Stability of Artificial Oil Bodies Constituted with Recombinant Caleosins

ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · MARCH 2009

Impact Factor: 2.91 · DOI: 10.1021/jf803566w · Source: PubMed

4 AUTHORS:

Ting-Hang Liu
University of California, Davis
5 PUBLICATIONS **36** CITATIONS
SEE PROFILE

Chia-Lin Chyan
National Dong Hwa University
33 PUBLICATIONS **638** CITATIONS
SEE PROFILE

Feng-Yin Li
National Chung Hsing University
57 PUBLICATIONS **828** CITATIONS
SEE PROFILE

Jason Tzen
National Chung Hsing University
155 PUBLICATIONS **3,403** CITATIONS
SEE PROFILE

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Stability of Artificial Oil Bodies Constituted with Recombinant Caleosins

TING-HANG LIU,[†] CHIA-LIN CHYAN,[‡] FENG-YIN LI,[§] AND JASON T. C. TZEN*,[†,||]

Graduate Institute of Biotechnology, National Chung-Hsing University, Taichung, Taiwan, Department of Chemistry, National Dong Hwa University, Hualien, Taiwan, Department of Chemistry, National Chung-Hsing University, Taichung, Taiwan, and Agricultural Biotechnology Research Center, Academia Sinica, Taipei, Taiwan

Caleosin is a unique calcium binding protein anchoring to the surface of seed oil bodies by its central hydrophobic domain composed of an amphiphatic α-helix and a proline-knot subdomain. Stable artificial oil bodies were successfully constituted with recombinant caleosin overexpressed in *Escherichia coli*. The stability of artificial oil bodies was slightly or severely reduced when the amphiphatic α-helix or proline-knot subdomain in the hydrophobic domain of caleosin was truncated. Deletion of the entire central hydrophobic domain substantially increased the solubility of the recombinant caleosin, leading to a complete loss of its capability to stabilize these oil bodies. A recombinant protein engineered with the hydrophobic domain of caleosin replaced by that of oleosin, the abundant structural protein of seed oil bodies, could stabilize the artificial oil bodies, in terms of thermo- and structural stability, as effectively as caleosin or oleosin.

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

INTRODUCTION

Plant seeds use triacylglycerols (TAGs) stored in discrete intracellular organelles, i.e., oil bodies, as the fuel for germination and subsequent seedling growth (*1, 2*). Oil bodies, averaging 0.5 to 2 μm in diameter and remarkably stable both in cells and isolated preparations (*3*−*5*), are composed of a TAG matrix, which is surrounded by a monolayer of phospholipids (PLs) embedded with proteins (*6, 7*). These oil-body associated proteins comprise abundant structural proteins, oleosin isoforms, and at least two minor ones, caleosin and steroleosin (*8*−*10*); oleosin and caleosin, but not steroleosin, are considered to contribute to the structural stability of oil bodies via steric hindrance and electronegative repulsion (*11*).

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 (*7*). The central hydrophobic anchoring domain of oleosin composed of approximately 70 nonhydrophilic residues is highly conserved, particularly in its relatively hydrophilic proline-knot motif at the middle of the sequence. Similar to oleosin, a caleosin molecule is proposed to comprise three structural domains: an N-terminal hydrophilic calcium-binding domain, a central hydrophobic oil-body anchoring domain, and a C-terminal hydrophilic phosphorylation domain (*8*). The central hydrophobic domain composed of approximately 36 residues is divided into an amphipathic α-helix and a proline-knot subdomain. Caleosin has been proposed to regulate some biological functions related to the synthesis or degradation of oil bodies (*12*−*14*).

It is technically feasible to reconstitute stable artificial oil bodies using the three essential constituents of oil bodies of diverse seeds, i.e., neutral lipids (mainly TAGs), PLs, and oil-body proteins (*15, 16*). Stable artificial oil bodies were successfully constituted with TAGs, PLs, and oleosin either extracted from seed oil bodies or expressed in *Escherichia coli* (*17*). Moreover, stable artificial oil bodies were also successfully constituted with TAGs, PLs, and caleosin (*11, 18*). Several application platforms for artificial oil bodies have been developed, including a bacterial expression/purification system for producing recombinant proteins, an oral delivery system for hydrophobic drugs, 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 (*19*−*24*).

Recently, we showed that stable artificial oil bodies could be constituted using the truncated form of the abundant oleosin, with its central hydrophobic domain of a minimal length of 36 residues, namely, half of the original length (*25*). In this study, we aimed to evaluate the indispensability of the central hydrophobic domain in caleosin, one of the minor proteins, for stabilizing oil bodies by constituting artificial oil bodies with recombinant caleosins whose amphipathic α-helix and/or proline-knot subdomain were truncated. In addition, the stability

* Corresponding author. Tel: 886-4-22840328. Fax: 886-4-22853527. E-mail: TCTZEN@dragon.nchu.edu.tw.
[†] Graduate Institute of Biotechnology, National Chung-Hsing University.
[‡] National Dong Hwa University.
[§] Department of Chemistry, National Chung-Hsing University.
[||] Academia Sinica.

10.1021/jf803566w CCC: $40.75
Published on Web 02/13/2009

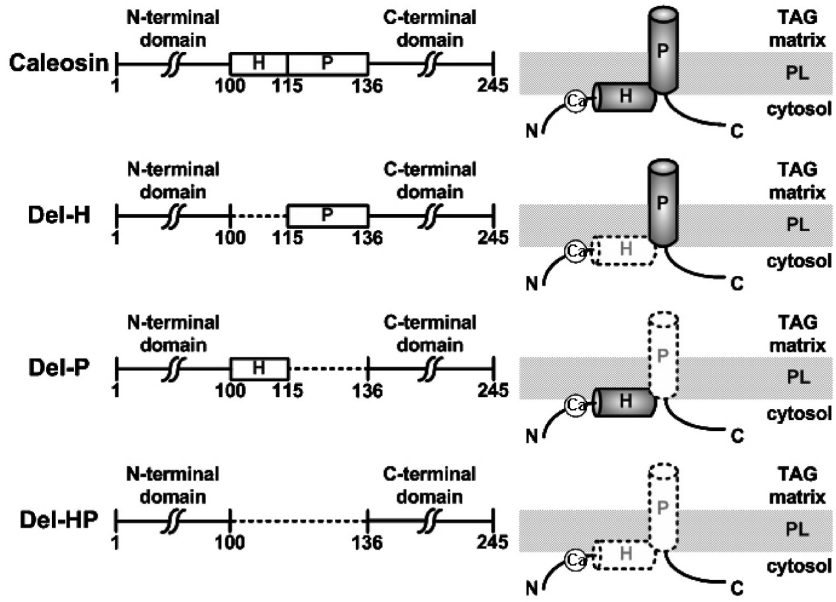

Figure 1. Schematic diagram of the recombinant caleosins with the central hydrophobic domain partly or completely truncated. Truncated caleosins lacking the amphipathic α -helix (Del-H), proline-knot subdomain (Del-P), and whole central anchoring domain (Del-HP) were obtained by PCR mutagenesis. Numbers under junction sites represent their relative positions counting from the N-terminus of the native sesame 27 kDa caleosin. A simplified structural model is shown on the right of each recombinant caleosin; a monolayer of PL, depicted by a shaded area, segregated the hydrophobic oil matrix of an oil body from the hydrophilic cytosol. The location of the calcium binding region is indicated in the N-terminal domain. Dashed lines represent truncated portions in the recombinant caleosins.

of artificial oil bodies constituted using a recombinant caleosin with its central hydrophobic domain replaced by that of oleosin was examined.

MATERIALS AND METHODS

Plasmid Construction for Recombinant Caleosins. The cDNA fragment encoding a sesame caleosin of 245 amino acid residues (accession number AF109921) was constructed in the nonfusion expression vector pET29a (Novagen), using an *Nde*I site at the initial methionine position and a *Xho*I site in the polylinker of the vector. The resulting plasmid pET29Cal was used to generate, by PCR-based mutagenesis, three constructs encoding truncated caleosins lacking the amphipathic α-helix (residues 101−115), the proline-knot subdomain (residues 116−136), and the entire central hydrophobic domain (residues 101−136) (**Figure 1**). For each construction, two 5′-phosphorylated divergent primers were designed to anneal to the desired mutation site: 5′-attgaaaccaatttggcgaagt-3′ and 5′-agttatcctactctcccgggtt-3′ for pETCal-Del-H (lacking the amphipathic α-helix); 5′-cagcgccacattaatgacgata-3′ and 5′-cacaaggccaaacatggaagcga-3′ for pETCal-Del-P (lacking the proline-knot subdomain); and 5′-attgaaaccaatttggcgaagt-3′ and 5′-cacaaggccaaacatggaagcga-3′ for pETCal-Del-HP (lacking the entire central hydrophobic domain).

To generate pETCal-Ole-Cal that encoded a recombinant caleosin with its hydrophobic domain replaced by the hydrophobic domain of oleosin, a cDNA fragment encoding the central hydrophobic region (residues 28−98) of a sesame oleosin (accession number AF091840) was obtained by PCR using 5′phosphorylated primers 5′-gcggccaccgccgtgacagccggc-3′ and 5′-aatccacgacagcacactcagcgccg-3′, and ligated with the PCR fragment of pETCal-Del-HP. To produce a recombinant fusion oleosin of 21 kDa as a positive control, the sesame 15 kDa oleosin clone was constructed in the fusion expression vector pET29a (Novagen), using an *Nco*I site in the polylinker of the vector (*24*). PCR amplification was carried out using ultra *pfu* (Stratagene) for 25 cycles at 94 °C, 1 min; 52 °C, 1 min; and 72 °C, 10 min. The blunt-ended linear PCR product was resolved in an agarose gel, purified by ethanol precipitation, and self-ligated to form a circular plasmid. These constructed plasmids were transformed and amplified in *E. coli* (DH10B) on a LB agar plate containing kanamycin of 50 mg/L, and the accuracy of plasmid construction was confirmed by direct sequencing.

Overexpression and SDS−PAGE Analysis of Recombinant Caleosins. Recombinant plasmids were transformed to *E. coli* BL21 (DE3), and the overexpression of recombinant caleosins was induced by adding 0.4 mM isopropyl β-D-thiogalactoside (IPTG) in a bacteriophage T7 RNA polymerase/promoter system. Three hours after induction, *E. coli* cells were harvested, lysed by sonication in 10 mM sodium phosphate buffer at pH 7.5. After centrifugation, the supernatant and the pellet were subjected to SDS−PAGE analyses. Protein samples 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 resolved by SDS−PAGE using 15% acrylamide, according to the Bio-Rad instruction manual. Following electrophoresis, the gel was stained with Coomassie blue R-250. The relative contents of recombinant caleosins in the supernatant and pellet of cell lysate were quantitated by using the ImageJ 1.41 program (http://rsb.info.nih.gov/ij/).

Reconstitution of Artificial Oil Bodies. Recombinant caleosins were eluted from SDS−PAGE gels and precipitated with equal amounts of acetone chilled at −20 °C. The acetone mixture was kept at −20 °C for 1 h and centrifuged at 10,000*g* for 30 min. The precipitate was suspended and precipitated three times in 1 mL 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 reconstitution of artificial oil bodies.

For reconstituting artificial oil bodies, TAG was obtained from sesame oil bodies by chemical extraction as reported previously (*17*), and dioleoyl phosphatidylcholine, the major PL found in oil bodies, was purchased from Sigma. Artificial oil bodies were constituted with 20 mg of TAG, 150 μg of PL, and 250 μg of each recombinant protein at a final volume of 1 mL in the sodium phosphate buffer. 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 the recombinant protein were incorporated, followed by sonication with a 3-mm-diameter probe in a Sonics & Materials VCX750 ultrasonic processor with 30% amplification for 20 s, and samples were then cooled in an ice bucket for 5 min. The sonication was repeated two more times to generate artificial oil bodies.

Turbidity Test for the Stability of Artificial Oil Bodies. Artificial oil bodies were suspended in 0.1 M sodium phosphate buffer, pH 7.5, and subjected to a stability test by measuring turbidity changes of a

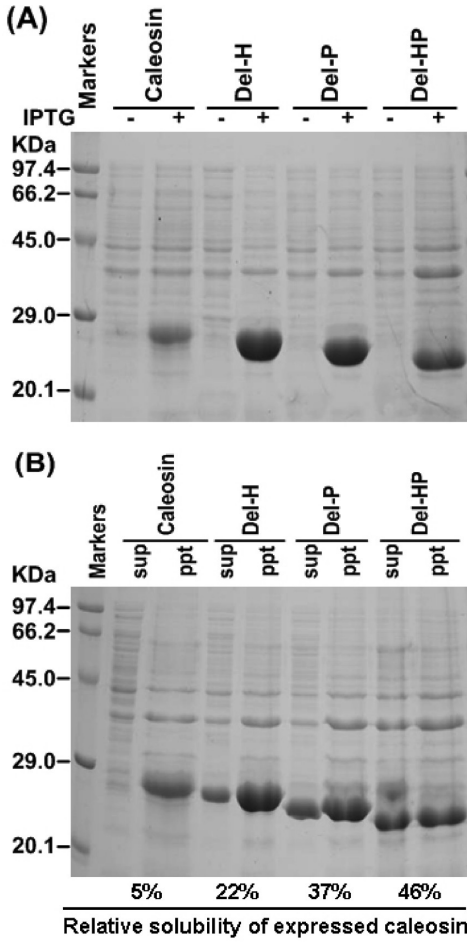

Figure 2. SDS−PAGE of recombinant caleosins overexpressed in *E. coli*. (**A**) Proteins lysed and extracted from *E. coli* cells with or without IPTG induction for overexpression of recombinant caleosins with the central hydrophobic domain partly or completely truncated were analyzed by SDS−PAGE. (**B**) Soluble (sup) and insoluble (ppt) proteins extracted from *E. coli* cells (20−30 μg total proteins) containing recombinant caleosins were analyzed by SDS−PAGE. Labels on the left indicate the molecular masses of marker proteins.

suspension of these oil bodies with time. The suspension was placed in a disposable 2-mL cuvette, which was covered with parafilm and 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 Jasco V-550 UV spectrophotometer. The turbidity (T) of the suspension was proportional to 10^A, and the relative turbidity was expressed as $T/T_o = 10^A/10^{A_o} = 10^A/10^{2.0}$, 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 0.1 M sodium phosphate buffer, pH 7.5, at 25, 50, 70, or 90 °C for 1 h, 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 (*7*). In this examination, artificial oil bodies were suspended in a medium of 0.1 M sodium phosphate buffer, pH 7.5 or 6.5, and left at 25 °C for 6 h before observation under a light microscope.

RESULTS

Production of Recombinant Caleosins with a Truncated Central Hydrophobic Domain. Recombinant caleosins with the intact central hydrophobic domain (molecular mass 27.6 kDa), deletion of the amphipathic α-helix (Del-H) (26.1 kDa), deletion of the proline-knot subdomain (Del-P) (25.1 kDa), and deletion of the whole central hydrophobic domain (Del-HP) (23.6 kDa) are shown in SDS−PAGE (**Figure 2A**). The solubility of the recombinant caleosin with the intact central hydrophobic domain was extremely low, but it increased to some

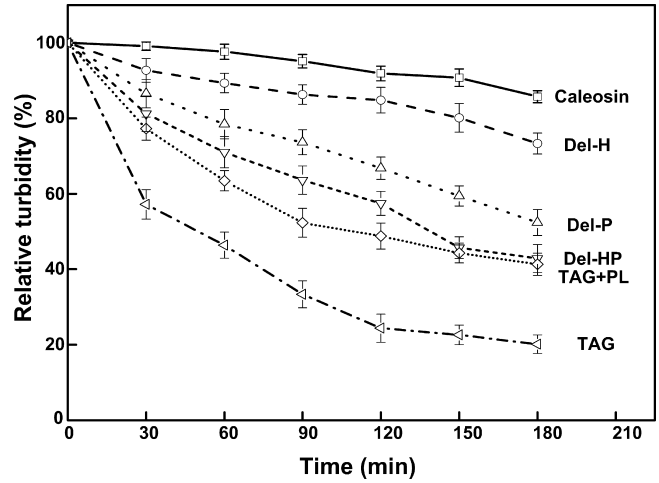

Figure 3. Turbidity tests for the stability of artificial oil bodies constituted with TAG, PL, and recombinant caleosins. Stability of artificial oil bodies constituted with TAG, PL, and recombinant caleosins with the central hydrophobic domain partly or completely truncated. A suspension (l mL) of artificial oil bodies was placed in a 2-mL cuvette and the relative turbidity (T/T_0) at 600 nm of the lower 0.5 mL of the suspension was measured at intervals. The data represent the mean ± SEM of three replicates.

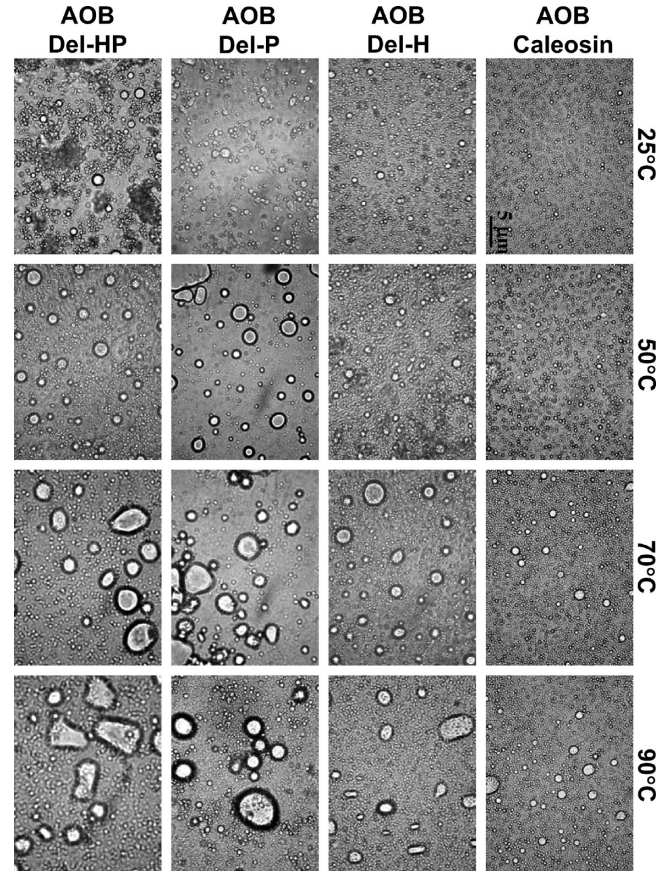

Figure 4. Light microscopy of artificial oil bodies with truncated caleosins under different temperatures. Artificial oil bodies constituted with recombinant caleosins with the central hydrophobic domain partly or completely truncated were left in 0.1 M sodium phosphate buffer, pH 7.5, at 25, 50, 70, and 90 °C for 1 h before taking the photos. All photos are of the same magnification. The bar represents 5 μm.

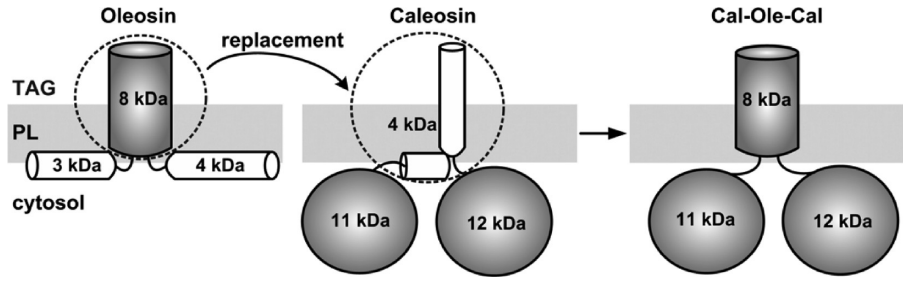

Figure 5. A cartoon picture showing the construction of the recombinant protein, Cal-Ole-Cal, from caleosin and oleosin. The recombinant Cal-Ole-Cal was constructed by replacing the 4 kDa central hydrophobic domain of caleosin with the 8 kDa central hydrophobic domain of oleosin.

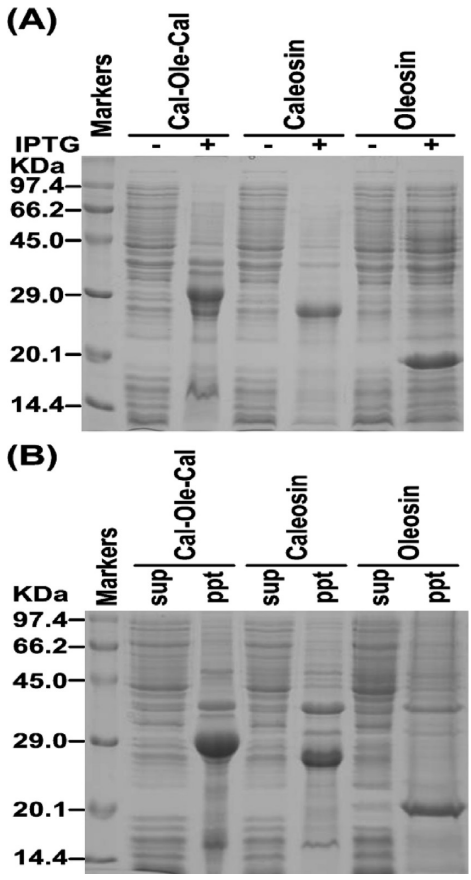

Figure 6. SDS−PAGE of recombinant Cal-Ole-Cal, caleosin, and oleosin overexpressed in *E. coli*. (**A**) Proteins lysed and extracted from *E. coli* cells with or without IPTG induction for overexpression of Cal-Ole-Cal, caleosin, and oleosin were analyzed by SDS−PAGE. (**B**) Soluble (sup) and insoluble (ppt) proteins extracted from *E. coli* cells (20−30 μg total proteins) containing Cal-Ole-Cal, caleosin, and oleosin were analyzed by SDS−PAGE. Labels on the left indicate the molecular masses of marker proteins.

extent when the amphipathic α-helix or the proline-knot subdomain was truncated (**Figure 2B**). The low solubility did not impair the capacity of recombinant caleosin in the following stabilization of artificial oil bodies. Deletion of the whole central hydrophobic domain substantially increased the solubility of the recombinant caleosin.

Stability of Artificial Oil Bodies Constituted with Truncated Caleosins. Results of a turbidity assay showed that the stability of artificial oil bodies was either slightly or severely reduced when they were constituted using recombinant caleosin with the amphipathic α-helix or proline-knot subdomain truncated, and a deletion of the entire central hydrophobic domain led to a complete loss of its capability to stabilize these oil bodies (**Figure 3**). Comparable thermostability was observed for

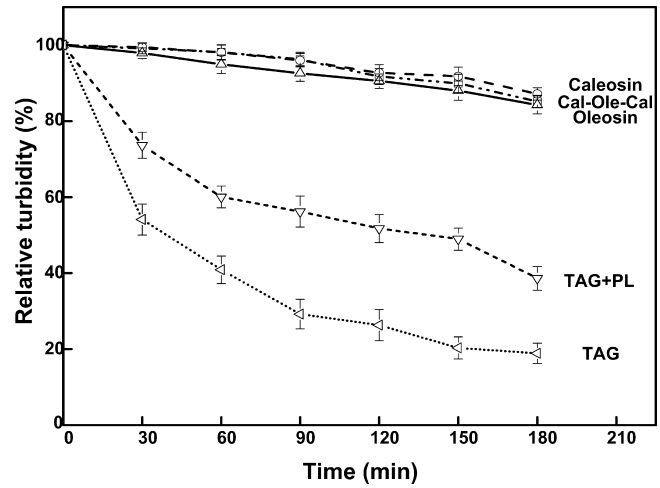

Figure 7. Turbidity tests for the stability of artificial oil bodies constituted with TAG, PL, and Cal-Ole-Cal, caleosin, or oleosin. Stability of artificial oil bodies constituted with TAG, PL, and Cal-Ole-Cal, caleosin, or oleosin. A suspension (l mL) of artificial oil bodies was placed in a 2-mL cuvette, and the relative turbidity (T/T_0) at 600 nm of the lower 0.5 mL of the suspension was measured at intervals. The data represent the mean ± SEM of three replicates.

artificial oil bodies stabilized by recombinant caleosins (**Figure 4**). Artificial oil bodies constituted by caleosin comprising the whole central hydrophobic domain remained stable up to 70 °C. When the amphiphatic α-helix in the central hydrophobic domain of recombinant caleosin was truncated, these oil-encapsulating particles stayed intact at temperatures lower than 50 °C, but started to decompose at temperatures higher than 70 °C. They were temporarily stable at room temperature but rapidly decomposed at higher temperatures when the proline-knot subdomain in the central hydrophobic domain of recombinant caleosin was truncated. No stable oil-encapsulating particles were observed when the whole central hydrophobic domain of recombinant caleosin was truncated.

Artificial Oil Bodies Constituted with the Recombinant Protein of Caleosin and Oleosin. To see if the central hydrophobic domain of caleosin could be replaced by that of oleosin for stabilizing artificial oil bodies, a recombinant protein Cal-Ole-Cal was engineered (**Figure 5**). As expected, the molecular mass of Cal-Ole-Cal (31 kDa) was higher than that of caleosin (27 kDa) or oleosin (21 kDa, 15 kDa sesame oleosin with an N-terminal fusion of 6 kDa) (**Figure 6A**). None of the recombinant Cal-Ole-Cal, caleosin and oleosin was soluble when overexpressed in *E. coli* (**Figure 6B**). A turbidity assay showed that artificial oil bodies constituted with Cal-Ole-Cal possessed stability equivalent to those constituted with recombinant caleosin or oleosin (**Figure 7**). As observed in a light microscope, artificial oil bodies constituted with Cal-Ole-Cal and those with recombinant caleosin or oleosin possessed similar ther-

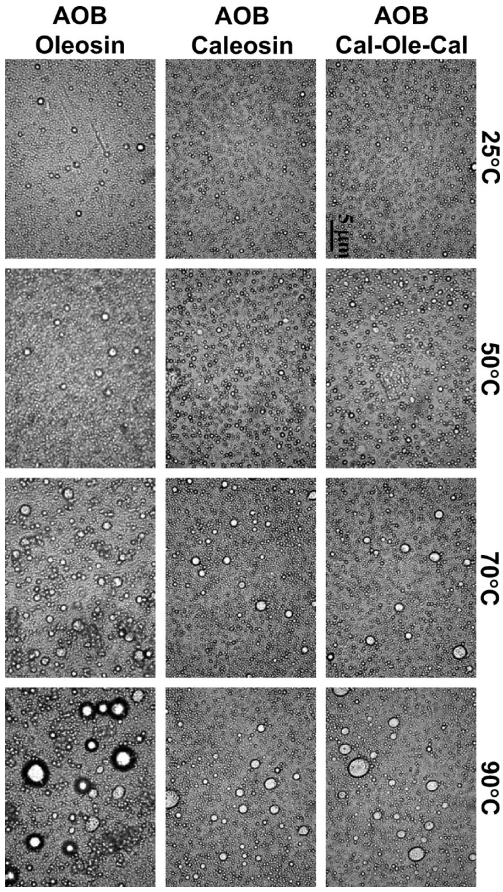

Figure 8. Light microscopy of artificial oil bodies with the recombinant protein of Cal-Ole-Cal, caleosin, or oleosin under different temperatures. Artificial oil bodies constituted with Cal-Ole-Cal, caleosin, or oleosin were left in 0.1 M sodium phosphate buffer, pH 7.5, at 25, 50, 70, and 90 °C for 1 h before taking the photos. All photos are of the same magnification. The bar represents 5 μm.

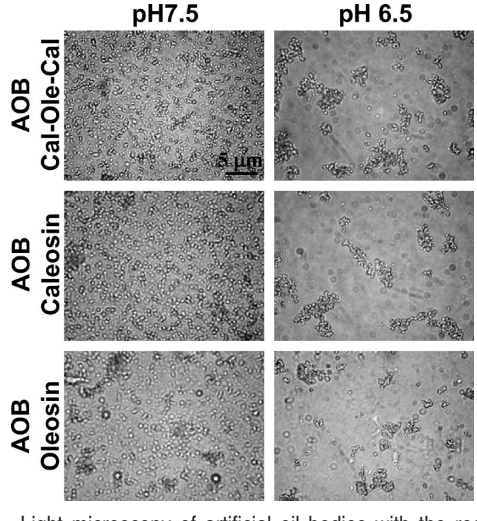

Figure 9. Light microscopy of artificial oil bodies with the recombinant protein of Cal-Ole-Cal, caleosin, or oleosin at pH 7.5 and 6.5. Artificial oil bodies constituted with Cal-Ole-Cal, caleosin, or oleosin were kept at 25 °C, pH 7.5, or 6.5 for 6 h before taking the photos. All photos are of the same magnification. The bar represents 5 μm.

mostability: they all remained stable up to 70 °C (**Figure 8**). The stability of these artificial oil bodies constituted with Cal-Ole-Cal, caleosin, or oleosin was presumably due to steric hindrance and electronegative repulsion since they maintained as individual discrete particles at pH 7.5, but aggregated without coalescence at pH 6.5 (**Figure 9**).

DISCUSSION

In this study, we observed that both structural stability and thermostability of artificial oil bodies were slightly or severely reduced when the amphiphatic α-helix or proline-knot subdomain of recombinant caleosin was truncated. These results are in agreement with the observations in an in vitro targeting analysis showing that the crucial structural portion required for caleosin targeting to artificial oil emulsion was the central hydrophobic domain, particularly the subdomain including the proline-knot motif (*26*). On the basis of these results, we speculate that the proline-knot subdomain may play a more important role than the amphiphatic α-helix in terms of anchoring caleosin to the surface of oil bodies. However, the proline-knot subdomain of 21 residues alone, i.e., without the amphiphatic α-helix of 15 residues, could not stabilize artificial oil bodies as effectively as the whole central hydrophobic domain of 36 residues in recombinant caleosins. Of course, it could not be ruled out that the different reduction of stabilization capacity in truncated caleosins was simply correlated to the length of the hydrophobic segment regardless of their secondary structures.

Among the three distinct protein classes identified so far from seed oil bodies, oleosin and caleosin, but not steroleosin, are able to stabilize artificial oil bodies (*11*). Both oleosin and caleosin are proposed to anchor to oil bodies by their central hydrophobic domains of 72 and 36 residues, respectively (*8*). In our previous study, we observed that stable artificial oil bodies could be constituted with truncated oleosins whose central hydrophobic domain had a minimal length of 36 residues including the proline-knot motif (*25*). In this study, we found that neither the amphiphatic α-helix nor proline-knot subdomain in the central hydrophobic domain of caleosin could be deleted without losing its capability to stabilize artificial oil bodies. In light of the results observed in both studies, we suggest that the minimal length of the hydrophobic domain in an oil-body anchoring protein is approximately 36 residues.

Artificial oil bodies with unique properties have recently been developed and utilized in several application platforms (*19*). The success of stabilizing artificial oil bodies with a recombinant protein, Cal-Ole-Cal, by replacing the central hydrophobic domain of caleosin with that of oleosin raised the possibility of stabilizing artificial oil bodies with another recombinant protein, Ole-Cal-Ole, by replacing the central hydrophobic domain of oleosin with that of caleosin. Moreover, strategies of engineering novel recombinant oil-body proteins with diverse properties as well as the capability to stabilize artificial oil bodies are also encouraged. Unfortunately, many constructs encoding nonfusion and fusion recombinant proteins containing Ole-Cal-Ole were not expressed, or their gene products were not accumulated in *E. coli* under our experimental conditions. It is unclear if any restrictions in protein structure or folding are present in the recombinant Ole-Cal-Ole. In our follow-up research, we aim to produce Ole-Cal-Ole and other recombinant proteins that are capable of stabilizing artificial oil bodies. Whether artificial oil bodies stabilized by Cal-Ole-Cal or other newly designed recombinant proteins, such as Ole-Cal-Ole, can be particularly suitable for some application platforms deserves further evaluation.

ABBREVIATIONS USED

IPTG, isopropyl β-D-thiogalactoside; PL(s), phospholipid(s); TAG(s), triacylglycerol(s).

ACKNOWLEDGMENT

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

LITERATURE CITED

(1) Huang, A. H. C. Oleosin and oil bodies in seeds and other organs. *Plant Physiol.* **1996**, *110*, 1055–1061.
(2) Napier, J. A.; Stobart, A. K.; Shewry, P. R. The structure and biogenesis of plant oil bodies: the role of the ER membrane and the oleosin class of proteins. *Plant Mol. Biol.* **1996**, *31*, 945–956.
(3) Slack, C. R.; Bertaud, W. S.; Shaw, B. P.; Holland, R.; Browse, J.; Wright, H. Some studies on the composition and surface properties of oil bodies from the seed cotyledons of safflower and linseed. *Biochem. J.* **1980**, *190*, 551–561.
(4) Frandsen, G. I.; Mundy, J.; Tzen, J. T. C. Oil bodies and their associated proteins, oleosin and caleosin. *Physiol. Plant* **2001**, *112*, 301–307.
(5) Murphy, D. J. The biogenesis and functions of lipid bodies in animals, plants and microorganisms. *Prog. Lipid Res.* **2001**, *40*, 325–438.
(6) Tzen, J. T. C.; Huang, A. H. C. Surface structure and properties of plant seed oil bodies. *J. Cell Biol.* **1992**, *117*, 327–335.
(7) Tzen, J. T. C.; Lie, G. C.; Huang, A. H. C. Characterization of the charged components and their topology on the surface of plant seed oil bodies. *J. Biol. Chem.* **1992**, *267*, 15626–15634.
(8) Chen, J. C. F.; Tsai, C. C. Y.; Tzen, J. T. C. Cloning and secondary structure analysis of caleosin, a unique calciumbinding protein in oil bodies of plant seeds. *Plant Cell Physiol.* **1999**, *40*, 1079–1086.
(9) Lin, L. J.; Tai, S. S. K.; Peng, C. C.; Tzen, J. T. C. Steroleosin, a sterol-binding dehydrogenase in seed oil bodies. *Plant Physiol.* **2002**, *128*, 1200–1211.
(10) Lin, L. J.; Tzen, J. T. C. Two distinct steroleosins are present in seed oil bodies. *Plant Physiol. Biochem.* **2004**, *42*, 601–608.
(11) Chen, M. C. M.; Chyan, C. L.; Lee, T. T. T.; Hung, S. H.; Tzen, J. T. C. Constitution of stable artificial oil bodies with triacylglycerol, phospholipid, and caleosin. *J. Agric. Food Chem.* **2004**, *52*, 3982–3987.
(12) Hanano, A.; Burcklen, M.; Flenet, M.; Ivancich, A.; Louwagie, M.; Garin, J.; Blée, E. Plant seed peroxygenase is an original heme-oxygenase with an EF-hand calcium binding motif. *J. Biol. Chem.* **2006**, *281*, 33140–33151.
(13) Poxleitner, M.; Rogers, S. W.; Samuels, A. L.; Browse, J.; Rogers, J. C. A role for caleosin in degradation of oil-body storage lipid during seed germination. *Plant J.* **2006**, *47*, 917–933.
(14) Purkrtova, Z.; d'Andrea, S.; Jolivet, P.; Lipovova, P.; Kralova, B.; Kodicek, M.; Chardot, T. Structural properties of caleosin: a MS and CD study. *Arch. Biochem. Biophys.* **2007**, *464*, 335–343.
(15) Tzen, J. T. C.; Cao, Y. Z.; Laurent, P.; Ratnayake, C.; Huang, A. H. C. Lipids, proteins, and structure of seed oil bodies from diverse species. *Plant Physiol.* **1993**, *101*, 267–276.
(16) Tai, S. S. K.; Chen, M. C. M.; Peng, C. C.; Tzen, J. T. C. Gene family of oleosin isoforms in sesame seed oil bodies and their structural stabilization to reconstituted oil bodies. *Biosci. Biotechnol. Biochem.* **2002**, *66*, 2146–2153.
(17) Peng, C. C.; Lin, I. P.; Lin, C. K.; Tzen, J. C. T. Size and stability of reconstituted sesame oil bodies. *Biotechnol. Prog.* **2003**, *19*, 1623–1626.
(18) Purkrtova, Z.; Le Bon, C.; Kralova, B.; Ropers, M. H.; Anton, M.; Chardot, T. Caleosin of *Arabidopsis thaliana*: effect of calcium on functional and structural properties. *J. Agric. Food Chem.* **2008**, *56*, 11217–11224.
(19) Roberts, N. J.; Scott, R. W.; Tzen, J. C. T. Recent biotechnological applications using oleosins. *Open Biotech. J.* **2008**, *2*, 13–21.
(20) Peng, C. C.; Shyu, D. J.; Chou, W. M.; Chen, M. J.; Tzen, J. T. C. Method for bacterial expression and purification of sesame cystatin via artificial oil bodies. *J. Agric. Food Chem.* **2004**, *52*, 3115–3119.
(21) Peng, C. C.; Chen, J. C. F.; Shyu, D. J. H.; Chen, M. J.; Tzen, J. T. C. A system for purification of recombinant proteins in *Escherichia coli* via artificial oil bodies constituted with their oleosin-fused polypeptides. *J. Biotechnol.* **2004**, *111*, 51–57.
(22) Chiang, C. J.; Chen, H. C.; Chao, Y. P.; Tzen, J. T. C. Efficient system of artificial oil bodies for functional expression and purification of recombinant nattokinase in Escherichia coli. *J. Agric. Food Chem.* **2005**, *53*, 4799–4804.
(23) Chen, M. C.; Wang, J. L.; Tzen, J. T. C. Elevating bioavailability of cyclosporine a via encapsulation in artificial oil bodies stabilized by caleosin. *Biotechnol. Prog.* **2005**, *21*, 1297–1301.
(24) Chiang, C. J.; Chen, H. C.; Chao, Y. P.; Tzen, J. T. C. One-step purification of insoluble hydantoinase overproduced in *Escherichia coli*. *Protein Expression Purif.* **2007**, *52*, 14–18.
(25) Peng, C. C.; Lee, V. S. Y.; Lin, M. Y.; Huang, H. Y.; Tzen, J. T. C. Minimizing the central hydrophobic domain in oleosin for the constitution of artificial oil bodies. *J. Agric. Food Chem.* **2007**, *55*, 5604–5610.
(26) Chen, J. C. F.; Tzen, J. T. C. An in vitro system to examine the effective phospholipids and structural domain for protein targeting to seed oil bodies. *Plant Cell Physiol.* **2001**, *42*, 1245–1252.

Received for review November 14, 2008. Revised manuscript received January 15, 2009. Accepted January 19, 2009. This work was supported by a grant from the Council of Agriculture, Taiwan, ROC (97AS-1.2.1-AD-U1 to J.T.C.T.).

JF803566W