SUBCELLULAR LOCALIZATION OF OLEIC ACID BIOSYNTHESIS ENZYMES IN THE DEVELOPING CASTOR BEAN ENDOSPERM

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Summary:

The complete enzyme complement for oleic acid synthesis from acetyl-CoA or malonyl-CoA was associated with subcellular particles (density 1.21g/cc) isolated from developing castor seed endosperm. These particles were either the proplastids or a new unique particle.

Unsaturated fatty acids are the major constituents of the oil from plant seeds (1). In developing oil seeds (2,3), the kinetics of incorporation of ^{14}C-acetate indicated that oleic acid was the initial free fatty acid formed, and that it was the precursor of other unsaturated fatty acids. Direct evidence that oleic acid is the precursor of linoleic and linolenic acids (4,5) and of ricinoleic acid (6), the major fatty acid of castor seed oil, has been reported.

Recently a 500 to 15,000 x G particulate enzyme fraction, which consistently synthesizes primarily oleic acid from ^{14}C-acetyl-CoA and malonyl-CoA was isolated and characterized from developing castor seeds (7). This particulate fraction was stabilized and separated on a sucrose density gradient into several distinct protein bands. Unsaturated fatty acid biosynthesis was associated with only one of these bands. Thus, it has been possible to demonstrate for the first time that the complete enzyme system for oleic acid synthesis in higher plants may be associated with a unique subcellular component. This component may be the proplastid.

Materials and Methods

Materials:

Developing castor oil seeds, Ricinus communis L., variety Baker 296, (Baker Castor Oil Co., Plainview, Texas), were harvested from greenhouse grown plants 25-30 days after pollination and the endosperm tissue was used for experimental purposes.

Methods:

Endosperm tissue (16g) was ground to a thin slurry in 0.5M sucrose - 0.1M phosphate buffer, pH 7.2 (24ml) with a mortar and pestle, and filtered through 2 layers of cheesecloth to yield the crude homogenate (C_I). After centrifuging C_I for 5 minutes at 500 x G, the supernatant (S_1) was recentrifuged at 15000 x G for 15 minutes resulting in a heterogenous pellet (P_2) and milky supernatant (S_2). P_2 was resuspended in the sucrose - phosphate grinding medium (4 ml).

An aliquot of P_2 containing 14-18 mg protein was layered on a linear sucrose gradient buffered with 0.01 M TES [N-tris (hydroxymethyl)-methyl-2-amino ethane sulfonic acid], pH 7.5, contained in Spinco SW 25.1 cellulose nitrate tubes. The slope of the gradient was determined by 24 ml of 35-60% sucrose cushioned on 6 ml of 62% sucrose. The gradients were centrifuged for 3 hours at 24,000 rpm in a Spinco L-2, type D, ultracentrifuge. Each gradient was separated into either seventeen 1.5 ml fractions for fatty acid synthetase assays or forty-eight 0.5 ml fractions for all other assays. The fractions were numbered in order of increasing density and stored at 0-4°C.

Aliquots of each fraction were assayed for protein by the Lowry method (8) and for percent sucrose with an Abbe refractometer. Acetyl-1-^{14}C-CoA incorporation into long chain fatty acids was assayed in Warburg flasks at 25°C for 30 minutes with optimum co-

factor concentrations (7). After saponification, ^{14}C-fatty acids were extracted with petroleum ether/ether (2:1), methylated (9), taken to dryness in a stream of nitrogen, dissolved in ether-heptane (1:2) and an aliquot counted in toluene scintillator. The fatty acid methyl esters were separated by gas liquid chromatography and the radioactivity in each mass peak was determined directly with a Nuclear-Chicago gas radio-chromatography counting system.

Succinic dehydrogenase was determined spectrophotometrically by the method of Hiatt (10), including a 15 minute substrate preincubation. Catalase and glycolic acid oxidase were assayed by methods outlined by Breidenbach et al. (11) using a Rank oxygen electrode. Triose phosphate isomerase was assayed by a modification of the method of Beisenherz (12). Protochlorophyllide was assayed according to the method of Anderson and Boardman (13).

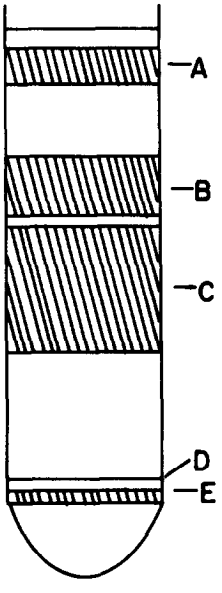

Figure 1. Separation of pellet fraction (P_2) components from developing castor bean endosperm on a linear sucrose density gradient. A and B. Mitochondria C. Fatty acid synthetase particles and peroxisomes. D and E. Unknown protein components.

Results:

The particulate pellet (P_2) was resolved into four distinct protein bands (Fig 1 and 2) with sucrose density gradient centrifugation. The uppermost band (A), near the soluble protein at the top of the gradient, and the next denser band (B) were identified as mitochondria based on density (11,14) and the presence of succinic dehydrogenase, an enzyme bound to mitochondrial membranes (11). The apparent isolation of two kinds of mitochondria (Fig. 2) may be due to a water elimination hypothesis suggested previously (14).

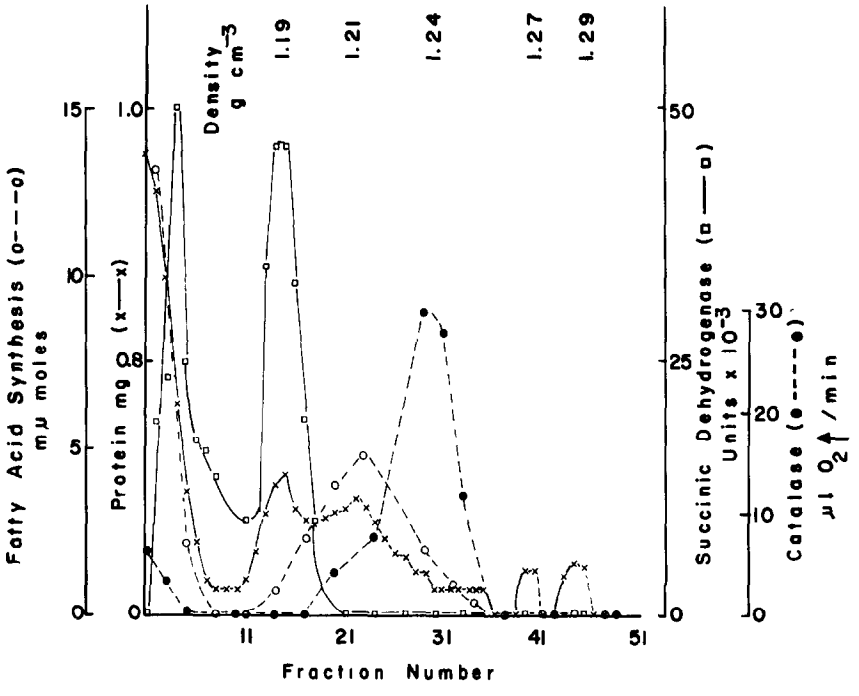

Figure 2. Distribution of protein, fatty acid synthetase, succinic dehydrogenase and catalase activity after sucrose density gradient separation of the components of the pellet fraction (P_2) derived from developing castor bean endosperm. Fatty acid synthesis-mμmoles acetyl-1-^{14}C-CoA incorporated x 1.5 ml fraction^{-1} x 30 min^{-1}; succinic dehydrogenase units - μmoles 2,6-dichlorophenolindophenol reduced x 0.5 ml fraction^{-1} x min^{-1}; catalase-μl O_2↑ x min^{-1} x 0.5 ml fraction^{-1}.

The third protein band (C) of density 1.21 g/cc, was visually resolved from B (Fig. 1) but a small amount of enzyme activity overlap did occur (Fig. 2). Particulate fatty acid synthesis was associated primarily with the protein band of density 1.21 g/cc (Fig. 2) and the unsaturated fatty acid, oleic acid, still constituted 40% of total fatty acids synthesized (Table I). The specific activity for fatty acid synthesis drops from 26.4 mμ moles x mg protein^{-1} x hr^{-1} in the P_2 pellet to 10.3 mμ moles x mg protein^{-1} x hr^{-1} for fractions 24-26 on the gradient. Triose phosphate isomerase also appeared to be associated with this region.

Acetyl-1-^{14}C-CoA was also incorporated into long chain fatty acids by the soluble protein at the top of the gradient (fractions 1-3) but only palmitic and stearic acids were formed (Table I). Most of this soluble protein probably arises due to particle disruption during resuspension of the P_2 pellet.

The peroxisome was demonstrated as a distinct protein shoulder of density 1.24 g/cc on the denser side of the fatty acid synthetase band (Fig 2) based on catalase localization (11,14, 15). Ninety-three percent of the P_2 catalase activity was localized

Table 1. Fatty acids synthesized by a particulate fraction (P_2) from developing castor bean endosperm and by various fractions separated from P_2 by sucrose density gradient centrifugation (See Figure 2).

Fraction	% distribution ^{14}C in fatty acids			
	palmitic	palmitoleic	stearic	oleic
P_2	8	0	12	80
1-3	25	0	75	0
20-23	55	trace	5	40

in the peroxisome. Glycolic acid oxidase and isocitritase activity could not be detected in any fraction derived from the developing castor endosperm. Two dense minor protein bands (D and E) have not been characterized (Fig. 1 and 2).

Discussion:

The enzymes required to synthesize oleic acid, the precursor of all other unsaturated fatty acids in developing oil tissues, and saturated fatty acids from acetyl-CoA are specifically associated with a non-mitochondrial particulate protein component in the developing castorbean endosperm. It has been shown that the microsomal fraction is the site of oleyl-CoA conversion to ricinoleic acid (6) the major fatty acid produced by this tissue (1,17). However, the density of the particulate oleic acid synthesis as well as the association of triose phosphate isomerase activity with the same gradient region suggest that the component is definitely not microsomal in origin. In personal communication Dr. M. Kobr and Dr. H. Beevers (Purdue University) report finding triose phosphate isomerase preferentially localized in the proplastid region on gradients used to study germinating castor bean endosperm organelles. We were unable to detect protochlorophyllide in this tissue but this may be due to the limited sensitivity of the absorption spectrophotometric method. Thus, the site of unsaturated fatty acid synthesis in this tissue remains open for further investigation as it may be proplastidic or a more specialized particle. The confinement of the enzymes required to convert acetyl-CoA to oleic acid in a compartment deficient in at least one of the oxidative enzymes of the TCA cycle also raises the question of how carbon flow to fatty acids and TCA cycle intermediates is regulated in this tissue.

The decrease in specific activity of fatty acid synthesis

when the P_2 pellet is separated into its protein components on the gradient may be due to denaturation of the particulate desaturase enzymes. The increase in palmitate and decrease in oleate synthesis associated with the lowered specific activity might also be due to the leaching out from the particles of sufficient quantities of enzymes or cofactors to make certain enzymic steps limiting in oleic acid synthesis. Further quantitative studies on the specifi enzymes in the fatty acid synthetase particles may help to outline the reason for the reduced fatty acid synthetase specific activity on the gradient. This information might also aid in elucidating the mechanism of unsaturated fatty acid biosynthesis in the castor bean endosperm.

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