JAOCS News Feature

Biosynthesis of Saturated and Unsaturated Fatty Acids by Maturing *Carthamus tinctorius* L. Seeds

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Recent advances in the biosynthesis of fatty acids by maturing safflower seeds are described. We now can define the systems responsible for (a) the conversion of CO_2 to sucrose to acetyl CoA; (b) the condensation of acetyl CoA via the de novo acyl carrier protein pathway to the terminal product, palmityl acyl carrier protein; (c) the elongation of palmityl acyl carrier protein to stearyl acyl carrier protein by a specific malonyl acyl carrier protein-elongation system; (d) the desaturation of stearyl acyl carrier protein to oleic acid by a highly specific, soluble stearyl acyl carrier protein desaturase; (e) the further desaturation of oleyl CoA to linoleyl CoA; and (f) the transfer of acyl CoAs to glycerol-3-phosphate to the final product, a triacylglycerol and its coalescence to the typical high lipid containing mature seed, engorged with oil droplets.

Future research should be directed to a fuller understanding of how the maturing seed triggers these complicated series of reactions, how all these enzymes are coordinated to allow a smooth flow of substrates for the formation of oil droplets, and, finally, how biochemical engineering can further improve oil crops in the ever growing demand for high yielding systems.

Introduction

The safflower (*Carthamus tinctorius* L.) is an intriguing ancient plant that has been grown for many centuries in a huge area of the world, ranging from the western borders of China to the Mediterranean Sea, as far north as Russia and as far south as Ethiopia. Its early use was as a source of orange dye for food and clothing, and only much later was it developed as a crop to produce edible oils. Of the total edible oils produced in the US, only 0.54% is derived from safflower with soybean (55%), cottonseed (21%), and maize (5%) being the major suppliers, in that order (1). Although safflower oil production, with respect to other oil crops, remains a minor crop, it was through the efforts of C.E. Claassen that the hull content of the seed was reduced with a parallel increase in protein and lipid content. In recent years, Knowles and his associates (2,3) have made major contributions in obtaining various genotypes of safflower with markedly different oil compositions. They have described 3 alleles at 1 chromosome locus that govern the proportions of linoleic and oleic acids in the mature seed. These genotypes are listed in Table I.

The allele, OL, relates to the formation of high levels of linoleic and concomitant low levels of oleic. The allele, ol, yields an oil with low linoleic and high oleic. The third allele, ol′, is associated with the formation of equal amounts of oleic and linoleic acids.

One of the goals of the plant biochemist is to relate these safflower genotypes to specific enzyme systems which participate in the formation of oleic and linoleic acids. As we shall see, recent biochemical advances in the knowledge of the biosynthesis of unsaturated fatty acids would suggest a much more complicated system than proposed in terms of the genetics of desaturation in the developing safflower.

To expand some of these problems, it will be worthwhile to review the present status of knowledge concerning fatty acid synthesis and desaturation in the developing safflower seed.

Relation of Maturation of Seed and Synthesis of Lipids

In the developing safflower seed, lipid synthesis begins about the tenth day after fertilization (DAF) and rises in a sigmoid fashion to about the 30th day, after which the seed begins to enter its period of dormancy (4). If the incorporation of [1-^{14}C] acetate into developing seed tissue is examined as a function of days after fertilization, a not unexpected initiation of vigorous incorporation of label into lipids is observed on the day 10 (DAF), with a sharp rise from the day 10 to the day 20 (DAF), and a rapid decline after the day 20 to a low level at about the day 26 (DAF) (5). These results readily explain the sigmoidal curve describing total lipid synthesis as a function of DAF, as observed by Sims, et al., (4). That is, at about the day 20 (DAF), the maximum rate of synthesis of fatty acids occurs; at this time, the point of inflection in Sims' oil synthesis curve occurs with a sharp fall in the rate of synthesis thereafter. Both oleic and linoleic acids are being intensively synthesized during the days 10-24 (DAF) with the highest rate observed again at the day 20. Thus, the enzymes for the formation of the fatty acids are evidently being synthesized in a coordinated manner in that critical period beginning around day 10 (DAF) and are then repressed or inactivated about the day 24 or 26.

The coordination of all the enzymes responsible for (a) the conversion of sucrose (synthesized in the leaf and transported to the newly fertilized seed) to acetyl CoA, (b) the formation

TABLE I

Composition of Safflower Fatty Acids as Related to Genotypes[a]

Genotypes	Linoleic (%)	Oleic (%)
OL OL	75-80	10-15
ol′ ol′	42-54	35-50
ol ol	12-30	64-83

[a]See Reference 2.

[1]Winner of the Award in Lipid Chemistry presented at the 48th Annual Fall Meeting, September, 1974, Philadelphia.

of stearyl acyl carrier protein (ACP), (c) the conversion to oleic and linoleic acids, and finally (d) the transfer of these 2 fatty acids as acyl CoAs to glycerol-3-PO_4 to form the final product, triacylglycerol, in the time interval from day 10 to day 24, must require a sophisticated program which is sufficiently stable not to be altered by external stresses of the environment.

It is now appropriate to outline the biochemical events that occur which make possible the biosynthesis of the final product, namely a triacylglycerol containing the desired oleyl and linoleyl functions as predicted by the genetics of the safflower plant.

Formation of Initial Substrates for Desaturation

The initial substrate for fatty acid synthesis is acetyl CoA. This important compound is undoubtedly formed by the glycolytic breakdown of sugar phosphates which are formed from sucrose. Sucrose is synthesized in the cytosolic compartment of the leaf cell from triose phosphates which were originally synthesized in the chloroplast and then transported out into the cytosol (6). It is not clear whether acetyl CoA is formed directly from pyruvate by oxidative decarboxylation, or indirectly by the transport of pyruvate into mitochondria, cleavage to acetyl CoA, condensation with oxaloacetate to citrate, transfer of citrate back to the cytosol, and finally cleavage to acetyl CoA and oxaloacetate. Nelson and Rinne (7) have recently shown that the citrate cleavage enzyme occurs in sufficient concentration in the developing soybean seed to allow for the generation of actyl CoA by the reaction:

$$\text{citrate} + \text{ATP} + \text{CoA} \rightarrow \text{acetyl CoA} + \text{oxaloacetate} + \text{ADP} + P_i.$$

If this observation can be extended to other plant tissues, it would demonstrate a great similarity between plant and animal cells in the mechanism of the generation of acetyl CoA. It has been known for a number of years that the citrate cleavage enzyme participates in the generation of acetyl CoA in animal cells (8).

Once acetyl CoA is formed in the cytosol, the conditions for synthesis must be met; namely, a sufficient concentration of adenosine triphosphate (ATP) to allow for the formation of malonyl CoA and the generation of reductants, i.e., NADPH and NADH, for the 2 reduction steps required for the addition of each C_2 unit to the growing fatty acid chain.

At least 9 proteins are required to convert acetyl CoA to palmitic acid. These proteins make up what is called the de novo system, and they specifically catalyze the conversion of C_2 units to a C_{16} unit. These proteins consist of the following:

(1) Acetyl CoA:ACP transacetylase:

$$\text{acetyl CoA} + \text{ACP} \rightleftharpoons \text{acetyl ACP} + \text{CoA}$$

(2) Acetyl CoA carboxylase, which consists, in turn, of 3 proteins, biotin carboxylase, biotinyl carboxyl carrier protein (BCCP), and transcarboxylase, which catalyzes the following set of reactions:

$$\text{ATP} + HCO_3 + \text{BCCP} \xrightarrow{\text{biotin carboxylase}} \text{ADP} + P_i + CO_2^- \sim \text{BCCP}$$

$$CO_2^- \sim \text{BCCP} + \text{acetyl CoA} \xrightarrow{\text{trans-carboxylase}} \text{malonyl CoA} + \text{BCCP}$$

(3) Malonyl CoA:ACP transacylase:

$$\text{malonyl CoA} + \text{ACP} \rightleftharpoons \text{malonyl ACP} + \text{CoA}$$

(4) β-ketoacyl ACP synthase:

$$\text{acetyl ACP} + \text{malonyl ACP} \rightarrow CO_2 + \text{acetoacetyl ACP} + \text{ACP}$$

(5) β-keto ACP reductase:

$$\text{acetoacetyl ACP} + \text{NADPH} + H^+ \rightarrow \text{D(-)}\beta\text{OH butyryl ACP} + \text{NADP}^+$$

(6) D(-)βOH acyl ACP dehydrase:

$$\text{D(-)}\beta\text{OH butyryl ACP} \xrightleftharpoons{-H_2O} \text{3-}\textit{trans}\text{-crotonyl ACP}$$

(7) Enoyl-ACP reductase:

$$\text{3-}\textit{trans}\text{-crotonyl ACP} + \text{NADPH} + H^+ \rightarrow \text{butyryl ACP} + \text{NADP}^+$$

Butyryl ACP now can return to step 4, replace acetyl ACP, and condense with malonyl ACP to form the C_6 intermediate, etc. The final product in the de novo synthesis of fatty acids is palmityl ACP. This system has been characterized in developing safflower seeds by Jaworski, Goldschmidt, and Stumpf (9). The system requires specifically ACP and both NADPH and NADH; the sole product is palmityl ACP. The system is stable at 37 C for 30 min, and it is completely inhibited by 1×10^{-5} M cerulenin. Its pH optimum is 7.0. It is a completely soluble system, i.e., prolonged centrifugation at 100,000 x g does not result in sedimenting the enzymes as happens with animal systems (8). The biochemical reactions in the de novo system include reactions 1-7 described previously with termination at the palmityl ACP level.

The second key system, also present in the developing safflower seed, is the elongation system (9). Its function is to convert palmityl ACP to stearyl ACP in the presence of malonyl ACP and NADPH. The elongation system requires the participation of a specific β-keto palmityl ACP synthase system which differs from the de novo synthase system (Reaction 4) in that it is far less sensitive to cerulenin than is the de novo system; a β-keto palmityl ACP reductase which requires NADPH specifically; a β-hydroxy palmityl ACP dehydrase; and, finally, a NADPH-linked Δ^2 trans-palmityl ACP reductase (9). The unusual aspect of this system is its strict specificity, i.e., only palmityl ACP is elongated to stearyl ACP, but the latter compound is not further converted to a C_{20} acid.

Thus, in the developing safflower seed, we have already identified at least 13 enzymes for the conversion of acetyl CoA to stearyl ACP. Each one of these enzymes is critical to the synthesis of fatty acids by the safflower. If any one of these were to be deleted by a mutation, the capacity of the safflower tissue to form stearyl ACP would be completely lost. Presumably all these enzymes appear, in a coordinated manner, on day 10 (DAF) in the cytosol of the developing safflower seed.

First Desaturation Step

In the developing safflower seed, McMahon and Stumpf (5) demonstrated that the 2 desaturases involved in the conversion of stearate to linoleate appear about day 10 (DAF) and reach a peak of activity about day 17-20. We now have a considerable amount of basic information concerning these 2 important steps.

The first desaturation step is catalyzed by a highly specific soluble enzyme called a stearyl ACP desaturase (10). It occurs in the cytosol of the developing seed. Molecular oxygen and a suitable reductant, usually NADPH, are required. Ferredoxin is the intermediate electron carrier coupling the reductant (NADPH) to the desaturase which presumably participates in the activation of molecular oxygen to form a species of oxygen capable of attacking and extracting a hydride ion and a proton from the C_9 and C_{10} of stearyl ACP. Once again, the substrate is highly specific, i.e., palmityl ACP, palmityl CoA, and stearyl CoA are totally unreactive. Because the enzyme is cyanide sensitive, the desaturase probably contains a prosthetic group which either binds to cyanide, or cyanide may be removing an essential component. Another interesting feature of the system is the consistent observation that while stearyl ACP readily accumulates in an enzyme system that is synthesizing this compound from ^{14}C-acetate or ^{14}C-malonate, the desaturation product, oleyl ACP, does not accumulate. Rather, free oleate is formed. These results pose the interesting possibility of oleyl ACP being formed from stearyl ACP, but being readily hydrolyzed to free oleic acid and free ACP with the ACP being recycled for the further synthesis of oleate. Free oleic acid is now converted to oleyl CoA by

acyl CoA synthetase which occurs in high activity on the endoplasmic reticuli of plant cells. Figure 1 summarizes some of these events.

Second Desaturation Step

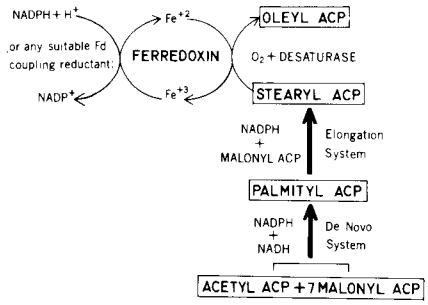
Why should the formation of oleyl CoA be important? First, oleyl CoA is the substrate for the acylation of glycerol-3-phosphate to phosphatidic acid which eventually is converted to the final product, triacylglycerol, the principal storage product of the mature safflower seed. Because oleyl ACP is unavailable as a substrate, no conclusion can be drawn about its role in acylation reactions. Second, oleyl CoA is the specific substrate for the final desaturation step, namely, the formation of linoleyl CoA. This product then is transferred very rapidly to suitable acceptors to form triacylglycerol. The second desaturation step, catalyzed by oleyl CoA desaturase, has been examined in some detail by Vijay and Stumpf (11,12), employing 14-18 day old maturing safflower seeds.

Associated very tightly with the endoplasmic reticuli of the developing safflower seed, the enzyme requires specifically molecular oxygen and NADPH. There is no evidence for an involvement of oleyl ACP as a substrate. Oleyl CoA is the specific substrate. Stearyl CoA and linoleyl CoA, as well as elaidyl CoA and *cis* vaccenyl CoA, are completely inert. These results are summarized in Figure 2. The product, linoleyl CoA, is rapidly transferred to suitable acceptors to form phospholipids and triacylglycerol.

The formation of the final oil droplet has not been clearly defined at the present writing. Oil droplets contain only triacylglycerols, presumably surrounded by a mono-molecular membrane. During maturation, oil droplets increase very rapidly in number and size. Their biogenesis must involve a very efficient delivery of (a) the necessary substrates which, in the case of safflower, are oleyl CoA and linoleyl CoA, and glycerol-3-phosphate which is the acceptor for the acyl CoAs, and (b) the prerequisite enzyme essential for the formation of triacylglycerols at a cellular site which allows the fusion of newly synthesized triacylglycerol molecules to yield the characteristic oil droplet. A number of studies have been conducted in this area, but the final description of the biogenesis of an oil droplet remains for further investigation (13-15).

Regulation of Lipid Biosynthesis in Developing Safflower Cell

With the background information now at hand concerning the basic steps required for the biosynthesis of the principal fatty acids, the biochemist must address himself (herself) to the basic questions concerning the regulation of lipid synthesis in these tissues. Why is it that the safflower accumulates large amounts of lipid as fat globules, whereas, another seed such as the sorghum or the pea has little, if any, oil droplet deposits, although the machinery for synthesis can be readily demonstrated in these low lipid-containing seeds (16). Would it be possible, by genetically manipulating the



FATTY ACID SYNTHESIS AND DESATURATION OF STEARYL ACP TO OLEYL ACP

FIG. 1. Mechanism of desaturation of stearyl acyl carrier protein (ACP) to oleyl ACP by a safflower stearyl ACP desaturase.

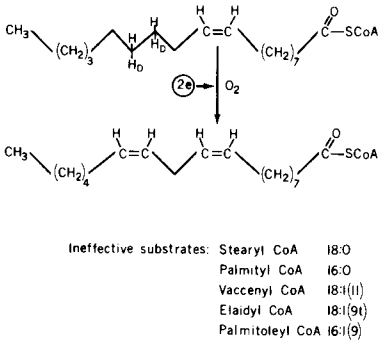


FIG. 2. Mechanism of desaturation of oleyl CoA to linoleyl CoA by a safflower oleyl CoA desaturase.

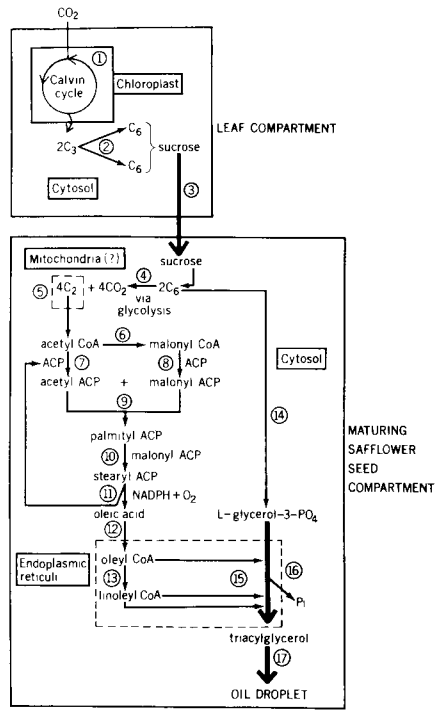


FIG. 3. Summary of events involved in the fixation of CO_2 in the chloroplast compartment to the synthesis of the final product, the oil droplet, in the maturing safflower seed: 1- conversion of CO_2 to triose phosphates by the Calvin cycle in the chloroplast and their transport into the cytosol of the leaf cell; 2- conversion of triose phosphates to hexose phosphates and their subsequent synthesis to sucrose; 3- transport of sucrose from leaf to maturing seed; 4- glycolytic breakdown of hexose monophosphate (derived from sucrose) to pyruvate and its transport into mitochondria to be converted to acetyl CoA; 5- subsequent transport out to cytosol (as citrate ?); 6- carboxylation of acetyl CoA to malonyl CoA by acetyl CoA carboxylase; 7- conversion of acetyl CoA to acetyl acyl carrier protein (ACP); 8- conversion of malonyl CoA to malonyl ACP; 9- de novo system converting substrates of palmityl ACP; 10- elongation system forming stearyl ACP; 11- desaturation of stearyl ACP to oleyl ACP and hence to oleic acid; 12- conversion of oleic acid to oleyl CoA; 13- desaturation of oleyl CoA to linoleyl CoA; 14- conversion of hexose monophosphate to L-3-glycerol phosphate; 15- transfer of acyl CoAs to L-3-glycerol-3-phosphate to form phosphatidic acid; 16- phosphatidate phosphatase cleavage to yield diacylglycerol, which is then acylated by acyl CoA to form triacylglycerol which is then acylated by acyl CoA to form triacylglycerol; 17- the coalescence of triacylglycerol to form the final product, an oil droplet.

TABLE II

Fatty Acid Composition of Seed and Leaf Lipids of Safflower

Safflower variety	Fatty acid				
	16:0	18:0	18:1	18:2	18:3
	(% Composition)				
US-10					
Seed (mature)	6	2	13	79	0
Cotyledenous leaf[a]	9	1	5	47	38
First foliage leaf[a]	20	t	4	26	50
UC-1					
Seed (mature)	6	7	75	12	0
Cotyledenous leaf[a]	11	t	10	24	55
First foliage leaf[a]	19	t	3	27	51

[a]Germinated seed, 3 weeks old, grown under 12 hr day:12 hr night conditions.

regulatory systems which turn on and offslipid biosynthesis, to convert low lipid containing seeds into high lipid seeds?

From the work of Knowles and others, 3 alleles have been identified which appear to affect the relative amounts of oleic and linoleic acids in the safflower seed. We now know that over 20 enzymes are directly involved in the synthesis of oleic and linoleic acids from acetyl CoA, and over 30 enzymes would have to be involved for the synthesis of triacylglycerols from acetyl CoA. Deletion of any of these enzymes would result in a lethal mutant. Furthermore, the alleles which appear to have an effect on the relative composition of oleic and linoleic acids in safflower are not absolute, i.e., the safflower variant simply has an altered ratio of oleic to linoleic acids. This result, therefore, implies that, as we have already indicated, the regulation of oleic and linoleic biosynthesis is complex, i.e., many factors are responsible for modifying the final composition of the fatty acids. However, in each variant, these factors are under strict control so that the phenotype always synthesizes the fatty acids programmed by its DNA.

None of the enzymes directly participating in the flow of acetyl CoA to the final product have been characterized as regulatory. No allosteric effectors have been described in plant systems which greatly accelerate or inhibit fatty acid synthesis. Therefore, unless future investigations reveal the presence of such effectors, direct kinetic modulation of key enzymes for synthesis may not be an important component of regulation.

One of the fascinating aspects concerning the problem of lipid biosynthesis is the mechansim by which lipid biosynthesis is turned on and off. Thus, the question arises, why in the maturation of the seed does vigorous lipid synthesis begin on day 10, to be shut down on about days 24-26. Information leading to an explanation of these events would be fundamental to all aspects of developmental plant biochemistry.

Another question which arises derived from the observation that the maturing seed genetically will always yield as its final oil product a fixed composition of oleic and linoleic acid with the complete absence of linolenic acid. Thus, one could conclude that the safflower DNA has no structural genes which can program for enzymes responsible for the synthesis of trienoic acids. Nevertheless, when leaf tissue lipids are examined, one is struck by the presence of a high amount of linolenic acid (Table II). Linolenic acid is wholly associated with chloroplast membrane lipids, and is an essential structural component of all eucaryotic chloroplast systems (17). Apparently, in the maturing seed, the structural genes responsible for the biosynthesis of linolenic acid are repressed. In contrast, in leaf tissue, these are derepressed so that a suitable translational process allows for the synthesis of the enzyme(s) required for linolenic synthesis.

Figure 3 brings together the several ideas discussed in this article, and presents the current thoughts concerning the biosynthesis of oleic and linoleic acids in the developing seed. With this information as a background, future research should elaborate and expand this figure so that biochemical engineering will not only increase the efficiency of synthesis, but also affect the type of fatty acid to be synthesized.

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Liverpool Polytechnic Holds Symposium

A symposium on 'Recent Advances in Chemistry and Technology of Fats and Oils' will be held December 11-12, 1975, at Liverpool Polytechnic.

Speakers will include B.J.F. Hudson (University of Reading), H.B.W. Patterson (Van den Bergh and Jurgens Ltd.), A. Turner and A.G. Marriner (Cadbury Typhoo Ltd.), A.S. Truswell (Queen Elizabeth College, London), S. Aneja and M.I. Gurr (Colworth-Welwyn Laboratory, Unilever Ltd.), C.W. Thomson (Guthrie Estates Ltd.), and A.E. Thomas (Glidden Durkee, Dwight P. Joyce Research Centre, Strongville, Ohio, USA). F.D. Gunstone (University of St. Andrews) will deliver the Hilditch Memorial Lecture entitled 'Natural and Unnatural Fatty Acids.'

For further details write: Head of Chemistry Department, Liverpool Polytechnic, Byrom Street, Liverpool, L3 3AF. Telephone, 051 207-3581 (Extn. 148). ■

Call for Papers

A symposium on Tall Oil, Oleoresins, and Naval Stores-Derived Products will be sponsored by the Cellulose, Paper, and Textile Division of the American Chemical Society. It will be held as part of the American Chemical Society National Meeting, March 20-25, 1977, in New Orleans, Louisiana.

The symposium will run from Wednesday morning through Friday noon, and will include both invited and contributed reviews and research papers of 20, 40, and 60 min (including question period). Authors will be free to publish their papers in the outlet of their choice. Titles, requested time, and a short abstract should be received by the Chairman as much before October 1, 1976, as possible. Send submitted work to Dr. John W. Rowe, Forest Products Laboratory, Forest Service, USDA, P.O. Box 5130, Madison, WI 53705. ■