Formation of Oleosomes in Maturing Safflower Seeds

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The accumulation of oil in maturing safflower seeds was studied with an electron microscope. It was suggested that net-like clusters of proteinaceous particles in the cytoplasm are the site of triacylglycerol synthesis. No evidence was obtained to support the hypothesis that oleosomes originate in the endoplasmic reticulum. A hypothesis has been proposed that triacylglycerols are initially synthesized by the net-like clusters in the cytoplasm to form a protein-oil complex, and the triacylglycerols are concentrated in the center of the cluster as their formation proceeds.

In oil-rich seeds, triacylglycerols are accumulated in a subcellular organelle which is named an oleosome (oil body). The origin and structure of oleosomes have been investigated and discussed by many workers. There are several papers on subcellular organelles catalyzing triacylglycerol synthesis in plant seeds. Up to the present, it has been believed that triacylglycerol synthesis in maturing seeds occurs in oleosomes or on the surface of oleosomes. The presence of other organelles catalyzing the reaction has been indicated by some workers. Frey-Wyssling et al. described that oleosomes are formed from spherosomes which themselves originate from the endoplasmic reticulum, and that oleosomes and spherosomes are surrounded by a unit membrane. Sorokin concluded that spherosomes and oleosomes represent separate entities, and that spherosomes are a common feature of most vegetative cells in higher plants, whereas oleosomes are restricted to cells producing oil. She reported that spherosomes have a limiting membrane, whereas oleosomes do not. It has previously been reported that a subcellular particulate fraction prepared from maturing safflower seeds catalyzes triacylglycerol synthesis from acyl-CoAs and 1,2-diacylglycerols.

The present paper deals with the ultrastructural development of oleosomes of safflower seeds (Carthamus tinctorius L.) during maturation and the chemical composition of the subcellular particulate fraction catalyzing triacylglycerol synthesis.

MATERIALS AND METHODS

Safflower plants were grown in a University field, and the maturing seeds were harvested at desired stages of development.

Electron microscopy of cotyledons. Cotyledons of safflower seeds were cut into small pieces and fixed in an aq. solution containing 3% glutaraldehyde and 0.05 M potassium-phosphate buffer (pH 6.8) for 2~3 hr at 0°C. The tissues were then rinsed for 2 hr in phosphate buffer and post-fixed in buffered 1.5% osmium tetroxide for 2 hr at room temperature. After rinsing with water, the fixed tissues were dehydrated through an ethanolic or an acetone series, transferred to propylene oxide, and then embedded in Spurr's low-viscosity epoxy resin. Sections were cut with glass knives using an LKB Ultratome and stained with lead citrate for 5 min. Samples were examined with a JEOL JEM 100B transmission electron microscope.

Electron microscopy of subcellular fractions. Safflower seeds 18 days after flowering were homogenized in 0.05 M potassium-phosphate buffer (pH 7.0) containing 0.4 M sorbitol with a Potter-Elvejem homogenizer. The ho-
mogenate was filtered through two layers of cotton cloth. The filtrate was centrifuged at 1000 x g for 10 min. The precipitate (particulate fraction) and the floating fat layer (fat fraction) obtained were suspended in the same buffer as used for homogenization. These fractions were fixed, post-fixed, dehydrated, embedded, sectioned, stained and examined by the same procedures as described above.

Chemical analysis of the subcellular fractions. Maturing seeds 18 days after flowering were homogenized in water and the homogenate was centrifuged at 40 x g for 2 min. The supernatant was centrifuged at 1000 x g for 10 min. The precipitate and the fat layer were washed with water three times. The lipid, protein, carbohydrate and RNA contents of these fractions were determined. Lipids were extracted from the fractions by the method of Bligh and Dyer,21) and determined gravimetrically. Protein was determined by the method of Lowry et al.22) Carbohydrate was determined by an anthrone method.23) RNA was determined by the methods of Kirby24) and Dieckert et al.25)

Lipid analysis. Lipids extracted from the subcellular fractions were analyzed by thin-layer chromatography. The lipids were separated into lipid classes on silica gel G with hexane-diethyl ether-acetic acid (70 : 30 : 1). A potassium bichromate-sulfuric acid reagent was sprayed on the thin-layer plates and the plates were heated at 180°C. Each lipid class was determined by densitometry with a Shimadzu TLC Scanner CS-900.

RESULTS AND DISCUSSION

Formation of the seed coat started after flowering (pollination). At 6 days after flowering, the seed was filled with clear liquid. At 9 days after flowering, a small thin embryo was observed. Oleosomes were already present in the cells at this early stage of embryo development (Fig. 1). The observations in this work are related only to the cotyledon cells of safflower; the radicle cells were not used. The cytoplasm of the cells is granular. A large nucleus is present with a well-defined nucleolus. Some cells have one or a few large vacuoles, and other cells have numerous small vacuoles. The oil content of the seeds including seed coats was 6~7% of the dry weight at 9 days after flowering. At 15~18 days after flowering, the cytoplasm develops and vacuoles become small (Fig. 2). The cytoplasm becomes densely packed with oleosomes during the middle stage of maturation (oil content, ca. 15%). Protein bodies develop in vacuoles and at the interfaces between vacuoles and the cytoplasm. Amyloplasts containing starch grains were observed in some cells, although they can not be seen in Fig. 2.

Schwarzenbach3) claimed that oil accumulated between the inner and outer layers of the unit membrane of the endoplasmic reticulum. Frey-Wyssling et al.1) described that oleosomes evolved from vesicles produced by the endoplasmic reticulum. However, there was no evidence to indicate that the oleosomes of maturing safflower seeds arose from the endoplasmic reticulum. If oleosomes originate in the endoplasmic reticulum, they should be surrounded by a limiting membrane. The oleosomes in maturing safflower seeds, however, do not have a densely staining limiting membrane (Figs. 2, 4a and 4b). Furthermore, no junction of oleosomes with the endoplasmic reticulum was found in safflower seed cells. In safflower seeds, oleosomes seem to arise directly from the cytoplasm with the participation of electron-dense particles (Fig. 4a and 4b).

Oleosomes further increased in number with seed maturation. At 28 days after flowering, oleosomes fill almost the entire remainder of the cell space not occupied by protein bodies, nucleus or residual cytoplasm (Fig. 3). The oil content of the mature seeds was 32%. Protein bodies are the second major storage products found in the cells. Most oleosomes are 0.5~1.5 μm in diameter. The electron density of oleosomes decreases with seed maturation. This is in agreement with the observations of Frey-Wyssling et al.1) and Rest and Vaughan.5) Oleosomes are compressed together, but they appear not to coalesce with adjacent oleosomes. No unit membrane structure can be seen on their surfaces, and there is no stainable contents within them.

It has previously been reported that a subcellular particulate fraction (1000 x g, precipitate) contains diacylglycerol acyltransferase, the last enzyme of the pathway of triacylglycerol synthesis, and that this fraction is able to catalyze triacylglycerol synthesis from 1,2-
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Fig. 1. Electron Micrograph of Safflower Seed Cells 9 Days after Flowering. The bar represents 1 μm. N, nucleus; n, nucleolus; O, oleosome; E, endoplasmic reticulum; V, vacuole; C, cell wall.

Fig. 2. Electron Micrograph of Safflower Seed Cells 18 Days after Flowering. The bar represents 1 μm. P, protein body. For other symbols, see the legend to Fig. 1.

Fig. 3. Electron Micrograph of Safflower Seed Cells 28 Days after Flowering. The bar represents 1 μm. For symbols, see the legends to Figs. 1 and 2.

Fig. 4a and 4b. Development of Oleosomes in situ (15 Days after Flowering). The bars represent 0.1 μm.

Fig. 5. Nascent Oleosomes (O) and Net-like Structure (N) in the 1000 × g Precipitate Fraction. The bar represents 0.2 μm.

Fig. 6. Nascent Oleosomes (O) and Net-like Structure (N) with One Nascent Oleosome and Several Hollows (H) in the 1000 × g Precipitate Fraction. The bar represents 0.2 μm.
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Table I. Chemical Composition of Subcellular Fractions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (Weight %)</th>
<th>Lipid (Weight %)</th>
<th>Carbohydrate (Weight %)</th>
<th>RNA &lt;0.07</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000 x g. ppt.</td>
<td>80.1</td>
<td>2.5</td>
<td>11.4</td>
<td>&lt;0.07</td>
</tr>
<tr>
<td>Fat layer</td>
<td>2.3</td>
<td>97.7</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

In the present work, the chemical composition of the particulate fraction and oleosomes (the floating fat fraction) was investigated, although the former fraction was still crude. As shown in Table I, the major component of the particulate fraction was protein. Carbohydrates probably indicate cellulose, because this fraction has been found to be contaminated with fragments of cell wall by electron microscopy. Since RNA was not detectable in this fraction, it is concluded that it does not contain ribosomes or rough-surfaced endoplasmic reticulum. The values in Table I were obtained with the fractions prepared with water as the solvent for homogenization and centrifugation. When the 1000 x g particulate fraction was prepared with a high-osmotic solution containing 0.4 M sorbitol, 0.05 M Tris-HCl (pH 7.6) and 1 mM EDTA, the ratio of the protein and lipid contents was nearly equal to that obtained with the particulate fraction prepared with water. Gurr et al. reported that the protein content of the fat fraction from mature safflower seeds was 10.9%, which was estimated from the nitrogen content. As shown in Table I, the protein content of the fat fraction was 2.3%, which was obtained with the fraction from maturing safflower seeds 18 days after flowering by the method of Lowry et al.

It is noteworthy that diacylglycerols and acyl-CoAs in the present work, the chemical composition of the particulate fraction and oleosomes (the floating fat fraction) was investigated, although the former fraction was still crude. As shown in Table I, the major component of the particulate fraction was protein. Carbohydrates probably indicate cellulose, because this fraction has been found to be contaminated with fragments of cell wall by electron microscopy. Since RNA was not detectable in this fraction, it is concluded that it does not contain ribosomes or rough-surfaced endoplasmic reticulum. The values in Table I were obtained with the fractions prepared with water as the solvent for homogenization and centrifugation. When the 1000 x g particulate fraction was prepared with a high-osmotic solution containing 0.4 M sorbitol, 0.05 M Tris-HCl (pH 7.6) and 1 mM EDTA, the ratio of the protein and lipid contents was nearly equal to that obtained with the particulate fraction prepared with water. Gurr et al. reported that the protein content of the fat fraction from mature safflower seeds was 10.9%, which was estimated from the nitrogen content. As shown in Table I, the protein content of the fat fraction was 2.3%, which was obtained with the fraction from maturing safflower seeds 18 days after flowering by the method of Lowry et al.

It is noteworthy that diacylglycerols, the precursors of triacylglycerols, are contained in the particulate fraction in a relatively high proportion, while the diacylglycerol content of the fat fraction is low (Table II). This may suggest that an organelle contained in the particulate fraction is the site of triacylglycerol synthesis. The triacylglycerol content of the fat fraction will become high with seed maturation.

The subcellular particulate fraction was further investigated by electron microscopy. Considerably homogenous particles were observed in the fraction with a light microscope. However, each of these particles was found, by electron microscopy, to be an aggregate of various organelles and cell wall fragments. The main content of the aggregates in this fraction was cross-linked net-like structures. Some of the net-like structures were bound to materials that seemed to be oil (Fig. 5). These binding substances may be nascent oleosomes. Other net-like structures had a few or several hollows from which nascent oleosomes had probably fallen off (Fig. 6). From the data in Table I, it is suggested that these net-like structures are almost completely composed of protein. It has been reported that the total activity of triacylglycerol synthesis of the particulate fraction is the highest of all the subcellular fractions tested. Therefore it is most likely that the net-like structures contained abundantly in the particulate fraction are the site of triacyl-
glycerol synthesis. Smith described that oleosomes were initially seen as a small electron-dense particulate mass which may be a cluster of specialized ribosomal material and that the particulate mass may be the site of enzymes required for oleosome synthesis. It is not clear whether or not the nature of this particulate mass found in Crambe seeds is identical with that of the net-like structure in safflower seeds. There is, at least, one discrepancy between the data in Table I and the opinion of Smith as to the participation of ribosomes in the biosynthesis of triacylglycerols. Rest and Vaughan reported that oleosomes appeared to arise directly from the cytoplasm. Some of the electron micrographs in this work are consistent with the observation of Rest and Vaughan. In such cases, the net-like structures will also participate in triacylglycerol formation. Harwood et al. suggested that vacuole-like inclusions within oleosomes are the site of lipid synthesis in the maturing castor bean endosperm. However, the present work has provided no evidence to support the theory of Harwood et al.

On the basis of the present and previous data and the results of Smith and Rest and Vaughan, I propose a hypothesis for the development of oleosomes, which is illustrated in Fig. 7. There is a reticular cluster of proteinaceous particles present in the cytoplasm (stage I). This net-like structure, which may be named "protein reticulum," has at least diacylglycerol acyltransferase. Triacylglycerols are initially formed by the protein reticulum and form a complex with the protein reticulum (stage II). This complex is a nascent oleosome. As triacylglycerol synthesis proceeds, oil separates from the protein reticulum to form an oil droplet (a small oleosome), but the interface between the oil and the protein reticulum is still obscure (stage III). With the increasing amount of triacylglycerols, the oleosome becomes large and the interface becomes clear (stage IV). The oleosome further develops with the accumulation of triacylglycerols (stage V). The fact that the activity of diacylglycerol acyltransferase occurs in all fractions of seed homogenates is expliable by this hypothesis. If the cells containing oleosomes of stage III or IV are homogenized and centrifuged, oleosomes and protein reticulum will separate from each other. Oleosomes with fragments of protein reticulum on their surfaces will be obtained as a floating fat layer on centrifugation, while pro-

![Fig. 7. Scheme for Oleosome Development. An explanation is given in the text.](image-url)
tein reticulum will precipitate as aggregates. Fragments of protein reticulum also may have diacylglycerol acyltransferase activity and they will precipitate on centrifugation at $10^4 \times g$ or $10^5 \times g$. Therefore diacylglycerol acyltransferase activity will mainly occur in the precipitate fractions, while the activity will be detectable in the fat fraction to some extent. From the data of McMahon and Stumpf,\textsuperscript{26,27} it is strongly suggested that this proteinaceous organelle contains the enzyme system for fatty acid synthesis and oleate desaturase. This organelle precipitates at 1000 $\times g$ as an aggregate with other cell fragments as described above. If the mass of the organelles and other cell fragments is disaggregated, these organelles may be obtained from the $10^5 \times g$ precipitate fraction on centrifugation. Methods for disaggregation are now under investigation.

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NOTE ADDED IN PROOF

Recently, another hypothesis has been proposed for the development of oleosomes [G. Wanner, H. Formanek and R. R. Theimer, Planta, 151, 109 (1981)].

REFERENCES

2) H. P. Sorokin, \textit{Amer. J. Bot.}, 54, 1008 (1967).