Differential Extraction of Eleostearic Acid-Rich Lipid–Protein Complexes in Tung Seeds

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ABSTRACT: Lipid–protein complexes were identified in the 104,000 × *g* supernatant fraction of developing tung seeds. Incubation of this fraction with linoleoyl-CoA promoted an increase of chloroform-extractable lipids in a time-dependent manner. High-performance liquid chromatography analysis indicated that the extracted lipids were similar to mature tung oil triglycerides. Differential extraction using chloroform or chloroform/methanol indicated that linoleoyl-CoA promoted extraction of pre-existing lipids rather than *de novo* synthesis. An increase in extractable lipids was also observed after incubation with proteinase K. Isolation of lipid–protein complexes by sucrose density centrifugation and analysis of proteins by gel electrophoresis revealed several proteins specifically associated with this lipid fraction.

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KEY WORDS: *Aleurites fordii*, differential extraction, eleostearic acid, linoleoyl-CoA, lipid–protein complexes, triglycerides, tung.

Eleostearic acid (9,11,13-octadecatrienoic acid) comprises approximately 80% of the fatty acid content in tung oil (1). It is also present to a lesser extent in a few other plants such as the Chinese melon *Momordica charantia* (2) and two species of *Ricinocarpus* (3). Tung oil has been used for many years in the paint and varnish industries because it forms tough, resistant coatings once it has dried. This drying property is the result of the high reactivity of the eleostearic acid conjugated bond system, which is easily oxidized to promote crosslinking and polymerization between triglyceride molecules.

Our long-term goal is to identify the enzyme systems responsible for the synthesis of tung oil. Cloning of the respective genes would provide an opportunity to modify other oils to increase their drying properties. This might be accomplished by using immobilized enzymatic systems *in vitro* (4) or expression of the genes in plants for *in vivo* modification of oils (5,6). In this paper we report the identification of lipid–protein complexes enriched in eleostearic acid and describe the linoleoyl-CoA dependent extraction of lipids from this fraction.

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EXPERIMENTAL PROCEDURES

Materials. Linoleoyl-CoA, cofactors, and general chemicals were purchased from Sigma (St. Louis, MO). Organic solvents were purchased from J.T. Baker (Phillipsburg, NJ) unless indicated otherwise. *Rainbow*™ protein molecular weight markers were purchased from Amersham Life Science Inc. (Arlington Heights, IL).

Preparation of tung seed homogenates and subcellular fractions. Tung nuts from the American Tung Oil Corporation in Lumberton, MS were harvested weekly throughout the developmental period from mid-March to mid-August, 1995. Tung seeds were excised from the fruit and stored at –80°C prior to use. All experiments were performed using 18 seeds (approximately 45 g) from nuts collected during the rapid onset of eleostearic acid synthesis (July 26 to August 13, 1995) (7). All homogenization procedures were carried out between 0 and 4°C. Seed coats were removed and kernels were equilibrated in ice-cold homogenization buffer (0.33 M sucrose, 0.1 M potassium phosphate pH 7.2, 1 mM dithiothreitol (DTT), and 35 μg/L phenylmethylsulfonyl fluoride (PMSF) at 2 mL/g tissue for 15 min. The sample was transferred to an ice-cold blending cup and homogenized by pulsing six times for 7 s using a Waring blender. The homogenate was filtered through four layers of cheesecloth (pre-wet with homogenization buffer) and centrifuged at 300 × *g* for 5 min to pellet large debris and unbroken cells. The 300 × *g* supernatant was centrifuged at 20,000 × *g* for 20 min in a Beckman SW28 swinging bucket rotor (Beckman Instruments, Palo Alto, CA) to remove excess oil by flotation (8). The 20,000 × *g* supernatant was centrifuged further at 104,000 × *g* for 1 h in an SW28 swinging bucket rotor. Protein concentrations were determined using the Bio-Rad protein assay with bovine serum albumin (BSA) as a standard (Bio-Rad Laboratories, Hercules, CA).

Measurement of extractable eleostearoyl lipids in the 104,000 × g *supernatant fraction using high-performance liquid chromatography (HPLC).* A typical 3 mL reaction mixture included 2.4 mL of 104,000 × *g* supernatant and cofactors at a final concentration of 0.2% BSA, 5 mM $MgCl_2$, and 1 mM each of ascorbic acid and propyl gallate as antioxidants. Reactions were initiated by adding linoleoyl-CoA to a final concentration of 600 μM. Incubations were carried out

with continual shaking for 30 min in a 30°C water bath. At designated times, 200 µL aliquots were removed in duplicate or triplicate and transferred to ice cold test tubes containing 65 µL of 0.5 N HCl and 735 µL water. Lipids were extracted using 3 mL of chloroform (hereafter called the chloroform method).

HPLC analysis was carried out using a Waters 600E Multisolvent Delivery System with 717 Plus Autosampler and 996 Photodiode Array Detector (Waters Associates, Milford, MA). The eluent was monitored over a wavelength range of 250–350 nm using Millennium 2010 Chromatography Software. Prior to injection, samples were reduced to dryness under a nitrogen stream, reconstituted in 1.0 mL methylene chloride (Burdick & Jackson, Muskegon, MI), and filtered. The lipid extracts were analyzed by nonaqueous reverse-phase HPLC using two Waters Nova-Pak C_{18} (3.9 × 150 mm) columns maintained at 35°C. The elution solvent was a gradient of (A) isopropanol (EM Science, Gibbstown, NJ) and (B) acetonitrile at a flow rate of 0.8 mL/min: 20–40% A over 7.5 min, hold 2.5 min; to 70% A over 5 min and hold 10 min; then return to initial conditions over 10 min. Total run time, including column reequilibration, was 40 min.

Differential extraction of lipids in the presence or absence of linoleoyl-CoA. Reaction mixtures containing 104,000 × *g* supernatant, with or without linoleoyl-CoA, were incubated as described above. Aliquots were removed at t = 0 and 30 min and lipids were extracted using the chloroform method or the chloroform/methanol procedure of Bligh and Dyer (9). Eleostearoyl content of the extracts was monitored by ultraviolet (UV) absorbance at the λ_{max} of eleostearic acid in chloroform (275.1 nm) as described previously (10).

Isolation of lipid–protein complexes using sucrose density gradient centrifugation. A sucrose step gradient was prepared using 4 mL of 2% sucrose (wt/vol), 8 mL of 5%, 8 mL of 104,000 × *g* supernatant (0.33 M sucrose = 11%), 8 mL of 20%, and 8 mL of 50% sucrose in buffer containing 0.1 M potassium phosphate pH 7.2, 1 mM DTT, and 35 µg/L PMSF. The sample was spun at 104,000 × *g* for 20 h in an SW28 rotor. Fractions were collected from the top and analyzed for eleostearic acid and protein content using the chloroform/methanol extraction procedure and the Bio-Rad protein assay, respectively. Fraction 3, which contained the largest pool of eleostearic acid, was diluted 1:10 with water to reduce density and centrifuged at 104,000 × *g* for 2 h to separate eleostearic acid-containing membranes from soluble proteins. The pellet was resuspended in 0.5 mL of buffer (20 mM potassium phosphate pH 7.2, 1 mM DTT, 1% sucrose, and 35 µg/L PMSF), and the protein and eleostearic acid content in the supernatant and pellet fractions were measured. Proteins from equivalent percentage volumes of each fraction were precipitated using 10% trichloroacetic acid (vol/vol) and analyzed using SDS-PAGE (11) and Coomassie brilliant blue R-250 staining.

RESULTS

Measurement of extractable eleostearoyl lipids in the 104,000 × g supernatant fraction using HPLC. Incubation of the 104,000 × *g* supernatant fraction of a tung seed homogenate with linoleoyl-CoA resulted in an increase in chloroform-extractable eleostearoyl lipids for roughly 20 min (Fig. 1). The increase was dependent upon addition of linoleoyl-CoA, and boiling the sample prior to addition of linoleoyl-CoA resulted in loss of activity (not shown). HPLC analysis demonstrated that the extracted lipids were qualitatively similar to mature tung oil (Fig. 1a and b). The major peaks in the tung oil standard, in increasing elution times, are trieleostearin, linoleoyl-dieleostearin, a combination of oleoyl-dieleostearin and palmitoyl-dieleostearin, and stearoyl-dieleostearin (12) . Inspection of the UV spectrum from 250–350 nm indicated that the large unretained peak in Figure 1b contained very little eleostearic acid.

Differential extraction of lipids in the presence or absence of linoleoyl-CoA. The chloroform/methanol method of Bligh

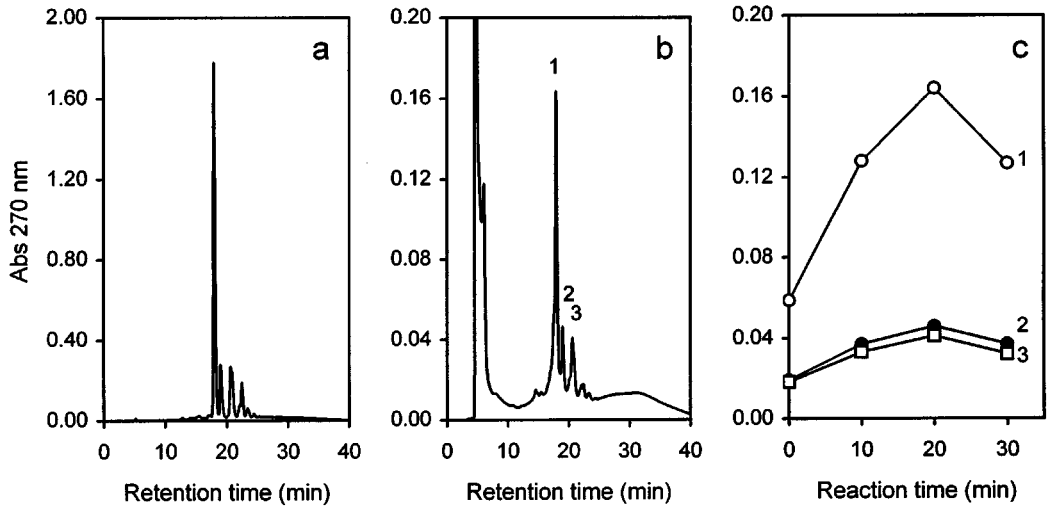

FIG. 1. High-performance liquid chromatography analysis of eleostearoyl lipids extracted after incubation of 104,000 × *g* supernatant with linoleoyl-CoA. (a) Tung oil standard. (b) Lipids extracted from the 104,000 × *g* supernatant after 20 min incubation. The three major peaks containing eleostearic acid are labeled 1, 2 and 3. (c) Changes in absorbance of each of the three major peaks observed in (b) throughout the course of the assay.

and Dyer extracted about four times as much eleostearic acid from the 104,000 × *g* supernatant as chloroform alone (Fig. 2). The amount of eleostearic acid extracted by chloroform/methanol, in the presence or absence of linoleoyl-CoA, did not change over time (Fig. 2). This demonstrated that linoleoyl-CoA did not promote the synthesis of eleostearic acid. However, linoleoyl-CoA did promote the chloroform extractability of pre-existing eleostearoyl lipids in a time-dependent manner (compare time 0 vs. 30 min, Fig. 2).

Incubation of the 104,000 × g supernatant with proteinase K. To determine if the chloroform-unextractable eleostearoyl lipids were associated with proteins, the 104,000 × *g* supernatant was incubated with increasing amounts of proteinase K. As shown in Figure 3, digestion of the 104,000 × *g* supernatant with proteinase K, in the absence of any exogenous linoleoyl-CoA, increased the amount of chloroform-extractable eleostearic acid in a dosage and time-dependent manner.

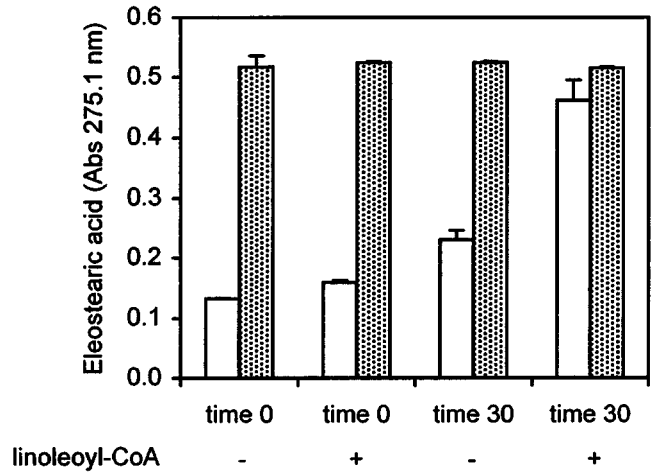

FIG. 2. Differential extraction of 104,000 × *g* supernatant lipids in the presence or absence of linoleoyl-CoA. Samples were extracted with chloroform (open bars) or chloroform/methanol (stippled bars).

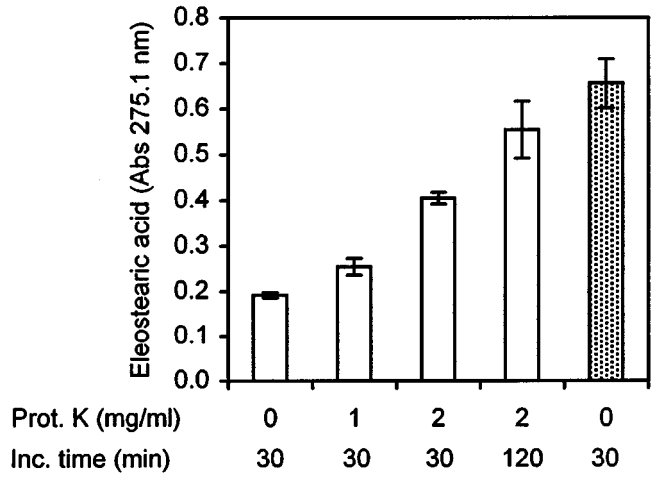

FIG. 3. Differential extraction of 104,000 × *g* supernatant lipids after incubation with proteinase K at 37°C. Samples were extracted with chloroform (open bars) or chloroform/methanol (stippled bars).

Isolation of lipid–protein complexes using sucrose density gradient centrifugation. Separation of the lipid components in the 104,000 × *g* supernatant by sucrose density centrifugation revealed two major bands in the gradient. The upper band was seen throughout the lightest sucrose fraction (2% wt/vol), while a thicker, more sharply defined band was observed at the 11–20% sucrose interface. Approximately 60% of the eleostearic acid in the upper band could be extracted with chloroform, while only about 20% could be extracted from the lower band, as compared to chloroform/methanol extraction efficiencies (not shown). Thus, the lower band contained the majority of the eleostearic acid that was not extractable by chloroform in the 104,000 × *g* supernatant. The proteins present in this fraction are shown in Figure 4 (F3).

To further concentrate the chloroform-unextractable lipid–protein complexes, the sucrose gradient fraction was diluted 1:10 to reduce density and centrifuged for 2 h at 104,000 × *g*. Organic extraction and protein measurement of the supernatant and pellet demonstrated that the pellet contained the majority of eleostearic acid but only 3% of the protein. SDS-PAGE analysis of the proteins in the pellet showed that there were only a few proteins associated with this fraction (Fig. 4, 10×). The 40 kDa and smaller bands associated with the pellet seem to be the same size as very abundant proteins in the supernatant, and therefore may represent slight contamination of the pellet. However, the largest protein of approximately 70 kDa, as well as the two prominent proteins of approximately 49 and 46 kDa, were selectively enriched upon pelleting and appear to be specifically associated with the chloroform-unextractable eleostearic acid fraction (Fig. 4, 10×).

DISCUSSION

The physiological role of the lipid–protein complexes identified here is presently unknown. Recently, Lacey and Hills

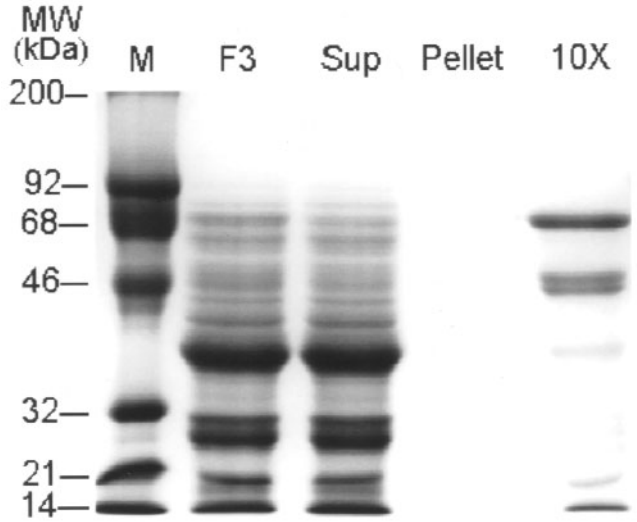

FIG. 4. SDS-PAGE of proteins from the indicated fractions. M, molecular weight standards; F3, sucrose gradient fraction 3; Sup and Pellet, supernatant and pellet obtained after dilution and centrifugation of fraction 3; 10×, 10-fold concentrate of the pellet fraction.

(13) have identified a subpopulation of microsomes from rapeseed that is also highly enriched in triacylglycerol content. Stobart *et al.* have demonstrated that safflower microsomes isolated *in vitro* are capable of synthesizing large amounts of triacylglycerols when incubated with linoleoyl-CoA and glycerol 3-phosphate (14). Since the triglycerides in nascent oil droplets are likely to be much easier to extract with chloroform, it is possible that our differential extraction assay is measuring this type of phenomenon. Alternatively, linoleoyl-CoA might act to solubilize lipid–protein complexes that formed adventitiously upon homogenization of the seed tissue. Further experiments will be required to determine how linoleoyl-CoA interacts with the lipid–protein complexes to promote the release of the eleostearoyl lipids, and what role the protein components might play in this process and/or in oil body biogenesis.

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