lipid droplets. We interpret Figure 4E to be further evidence for the existence of membranes surrounding lipid bodies and to be evidence that those membranes contain protein as an essential part of their normal structure—not randomly adhering protein.

Analyses for acid phosphatase showed no activity in any of the lipid body preparations, but positive evidence of activity (deposition of lead salts) was found in soybean protein bodies.

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Triglyceride Analysis with Glass Capillary Gas Chromatography

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ABSTRACT

The analyses of triglycerides on capillary columns is reported. Applications in which this technique can be used include: rapid identification of fats and oils, measurement of butter fat or coconut oil content in margarine or chocolate, monitoring of processes such as fractionation, transesterification or heat treatment. Although separation of all isomers within a group of triglycerides with identical carbon numbers is not usually feasible by gas chromatography (GC) alone, the resolution obtained with capillary columns gives more information than that obtained with packed column GC. The conditions used in this work are described. Persilylated columns coated with nonpolar gum phases could be used for ca. 1 yr with hundreds of injections.

INTRODUCTION

Fats and oils are largely characterized by highly developed procedures to analyze the fatty acid methyl esters (FAME), (1). These relatively volatile compounds are amenable to gas chromatography (GC) analyses and give good fingerprint chromatograms for different fats and oils. Simultaneously, this GC method has been widely used to determine the *trans* isomers of 18:1 fatty acids produced by hydrogenation or prolonged heat-treatment of fats or oils, despite the fact that the *cis* and *trans* isomers cannot be satisfactorily separated even with capillary columns (2). HPLC on $AgNO_3$-coated silica gel gives better results for these separations (3).

The analysis of triglycerides complements the chromatography of FAME if it is used for identification purposes or to measure butterfat or coconut oil content. The method is extremely simple, since sample preparation consists of merely diluting the fat with a solvent.

The technical aspect of the GC analysis requires some attention. As soon as a suitable column and an appropriate injection technique is used, the method becomes routine, even though molecular weights of some triglycerides exceed 1000 daltons.

Triglyceride analysis on packed columns has been done for nearly 20 years (4,5) and is a rapid, routine method (6). On nonpolar stationary phases, it is possible to separate the triglycerides according to their total carbon number (not including the odd-numbered ones). An attempt has been made to separate triglycerides based on their degree of unsaturation (7). However, the Silar 10C-phase used for this purpose has to be operated at its temperature limit.

The use of capillaries provides the well known advantages of increased resolution and relatively short analysis time. Little has been published on this technique: Schomburg et al. (8) shows some chromatograms; Schulte reports some applications (9); and a recent paper (10) shows a number of capillary GC runs on very short columns and gives information on the column and chromatographic conditions in detail. Although separations on 4-6-m capillaries are much better than on packed columns, we believe that considerably more information could be obtained using 15-20-m columns.

Apolar stationary phases such as OV 1 and 101, SE 30 and 52 separate the triglyceride peaks according to the total number of carbon atoms. The composition of these groups is complex; (a) there are many isomers of the same molecular weight with different combinations of various fatty acids, e.g., 12-12-12, predominate in coconut oil (11), which is well separated from 4-14-18, predominate in butter fat (12) (Fig. 1); (b) triglycerides composed of the same fatty acids may exist as stereoisomers, differing in the position of the fatty acids on the glyceride moiety; (c) a considerable proportion of the fatty acids is unsaturated, giving a large number of possible isomers differing in the number of double bonds as well as their distribution in the triglyceride molecule.

With the separation technique used presently, it is impossible to separate more than a small number of these isomeric triglycerides within one run. Nevertheless our experiments have shown that characteristic peak patterns are obtained for most fats.

At this point, it might be asked why we prefer nonpolar phases over polar ones. Nonpolar phases have good selectivity for structural and stereoisomers as they are characteristic in butter fat e.g., But the separation of unsaturated species is less satisfactory: resolution may be quite high, but peaks of different identity are mixed, not grouped according to the number of double bonds as on polar phases. There is little chance to develop simple rules to interpret or predict the appearance of unsaturated trigly-