Elongases Synthesizing Very Long Chain Monounsaturated Fatty Acids in Developing Oilseeds and Their Solubilization*

Denis J. Murphy

University of Durham, Department of Biological Sciences, Durham DH1 3LE, U.K.

Kumar D. Mukherjee

Bundesanstalt für Fettforschung, Institut für Biochemie und Technologie – H. P. Kaufmann-Institut –, Piusallee 68, D-4400 Münster, Bundesrepublik Deutschland

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Particulate ($15{,}000 \times g$) fractions from developing seeds of mustard *(Sinapis alba)*, honesty *(Lunaria annua)*, and nasturtium *(Tropaeolum majus)* synthesize radioactive very long chain (n-9)-(Z)-monounsaturated fatty acids, *e.g.* gadoleic (20:1), erucic (22:1), and nervonic (24:1) acids from [1-^{14}C]oleoyl-CoA with malonyl-CoA or from [2-^{14}C]malonyl-CoA with oleoyl-CoA. Pyridine nucleotides are required for the elongation reactions. Both NADH and NADPH are equally effective for the formation of 20:1, but NADPH is the preferred reductant for the formation of 22:1, which suggest the existence of separate elongase systems. Detergents, such as Triton X-100 and octyl thioglucoside, stimulate the elongation at low concentrations, but they are inhibitory at high concentrations. Partial solubilization of the elongase system using these detergents has been achieved.

Introduction

Seeds of certain plants are rich in triacylglycerols containing very long chain (n-9)-(Z)-monounsaturated fatty acids, such as gadoleic (20:1), erucic (22:1), and nervonic (24:1) acids, which are of interest as renewable raw materials for the oleochemical industry [1]. Little is known so far about the subcellular localization and other characteristics of the elongase(s) catalyzing the synthesis of very long chain monounsaturated fatty acids in higher plants.

Compositional changes in lipids of seeds during the course of maturation and the pattern of radiolabeling of lipids in developing seeds have shown for rape, *Brassica napus* [2], crambé, *Crambé abyssinica* [3, 4], mustard, *Sinapis alba* [5, 6], nasturtium, *Tropaeolum majus* [7], and honesty, *Lunaria annua* [8] that very long chain monounsaturated fatty acids are formed by sequential elongations of oleic acid, very likely by the condensations of oleoyl-CoA with malonyl-CoA. Such elongation reactions have also been observed in cell-free homogenates from developing seeds of jojoba, *Simmondsia chinensis* [9] and in subcellular fractions from developing seeds of brown mustard, *Brassica juncea* [10].

We have shown recently that subcellular fractions from developing seeds of *S. alba*, *L. annua*, and *T. majus*, and especially the $15{,}000 \times g$ particulate fractions, are highly active in the synthesis of very long chain (n-9)-(Z)-monounsaturated fatty acids by condensation of exogenous oleoyl-CoA or of endogenous acyl-CoA thioesters with malonyl-CoA [11]. It appeared from these studies that very long chain (n-9)-(Z)-monounsaturated fatty acids are synthesized from oleoyl-CoA in a manner similar to that by which very long chain saturated fatty acids are synthesized from stearoyl-CoA, *i.e.* by condensation with malonyl-CoA, as observed in leek *(Allium porrum)* epidermal cell microsomes [12–16].

Here we report some properties of the elongase(s) in $15{,}000 \times g$ particulate fractions from developing seeds of *S. alba*, *L. annua*, and *T. majus*. Solubilization of the membrane-bound elongases catalyzing the synthesis of very long chain monounsaturated fatty acids in higher plants is reported for the first time.

Materials and Methods

Chemicals

[1-^{14}C]Oleoyl-CoA, 1.85 GBq·mmol^{-1} and [2-^{14}C]malonyl-CoA, 1.59 GBq·mmol^{-1}, were purchased from NEN Chemicals, D-6072 Dreieich. All other substrates and cofactors were from Sigma

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Abbreviations: Fatty acids having X carbon atoms and Y (n-9)-(Z)-double bonds are designated as X:Y.

Reprint requests to K. D. Mukherjee.

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Chemie GmbH, D-8024 Deisenhofen. Reagents and adsorbents for thin layer chromatography were obtained from E. Merck AG, Darmstadt, Federal Republic of Germany. Distilled solvents were used throughout. Pure lipids used as references for chromatography were from Nu-Chek-Prep, Elysian, Mn., U.S.A.

Plant material and preparation of particulate fractions

Mustard (*Sinapis alba*, variety Albatros), honesty (*Lunaria annua*, garden variety), and nasturtium (*Tropaeolum majus*, garden variety) were grown outdoors. The developing seeds, collected between 5 and 6 weeks after flowering, were either used immediately or kept frozen at −70 °C prior to use. The 15,000 × *g* particulate fractions were prepared from the cotyledons of the developing seeds as described earlier [11].

Enzyme assays

Aliquots of the 15,000 × *g* particulate fraction, suspended in the homogenization medium, were incubated with either 1.85 KBq [1-^{14}C]oleoyl-CoA contained in 17 μM oleoyl-CoA and 1 mM malonyl-CoA, or 1.85 KBq [2-^{14}C]malonyl-CoA contained in 17 μM malonyl-CoA with or without 100 μM oleoyl-CoA as described previously [11]. The effects of cofactors on the elongase reaction were investigated by omission of the corresponding cofactor(s) from the incubation medium. The effects of detergents were studied by adding solutions of Triton X-100 or octyl thioglucoside at various concentrations to the incubation medium.

Solubilization experiments

In several experiments the 15,000 × *g* particulate fractions obtained from 50 cotyledon pairs of *S. alba* (fresh seeds) or 50 cotyledon pairs of *L. annua* (frozen seeds) were suspended in 750 μl of a solution containing 50 mM HEPES, pH 7.0, 10 mM β-mercaptoethanol, and 20%, w/v glycerol. Aliquots, 250 μl each, of this suspension were shaken, as described earlier [11], for various periods at 4 °C with 250 μl, each, of either 0.3%, w/v of Triton X-100 or 30 mM octyl thioglucoside. In control experiments, the suspensions were shaken under identical conditions but without detergents. In each case, the suspensions (total fraction) were assayed for elongase activity in the incubation medium as described above. Alternatively, the suspensions were centrifuged at 150,000 × *g* for 1.5 h and the resulting supernatant (solubilized fraction) assayed for elongase activity in the incubation medium.

Analytical procedures

Methods described in previous communications were used to saponify the lipids contained in the incubation mixture, isolate the fatty acids and convert them to methyl esters, and analyze the methyl esters by radio gas chromatography [6, 11].

Protein content was determined according to Markwell *et al.* [17]. Radioactivity in lipid samples was measured in a Packard Tri-Carb C2425 liquid scintillation spectrometer using toluene scintillator (Packard Instruments Company Ltd., Downers Grove, Ill., U.S.A.).

Results and Discussion

Earlier experiments have revealed that, in subcellular fractions from developing seeds of *S. alba*, *L. annua*, and *T. majus*, oleoyl-CoA is elongated by malonyl-CoA to form gadoleoyl-CoA, erucoyl-CoA, and nervonoyl-CoA; moreover, condensation of malonyl-CoA with exogenous oleoyl-CoA and endogenous acyl-CoA thioesters has been found to occur [11]. As an extension of these studies, Table I shows the time course of formation of very long chain (*n*-9)-(*Z*)-monounsaturated fatty acids (as acyl-CoA and/or acyl moieties of lipids) from radioactive oleoyl-CoA or malonyl-CoA by the 15,000 × *g* particulate fractions from the above seeds.

The particulate fraction from *S. alba* synthesizes very long chain (*n*-9)-(*Z*)-monounsaturated fatty acids at a higher rate from [2-^{14}C]malonyl-CoA than from [1-^{14}C]oleoyl-CoA; predominantly erucic acid is formed from [2-^{14}C]malonyl-CoA, irrespective of whether or not unlabeled oleoyl-CoA is added to the incubation mixture (Table I).

The particulate fraction from *L. annua* synthesizes little gadoleic acid, but comparatively larger proportions of erucic and nervonic acids from [2-^{14}C]malonyl-CoA in conjunction with exogenous oleoyl-CoA (Table I).

In the particulate fraction from *T. majus* the extent of formation of very long chain (*n*-9)-(*Z*)-monounsaturated fatty acids from [2-^{14}C]malonyl-CoA is less compared to the particulate fractions from *S. alba* or *L. annua* (Table I).

Effects of cofactors on the elongation by condensation of [1-^{14}C]oleoyl-CoA with malonyl-CoA by the particulate fractions are shown in Table II. It is evi-

Table I. Formation of very long chain monounsaturated fatty acids from [1-^{14}C]oleoyl-CoA or [2-^{14}C]malonyl-CoA by 15,000 × *g* particulate fractions from developing seeds[a].

Seed	Substrate	Incubation time [h]	Elongation products [nmol]			Total elongation of substrate	
			20:1	22:1	24:1	(nmol · assay^{-1})	(nmol · mg^{-1} protein)
S. alba (fresh)	[1-^{14}C]oleoyl-CoA + malonyl-CoA	1	0.38	0.03	0.0	0.41	8.2
		2	0.26	0.14	0.0	0.40	8.2
		3	0.22	0.26	0.0	0.48	9.6
		5	0.37	0.35	0.0	0.72	14.4
	[2-^{14}C]malonyl-CoA + oleoyl-CoA	1	0.07	0.67	0.0	0.74	14.8
		2	0.18	0.93	0.0	1.11	22.2
		3	0.07	1.43	0.0	1.50	30.0
		5	0.13	1.49	0.0	1.62	32.4
	[2-^{14}C]malonyl-CoA	2	0.22	0.82	0.0	1.04	20.8
L. annua (frozen)	[1-^{14}C]oleoyl-CoA + malonyl-CoA	2	0.14	0.10	0.05	0.28	4.1
	[2-^{14}C]malonyl-CoA + oleoyl-CoA	1	0.03	0.13	0.14	0.30	4.4
		3.5	0.06	0.30	0.33	0.69	10.1
	[2-^{14}C]malonyl-CoA	1	0.06	0.15	0.06	0.27	4.0
T. majus (fresh)	[2-^{14}C]malonyl-CoA	2	0.07	0.01	0.06	0.14	1.9

[a] Preparation of the particulate fractions, incubations, and analysis of the reaction products were carried out as described in Materials and Methods section. The results are means of two independent determinations.

Table II. Effects of cofactors on elongation by condensation of oleoyl-CoA with malonyl-CoA by 15,000 × *g* particulate fractions from developing seeds.

Cofactor	Elongation by particulate fraction from *S. alba* (fresh)					*L. annua* (frozen)					
	Assay time [h]	Elongation products [nmol]		Total elongation		Assay time [h]	Elongation products [nmol]			Total elongation	
				(nmol · assay^{-1})	(nmol · mg^{-1} protein)					(nmol · assay^{-1})	(nmol · mg^{-1} protein)
		20:1	22:1				20:1	22:1	24:1		
All (control)[a]	1	0.32	0.0	0.32	6.4	2	0.22	0.16	0.08	0.46	6.8
− NADH/NADPH	1	0.01	0.0	0.01	0.2	2	0.05	0.0	0.0	0.05	0.7
− ATP						2	0.08	0.03	0.02	0.13	1.9
− CoASH						2	0.11	0.08	0.02	0.21	3.1
− ATP/CoASH	1	0.15	0.0	0.15	3.0	2	0.09	0.04	0.0	0.13	1.9
All (control)[a]	2	0.26	0.14	0.40	8.0						
− NADH	2	0.02	0.30	0.32	6.4						
− NADPH	2	0.16	0.21	0.37	7.4						
− NADH/NADPH	2	0.06	0.06	0.12	2.4						
All (control)[b]	2	0.35	0.76	1.11	20.4						
− NADH	2	0.08	0.69	0.77	14.1						
− NADPH	2	0.44	0.18	0.62	11.4						
− NADH/NADPH	2	0.04	0.14	0.18	3.4						
− ATP	2	0.16	0.50	0.66	12.2						
− CoASH	2	0.30	0.78	1.08	19.9						
− ATP/CoASH	2	traces	0.23	0.23	4.2						

[a] Substrate [1-^{14}C]oleoyl-CoA + malonyl-CoA.
[b] Substrate oleoyl-CoA + [2-^{14}C]malonyl-CoA.

dent from these results that pyridine nucleotides are required for these elongation reactions, because little elongation of oleoyl-CoA occurs in the absence of both NADH and NADPH. The total amount of very long chain (*n*-9)-(*Z*)-monounsaturated fatty acids formed is similar with either NADH or NADPH, however, with NADPH distinctly higher amounts of erucic acid are formed than with NADH. In the particulate fraction from *L. annua*, omission of either ATP or CoASH from the incubation mixture distinctly reduces the extent of elongation of oleoyl-CoA.

The cofactor requirement for elongation of exogenous oleoyl-CoA by condensation with [2-^{14}C]malonyl-CoA is similar to that in the elongation of [1-^{14}C]-oleoyl-CoA as shown in Table II. Thus, the extent of total elongation is similar with either NADH or NADPH, whereas NADPH is the preferred reductant for the formation of erucic acid. The rate of synthesis of very long chain monounsaturated fatty acids from malonyl-CoA is severely reduced in the absence of CoASH and ATP.

The fact that NADPH is the preferred reductant for the formation of erucic acid, whereas the total elongation, which includes the intermediate formation of gadoleic acid, is similar with either NADH or NADPH (Table II), supports the idea of the existence of more than one elongase systems having different cofactor requirement. Similar findings have also been reported for particulate fractions from *B. juncea* seeds [10]. In this context it is of interest to note that evidence has accumulated lately on the existence in plant tissues of separate elongases that catalyze the formation of very long chain saturated fatty acids by successive condensations of stearoyl-CoA, and its elongation products, with malonyl-CoA [12, 14, 18].

The effect of detergents on the elongation by condensation of [1-^{14}C]oleoyl-CoA with malonyl-CoA was examined in the 15,000 × *g* particulate fractions from *S. alba* seeds with a view to solubilizing these membrane-bound elongases. The results, given in Table III, show that Triton X-100 at low concentrations (0.05%, w/v) stimulates the total elongation by strongly increasing the extent of formation of erucic acid. At a higher concentration (0.15%, w/v), the total elongation remains almost unaffected, however, the elongation to erucic acid is greatly impaired with a concomitant increase in the formation of gadoleic acid. These findings again support the existence of separate elongases. The elongase system catalyzing the formation of erucic acid seems to be stimulated at low concentrations (0.05%, w/v) of Triton X-100 and inhibited at higher concentrations (0.15%, w/v) of this detergent, whereas at the higher concentration the elongase system catalyzing the formation of gadoleic acid does not seem to be affected. It cannot be ruled out though, that the effects observed are due to differential substrate availability in the presence of detergent. Further increase in the concentration of Triton X-100 to 0.3%, w/v, however, results in a sharp decline in total elongation. The data given in Table III also show that octyl

Table III. Effect of detergents on elongation by condensation of [1-^{14}C]oleoyl-CoA with malonyl-CoA by 15,000 × *g* particulate fraction from developing seeds of *S. alba*[a].

Detergent	Concentration	Elongation products [nmol]		Total elongation	
		20:1	22:1	(nmol · assay^{-1})	(nmol · mg^{-1} protein)
None		0.26	0.14	0.40	11.7
Triton X-100	0.05% (w/v)	0.17	0.36	0.53	19.4
Triton X-100	0.15% (w/v)	0.38	0.11	0.49	18.6
Triton X-100	0.30% (w/v)	0.13	traces	0.13	6.9
Octyl thioglucoside	5 mM	0.21	0.02	0.23	14.9
Octyl thioglucoside	15 mM	0.12	traces	0.12	13.0
Octyl thioglucoside	30 mM	0.03	0.0	0.03	5.6

[a] Assay time 2 h in all experiments.

thioglucoside, as opposed to Triton X-100, inhibits the elongase(s) even at low detergent concentrations.

The results of experiments designed to solubilize the membrane-bound elongase(s) from the $15,000 \times g$ particulate fractions of *S. alba* and *L. annua* seeds are summarized in Table IV. The data show the formation of very long chain (*n*-9)-(*Z*)-monounsaturated fatty acids from [1-^{14}C]oleoyl-CoA and malonyl-CoA by the particulate fractions (controls), particulate fractions treated with detergents for various periods (total fractions), and the $150,000 \times g$ supernatant fraction (solubilized fractions) obtained by centrifugation of the total fractions. It is evident from these results that after 22 h of treatment of the particulate fraction from *S. alba* with either Triton X-100 or octyl thioglucoside, considerable elongase activity is found in the solubilized fraction, as compared to the control experiment. Treatment of the particulate fraction from *L. annua* seeds for 3.5 h with either Triton X-100 or octyl thioglucoside also yields solubilized fractions having substantial elongase activity compared to the controls (Table IV). The pellet resulting after treatment of the $15,000 \times g$ particulate fraction from *L. annua* seeds with Triton X-100 exhibited about 18% elongase activity as compared to the solubilized fraction (data not shown).

For the particulate fractions from both *S. alba* and *L. annua,* the solubilized fractions obtained using Triton X-100 exhibit higher elongase activity than those prepared using octyl thioglucoside (Table IV). These findings agree with the data on inhibition by the two detergents, as given in Table III.

The data presented in Table IV demonstrate, to our knowledge for the first time, the solubilization of membrane-bound elongase(s) catalyzing the synthesis of very long chain (*n*-9)-(*Z*)-monounsaturated fatty acids in higher plants. Although such elongase(s) have not been characterized so far, it is quite likely that, similar to other organisms, the elongase complex catalyzing the condensation reaction is composed of β-ketoacyl-CoA synthase, β-ketoacyl-CoA reductase, β-hydroxyacyl-CoA dehydratase, and enoyl-CoA reductase. It is quite obvious from the results given in Table IV that the entire elongase complex has been solubilized.

It is also striking from the data presented in Table IV that the solubilized fraction from *L. annua* synthesizes only gadoleic acid, although the corresponding controls as well as the total fractions synthesize substantial proportions of erucic and nervonic acids in addition to gadoleic acid. In this context it is of interest to note that the elongase solubilized from *A. porrum* epidermal cell microsomes specifically catalyzes the condensation of stearoyl-CoA with malonyl-CoA to yield arachidoyl-CoA [12, 16].

Although the nature of the $15,000 \times g$ particulate fractions used in these studies and the subcellular

Table IV. Elongation by condensation of [1-^{14}C]oleoyl-CoA with malonyl-CoA by $15,000 \times g$ particulate fractions from developing seeds before and after detergent treatment and by the detergent-solubilized fractions prepared therefrom[a].

Seed	Detergent	Time of detergent treatment [h]	Fraction	Elongation products [nmol]			Total elongation (nmol · assay^{-1})
				20:1	22:1	24:1	
S. alba (fresh)	None	22	control	0.12	0.0	0.0	0.12
	Triton X-100	22	total	0.10	0.0	0.0	0.10
	Triton X-100	22	solubilized	0.12	0.0	0.0	0.12
	Octyl thioglucoside	22	total	0.06	0.0	0.0	0.06
	Octyl thioglucoside	22	solubilized	0.08	0.0	0.0	0.08
L. annua (frozen)	None	3.5	control	0.23	0.16	0.07	0.46
	Triton X-100	3.5	total	0.09	0.01	0.03	0.13
	Triton X-100	3.5	solubilized	0.09	0.0	0.0	0.09
	Octyl thioglucoside	3.5	total	0.08	0.0	0.0	0.08
	Octyl thioglucoside	3.5	solubilized	0.02	0.0	0.0	0.02

[a] Assay time 2 h in all experiments.

localization of elongase activity are not clear at the moment, the analogy with the 20,000 × *g* particulate fraction from safflower cotyledons, which exhibits strong activity of acyl-CoA : 1-acyl-*sn*-glycerol-3-phosphate acyltransferase [19] deserves further studies on characterization of such fractions from developing seeds.

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