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Characterization of Seed Oil Bodies and Their Surface Oleosin Isoforms from Rice Embryos[1]

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Plant seeds store triacylglycerols in discrete organelles called oil bodies. An oil body stores a matrix of triacylglycerols surrounded by phospholipids and alkaline proteins termed oleosins. Oil bodies in rice seeds are present in embryos and aleurone layers. They do not coalesce in crowded environments, as observed on electron microscopy. The detected isoelectric point of purified rice oil bodies is pH 6.2. This implies that rice oil bodies possess a negatively charged surface at neutral pH. The suspension of rice oil bodies in pH 6.5 buffer induces aggregation. Presumably, the negatively charged surface causes electrostatic repulsion that maintains rice oil bodies as discrete organelles. Rice oil bodies lose their integrity on trypsin treatment. Undoubtedly, oleosins play an important role in the stability of oil bodies. There are two oleosin isoforms in rice oil bodies. Antibodies raised against these two homologous isoforms do not cross-recognize each other. Both isoforms are restricted to oil bodies, as detected on immuno-assaying. Partial amino acid sequences of these two isoforms were obtained, and compared with the deduced sequences of two maize and two rice oleosin genes. The comparison confirmed that the two major proteins in rice oil bodies are the two oleosin isoforms.

Key words: isoform, oil body, oleosin, rice, structure-function.

Vegetable cooking oils used for human consumption comprise triacylglycerols (TAGs) extracted from various plant seeds. Plant seeds store TAGs as energy sources for the germination and postgerminative growth of seedlings. The TAGs are stored in small discrete intracellular organelles called oil bodies (*1-4*). 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 (*5*). Oleosins are alkaline proteins with molecular masses of 15 to 26 kDa, depending on the species (*6*). The amino acid sequences of oleosins from several plant species have been determined from the corresponding cDNA and genomic DNA, and by direct amino acid sequencing (*7*).

Among all known eukaryotic organelles, the plant seed oil body possesses the simplest organization. This character allows oil bodies to serve as a model system for the investigation of organelles. Similar or equivalent organelles are present in the pollen of angiosperms (*8*) as well as in the tissues of more primitive plants, such as the megagametophytes of gymnosperms (*9*), and the spores of ferns (*10*). Other lipid-storage organelles of similar structure or function are present in nonplant species, such as brown adipose tissue of mammals (*11*), eggs of some nematodes (*12*), yeast (*13*), *Euglena* (*14*), and algae (*15*). Similar structural organization is also found in mammalian lipoproteins, the vesicles for transportation of TAGs and cholesterol esters in the blood (*16*).

Oil bodies are maintained as individual small organelles even after a long period of storage in plant seeds. This stability is a consequence of the steric hindrance and electronegative repulsion caused by oleosins on the surface of oil bodies (*17*). An oleosin molecule is proposed to comprise three distinct structural domains: an N-terminal amphipathic domain, a central hydrophobic antiparallel β-strand domain, and a C-terminal amphipathic α-helical domain. The proposed secondary structures of oleosins are in agreement with the results of spectrum analyses (*18*). The central hydrophobic domain of oleosins is highly conserved among diverse species. The possible structural role of the central hydrophobic domain is to anchor the oleosins on the surface of oil bodies. It has been suggested that the entire surface of an oil body is covered by oleosins (*19*). Therefore, the profound and compressed oil bodies in the cells of mature seeds would never coalesce or aggregate. The advantage of maintaining oil bodies as small individual particles is to provide a sufficient surface area, such that the TAGs can be mobilized rapidly as an energy supply.

In this study, we examined rice oil bodies by electron microscopy, purified oil bodies from rice embryos, and, furthermore, purified the two oleosin isoforms to homogeneity from these oil bodies, respectively. Antibodies against each isoform were raised in chickens. The surface properties of rice oil bodies were examined. The presence of these two oleosin isoforms in the oil bodies was confirmed by their subcellular location, and sequence comparison with maize oleosins and rice cDNA clones.

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Abbreviation: TAGs, triacylglycerols.

EXPERIMENTAL PROCEDURES

Plant Materials—Mature seeds of rice (*Oryza sativa*) and maize (*Zea mays*) were purchased from local seed stores. Embryos of mature seeds were freshly used (maize) or soaked in water for 2 h (rice) before use. After dissection from seeds, embryos were collected at 4°C and subjected to oil body preparation immediately.

Preparation of Oil Bodies—The tissue was homogenized at 4°C in a grinding medium (5 g tissue per 20 ml) with a Polytron at 7,000 rpm for 40 s. The grinding medium comprised 0.6 M sucrose and 0.01 M sodium phosphate buffer, pH 7.5. The homogenate was filtered through three layers of cheese cloth. After filtration, a 25-ml portion of the homogenate was placed at the bottom of a 50-ml centrifuge tube, and then 25 ml of a flotation medium (the grinding medium containing 0.4 instead of 0.6 M sucrose) was layered on top. The tube was centrifuged at 10,000 × *g* for 20 min in a swinging-bucket rotor. The oil bodies on top were collected and resuspended in 25 ml of the grinding medium containing an additional 2 M NaCl. The suspension was then placed at the bottom of a 50-ml centrifuge tube, 25 ml of a floating medium (the grinding medium containing 2 M NaCl and 0.25 M instead of 0.6 M sucrose) was layered on top, and then the tube was centrifuged. The oil bodies on top were collected and resuspended in 25 ml of grinding medium. The suspension was then placed at the bottom of a 50-ml centrifuge tube, 25 ml of a floating medium (the grinding medium containing 0.4 instead of 0.6 M sucrose) was layered on top, and then the tube was centrifuged. This last flotation wash was repeated once. The oil bodies on top were collected and resuspended in the grinding medium to give a concentration of about 100 mg lipid/ml.

Electron Microscopy of Oil Bodies—A rice embryo or embryo axis was fixed in 2.5% glutaraldehyde in 50 mM sodium phosphate buffer, pH 7.5, for 3 h. After several rinses with the buffer, it was postfixed in 1% OsO_4 in the buffer overnight. Dehydration was carried out in a graded ethanol series, and the sample was embedded in Spurr's resin. Sections of 75 nm were stained with uranyl acetate and lead citrate, and observed under a Nikon type 104 light microscope and a Hitachi H-300 electron microscope.

Isoelectrofocusing of Rice Oil Bodies—This was performed in a Bio-Rad Rotofor Cell. The horizontal cylindrical focusing cell of 55 ml was divided vertically into 20 chambers by partitions in order to minimize diffusion during electrophoresis and disturbance of the gradient during fractionation. Each partition was made of a monofilament polyester screen of 6 μm × 6 μm pore size. During electrofocusing, the Rotofor Cell was rotated at 1 rpm, which prevented the flotation of oil bodies. Fractionation of the contents of each chamber was achieved simultaneously within 1 s with a vacuum harvesting system. The pH gradient, made from 1% Ampholyte (0.5% Bio-Lyte 6-8 and 0.5% Bio-Lyte 3-10; both from Bio-Rad), was preformed by applying an electric field of constant power (12 W) and 400-500 V for 10 min. Isolated rice oil bodies (about 20 mg lipid) in 200 μl of a 10 mM KCl suspension were applied to the gradient at the high pH end (approximately pH 8) of the chamber. An electric field of the same power (12 W) was applied for 20 min to focus the oil bodies. Movement of the oil bodies was observed visually. After fractionation, the oil bodies and the pH along the gradient were detected at 600 nm with a spectrophotometer and a pH meter, respectively.

Trypsin Digestion of Rice Oil Bodies—Trypsin (4 μg; bovine pancreas type III) was added to a 2-ml oil body suspension containing 3 mg lipids in the grinding medium. The reaction mixture was kept at 23°C for 30 min. After trypsin digestion, the stability and size of rice oil bodies were examined by light microscopy and then photography.

Purification of Oleosins by SDS-PAGE—The proteins in the oil body preparation were resolved by SDS-PAGE (*20*). A 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 then the mixture was boiled for 5 min. The electrophoresis system consisted of 12.5 and 4.75% polyacrylamide in the separating and stacking gels, respectively. After electrophoresis, the gel was stained with Coomassie Blue R-250, and then destained.

In the purification of oleosins from rice for antibody preparation, the oil body sample was subjected to SDS-PAGE as described in the preceding paragraph, but without the use of gel combs. After Coomassie Blue staining, two major bands (oleosins) of 18 and 16 kDa were cut from the gel, respectively. These two oleosin gel slices were separately homogenized in an extraction buffer (0.125 M Tris-HCl, pH 8.0, 1 mM EDTA, 0.1% SDS) using a mortar and pestle. The homogenate was centrifuged at 10,000 × *g* for 10 min. The supernatant was retained, and the pellet was re-extracted with the extraction buffer. The re-extraction was performed two more times. The oleosin in the combined supernatants was precipitated with an equal amount of acetone pre-chilled at −20°C. The acetone mixture was kept at −20°C for 30 min and then centrifuged at 10,000 × *g* for 30 min to obtain a pellet. The pellet was dissolved in extraction buffer to a concentration of 1 mg/ml.

Preparation of Antibodies—Antibodies against each rice oleosin (18 or 16 kDa) were raised in two chickens. Pre-immune eggs were taken from the chickens a week before antigen injections, and were used as pre-immune blotting controls. Hens were kept in cages individually for immunization and egg production. The antigen (oleosin in a solution of 1 mg/ml) was mixed with an equal volume of complete Freund's adjuvant. The antigen mixture (1.0 ml) was injected into the thigh muscle of each chicken. Booster injections of equal amounts of the antigen were given 10 and 20 days after the first injection, except for the use of incomplete Freund's adjuvant instead of complete Freund's adjuvant. A week after the second booster injection, eggs were collected daily. Immunoglobulins were purified from the egg yolks (*21*), aliquoted, and stored at −80°C in the presence of 0.1% sodium azide.

Western Blotting—The proteins in the SDS-PAGE gel were transferred to a PVDF membrane for 2 h at 0.25 A in a Bio-Rad Trans-Blot system. The transfer buffer comprised 25 mM Tris, 192 mM glycine (pH 8.3), and 20% methanol. The membrane was blocked with 3% gelatin in Tris-buffered saline (TBS) containing 10 mM Tris-HCl, pH 7.5, and 150 mM NaCl for 30 min. It was then incubated with the antibody-containing immunoglobulin or pre-immune-immunoglobulin (control) diluted in TBS containing 1% gelatin at room temperature for 2 h. The membrane was rinsed with distilled water and then washed twice (10 min

each) in TBS containing 0.05% Tween-20 before the addition of the peroxidase-conjugated goat anti-chicken IgG in TBS containing 1% gelatin. After 1 h incubation, the membrane was briefly rinsed with a large volume of water, and then washed twice (10 min each) in TBS containing 0.05% Tween-20. It was then incubated with 4-chloro-1-naphthol containing H_2O_2 for color development (*22*).

Fractionation of the Extract of Rice Embryos—Following the preparation of oil bodies, the rice embryo extract was separated into three fractions (oil bodies, supernatant, and pellet) by the first centrifugation. The oil bodies were subjected to further purification as described above. The supernatant was centrifuged at 100,000×*g* for 90 min to yield a 100,000×*g* supernatant and a 100,000×*g* pellet.

Partial Amino Acid Sequencing of Rice Oleosins—Each pure rice oleosin (18 or 16 kDa) eluted from the gel was subjected to trypsin digestion. In the reaction mixture, each oleosin, 20 μg, was digested with 5 μg trypsin at 37°C for 30 min in a buffer, 50 mM Tris-HCl, pH 7.5. After digestion, the reaction mixture was mixed with an equal volume of 2× SDS-PAGE sample buffer, and then boiled for 5 min. The hydrolysis products were resolved in a SDS-PAGE gel, with 15% and 4.75% polyacrylamide in the separating and stacking gels, respectively. The electrophoresis system was similar to that for the SDS-PAGE described previously except for the addition of 0.1 mM sodium thioglycolate to the gel and running buffer. After electrophoresis, polypeptide fragments were transferred to a PVDF membrane at a current of 0.5 A for 30 min at 4°C in the blotting buffer, *i.e.* 10% methanol and 10 mM Caps-NaOH, pH 11. After blotting, the PVDF membrane was stained with Coomassie Blue for 5 min, destained for 5 min, rinsed with water three times, and then left to dry in the air. The major stained band was chosen for sequencing from the N-terminus with an Applied Biosystems 476A Protein Sequencer at Chung-Hsing University, Taiwan.

RESULTS

Examination of Oil Bodies in Rice Seeds—In the mature rice seed, oil bodies are present in both the embryos and aleurone layers. In our preparation, oil bodies extracted from rice embryos formed a compact layer on top on centrifugation. Rice oil bodies were present in both the embryos and embryo axis (Fig. 1). These oil bodies were present in discrete organelles of regular size, even in the compressed environment of mature seeds, as observed on electron microscopy (Fig. 2).

Isoelectrofocusing of Rice Oil Bodies—In order to determine the isoelectric point, isolated rice oil bodies were applied to a horizontal Bio-Rad Rotofor Cell. The pH focusing chamber of the Rotofor Cell contained vertical partitions along the pH gradient to reduce diffusion during isoelectrofocusing and disturbance during fractionation. A pH gradient of 3 to 10 was preformed before loading oil bodies. Isolated oil bodies suspended in 10 mM KCl buffer were applied to the pH 8 area of the preformed gradient, and then moved toward their isoelectric point under an electric field. After electrofocusing, oil bodies in the pH gradient were fractionated into 20 tubes of about the same volume. The pH value and OD reading at 600 nm (an index of the distribution of oil bodies) in each tube were determined and plotted (Fig. 3). Most oil bodies moved toward the lower pH area in the gradient and stopped at about pH 6.2. Apparently, rice oil bodies maintain a negatively charged surface at neutral or higher pH, and the isoelectric point of this oil-storage organelle is pH 6.2. It is supposed that the negatively charged surface results in electrostatic repulsion that maintains rice oil bodies as discrete organelles.

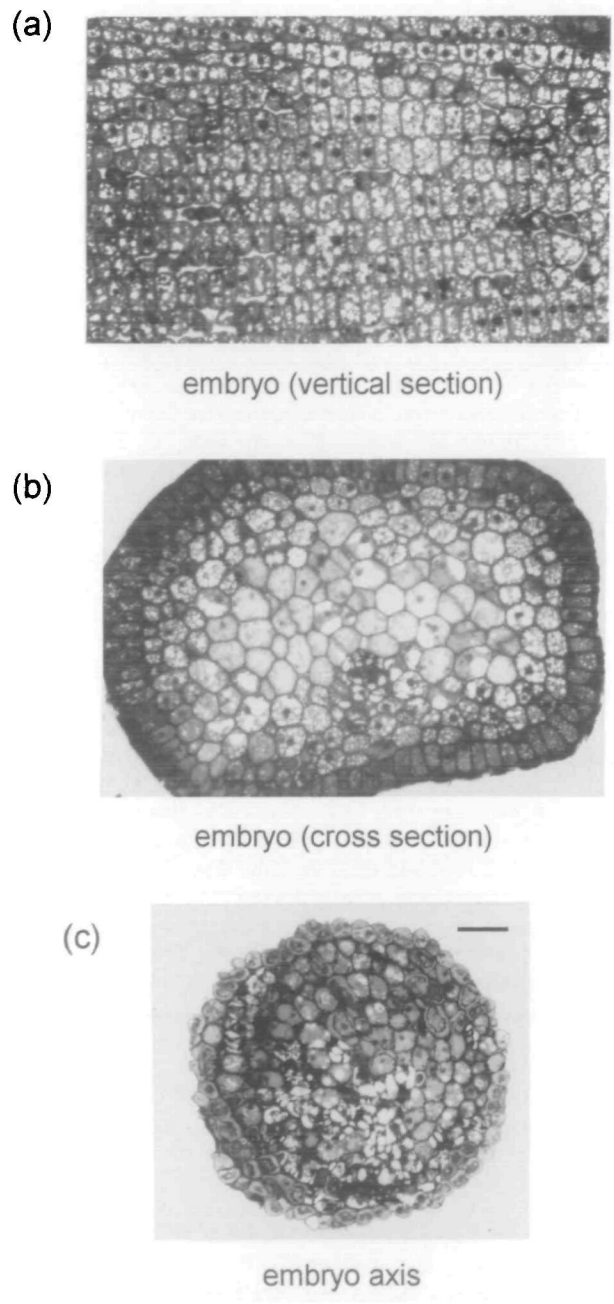

Fig. 1. **Light microscopy of a rice embryo and an embryo axis.** A mature rice embryo was fixed in 2.5% glutaraldehyde and postfixed in 1% OsO_4. A vertical section of the embryo (a), a cross section of the embryo (b), and a cross section of the embryo axis (c) were examined and photographed through a light microscope. The light transparent spherical particles are oil bodies. More detailed photos obtained on electron microscopy are shown in Fig. 2. All three photos are of the same magnification. Bar, 20 μm.

Aggregation and Coalescence of Rice Oil Bodies—Puri-

fied rice oil bodies were maintained as stable individual particles in a medium of pH 7.5 at 23°C for several days (data not shown). Aggregation of rice oil bodies could be induced by lowering the pH of the medium from 7.5 to 6.5 (Fig. 4). Since the isoelectric point of rice oil bodies is pH 6.2, the aggregation was probably a consequence of attenuation of the electronegative repulsion on the surface of these organelles. Interestingly, these aggregated oil bodies did not coalesce in a medium of pH 6.5, even after the preparation had been left at 23°C for 12 h (Fig. 4b). The lack of coalescence was presumably due to the steric hindrance caused by the protrusive N-terminal and C-terminal domains of oleosins on the surface of oil bodies, since removal of these two surface domains by trypsin digestion led to the coalescence of oil bodies (see the next paragraph).

The coalescence of rice oil bodies could be induced by trypsin digestion in the medium of pH 7.5 (Fig. 4c). On trypsin treatment, rice 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 floating oil bodies were much larger than the native ones in diameter. According to our analyses of the two deduced oleosin sequences, the junction between each of the two surface domains (N- or C-terminal domain) and the central hydrophobic domain of oleosins possesses positively charged residues (Arg and/or Lys residues). It is likely that trypsin cleaved both the N-terminal and C-terminal domains, abolished the steric hindrance on the surface of oil bodies, and thus induced coalescence. Obviously, rice oleosins play an important structural role in the stability of rice oil bodies.

Non-Cross Recognition of Antibodies against Two Rice Oleosins—The two rice oleosin isoforms (18 and 16 kDa) were separated from an oil body preparation by SDS-PAGE. The purity of each oleosin preparation was examined by SDS-PAGE (Fig. 5a) before injection into chickens for antibody production. Antibodies against oleosins 18 and 16 kDa were purified from yolks, individually. After Western blotting, the purified oleosins and proteins extracted from oil bodies were resolved by SDS-PAGE and then transferred to a PVDF membrane, which was further incubated with antibodies against oleosins and then with secondary antibodies against chicken IgG before color development. Under the conditions of our experiments, antibodies raised against rice oleosin 16 kDa could recognize the original antigen as well as the corresponding

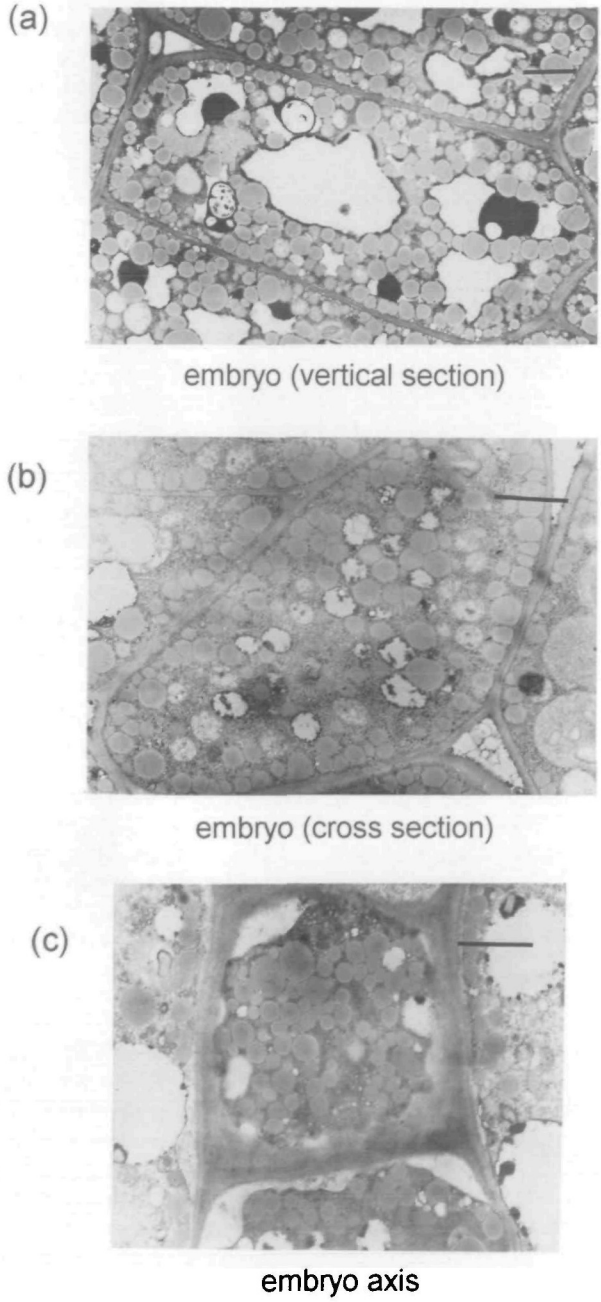

Fig. 2. **Electron microscopy of a rice embryo and an embryo axis.** The same samples as in Fig. 1 were sectioned at 75 nm thickness and used for photography under an electron microscope. The most abundant gray spherical particles are oil bodies. Bars, 2 μm.

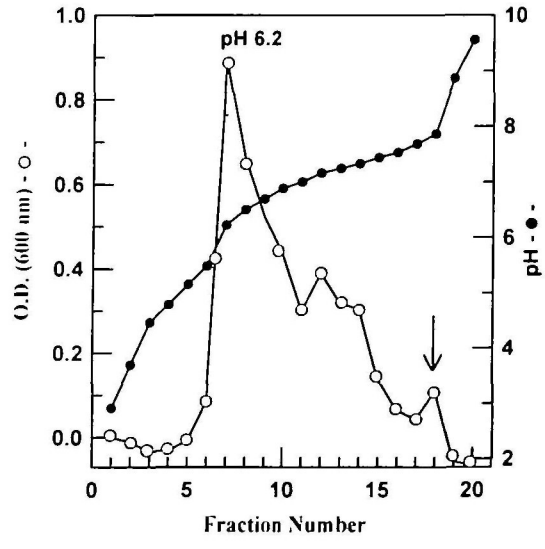

Fig. 3. **Isoelectrofocusing of rice oil bodies.** Isoelectrofocusing was performed in a horizontal Bio-Rad Rotofor Cell. The pH gradient was preformed before loading rice oil bodies into the chamber. Rice oil bodies were loaded at the pH 8 region, as indicated by the arrow. After electrofocusing, the rice oil bodies in the pH gradient were fractionated into 20 tubes. The distribution of oil bodies detected at OD 600 nm (open circles) and the pH gradient (close circles) were recorded for each tube.

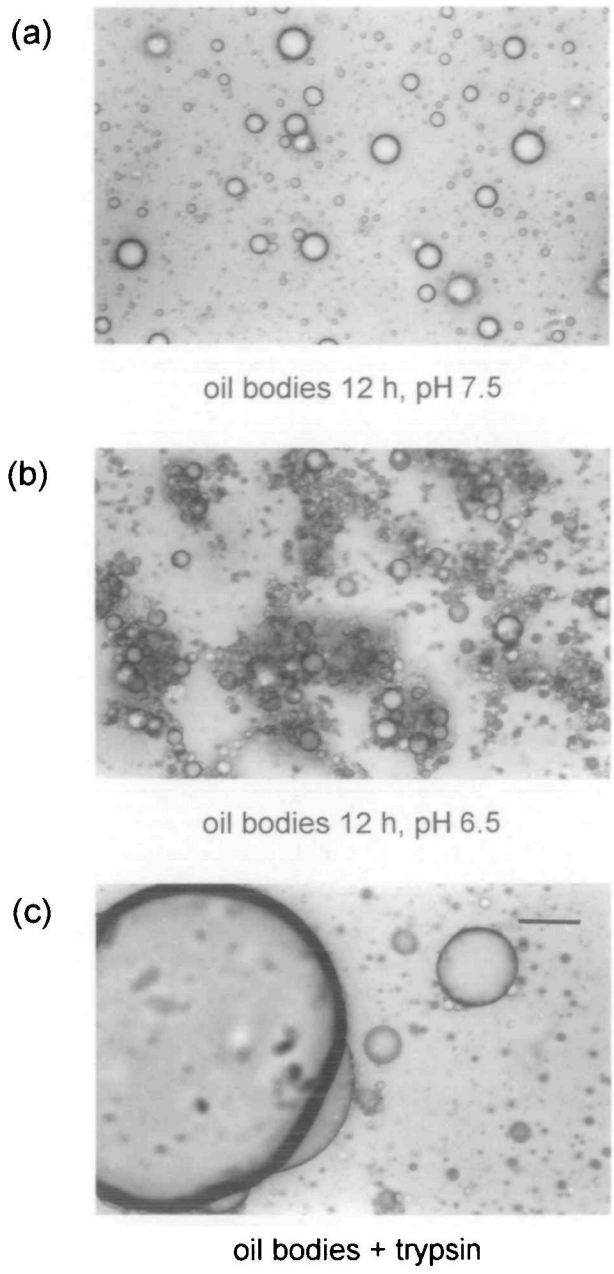

Fig. 4. Light microscopy of rice oil bodies after different treatments. An oil body preparation containing 3 mg lipids was suspended in a medium containing (a) 0.6 M sucrose and 0.1 M potassium phosphate buffer, pH 7.5, (b) 0.6 M sucrose and 0.1 M potassium phosphate buffer, pH 6.5, or (c) 4 μg trypsin, 0.6 M sucrose, and 0.1 M potassium phosphate buffer, pH 7.5. Preparations (a) and (b) were left at 23°C for 12 h, while preparation (c) was digested with trypsin at 23°C for 30 min before taking photos. All three photos are of the same magnification. Bar, 20 μm.

protein band (oleosin 16 kDa) of the oil body preparation, but did not recognize rice 18 kDa oleosin (Fig. 5b). In a similar manner, antibodies against rice oleosin 18 kDa could only recognize their antigen, not rice oleosin 16 kDa (Fig. 5c).

The maize oil bodies we prepared gave two putative oleosin bands on SDS-PAGE (Fig. 5a). The estimated molecular weights of these two maize oleosins were 16.5 and 18.5 kDa. In the following text, we denote these two maize oleosins as maize oleosin 16 kDa and maize oleosin 18 kDa, respectively. Under the conditions of our experiments, antibodies against rice 16 kDa did not cross-recognize maize oleosins (Fig. 5b), while antibodies against rice 18 kDa cross-recognized maize oleosin 18 kDa strongly but not maize oleosin 16 kDa (Fig. 5c).

Unique Localization of Oleosins in Oil Bodies—The separation of a rice seed extract into four fractions (supernatant, oil bodies, 10,000 × *g* ppt, and 100,000 × *g* ppt) was achieved by 10,000 × *g* centrifugation of a seed extract, followed by 100,000 × *g* centrifugation of the supernatant obtained. These four fractions represented the water soluble content, oil bodies, cell debris, and microsomes, successively. The proteins in these four fractions were resolved on SDS-PAGE (Fig. 6a). Apparently, most proteins were located in the supernatant fraction, and the two oleosins were predominantly present in the oil body fraction. The location of the two oleosins in these four fractions was further determined by immunoassaying with antibodies raised against rice oleosin 16 kDa (Fig. 6b) and ones raised against rice oleosin 18 kDa (Fig. 6c), individually. The results confirmed that both oleosins were exclusively present in the oil body fraction.

Partial Amino Acid Sequences of Rice Oleosins—For amino acid sequencing, both rice oleosins were resolved by SDS-PAGE and then transferred to a PVDF membrane. The N-terminus of each rice oleosin seemed to be blocked since sequencing could not be performed when the purified oleosins were used directly. In order to obtain partial sequences, the purified rice oleosin 16 and 18 kDa were partially digested with trypsin, respectively. The digested fragments of oleosins were resolved by SDS-PAGE and then transferred to a PVDF membrane for amino acid sequencing. A sequence of 22 residues homologous to a segment at the N-terminal domain of maize 16 kDa (*23*) was obtained for one digested fragment of rice oleosin 16 kDa (Fig. 7a). Comparison with the deduced sequence for the rice oleosin 16 kDa cDNA clone (GenBank U43930) also confirmed that the digested 16 kDa protein is an oleosin. A sequence of 19 residues was obtained for one digested fragment of rice oleosin 18 kDa (Fig. 7b). This sequence is almost identical (18 out of 19 residues) to a segment at the C-terminal domain of maize 18 kDa (*24*), and 100% identical to the deduced sequence for the rice oleosin 18 kDa cDNA clone (GenBank U43931). This high homology implies that the digested 18 kDa protein is also an oleosin.

DISCUSSION

Rice oil bodies are negatively charged at near their physiological pH (pH 7.2–7.5). Presumably, the electrostatic repulsion on the surface of oil bodies maintains them as discrete organelles. Aggregation of oil bodies could be induced when the oil bodies were suspended in a medium of a pH slightly lower than 7. This phenomenon probably resulted from the attenuation of the surface repulsion due to protonation of amino acid residues of oleosins at lower pH. It also implied that the electrostatic repulsion on the surface of oil bodies was close to the minimum force for keeping these organelles apart from each other. Moreover, this electronegative repulsion on the surface of oil bodies agrees with the general concept that the surface of mem-

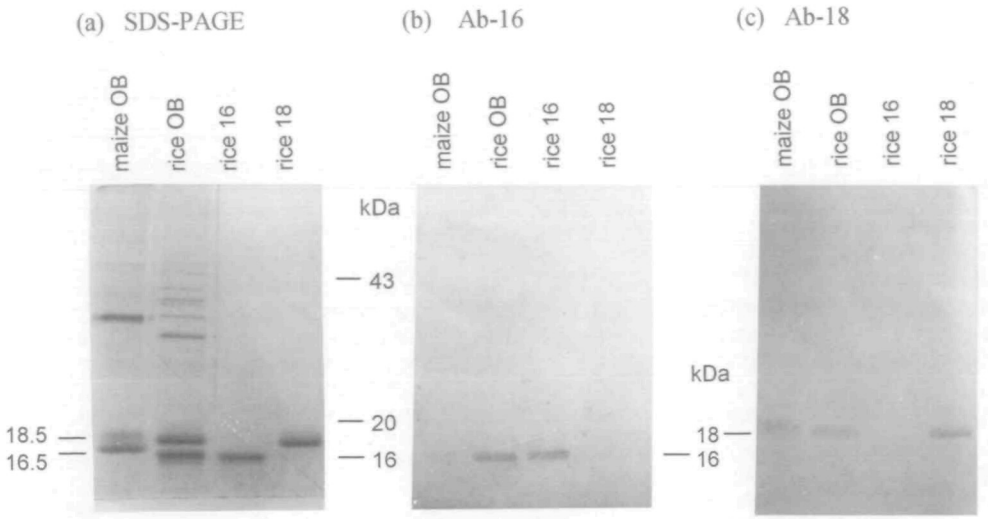

Fig. 5. SDS-PAGE and Western blots of maize oil bodies, rice oil bodies, and purified rice oleosins. (a) Maize oil bodies (10 μg proteins), rice oil bodies (10 μg proteins), rice oleosin 16 kDa (3 μg), and rice oleosin 18 kDa (3 μg) were resolved by SDS-PAGE. The molecular size marker proteins were ovalbumin (43 kDa), rapeseed oleosin (20 kDa), and rice oleosin (16 kDa). The estimated molecular weights of the two maize oleosins (16.5 and 18.5 kDa) are indicated on the left. (b) and (c) Duplicate SDS-PAGE gels were transferred to two piece of PVDF membranes and then subjected to immunoassaying using antibodies raised against rice oleosin 16 kDa and against rice oleosin 18 kDa, respectively.

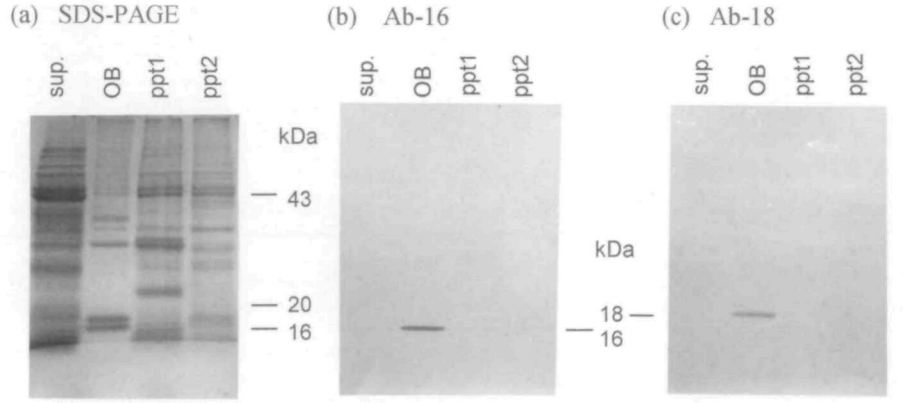

Fig. 6. **SDS-PAGE and Western blots of rice seed proteins fractionated by centrifugation.** (a) The total extract of rice seed proteins was subjected to differential centrifugation to yield a 100,000 × *g* supernatant, an oil body fraction, a 10,000 × *g* pellet (ppt 1), and a 100,000 × *g* pellet (ppt 2). The proteins in these four fractions were resolved by SDS-PAGE with loading samples adjusted to represent amounts derived from equal quantities of the seed extract, except in the case of the oil body fraction, for which a two times amount of proteins was loaded in order to show clear oleosin bands. (b) and (c) Duplicate SDS-PAGE gels were transferred to two piece of PVDF membranes and then subjected to immunoassaying using antibodies against rice oleosin 16 kDa and against rice oleosin 18 kDa, respectively.

(a)

Maize 16 kDa	KAATAATFGGSMLVLSGLLLAGT
Rice 16 kDa	KTVTAATAGGSMLV-SGLIVAGT
DNA deduced	KTVTAATAGGSMLVLSGLILAGT

(b)

Maize 18 kDa	QRTPDYVEEARRRMAEAAA
Rice 18 kDa	QRTPDYVEQARRRMAEAAA
DNA deduced	QRTPDYVEQARRRMAEAAA

Fig. 7. Sequence comparisons of partial amino acid sequences of rice oleosins with maize oleosins and cDNA deduced rice oleosins. (a) A partial sequence of rice oleosin 16 kDa (22 residues) was aligned with the sequence for maize oleosin 16 kDa (*23*), and compared with the deduced rice oleosin 16 kDa sequence (GenBank U43930). (b) A partial sequence of rice oleosin 18 kDa (19 residues) was aligned with the sequence for maize oleosin 18 kDa (*24*), and compared with the deduced rice oleosin 18 kDa sequence (GenBank U43931). Identical residues in the rice partial sequence and the maize sequence are boxed. The differences between the rice oleosin 18 kDa sequences obtained on partial sequencing and cDNA deduction are underlined in the sequence of cDNA-deduced residues.

brane-confined compartments (*e.g.*, organelles and cell surface) is negatively charged. It is likely that electronegative repulsion is one of the forces, if not the only one, that prevent organelles from self or non-self aggregation.

In general, the epitopes of a protein are present in the regions exposed to an aqueous environment, and thus are predominantly localized in the hydrophilic residues on the protein surface. Hydrophobic residues are commonly embedded in the interior portion of a protein and therefore represent no or only a very weak epitope site for the induction of antibodies. Based on our analysis of the two rice oleosin sequences deduced from cDNA clones, rice oleosin 16 and 18 kDa are two homologous proteins. The homology is primarily located in the central hydrophobic domain, not in the amphipathic N- and C-terminal domains. We expected no or only weak cross-recognition of the antibodies raised against each of the two oleosin isoforms. As we predicted, the respective antibodies raised against rice oleosin 16 kDa and rice oleosin 18 kDa did not cross-recognize each other to their antigens in our immunoassaying. This indicates that the whole central hydrophobic domain of oleosins (approximately 70 residues) represents a poor epitope and induced no or non-detectable antibodies

in our experiment.

Sequence comparison of rice and maize oleosins revealed high homology in all of the three structural domains between rice oleosin 18 kDa and maize oleosin 18 kDa (82.6% identity). Indeed, antibodies raised against rice oleosin 18 kDa strongly recognized maize oleosin 18 kDa in our immunoassaying. Meanwhile, high homology was also present between rice oleosin 16 kDa and maize oleosin 16 kDa (88.5% identity) in the three structural domains. However, antibodies raised against rice oleosin 16 kDa did not cross-recognize maize oleosin 16 kDa in our immunoassaying. We are sure that the non-recognition was not due to deficient transfer from the SDS-PAGE gel to the PVDF membrane since a duplicate PVDF membrane subjected to protein staining showed the adequate transfer of the bands of all four oleosins (data not shown). This may imply an inflexible conformation difference between rice oleosin 16 kDa and maize 16 kDa or extensive variation between the maize variety we used in Taiwan and the maize inbred line (MO 17) used for the sequences published in literature (*23*).

In the experiment on oleosin localization in different cellular fractions, antibodies against rice oleosin 16 kDa strongly recognized the oil body fraction, and also weakly the ppt and supernatant. The weak recognition may be a consequence of contamination during fractionation; or it may indicate a minor amount of oleosin in the assembly factory, probably the ER membrane, for the formation of oil bodies. It seems reasonable that the recognition was weak in the non-oil body fractions since the biosynthesis of oil bodies does not actively occur in mature seeds.

In addition to the two abundant oleosin bands, the oil body fraction gives several bands of higher molecular weights (30-70 kDa) (Fig. 5a). Most of these proteins were consistently found in our oil body preparations. It is possible that some minor proteins with unidentified functions are present in/on the oil bodies. Whether these proteins are minor constituents of oil bodies or contaminants during fractionation remains to be determined.

To date, all of the examined oil bodies from diverse species of angiosperms contain, at least, two isoforms of oleosin (*25, 26*). The physiological role of the presence of these two isoforms remains unknown. It is likely that there are only two oleosin isoforms in rice, according to the results of SDS-PAGE of oil body proteins (Fig. 5a). Compared with the complicated oleosin family in most other species (*25*), the simplicity of rice oleosins may allow rice oil bodies to serve as a model system for the investigation of the structure-function relationship of the two oleosin isoforms on the surface of oil bodies. However, we should not ignore the discrepancy between the partial sequence of rice oleosin 16 kDa and the sequence deduced from the cDNA clone (Fig. 7a), although it may be due to wrong identification of residues or nucleotides during the sequencing assignment. It is still possible that there are two oleosins of similar sequences and molecular weights (16 kDa), which can not be resolved by SDS-PAGE under the conditions in our experiments.

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