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The Enzymology of Castor Oil Biosynthesis

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The castor plant *Ricinus communis* L., Euphorbiaceae produces seeds containing a unique oil. Castor oil is the only commodity vegetable oil that contains significant amounts of hydroxy fatty acid. This fatty acid is ricinoleic acid (12-hydroxy oleic acid) (Fig. 1) and it comprises up to 90% of the total fatty acid (FA) content of castor oil. Due to the physical and chemical properties imparted by the mid-chain hydroxy group, the oil has many important industrial uses. However, due to the presence of the toxic protein ricin and hyperallergenic 2S albumins, the production of castor oil is problematic. One logical approach to solving the biohazard problem presented by castor meal is to produce ricinoleate in plants lacking these noxious components. Several researchers have attempted to produce a ricinoleate oil by transgenic expression of the oleoyl-12-hydroxylase, the enzyme that is responsible for ricinoleate biosynthesis. This approach of introducing a single gene to engineer a novel oil composition was pioneered by the Calgene company the in order to develop laurate canola (Voelker et al. 1992).

Expression of the cDNA that encodes the oleoyl-12-hydroxylase (or FAH, for fatty acyl hydroxylase), resulted in the accumulation of a low level of hydroxy fatty acids in tobacco and *Arabidopsis* (Broun and Somerville 1997). These results suggested that the FAH gene by itself is not sufficient to produce high levels of ricinoleate in plants other than castor (McKeon and Lin 2002). An in vitro system using microsomes isolated from developing castor seed endosperm provided an effective means for following fatty acid hydroxylation and castor oil biosynthesis (McKeon et al. 1997; Lin et al. 1998a). Using metabolic profiling tools for analyzing lipid biosynthetic products (Lin et al. 1998a, 2002), the analysis of radiolabeled products from fatty acids incubated in the microsomal system enabled identification of several enzyme activities that give castor its unique ability to produce a high ricinoleate oil (McKeon and Lin 2002). Based on these studies, the preferential incorporation of ricinoleate into triacylglycerol (TG) led us to identify the final step in oil biosynthesis (Fig. 2) as a key step in maintaining high ricinoleate content while minimizing oleate incorporation into the TG fraction. Table 1 shows that the microsomes incorporate ricinoleate preferentially by a factor of 6-fold. The diacylglycerol acyltransferase (DGAT) is a transmembrane enzyme that catalyzes the acylation of diacylglycerol (DG) to TG, using acylCoA as the source for the final acyl group. This step has long been considered to be a rate limiting step in oil biosynthesis, and considerable evidence has accumulated to indicate that altered DGAT activity levels dramatically affect the yield of oil (He et al. 2004). AcylCoA synthetases (ACS) produce the acyl-donor

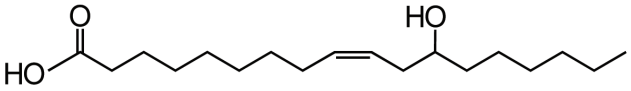

Fig. 1. Ricinoleic acid, the major component fatty acid of castor oil.

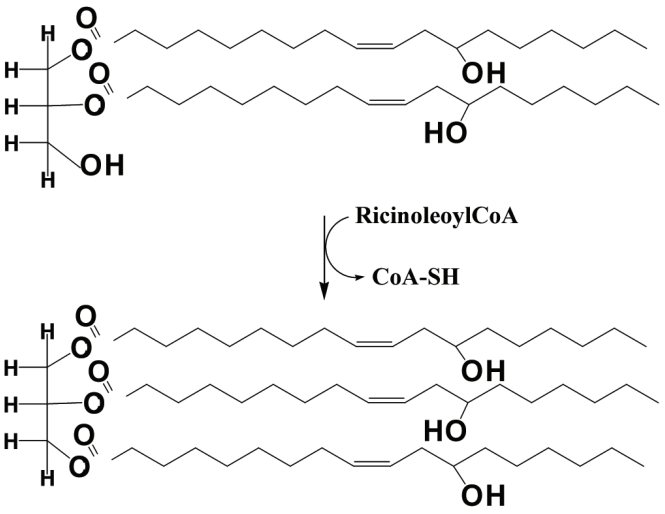

Fig. 2. Reaction catalyzed by Castor DGAT.

Table 1. Incorporation of ^{14}C-fatty acids into triacylglycerol by castor microsomes.

Fatty acid incubated (nmol)	Triacylglycerol produced, nmol
Ricinoleate(91)	48
Oleate(96)	8.3
Linoleate(98)	13
Linolenate(96)	10
Palmitate(89)	2.9
Stearate(114)	3.3

for this and many other acyltransferase reactions. The ACS have been cloned from *Arabidopsis* and identified in sequence databases. They are present as gene families of partially homologous sequences. In this study, we describe the activity of the castor DGAT encoded by a cDNA (He et al. 2004) from *Ricinus communis* (RcDGAT) and provide preliminary findings on the activity of a castor ACS expressed in yeast transformed with a cloned cDNA.

MATERIALS AND METHODS

Materials Used

Yeast strains and kits for constructing vectors were obtained from Invitrogen (Carlsbad, California) and used as previously described (He et al. 2004) to construct vectors, transform yeast, and express cloned cDNAs. Dipalmitolein and diolein were obtained from Larodan Fine Chemicals (Malmo, Sweden) and diricinolein was prepared by lipase digestion of triricinolein (Turner et al. 2003).

Assays

Yeast microsomes were prepared as described and DGAT assays performed (He et al. 2004) using 1-[^{14}C]-oleoylCoA as the acyl donor. The 1-[^{14}C]-oleoylCoA was synthesized from 1-[^{14}C]-oleic acid (58 mCi/mmole, ICN Radiochemicals, Irvine, California) using acylCoA synthetase (Sigma Chemical, St. Louis, Missouri) as described (McKeon et al. 1997). The ACS assays were based on the method of McKeon et al. (1997) with extraction of the acylated CoA into the aqueous phase of a Bligh-Dyer extraction.

Cloned Genes

The cDNAs for DGAT type 1, DGAT type 2 (Lin-Ma and McKeon, unpubl.), and the two ACS clones (He and McKeon, unpubl.), ACS 2 and ACS 4 were cloned based on conserved sequences derived from corresponding genes in other plants. The corresponding GenBank numbers are AY366496, AY916129, DQ300358, and DQ300359, respectively.

Chromatography

HPLC was carried out on a liquid chromatograph (Waters Associates, Milford, Massachusetts) using a flow scintillation analyzer (Model 150TR; Packard Instrument Co., Downers Grove, Illinois) to detect [^{14}C]-labeled TG at a flow rate of 1 ml/min. After lipid preparation from microsomal incubations, individual TG were separated on C18 reverse phase columns eluted with a linear gradient of 100% methanol to 100% isopropanol over 40 min (Lin et al. 1998b).

RESULTS AND DISCUSSION

The RcDGAT was cloned and expressed in yeast as described (He et al. 2004), with transcriptional expression of the RcDGAT under control of a galactose-inducible promoter. When the introduced gene is upregulated by the addition of galactose, DGAT activity detected is seven-fold that of the background DGAT activity present in the uninduced yeast with the DGAT gene. The control yeast carrying the same plasmid lacking the DGAT gene has DGAT activity similar to that seen in the uninduced yeast (Fig. 3) (He et al. 2004).

The products detected from RcDGAT activity assayed in yeast microsomes using added 1-[^{14}C]-oleoylCoA are radiolabeled TG derived from endogenous DG. Data presented in Fig. 4 demonstrate that when 1 mM diricinolein is added exogenously to yeast microsomes, the diricinolein is preferentially acylated compared to the endogenous DG, and the labeled product diricinoleoyl, oleoyl-TG represents twice the labeled TG observed compared to the trioleoyl-TG or diplamitoleoyl, oleoyl-TG when diolein or dipalmitolein are added at 1 mM. This indicates that the RcDGAT has a preference for acylating diricinolein as opposed to other DG. As a result of the efficient conversion of oleate to ricinoleate in castor, these DG would not normally be present in developing castor seed endosperm.

We have cloned two ACS from castor and expressed them as active enzymes in yeast (He and McKeon, unpubl.). When assayed in the presence of ricinoleate and oleate, the two ACS display different substrate preferences. The ACS 2 enzyme displayed approximately a three-fold preference for incorporating the ricinoleate into

acylCoA while the ACS 4 enzyme showed greater than 2-fold preference for oleate (Table 2). Taken together with the DGAT preference for acylating a ricinoleoyl substrate, the combined preferences of DGAT type 1 and ACS 2, two of the "final step" enzymes, could account for a significant portion of the 6-fold difference observed for incorporating ricinoleate vs. oleate into TG by castor microsomes.

We predict that other enzymes in the pathway will also display a preference for using ricinoleate, and that the combined effect of these preferences ultimately results in the high proportion of ricinoleate in castor oil. The pathway depicted in Fig. 5 indicates several enzymes that contribute to the ricinoleate content of castor oil. These enzymes serve two basic roles: high incorporation of ricinoleate into the acylglycerol fraction, and exclusion of oleate from the triacylglycerol fraction. These steps have been previously described (McKeon and Lin 2002), but are summarized here. Oleate is produced in the plastid and exported into the cytoplasm forming oleoyl-CoA. This product of fatty acid biosynthesis initiates the process of oil production. The lyso-phosphatidylcholine acyltransferase (LPCAT) transfers the oleoyl- moiety from oleoyl CoA into the *sn*-2 position of PC to make the substrate for the oleoyl-12-hydroxylase, which hydroxylates the *sn*-2 oleate to form *sn*-2 ricinoleoyl-PC. Phospholipase A_2 preferentially removes ricinoleate from the *sn*-2 position of PC and releases lyso-PC which is then recycled into the hydroxylase substrate by LPCAT. The ricinoleate is converted into ricinoleoyl-CoA, which

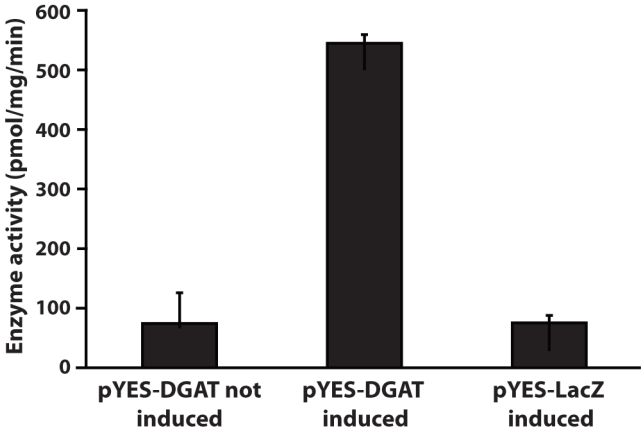

Fig. 3. Castor DGAT activity in yeast microsomes. Batches of microsomes (50 µg of total protein) extracted from yeast cells carrying the indicated plasmids were incubated with 14C-oleoyl-CoA Enzyme activities were measured based on the 14C-label incorporated into the TAG products.

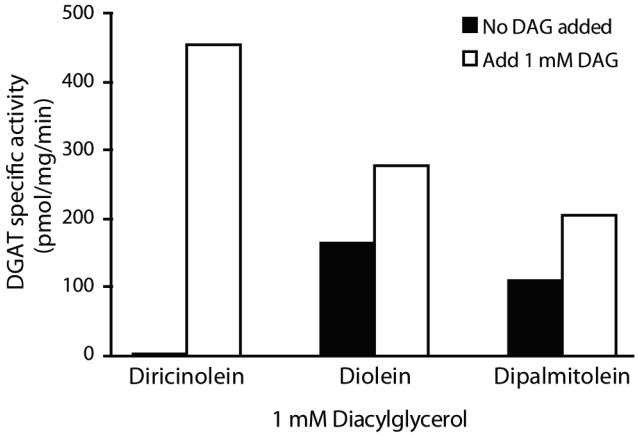

Fig. 4. Selective acylation of different DAG substrates by RcDGAT with ^{14}C-oleoyl-CoA. Aliquots of diacylglycerol in methanol were added to assay cocktails and reactions initiated by addition of ^{14}C-oleoyl-CoA. Lipids were extracted, then TG separated, identified and label quantified by HPLC with radioflow detection. These results have previously appeared (He et al. 2004). The bar for each DG represents the increase in labeling of that DG vs. total labeling of DG.

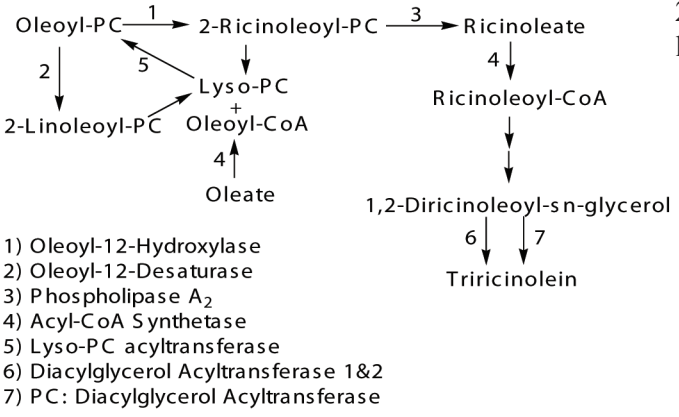

Fig. 5. Abbreviated version of castor oil biosynthetic pathway with key enzymes noted.

Table 2. AcylCoA synthetase activity based on amount of labeled FA incorporated into Acyl-CoA by 50 µg yeast microsomal protein in a 15 minute incubation.

	AcylCoA synthetase activity (nmol/min/mg)	
	Substrate	Substrate
Enzyme	1-^{14}C-oleate	1-^{14}C-ricinoleate
ACS 2	4.1	11.8
ACS 4	2.3	1.0

is then used as the acyl donor to incorporate preferentially the ricinoleate into phosphoglycerolipids to produce ricinoleoyl-containing diacylglycerols. The diricinoleoyl DG are then preferentially acylated by the DGAT to form diricinoleins and triricinolein, which make up castor oil. The phospholipid-diacylglycerol acyltransferase (PDAT) incorporates the *sn*-2 ricinoleate directly from the ricinoleoyl-PC product of the hydroxylase reaction into the triacylglycerol end product, and may serve as a "proofreading" mechanism to restructure acylglycerols and assure high ricinoleate content and low oleate content. We are further evaluating the role that these other enzymes play in directing the production of a high ricinoleate oil in castor. In addition to the family of acylCoA synthetases, we have cloned and are characterizing a second type of DGAT enzyme also cloned from the developing castor seed (Lin-Ma and McKeon, unpubl.). A recent report described the activity of this enzyme, the DGAT type 2 from castor (Kroon et al. 2006). The activity as expressed in the yeast was considerably lower, approximately 2% that of the DGAT type 1 from castor.

Because the physical behavior of hydroxylated FAs, such as ricinoleate, is so different from that of the saturated and unsaturated FA, the pathway that has evolved in castor may have flexibility in being able to incorporate other types of hydroxy FA and FA with other polar functional groups, e.g., epoxy. We are, therefore, evaluating the specificity of our cloned and expressed genes for using other unusual FAs. Since we are also evaluating transformation of the castor plant as an alternative for eliminating the noxious proteins expressed during seed development (Auld et al. 2001; McKeon et al. 2002), we hope to have the means to introduce such genes into castor. We believe that such genetic engineering can lead to development of castor as a crop that will meet the needs of the chemical industry for renewable resources that provide chemical feedstock.

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