JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Minimizing the Central Hydrophobic Domain in Oleosin for the Constitution of Artificial Oil Bodies

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Oleosin, a unique structural protein anchoring onto the surface of seed oil bodies by its central hydrophobic domain, stabilizes these lipid-storage organelles as discrete entities. Stable artificial oil bodies have been successfully constituted with native or recombinant oleosins. In this study, recombinant sesame oleosin with 12 residues stepwise truncated from its central hydrophobic domain of 72 residues was overexpressed in *Escherichia coli*, was purified to homogeneity, and was used for the constitution. Artificial oil bodies constituted by truncated oleosins with the central hydrophobic domain longer than 36 residues were as stable as native sesame oil bodies, and those constituted by truncated oleosins lacking more than half of the original central hydrophobic domain inclined to coalesce upon collision or aggregation.

KEYWORDS: Artificial oil bodies; oleosin; sesame; structural stability; thermostability

INTRODUCTION

Seeds store triacylglycerols (TAGs) in discrete intracellular organelles termed oil bodies and use them as the fuel for germination and subsequent seedling growth (*1*, *2*). An oil body is made of a TAG matrix, which is surrounded by a monolayer of phospholipids (PLs) embedded with abundant structural proteins, oleosin isoforms, and some minor proteins of higher molecular mass (*4*−*6*). Oil bodies, averaging 0.5−2 μm in diameter, are remarkably stable both in cells and in isolated preparations as a consequence of the steric hindrance and electronegative repulsion mainly provided by oleosin on the surface of oil bodies (*7*, *8*).

It is technically feasible to reconstitute stable artificial oil bodies using the three essential constituents that comprise oil bodies of diverse seeds, that is, neutral lipids (mainly TAGs) (94−98%), PLs (0.5−2%), and proteins (mainly oleosin isoforms) (0.5−3.5%) (*9*, *10*). Sizes of artificial oil bodies, which are related to their thermo- and structural stability, could be controlled by varying the ratio of matrix oil over oil-body protein (*11*). Artificial oil bodies stabilized by recombinant oleosin expressed in *Escherichia coli* were comparable in size, topology, and stability to those encapsulated with native oleosin isolated from seed oil bodies (*12*). Recently, several application platforms for artificial oil bodies have been developed, including a bacterial expression/purification system for producing recombinant proteins (*13*, *14*), an oral delivery system for hydrophobic drugs (*15*), and a new technique of enzyme fixation designed to achieve, in one step, protein refolding and immobilization by linking a target enzyme to oleosin on the surface of artificial oil bodies (*16*).

An oleosin molecule is proposed to comprise three structural domains: an N-terminal amphipathic domain, a central hydrophobic oil-body anchoring domain, and a C-terminal amphipathic α-helical domain (*17*). Both N- and C-terminal domains of oleosins have been proposed to reside on the oil-body surface to stabilize this organelle via steric hindrance and electronegative repulsion (*18*). The central hydrophobic anchoring domain of oleosins from diverse plant species, with approximately 70 amino acid residues representing the longest hydrophobic segment ever found in natural proteins (*19*), is highly conserved, particularly in its relatively hydrophilic proline knot motif at the middle of the sequence (*10*).

We aimed to minimize the length of the central hydrophobic domain in oleosin for the constitution of stable artificial oil bodies that might be used in diverse applications. In this study, artificial oil bodies were constituted with recombinant oleosins whose central hydrophobic domain was subsequently truncated by 12 residues. Stability of these artificial oil bodies was examined and compared with that of native oil bodies purified from sesame seed.

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10.1021/jf070977o CCC: $37.00
Published on Web 06/14/2007

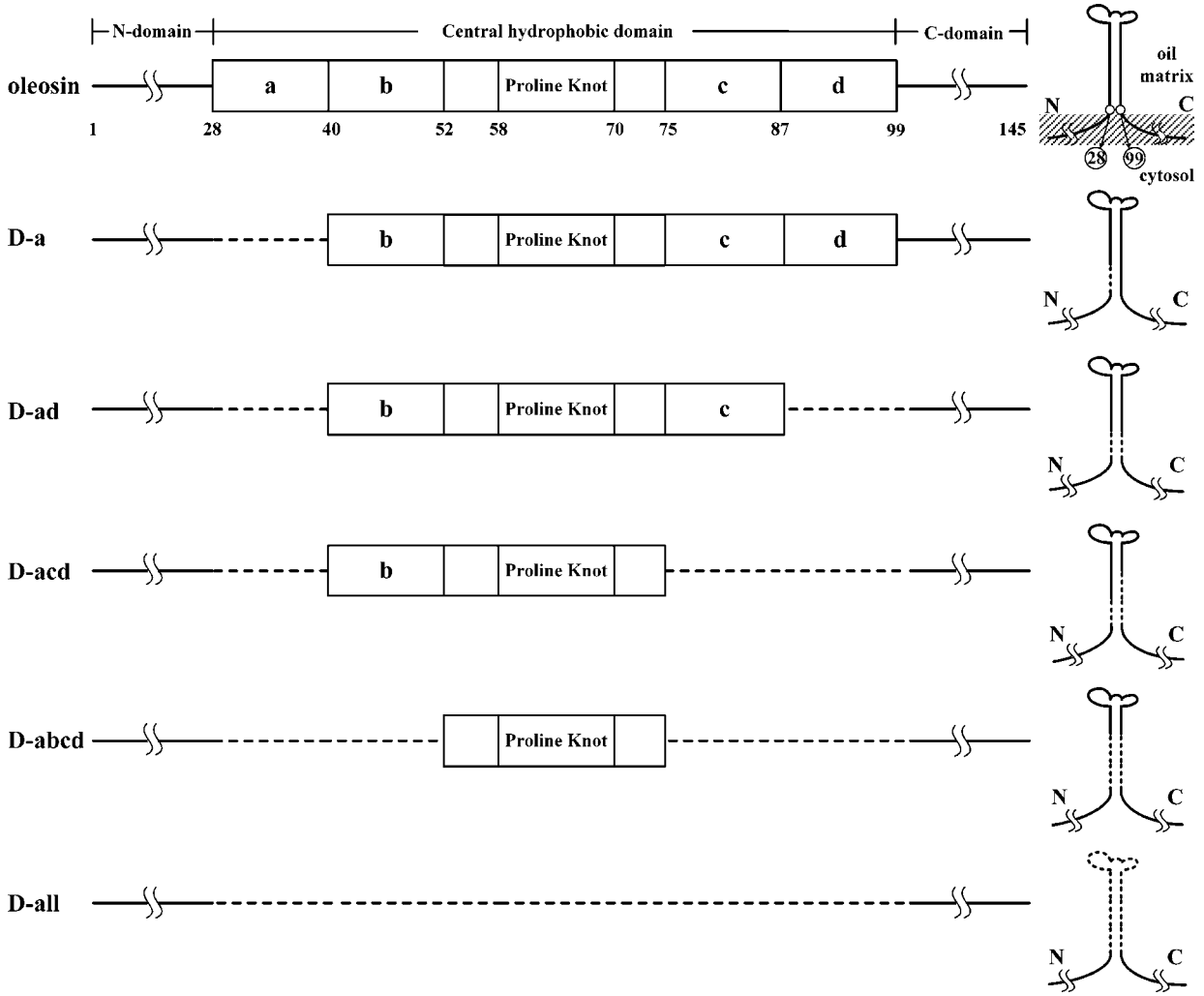

Figure 1. Schematic diagram showing all the recombinant oleosins expressed in this study. Five truncated oleosins lacking 12, 24, 36, 48, and all the 72 residues of the central hydrophobic domain were obtained by subsequent deletion of 12 residues from both borders. Numbers under junction sites represent their relative positions counting from N-terminus of the native sesame 15 kDa oleosin. A simplified structural model is shown on the right of each recombinant oleosin; a monolayer of PL, depicted by a shaded area, segregated the hydrophobic oil matrix of an oil body from the hydrophilic cytosol. Dashed lines represent the truncated portions in the recombinant oleosins.

MATERIALS AND METHODS

Purification of Sesame Oil Bodies. Mature seeds of sesame (*Sesamum indicum* L.), a gift from the Crop Improvement Department, Tainan District Agricultural Improvement Station, were soaked in water for 15 min prior to the purification of oil bodies. Oil bodies were purified according to the protocol developed by Tzen et al. (*20*), including a two-layer flotation by centrifugation, detergent washing, ionic elution, treatment of chaotropic agent, and integrity testing with hexane.

Plasmid Construction for Recombinant Oleosins. cDNA fragment encoding the first 140 amino acids of sesame 15 kDa oleosin (accession number AF091840) was constructed in the fusion expression vector pET29a (Novagen), using an *Nco*I site in the polylinker of the vector. The resulting plasmid pET29Ole was used to generate, by PCR-based mutagenesis, five other constructs corresponding to truncated oleosins lacking 12, 24, 36, 48, and all 72 residues of the central hydrophobic domain. The strategy used to produce various recombinant oleosins with stepwise deletion of 12 residues from either or both borders of their central hydrophobic domain is shown in **Figure 1**. For each construction, two 5′phosphorylated divergent primers were designed to anneal to the desired mutation site, and the resulting construct was used as the template for the next construction. Paired primers used to generate constructs encoding the truncated oleosins are listed in **Table 1**. PCR amplification was carried out using ultra *pfu* (Stratagene) for 15 cycles at 94 °C, 1 mim; 50 °C, 1 min; 72 °C, 10 min. The blunt-ended linear PCR product was resolved in an agarose gel, was purified by ethanol precipitation, and was self-ligated to form a circular plasmid. The resulting plasmids were used to transform *E. coli* DH5 competent cells on an LB agar plate containing kanamycin of 50 mg/L, and the accuracy of plasmid construction was confirmed by direct sequencing.

Table 1. Paired Primers Used to Generate Constructs for Truncated Oleosins

construct	paired primer sequences
pET29OleD-a	5′ CTCTCTGGCCTCACTTTAGCC 3′
	5′ CTTCACTACCCGCTGGGC 3′
pET29OleD-ad	5′ AGATATCTGACAGGGAAACAC 3′
	5′ GCCTCCGGATGCCAGAAAAC 3′
pET29OleD-acd	5′ AGATATCTGACAGGGAAACAC 3′
	5′ AATGGTTATGACCGCCGG 3′
pET29OleD-abcd	5′ CTCACCATCGCCACTCCG 3′
	5′ CTTCACTACCCGCTGGGC 3′
pET29OleD-all	5′ AGATATCTGACAGGGAAACAC 3′
	5′ CTTCACTACCCGCTGGGC 3′

Overexpression of Recombinant Oleosins. Recombinant plasmids were transformed to *E. coli* BL21 (DE3), and the overexpression of

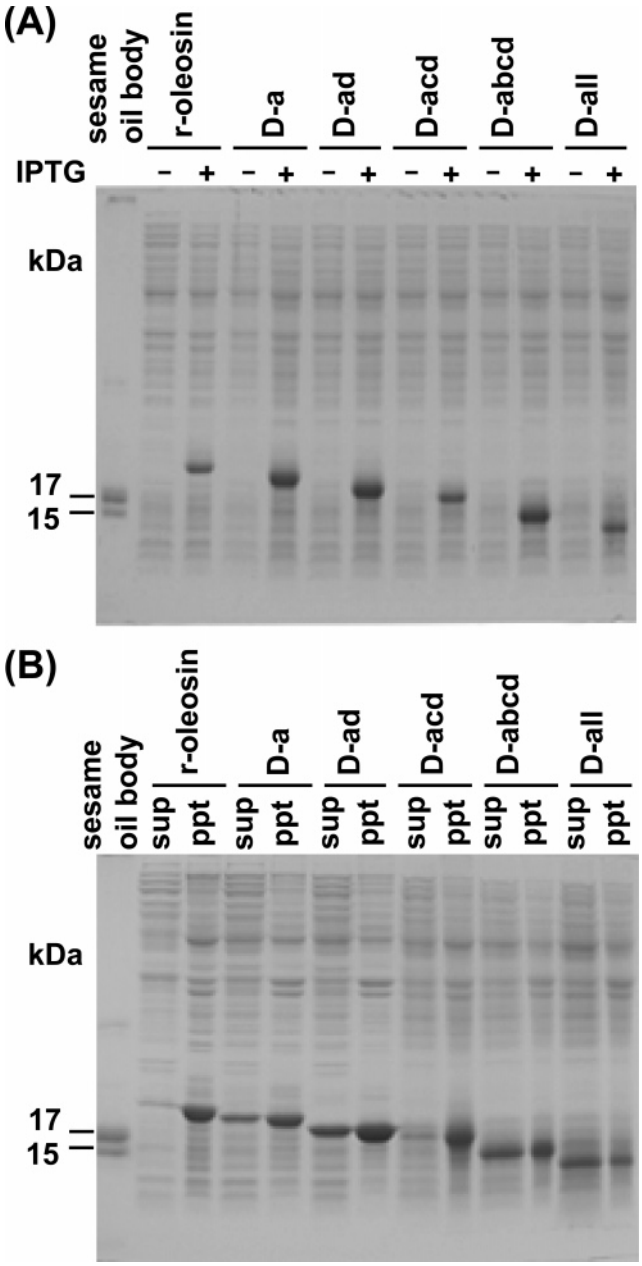

Figure 2. SDS-PAGE of recombinant oleosins overexpressed in *E. coli*. (**A**) Proteins extracted from sesame oil bodies and from *E. coli* cells with or without IPTG induction for overexpression of recombinant oleosins lacking various lengths of the central hydrophobic domain were analyzed by SDS-PAGE. (**B**) Soluble (sup) and insoluble (ppt) proteins extracted from *E. coli* cells (20–25 μg total proteins) containing recombinant oleosins were analyzed by SDS-PAGE. Labels on the left indicate the molecular masses of two oleosin isoforms (15 and 17 kDa) in sesame oil bodies.

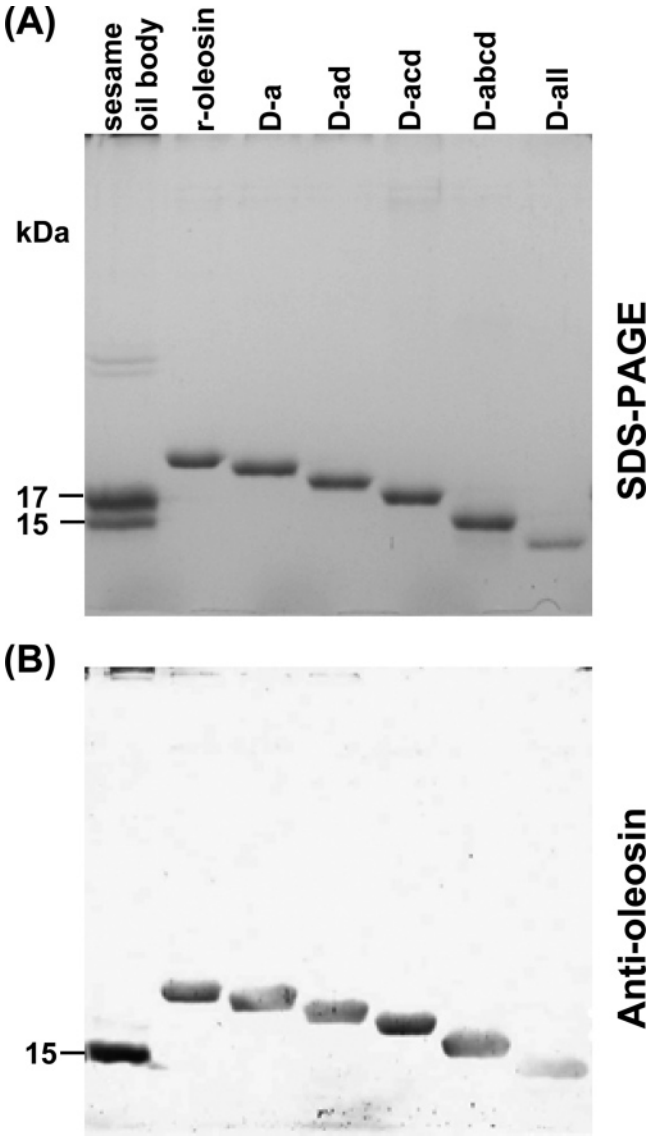

Figure 3. SDS-PAGE and Western blotting of the recombinant oleosins lacking various lengths of the central hydrophobic domain. Along with sesame oil-body proteins, the purified recombinant oleosins were analyzed by SDS-PAGE (**A**). A duplicate gel was transferred onto nitrocellulose membrane and then was subjected to immunoassay using antibodies against sesame 15 kDa oleosin (**B**). Labels on the left indicate the molecular masses of two oleosin isoforms (15 and 17 kDa) in sesame oil bodies.

recombinant oleosins was induced by adding 0.1 mM isopropyl β-D-thiogalactoside (IPTG) in a bacteriophage T7 RNA polymerase/promoter system. Three hours after induction, *E. coli* cells were harvested and were lysated by sonication in 10 mM sodium phosphate buffer, pH 7.5, and after centrifugation the supernatant was subjected to SDS-PAGE and western blotting analyses.

SDS-PAGE and Western Blot Analyses. Proteins in the supernatant were mixed with the sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 0.02% bromophenol blue, 10% glycerol, and 5% β-mercaptoethanol and were resolved by SDS-PAGE using 12.5% acrylamide, according to Bio-Rad instruction manual. Following electrophoresis, the gel was stained with Coomassie blue R-250. Antibodies against oleosin were raised in chicken using purified sesame 15 kDa oleosin as described previously (*21*). For Western blotting, proteins were transferred from SDS-PAGE gel onto nitrocellulose membrane using a Bio-Rad Trans-Blot system (Bio-Rad) according to the manufacturer's instructions. The membrane was subjected to immunodetection using secondary antibodies conjugated with horseradish peroxidase and then was incubated with 3 mM 4-chloro-1-naphthol containing 0.015% H_2O_2 for color development.

Constitution of Artificial Oil Bodies. Recombinant oleosins were eluted from SDS-PAGE gels and were precipitated with equal amounts of acetone chilled at −20 °C. The acetone mixture was kept at −20 °C for 30 min and was centrifuged at 10 000*g* for 30 min. The precipitate was suspended and precipitated three times in 500 μL of 0.1 M sodium phosphate buffer, pH 7.5, to remove SDS. Subsequently, the insoluble protein pellets were sonicated in 500 μL of sodium phosphate buffer prior to the constitution of artificial oil bodies.

For constituting artificial oil bodies, TAG was obtained from sesame oil bodies by chemical extraction as reported previously (*7*), and

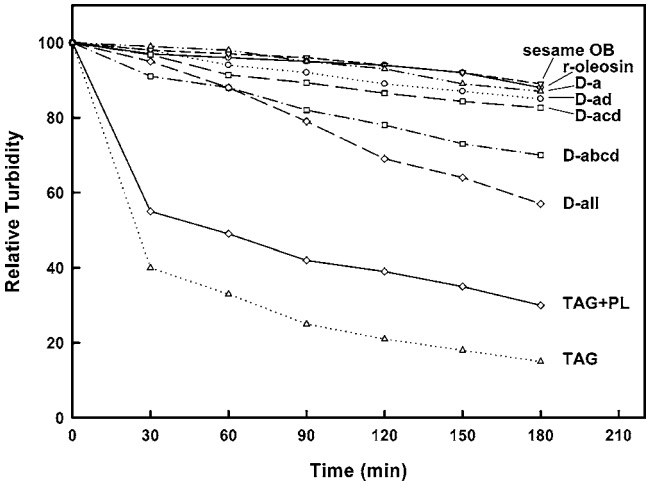

Figure 4. Turbidity tests for stability of artificial oil bodies constituted with TAG, PL, and recombinant oleosins. Along with TAG and PL, artificial oil bodies were constituted with the recombinant oleosin lacking various lengths of the central hydrophobic domain. A suspension (l mL) of sesame oil bodies or artificial oil bodies was placed in a 2 mL cuvette. The relative turbidity (T/T_0) at 600 nm of the lowest 0.5 mL of the suspension was measured at intervals.

dioleoyl phosphatidylcholine, the major PL found in oil bodies, was purchased from Sigma. Artificial oil bodies were constituted with 15 mg of TAG, 150 μg of PL, and recombinant oleosin (160, 150, 140, 132, 124, and 107 μg for oleosins lacking 0, 12, 24, 36, 48, and all 72 residues of the central hydrophobic domain). PL dissolved in chloroform was placed at the bottom of an Eppendorf tube, and the solvent was allowed to evaporate in a chemical hood overnight. Then, TAG and recombinant oleosin were incorporated, followed by sonication with a 3 mm diameter probe in an ultrasonic processor GE 601 with 30% amplification for 20 s, and samples were then cooled in an ice bucket for 5 min. The sonication was repeated twice.

Turbidity Test for Stability of Artificial Oil Bodies. Artificial oil bodies or native oil bodies isolated from sesame seeds were suspended in 0.1 M sodium phosphate buffer, pH 7.5, and were subjected to a stability test by measuring turbidity changes of a suspension of these oil bodies with time. The suspension was placed in a disposable 2 mL cuvette which was covered with Parafilm and was kept undisturbed. The absorbance (A) at 600 nm of the suspension in the lower portion of the cuvette was recorded at time intervals with a Beckmen DU 530 spectrophotometer. The turbidity (T) of the suspension was proportional to 10^A, and the relative turbidity was expressed as $T/T_o = 10^A/10^{Ao} = 10^A/10^{2.0}$, where A_o was 2.0.

Thermostability and Structural Stability of Artificial Oil Bodies. The thermostability was measured by leaving suspensions of artificial oil bodies in the sodium phosphate buffer (pH 7.5) at 40, 50, 60, or 70 °C for 30 min, prior to observation under a Nikon type-104 light microscope. The structural stability of these oil bodies was examined by analyzing their surface properties (steric hindrance and electrostatic repulsion) that accounted for the aggregation of oil bodies without fusion at pH lower than 6.5 (*18*). In this examination, artificial or native oil bodies were suspended in a medium of 0.1 M sodium phosphate buffer, pH 7.5 or 6.5, and were left at room temperature for 6 h before observation under the light microscope.

RESULTS

Production of Recombinant Oleosins with Truncated Central Hydrophobic Domain. As a result of an N-terminal fusion with S-Tag and a C-terminal fusion with His-Tag, the expected molecular masses of the six recombinant oleosins withstepwise deletion of 12 resides, that is, r-oleosin, D-a, D-ad, D-acd, D-abcd, and D-all, were 21.3, 20.3, 19.2, 18.1, 16.6, and 14.4 kDa, respectively (**Figure 2A**). Recombinant oleosins with a central hydrophobic domain longer than 36 residues (r-oleosin, D-a, D-ad, and D-acd) were predominantly found in the insoluble fraction of cell lysate (**Figure 2B**). Solubility of the recombinant oleosin increased substantially when more than half of its central hydrophobic domain was truncated, and the oleosin with its central hydrophobic domain completely deleted became practically soluble. The homogeneity of all six recombinant oleosins purified by acetone precipitation was examined by SDS-PAGE (**Figure 3A**) and was confirmed by immunodetection using antibody against sesame 15 kDa oleosin (**Figure 3B**).

Stability of Artificial Oil Bodies Constituted with Truncated Oleosins. Since unstable artificial oil bodies, such as the emulsion composed of only TAG and PL but not oleosin, would fuse to form large irregular oil drops, a turbidity test was used to examine the stability of oil-body preparations using oleosins with various lengths of the central hydrophobic domain truncated (**Figure 4**). The results have shown that successive shortening of the central hydrophobic domain in oleosin by 12, 24, and 36 residues altered its capability of stabilizing artificial oil bodies at a negligible level but drastically reduced this capability thereafter when more than half (48 and 72 residues) of the central domain in oleosin was truncated.

Comparable thermostability was observed between native oil bodies purified from sesame seeds and those artificial oil bodies stabilized by recombinant oleosins with a central hydrophobic domain longer than 36 residues; these oil-encapsulating particles remained intact at temperatures lower than 50 °C but started to decompose at temperatures higher than 60 °C (**Figure 5**). In contrast, those artificial oil bodies constituted with truncated oleosins lacking more than half of the central hydrophobic domain were less stable and coalesced at temperatures lower than 50 °C.

Similar structural stability was observed between native sesame oil bodies and artificial ones stabilized by recombinant oleosins with a central hydrophobic domain longer than 36 residues; these oil-encapsulating particles maintained as individual discrete particles at pH 7.5 but aggregated without coalescence at pH 6.5 because of the steric hindrance of surface oleosins (**Figure 6**). In contrast, when half of the central hydrophobic domain was removed from oleosins used in the constitution, the surface of the resulting emulsion was not properly shielded. An aggregation of the oil bodies induced at pH 6.5 expedited their coalescence.

DISCUSSION

To define the minimal central hydrophobic domain of oleosin required for the constitution of stable artificial oil bodies, recombinant oleosins with various lengths deleted from the central domain were produced, and three independent observations, that is, turbidity test, thermal stability, and structural integrity inspection, were made to assess the stability of artificial oil bodies constituted with these truncated oleosins. The results have shown that stable artificial oil bodies could be constituted with truncated oleosins whose central hydrophobic domain had a minimal length of 36 residues, namely, half of the original length.

Among the three distinct proteins identified so far from seed oil bodies (*22*), oleosin and caleosin, but not steroleosin, are able to stabilize artificial oil bodies (*23*). Just like oleosin,

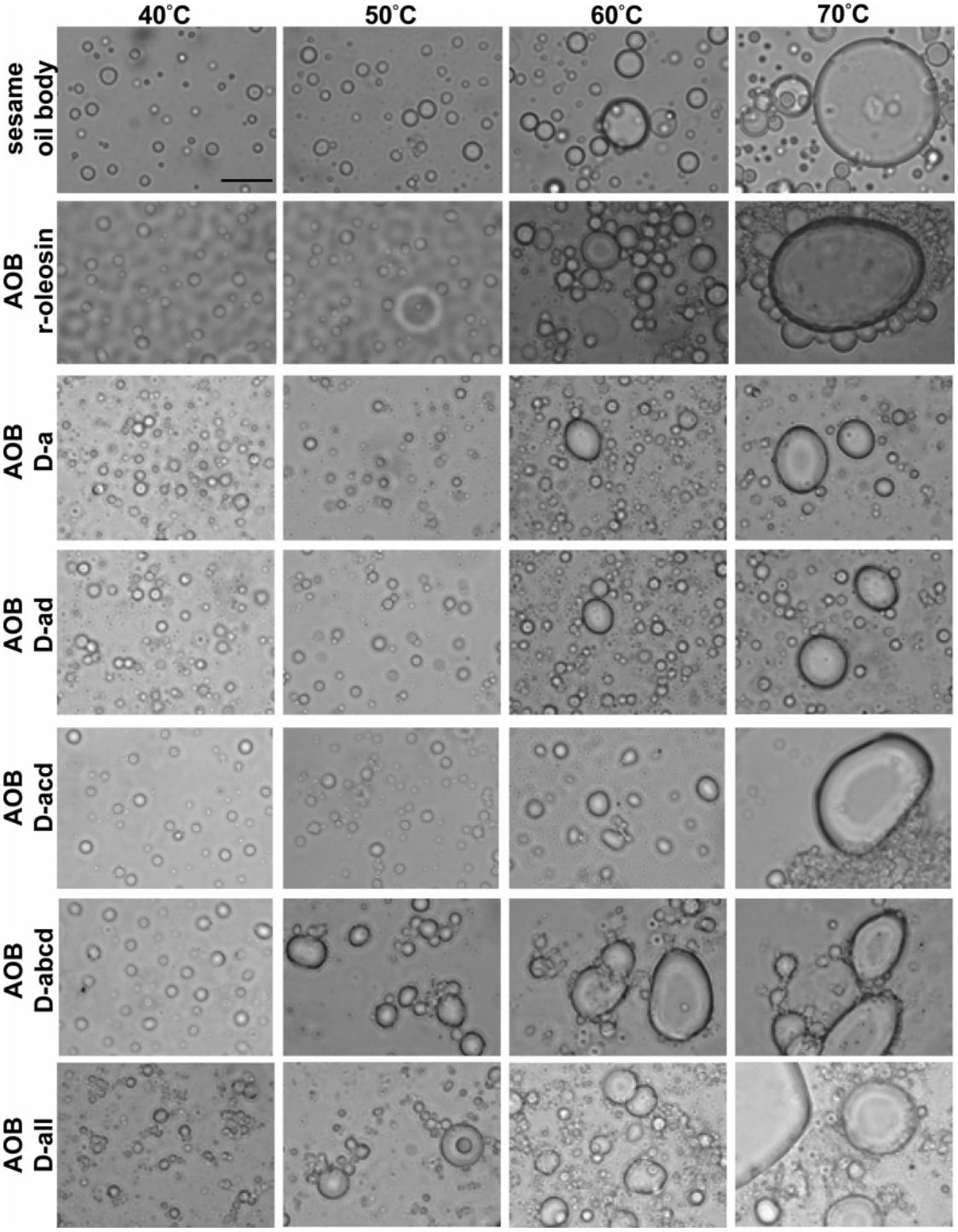

Figure 5. Light microscopy of native and artificial oil bodies under different temperatures. Native sesame oil bodies and artificial oil bodies constituted with recombinant oleosins lacking various lengths of the central hydrophobic domain were left in 0.1 M sodium phosphate buffer, pH 7.5, at 40, 50, 60, and 70 °C for 30 min before taking the photos. All photos are of the same magnification. Bar represents 10 μm.

caleosin is proposed to comprise three structural domains: an N-terminal hydrophilic calcium-binding domain, a central hydrophobic domain, and a C-terminal hydrophilic domain (*4*). The central hydrophobic domain of 36 residues responsible for anchoring caleosin to oil bodies comprises an amphipathic α-helix and a pair of antiparallel β-strands connected with a proline knotlike motif. The minimal oleosin central domain observed in this study for constituting stable oil bodies is similar in both length and structural organization to the central oil-body anchoring domain of caleosin. This observation implies the possibility that oleosin and caleosin could be evolutionarily related.

The characteristic of oil bodies lies in forming oil-in-water microencapsulated emulsions, and artificial oil bodies with unique properties have recently been developed and utilized in several application platforms (*13*−*16*). Whether artificial oil bodies stabilized by the minimal oleosin central domain described in this study can be equivalently used in these

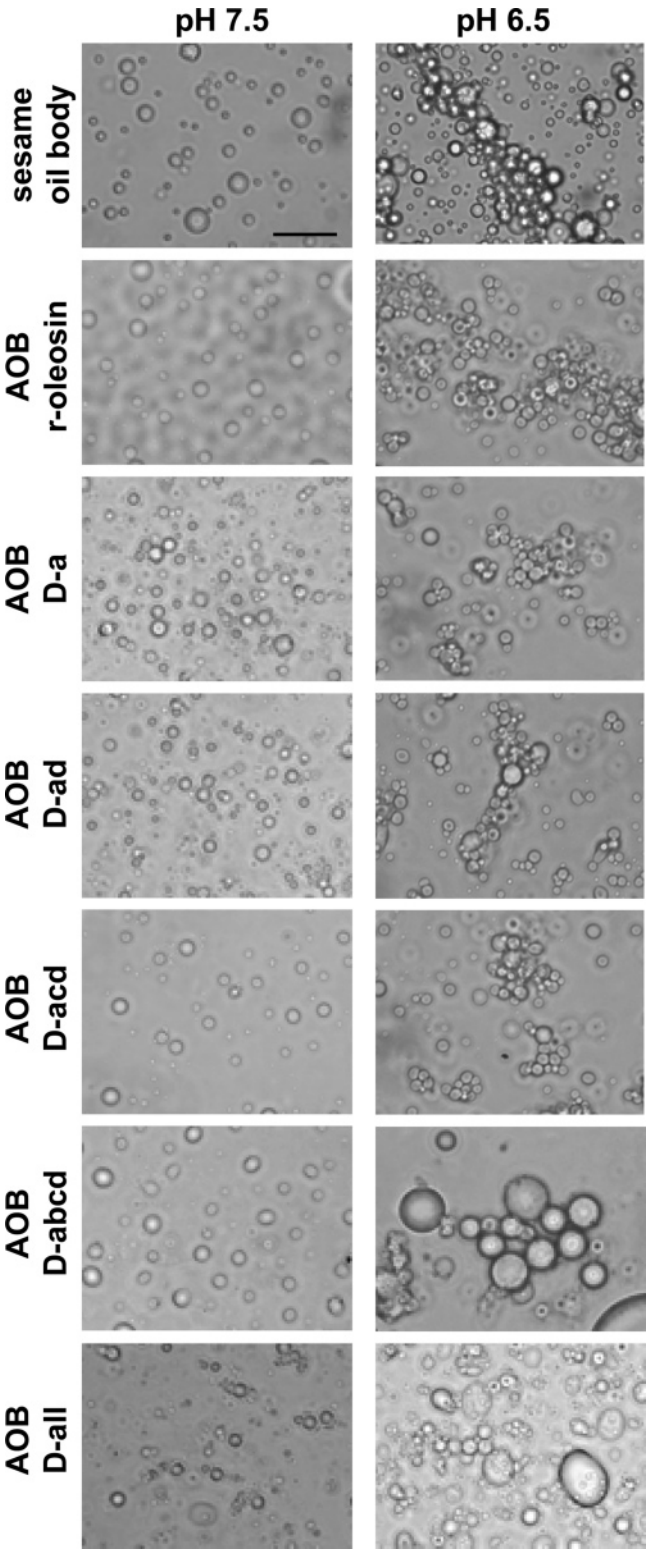

Figure 6. Light microscopy of native and artificial oil bodies at pH 7.5 and 6.5. Native sesame oil bodies and artificial oil bodies constituted with recombinant oleosins lacking various lengths of the central hydrophobic domain were kept at room temperature, pH 7.5 or 6.5, for 6 h before taking the photos. All photos are of the same magnification. Bar represents 10 μm.

application platforms or in other specialized applications deserves further investigation.

ABBREVIATIONS USED

IPTG, isopropyl β-D-thiogalactoside; PLs, phospholipids; TAGs, triacylglycerols.

ACKNOWLEDGMENT

We thank Professor Chih-Ning Sun for critical reading of the manuscript.

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Received for review April 3, 2007. Revised manuscript received May 4, 2007. Accepted May 6, 2007. The work was supported by a grant from the Council of Agriculture, Taiwan, ROC (96AS-1.2.1-AD-U1 to J.T.C.T.).

JF070977O