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Review Article

Integral Proteins in Plant Oil Bodies

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Hydrophobic storage neutral lipids are stably preserved in specialized organelles termed oil bodies in the aqueous cytosolic compartment of plant cells via encapsulation with surfactant molecules including phospholipids and integral proteins. To date, three classes of integral proteins, termed oleosin, caleosin, and steroleosin, have been identified in oil bodies of angiosperm seeds. Proposed structures, targeting traffic routes, and biological functions of these three integral oil-body proteins were summarized and discussed. In the viewpoint of evolution, isoforms of oleosin and caleosin are found in oil bodies of pollens as well as those of more primitive species; moreover, caleosin- and steroleosin-like proteins are also present in other subcellular locations besides oil bodies. Technically, artificial oil bodies of structural stability similar to native ones were successfully constituted and seemed to serve as a useful tool for both basic research studies and biotechnological applications.

1. Introduction

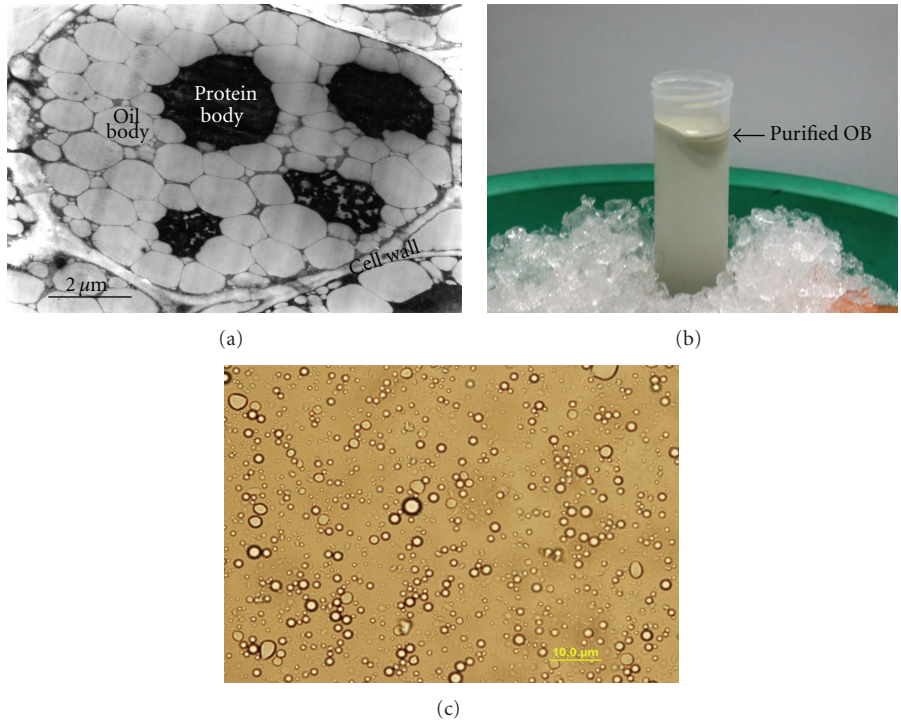
The stored energy in plant tissues is occasionally preserved in the form of proteins, yet much more commonly in the form of carbohydrates or lipids. Plant cells deposit storage resources of carbohydrates, proteins, and neutral lipids in subcellular particles termed starch granules, protein bodies, and oil bodies, respectively. In contrast with the active studies of protein bodies and starch granules [1–6], research progress on oil bodies is relatively late and slow presumably due to less research input and inevitable technical problems caused by the hydrophobic features of these lipid-storage organelles.

Oil bodies are intracellular organelles for storing neutral lipids, mainly triacylglycerols and sterol esters, and they are also referred to as lipid bodies, lipid droplets, oil globules, oleosomes, and spherosomes. These organelles have been found across a wide range of plant cells, from microalgae to the most complex angiosperms; among them, oil bodies obtained from seed cells have been studied most intensively [7]. According to the cumulative research outcome in the past three decades, it is generally assumed that an oil body is composed of a neutral lipid matrix surrounded by a monolayer of phospholipids embedded with some unique integral proteins [8–10]. This paper focused on the integral proteins of oil bodies in terms of their proposed structures, organelle targeting, biological functions, homologous isoforms, and utilization of artificial oil bodies.

2. Identification of Integral Proteins in Oil Bodies of Angiosperm Seeds

Constituents of oil bodies in angiosperm species, particularly those in oily seeds, have been continually investigated in the past three decades [11–13]. Research approaches by using tools of molecular biology and protein chemistry were relatively active in this research area in the past two decades.

2.1. Structural Components of Seed Oil Bodies. Vegetable cooking oils commonly extracted from various oily seeds are triacylglycerol molecules that tend to segregate from aqueous solution and form a transparent layer on the top. These hydrophobic triacylglycerol molecules are originally assembled in specialized organelles termed oil bodies, and these lipid storage organelles are stably packed in aqueous environments, that is, the cytosolic compartment of seed cells, with sizes mostly ranging from 0.5 to 2 μm (Figure 1(a)) [14, 15]. Intact oil bodies isolated from oily seeds, such as sesame, form a milky layer on top of the solution after centrifugation (Figure 1(b)), and they look drastically different from the transparent vegetable cooking oils that



(a) (b)

(c)

Figure 1: Visualizations of oil bodies. (a) Electron microscopy of a seed cell of mature sesame (adopted and modified from Figure 5 of Peng and Tzen, [14]). The most abundant gray spherical particles are oil bodies. (b) A photo of isolated sesame oil bodies. Purified oil bodies floated on the top and formed a milky layer after centrifugation. (c) Light microscopy of isolated sesame oil bodies. Isolated oil bodies were kept in 0.1 M sodium phosphate buffer, pH 7.5 for 30 min before taking the photo.

are generally extracted from seed oil bodies under relatively stringent conditions (high temperature or organic solvent). The isolated oil bodies remained maintaining their structural integrity and stability when they were suspended in an aqueous solution as observed under a light microscope (Figure 1(c)). Evidently, oil bodies are remarkably stable both in vivo and in vitro as compressed oil bodies in cells of a mature seed or in the milky layer during isolation never coalesce or aggregate. The remarkable stability of oil bodies in aqueous environments implies that surfactant molecules are present on the surface of oil bodies [16–19]. Chemical analyses suggested that phospholipids and proteins might be minor constituents (one to a few percent by weight) of oil bodies and served as surfactants to encapsulate abundant hydrophobic neutral lipids into many relatively small hydrophilic particles.

2.2. Identification of a Major Integral Protein in Seed Oil Bodies. According to the chemical detection of phospholipids in oil bodies as well as the observation of one single boundary line on the surface of oil bodies under an electron microscope, it is generally accepted that the matrix neutral lipids of seed oil bodies are encapsulated by a monolayer of phospholipids [20–24]. In contrast, the presence of unique integral proteins, rather than nonspecifically associated contaminants of purification, in seed oil bodies was not confirmed until the striking discovery of oleosin, a major surfactant protein in seed oil bodies of maize (*Zea mays* L.) [25]. The amino acid sequences of two maize oleosins deduced from their cDNA clones show a conservative central hydrophobic domain of approximately 70 residues that is the longest hydrophobic segment found in natural proteins so far, and apparently responsible for the anchorage of the proteins on the surface of oil bodies [26]. Oleosin was named in 1990 taking its meaning of an oil (oleo-) protein (-sin). Right after the kick-off studies on maize oleosins, homologous oleosin isoforms were subsequently identified in oil bodies of rapeseed (*Brassica napus*), soybean (*Glycine max* L.), carrot (*Daucus carota*), sunflower (*Helianthus annuus*), *Arabidopsis thaliana*, and cotton (*Gossypium hirsutum*) with their corresponding cDNA fragments cloned [27–34]. It seems that oleosin isoforms are universally present in oil bodies of angiosperm seeds including both monocotyledonous and dicotyledonous species [35–39]. Furthermore, oleosin isoforms found as the major proteins (approximately 80–90%) in seed oil bodies were demonstrated to shield the whole surface of the organelles in the company of phospholipids [40]. The structural integrity and stability of seed oil bodies were assumed to be provided by abundant oleosins via two factors, steric hindrance and electronegative repulsion [41].

2.3. Identification of Two Minor Integral Proteins in Seed Oil Bodies. After the identification of the major protein, oleosin in seed oil bodies, whether minor integral proteins could also

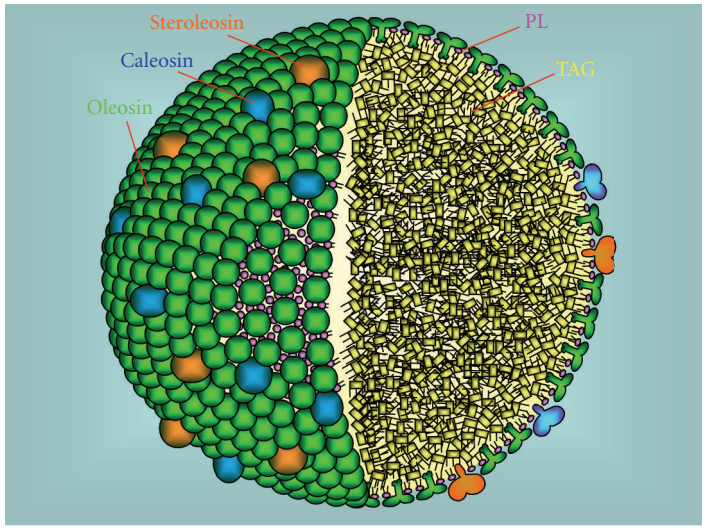


Figure 2: A structural model of an oil body with three types of integral oil-body proteins. An oil body is proposed to contain a triacylglycerol (TAG) matrix surrounded by a monolayer of phospholipids (PL) embedded with three classes of proteins, oleosin, caleosin, and steroleosin.

be found in these organelles became the next challenge. The approach to this challenge was severely impeded since many non-specifically associated proteins were contaminated in the preparation of oil bodies. The impediment was overcome by the development of a purification protocol that removed almost all the contaminated proteins on the surface of isolated oil bodies by washing harshly with detergent, high salt, chaotropic agent, and hexane [42]. As exemplified by seed oil bodies of sesame (*Sesamum indicum* L.), besides abundant oleosin isoforms, three minor protein bands of relatively high molecular masses were found after the harsh washing [43]. These three minor proteins were later confirmed as two classes of integral oil-body proteins named caleosin and steroleosin [44, 45]. Caleosin was named in 1999 taking its meaning of a calcium-binding (cal-) oil protein (-leosin) [46], and steroleosin was named in 2002 as a sterol-regulatory (sterol-) oil protein (-leosin) [47]. Taken together, a structural model of seed oil body was depicted in Figure 2.

2.4. Identification of Other Potential Oil-Body Proteins. Searching for more oil-body proteins has been approached by subproteomic analysis under the assistance of liquid chromatography electrospray ionization tandem mass spectrometry in the past few years [48–54]. In addition to the three known integral oil-body proteins, oleosin, caleosin, and steroleosin, several proteins were detected as potential oil-body proteins in seed oil bodies of maize and rapeseed. However, none of these newly identified proteins have been confirmed as integral oil-body proteins or peripheral proteins associated with some surface components of oil bodies for particular physiological functions. It remains to be clarified if proteins other than oleosin, caleosin, and steroleosin are embedded or peripherally associated on the surface of seed oil bodies.

3. Proposed Structures of Integral Oil-Body Proteins

Due to the insolubility of oleosin, caleosin, and steroleosin possibly caused by their hydrophobic oil-body anchoring domains, no three-dimensional structures derived from X-ray or NMR are available at the present time. Proposed structures of these three oil-body proteins are predicted based on their sequence analyses and spectrometric determination.

3.1. Proposed Oleosin Structure. 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 (Figure 3(a)) [55]. The N-terminus of oleosin is blocked with acetylation after the removal of the first methionine, a cotranslational modification presumably related to the enhancement of protein structural stability to fulfill the long-term storage of oil bodies within seed cells [56]. Both N- and C-terminal domains are not conserved among oleosins of diverse species, and even their lengths are quite variable in different oleosin isoforms. It is generally agreed that these two domains are putatively resided on the surface of oil bodies and stabilize the organelles via steric hindrance and electronegative repulsion [57–59]. In contrast, the central anchoring domain of oleosins is highly conserved among diverse species, particularly in a relatively hydrophilic proline knot motif at the middle of the sequence [60–63]. To date, controversial secondary structures of this domain have been proposed or determined in the past two decades, yet the contents of α-helical and β-stranded structures in these proposed models are extremely different [41, 64–72]. The controversy among these proposed structures does not seem to be receded unless a convincing three-dimensional structure of oleosin or at least its central hydrophobic domain is resolved.

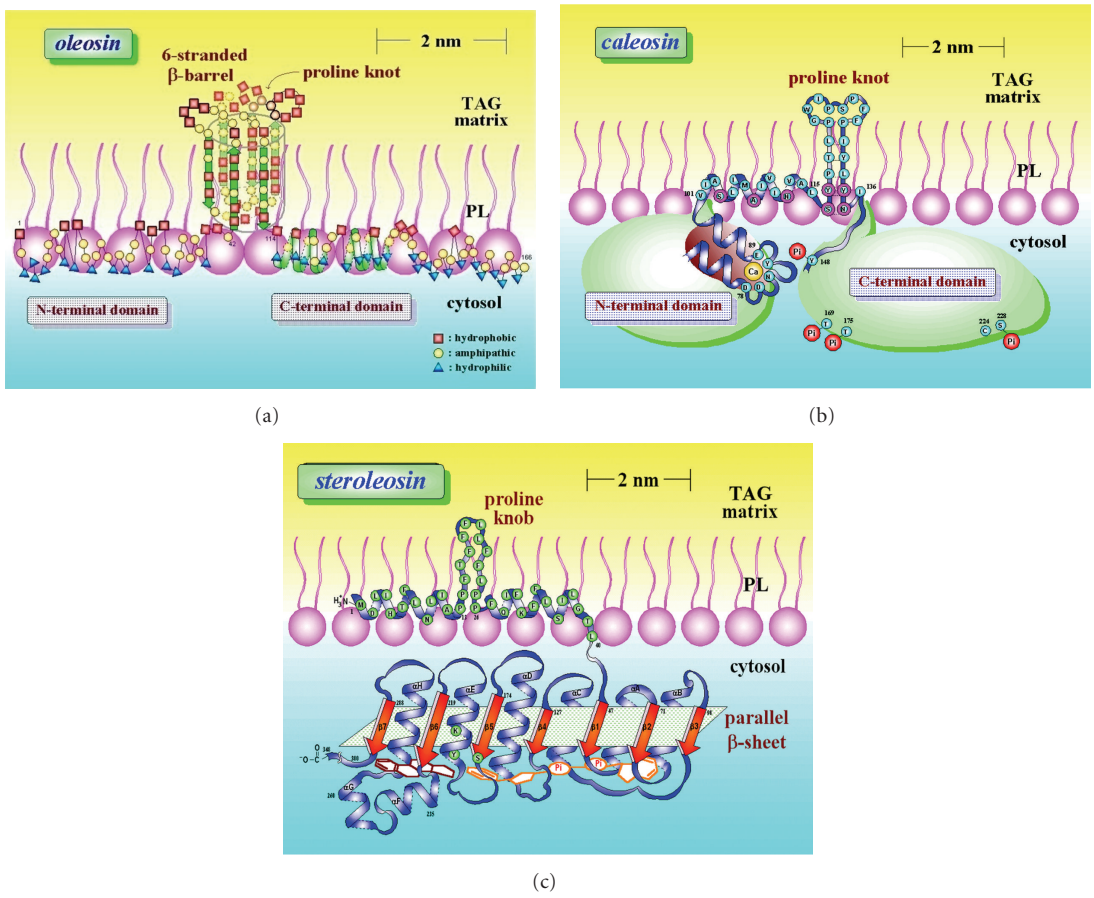


FIGURE 3: Secondary structure organizations of integral oil-body proteins on the surface of an oil body. Proposed secondary structures of oleosin (a), caleosin (b), and steroleosin (c) are adopted and modified from Figure 5 of Tzen et al., [55], Figure 7 of Chen et al., [46], and Figure 6 of Lin et al., [47], respectively.

3.2. Proposed Caleosin Structure. A caleosin molecule is also 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 (Figure 3(b)) [46]. The N-terminus of caleosin is also blocked with acetylation after the removal of the first methionine, presumably modified by the same co-translational mechanism found in oleosin [56]. The N-terminal hydrophilic domain consists of an EF hand calcium-binding motif of 28 residues including an invariable glycine residue as a structural turning point and five conserved oxygen-containing residues as calcium-binding ligands [23, 46]. Owing to the presence of this calcium-binding domain, calcium ion affected electrophoretic mobility of native and recombinant caleosin on SDS-PAGE [46, 73, 74]. The calcium-binding capacity of caleosin is in agreement with the observation of calcium staining on the surface of oil bodies in electron microscopy prior to the discovery of caleosin in these lipid storage organelles [75]. The central hydrophobic domain of caleosin is relatively short in comparison with that of oleosin, and comprises an amphipathic α-helix and an anchoring region. The amphipathic α-helix is assumed to be located in the interface between hydrophobic and hydrophilic environments while the anchoring region is predicted to comprise a pair of antiparallel β-strands connected with a proline knot motif. The similarity of proline knot motifs of oleosin and caleosin seems to imply a significant role associated with this unique motif, such as protein folding, intermolecular assembly, or specific targeting to oil bodies [76–78]. The C-terminal hydrophilic domain of caleosin contains several potential phosphorylation sites. The native caleosin in seed oil bodies of *Arabidopsis thaliana*, but not bacterially expressed caleosin, has been found partially phosphrylated [79]. An invariable cysteine residue is present near the C-terminus of caleosin and unlikely involved in any interdisulfide linkage with another caleosin molecule or other proteins on the surface of oil bodies.

3.3. Proposed Steroleosin Structure. A steroleosin molecule is proposed to comprise a relatively small N-terminal oil-body anchoring domain and a relatively large soluble sterol-binding dehydrogenase domain (Figure 3(c)) [47]. Free N-terminus occurs in steroleosin with the translation-initiating methionine as the first residue, in contrast with the acetylation-blocked N-termini of oleosin and caleosin [56]. So far, less research investigation has been executed on the structure of steroleosin in comparison with that

of oleosin or caleosin. The N-terminal anchoring segment comprises two amphipathic α-helices (12 residues in each helix) connected by a hydrophobic sequence of 14 residues bordered by 1-2 proline residues at each end. The relatively hydrophilic proline residues located in both ends of the 14-residue hydrophobic sequence are proposed to aggregate in the hydrophobic surroundings and form a unique structure, termed proline knob motif, for the integrity and stability of steroleosin anchorage on the surface of oil bodies. The soluble sterol-binding dehydrogenase domain contains an NADPH-binding subdomain, an active site region, and a sterol-binding subdomain. Three-dimensional structure of the dehydrogenase domain has been simulated by homology modeling [47]. The modeling structure reveals that the NADPH-binding region, active site, and sterol-binding region are located in the C-terminal ends of a parallel β-sheet and that the NADPH-binding region is expectably located in the crevice region, termed topological switch point, as observed in all similar α/β structures [80].

4. Possible Biological Functions of Integral Oil-Body Proteins

Except for the structural role, it is reasonable that oil-body proteins may exert some biological functions related to the synthesis or degradation of oil bodies, for example, signaling for the formation, assembly, fusion, or mobilization of these lipid storage organelles. Indeed, some physiological functions have been proposed for oil-body proteins in the past decade [81]. Further verification of these proposed physiological functions is anticipated in the come-up research progress.

4.1. Proposed Functions of Oleosin. Oleosin has been proven to play a key role in the stability of seed oil bodies via electronegative repulsion and steric hindrance [41]. This structural role prevents coalescence of oil bodies during seed desiccation and maintains them as discrete and relatively small organelles. It is demonstrated that the structural role of oleosin also protects *Arabidopsis thaliana* seeds against freeze/thaw-induced damage of their cells in vivo [82]. The contents of oleosins are found to determine sizes of seed oil bodies; presumably the ratio of oleosin over triacylglycerol is inversely proportional to the sizes of oil bodies [83–88]. The stability of isolated oil bodies could be substantially enhanced after their surface proteins were cross-linked by linker molecules, such as glutaraldehyde or genipin [87]. Being structural proteins, oleosin isoforms as well as caleosin ones are partially degraded by a special thiol-protease, thioredoxin h, after germination, and this specific degradation of oil-body structural proteins is proposed to be associated with mobilization of oil bodies in seedlings [89–92].

Furthermore, oleosin is suggested to be a bifunctional enzyme that has both monoacylglycerol acyltransferase and phospholipase activities during seed germination [93]. The regulation of these distinct dual activities seems to be controlled by the phosphorylation of oleosin presumably by a serine/threonine/tyrosine protein kinase, and the oleosin phosphorylation is also found to be activated by phosphatidylcholine and diacylglycerol, but inhibited by lysophosphatidylcholine, oleic acid, and calcium ion [94]. It will be interesting to see if these two enzymatic activities are universally detectable in oleosin isoforms of diverse species since both N- and C-terminal domains are not conserved among these oleosin isoforms. Meanwhile, most oleosins are relative small proteins of 15–20 kDa, particularly their N- and C-terminal domains are quite tiny (3 to 5 kDa for each domain; Figure 3(a)). In terms of structure-function relationship, it is a challenging task to reveal how the small structural domain(s) of oleosins construct the three dimensional active sites for the two observed enzymatic activities.

4.2. Proposed Functions of Caleosin. Having a structural organization and oil-body anchorage similar to oleosin, caleosin has been demonstrated to stabilize seed oil bodies as efficiently as oleosin [76]. The structural role of caleosin is clearly verified by the observation of stable cycad (*Cycas revoluta*) seed oil bodies that are mainly sheltered by caleosin without the presence of any oleosin isoform [95]. The investigation also invalidates a prevalent concept in this research area for two decades, declaring that oleosin is an essential constituent and can be regarded as a marker protein of plant oil bodies.

Caleosin comprises a calcium-binding motif and several potential phosphorylation sites, that is, well-known candidates involved in signal transduction, and thus may possess biological function(s) in addition to its structural role for the stability of oil bodies. According to the characterization of two independent insertion mutants lacking caleosin, it was proposed that caleosin might play a role in the degradation of storage lipids in oil bodies by inducing the interaction of oil bodies with vacuoles during germination [96]. Putative interaction between oil bodies and vacuoles was also observed in pollen cells after germination under electron microscopy; and the pollen oil bodies were presumably surrounded by tubular membrane structures and encapsulated in the vacuoles after germination [97–99]. The detailed molecular interaction between caleosin on the surface of oil bodies and its specific partner protein on the membrane of vacuoles remains to be studied.

Caleosin isoforms or caleosin-like proteins are not only localized in oil bodies but also found as membrane-bound proteins in other subcellular fractions, such as microsomal membrane; moreover, they were demonstrated to possess different biological functions, such as peroxygenase activity in biotic and abiotic stress responses in their phosphorylated forms [100–102]. Site-directed mutagenesis studies revealed that the peroxygenase catalytic activity of caleosin, an original heme-oxygenase, was dependent on two highly conserved histidines [100]. It was proposed that caleosin-like proteins might be involved in the plant-pathogen recognition, symptom development, and the basal tolerance to biotic and abiotic stresses through the salicylic acid signaling pathway [102]. In *Arabidopsis*, a stress-responsive

caleosin-like protein, AtCLO4, was demonstrated to act as a negative regulator of ABA responses [103], whereas another caleosin-like protein, RD20, was involved in ABA-mediated inhibition of germination but did not response to biotic or abiotic stresses [104]. Recently, a wheat caleosin-like protein was proposed to play a role in the Ca^{2+}-triggered feedback regulation of both the canonical Gα subunit of the heterotrimeric G protein complex and phosphoinositide-specific phospholipase C [105], and a noncanonical caleosin from *Arabidopsis* was found to epoxidize unsaturated fatty acids efficiently with complete stereoselectivity [106]. Taken together, the recent research progress on the identification of caleosin functions is encouraging. However, it also raises a puzzle how the highly conserved caleosin isoforms execute several diverse functions that may require a well-structured active site for an enzymatic reaction and a well-featured binding surface for a specific protein-protein interaction.

4.3. Proposed Functions of Steroleosin. Steroleosin possesses a sterol-regulatory dehydrogenase domain that belongs to a superfamily of presignal proteins involved in signal transduction via activation of its partner receptor after binding to a regulatory sterol [45]. Besides dehydrogenase activity, no other biological functions have been experimentally proven for steroleosin so far [44, 47]. As caleosin and steroleosin are minor integral proteins of comparable contents in oil bodies of sesame seeds, it is speculated that caleosin may be regulated by a pre-signal partner, such as a sterol-activated steroleosin, to serve as a receptor or signaling molecule on the surface of oil bodies. Meanwhile, two steroleosin isoforms having distinct sterol-binding sites are found in oil bodies of angiosperm seeds, and may be involved in the activation of sterol signal transduction that regulates specialized biological functions related to the mobilization of oil bodies during seed germination.

5. Targeting of Integral Oil-Body Proteins

Targeting of oil-body proteins, particularly oleosin, has been extensively investigated via in vivo systems using transgenic techniques and in vitro systems using microsomal membranes for integration of translated proteins [107–111]. The signal segment for specific targeting of oleosin to oil bodies is apparently located within the protein itself since recombinant oleosins are able to target correctly oil bodies in different transgenic plants and yeasts [112–114]. The central hydrophobic domain, particularly the conservative proline knot motif, of oleosin or caleosin has been demonstrated to play an essential role for the protein targeting oil bodies [60–63, 78]. According to the studies with an in vitro system using microsomal membranes as targets for integration of translated proteins, it has been suggested that oleosin might target endoplasmic reticulum (ER) under the assistance of the signal recognition particle (SRP) prior to transportation to maturing oil bodies [115–119]. However, neither oleosin nor caleosin possesses a cleavable or noncleavable N-terminal signal sequence required for the SRP-dependent pathway of targeting to the ER [120, 121]. Thus, it is proposed that oleosin contains several segments that are capable of interacting with SRP to direct the protein to the ER membrane [122].

An in vitro system was established to evaluate the targeting traffic routes of oleosin, caleosin, and steroleosin by constituting artificial oil emulsions (generated by sonication of triacylglycerol and phospholipid in a buffer solution) to mimic maturing oil bodies for integration of translated oil-body proteins [9, 78]. The results suggest that steroleosin and caleosin/oleosin may be assembled to maturing oil bodies through different locations of ER membrane, that is, caleosin/oleosin directly target maturing oil bodies where ER membranes are enlarged with deposited triacylglycerol molecules, whereas steroleosin is recognized by SRP and guided to integrate into the phospholipid bilayer of ER membrane prior to its lateral migration to maturing oil bodies (Figure 4). The distinct targeting traffic routes between steroleosin and caleosin/oleosin are in agreement with the following two observations. Firstly, steroleosin and caleosin/oleosin target and anchor to oil bodies via different structural organizations. Steroleosin possesses a non-cleavable N-terminal signal sequence putatively responsible for ER targeting via SRP dependent pathway, and its anchoring to oil bodies lies mainly in the N-terminal hydrophobic domain; caleosin and oleosin, lacking an N-terminal signal sequence, target/anchor to oil bodies via their central hydrophobic domains. Secondly, steroleosin possesses a free methionine at its N-terminus while caleosin and oleosin are N-terminally blocked by acetylation after the removal of the first methionine residue [56]. Presumably, the N-terminus of steroleosin is protected by SRP complex and/or embedded in ER membrane during its synthesis and targeting while the N-termini of caleosin and oleosin are freely exposed to cytosol during their synthesis and targeting to maturing oil bodies via central hydrophobic domains.

Negatively charged phospholipids (phosphatidylserine and phosphatidylinositol) are present in a consistent amount (30–40%) in the phospholipids of oil bodies from diverse seeds [15]. According to an in vitro targeting study, inclusion of negatively charged phospholipids in artificial oil emulsions substantially enhanced the targeting efficiency of oleosin and caleosin to these emulsions [78]. It is assumed that negatively charged phospholipids in the surface area of oil bodies are involved in targeting or assembling of oil-body proteins to these organelles. Definitely, it is an important task to figure out the specific targeting interaction between the unique segments of oil-body proteins and the negatively charged phospholipids of maturing oil bodies.

6. Isoforms of Integral Oil-Body Proteins in Evolution

6.1. Oleosin Isoforms. Oleosin is an alkaline protein unique to oil bodies and has been found exclusively in plant species. Two distinct classes, H- and L- (high and low molecular weight) oleosins, are present in seed oil bodies of diverse angiosperms, and one or more isoforms may occur in each oleosin class of the same species [123].

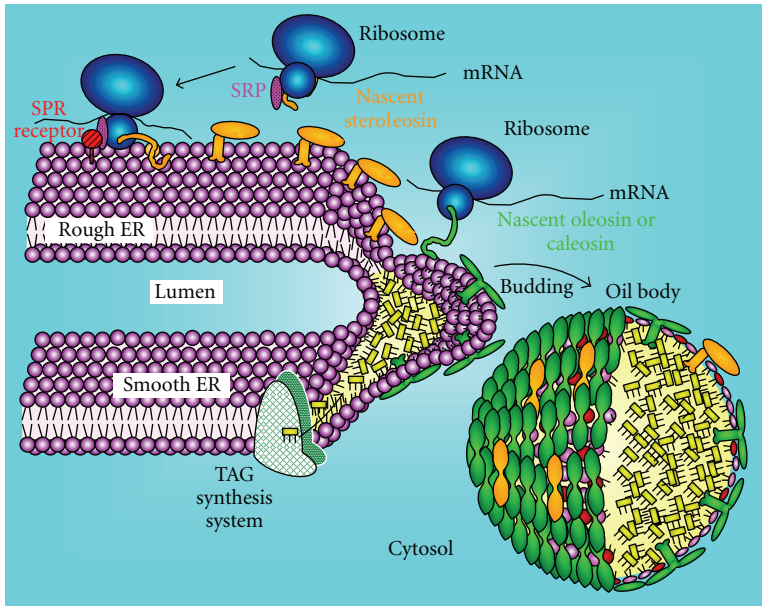


Figure 4: Targeting model of oleosin, caleosin, and steroleosin to a maturing oil body. Oleosin and caleosin are proposed to target directly a maturing oil body. In contrast, steroleosin is assumed to be integrated into the phospholipid bilayer of endoplasmic reticulum (ER) membrane prior to its lateral migration to a maturing oil body (adopted and modified from Figure 7 of Chen and Tzen, [78]).

It has been shown that H- and L-oleosins coexist on the surface of each oil body in seed cells of sesame [124]. The main difference between these two oleosin isoforms is an insertion of 18 residues in the C-terminal domain of H-oleosin, accounting for a 2 kDa difference in mass between the two classes found in many species [125–127]. The physiological significance of the presence of these two oleosin isoforms in oil bodies of angiosperm seeds remains to be elucidated. L-oleosin but not H-oleosin is found in megagametophytes of two gymnosperm species, pine (*Pinus koraiensis*) and ginkgo (*Ginkgo biloba*); it may imply that L-oleosin is a more primitive isoform class, with H-oleosin derived from L-oleosin before the divergence of monocot and dicot species during evolution [128]. Moreover, cDNA fragments encoding putative oleosin isoforms were found in pollen of rapeseed [129, 130]. Recently, stable oil bodies were successfully isolated from lily (*Lilium longiflorum* Thunb.) pollen, and a unique P-oleosin was found as the major integral oil-body protein [97]. Three oleosin genes, representative of early trends in evolution, were found in the model moss, *Physcomitrella* with a complex pattern of expression based on gene splicing [131]. Moreover, oleosin-like proteins, forming a distinct class termed oleopollenin, were found in tapetum and external surfaces of pollen grains [132–134].

6.2. Caleosin Isoforms. In angiosperm species, sequence alignment shows that caleosins in monocot seed oil bodies seem to possess an additional N-terminal appendix of approximately 40–70 residues, and thus are larger than those in dicotyledonous seed oil bodies [135–137]. Recently, a distinct P-caleosin isoform was also identified in pollen oil bodies of lily and olive (*Olea europaea* L.) [97, 98, 138]. Caleosin is also found in more primitive species, such as cycad and microalgae, and thus is assumed to be an oil-body protein more primitive than oleosin in evolution [95, 139]. Phylogenetic tree analysis supports that microalgal caleosin is the most primitive caleosin found in oil bodies to date [8, 23, 139]. The additional N-terminal appendix found in monocot caleosins is not present in pollen or cycad caleosin. Therefore, the additional N-terminal appendix found in monocot caleosins seems to be resulted from an insertion mutation in monocot seed caleosin in evolution. Of course, this hypothetic evolutionary event should be verified by further molecular evidence. Since caleosin is more primitive than oleosin, it is reasonable to speculate that caleosin is an essential integral protein of plant oil bodies [76]. However, this speculation has been invalidated as stable oil bodies located in rice aleurone layer are composed of H- and L-oleosin but not caleosin [135]. Interestingly, those rice oil bodies lacking caleosin are not mobilized after germination [39]. It remains to be studied whether caleosin is indispensable for the mobilization of oil bodies. In contrast with oleosin isoforms that are unique to oil bodies, caleosin isoforms or caleosin-like proteins are possibly present in other cellular locations, for example, ER membrane [140]. Moreover, the same caleosin isoform is possibly present in both seed oil bodies and membrane-bound fractions of other tissues [99].

6.3. Steroleosin Isoforms. Limited research progress has been advanced in the identification of steroleosin isoforms so far. Similar to caleosin, steroleosin isoforms or steroleosin-like proteins are possibly present not only in oil bodies but also in other subcellular locations [44]. Homologous proteins of steroleosin are presumably present in all kinds

of living organisms including bacteria and humans [47]. Most steroleosin-like proteins are lack of the N-terminal hydrophobic anchoring domain, and they all possess the highly conservative NADPH-binding subdomain and active site, but diverse sterol-binding subdomains. Diverse sterol-binding subdomains are also found in the two steroleosin isoforms located in sesame oil bodies, implying that different sterols may regulate these two steroleosin isoforms to conduct distinct biological functions related to the formation or degradation of seed oil bodies [9].

7. Artificial Oil Bodies in Basic Research Studies

Artificial oil bodies of similar sizes (0.5–2 μm) and structural stability have been successfully reconstituted with triacylglycerols, phospholipids, and integral oil-body proteins under the same proportions as they are found in native oil bodies [125]. The sizes of artificial oil bodies could be controlled by changing the ratio of triacylglycerol over oil-body protein, whereas both thermostability and structural stability of artificial oil bodies decreased as their size increased, and vice versa [87]. For encapsulation of artificial oil bodies, recombinant oleosins expressed in *Escherichia coli* were found comparable to native oleosins isolated from seed oil bodies [141, 142]. It has been demonstrated that artificial oil bodies could be stabilized by oleosin or caleosin, but not steroleosin, and the average sizes (50–200 nm) of artificial oil bodies constituted with caleosin were 10-times smaller than those (0.5–2 μm) constituted with oleosin (Figure 5) [143].

Since stable artificial oil bodies could be simply generated with triacylglycerol, phospholipid, and oil-body protein, but not any two of them, it is apparent that these three constituents are essential components for the construction of oil bodies [41]. Artificial oil bodies could be stabilized by the combination of sesame oleosin isoforms or any oleosin isoform alone, that is, H1-oleosin, H2-oleosin, or L-oleosin; however, a slightly better structural stability was observed in artificial oil bodies constituted with L-oleosin than those constituted with either of the two H-oleosin isoforms [125]. Similar results were observed for the artificial oil bodies constituted with L-oleosin or H-oleosin extracted from oil bodies of rice seeds [124]. Obviously, L-oleosin is a better oil-body structural protein than H-oleosin. The relative small artificial oil bodies constituted with caleosin possessed a better thermostability (up to 70°C) than native oil bodies or artificial oil bodies stabilized with oleosin (lower than 50°C) [143]. The observation was in accordance with a later investigation on the relatively high thermostability (up to 70°C) of small cycad oil bodies that were mainly sheltered by a unique caleosin [95]. Evidently, caleosin is a better oil-body structural protein than oleosin in terms of thermal tolerance.

Artificial oil bodies constituted with truncated oleosin and caleosin have been utilized to evaluate the segments responsible for the structural stability of oil bodies. Artificial oil bodies constituted with truncated oleosins of the central hydrophobic domain longer than 36 residues were as stable as native sesame oil bodies, and those constituted with truncated oleosins lacking more than half of the original central hydrophobic domain inclined to coalesce upon collision or aggregation [60]. Both structural stability and thermostability of artificial oil bodies were slightly or severely reduced when the amphiphatic α-helix (15 residues) or proline-knot subdomain (21 residues) of recombinant caleosin was truncated, and thus the whole central hydrophobic domain of 36 (15 + 21) residues is crucial for the stability of oil bodies [77]. Taken together, the minimal length of hydrophobic domain to serve as an oil-body anchoring segment is approximately 36 residues (mainly the proline-knot regions shown in the secondary structures of oleosin and caleosin in Figure 3).

8. Artificial Oil Bodies in Biotechnological Applications

Several biotechnological applications have been developed by using the unique characteristics of oil bodies, such as new ingredients for flavoring or emulsifying agents, affinity matrices for enzyme fixation/purification, and expression/purification systems for producing recombinant proteins via transgenic plants [144–154]. Many applications related to the utilization of oil bodies have been patented [155–163]. These applications of seed oil bodies can be authentically applied to the utilization of artificial oil bodies. Moreover, some novel usages of artificial particles have also been developed in the past decade.

8.1. Protein Expression/Purification System. A bacterial expression/purification system to produce recombinant proteins was developed by using artificial oil bodies [141, 164–169]. In this system, a target protein was first overexpressed as an insoluble oleosin-fused polypeptide, collected from the pellet of cell lysate simply by centrifugation, assembled into artificial oil bodies, separated from oleosin, and then harvested by concentrating the ultimate supernatant. This technique offers a powerful and competitive option to replace the conventional affinity chromatography used for protein purification. However, the requirement of using a relatively expensive endopeptidase, for example, factor Xa, for specific release of the target protein from the recombinant oleosin-fused polypeptide raises the processing cost substantially, and thus severely restricts its potential applications. To cost down this process, an improved system was developed by replacing the specific proteolytic cleavage sequence between oleosin and the target protein with an intein (an inducible self-splicing polypeptide) linker. In this revised system, the target protein was released from artificial oil bodies via self-splicing of the intein linker, induced by temperature alteration or dithiothreitol supplement, without using the expensive endopeptidase.

8.2. Matrix for Enzyme Immobilization. A new technique of enzyme fixation was designed to achieve, in one step, protein refolding and immobilization by linking a target enzyme, for example, D-hydantoinase, to oleosin on the surface of artificial oil bodies [170, 171]. The immobilized enzyme remained stable for at least 15 days when stored at

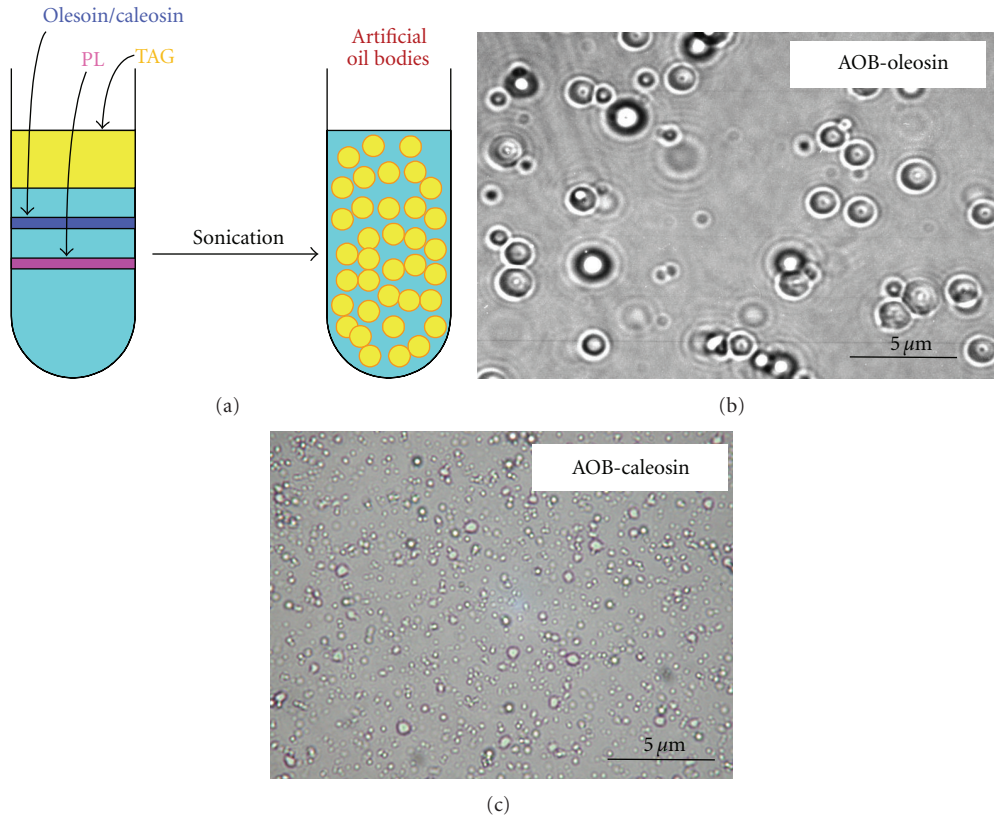


Figure 5: Constitution of artificial oil bodies. A cartoon diagram showed the preparation of artificial oil bodies by sonication (a). Artificial oil bodies (AOB) were generated with triacylglycerol (TAG) and phospholipid (PL) in the presence of oil-body protein, oleosin (b), or caleosin (c). Photos of (b) and (c) are adopted from Figure 3 of Chen et al., [143].

4°C, and its conversion yield exceeded 80% after 7 cycles of repeated use. Apparently, the simple and effective system by fixing target enzymes on the surface of artificial oil bodies is practical and useful for the routine operation of industrial enzymatic reactions.

8.3. Formula for Encapsulation of Bacteria. Numerous healthy and nutritional benefits have been ascribed to probiotics, such as lactic acid bacteria. Since probiotics may not survive in sufficient number to retain their functionality in human gastrointestinal tract, many approaches have been explored to increase their viability when used as food supplements. A technique was developed to protect lactic acid bacteria against simulated gastrointestinal conditions by encapsulation of bacterial cells within artificial oil bodies [172]. Compared with nonencapsulated cells, the entrapped bacteria demonstrated a significant increase (approximately 10,000 times) in survival rate in the presence of simulated high acid gastric or bile salt conditions. It is recommended that artificial oil bodies may represent a suitable formula of biocapsule to encapsulate bacteria for commercial utilization in dairy products.

8.4. Carrier for Drug Delivery. Relatively small artificial oil bodies stabilized with caleosin have been used to develop an oral delivery system for hydrophobic drugs, for example, cyclosporine A, a drug commonly utilized as a clinical immunosuppressant to prevent transplant rejection and to treat several autoimmune diseases [173]. Cyclosporine A efficiently encapsulated in artificial oil bodies stabilized with caleosin could be stably stored for weeks at 4°C. An oral delivery formulation with cyclosporine A in artificial oil bodies was demonstrated to exhibit satisfactory bioavailability in an animal test [173]. This drug delivery system or its improved formula may also be used as an adequate carrier for many other hydrophobic drugs, such as antitumor drugs [174–177].

8.5. Antibody Generation System. Recently, a system of generating antibodies against small molecules (haptens) was established under the assistance of artificial oil bodies [178]. To develop this system, a series of recombinant caleosins were engineered with more Lys residues to link and render small molecules on the surface of artificial oil bodies for antibody production. In this design, covalently conjugated haptens were anticipated to cover the whole surface of artificial oil bodies constituted with hapten-charged caleosins. The results indicate that engineered Lys-rich caleosins are suitable carrier proteins for the production of monospecific antibodies against small molecules, such as drug, herbal compounds, pesticides, herbicides, antibiotics, and hormones.

9. Perspective

In the past three decades, continual research advancement has confirmed the presence of three classes of integral proteins, oleosin caleosin, and steroleosin on the surface of oil bodies. A lot of potential oil-body proteins have been recently screened by the subproteomic approaches under the assistance of mass spectrometry; though a few of them seem to be contaminants apparently, some candidate proteins are waiting for further verification to see if they are real integral oil-body proteins or peripheral proteins associated with some surface components of oil bodies for particular physiological functions. Controversial structures have been proposed for oleosin, and the controversy cannot be receded until a convincing three-dimensional structure of oleosin is determined. Several physiological functions other than structural role have been actively demonstrated or proposed for oleosin and caleosin in the past decade. Taken together, it seems unlikely that these two relatively small proteins are capable of executing several diverse biological functions jointly, and thus some of the proposed functions may not be correct and should be ruled out in the follow-up researches. Oleosin- and caleosin-stabilized artificial oil bodies have been successfully constituted and used to develop various systems for biotechnological applications. Further investigation and technical improvement will create novel artificial oil bodies as versatile vehicles to fulfill many other requirements for specialized applications.

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