Very Long Chain Fatty Acid Synthesis in Sunflower Kernels

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Most common seed oils contain small amounts of very long chain fatty acids (VLCFAs), the main components of oils from species such as *Brassica napus* or *Lunnaria annua*. These fatty acids are synthesized from acyl-CoA precursors in the endoplasmic reticulum through the activity of a dissociated enzyme complex known as fatty acid elongase. We studied the synthesis of the arachidic, behenic, and lignoceric VLCFAs in sunflower kernels, in which they account for 1−3% of the saturated fatty acids. These VLCFAs are synthesized from 18:0-CoA by membrane-bound fatty acid elongases, and their biosynthesis is mainly dependent on NADPH equivalents. Two condensing enzymes appear to be responsible for the synthesis of VLCFAs in sunflower kernels, β-ketoacyl-CoA synthase-I (KCS-I) and β-ketoacyl-CoA synthase-II (KCS-II). Both of these enzymes were resolved by ion exchange chromatography and display different substrate specificities. While KCS-I displays a preference for 20:0-CoA, 18:0-CoA was more efficiently elongated by KCS-II. Both enzymes have different sensitivities to pH and Triton X-100, and their kinetic properties indicate that both are strongly inhibited by the presence of their substrates. In light of these results, the VLCFA composition of sunflower oil is considered in relation to that in other commercially exploited oils.

KEYWORDS: *Helianthus annuus*; kernel; sunflower oil; arachidic; behenic; fatty acid elongase

INTRODUCTION

Very long chain fatty acids (VLCFAs) are those that contain more than 18 carbon atoms. These are common components of plant waxes and surface coverings, either in their free form or when they are esterified to fatty alcohols, and they are also the precursors to fatty alcohols, ketones, and hydrocarbons (*1*). In the seed oil of the *Brassicaceae* family, these fatty acids are found in high amounts as monounsaturated 20:1 or 22:1 derivatives. Indeed, in rapeseed oil, erucic acid (22:1) accounts for between 30 and 60% of the fatty acid content. Since a high intake of this type of fatty acid produces damaging effects on the cardiovascular system in some animals (*2*), erucic acid was removed from this oil by plant breeding techniques, giving rise to the rape cultivar known as canola in the early 1960s. Nevertheless, VLCFA are present in most common seed oils, albeit in small amounts.

These fatty acids are synthesized in the endoplasmic reticulum from acyl-CoAs exported from plastids and malonyl-CoA by the action of a fully dissociated enzyme complex called fatty acid elongase (FAE; ref *3*). The first enzyme of this complex is the 3-ketoacyl-CoA synthase (KCS, not yet classified by the enzyme commission), which condenses the aforementioned substrates to yield a β-ketoacyl-CoA, with the concomitant release of CO_2. Then, reduction and dehydration reactions yield a two carbon elongated acyl-CoA. KCS is important in this process. Indeed, the KCS responsible for the synthesis of the VLCFAs that contribute to the waxes of *Sidmonia chinensis*

could complement the mutation responsible for the absence of erucic acid in canola (*4*). The β-ketoacyl-CoAs produced by KCS are reduced to their hydroxyl derivatives by the action of the β-ketoacyl-CoA dehydrogenases (E.C. 1.1.1.35 and E.C. 1.1.1.36). The β-hydroxy-CoA products are then dehydrated and further reduced by the successive actions of a dehydratase (E.C. 4.2.1.17) and a reductase (E.C. 1.3.1.38) to yield the final 2C-elongated acyl-CoA (*5*). Some of these latter enzymes have already been described, and their cofactor dependence has been investigated (*6, 7*).

VLCFA synthesis has been studied extensively in species that produce unsaturated derivatives of these fatty acids such as *Brassica napus*, *Lunaria annua*, and *Limnantes alba*. These species were studied due to the intense biosynthetic activity in developing seeds (*7−9*) and the active synthesis in other tissues such as the epidermal cells where they are important for the synthesis of saturated VLCFAs destined for wax production (*10*). However, little attention has been paid to the synthesis of the VLCFAs in oils of other commercial species. Common sunflower oil contains three main saturated VLCFAs: arachidic (20:0), behenic (22:0), and smaller amounts of lignoceric (24: 0) acids. These fatty acids account for 1−3% of the total fatty acids in sunflower seeds (*11*), their content being augmented in high-saturated sunflower lines developed by breeding and mutagenesis (*12*). Although these fatty acids are a relatively minor component of these oils, their high melting points could affect the properties of the triacylglycerols in the oil. In this regard, their presence could improve the properties of plant oils destined for the production of plastic fats.

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In the present study, acyl-CoA elongase activity was characterized in microsomes of developing sunflower kernels. The VLCFA composition of sunflower oil is discussed in function of these results.

EXPERIMENTAL PROCEDURES

Microsome Preparation. Microsome fractions were prepared by differential centrifugation of fresh developing sunflower kernels from the commodity-type sunflower line CAS-6, using a variation of the method described by Sarmiento et al. (*13*). Approximately 5 g of endosperm was ground with a mortar and pestle in the presence of 50 mL of ice-cooled grinding buffer that contained 50 mM potassium phosphate pH 7.5, 0.6 M sorbitol, 1 mM EDTA, and 1 mM MgCl$_2$. The grinding was carried out in three steps with the addition of a fraction of the grinding buffer followed by the filtration of the homogenate through two layers of miracloth. The filtrate was then centrifuged for 20 min at 38 000g, and the resulting supernatant was centrifuged again for 2 h at 150 000g. This allowed complete recovery of the microsomal fraction of sunflower kernels. The resulting pellets were washed three times with 10 mL of fresh 50 mM potassium phosphate, pH 7.0 cooled on ice, and resuspended in a final volume of 2 mL of the same buffer using a glass homogenator. The microsome suspensions were stored at −80 °C, and thaw/freezing cycles were avoided because they promote inactivation of fatty acid elongase (data not shown).

Fatty Acid Elongase Activity Assay. Total FAE activity was assayed by incubating 500 μg of microsomal protein with 50 mM potassium phosphate buffer pH 7.5, 0.5 mM NADH, 0.5 mM NADPH, 25 μM acyl-CoA, 2 mM DTT, and 2 kBq [2-^{14}C]malonyl-CoA (2.0 GBq/mmol) in a final volume of 0.2 mL for 2 h at 25 °C. The assay was stopped by adding 0.85 mL of 2.35 M NaOH and allowing saponification to proceed for 30 min at 80 °C. A 0.35 mL volume of 4 M H$_2$SO$_4$ was added, and the acyl fragments were extracted three times with 2 mL aliquots of hexane. Radioactivity in the pooled organic fractions was counted using an aliquot of 1/10 the total volume. The remaining extract was evaporated under a stream of nitrogen gas, and the residue was methylated with 1 mL of 2.5% methanolic H$_2$SO$_4$ at 80 °C for 10 min. This reaction was stopped by adding 3 mL of 5% NaCl, and the fatty acid methyl esters were extracted twice with 2 mL aliquots of hexane. The combined hexane fraction was evaporated under nitrogen, and the fatty acid methyl esters were fractionated by reversed-phase thin-layer chromatography (TLC) using methyl stearate and methyl arachidate as standards. Radioactive bands were identified and quantified in an Instant Imager radioactivity scanner (Packard, Canberra, Australia).

In experiments intended to study the effect of the different cofactors, methylated acyl fragments were fractionated by normal phase TLC using methyl stearate, methyl ricinoleate, and 2-decanone as standards.

Thin-Layer Chromatography. Direct phase thin-layer chromatography was run on 20 cm × 20 cm G60 silica gel plates, using hexane/diethyl ether/formic acid (90:10:1) as the solvent. For reverse-phase TLC, the silica gel plates were coated with 2.5% vaseline oil (mixture of purified hydrocarbons of a high molecular weight) in hexane and activated for 1 h at 80 °C. These plates were developed with acetonitrile/hexane (90:10). Argentation TLC of methyl esters of the trans unsaturated intermediates was performed on silica gel plates coated with a solution of 15% of AgNO$_3$ in acetonitrile, using hexane/ether (80:20) as the developing solvent.

Enzyme Purification. Sunflower kernel microsomes, prepared as indicated previously, were centrifuged for 2 h at 150 000g, and the pelleted microsomes were homogenized in 20 mM potassium phosphate pH 7.5, 10 mM DTT, and 0.5% Triton X-100 and kept on ice for 2 h with mild stirring. The solubilized membranes were recentrifuged for 2 h at 150 000g, and while the pellet, containing the insoluble debris, was discarded, the supernatant was submitted to ion exchange chromatography. Eighty milligrams of the solubilized microsomal protein was loaded on a HiTrap DEAE FF (5 mL) ion exchange column attached to an AKTA PRIME protein separation unit (Amersham, Upsala, Sweden) previously equilibrated with 10 column volumes of elution buffer that contained 20 mM potassium phosphate pH 7.5, 0.5% Triton X-100, and 1 mM DTT. Following binding of the protein, the

column was eluted with 10 mL of elution buffer followed by a 60 mL gradient of 0−0.5 M NaCl prepared in the same buffer. The purification was terminated by the column being washed with 20 mL of elution buffer containing 0.5 M NaCl. Fractions of 1.5 mL were collected and immediately transferred to ice before the FAE activity was assayed.

Preparation of 20:0-CoA. Noncommercial arachidyl-CoA (20:0-CoA) was necessary to study the 22:0 biosynthesis. This compound was synthesized by reacting the *N*-hydroxysuccinimide ester of arachidic acid (NHS-20:0) with fresh reduced coenzyme A (*14*). NHS-20:0 was prepared by reacting arachidic acid with *N*-hydroxysuccinimide in the presence of an equimolar amount of dicyclohexylcarbodiimide in dry ethyl acetate for 16 h at 25 °C (*15*). The substitution reaction was started by mixing 50 mg of coenzyme A dissolved in 3 mL of water with an excess of 0.8 g of NHS-20:0 dissolved in 6 mL of tetrahydrofuran in the presence of 50 μL of thioglycolic acid and 168 mg of sodium bicarbonate for 4 h at 25 °C. After the remaining CO$_2$ was removed under vacuum, the reaction product was precipitated by adding 12 mL of 5% HClO$_4$. The white precipitate was filtered and washed with 0.8% HClO$_4$ and then washed again with acetone and ethyl ether. The dry 20:0-CoA was resuspended in water and precipitated again by raising the percentage of HClO$_4$ in the solution to 0.8%. The purified product was washed with acetone and dried under nitrogen. The purity of 20:0-CoA was assessed by normal phase TLC, using *n*-butanol/water/acetic acid (8:8:1) as the solvent. Commercial 18:0-CoA was used as the standard. Exact quantification of the 20:0-CoA produced was achieved by gas chromatography of its methylated acyl moiety using heptadecanoic acid as the internal standard.

Analysis of Fatty Acid Methyl Esters. Fatty acid methyl esters were prepared by treating the sunflower endosperm or the lipid sample with 3 mL of methanol/toluene/sulfuric acid (88:10:2 v/v) for 1 h at 80 °C (*16*). After cooling, fatty acid methyl esters were extracted twice with 2 mL aliquots of heptane and analyzed by gas chromatography using a Supelco SP-2380 capillary column (30 m, 0.25 mm i.d., 0.20 μm film thickness; Bellefonte, PA). Hydrogen at 28 cm/s was used as the carrier gas. The detector and injector temperatures were 200 °C, and the oven temperature was 170 °C. Methyl esters were identified by comparison with known standards.

Protein Determination. Protein determination was carried out by the method described by Dulley et al. (*17*) using BSA as the standard.

RESULTS AND DISCUSSION

General Aspects of Fatty Acid Elongase Activity in Sunflower Kernels. Fatty acid elongase activity is due to a dissociated complex of enzymes that includes β-ketoacyl-CoA synthase, β-ketoacyl-CoA dehydrogenase, β-hydroxyacyl-CoA dehydratase, and acyl-CoA reductase. The total FAE activity in sunflower kernels was about 3 orders of magnitude lower than that of the soluble condensing activity in plastids such as that of β-ketoacyl-ACP synthases (*18*). Similarly, FAE activity in sunflower was lower than that found in species displaying a high content of erucic acid in their oils (*7*), which probably accounts for the low VLCFA content in sunflower oils from commodity-type lines (*11*). Therefore, it was very important to carefully rinse the microsomes with fresh phosphate buffer to completely remove the residual soluble ketoacyl-ACP synthases (KAS) prior to assaying FAE elongase activity. If not adequately removed, soluble KAS activity will hamper the correct determination of FAE activity due to consumption of the malonyl-CoA substrate into palmitate, stearate, and oleate.

A time course of the generation of 18:0-CoA and 20:0-CoA provided a first approximation to understand the mechanism of fatty acid elongation in sunflower kernels substrates analyzed. The generation of both products displayed linear kinetics during the first 2 h, reaching saturation at longer incubation times (data not shown). The subcellular localization of FAE activity was also determined, and most acyl-CoA elongase activity was found in the microsomal fraction isolated by centrifugation at 150 000g. Higher density membranes isolated by centrifugation at 38 000g
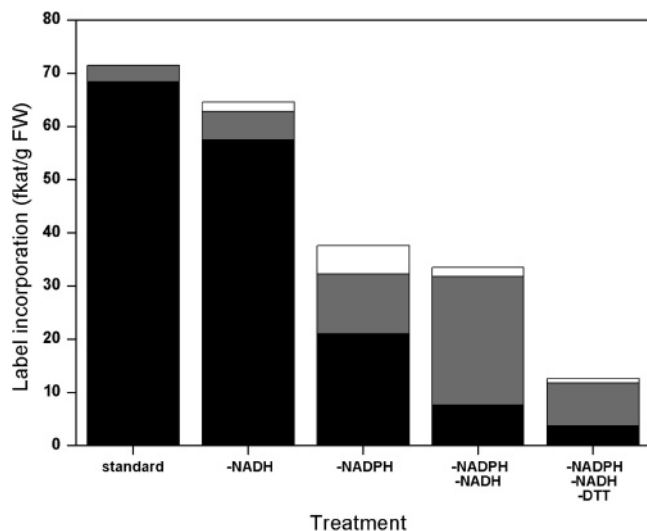
Figure 1. Effect of the addition of cofactors on the accumulation of 18:0-CoA elongation intermediates in microsomes isolated from sunflower kernels. (■) Very long chain fatty acids, (gray square) β-ketoacyl derivatives, and (□) β-hydroxiacyl derivatives. Data corresponded to the average of three separate experiments, and standard deviations were lower than 10% of the average values in all cases.

displayed only trace FAE activity. This subcellular distribution differed considerably from that found in *B. napus*, in which erucic acid synthesis is found in high-density membranes obtained by centrifugation at 15 000*g* (*7*) but resembles the one found in *Allium porrum* (*10*).

Cofactor Dependence. Although the condensing enzymes that are responsible for the synthesis of VLCFAs have been studied extensively, little attention has been paid to the enzymes that catalyze the intermediate steps of the elongation reaction. Unlike KCSs, these enzymes require a reducing potential in the form of pyridine nucleotides, when uncoupled from the β-hydroxyacyl-CoA dehydratase. Furthermore, it has been suggested that ATP-dependent elongation reactions may exist (*19*). Hence, the cofactor dependence of the dehydrogenases and reductases involved in the biosynthesis of arachidic and behenic acids was analyzed in sunflowers. This was studied by determining the intermediates that accumulated upon removal of NADPH or NADH from the standard elongation assay mixture. The intermediate metabolites of the elongation reaction are β-ketoacyl-CoA, β-hydroxyacyl-CoA, and β-trans-alkenyl-CoA derivatives. The first compound undergoes decarboxylation during the saponification step at 80 °C producing a labeled ketone that was identified using the analogous compound 2-decanone as a standard. The other compounds were identified as their methyl esters using ricinoleic and stearic acids as the standards.

The elongation of 18:0-CoA was not dependent on the presence of ATP. Indeed, the highest total incorporation of radiolabel was observed in the presence of both NADH and NADPH. Under these conditions, the lowest level of intermediates was found, and VLCFA accounted for 95% of the label with only small amounts of ketoacyl derivatives being detected (**Figure 1**). This is in contrast to the FAE activity found in pea seedlings that were inhibited when NADH was added to the reaction mixture (*6*). The total FAE activity in sunflowers was similar when NADH was removed from the assay mix, although a more important accumulation of intermediates was observed. Thus, the levels of ketoacids and hydroxy acids observed rose to 8 and 3% in relation to total incorporated malonyl-CoA into lipid, respectively. The removal of NADPH produced a 25%

decrease of the overall radiolabel incorporation into lipids, which was concomitant with an important increase in the presence of intermediaries. Thus, incorporation of the label into ketoacids and hydroxyl acids reached 30 and 14% respectively. However, significant quantities of elongated fatty acids were still produced, indicating that there were forms of reductases and dehydrogenses that were dependent on NADH. The removal of both NADH and NADPH largely blocked the synthesis of VLCFA. As expected, the ketoacyl derivatives were the main products that accumulated due to the activity of the KCSs present in the extract. Nevertheless, the weakest incorporation was observed when DTT, NADH, and NADPH were all removed.

Taken together, these data indicate that the dehydrogenation and reduction steps of VLCFA synthesis in sunflower kernels are dependent mainly on the presence of NADPH. However, accumulation of metabolites in the absence of NADH, as well as the incorporation of the label into fatty acids observed when NADH was the only exogenous source of reducing potential, is evidence of the contribution of this cofactor to extraplastidial elongation of fatty acids. In the total absence of exogenous reducing power, the main product detected was the ketoacyl derivative that resulted from the condensation of 18:0-CoA and malonyl-CoA by the action of KCSs. The low total label incorporation observed under these conditions suggests that the accumulated ketoacyl products inhibited the condensing enzymes, a result similar to that found in many previous studies (*8, 20, 21*). The cofactor dependence of sunflower kernels was similar to that found in developing seeds of *B. juncea*, *Sinapsis alba*, and in epidermal cells from leek (*22*), in which a preference for NADPH was observed. This situation is in contrast to that in *B. napus* embryos, in which the contribution of NADH to the production of VLCFAs was much more important (*3*). Finally, the condensation reaction was even slower when the reducing agent DTT was removed from the reaction mixture, indicating a role for reduced thiol groups in this catalytic step (*3*). No accumulation of trans acyl derivatives was observed under any of the experimental conditions, indicating that the reduction step was rapid and that it was controlled by the prior hydroxyacyl-CoA dehydratation step.

Influence of pH. Elongation of 18:0-CoA in fresh microsomes of developing sunflower kernels was assayed within a pH value range of 4.5−9.5. Formation of the elongation products arachidic acid (20:0), behenic acid (22:0), and lignoceric acid (24:0) were monitored as a function of the pH of the assay media (**Figure 2**). The label was incorporated into arachidic acid even at the most extreme pH values assayed, with maximum incorporation being reached between pH 6.0 and 7.5 using MES and phosphate buffers, respectively. In contrast, maximum synthesis of behenic and lignoceric acids occurred at pH 7.5 with phosphate buffer, and the amount of label incorporation into these two VLCFAs was reduced abruptly at pH values above or below this optimum value.

The pH dependency displayed by this activity seemed to indicate the existence of at least two forms of condensing activities. One of these has an acidic optimum pH value that preferentially catalyzed the elongation of 18:0-CoA to 20:0-CoA, and the other displays an alkaline optimum pH value that is more active in the elongation of 20:0-CoA to 22:0-CoA. The presence of KCSs forms with different optimum pH values has previously been reported by Barrett and Harwood (*6*) in pea seedlings. In this species, 18:0-CoA elongation occurred best at pH 7.0, whereas the elongation of 20:0-CoA reached its optimum at the slightly acidic pH value of 6.6.
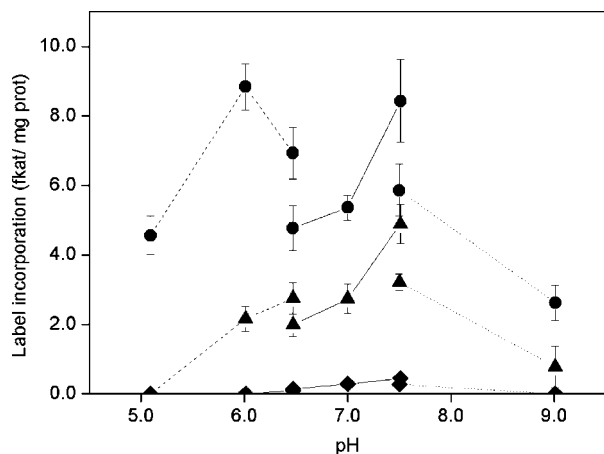
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Figure 2. Influence of pH on the elongation products. Three buffers were used to embrace the pH range studied: MES (dashed line), potassium phosphate (solid line), and TRIS (dotted line). Incorporation of radiolabel into 20:0 (●), 22:0 (▲), and 24:0 (◆). Data correspond to the average of three separate experiments.
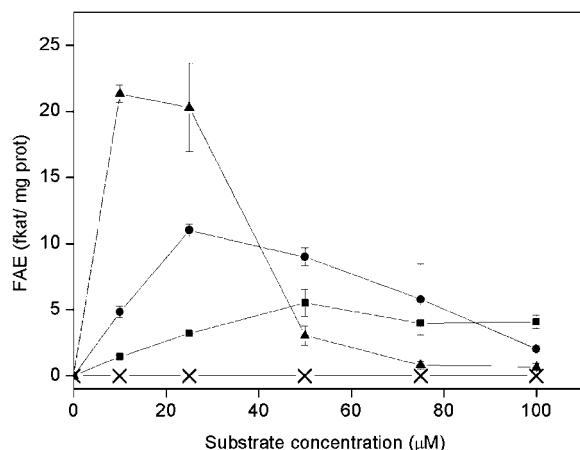

Figure 3. Effect of the concentration of acyl-CoAs on the fatty acid elongase activity in crude sunflower kernel microsomes. Kinetics of 16:0-CoA (–■–), 18:0-CoA (–●–), 20:0-CoA (–▲–), and 18:1-CoA (–×–) elongation were investigated. Data correspond to the average of three independent determinations.

Kinetics and Substrate Specificity. Fatty acid elongase activity in crude microsomes from developing sunflower kernels was characterized at different concentrations of coenzyme A esters of the oleic, palmitic, stearic, and arachidic acids. Elongation occurred with all substrates except 18:1-CoA (**Figure 3**), indicating that the reticular condensing enzymes present in sunflower are specific for saturated acyl-CoA substrates. The inability to elongate unsaturated substrates probably explains the presence of most trace amounts of erucic acid in sunflower oil (*11*). Furthermore, all the saturated acyl-CoA assayed displayed elongation dynamics that differed considerably from regular Michaelis−Menten enzyme kinetics. In each case, a maximal peak of activity was observed rather than the more common saturation curves. The observed enzyme kinetics indicates substrate inhibition, as has been reported for FAE from *B. napus* and *A. porrum* (*7*, *23*). This effect was more intense as the acyl chain length increased. Thus, it appears that the acyl-CoA pool size in sunflowers may be at least as important as the level of FAE enzyme activity in determining the amount of VLCFAs in the final oil.

In this regard, these results might explain the high ratio 22:0/20:0 found in common sunflower lines since the nascent
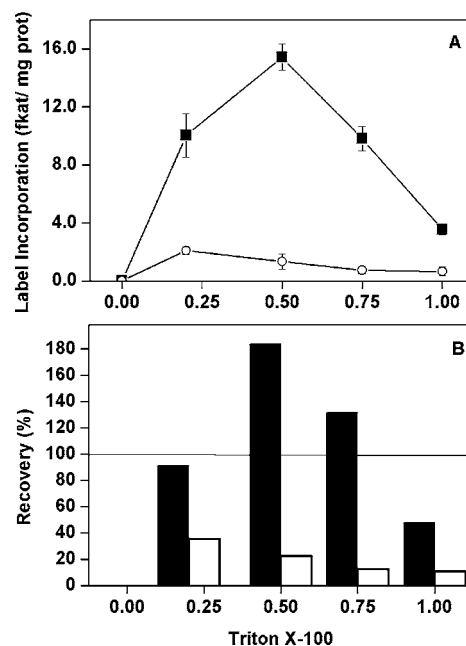

Figure 4. Solubilization of the fatty acid elongase complex from sunflower kernels with Triton X-100. (**A**) Incorporation of radiolabel into 20:0 (–■–) and 22:0 (–○–). (**B**) Fatty acid elongase recovered in the solubilized supernatant relative to the initial activity in the particulate fraction was measured by the incorporation of radiolabel into 20:0 (■) or 22:0 (□). Data correspond to the average of three determinations.

20:0-CoA produced from 18:0-CoA can be efficiently elongated at very low concentrations by the sunflower FAE complex (**Figure 3**). This activity profile differs from those found in seeds from other species such as *L. annua*, *B. napus*, and *Limnantes alba* (*7*−*9*) or in the roots of *Zea mays* (*24*), in which fatty acid elongase acts on 18:1-CoA as well as saturated susbtrates. The specificity of sunflower kernel FAE resembles that of leek FAEs that are specific in vivo for saturated substrates (*25*). However, specificity of leek fatty acid elongase is due to the organization of the membrane and competition with other enzymes since purified leek KCSs display activity toward C18 unsaturated acyl-CoAs (*26*). On the other hand, the fact that 16:0-CoA was a substrate for reticular fatty acid elongases indicates that not all the C18 fatty acids are synthesized in the plastids. Rather, a fraction of the stearate found in seed oil and in other tissues could be produced in the endoplasmic reticulum by the action of this enzyme complex.

Solubilization of the FAE Complex. Although membrane localization often hampers enzyme studies, many microsomal enzymes can be solubilized with surfactants prior to their characterization. Triton X-100 has been used extensively to solubilize the FAE complex since it does not inactivate it and may even be stimulatory (*27*). The effect of different concentrations of this solubilizing agent on the synthesis of 20:0 and 22:0 from 18:0-CoA was determined. While Triton X-100 efficiently disaggregated the kernel microsomes after a 2 h incubation at 0 °C, its efficiency in solubilizing FAE was strongly dependent on its concentration. Maximum radiolabel incorporation into 20:0 occurred with 0.5% Triton X-100 (**Figure 4A**), whereas at higher concentrations, label incorporation decreased abruptly. Minimum incorporation was observed at a concentration of 1% Triton X-100, indicating that excess surfactant affected the function of the condensing enzymes. In addition, the incorporation of 18:0-CoA incorporation into 22:0 was maximal at the lowest concentration of Triton X-100 assayed (0.2%), further

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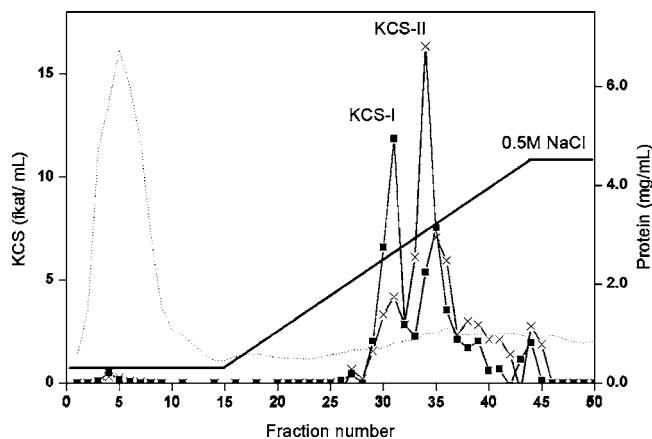
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Figure 5. Purification of ketoacyl-CoA synthases in solubilized microsomes isolated from sunflower kernels. Fractions were assayed for 18:0-CoA (–■–) and 20:0-CoA (–×–) synthesis. Protein was also quantified (dotted line).

Figure 6. FAE activity in sunflower kernels at different developmental stages. Incorporation of radiolabel into 18:0-CoA (■) and 20:0-CoA (□). Data correspond to the average of three separate experiments.

suggesting that a second form of KCS exists that is more active toward 20:0-CoA and acts or is recovered more efficiently at lower concentrations of Triton X-100.

The results of the specific FAE activity in the solubilized microsomes were complemented with the total activity recovered (**Figure 4B**). Concentrations of 0.50 and 0.75% Triton X-100 increased 18:0-CoA elongation to 20:0, 83%, and 30%, respectively, as compared to that present initially in the crude microsomes. This indicates stable soluble condensing enzymes. Similar results were reported by Creach and Lessire (*27*), although they used lower amounts of Triton X-100, from 0.2 to 1 mM (equivalent to between 50 and 250 ppm). However, the levels of FAE activity that are recovered were not attained in that study. The activity responsible for precursor incorporation into 22:0 was not recovered to such a high extent and was in all cases lower than 100%, with the highest degree of recovery at 0.2% Triton X-100. These data indicated that FAE activity in sunflower kernels can be solubilized with Triton X-100 and that this solubilization affects differently the production of 20:0 and 22:0, again suggesting the existence of two forms of condensing enzymes in sunflower kernels.

Ion Exchange Fractionation of Sunflower Kernel KCSs. A combined sample of active sunflower kernel microsomes was solubilized and fractionated by ion exchange chromatography. The resulting fractions were assayed for both 18:0-CoA and 20:0-CoA elongating activities. Since it was possible that these fractions did not contain all the enzymes of the FAE complex, only the condensing activities were measured. Two peaks of condensing activity were eluted, each with different substrate specificities (**Figure 5**). The first peak (KCS-I) was eluted with approximately 0.25 M NaCl and displayed a higher specificity toward 20:0-CoA. In contrast, the second peak (KCS-II) eluted with 0.35 M NaCl and presented a preference for elongation of 18:0-CoA.

The substrate specificity of these KCS enzymes was not absolute; thus, both KCS-I and KCS-II acted on both substrates. However, the activity of KCS-I toward 18:0-CoA was 25% of that observed on 20-CoA and that of KCS-II for 20:0-CoA was likewise a quarter of that toward its preferred substrate, 18:0-CoA (**Figure 5**). These data confirm that the VLCFAs present in sunflower oil are synthesized by the successive activity of two condensing enzymes: one that elongates mainly 18:0-CoA and other that acts on the resulting 20:0-CoA. This situation is identical to that found in leek epidermal cells, where the two forms of KCS exist with specificity toward 18:0-CoA and
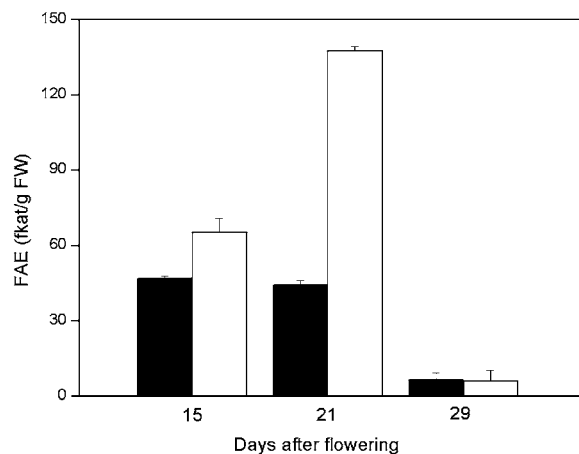
20:0-CoA. Indeed, in leeks, both forms of KCS were resolved from solubilized membranes using sucrose gradients (*20*, *28*), and it appeared that they were possibly bound to different types of membranes (*29*). In contrast, in seeds from *B. napus*, a single form of KCS is responsible for the synthesis of erucic acid by sequential elongation of 18:1-CoA (*7*). Similarly, a single KCS was purified from solubilized membranes from *L. alba* seeds by ion exchange chromatography (*9*). Among the seeds that produce 22:1 so studied, only *L. annua* had two forms of KCS with different substrate specificities (*30*). On the other hand, the multiplicity of KCS forms in *Arabidopsis* seeds had been demonstrated through molecular biology. The characteristics of these KCSs have been contemplated considering the VLCFA composition of plants transformed with them (*31*).

Activity during Seed Formation. Sunflower kernel FAE was characterized further by studying the activity of both 18:0- and 20:0-specific elongation complexes during seed formation. These activities were measured in microsomes prepared from seeds harvested at the beginning, the middle, and the end of the period of oil accumulation, from day 14−30 after flowering (DAF). Peak 18:0-CoA elongation activity was found at DAF 15 and 21 and decreased abruptly at later developmental stages (**Figure 6**). In contrast, 20:0-CoA elongase activity increased from 15 DAF to 21 DAF before diminishing abruptly. The distinct ratios of 18:0/20:0 elongating ratios found indicate that KCS-I and KCS-II display different expression profiles. In this regard, the developmental time course of 18:0 and 20:0 elongation activity was similar to that found previously for the plastidial condensing activity (KAS II) in CAS-6 (*18*). KAS II was most active in CAS-6 seeds that were 14 and 19 DAF, at the beginning of the active period of oil synthesis.

Conclusion. VLCFA fatty acids are produced in sunflower by extraplastidial elongation of 18:0-CoA due to the action of the FAE complex. This reaction depends strongly on the supply of reducing agents in the form of NADPH, although a degree of dependence on NADH can also be observed. The primary condensation reactions associated with this process are catalyzed by two KCS forms that are present in the membranes of the developing seed kernel and that can be separated by ion exchange chromatography. The first of these, called KCS-II, is mainly responsible for the elongation of 18:0-CoA to 20:0-CoA, whereas KCS-I catalyzes the subsequent elongation to 22:0-CoA. The behavior of these KCS isoforms differs with respect to their pH dependency and solubilization. These two enzymes were strongly inhibited by the presence of their substrate, which

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makes it important to consider the size of the cytosolic acyl-CoA pool when attempting to estimate their activity in vivo.

Sunflower kernel FAE was more similar to the fatty acid elongase activity found in leek epidermis and pea seedlings than to that responsible for erucic acid synthesis found in rapeseed. This probably means that sunflower lacks a mechanism to specifically synthesize VLCFA destined for triacylglyceride production, but the mechanism of the arachidic and behenic present in sunflower oil is the same as that involved in the synthesis of the seed waxes. On the other hand, different sunflower mutants with a variety of phenotypes have been isolated and are being cultivated for their oils. These phenotypic variants included lines with high oleic, high palmitic, or high stearic traits. The results presented here could be of interest in the production of new sunflower lines with modified fatty acid compositions. Although recent studies have reported that a mild daily intake of saturated VLCFA produces benefits in terms of health (*32*), there is still little data available regarding the impact of the higher consumption of these fatty acids. However, the negative effects in human health reported for the structurally related erucic acid should be considered when increasing the content of VLCFA in plant oils, either by breeding or genetic engineering. Furthermore, removing VLCFAs may be of interest in producing improved sunflower oils, accentuating the need to clone the genes encoding KCS-I and KCS-II. In this regard, the suppression of KCS-II activity would prevent 18:0-CoA elongation to 20:0, the precursor for the most abundant VCLFA, behenic acid. Furthermore, the possible impact of these changes on the seed waxes should be also investigated. Obtaining sequence data from these genes would also be of interest to establish which residues determine substrate specificity by comparing their sequences to those of other known KCSs with defined biochemical parameters such as the KCSs from *Brassica* and *Arabidopsis* (*31*). The biochemical data regarding substrate specificity and inhibition presented in this work should be considered at the time of modifying the VLCFA content in sunflower oil.

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