

# Surface Structure and Properties of Plant Seed Oil Bodies

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**Abstract.** Storage triacylglycerols (TAG) in plant seeds are present in small discrete intracellular organelles called oil bodies. An oil body has a matrix of TAG, which is surrounded by phospholipids (PL) and alkaline proteins, termed oleosins. Oil bodies isolated from mature maize (*Zea mays*) embryos maintained their discreteness, but coalesced after treatment with trypsin but not with phospholipase A2 or C. Phospholipase A2 or C exerted its activity on oil bodies only after the exposed portion of oleosins had been removed by trypsin. Attempts were made to reconstitute oil bodies from their constituents. TAG, either extracted from oil bodies or of a 1:2 molar mixture of triolein and trilinolein, in a dilute buffer were sonicated to produce droplets of sizes similar to those of oil bodies; these droplets were unstable and coalesced rapidly. Addition of oil body PL or dioleoyl phosphatidylcholine, with or without charged stearylamine/stearic acid, or oleosins, to the medium before sonication provided

limited stabilization effects to the TAG droplets. High stability was achieved only when the TAG were sonicated with both oil body PL (or dioleoyl phosphatidylcholine) and oleosins of proportions similar to or higher than those in the native oil bodies. These stabilized droplets were similar to the isolated oil bodies in chemical properties, and can be considered as reconstituted oil bodies. Reconstituted oil bodies were also produced from TAG of a 1:2 molar mixture of triolein and trilinolein, dioleoyl phosphatidylcholine, and oleosins from rice (*Oryza sativa*), wheat (*Triticum aestivum*), rapeseed (*Brassica napus*), soybean (*Glycine max*), or jojoba (*Simmondsia chinensis*). It is concluded that both oleosins and PL are required to stabilize the oil bodies and that oleosins prevent oil bodies from coalescing by providing steric hindrance. A structural model of an oil body is presented. The current findings on seed oil bodies could be extended to the intracellular storage lipid particles present in diverse organisms.

PLANT seeds store triacylglycerols (TAG)<sup>1</sup> as food reserves for germination and postgerminative growth of the seedlings. The TAG are present in small discrete intracellular organelles called oil bodies (11, 12, 25, 30). Isolated oil bodies have a spherical shape and possess diameters ranging from 0.5 to 2.5  $\mu\text{m}$ . They contain mostly TAG and small amounts of phospholipids (PL) and proteins. EM of the oil bodies in situ or in isolated preparations fixed with glutaraldehyde and osmium tetroxide shows that the organelle has an electron-opaque matrix of TAG surrounded by one electron-dense layer (30). This single electron-dense layer is in contrast to the two closely situated, parallel electron-dense layers of a unit membrane of two PL layers in other cellular membrane in the same electron micrograph. The idea has been put forth that the oil body is surrounded by a "half-unit" membrane of one PL layer, in which the two acyl moieties of a PL molecule face inward to interact with the hydrophobic TAG in the matrix, and the hydrophilic PL head group is exposed to the cytosol.

Oil bodies contain a few major and related proteins unique to the organelles (12, 26). These proteins are called oleosins

which are alkaline proteins of small molecular mass, ranging from 15 to 26 kD. The amino acid sequences of the oleosins from several plant species have been obtained via the corresponding cDNA or genomic DNA (10, 16, 19, 27). As deduced from computer model analyses of the amino acid sequences, each oleosin consists of three structural domains, including an amphipathic  $\text{NH}_2$ -terminal domain, a central hydrophobic domain, and an amphipathic  $\alpha$ -helical domain at or near the COOH-terminus. These secondary structures apparently enable the protein to reside stably on the surface of the oil bodies. The function of oleosins has not been previously reported.

Oil bodies are remarkably stable either inside the cells or in isolated preparations. In both situations, the organelles occur as individual entities, and when they are pressed against one another in vivo due to seed desiccation or in vitro after flotation centrifugation, they do not aggregate or coalesce, even after prolonged storage (11, 30). The physiological significance of maintaining the oil bodies as small entities is to provide ample surface areas for the attachment of lipase to the organelles during postgerminative growth so that the reserve TAG can be mobilized rapidly. How the oil bodies maintain their small sizes without coalescing is unknown.

Oil bodies are abundant in plant seeds, and are among the

1. Abbreviations used in this paper: PL, phospholipid(s); TAG, triacylglycerol(s).

simplest organelles in eukaryotes. Similar organelles can be found in the pollens (24) and the roots (13) of angiosperms as well as in tissues of more primitive plants, such as the megagametophytes of gymnosperms (1) and the spores of ferns (7). Intracellular storage lipid organelles of similar structure are also present in tissues of nonplant species, including the brown adipose (8) and other tissues (6) of mammals, eggs of some nematodes and other nonmammals (21), and unicellular organisms such as yeast (3), *Euglena* (17), and algae (20). The surface properties and the structure of the lipid particles in all the above organisms have not been previously explored in detail by biochemical means. We did such a study on the oil bodies in plant seeds, and our findings could be extended to other nonplant systems.

In this paper, we provide experimental evidence to show that on the surface of seed oil bodies, amphiphilic oleosins and PL work cooperatively to stabilize the organelles, and oleosins further prevent the organelles from coalescing by providing steric hindrance. We also report successes in producing reconstituted oil bodies using natural or artificial TAG, natural or artificial PL, and oleosins. Based on the available information, we propose a model of the structure of an oil body.

## Materials and Methods

### Plant Materials

Embryos of maize (*Zea mays*, inbred line MO 17), rice (*Oryza sativa*), soybean (*Glycine max*, var. York), rapeseed (*Brassica campestris*, var. R-500), and jojoba (*Simmondsia chinensis*) were obtained from the mature seeds. The mature seeds were either used directly (rapeseed) or soaked in water for 1 h (soybean and wheat bran) or 24 h (maize, rice, and jojoba) before use. Bran of wheat (*Triticum aestivum*) were obtained from a local market.

### Isolation of Oil Bodies

The plant material was homogenized at 4°C in grinding medium (5 g material per 20 ml) with a Polytron (Brinkmann, Westbury, NY) fitted with a PTA 10 generator at high speed for 40 s (26). The grinding medium contained 0.6 M sucrose, 1 mM EDTA, 10 mM KCl, 1 mM MgCl<sub>2</sub>, 2 mM DTT, and 0.15 M TRICINE adjusted to pH 7.5 with KOH. The homogenate was filtered through a Nitex cloth having 20 μm × 20 μm pores (Tetko, Elmsford, NY). After filtration, each 15-ml portion of the homogenate was placed at the bottom of a 30-ml centrifuge tube, and 15 ml of flotation medium (grinding medium containing 0.4 instead of 0.6 M sucrose) was layered on top. The tubes were centrifuged at 10,000 g for 30 min in a swinging-bucket rotor. The oil bodies on top were collected, and resuspended in 15 ml of grinding medium containing an additional 2 M of NaCl. The resuspension was placed at the bottom of a 30-ml centrifuge tube, and 15 ml of floating medium (grinding medium containing 2 M NaCl and 0.25 M instead of 0.6 M sucrose) was layered on top. The tubes were centrifuged. The oil bodies on top were collected, and resuspended in 15 ml grinding medium. The resuspension was placed at the bottom of a 30-ml centrifuge tube, and 15 ml of floating medium (grinding medium containing 0.4 instead of 0.6 M sucrose) was layered on top. The tube was centrifuged. This last step of flotation wash was repeated once. The oil bodies on top were collected and resuspended with grinding medium to give a concentration of 100 mg lipid/ml.

### Separation of Neutral Lipids, PL, and Proteins from Isolated Oil Bodies

A 500-μl preparation of isolated oil bodies was extracted with 1 ml diethyl ether in a 1.5-ml Eppendorf tube. After centrifugation at 13,600 g for 4 min, the upper ether layer was collected. The lower aqueous layer and the interfacial materials were extracted with 1 ml diethyl ether two additional times. The ether fractions were pooled (3 ml), and the ether was evaporated under nitrogen gas.

The aqueous layer, together with the interfacial materials, was placed under nitrogen for 1 h in order to evaporate the remaining ether. A volume of 750 μl chloroform/methanol (2:1, vol/vol) was added. After gentle shaking, the tube was centrifuged at 13,600 g for 4 min. The lower chloroform layer, the upper methanol-water layer, and the interfacial materials were collected individually.

The chloroform fraction (which contained PL) was washed three times, each with 1 ml methanol/water (1:1, vol/vol) followed by centrifugation.

The interfacial materials were washed three times by the following procedure. They were mixed with 250 μl water and 750 μl chloroform/methanol (2:1, vol/vol), and the mixture was centrifuged. The interfacial materials were collected and resuspended in 0.5 ml water. Since the interfacial materials existed in aggregates, they were sonicated to yield a more uniform sample. Sonication was performed with a 4-mm diameter probe in a Braun-Sonic 2000 ultrasonic generator (Freeport, IL) with a digital meter reading of 200 for 15 s.

### Analyses of Oil Body Constituents

The lipid and protein contents in the above ether fraction, the chloroform fraction, the methanol-water fraction, and the interfacial fraction were analyzed by TLC and SDS-PAGE, respectively. The TLC plate (Silica Gel 60A from Whatman) was developed in hexane/diethyl ether/acetic acid (80:20:2; vol/vol/vol) for the separation of neutral lipids. After drying, the plate was further developed briefly in chloroform/acetic acid/methanol/water (70:25:5:2; vol/vol/vol/vol) in order to allow the separation of PL from the origin. The plate was allowed to react with iodine. For SDS-PAGE, the separating gel and the stacking gel consisted of 12.5 and 4.75% polyacrylamide, respectively (26). After electrophoresis, the gel was stained with Coomassie blue R-250, and destained.

The neutral lipids in the ether fraction were weighed directly (~1 g from 10 ml of oil body suspension) after exhaustive evaporation of the ether. The PL in oil body fraction were quantitated by their phosphate content (5) using KH<sub>2</sub>PO<sub>4</sub> as a reference. The proteins in the oil body fraction after ether extraction were quantitated by the Bradford method (23) using BSA, Fraction V, as standard.

### Reconstitution of Oil Bodies from Their Constituents

The three major constituents (TAG, PL, and proteins) of the oil bodies were used. Neutral lipids from the ether fraction (containing ~95% TAG and 5% other neutral lipids composed largely of diacylglycerols, to be described in Fig. 1) or commercially prepared triolein and trilinolein (from Nu-Chek, Elysian, MN) in a 1:2 molar ratio were used. PL in the chloroform fraction or commercially prepared dioleoyl phosphatidylcholine (from Sigma, St. Louis, MO) were used. The interfacial fraction was used as a source of protein.

A 1-ml suspension containing 0.25 M sucrose, 50 mM Bis-Tris, pH 7.2, and TAG (15 mg), PL (135 μg) and/or (as specified in Results) proteins (210 μg) in a 1.5-ml Eppendorf tube was prepared. PL dissolved in chloroform were placed at the bottom of an Eppendorf tube, and the chloroform was allowed to evaporate under nitrogen. A volume of 16 μl TAG was added. Proteins in a sonicated suspension in 100 μl water were added. Buffer and water were added to make a final volume of 1 ml. The mixture was vortexed, and then sonicated with a 4-mm-diameter probe in a Braun-Sonic 2000 ultrasonic generator with a digital meter reading of 50 for 20 s. The sample was cooled in an ice bucket for 5 min. A second sonication with a digital meter reading of 200 for 20 s was applied.

### Turbidity Test

Oil bodies or reconstituted oil bodies, with or without enzymatic treatment, in a suspension in 0.25 M sucrose and 50 mM Bis-Tris buffer, pH 7.2, floated to the top of the mixture. As a consequence, the suspension below the floated oil body layer decreased in turbidity. The turbidity below the oil body layer was measured at time intervals by the following method. The mixture of 1.4 ml was placed in a disposable cuvette of 1.8 ml capacity (from Fisher Corp., Tustin, CA), which consisted of a lower portion of 1 cm (light path) × 0.4 cm (width) × 3.2 cm (height) for a volume of 1.28 ml, and an upper portion of enlarged width for an additional volume of 0.52 ml. The cuvette was covered with parafilm and subjected to minimal disturbance. The absorbance of the suspension in the lower portion of the cuvette was read at 600 nm in a Beckman DU spectrophotometer at time intervals. At the start of the measurement, the absorbance ( $A$ ) was 2.4 ( $A_0$ ). The turbidity ( $T$ ) of the suspension was proportional to  $10^4$ , and the relative turbidity was expressed as  $T/T_0 = 10^4/10^{4.0} = 10^4/10^{2.4}$ .

## Treatment of Native or Reconstituted Oil Bodies with Protease or Phospholipases

All treatments were performed at 24°C. Trypsin (4 µg; bovine pancreas type III; Sigma Chemical Co., St. Louis, MO), phospholipase A2 (40 µg, from *Naja naja* venom; Sigma Chemical Co.), or phospholipase C (40 µg, from *Clostridium perfringens*, Type XIV; Sigma Chemical Co.) was added to a 2-ml oil body suspension containing 3 mg lipids in 0.25 M sucrose and 50 mM Bis-Tris, pH 7.2. Duplicate reaction mixtures were set up for the turbidity test, the monitoring of compositional changes, and light microscopy. For the turbidity test, a volume of 1.4 ml of the reaction mixture was added to a cuvette, and the decrease in turbidity at the lower portion was recorded at time intervals. For the monitoring of compositional changes and light microscopy, the reaction with trypsin was stopped after 30 min by adding 4 µg of trypsin inhibitor (soybean, type I-S; Sigma Chemical Co.), and the reaction with phospholipase was terminated after 2 h by adding 3 ml chloroform/methanol (2:1, vol/vol). Protein composition was analyzed by SDS-PAGE with a 14% acrylamide gel. Lipids were assessed by TLC developed in chloroform/acetone/methanol/water (70:25:5:2; vol/vol/vol/vol) for PL, and hexane/diethyl ether/acetic acid, (80:20:2; vol/vol/vol) for neutral lipids. For light microscopy, the mixture was shaken gently for uniform sampling, and the oil bodies were observed under a light microscope with Nomarski optics.

## EM of Isolated Oil Bodies

The oil body preparation was fixed in 2.5% glutaraldehyde in 50 mM potassium phosphate buffer, pH 7.5, for 3 h. After several rinses with the buffer, it was postfixed in 1% OsO<sub>4</sub> in the buffer overnight. Dehydration was carried out in a graded acetone series, and the sample was embedded in Spurr's resin. 100-nm serial sections were stained with uranyl acetate and lead citrate, and observed in a Philips EM400 electron microscope. Each of the 195 oil bodies within a defined area was followed throughout the serial sections, and its largest diameter was recorded. The average diameter of these 195 oil bodies was 1.45 ± 0.06 µm; 184 out of the 195 oil bodies fell within the range of 0.6 to 2.1 µm.

## Results

### Oil Bodies from Mature Maize Embryos Possessed Special Chemical Characteristics

Although oil bodies in seeds are abundant and important organelles, a comprehensive analysis of their chemical features has not been previously reported. Here, we report such an analysis of the oil bodies isolated from maize embryos.

Isolated maize oil bodies maintained a hydrophilic surface. This was shown in our attempts to extract the matrix TAG with the following organic solvents of ascending solubilities in water: hexane (<0.02% in water at 25°C), benzene (<0.1%), chloroform (<0.5%), diethyl ether (6%), and acetone (miscible). The extraction was performed with a 1:1 (vol/vol) ratio of solvent to oil body resuspension (100 mg lipids per ml) in a horizontal shaker with gentle shaking for 12 h. Whereas all the solvents dissolved commercially prepared triolein or TAG previously extracted from isolated oil bodies, only diethyl ether or acetone was able to extract the matrix TAG from isolated oil bodies. The moderately water-soluble chloroform and benzene were unable to extract the lipids, and converted the oil body suspension into a milky jelly phase. The least water-soluble hexane, in a 1:1 and even 100:1 (vol/vol) ratio of solvent to oil body suspension, failed to extract the lipids, leaving a clear hexane phase and a milky oil body resuspension. Apparently, only diethyl ether or acetone was able to penetrate the hydrophilic surface, presumably of a layer of PL and proteins with associated water molecules, and dissolve the matrix TAG.

Isolated maize oil bodies contained about 97.7% TAG and

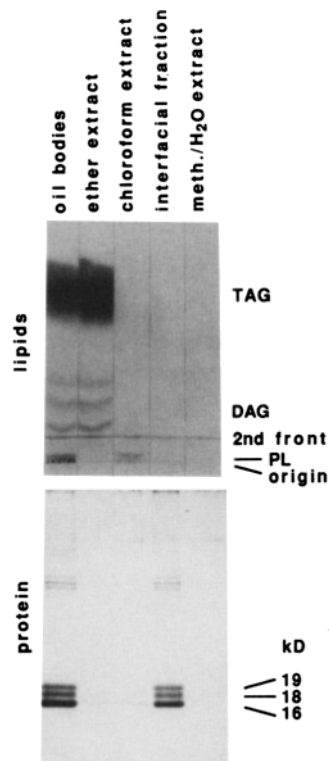


Figure 1. Thin layer chromatography (upper photo) and SDS-PAGE (lower photo) of the various fractions obtained in a fractionation of a maize oil body preparation. The three major components (TAG and other neutral lipids, PL, and proteins) of the oil bodies were separated from one another by fractionation with diethyl ether followed by chloroform/methanol. The diethyl ether fraction contained TAG, diacylglycerols, and other minor neutral lipids. The chloroform fraction retained the PL. The interfacial fraction in the chloroform/methanol extraction had the proteins. Each of the three fractions contained one component with negligible portions of the other two components. Of each fraction, the amount applied to the TLC plate or PAGE gel was adjusted to represent that derived from an equal quantity of the oil body fraction. The TLC plate was developed in a solvent for

the separation of neutral lipids; after drying, it was further developed in another solvent for the separation of the PL from the origin. The second front denotes the solvent front of the second plate development. Labels on the right indicate the types of lipids or the molecular mass of the oleosins.

other minor neutral lipids (~95% TAG, 4% diacylglycerols [Fig. 1]; for convenience in description, these neutral lipids are referred to as native TAG in this report), 0.9% PL, and 1.4% proteins (Table I). The TAG and other acyl-lipids included ~65% linoleoyl, 22% oleoyl, and 10% palmitoyl

Table I. Parameters of an Average Spherical Maize Oil Body. It has a Diameter of 1.45 µm, and a Volume of  $1.63 \times 10^{-12} \text{ cm}^3$

	Total	TAG	PL	Protein
Weight (% wt/wt)	100	97.66	0.91	1.43
Density (g/cm <sup>3</sup> )	0.926	0.92	1.03	1.3
Weight ( $\times 10^{-14}$ g)	148	144.5	1.35	2.12
# of molecules ( $\times 10^5$ )	—	10,000	107	8
Ratio of molecules	—	1,250	13	1

Values are obtained by the following methods:

Diameter of oil body, by measurement (this report).

Volume of oil body, calculated from the diameter.

Percent weight, by chemical determination (this report).

Density of oil body, by adding (weight %  $\times$  density of individual molecules).

Density of individual molecules: triolein or trilinolein, 0.92 g/cm<sup>3</sup>; dioleoyl-phosphatidylcholine, 1.03 g/cm<sup>3</sup> (both from Merck index); and protein, assumed to be 1.3 g/cm<sup>3</sup>.

Total weight of oil body, by (volume  $\times$  density).

Weight of individual molecules, by (weight %  $\times$  total oil body weight).

Number of individual molecules, by [(weight/molecular weight)  $\times$  Avogadro number]; Molecular Weight of TAG, PL, and oleosin assumed to be 880, 759, and 16,000, respectively.

Ratio of molecules, calculated from the number of individual molecules.

acyl moieties (29). More than 90% of the proteins are oleosins of 16, 18, and 19 kD (Fig. 1), as reported earlier (26).

The diameters of seed oil bodies from several plant species as measured from electron micrographs have been reported (8). However, they were all underestimated values, since the oil bodies in an electron micrograph are mostly not viewed in their actual equatorial planes but rather at randomly cut planes of the organelles. To obtain a true value, we did a serial sectioning of a preparation of isolated maize oil bodies, and followed and measured the largest diameters of all the 195 oil bodies within a defined area. An average diameter of 1.45  $\mu\text{m}$  was obtained. Using this value, we were able to calculate the physical parameters of the organelles (Table I). The latter parameters were used to construct a structural model of the organelle (see Discussion).

The three major components of the oil bodies (neutral lipids, PL, and proteins) were separated from one another by diethyl ether extraction followed by chloroform/methanol extraction (Fig. 1). These three separated components were used to reconstitute oil bodies of characteristics similar to those of the native oil bodies (to be described).

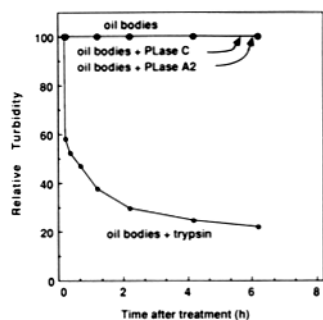
### **The Size and Integrity of Maize Oil Bodies in a Suspension Were Monitored by Following the Turbidity Spectrophotometrically**

When oil bodies freshly isolated from mature maize embryos were resuspended in a dilute buffer at pH 7.5 and at 24°C, the suspension remained quite stable for the first 24 h. Although the oil bodies slowly floated to the top of the solution, negligible change in the turbidity was observed. When the oil bodies were treated such that they coalesced (to be described), the larger oil particles floated to the top of the solution at a higher rate. As a consequence of the flotation of the oil bodies, the suspension below the floated oil body layer decreased in turbidity. This decrease in turbidity below the oil body layer was measured at time intervals at 600 nm in a spectrophotometer (see Materials and Methods). The rate of decrease in the relative turbidity, ( $T/T_0 = 10^A/10^{A_0}$ ), reflected a combination of (a) the rate of the enzymatic reaction (e.g., trypsin digestion, to be described), (b) the change in the size of the oil bodies (change in light scattering), and (c) the floating rate of the original or coalesced oil bodies.

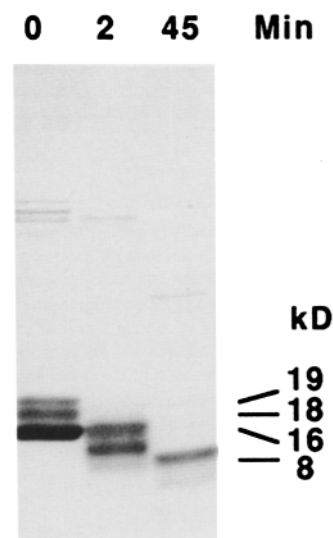
A suspension of freshly isolated oil bodies remained unchanged in its turbidity for 6 (Fig. 2) to more than 24 h (data not shown). At the end of the incubation, there was no change in the size of the oil bodies, and the oil bodies also did not aggregate (Fig. 3).

### **Trypsin Did, and Phospholipase A2 or Phospholipase C Did Not, Induce the Coalescence of Maize Oil Bodies**

The possible structural role of the surface oleosins and PL in maintaining the integrity of the oil bodies was probed. The oil bodies were treated with either trypsin, phospholipase A2, or phospholipase C, and the turbidity of the oil body suspension was monitored. With trypsin treatment, the oil bodies floated rapidly to the top of the solution (visual observation), and this flotation rate was reflected in the rapid decrease in the turbidity of the suspension below the floated oil bodies (Fig. 2). Coalescence was observed after only 1 min of trypsin treatment. After 6 h of treatment, the oil bod-



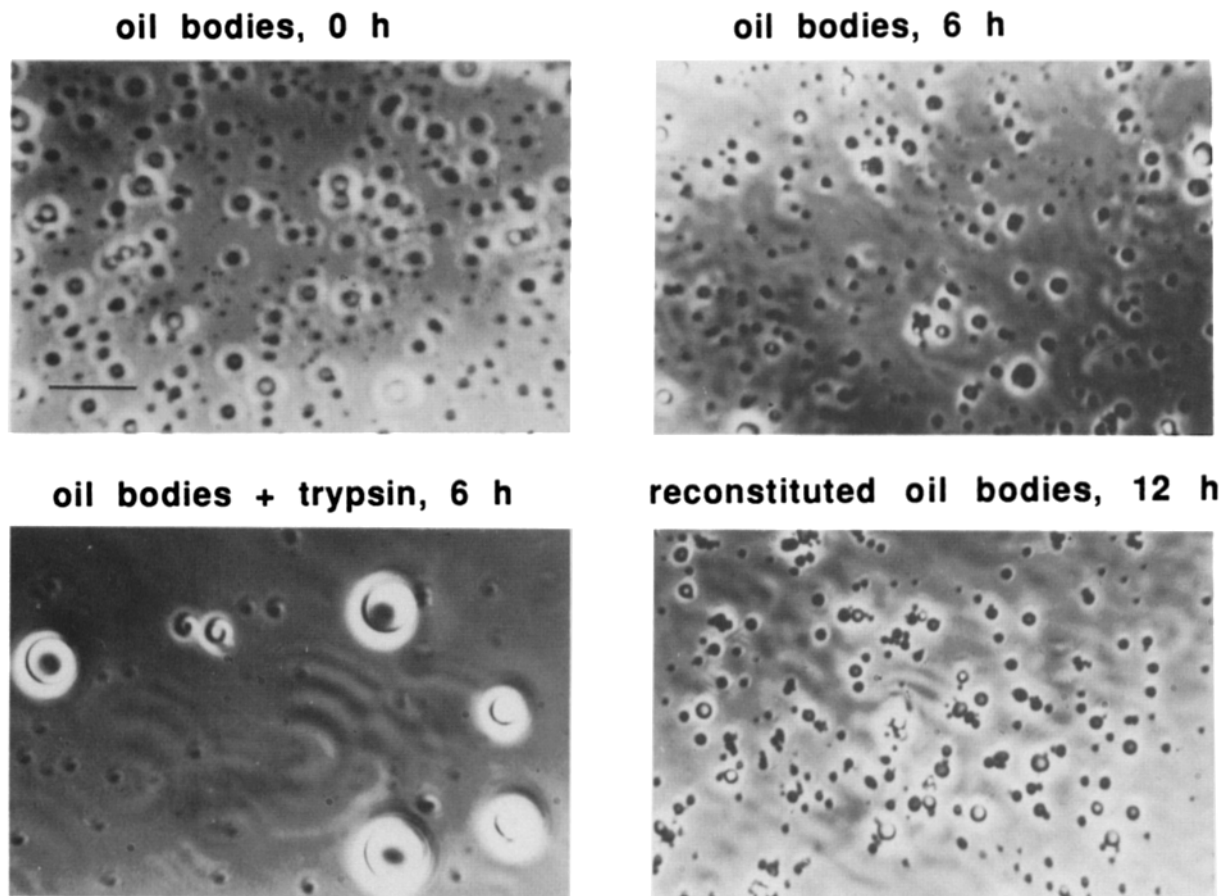
### **SDS-PAGE**



**Figure 2.** Effects of trypsin, phospholipase A2, and phospholipase C on isolated maize oil bodies. The enzyme was added to an oil body suspension of 1.4 ml in a 1.8-ml cuvette, and the relative turbidity ( $T/T_0$ ) at 600 nm of the lower portion ( $\sim 0.5$  ml) of the suspension was measured at time intervals. The SDS-PAGE protein profile of the suspension after 0, 2, and 45 min of trypsin treatment is shown at the lower portion of the figure; the three major oleosins of 19, 18, and 16 kD were converted to a major band representing polypeptides of 8 kD.

ies could be seen as much larger particles (Fig. 3). The enzymatic activity of trypsin on the oleosins on the surface of the oil bodies during the incubation time resulted in the production of smaller polypeptides as revealed by SDS-PAGE (Fig. 2). The remaining major band of polypeptides of  $\sim 8$  kD probably represented the central hydrophobic domain of the oleosins (19, 27) which would not have been accessible to the external trypsin and which contained no arginine or lysine.

Contrary to trypsin treatment, phospholipase A2 treatment up to 6 h did not result in an appreciable change in the turbidity of the oil body suspension (Fig. 2) as well as the size of the oil bodies (light microscopy, not shown). This lack of effect was also shown in the absence of detectable phospholipase A2 enzymatic activity in terms of the disappearance of PL and the appearance of product lysophosphatidyl lipids (Fig. 4). On the contrary, phospholipase A2 did exert its enzymatic activity on (a) PL previously extracted from the oil bodies, (b) combined PL and TAG previously extracted from the oil bodies, and (c) oil bodies that had been first treated with trypsin for 30 min (Fig. 4). Very similar results were obtained with phospholipase C whose activity would hydrolyze PL to 1,2-diacylglycerol (Figs. 2 and 4). The inaccessibility of the PL on oil bodies to the two phospholipases was not due to the positive charges associated with the alkaline oleosins (19, 27), since the PL in reconstituted oil bodies (next section) containing no oleosins but



**Figure 3.** Light microscopy of maize oil bodies untreated for 0 and 6 h, treated with trypsin for 6 h, and of reconstituted maize oil bodies 12 h after preparation. Light microscopy was performed with Nomarski optics. All the four photos are of the same magnification. Bar, 10  $\mu\text{m}$ .

stearylamine with equivalent positive charges were susceptible to phospholipases A2 and C (Fig. 4). The results strongly suggest that the PL on the surface of oil bodies were not accessible to external phospholipase A2 (molecular mass, 13 kD; ref. 4) or phospholipase C (molecular mass, 90 kD; ref. 2) due to the shielding effect of oleosins.

#### ***Reconstitution of Oil Bodies from TAG and Either PL or Oleosins Was Unsuccessful***

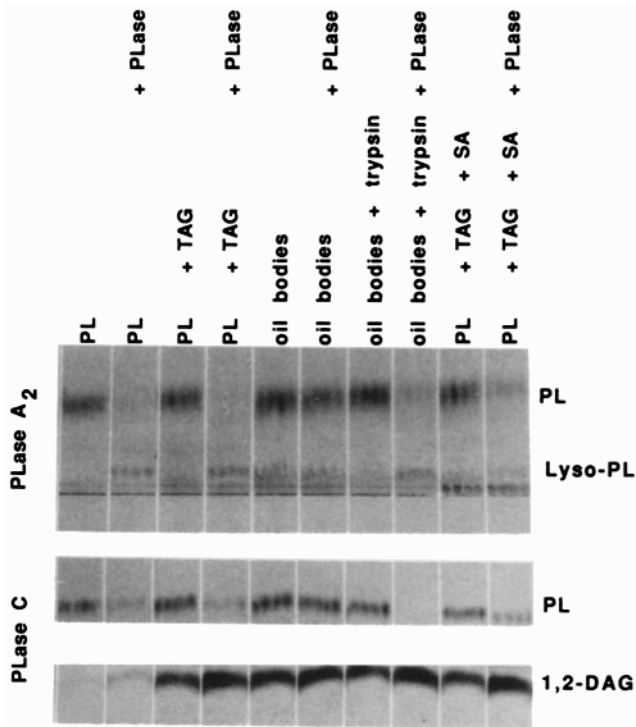
When TAG extracted from isolated maize oil bodies were sonicated in a dilute buffered solution of pH 7.5, small oil droplets were formed; these droplets were of sizes similar to those of the native oil bodies (light microscopy, not shown). The TAG droplets quickly coalesced and floated to the top of the solution (Fig. 5 A). Inclusion of maize oil body PL or maize oleosins into the solution before sonication produced TAG droplets which were slightly more stable (Fig. 5 A). The droplets reconstituted with TAG and oleosins coalesced, and thus floated, substantially faster than those with TAG and PL, even though the denser oleosins would have contributed to a slower flotation. This faster coalescence is likely due to the instability of the oleosins, relative to that of the PL, on the surface of the TAG droplet, since the stability of the oleosin requires the protein to interact closely with PL (19, 27). Inclusion of positively charged stearylamine or negatively charged stearic acid in addition to PL before soni-

cation did further stabilize the TAG droplets (Fig. 5 A). Still, the stability of the TAG droplets in the presence of PL and stearylamine/stearic acid was far from that of isolated native oil bodies. In the above reconstitutions, the amount of PL or oleosins in proportion to TAG was similar to that in native oil bodies. Also, the amount of positive charges derived from stearylamine in proportion to TAG was that derived from oleosins (five charges in one oleosin molecule, as judged from the amino acid sequences; 19, 27) in native oil bodies, and the amount of negative charges derived from stearic acid was similar to the positive charges from stearylamine. Using twice the amounts of PL and stearylamine/stearic acid, or of oleosins, in proportion to TAG as those in native oil bodies did not substantially alter the patterns of stability of the TAG droplets (data not shown).

The above reconstitution was also performed with commercially prepared TAG of a 1:2 molar mixture of triolein and trilinolein instead of maize TAG, or with dioleoyl phosphatidylcholine instead of maize oil body PL. A similar pattern of minimal stabilization effect exerted by dioleoyl phosphatidylcholine or oleosins on the commercially prepared TAG was observed (Fig. 5 B).

#### ***Reconstitution of Oil Bodies from TAG, PL, and Oleosins Was Successful***

When both maize PL (or dioleoyl phosphatidylcholine) and

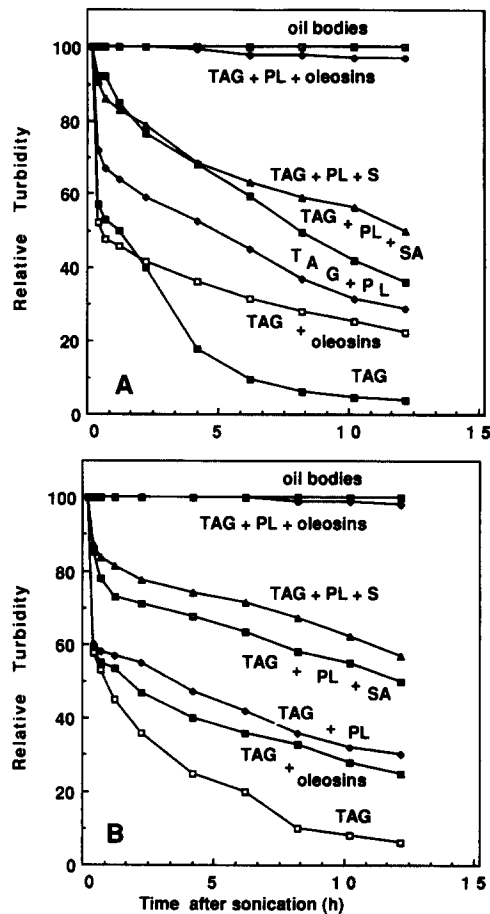


**Figure 4.** Thin layer chromatography of the reactants of phospholipase A2 and phospholipase C treatment of maize oil bodies and extracted lipids. The PL and PL plus TAG previously extracted from isolated oil bodies, with or without the addition of stearylamine (SA), were sonicated before use. The lipid components on the plate are identified on the right. In the phospholipase A2 study, the plate was developed in chloroform/acetic acid/methanol/water (70:25:5:2). In the phospholipase C study, the plate was developed in either the above developing solvent for the separation of PL or hexane/diethyl ether/acetic acid (80:20:2) for the separation of neutral lipids (diacylglycerols). Only the portions of the plates showing the reactants (PL and lysoPL in the phospholipase A2 reaction, and PL and diacylglycerols in the phospholipase C reaction) are shown. Isolated maize oil bodies contained a few percent of diacylglycerols which were several times higher than that of PL (see Fig. 1); these internal diacylglycerols masked the diacylglycerols released from PL in the phospholipase C reaction on extracted PL plus TAG, trypsin-treated oil bodies, and PL plus TAG plus stearylamine.

maize oleosins were added to maize TAG (or a 1:2 molar mixture of triolein and trilinolein) in a solution before sonication, TAG droplets of stability very close to that of isolated oil bodies were produced (Fig. 5). These droplets, or reconstituted oil bodies, were similar to the native oil bodies in their sizes as observed under a light microscope (Fig. 3). In addition, they were similar to the native oil bodies in that they were destabilized by treatment with trypsin but not with phospholipase A2 or phospholipase C (data not shown).

***In the Reconstitution of Stable Maize Oil Bodies with TAG, the Minimal Amounts of PL and Oleosins Required Were Similar to Those in Native Oil Bodies***

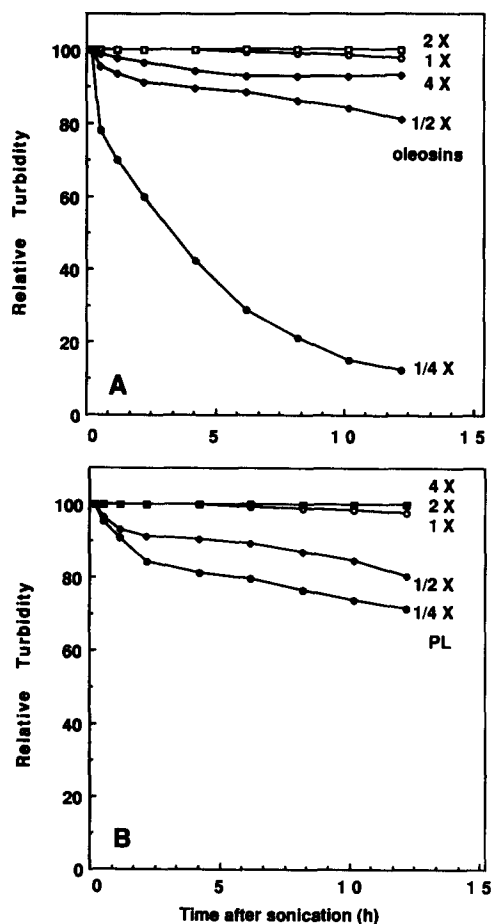
In the above preparations of reconstituted oil bodies, the amounts of the three basic ingredients of TAG, PL, and oleosins were in proportions similar to those in the native oil bodies. To test if this proportion was critical to the successful



**Figure 5.** Stability of oil bodies reconstituted from TAG, PL, and/or maize oleosins. A suspension of the reconstituted oil bodies of 1.4 ml was placed in a cuvette, and the relative turbidity ( $T/T_0$ ) at 600 nm of the approximately lower 0.5 ml of the suspension was measured at time intervals. The proportion of TAG (97.7%), PL (0.9%), and oleosin (1.4%) used was similar to that in the native maize oil bodies. The amount of stearylamine (SA) used (10% of PL, wt/wt) was similar to the amount of the calculated positive charges in oleosins in native oil bodies. The amount of stearic acid (S) used was similar in molarity to that of stearylamine. In A, the TAG and PL were the native components extracted from isolated maize oil bodies. In B, the TAG was a 1:2 molar mixture of triolein and trilinolein, and the PL was dioleoyl phosphatidylcholine.

formation of reconstituted oil bodies, a series of preparations of reconstituted oil bodies was made by maintaining the same amounts of TAG and either PL or oleosins, and changing the amount of oleosins or PL, respectively.

In one set of preparations of reconstituted oil bodies, maize TAG and PL in their native proportion (97.7 and 0.9% wt/wt, respectively) were used with increasing amounts of oleosins (1/4, 1/2, 1, 2, and 4  $\times$  of the native 1.4%). Only those preparations made with 1, 2, and 4  $\times$  of the native proportion of oleosins contained reconstituted oil bodies that were stable (Fig. 6 A). Those preparations made with 1/4 or 1/2 of the native proportion of oleosins contained reconstituted oil bodies that were unstable and tended to coalesce and float to the top of the solution. In the preparations made with 2 and 4  $\times$  of the native proportion of oleosins, the ex-



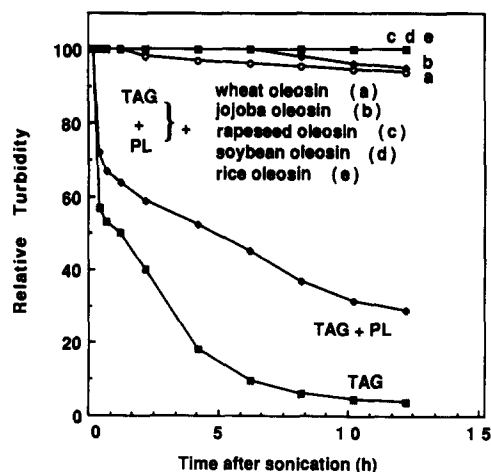
**Figure 6.** (A) Stability of maize oil bodies reconstituted from native TAG, native PL, and increasing amounts of oleosins. The amounts of TAG (97.7%, wt/wt), PL (0.9%), and oleosins (1× representing 1.4%) were of the same proportion as that in native maize oil bodies. (B) Stability of maize oil bodies reconstituted from native TAG, oleosins, and increasing amounts of native PL. The amounts of TAG (97.7%, wt/wt), oleosins (1.4%), and PL (1× representing 0.9%) were of the same proportion as that in native maize oil bodies. A suspension of the reconstituted oil bodies of 1.4 ml was placed in a 1.8-ml cuvette, and the relative turbidity (*T/T*<sub>0</sub>) at 600 nm of the approximately lower 0.5 ml of the suspension was measured at time intervals.

cess oleosins were not associated with the reconstituted oil bodies but could be seen as precipitates (aggregates) at the bottom of the tubes minutes after the preparation.

In a complementary set of preparations of reconstituted oil bodies, TAG and oleosins in their native proportion (97.7 and 1.4% wt/wt, respectively) were used with increasing amounts of PL (1/4, 1/2, 1, 2, and 4 × the native 0.9%). Only those preparations made with 1, 2, and 4 × of the native proportion of PL contained reconstituted oil bodies that were stable (Fig. 6 B). Those preparations made with 1/4 or 1/2 of the native proportion of PL contained reconstituted oil bodies that were unstable and tended to coalesce and float to the top of the solution.

#### **Stable Reconstituted Oil Bodies Were Made Similarly from TAG, PL, and Oleosins from Other Plant Species**

Stable reconstituted oil bodies were made from TAG of a 1:2



**Figure 7.** Stability of oil bodies reconstituted from TAG (a 1:2 molar mixture of triolein and trilinolein), PL (dioleoyl phosphatidylcholine), and oleosins extracted from oil bodies isolated from the seeds of various plant species. The proportion of TAG, PL, and oleosin were 97.7, 0.9, and 1.4%, wt/wt, respectively. A suspension of the reconstituted oil bodies of 1.4 ml was placed in a 1.8-ml cuvette, and the relative turbidity (*T/T*<sub>0</sub>) at 600 nm of the approximately lower 0.5 ml of the suspension was measured at time intervals.

molar mixture of triolein and trilinolein, dioleoyl phosphatidylcholine, and oleosins (97.7, 0.9, and 1.4%, wt/wt, respectively) extracted from oil bodies isolated from wheat, rice, rapeseed, soybean, or jojoba (Fig. 7). Thus, the results of our detailed study with the oil bodies from maize could be extended to other plant species.

#### **Discussion**

The surface of a seed oil body is hydrophilic and hydrated. This hydrophilicity is due to the presence of PL and oleosins. The two types of molecules interact not only with the hydrophobic TAG core, but also between themselves. The interaction between oleosin and PL is specific due to the charge and polarity distribution within the molecules (19, 27). It is likely that an oil body contains an optimal but minimal amount of oleosins for maximal efficiency, and so the molar ratio of PL to oleosins within an oil body is defined. Artificially prepared particles of TAG surrounded by a layer of PL with or without stearylamine/stearic acid coalesce rapidly. However, when these particles contain additional oleosins on the surface, as in the case of seed oil bodies, they become stable and do not aggregate or coalesce, even when they are brought to press against one another *in vivo* and *in vitro*. The maintenance of individuality is due in part to the oleosins which provide not only an amphipathic surface but also steric hindrance. These characteristics of the oleosins are important to the functioning of the molecules, and therefore are preserved among the oleosins from diverse plant species (12, 14).

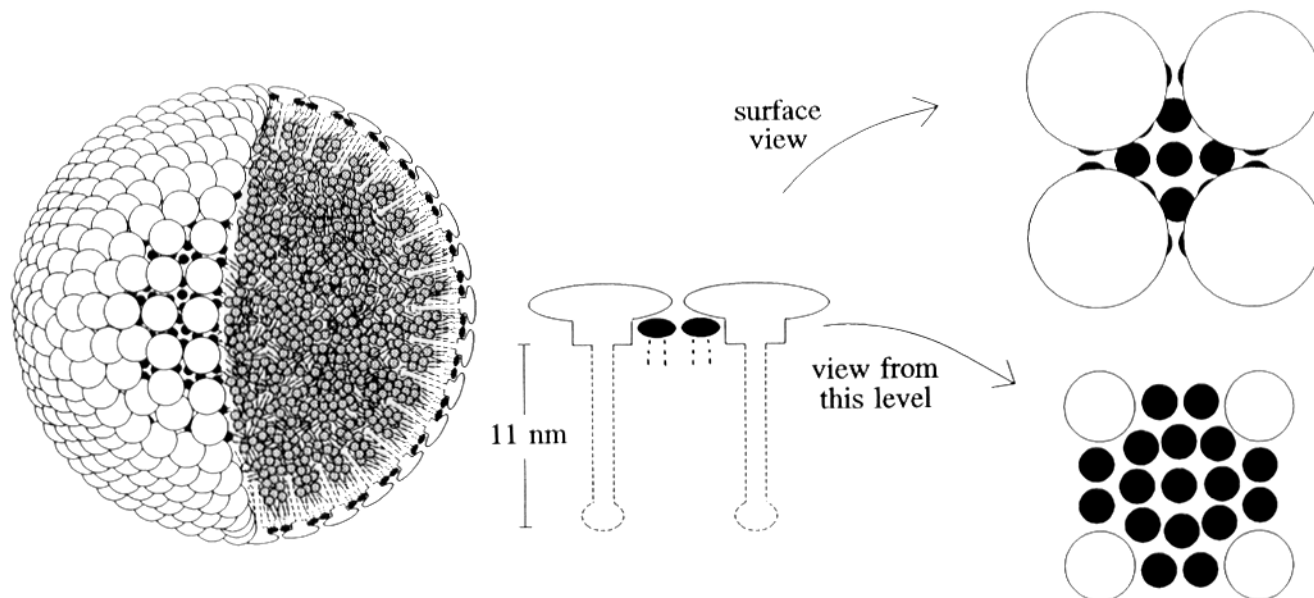
The following calculations by two different approaches show that the PL on the surface of an average maize oil body are sufficient to occupy 80% of a 2.5-nm-thick shell (thickness of a monolayer of PL). (a) The volume of such a shell on the surface of an average maize oil body of 1.45 μm diameter is  $\frac{4}{3} \pi (725)^3 - \frac{4}{3} \pi (725 - 2.5)^3 = 1.65 \times 10^7$

nm<sup>3</sup>. The amount of PL in an oil body ( $1.35 \times 10^{-14}$  g; Table I) can occupy  $1.35 \times 10^{-14}$  g/1.03 g/cm<sup>3</sup> (density of PL) =  $1.31 \times 10^7$  nm<sup>3</sup>, or 80% of the volume of the shell. (b) The surface of the oil body has an area of  $4\pi r^2 = 4\pi(725)^2 = 6.6 \times 10^6$  nm<sup>2</sup>. To occupy 80% of this area by  $1.07 \times 10^7$  PL molecules (Table I), each PL molecule will need a space with an area of 0.49 nm<sup>2</sup>, or a square with a width of 0.7 nm. Such a value for a PL molecule with a length of 2.5 nm is in agreement with that reported earlier on a PL bilayer (15, 18) and that calculated based on the density and molecular mass of PL being 1.03 g/cm<sup>3</sup> and 759 D, respectively. A PL monolayer in an aqueous environment is unstable and collapses to generate a larger area of 0.6–0.7 nm<sup>2</sup> per PL molecule; however, with the presence of TAG underlining the PL layer in an oil body and with the interaction between the oleosin and PL, the more compact value of 0.49 nm<sup>2</sup> is expected. The above calculations show that the oil body surface is covered by only one layer of PL. It is likely that the remaining 20% of the surface shell is occupied by oleosins. This 20% value matches well with the amount of oleosins in an oil body ( $2.12 \times 10^{-14}$  g; Table I), roughly 1/5 of which is predicted to be buried within the PL monolayer (19, 27). This 1/5 portion of the oleosin occupies  $(1/5 \times 2.12 \times 10^{-14} \text{ g}/1.3 \text{ g/cm}^3) = 3.26 \times 10^6$  nm<sup>3</sup>, or 20% of the outer shell.

Our studies show that the monolayer of PL on the surface of the oil body is covered by oleosins such that they are not

accessible to phospholipase of a small molecular mass. This experimental finding is in agreement with the following theoretical calculation. In the maize oleosin, the NH<sub>2</sub>-terminal domain and the COOH-terminal domain, each of ~45 residues, are amphipathic (19, 27). The NH<sub>2</sub>-terminal domain is likely arranged into an amphipathic helical or random structure, and the COOH-terminal domain is well documented to have an amphipathic  $\alpha$ -helical structure. To calculate the minimal area covered by the oleosins, let us assume the two domains of 90 residues to have the relatively compact  $\alpha$ -helical structure (22). A stretch of 90 residues occupies  $90 \times 0.6$  nm (width of the  $\alpha$ -helix)  $\times$  0.15 nm (surface length of one residue in the  $\alpha$ -helix), or 8.1 nm<sup>2</sup>. Since there are  $8 \times 10^5$  oleosins in a maize oil body (Table I), the total surface area occupied by oleosins is  $8.1 \text{ nm}^2 \times 8 \times 10^5 = 6.48 \times 10^6$  nm<sup>2</sup>. This value is very close to the surface area of the oil body ( $6.6 \times 10^6$  nm<sup>2</sup>) as described in the preceding paragraph.

A model of the oil body structure is shown in Fig. 8. The basic structural unit of the oil body surface consists of 13 PL molecules and one oleosin molecule (Table I). About 2/5 of the oleosin is the hydrophobic stalk of ~11 nm embedded in the hydrophobic acyl moieties of PL and the TAG matrix; this stalk is depicted as a hairpin structure of ~70 amino acid residues. The remaining 3/5 of the oleosin molecule covers or protrudes outward from the oil body. The covering portion is part of an amphipathic  $\alpha$ -helix of ~33 amino acid



**Figure 8.** A model of a maize oil body. All molecules are drawn roughly in proportional sizes. The shape of the oleosin molecule is depicted as a stalk of 11 nm (equivalent to the central hydrophobic hairpin domain) attached to an amphipathic globular structure (equivalent to the NH<sub>2</sub>-terminal amphipathic domain of unknown secondary structure and the COOH-terminal amphipathic  $\alpha$ -helical domain, respectively). Solid lines and circles represent hydrophilic components, and dotted lines denote hydrophobic components. Dark spheres attached to two lines and shaded spheres attached to three lines represent PL and TAG, respectively. (Left) A whole oil body with a quarter cut open. To show the whole organelle in this drawing, the dimension of the oil body in proportion to that of the molecules has been reduced 24 times; the curvature of the surface molecules is much sharper (576 times) than that of a native oil body; the molar proportion of PL to oleosin remains as that in the native oil bodies; and the molar proportion of TAG to PL and oleosin is drastically reduced. (Middle) A small surface section cut perpendicular to the surface, showing two oleosin molecules and two PL molecules. (Right) A surface view of a structural unit consisting of 13 PL molecules and 1 oleosin molecule. To illustrate the structural unit clearly, the adjacent three other oleosin molecules and four PL molecules are also shown. The upper figure is the surface view above the oleosin globe, and the lower figure is the view at level of the PL head group.



residues, and the protruding portion is of unknown secondary structures (19, 27). Without further information, the covering portion and the protrusion portion of the oleosin are tentatively depicted as a spherical structure (Fig. 8). Together, they shield the PL shell such that the PL are not accessible to external phospholipase A2 (13 kD) and phospholipase C (90 kD). The oil body model represents the basic structure in a mature seed, and it does not take into account some minor components. These latter components include diacylglycerols (~4% of TAG, see Fig. 1), the surface TAG and diacylglycerols (a few % of the PL; 9, 15) whose presence are due to molecular partitioning between the hydrophobic core and the hydrophilic exterior, and lipase which is added to the oil body during postgerminative growth (28).

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