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A New Method for Seed Oil Body Purification and Examination of Oil Body Integrity Following Germination[1]

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Plant seeds store triacylglycerols as energy sources for germination and postgerminative growth of seedlings. The triacylglycerols are preserved in small, discrete, intracellular organelles called oil bodies. A new method was developed to purify seed oil bodies. The method included extraction, flotation by centrifugation, detergent washing, ionic elution, treatment with a chaotropic agent, and integrity testing by use of hexane. These processes subsequently removed non-specifically associated or trapped proteins within the oil bodies. Oil bodies purified by this method maintained their integrity and displayed electrostatic repulsion and steric hindrance on their surface. Compared with the previous procedure, this method allowed higher purification of oil bodies, as demonstrated by SDS-PAGE using five species of oilseeds. Oil bodies purified from sesame were further analyzed by two-dimensional gel electrophoresis and revealed two potential oleosin isoforms. The integrity of oil bodies in germinating sesame seedlings was examined by hexane extraction. Our results indicated that consumption of triacylglycerols reduced gradually the total amount of oil bodies in seedlings, whereas no alteration was observed in the integrity of remaining oil bodies. This observation implies that oil bodies in germinating seeds are not degraded simultaneously. It is suggested that glyoxisomes, with the assistance of mitochondria, fuse and digest oil bodies one at a time, while the remaining oil bodies are preserved intact during the whole period of germination.

Key words: oil body, oleosin, organelle integrity, purification, sesame.

Plant seeds store triacylglycerols (TAGs) as energy sources for germination and postgerminative growth of seedlings. The storage TAGs are confined to discrete, spherical organelles called oil bodies, lipid bodies, oleosomes, or spherosomes (*1-3*). An oil body is 0.5 to 2.5 μm in diameter and contains a TAG matrix surrounded by a monolayer of phospholipids and abundant proteins termed oleosins (*4*). Oleosins are alkaline proteins of molecular mass 15 to 26 kDa, depending on the species (*5, 6*). There are at least two isoforms of oleosins present in seed oil bodies (*7, 8*). The organization and biological meanings of the presence of these two isoforms are still unknown.

In addition to oleosins, many minor proteins are present in seed oil bodies of most species obtained by the traditional method of purification (*7, 9*). Whether these minor proteins are real constituents of oil bodies or contaminants from preparation is not clear. To investigate the properties and organization of oil bodies, a method of preparation to yield high purity of these organelles is indispensable. Recently, Millichip *et al.* (*10*) successfully reduced these minor proteins in oil body preparations of sunflower seeds by incubating oil bodies with 9 M urea for 30 min at room temperature. However, no experimental data was provided to inspect the integrity of oil bodies after urea treatment. Urea is a chaotropic agent and classified as a denaturing agent for proteins. Whether the proteinaceous surface (*11*) of oil bodies is denatured or maintained intact after urea incubation requires further investigation.

Oil bodies remain as individual small organelles even after a long period of storage in plant seeds (*12*). This stability is a consequence of the steric hindrance and electronegative repulsion provided by oleosins on the surface of oil (*13*). It has been suggested that the entire surface of an oil body is covered by oleosins (*11*). Therefore, the abundant and compressed oil bodies in the cells of a mature seed would never coalesce or aggregate. It is generally accepted that the physiological significance of maintaining oil bodies as small, individual entitles is to provide sufficient surface area for the attachment of lipase during germination so that the TAGs can be mobilized rapidly (*4, 14, 15*). So far, however, this notion has not been experimentally validated.

In this report, we developed a new method of preparation of oil bodies and examined their purity and integrity. We also inspected the integrity of oil bodies during seed germination. Based on our observation, we revised the current theory of the physiological significance of maintaining oil bodies as small, individual entitles.

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Abbreviations: TAGs, triacylglycerols; TLC, thin layer chromatography.

MATERIALS AND METHODS

Plant Materials—Mature seeds of peanut (*Arachis hypogaea* L.), soybean (*Glycine max* L.), sunflower (*Helianthus annus* L.), and rape (*Brassica campestris* L.) were purchased from local seed stores. Mature seed of sesame (*Sesamum indicum* L., Tainan1) was a gift from the Crop Improvement Department, Tainan District Agricultural Improvement Station. The mature seeds were either used directly (rape and sesame) or soaked in water for 6 h (peanut, soybean, and sunflower) before use.

Electron Microscopy of Mature and Germinating Sesame Seeds—Mature and germinating (3 days in dark at 27°C) sesame seeds were fixed in 2.5% glutaraldehyde in 50 mM sodium phosphate buffer, pH 7.5 for 3 h (*16*). After several rinses with the buffer, they were postfixed in 1% OsO_4 in the buffer overnight. Dehydration was carried out in a graded ethanol series, and the samples were embedded in Spurr's resin. Sections of 75 nm were stained with uranyl acetate and lead citrate, and observed in a Hitachi H-300 electron microscope.

Purification of Oil Bodies—Mature seed was homogenized at 4°C in grinding medium (10 g seed per 50 ml) with a polytron at 8,000 rpm for 40 s. The grinding medium contained 0.6 M sucrose and 10 mM sodium phosphate buffer pH 7.5. The homogenate was filtered through three layers of cheesecloth. After filtration, each 20-ml portion of the homogenate was placed at the bottom of a 50-ml centrifuge tube, and 20 ml of flotation medium (grinding medium containing 0.4 M instead of 0.6 M sucrose) was layered on top. The tube was centrifuged at $10,000 \times g$ for 20 min in a swinging-bucket rotor. The oil bodies on top were collected and resuspended in 40 ml of detergent washing solution containing 0.1% Tween-20, 0.2 M sucrose, and 5 mM sodium phosphate buffer pH 7.5. The resuspension was placed at the bottom of two 50-ml centrifuge tubes (20 ml in each), 20 ml of 10 mM sodium phosphate buffer pH 7.5 was layered on top, and the tubes were centrifuged. The oil bodies on top were collected and resuspended in 40 ml of ionic elution buffer (grinding medium additionally containing 2 M NaCl). The resuspension was placed at the bottom of two 50-ml centrifuge tubes (20 ml in each), 20 ml of floating medium (grinding medium containing 2 M NaCl and 0.25 M instead of 0.6 M sucrose) was layered on top, and the tubes were centrifuged. The oil bodies on top were collected and resuspended in 20 ml of 9 M urea. The resuspension was left on a shaker (60 rpm) at room temperature for 10 min, then placed at the bottom of a 50-ml centrifuge tube, 20 ml of 10 mM sodium phosphate buffer pH 7.5 was layered on top; and the tube was centrifuged. The oil bodies on top were collected and resuspended in 20 ml of grinding medium. The resuspension was mixed with 20 ml of hexane and the tube was centrifuged. After removal of the upper hexane layer, the oil bodies were collected and resuspended in 20 ml of grinding medium. The resuspension was placed at the bottom of a 50-ml centrifuge tube, 20 ml of flotation medium was layered on top, and the tubes were centrifuged. The oil bodies on top were collected and resuspended with grinding medium to give a concentration of about 100 mg lipid/ml.

Solubilization Test of Oil Bodies with Various Detergents—Sesame oil bodies collected from first flotation by centrifugation were used for solubilization tests with various detergents. The detergents applied to solubilize oil bodies were deoxycholic acid, sodium choleate, Tween-20, Triton X-100, and SDS at a final concentration of 1, 0.2, 0.05, or 0.01%. In our analyses, the integrity of oil bodies could be destroyed by SDS at a concentration higher than 0.05% but not any other detergents at the concentrations examined. The two non-ionic detergents (Tween-20 and Triton X-100) revealed a slightly higher capacity to remove contamination from oil bodies than the ionic detergents, deoxycholic acid and sodium choleate (data not shown). Inasmuch as Tween-20 possesses a lower critical micelle concentration (0.059%) than Triton X-100 (0.25%), we selected 0.1% Tween-20 for detergent washing during oil body purification. Under these conditions, Tween-20 molecules formed micelles which had a better potential to solubilize proteins. After detergent washing, Tween-20 micelles broke down into single molecules which were easily removed by extensive dilution when the oil bodies were subjected to further purification.

Determination of Oil Body Recovery and Protein Content—A sample of 50 μl from each step of purification was extracted with 150 μl of diethyl ether. After centrifugation, the upper ether fraction and the lower aqueous fraction were separately saved for determination of oil body recovery and protein content, respectively. Oil body recovery was estimated by TAG content in the samples. The ether fraction of each sample was spotted onto a TLC (thin layer chromatography) plate coated with silica gel and developed in hexane:diethyl ether:acetic acid (80 : 20 : 2, v/v/v). After development and drying, TAG content was visualized by reacting with iodine. A serial dilution (10-90 in 10% steps) of the total extract (step 1) was spotted onto the same TLC plate to simply estimate the relative amounts of TAG in different steps of purification.

The lower equeous fraction was subjected to protein assay. The aqueous fraction of each step was mixed with a reaction reagent containing 2% sodium carbonate, 0.02% sodium tartrate, and 0.01% cupric sulfate for 20 min at room temperature. The mixture was further reacted with Folin & Ciocalteu's Phenol Reagent (Sigma) for 30 min. By reading sample absorption at 500 nm, the protein content was calculated from a linear standard equation derived from the absorption readings of a serial dilution of known BSA concentrations.

Trypsin Digestion of Sesame Oil Bodies—Trypsin (5 μg; bovine pancreas type III) was added to a 2-ml oil body suspension containing 3 mg of lipids in grinding medium (*8*). The reaction mixture was kept at 23°C for 30 min. After trypsin digestion, stability and size of sesame oil bodies were observed under a Nikon type 104 light microscope and then photographed.

Analysis on the Purity of Oil Body Proteins by SDS-PAGE—The oil body proteins obtained from the previous (*7*) and the new methods were resolved by SDS-PAGE (*17*). The sample, at a concentration of 1 mg protein/ml, was mixed with an equal volume of 2×sample buffer according to the suggestion in the Bio-Rad instruction manual, and the mixture was boiled for 5 min. The electrophoresis system consisted of 12.5 and 4.75% polyacrylamide in the separating gel and stacking gel, respectively. After electrophoresis, the gel was stained with Coomassie Blue R-250, and destained.

Two-Dimensional Gel Electrophoresis—Purified sesame oil bodies were further concentrated to 500 mg lipid/ml. Oil bodies of 250 mg of lipids in 0.5 ml of preparation were mixed with 1 ml of diethyl ether and vortexed for 1 min. After centrifugation, the upper ether fraction was discarded. Ether remaining in the water-soluble fraction and the interfacial insoluble layer was extensively evaporated under nitrogen gas. The sample was then mixed with two volumes of Lysis Buffer containing 9.5 M urea, 2% Triton X-100, 2% 3-10 ampholite, and 5% β-mercaptoethanol (*18*). The mixture was boiled for 5 min and centrifuged to collect soluble supernatant as the sample for isoelectrofocusing (first dimensional gel). The isoelectrofocusing apparatus was purchased from Hoefer (GT1 Tube Gel Unit) and employed 15 cm (length) $\times$1.5 mm (inner diameter) cylinder tubes. Tube gels of 4% acrylamide, 2% Triton X-100, 9 M urea, and 0.5% ampholite (2/3 Biolyte 3-10 and 1/3 Biolyte 6-8 from Bio-Rad) were cast for 12 cm height. The gels were prefocused at 200 V for 10 min, 300 V for 15 min, and 400 V for 15 min before loading 20 μl of sample to each tube gel. The loaded sample was electrofocused at 700 V for 15 h. After electrofocusing, the tube gels were ejected into the SDS-PAGE sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 0.004% bromophenol blue, and 10% glycerol, and incubated at room temperature for 30 min. The stained tube gels were mounted onto SDS-PAGE gels (described previously) and subjected to the second dimensional electrophoresis.

Germination of Sesame Seeds—Mature sesame seeds were spread in five trays for germination at 27°C in the dark. One thousand seeds were harvested from one of the trays 1, 2, 2.5, 3, or 4 days after germination. The harvested seeds were weighed and homogenized with 20 ml of grinding medium. Further purification of oil bodies in different stages of germination followed the new method developed in this report.

Integrity Test by Hexane Treatment and Analysis of Oil Content by TLC—The total extracts or freshly purified oil bodies of different germination stages (200 μl each) were treated with 200 μl of hexane to test the integrity of oil bodies, since defective oil bodies which were not entirely covered by oleosins would be susceptible to hexane extraction. After centrifugation, the upper hexane fraction was saved for further analysis and the remaining sample was treated with 300 μl of chloroform : methanol (2 : 1, v/v) for extraction of lipids. The mixture was vortexed for 1 min to enhance the extraction. After centrifugation, the lower chloroform fraction was collected. Both hexane and chloroform fractions from different stages of germination were subjected to lipid analysis by TLC as described earlier in "Determination of Oil Body Recovery and Protein Content."

RESULTS

Mature and Germinating Sesame Oil Bodies under Electron Microscopy—In a mature sesame seed, abundant oil bodies were compressed and crowded together but remained as individual discrete organelles (Fig. 1a). The

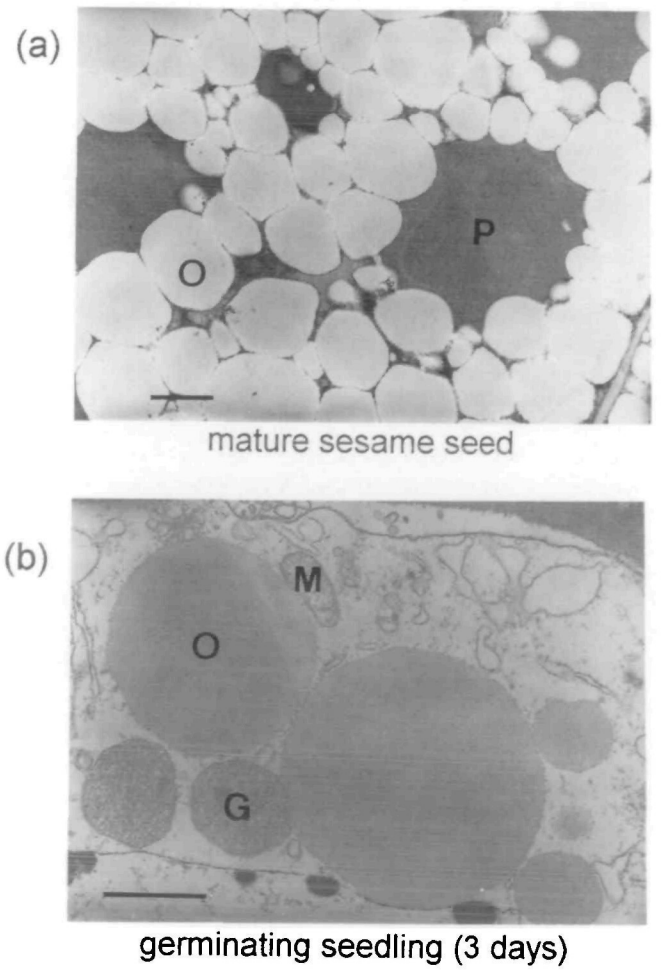

Fig. 1. **Electron microscopy of a mature sesame seed and a germinating seedling.** Samples of a mature sesame seed (a) and a germinating seedling (b) were fixed in 2.5% glutaraldehyde, postfixed in 1% OsO_4, sectioned at 75 nm thickness and photographed under an electron microscope. Bars represent 1 μm. The letter symbols are: O for oil body; P for protein body; G for glyoxisome; M for mitochondrion.

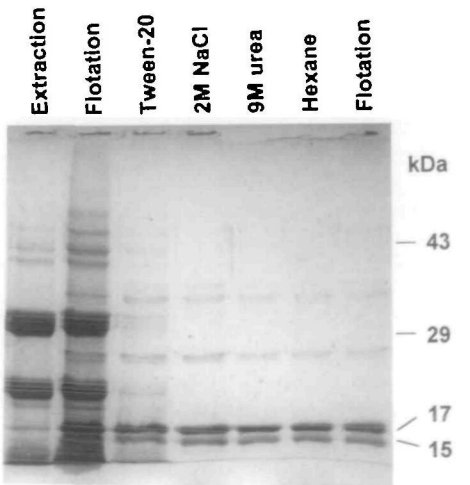

Fig. 2. **SDS-PAGE of proteins of oil bodies at different steps of purification.** To compare the relative purity of oil bodies at different steps of purification, the loaded protein amount of each sample was adjusted to display equal oleosin content, except for the first step (extraction). Labels on the right indicate the molecular masses of proteins.

compression did not lead to coalescence or aggregation of oil bodies within the cell environments. In a germinating sesame seed, oil bodies contacted and/or fused with glyoxisomes and mitochondria (Fig. 1b), similar to the observation reported in a cucumber seedling (*19*). Our observation in the germinating sesame seeds agrees with the current model for TAG degradation connecting the above three organelles *via* β-oxidation and the glyoxylate cycle in glyoxisomes (*20*).

Purification of Oil Bodies—Proteins non-specifically associated with or trapped within oil bodies were subsequently removed by flotation, detergent washing, ionic elution, and urea treatment (Fig. 2). TAGs in defective or broken oil bodies were removed by hexane extraction. The oil body recovery and protein content are recorded in Table I for each step. In our preparation, 60% of oil bodies were recovered at the end of purification. In the mature seeds of sesame (Tainan1), oil body proteins represent approxi-

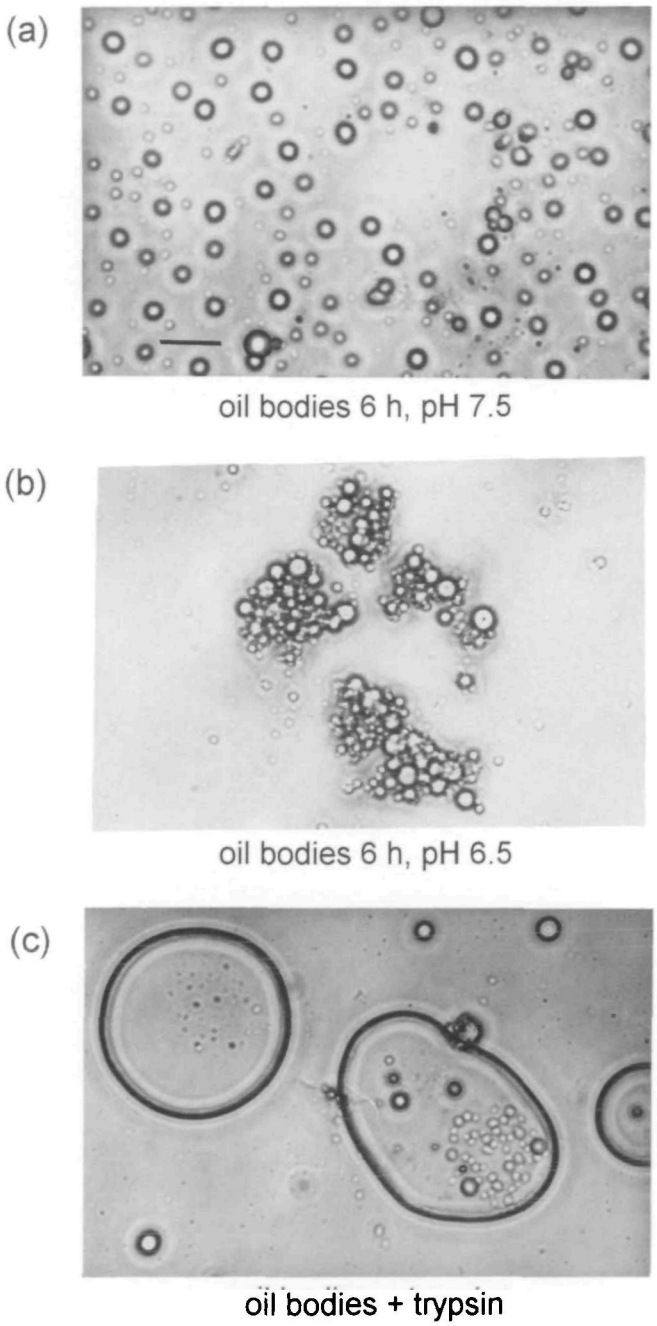

Fig. 3. **Light microscopy of purified sesame oil bodies after different treatments.** An oil body preparation of 3 mg of lipids was suspended in a medium containing (a) 0.6 M sucrose and 0.1 M potassium phosphate buffer pH 7.5, (b) 0.6 M sucrose and 0.1 M potassium phosphate buffer pH 6.5, and (c) 5 μg trypsin, 0.6 M sucrose and 0.1 M potassium phosphate buffer pH 7.5. Preparations (a) and (b) were left at 23°C for 6 h, while preparation (c) was digested with trypsin at 23°C for 30 min before taking photos. All the three photos are of the same magnification. Bar represents 5 μm.

TABLE I. **Purification of seed oil bodies.**

Step	Process	Oil body recovery[a] (%)	Total protein[b] (mg)
Extraction	Homogenization (20 g of mature sesame seed)	100	940
Flotation	Centrifugation on a two-layer density gradient	90	514
Detergent washing	0.1% Tween-20 at 4°C	85	115
Ionic elution	2 M NaCl at 4°C	70	50.0
Chaotropic treatment	9 M urea for 10 min at room temperature	60	37.7
Integrity test	Equal amount of hexane at room temperature	ND	ND
Resuspension (flotation)	Centrifugation to remove remaining hexane	60	35.6

[a]Estimated by TAG content. [b]Determined by protein assay. ND: not determined.

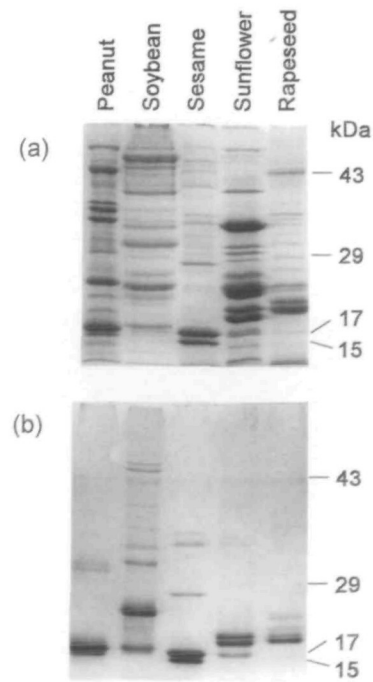

Fig. 4. **SDS-PAGE of proteins of oil bodies from various oilseeds purified by the previous and the new methods.** Proteins extracted from oil bodies purified by the previous method (a) and the new method (b) were resolved by 12.5% SDS-PAGE. The loaded protein amount of each sample was adjusted to show comparable oleosin content in both methods. Labels on the right indicate the molecular masses of proteins.

mately 6.3% of total proteins, which comprise around 4.7% of seed weight. In addition to the two potential oleosin isoforms of 17 and 15 kDa, three minor protein bands of 38, 36, and 27 kDa were consistently present in our preparations of sesame oil bodies. These three proteins seem to be oil body constituents that are embedded in the surface of oil bodies.

No Observable Alteration in the Surface Properties of Purified Oil Bodies—Oil bodies are known to maintain their integrity by steric hindrance and electrostatic repulsion on the surface of the organelles (*13*). It appeared that oil bodies purified by the new method also possessed this integrity and remained as small, discrete entities in the medium of pH 7.5 (Fig. 3a). Aggregation of oil bodies occurred as a result of attenuation of electrostatic repulsion by lowering pH of the medium from 7.5 to 6.5 (Fig. 3b). But aggregated oil bodies did not coalescence, presumably due to the steric hindrance of surface oleosins. Coalescence of oil bodies could be induced by trypsin digestion, which eliminates steric hindrance by destroying surface portions of oleosins (Fig. 3c). In the trypsin treatment, oil bodies floated rapidly (visible within 1 min) to the top of the solution. The milky oil bodies coalesced and formed a transparent layer on top. These results were consistent with those obtained with oil bodies purified by the previous method. We did not observe any alteration in the surface properties of oil bodies purified by this new method in terms of steric hindrance and electrostatic repulsion.

Comparison of Oil Body Proteins between Previous and New Preparations—Sesame, peanut, soybean, sunflower seed, and rapeseed, which are commonly used for oil consumption, were subjected to oil body preparation by the previous and the new methods. The proteins of oil bodies in these preparations were resolved by SDS-PAGE (Fig. 4). The potential oleosins of low molecular weight were present in both preparations, whereas most proteins of high molecular weight that were present when the previous method was used were absent when the new method was used. The new method thus showed a substantial improvement in the removal of non-specifically associated proteins from the oil bodies. The integrity of oil bodies from these oilseeds was also confirmed by the examination of their surface properties (steric hindrance and electrostatic repulsion) as described in the preceding paragraph (data not shown).

Two Potential Oleosin Isoforms Present in Sesame Oil Bodies—Proteins extracted from sesame oil bodies were further analyzed by two-dimensional gel electrophoresis. Two protein spots representing two potential oleosin isoforms were found in the basic pH range of the electrofocusing (Fig. 5). This observation was in accord with the alkaline property of oleosins based on the analysis of amino acid sequences deduced from the corresponding nucleotide sequences of known oleosin genes (*21*). The simplicity of oleosin isoforms in sesame oil bodies renders the organelle a model system for the investigation of oleosin isoforms in dicot species.

Integrity of Oil Bodies Remaining in Germinating Sesame Seedlings—The integrity of oil bodies in germinating sesame seedlings was examined by hexane extraction. Oil bodies present in the crude extract (step 1 in Table I) or freshly purified from the different stages of germination were resistant to hexane extraction (no detectable TAG in hexane extract analyzed by TLC; data not shown). The TAG content of those purified oil bodies after hexane extraction was extracted with chloroform : methanol (2 : 1, v/v) and analyzed in a TLC plate (Fig. 6a). Our analyses indicated that more than 85% of TAG was depleted within 4 days of germination, while the remaining oil bodies

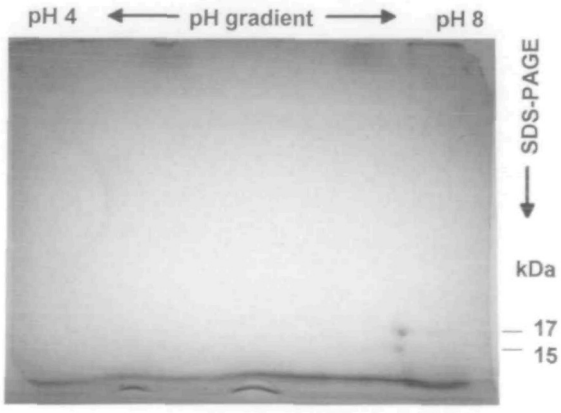

Fig. 5. **Two-dimensional gel electrophoresis of proteins of sesame oil bodies.** The first dimensional gel (isoelectrofocusing) resulted in a pH gradient horizontally ranging from pH 4 to 8. The second dimensional gel (SDS-PAGE) was performed vertically under similar conditions to those used in the SDS-PAGE in Fig. 2. The molecular masses of the two potential oleosins (17 and 15 kDa) are marked on the right.

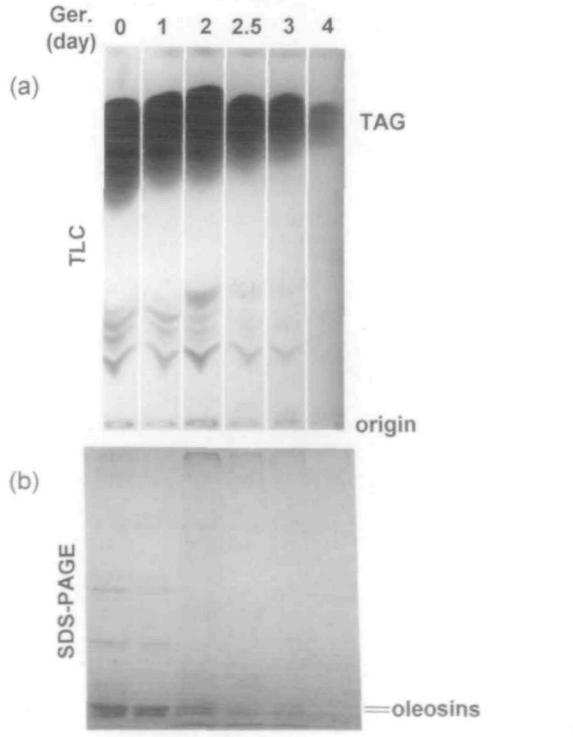

Fig. 6. **TAG and protein contents of purified oil bodies in different stages of germination.** Oil (TAG) and oleosin (protein) contents of oil bodies in different stages of germination were analyzed by TLC (a) and SDS-PAGE (b), respectively. The origin before solvent development and the TAG position after solvent development are labeled on the right of the TLC. The two potential oleosins are labeled with a double line on the right of the SDS-PAGE.

continued to maintain their integrity. The integrity was also confirmed by examination of the surface properties (steric hindrance and electrostatic repulsion) of oil bodies as described previously, including low pH aggregation and trypsin-induced coalescence (data not shown).

Oleosins in the oil bodies from different stages of germination remained intact, as revealed by SDS-PAGE (Fig. 6b). The intactness of oleosins was consistent with the integrity of remaining oil bodies, since the surface properties of oil bodies are essentially provided by oleosins (*13*). Roughly estimated from a comparison with a serial dilution (10-90% in 10% steps) of mature seed oil bodies, the ratio of TAG content to protein (mainly oleosins) content remained constant during the germination (data not shown). The constant ratio of TAG/oleosins, together with the maintenance of oil body integrity, implied that the remaining oil bodies remained unaltered during germination.

DISCUSSION

Traditionally, detergents, chaotropic agents, and organic solvents are considered as harsh chemicals to biomolecules and may induce denaturation of the biosystems they constitute. In this newly developed method, we took advantage of the stable surface organization of seed oil bodies and purified these organelles using relatively strong conditions to wash their surface briefly. The high purity of oil bodies obtained by this new method provides a better source for the investigation on the organization of the organelle. It is likely that the proteins obtained from oil bodies purified by this new method are integral proteins embedded in the organelles. Therefore, we should not eliminate the possibility that some non-covalently bonded proteins peripherally associated with oil bodies may be washed away in these harsh conditions of our purification.

To date, seed oil bodies have been considered as storage organelles destined exclusively to supply energy for germination and postgerminative growth of seedlings (*22*). No other function has been ascribed to this tissue-specific organelle. According to the SDS-PAGE analysis, oil bodies purified by the new method were composed of only a few polypeptides, and potential oleosins represented 80-90% of the proteins. This simple organization is consistent with the single function of the organelle. To our knowledge, oil bodies represent the simplest assembly among all known organelles.

In addition to the structural proteins, oleosins, which give rise to steric hindrance and electrostatic repulsion on the surface of the organelle, the minor proteins present on oil bodies may participate in other biological functions related to seed oil biosynthesis or degradation. These may include ER membrane proteins (enzymes), glyoxisome receptor, inactive lipase, or lipase receptor. Of course, some of these functions may be carried out by the oleosin isoforms or peripherally associated proteins washed away during our purification.

Seed oil bodies are stable both inside the cells and in the purified form. It is generally accepted that the physiological significance of maintaining the oil bodies as small individual entities is to provide ample surface area for the attachment of lipase during germination so that the reserve TAG can be mobilized rapidly (*14*). In this case, most oil bodies should be utilized at the same time (to contact most of the surface simultaneously) during germination. This rationale may be correct if the utilization of oil bodies is simply executed by an enzyme, lipase. However, the degradation of oil bodies seems to be completed by an organelle, glyoxisome, with the assistance of mitochondria. Indeed, our observation of the mobilization of sesame oil bodies was at variance with the generally accepted rationale, since oil bodies were mobilized one after another *via* β-oxidation and the glyoxylate cycle in glyoxisomes. Actually, we should not expect an excess amount of glyoxisomes to be synthesized and to mobilize oil bodies simultaneously during germination. A relatively small amount of glyoxisomes should be sufficient for the mobilization of oil bodies in a manner similar to enzyme-substrate relationship. Inasmuch as the germination process commonly takes several days and the degraded oil bodies cannot be kept stable for several days, we propose that the physiological significance of maintaining the oil bodies as small individual entities is to retain the integrity of remaining oil bodies such that energy supply can be distributed to each stage of germination.

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