Gluconeogenesis from Storage Wax in the Cotyledons of Jojoba Seedlings

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ABSTRACT

The cotyledons of jojoba (Simmondsia chinensis) seeds contained 50 to 60% of their weight as intracellular wax esters. During germination there was a gradual decrease in the wax content with a concomitant rise in soluble carbohydrates, suggesting that the wax played the role of a food reserve. Thin layer chromatography revealed that both the fatty alcohol and fatty acid were metabolized. The disappearance of wax was matched with an increase in catalase, a marker enzyme of the gluconeogenic process in other fatty seedlings. Subcellular organelles were isolated by sucrose gradient centrifugation from the cotyledons at the peak stage of germination. The enzymes of the β oxidation of fatty acid and of the glyoxylate cycle were localized in the glyoxysomes but not in the mitochondria. The glyoxysomes had specific activities of individual enzymes similar to those of the castor bean glyoxysomes. An active alkaline lipase was detected in the wax bodies at the peak stage of germination but not in the ungerminated seeds. No lipase was detected in glyoxysomes or mitochondria. After the wax in the wax bodies had been extracted with diethyl ether, the organelle membrane was isolated and still retained the alkaline lipase. The gluconeogenesis from wax in the jojoba seedling appears to be similar, but with modification, to that from triglyceride in other fatty seedlings.

The jojoba plant is native to arid regions of southwestern North America. It may be a unique member of the plant kingdom, in that it is the only known plant whose seeds contain a large amount (50-60% of the fresh weight) of intracellular wax esters in the cotyledons (18, 19, 21, 27). The industrial application of the seed wax is being actively explored since its chemical structure is very similar to the oil of the sperm whale. Agricultural programs have been started in an attempt to establish a high productivity of the seeds (26). On the other hand, the physiology of the seed wax has not been previously studied. The jojoba seed may be a useful system for the study of wax metabolism in higher plants since during seed development and germination, wax metabolism should be very active. In this paper, we establish the role of the jojoba wax as a food reserve for germination and elucidate the gluconeogenic process from wax in the jojoba seedling. The findings are compared with the metabolic pathways of storage triglyceride in other fatty seedlings.

Materials and Methods

Plant Materials. Seeds of jojoba (Simmondsia chinensis [Link] Schneider) were first surface-sterilized in 5% Clorox for 1 hr and soaked in running tap water for 16 hr. Germination was carried out in moist vermiculite in darkness at 28 C. In the germination study, plant materials were carefully selected for uniformity. Only those seeds weighing between 0.5 and 0.7 g were used. At 5-day intervals, seedlings were selected by both chronological age and hypocotyl length. Seedlings with the following hypocotyl length were used: day 0 (dry seed), day 5 (0.1-1 cm radical), day 10 (6-8 cm), day 15 (10-12 cm), day 20 (13-15 cm), day 25 (16-21 cm), and day 30 (22-30 cm).

Compositional Analysis. Five seedlings from each growth stage were separated into cotyledons and embryonic axes. The fresh weights were recorded. The cotyledons were ground in ice-cold methanol in the ratio of 1:10 (w/v) for 10 min at high speed in a VirTis-45 homogenizer. Two volumes of chloroform were added and the mixture was kept at room temperature for 2 hr to extract the lipids. The mixture was filtered and the extraction procedure was repeated twice. The combined extract was mixed with 2 volumes of water in a separatory funnel. The lower organic phase was separated and dried at 70 C for 24 hr. The resulting weight was recorded as that of the total lipids. The chloroform-methanol-extracted residue on the filter was dried at 70 C for 24 hr and weighed. An aliquot of this residue was added to 80% (v/v) ethanol and ground for 10 min at high speed in a VirTis-45 homogenizer. The homogenate was filtered and the extraction was repeated twice. The ethanol-extracted residue was dried at 80 C for 24 hr and weighed. Soluble carbohydrates and amino acids were determined in the methanol-water and ethanol extractions by the anthrone method (25) and the ninhydrin method (1), respectively. Starch and other easily hydrolyzable carbohydrates were determined in the ethanol-extracted residue by a modified anthrone method (4).

For the determination of protein and catalase activity, the cotyledons from five seedlings of the same age were ground for 10 min at high speed in a VirTis-45 homogenizer in 25 ml of grinding medium composed of 0.6 M sucrose, 1 M EDTA, 10 mM KCl, 1 mM MgCl2, 2 mM dithiothreitol, 0.15 M Tricine buffer, adjusted with KOH to a pH of 7.5. The homogenate was filtered through a Nitex cloth with pore size 102 μm2 and assayed directly for catalase activity and protein.

Thin Layer Chromatography. A small aliquot of the chloroform-methanol-extracted lipids was removed before the samples were dried. These samples were spotted onto a TLC plate coated with 250 μm of Silica Gel G. The plate was developed in 70:30:1.5 (v/v/v) hexane-diethyl ether-acetic acid. Unsaturated lipids were detected by spraying with 5% phosphomolybdic acid in 95% ethanol and heating at 110 C for 10 min. Oleoyl palmitate, oleic acid, and oleoyl alcohol were used as standards for wax ester, fatty acid, and fatty alcohol, respectively. TLC plates were obtained from Applied Science Laboratories, and the lipid standards from Sigma.

Organelle Preparation. The preparation of organelles from 20-day-old cotyledons followed the procedure described earlier (10, 11). The cotyledons were first chopped into small pieces with an onion chopper, and then ground with a mortar and

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pestle. The grinding medium was the same as described above. The homogenate was filtered through eight layers of cheesecloth and centrifuged at 270g for 10 min. The supernatant fraction was layered directly onto a sucrose gradient which was composed of 5 ml 20% (w/w) sucrose on top of 24 ml of a 30 to 60% linear sucrose gradient. The gradient was centrifuged at 21,000 rpm for 4 hr in a Beckman L2-65B ultracentrifuge with Spinco rotor 27, and fractionated.

In the preparation of wax bodies for electron microscopy and for the separation of the organelle membranes, the cotyledons were ground and the homogenate was filtered as described. The filtrate was centrifuged at 10,000g for 30 min and the lipid pad was removed with a spatula. The lipid pad was resuspended in grinding medium, shaken with a Vortex mixer, and recentrifuged at 10,000g for 30 min. The lipid pad was again removed, and was used as a wax body fraction for electron microscopy and membrane extraction. In the preparation for electron microscopy, the Tricine buffer in the grinding medium, which would have interfered with OsO₄ postfixation, was replaced by K-phosphate buffer.

In the extraction of the wax body membrane, the wax bodies were resuspended in grinding medium and extracted three times with diethyl ether to remove the wax. The trace amount of diethyl ether remaining was evaporated under a stream of N₂. The membrane fraction was centrifuged in a sucrose gradient as described above.

Assays. The assays of catalase, isocitrate lyase, NAD-malate dehydrogenase, NADH-Cyt reductase, malate synthetase, fatty acyl CoA dehydrogenase, Cyt oxidase, citrate synthetase, and glycerol phosphate oxidoreductase followed those described earlier (10, 11). Lipase was assayed in a reaction mixture of 4 ml containing 0.1 m tris-HCl buffer (pH 9), 2 mM dithiothreitol, and 0.83 mM N-methylnitroxylinst rate in ethylene glycol monomethyl ether (0.1 ml) (22). The fluorophotometer was set up with filters as described (22). In protein determination, the protein was precipitated with 10% trichloroacetic acid and assay by the Lowry method (16). Phospholipids were assayed by digesting the samples with 70% perchloric acid and measuring the resulting phosphates by the Bartlett method (7).

Electron Microscopy. The lipid pad was placed in a chilled Petri dish and cut into small pieces with a razor blade. The samples were fixed in 3.5% glutaraldehyde in grinding medium for 2 hr. After several rinses with the grinding medium, the samples were postfixed in 1% OsO₄ in grinding medium for 2 hr. Dehydration was carried out in a graded ethanol series, and the samples were embedded in Spurr. Sections were stained with uranyl acetate and lead citrate and observed in a Zeiss EM 9 electron microscope.

RESULTS AND DISCUSSION

Compositional Changes during Germination. In the cotyledons of ungerminated seeds, approximately 50 to 60% of the total fresh weight was lipid and 15% was protein (27). Most of the remaining fresh weight was attributed to soluble carbohydrate and starch (10%), water (5–10%), and seed coat (5–10%). During germination, there was a gradual increase in the fresh weight of the cotyledons as well as the growing embryonic axis (Fig. 1). In the cotyledons, the content of lipid gradually decreased with a concomitant increase in the amount of soluble carbohydrates. Jojoba seeds contained a very low level of starch (less than 2.5% of the fresh weight) which should not be a significant source of soluble carbohydrates. During the period of germination, there was actually an increase in starch from 14 to 28 mg/pair of cotyledons. The quantitative recovery of carbohydrates from the lost wax suggests a highly efficient gluconeogenic process, as is the case in castor bean (6). Since there should have been a transport of the soluble carbohydrates from the cotyle-

dons to the growing embryonic axis, the quantitative recovery suggests that some soluble carbohydrates may also be derived from amino acids, as in the castor bean endosperm (24). There was a gradual decrease in the total protein and a rise in the amino acids. The accumulated amino acids represented less than 10% of the hydrolyzed protein, presumably due to a transport of the amino acids to the growing embryonic axis and a transformation of the amino acids to carbohydrates. In essence, the mobilization of food reserve in the jojoba cotyledon appeared to be very similar to that in the castor bean endosperm. Furthermore, the activity of catalase, a marker enzyme of the gluconeogenic process in castor bean seedlings (28), increased 14-fold during the period of germination.

Changes in Lipid Components during Germination. The change in the lipid components during germination was studied by TLC (Fig. 2). In the dry seeds, most of the lipid appeared as wax ester of fatty acid and fatty alcohol, as reported (18). There were trace amounts of fatty acid and fatty alcohol and two unidentified components. During the germination period, even though there was a 50% reduction in the total lipid, there was no drastic change in the lipid composition. The finding indicates that both the hydrolyzed products of the wax, fatty acid and fatty alcohol, were rapidly metabolized.

Subcellular Organelles Involved in Gluconeogenesis from Wax. In fatty seeds containing triglycerides as a food reserve, the fatty acids released from the triglycerides are metabolized to hexaso by a N-long sequence of metabolic pathways involving the enzymes of the β oxidation and the glyoxylate cycle in the glyoxysomes, the enzymes of the tricarboxylic acid cycle in the mitochondria, and the enzymes of the reversed glycolysis in the cytosol (2, 12). Such a gluconeogenic pathway appeared to be present in the jojoba cotyledons, even though the reserve lipid
Fig. 2. Thin layer chromatogram of the total lipids during germination. Applied sample was in an amount proportional to the lipid content per seed in each germination period. Standards were oleoyl palmitate (wax), oleic acid (acid), and oleoyl alcohol (alc). 11-Eicosenol and erucyl alcohol (not shown) migrated to a similar position as oleoyl alcohol. The lower two components were not identified.

was wax rather than triglyceride. The various organelles from the cotyledon extract of the 20-day-old jojoba seedlings were isolated by sucrose gradient centrifugation (Fig. 3). Four major organelles were identified in the gradients. The wax bodies floated on top of the gradient while the glyoxysomes (catalase as marker), the mitochondria (Cyt oxidase as marker), and a membrane fraction (NADH-Cyt reductase as marker) were located at densities 1.25, 1.17, and 1.12 g/cm³, respectively. Isocitrate lyase of the glyoxylate cycle (not shown) and fatty acyl CoA dehydrogenase of the β oxidation sequence occurred in the glyoxysomes and were absent in the mitochondria. The enzymes at the top of the gradient were presumably derived from broken organelles during the preparation.

Isolated glyoxysome from jojoba cotyledons had an equilibrium density of 1.25 g/cm³ in sucrose gradient, similar to that of glyoxysomes from other fatty seedlings (10). In addition, individual enzymes in jojoba glyoxysomes had specific activities similar to those in glyoxysomes of castor bean (Table I) and of other fatty seedlings (10). The jojoba mitochondria contained specific activities of Cyt oxidase and citrate synthetase similar to those in the castor bean mitochondria. Surprisingly, the jojoba mitochondria had a glycerophosphate oxidoreductase activity as high as that in the castor bean mitochondria. The castor bean enzyme was suggested to carry out the metabolism of the glycerol moiety of the triglycerides (11). Since the jojoba lipid is an ester of fatty acid and fatty alcohol and no glycerol is present, the functional role of this enzyme in the jojoba mitochondria is unknown.

Apparently, the fatty alcohol derived from the wax ester was also metabolized to give rise to soluble carbohydrates. In microorganisms grown on fatty alcohols or alkanes as the sole carbon source, the fatty alcohols go through two oxidative enzymic reactions each of which utilizes NAD as the coenzyme (9, 15). Conversion of hexadecane to hexadecanoic acid has also been demonstrated in leaf slices (14). If the two enzymes are present in the jojoba cotyledon, they may be localized in the wax bodies or in the glyoxysomes. The glyoxysomes are the more likely candidates since they carry out several oxidation-reduction reactions of NADH/NAD (2) and have the capacity to modify the substituted and unsaturated fatty acids into soluble substrate for β oxidation (13).

Fig. 3. Separation of organelles from the cotyledon extract of 20-day-old jojoba seedlings on sucrose gradient. The supernatant fraction obtained after centrifugation of the crude homogenate for 10 min at 27,000 g was layered onto the gradient. The lipid pad floated on the gradient and was removed before fractionation. It was resuspended in 2 ml grinding medium and is presented as fractions 32 and 33. Units/gradient fraction: absorbance at 280 nm, relative; Cyt oxidase, NADH-Cyt reductase, and fatty acyl CoA dehydrogenase, μmol/min; catalase, mmol/min; lipase, mmol/min.

Alkaline Lipase in the Wax Bodies. Using an artificial substrate, N-methylindoxylmyristate, lipase activity was examined in the total cotyledon extract. No activity was detected in the extract of dry seeds at pH 5, 7, and 9. In the cotyledon extract from 20-day-old seedlings, an alkaline lipase was detected with an optimal pH at 9; the activity was only 50% at pH 7 and not
detectable at pH 5. The alkaline lipase in the 20-day-old seedlings had an activity of 236 nmol/min/cotyledon pair, which was roughly 50 times the rate of lipid degradation in vivo as calculated from Figure 1. We assayed the lipase activity with an artificial substrate (N-methyldioxynymristate), and thus the activity of the enzyme on the native substrate (wax ester) may be quite different.

When the alkaline lipase was assayed along the sucrose gradient containing the various organelles (Fig. 3), approximately 40% of the activity was found in the wax bodies, 20% in the soluble fraction, and 40% in the membrane fraction at density 1.12 g/cm³, and the activity was absent in the mitochondria and glyoxysomes. When the wax body fraction was examined under the electron microscope, a pure preparation was observed (Fig. 4). The wax bodies had a diameter of roughly 1 µm and were surrounded by a distinct membrane. They appeared to be similar to those wax bodies of the dry seeds observed in situ (21). In some preparations, certain wax bodies contained one or several electron-dense cores per organelle; we also observed this kind of wax bodies in situ in some cells. At this time, we do not know the composition of the core-containing wax bodies or the extent of their occurrence.

In the endosperm of dry castor bean, an acid lipase is present in the membrane of lipid bodies (23). If the alkaline lipase in the jojoba wax bodies is also localized in the membrane, its activity in the membrane fraction at density 1.12 g/cm³ (Fig. 3) may represent the membrane ghosts of the wax bodies. These membrane ghosts may be derived either from organelle breakage during the preparation or from remnants of wax bodies after the wax had been used up in vivo. The latter phenomenon was observed in the cotyledons of germinating legume seeds where the spherosomes were transformed into flattened sacs after the lipid had been exhausted (20). The situation in jojoba cotyledon was examined by the following method. The wax in the wax bodies was extracted with diethyl ether and the remaining solution was centrifuged in a sucrose gradient similar to the one used earlier. Most of the alkaline lipase of the wax bodies appeared at a membrane fraction at density 1.12 g/cm³, and the rest was present as a soluble enzyme at the top of the gradient (Fig. 5). The membrane fraction at 1.12 g/cm³ contained the phospholipid of the wax bodies. The treatment with diethyl ether and the subsequent gradient centrifugation did not inactivate the enzyme since 85% of the enzyme activity was recovered in the gradient. The data indicate that the alkaline lipase in the soluble fraction and the membrane fraction at density 1.12 g/cm³ in the sucrose gradient of the total extract (Fig. 3) were derived from the wax bodies. Thus, the alkaline lipase was restricted to the wax bodies, and more specifically, to the membrane of the organelles.

In many plant tissues, Cyt c reductase is present mainly in the ER which has an equilibrium density of 1.12 g/cm³ in the conventional sucrose gradient centrifugation (3, 10, 22). Thus, most of the Cyt c reductase activity at density 1.12 g/cm³ in the sucrose gradient of the total extract (Fig. 2) should represent that of the organelle. However, a small portion of the enzyme activity may also represent the membrane fragments derived from other organelles. The membranes of tonoplast (17), glyoxysomes (8), mitochondria (3, 8), leaf peroxisomes (8), and

### TABLE I.

<table>
<thead>
<tr>
<th>Glyoxysomes</th>
<th>Jojoba</th>
<th>Castor bean</th>
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<tbody>
<tr>
<td>Catalase</td>
<td>9.100</td>
<td>13.000</td>
</tr>
<tr>
<td>Lipase</td>
<td>1.1</td>
<td>0.93</td>
</tr>
<tr>
<td>NAD-malate dehydrogenase</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>Melate synthetase</td>
<td>1.6</td>
<td>2.1</td>
</tr>
<tr>
<td>Fatty acyl CoA dehydrogenase</td>
<td>4.7</td>
<td>6.5</td>
</tr>
<tr>
<td>Mitochondria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome oxidase</td>
<td>1.5</td>
<td>2.3</td>
</tr>
<tr>
<td>Citrate synthetase</td>
<td>0.40</td>
<td>0.45</td>
</tr>
<tr>
<td>Glycerophosphate oxid-reductase</td>
<td>0.32</td>
<td>0.22</td>
</tr>
</tbody>
</table>

The data on castor bean glyoxysomes are those reported previously (8).
protein bodies (5) have been reported to contain the enzyme. We also detected a trace amount of the enzyme activity in the membrane of the isolated wax bodies. Indeed, the observation that so many organelle membranes contain the enzyme may merely indicate the close biogenetic relationship of the various cellular membranes (3, 17). Mitochondrial membrane should be the exception since the properties of its enzyme are different from those of the enzymes of other organelles (8).

When the information we have presented is added to the current knowledge of gluconeogenesis from storage triglycerides in other fatty seedlings, we propose a pathway for the metabolism of storage wax in jojoba cotyledons. The wax in the wax bodies is hydrolyzed by an alkaline lipase which is associated with the membrane of the organelle. The resulting fatty acid and fatty alcohol would move to the glyoxysomes where they are modified and activated before entering the β oxidation sequence. The reactions of β oxidation and the rest of the gluconeogenic pathway are similar to those described in great detail in the castor bean endosperm. Further study is required in order to determine the enzymatic reactions and subcellular site of fatty alcohol modification.

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