

Identification of Three Novel Unique Proteins in Seed Oil Bodies of Sesame

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Plant seeds store triacylglycerols in discrete organelles called oil bodies. An oil body preserves a matrix of triacylglycerols surrounded by a monolayer of phospholipids embedded with abundant structural proteins termed oleosins and probably some uninvestigated minor proteins of higher molecular mass. Three polypeptides of 27, 37, and 39 kDa (temporarily denominated as Sop1, Sop2, and Sop3) were regularly co-purified with seed oil bodies of sesame. Comparison of amino acid composition indicated that they were substantially less hydrophobic than the known oleosins, and thus should not be aggregated multimers of oleosins. The results of immuno-recognition to sesame proteins extracted from subcellular fractions of mature seeds, various tissues, and oil bodies purified from different stages of seed formation revealed that these three polypeptides were unique proteins gathered in oil bodies, accompanying oleosins and triacylglycerols, during the active assembly of the organelles in maturing seeds. Both *in vivo* and *in vitro*, immunofluorescence labeling using secondary antibodies conjugated with FITC (fluorescein isothiocyanate) confirmed the localization of these three polypeptides in oil bodies.

Key words: Immunofluorescence — Oleosin — Seed oil body — Sesame — Triacylglycerol.

Vegetable cooking oils are triacylglycerols (TAGs) extracted from various plant seeds. Plant seeds store TAGs as food 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 (Yatsu and Jacks 1972, Stymne and Stobart 1987, Huang 1996). An oil body, 0.5 to 2.5 μm in diameter (Tzen et al. 1993), contains a TAG matrix surrounded by a monolayer of phospholipids embedded with abundant structural proteins termed oleosins and probably some minor uninvestigated proteins of higher molecular mass (Tzen et al. 1997). Oil bodies maintain as individual small organelles even after a long period of storage in plant seeds (Slack et al. 1980). This stability is a consequence of the steric hindrance and electronegative repulsion provided by proteins (mostly oleosins) on the surface of oil bodies

(Tzen et al. 1992). It has been suggested that the entire surface of an oil body is covered by proteins (Tzen and Huang 1992). Therefore, the compressed oil bodies in the cells of a mature seed never coalesce or aggregate.

Oleosins are alkaline proteins of molecular mass 15 to 24 kDa, depending on species (Qu et al. 1986, Murphy and Au 1989). There are at least two isoforms of oleosins present in seed oil bodies from diverse angiosperm species (Tzen et al. 1990, Murphy et al. 1991). It has been demonstrated that both oleosin isoforms coexist in each oil body via immunofluorescence labeling (Tzen et al. 1998). In the past decade, extensive investigation has been achieved on oleosins including cloning of more than thirty genes, relationship of protein structure-function (Tzen et al. 1992, Li et al. 1992), targeting of the proteins to oil bodies (Hills et al. 1993, Loer and Herman 1993, Thoyts et al. 1995, Abell et al. 1997), gene transformation (Lee et al. 1991, Plant et al. 1994, Sarmiento et al. 1997), and potential applications of transgenic plants (van Rooijen and Moloney 1995, Chaudhary et al. 1998). However, no other protein resided in seed oil bodies has been identified or investigated at the present time. Oil bodies of diverse seeds purified with various methods (Murphy and Au 1989, Millichip et al. 1996, Tzen et al. 1997) often contain minor proteins other than oleosins. Whether these minor proteins co-purified with oil bodies are real constituents of the organelles or contaminants in purification has not been verified.

In this study, we identified three minor proteins unique to the oil bodies of sesame seeds via immunodetection. To the best of our knowledge, this is the first report that provides experimental evidence for the presence of seed oil body proteins other than oleosins.

Materials and Methods

Plant materials—Mature and fresh maturing sesame (*Sesamum indicum* L.) seeds were gifts from the Crop Improvement Department, Tainan District Agricultural Improvement Station. The seeds were soaked in water for 10 min prior to purification of oil bodies.

Purification of oil bodies—Oil bodies were extracted from sesame seeds and subjected to further purification using the protocol developed by Tzen et al. (1997) including two-layer flotation by centrifugation, detergent washing, ionic elution, treatment of chaotropic agent, and integrity testing with hexane.

Purification of Sop1, Sop2, Sop3, 15 kDa oleosin, and the 23 kDa subunit of 11S storage protein from sesame by SDS-PAGE—The proteins in the oil body preparation or 11S storage protein preparation (Okubo et al. 1979) were resolved by SDS-PAGE (Laemmli 1970). The sample, at a concentration of 1 mg protein

Abbreviations: FITC, fluorescein isothiocyanate; TAG(s), triacylglycerol(s); TLC, thin layer chromatography.

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ml⁻¹, 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 and stacking gels, respectively. After electrophoresis, the gel was stained with Coomassie Blue R-250, and then destained. After destaining, protein bands corresponding to Sop1, Sop2, Sop3, 15 kDa oleosin, and the 23 kDa subunit of 11S storage protein were cut from the gels, separately. The proteins in gels were separately homogenized in extraction buffer (0.125 M Tris-HCl, pH 8.0, 1 mM EDTA, 0.1% SDS) using a pestle and mortar (Chuang et al. 1996). The homogenate was centrifuged at 10,000 × g for 10 min. The supernatant was retained, and the pellet was re-extracted with extraction buffer. The re-extraction was performed two more times. The eluted proteins in the combined supernatants were 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 collect pellet. The pellet of each protein was dissolved in the extraction buffer to a concentration of 1 mg ml⁻¹.

Analysis of amino acid composition of Sop1, Sop2, and Sop3—Sop1, Sop2, and Sop3 eluted from gels were separately precipitated with an equal amount of acetone to remove SDS in the solution. The pellets were suspended in a buffer of 10 mM sodium phosphate, pH 7.5, and the acetone precipitation was executed two more times. The washed pellets were subjected to analysis of amino acid composition by the Pico Tag System in National Tsing-Hua University in Hsinchu, Taiwan.

Preparation of antibodies—Antibodies against Sop1, Sop2, Sop3, 15 kDa oleosin, and the 23 kDa subunit of 11S storage protein were individually raised in chickens. Pre-immune eggs were taken from the chickens a week before antigen injections, and used as pre-immune blotting controls. Hens were kept individually in cages for immunization and egg production. The antigen (protein in a solution of 1 mg ml⁻¹) was mixed with an equal volume of complete Freund's adjuvant. A volume of 1 ml of the antigen mixture was injected into the chest muscle of each chicken. Booster injections of equal amounts of the antigen were given 10 and 20 d 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 (Polson 1990), 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 onto a nitrocellulose 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 antibodies or pre-immune antibodies (control) diluted with TBS containing 1% gelatin at room temperature for 2 h. The membrane was rinsed with distilled water and then washed twice (10 min each) with 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 in 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 H₂O₂ for color development (Wilson and Nakane 1978).

Fractionation of the extract from sesame seeds—Followed the procedure for preparation of oil bodies, sesame extraction was

separated into three fractions (oil bodies, supernatant and pellet) after the first centrifugation (10,000 × g for 20 min). Oil bodies were subjected to further purification as described previously. Supernatant was centrifuged at 100,000 × g for 90 min to yield a 100,000 × g supernatant (soluble proteins) and a 100,000 × g pellet (microsomal fraction).

Extraction of proteins from different tissues—Total proteins were extracted from stem, leaf and root from sesame plants a month after germination. The tissues were cut into small pieces and homogenized at 4°C in a grinding medium (30 ml per 10 g tissue) with a Polytron at 9,000 rpm for 1 min. The grinding medium contained 0.6 M sucrose and 0.01 M sodium phosphate buffer, pH 7.5. The homogenate was filtered through three layers of cheese cloth. The total extracted proteins were analyzed by SDS-PAGE and subjected to Western blots using antibodies against Sop1, Sop2, Sop3, and 15 kDa oleosin respectively.

Oil bodies from different stages of maturing sesame seeds—Sesame was planted in an experimental field of the Crop Improvement Department, Tainan District Agricultural Improvement Station, Taiwan. The growing season was from early March to late May in 1996. Flowers were tagged for sampling different stages of maturing seeds. Three thousand seeds of each stage were harvested and subjected to purification of oil bodies. As a consequence of the limited samples, oil bodies from various maturing stages were partially purified by centrifugation and ionic elution to attenuate the loss of oil bodies during purification.

Analysis of TAG content in maturing seeds—Oil-body fractions obtained from various stages of developing seeds were subjected to analysis of TAG content. Each sample of 50 µl was extracted with 150 µl diethyl ether. After centrifugation, the upper ether fraction was collected and spotted onto a TLC (thin layer chromatography) plate coated with silica gel. The TLC plate was developed in a solvent system of hexane: diethyl ether: acetic acid (80:20:2, v/v/v) (Yao et al. 1991). After development and drying, TAG content was visualized by reacting with iodine.

Electron microscopy of mature sesame seeds—Sesame seeds were fixed overnight in 2.5% glutaraldehyde in 50 mM sodium phosphate buffer, pH 7.5 (Peng and Tzen 1998). After several rinses with the buffer, they were postfixed in 2% OsO₄ in the buffer for 2 h. Dehydration was carried out in a graded ethanol series, from 50 to 100%, and the sample was embedded in LR-White resin. Sections of 75 nm were stained with uranyl acetate and lead citrate and observed in a Hitachi H-300 electron microscope.

Paraffin section of sesame seeds—Sesame seeds were fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. After several rinses with the phosphate buffer, each fixed sample was dehydrated with a graded ethanol series, from 50 to 100%. After dehydration, the sample was rinsed with xylene, soaked in wax at 55°C overnight, and finally embedded in wax at room temperature. Sections of 2 mm were sliced in distilled water and layered on slide glasses coated with 2% polyvinyl alcohol overnight.

Immunofluorescence detection—Paraffin sections of sesame seeds fixed on slide glasses were wax-deprived with xylene, rinsed with a graded ethanol series, from 100 to 50% to wash away the xylene, and then soaked in TBS buffer for 15 min. Paraffin sections and purified oil bodies in Eppendorf tubes were separately blocked in 3% gelatin in TBS buffer for 1 h at 37°C. The samples were individually incubated with chicken antibodies against Sop1, Sop2, Sop3, 15 kDa oleosin, and the 23 kDa subunit of 11S storage protein or the respective pre-immune antibodies in 1% gelatin in TBS buffer for 1.5 h at 37°C. After washing twice (10 min each) in TBS containing 0.05% Tween-20, the samples were incubated

with FITC (fluorescein isothiocyanate)-conjugated anti-chicken IgG from mouse (Sigma) in 1% gelatin in TBS buffer for 1.5 h at 37°C. After washed twice (10 min each) in TBS containing 0.05% Tween-20, paraffin sections fixed on slide glasses were covered with glycerol. The paraffin sections and purified oil bodies layered on slide glasses were observed under a Nikon type 104 light microscope with radiation to detect green fluorescence.

Results

Three potential oil-associated proteins co-purified with oil bodies of sesame—In addition to the abundant oleosins of molecular masses 15–17 kDa, three minor proteins of regular proportion were consistently found in sesame oil bodies purified via two-layer flotation by centrifugation, detergent washing, ionic elution, treatment of chaotropic agent, and integrity testing with hexane (Fig. 1). The molecular masses of these three potential minor seed oil proteins, temporarily denominated as Sop1, Sop2 and Sop3, are 27, 37 and 39 kDa estimated on SDS-PAGE. These three polypeptides were purified to apparent homogeneity and utilized to generate antibodies in chickens, respectively.

Comparison of amino acid composition among Sop1, Sop2, Sop3, and oleosins—Amino acid composition of Sop1, Sop2, and Sop3 was determined and compared with that of a known 15.5 kDa sesame oleosin (Chen et al. 1997) deduced from its nucleotide sequence (Table 1). Similar to all the other known oleosins from diverse species (data not shown), sesame 15.5 kDa oleosin possessed less hydrophilic residues (Asp, Asn, Glu, Gln, His, Arg, and Lys) than hydrophobic residues (Val, Met, Ile, Leu, and Phe), predominately due to the environmental selection against hydrophilic residues in its central hydrophobic domain (more than 70 residues with no hydrophilic residue). In contrast, Sop1 and Sop2 possessed more hydrophilic residues than

Table 1 Amino acid composition of sesame Sop1, Sop2, Sop3, and 15.5 kDa oleosin

	Amino acid composition (%)			
	Sop1	Sop2	Sop3	Oleosin 15.5
Asx	7.6	10.0	5.2	4.2
Glx	10.8	10.7	8.5	9.0
Ser	7.0	6.2	5.0	6.9
Gly	11.5	10.5	38.5	7.6
His	0.9	3.3	1.2	1.4
Arg	7.2	4.7	3.5	6.9
Thr	5.5	5.6	4.0	7.6
Ala	8.6	10.7	5.2	9.7
Pro	7.1	7.7	3.8	6.9
Tyr	1.2	1.8	1.2	1.4
Val	7.6	4.8	5.2	9.0
Met	2.7	3.0	1.6	1.4
Cys	0.8	0.6	0.7	0.7
Ile	4.4	5.1	3.6	6.9
Leu	7.6	8.5	6.9	13.9
Phe	5.6	4.0	3.8	3.5
Lys	3.9	2.8	2.1	2.1
Trp	—	—	—	0.7
H-phobic ^a	27.9	25.4	21.1	34.7
H-philic ^b	30.4	31.5	20.5	23.6

The residue composition of Sop1, Sop2, and Sop3 was determined by the Pico Tag System after acidic hydrolysis of the proteins while that of 15.5 kDa oleosin was derived from its corresponding gene (Chen et al. 1997).

^a H-phobic (hydrophobic) = Val + Met + Ile + Leu + Phe.

^b H-philic (hydrophilic) = Asx + Glx + His + Arg + Lys.

hydrophobic residues while Sop3 possessed comparable percentage of hydrophilic and hydrophobic residues. As a consequence of this apparent distinction in hydrophobicity, Sop1, Sop2, and Sop3 should not be aggregated multimers of oleosins but might represent novel proteins located in oil bodies. Meanwhile, Sop3 was composed of 38.5% glycine residue, and obviously a glycine-rich protein.

Unique localization of Sop1, Sop2, and Sop3 in sesame oil bodies—Total extract of sesame seeds was fractionated into cell debris (10,000 × g pellet), soluble proteins (100,000 × g supernatant), microsomes (100,000 × g pellet), and oil bodies by two steps of centrifugation. Sop1, Sop2, and Sop3 were uniquely located in oil bodies (Fig. 2). The location of these three polypeptides in the above four fractions was further detected by Western blots using antibodies raised against each protein. The results confirmed that these three Sop proteins as well as oleosins were exclusively present in the oil bodies.

Sesame proteins extracted from leaf, stem, and root as well as those extracted from seed oil bodies were resolved by SDS-PAGE (Fig. 3). The location of Sop1, Sop2, and

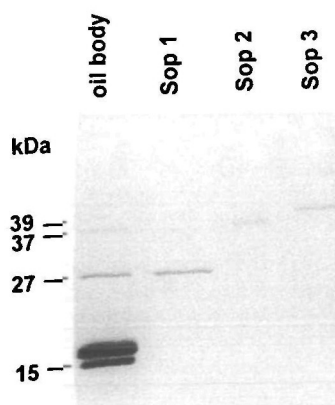


Fig. 1 SDS-PAGE of purified Sop1, Sop2, and Sop3 compared with oil body proteins in sesame seeds. Labels on the right indicate the molecular masses of proteins.

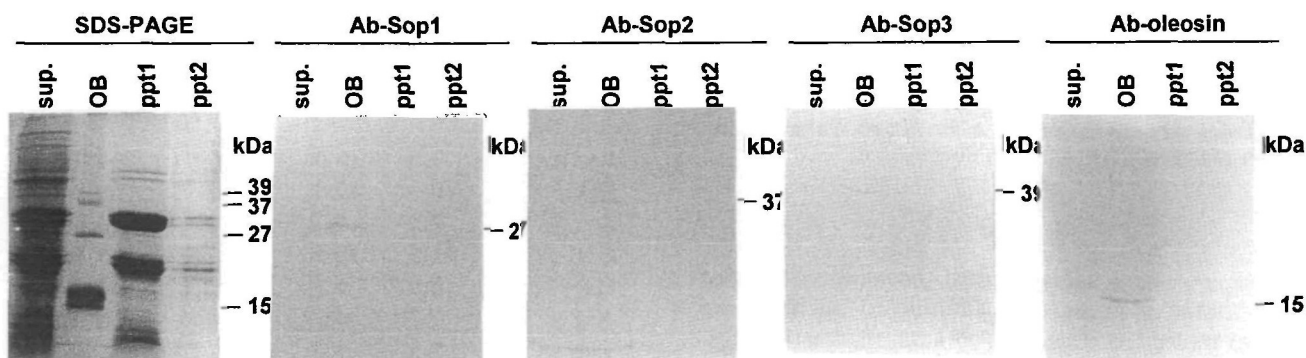


Fig. 2 SDS-PAGE and Western blots of seed proteins of sesame fractionated by centrifugations. Total extract of mature seeds was subjected to differential centrifugations to yield a $100,000 \times g$ supernatant (sup.), an oil body fraction (OB), a $10,000 \times g$ pellet (ppt 1), and a $100,000 \times g$ pellet (ppt 2). The proteins in these four fractions were resolved in SDS-PAGE with loading samples adjusted to represent amounts derived from an equal quantity of the seed extract. Replicate SDS-PAGE gels were transferred onto four pieces of nitrocellulose membrane and subjected to Western blots using antibodies against Sop1, Sop2, Sop3, and 15 kDa oleosin, respectively.

Sop3 in these extracts was detected by Western blots using their individual antibodies. The results confirmed that these three Sop proteins as well as oleosins were present in seed oil bodies but not in the other three tissues.

Exclusive accumulation of Sop proteins, oleosins, and TAGs in oil bodies of maturing sesame seeds—The protein extracts from various stages of maturing sesame seeds were fractionated into four subcellular fractions (cell debris, soluble proteins, microsomes, and oil bodies) by centrifugation. Sop1, Sop2, and Sop3 were exclusively found in oil bodies (Fig. 4) but not in the other three fractions (data not shown). The exclusive accumulation of these three polypeptides in seed oil body fractions of various maturing stages was further detected by Western blots. The accumulation of 15 kDa oleosin was also detected by the Western blot using antibodies raised against the 15 kDa oleosin, a major structural protein exclusively present in seed oil bodies of sesame (Peng and Tzen 1998). The results of the immunode-

tection revealed that Sop1 and oleosin 15 kDa were accumulated simultaneously (initiated at 18 d after flowering) while Sop2 and Sop3 were accumulated slightly later than Sop1 during seed formation. The TAG content in various stages of maturing seeds was analyzed by TLC (Fig. 4, bottom panel). It appeared that the three Sop proteins were unique proteins gathered in oil bodies, accompanying oleosins and TAGs, during the active assembly of the organelles in maturing seeds.

Localization of Sop1, Sop2, and Sop3 in oil bodies via immunofluorescence labeling—In a mature sesame seed, most of the cell space was occupied by a few protein bodies and abundant oil bodies (Fig. 5). The larger protein bodies were surrounded by the abundant oil bodies, which were compressed and crowded together but remained as individual discrete organelles. In paraffin sections of a sesame seed observed under a light microscope, abundant oil bodies surrounding protein bodies were not visibly imag-

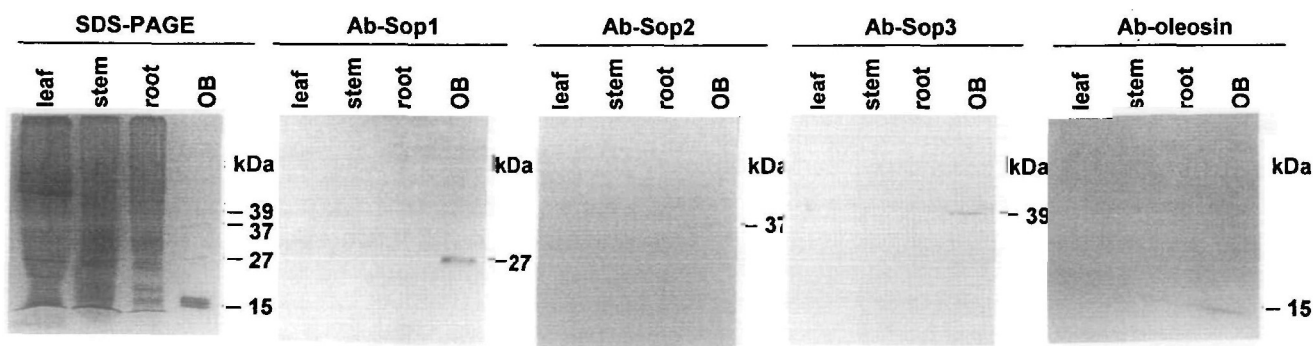


Fig. 3 SDS-PAGE and Western blots of sesame proteins extracted from leaf, stem, root, and seed oil bodies. Total extracts of leaf, stem, and root of approximately $30 \mu\text{g}$ proteins as well as proteins extracted from seed oil bodies were resolved by SDS-PAGE. Replicate SDS-PAGE gels were transferred onto four pieces of nitrocellulose membrane and subjected to Western blots using antibodies against Sop1, Sop2, Sop3, and 15 kDa oleosin, respectively.

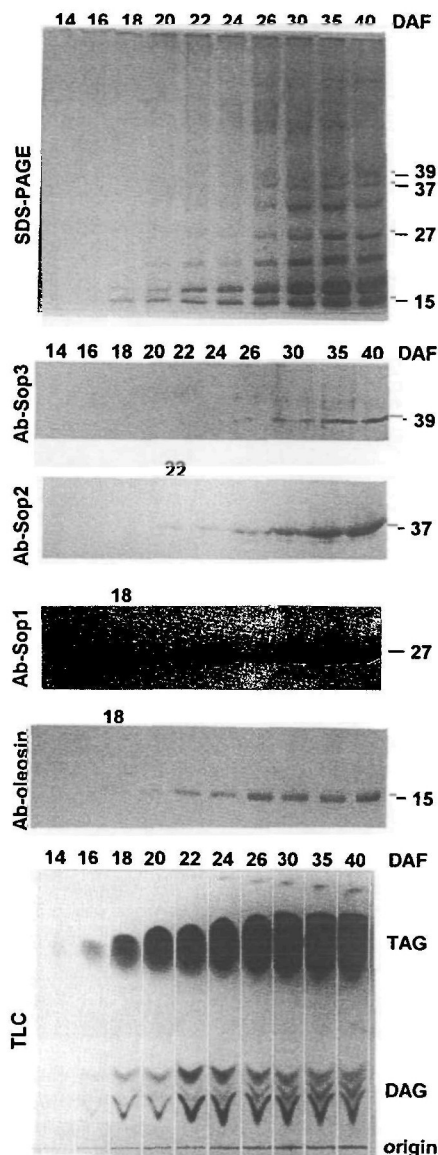


Fig. 4 SDS-PAGE, Western blots, and TLC analysis of oil-body fractions in various stages of maturing sesame seeds. The proteins extracted from oil-body fractions in various stages of seed formation were resolved by SDS-PAGE with loading samples adjusted to represent amounts derived from an equal quantity of the seed extract. Replicate SDS-PAGE gels were transferred onto four pieces of nitrocellulose membrane and subjected to Western blots using antibodies against Sop1, Sop2, Sop3, and 15 kDa oleosin, respectively. TAG (triacylglycerol) content in these stages was analyzed by TLC (bottom panel) developed in a solvent system of hexane : diethyl ether : acetic acid (80 : 20 : 2, v/v/v). The positions of TAG and DAG (diacylglycerol) are indicated in the right margin. DAF represents days after flowering.

ed (Fig. 6, left panels), but could be tracked with antibodies against Sop proteins (Fig. 6, right panels). In the immunofluorescence detection using Sop antibodies, corresponding

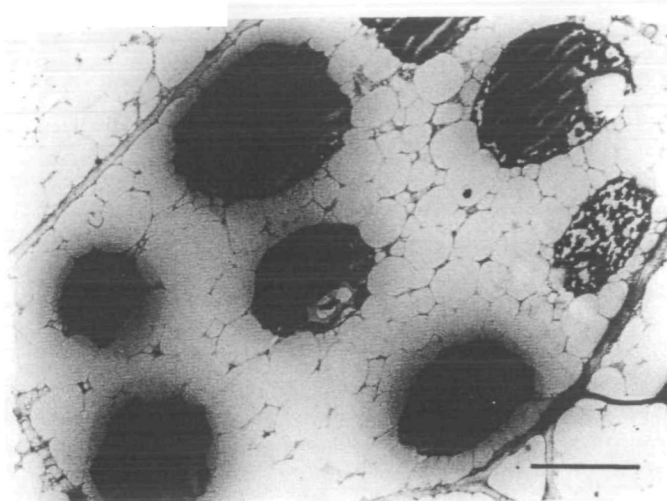


Fig. 5 Electron microscopy of a mature sesame seed. The seed was fixed in 2.5% glutaraldehyde, postfixed in 1% OsO₄, sectioned at 75 nm thickness and used for photography under an electron microscope. The larger dark-stained protein bodies are embedded in the smaller abundant oil bodies. Bar represents 2 μ m.

positions of protein bodies were lack of fluorescence while their surrounded areas, presumably full of oil bodies, illuminated green fluorescence. In contrast, the locations of protein bodies, but not their surrounded areas, illuminated fluorescence by replacing antibodies against Sop proteins with antibodies against the 23 kDa subunit of 11S storage protein (bottom right panel). Consistent results were observed using oil bodies purified from sesame seeds instead of paraffin tissue sections when treated in the same condition (Fig. 7). Purified oil bodies could be tracked with antibodies against Sop proteins, but not those against the 23 kDa subunit of 11S storage protein. In both paraffin sections and purified oil bodies, no detectable fluorescence was observed using pre-immune chicken antibodies (data not shown).

Discussion

Oleosins have been proposed to be embedded in the TAG matrix via their central hydrophobic domain and associated with the phospholipid layer of oil bodies. Thus, oleosins possess limited free residues exposed to the surface region of the organelles (Tzen et al. 1992). The three novel polypeptides identified by immunodetection as unique proteins to the sesame oil bodies in this study, i.e., Sop1, Sop2, and Sop3 had a higher percentage of hydrophilic residues than oleosins (Table 1). This observation suggests that these three Sop proteins might comprise a significant portion of hydrophilic residues presumably located in the surface region of oil bodies, and could have some biological functions different from the structural functions, e.g., steric hindrance and electronegative repulsion, conferred

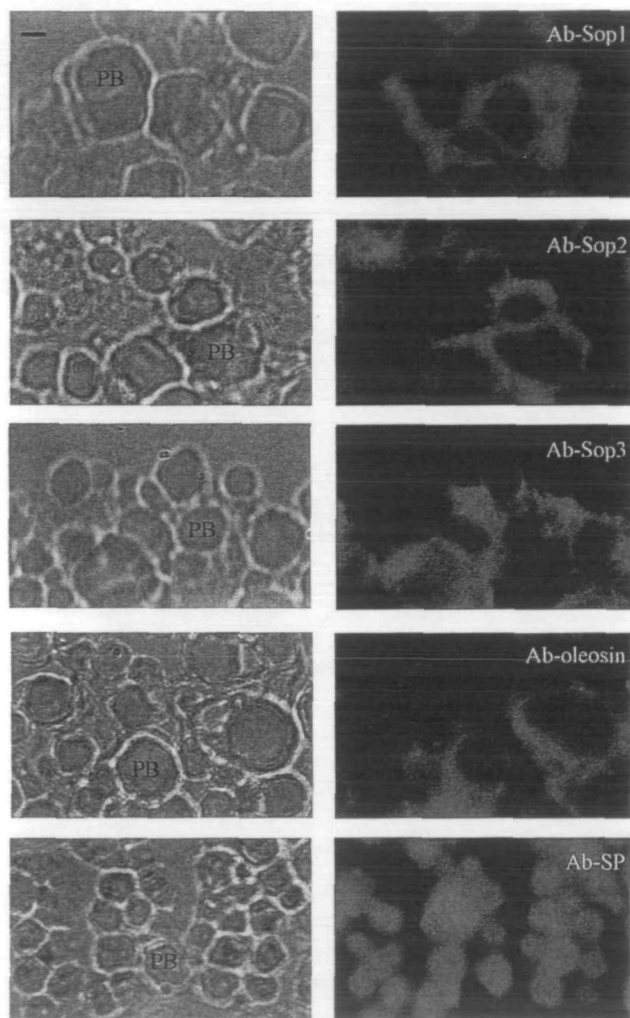


Fig. 6 Fluorescence microscopy of paraffin sections of sesame seeds. Paraffin sections of sesame seeds were observed in a light microscope (left panels) or incubated with chicken antibodies against Sop1, Sop2, Sop3, 15 kDa oleosin, or the 23 kDa subunit of 11S storage protein (abbreviated as SP), and then with anti-chicken IgG conjugated with FITC. After incubation, the sections were observed in a microscope with radiation to detect green fluorescence (right panels). PB represents protein body. Abundant oil bodies surrounding protein bodies were not visibly imaged in these paraffin sections, but could be tracked with antibodies against Sop proteins or 15 kDa oleosin, and then visualized via fluorescence. All photos are of the same magnification. Bar represents 2 μ m.

by oleosins. These three novel oil body proteins may be involved in seed oil biosynthesis or degradation serving as ER membrane proteins (enzymes), glyoxisome receptor, inactive lipase, or lipase receptor. Of course, it cannot be excluded that some of the above functions may be conferred by oleosins or peripherally associated proteins, if any, lost due to the stringent conditions of oil body preparation. We are currently cloning the pertinent genes in order to explore

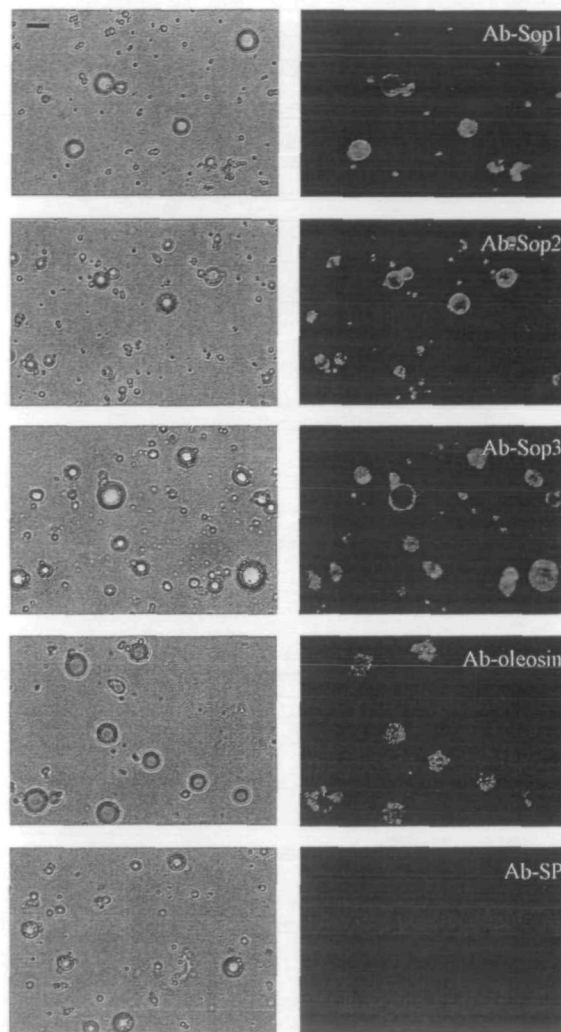


Fig. 7 Fluorescence microscopy of purified sesame oil bodies. Purified sesame oil bodies were treated in the same process as described in Fig. 6 and then observed in a microscope (left panels) or examined via immunofluorescence detection using the five antibodies used in Fig. 6, followed by secondary antibodies conjugated with FITC (right panels). All photos are of the same magnification. Bar represents 2 μ m.

the structure and function of Sop1, Sop2, and Sop3 from their deduced amino acid sequences.

A concomitant accumulation of TAGs and oleosins has been found in the maturing seeds of maize (Qu et al. 1986), soybean (Herman 1987), rapeseed (Tzen et al. 1993), sunflower (Thoyts et al. 1995), sesame (Peng and Tzen 1998), and rice (Wu et al. 1998). The accumulation of Sop1, Sop2, and Sop3 in oil bodies during seed formation is similar to or slightly later than that of oleosins (Fig. 4), and thus presumably accompanies the active synthesis of TAGs for the assembly of oil bodies. However, quantitative variance and the slightly temporal deviation in the accu-

mulation of these oil body proteins (oleosins and Sop proteins) implies a non-uniformity of protein constituents for oil bodies assembled at different stages of seed maturation. Whether this non-uniformity of protein constituents in oil bodies associates with any biological phenomenon or function remains to be studied.

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