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Biosynthesis of very long chain monounsaturated fatty acids by subcellular fractions of developing seeds

Denis J. Murphy and Kumar D. Mukherjee*

University of Durham, Department of Botany, Science Laboratories, South Road, Durham DH1 3LE, England and **Bundesanstalt für Fettforschung, Institut für Biochemie und Technologie, H.P. Kaufmann-Institut, Piusallee 68, D-4400 Münster, FRG*

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Subcellular fractions from developing seeds of mustard (*Sinapis alba*), honesty (*Lunaria annua*) and nasturtium (*Tropaeolum majus*) synthesize very long chain *cis* (*n*–9) monounsaturated fatty acids, e.g. gadoleic (20:1), erucic (22:1) and nervonic (24:1) acid, from oleoyl-CoA and malonyl-CoA by condensation reactions. The particulate $2000 \times g$ and $15\,000 \times g$ fractions exhibit considerably higher elongase activities compared to the microsomal or oil body fractions, whereas the soluble ($150\,000 \times g$ supernatant) fraction is devoid of such activities.

Very long chain monounsaturated fatty acid; Elongase; Gadoleic acid; Erucic acid; Nervonic acid; (Developing seed)

1. INTRODUCTION

Very long chain monounsaturated fatty acids, such as gadoleic, $(n-9)$, *cis*-icosenoic (20:1), erucic, $(n-9)$ *cis*-docosenoic (22:1), and nervonic, $(n-9)$ *cis*-tetracosenoic acid (24:1), are major constituents of certain seed oils. These fatty acids are of considerable interest as renewable raw materials for oleochemicals [1]. Knowledge of the enzymes involved in the biosynthesis of very long chain monounsaturated fatty acids and isolation of these enzymes are prerequisites for genetic manipulation of oilseed plants for efficient production of such fatty acids.

In higher plants, the synthesis of very long chain saturated fatty acids has been shown to occur by the condensation of stearoyl-CoA with malonyl-CoA [2–5]; these membrane-bound elongases have been solubilized and partially purified from leek (*Allium porrum* L.) epidermal cell microsomes [6,7].

Correspondence address: K.D. Mukherjee, Bundesanstalt für Fettforschung, Institut für Biochemie und Technologie, H.P. Kaufmann-Institut, Piusallee 68, D-4400 Münster, FRG

Little is known so far about the elongases involved in the formation of very long chain monounsaturated fatty acids in plants. Developing seeds of *Crambe abyssinica* [8], *Tropaeolum majus* [9], and *Sinapis alba* [10] have been shown to elongate oleic acid to 20:1 and 22:1, apparently by successive condensations of oleoyl-CoA with malonyl-CoA, as shown recently for subcellular fractions from developing *Brassica juncea* seeds [11].

We report here the biosynthesis of very long chain monounsaturated fatty acids by subcellular fractions from developing seeds of *S. alba*, *T. majus* and *Lunaria annua*, which have been found to contain large proportions of such fatty acids in the storage triacylglycerols [12–14].

2. MATERIALS AND METHODS

Plants of mustard (*S. alba*, variety Albatros), honesty (*L. annua*, garden variety) and nasturtium (*T. majus*, garden variety) were grown outdoors, and the developing seeds, collected between 4 and 6 weeks after flowering, were either used immediately or kept frozen at −70°C before use.

[1-^{14}C]Oleoyl-CoA (18:1-CoA) (1.85 GBq·mmol^{-1}) and [2-^{14}C]malonyl-CoA (1.59 GBq·mmol^{-1}), both from NEN,

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were used as radioactive substrates. The unlabeled substrates and the cofactors were purchased from Sigma. All reagents, adsorbents, and solvents were from Merck.

The cotyledons of the developing seeds were separated from the seed coat. Subcellular fractions were prepared from the cotyledons (30 pairs, 200 mg from *S. alba*, 25 pairs, 770 mg, from *L. annua*, and 7 pairs, 570 mg from *T. majus*). All operations were carried out at 4°C as follows. The cotyledons were gently homogenized with a mortar and pestle in 3–5 ml of a solution consisting of 80 mM Hepes (pH 7.2), 0.32 mM sucrose, 10 mM β-mercaptoethanol, and 150 mg·ml^{-1} polyvinyl pyrrolidone. The homogenate was filtered through two layers of miracloth, and the filtrate centrifuged at 300 × *g* for 5 min to yield a pellet and a supernatant. The latter was further centrifuged at 2000 × *g* for 20 min. The 2000 × *g* pellet and the floating oil body fraction were recovered and the supernatant centrifuged again at 15000 × *g* for 25 min. After removing the 15000 × *g* pellet, the resulting supernatant was subjected to a final centrifugation at 150000 × *g* for 1 h which yielded the 'microsomal' pellet and the 'soluble' supernatant fraction.

The protein content of the fractions was determined [15] and aliquots of each fraction were incubated with either of the radioactive substrates, diluted to known specific activity, in a gyratory shaker (120 gyrations·min^{-1}) at 30°C for 1 or 2 h. Incubations with [1-^{14}C]oleoyl-CoA contained 17 μM oleoyl-CoA and 1 mM malonyl-CoA; those with [2-^{14}C]malonyl-CoA contained 17 μM malonyl-CoA and 100 μM oleoyl-CoA. The incubation medium consisted of 50 μl of the homogenization solution and 50 μl of a solution containing 1 mM ATP, 1 mM CoASH, 0.5 mM NADPH, 0.5 mM NADH, 2 mM $MgCl_2$ in 80 mM Hepes, pH 7.2.

The incubations were halted by adding 2 ml of 10% ethanolic KOH to the mixture and heating at 80°C for 30 min, in order to saponify the acyl-CoA derivatives and the acyl lipids. The resulting soaps were acidified with 5 M H_2SO_4 and the fatty acids recovered by extracting three times with 2 ml hexane per extraction. The hexane extract was washed with water till neutral, hexane evaporated to dryness with nitrogen, and the fatty acids were converted to methyl esters using diazomethane.

The methyl esters were analyzed by radio gas chromatography on glass columns (1.8 m × 4 mm), packed with silar 5CP/gas-chrom Q, 80–100 mesh (Applied Science Laboratories) isothermally at 230°C using helium as carrier gas in the equipment described earlier [10]. The extent of elongation was measured from the radioactivity in the peaks of methyl esters of 20:1, 22:1 and 24:1 fatty acids. Identity of the very long chain monounsaturated fatty acids was deduced both from retention times in radio gas chromatography and by degradation techniques, such as reductive ozonolysis and α-oxidation, as detailed elsewhere [10].

In several experiments, the chloroform-soluble lipids were extracted from the incubation mixture and the acyl-CoA derivatives, partitioning into the aqueous phase, were isolated [16]. The composition of the acyl moieties of these acyl-CoA derivatives was determined, after hydrolysis and conversion into methyl esters, by radio gas chromatography as described above.

3. RESULTS AND DISCUSSION

Radiolabeled very long chain ($n-9$) *cis*-monounsaturated fatty acids (20:1, 22:1, 24:1) are obtained when subcellular fractions from the developing seeds examined are incubated, in the presence of ATP, CoASH, Mg^{2+}, NADPH, and NADH, with [1-^{14}C]18:1-CoA together with malonyl-CoA and the products formed are hydrolyzed (table 1). Analysis of the very long chain monounsaturated fatty acids formed using degradation techniques [10] revealed that [1-^{14}C]18:1 moieties of oleoyl-CoA are successively elongated to [3-^{14}C]20:1, [5-^{14}C]22:1, and [7-^{14}C]24:1 moieties. These radioactive acyl moieties were also found in the acyl-CoA derivatives recovered from the aqueous phase after extraction of the chloroform-soluble lipids. Taken together, these findings show that in the subcellular fractions of the developing seeds examined, oleoyl-CoA is condensed with malonyl-CoA to yield very long chain ($n-9$) *cis*-monounsaturated acyl-CoA derivatives in a manner similar to that shown for *B. juncea* seeds [11]. The mechanism of these elongation reactions appears to be similar to those observed in the formation of very long chain saturated fatty acids in microsomal fractions of *Allium porrum* [2–5]. Analogy in the mode of elongation of ($n-9$) *cis*-monounsaturated and saturated fatty acids has been observed recently in developing seeds of *S. alba* [10].

The results listed in table 1 show the elongation of oleoyl-CoA by the subcellular fractions of developing seeds of *S. alba*, *L. annua* and *T. majus*. They indicate that the soluble (150000 × *g*) fraction is devoid of elongase activity, whereas the particulate fractions exhibit substantial activities in elongating 18:1-CoA. Among the particulate fractions, the 2000 × *g* and 15000 × *g* pellets exhibit high elongase activities, with respect to both total activity and specific activity (table 1). Similar findings have been reported for *B. juncea* seeds [11]. Only in the case of fresh seeds does the microsomal (150000 × *g*) fraction show substantial elongase activity (table 1). The oil body fractions from both fresh and frozen *S. alba* seeds are devoid of elongase activity, whereas some is exhibited by the oil body fractions from *L. annua* and *T. majus* seeds (table 1). In general, the subcellular fractions from fresh seeds have higher elongase activity than those from frozen seeds. The elongase activities of the particulate fractions from

Table 1

Elongation of [1-^{14}C]oleoyl-CoA by subcellular fractions of developing seeds

Fraction	*S. alba* (fresh) (1 h incubation)			*S. alba* (frozen) (2 h incubation)			*L. annua* (frozen) (2 h incubation)				*T. majus* (fresh) (2 h incubation)			
	Elongation products (nmol · fraction^{-1})		Total elongation (nmol · mg^{-1} protein)	Elongation products (nmol · fraction^{-1})		Total elongation (nmol · mg^{-1} protein)	Elongation products (nmol · fraction^{-1})			Total elongation (nmol · mg^{-1} protein)	Elongation products (nmol · fraction^{-1})			Total elongation (nmol · mg^{-1} protein)
	20:1	22:1		20:1	22:1		20:1	22:1	24:1		20:1	22:1	24:1	
300 × *g* pellet	tr	tr	tr	33	7	3.9	182	278	139	34.9	tr	tr	tr	tr
2000 × *g* pellet	1400	0	51.0	460	tr	29.7	250	213	87	39.0	30	30	tr	8.0
15000 × *g* pellet	1520	0	60.0	260	tr	29.6	169	31	tr	22.3	268	172	tr	65.8
150000 × *g* pellet	660	0	43.0	70	tr	9.5	20	tr	tr	2.9	144	20	135	45.7
Oil bodies	0	0	0	tr	tr	tr	41	60	19	23.0	1	1	0	14.8
150000 × *g* supernatant	0	0	0	0	0	0	0	0	0	0	0	0	0	0

tr, trace

the developing seeds (table 1) are of the same order of magnitude as the activity of stearoyl-CoA elongase and arachidoyl-CoA elongase from the microsomal fractions of epidermal cells of *Al. porrum* [7]. However, unlike the long chain acyl-CoA elongases of *Al. porrum* epidermal cells [7] and animal cells [17], where these enzymes were found to be microsomal, the oilseed elongases are mostly associated, on the basis of both total activity and specific activity, with the 2000 × *g* and 15000 × *g* particulate fractions (table 1).

Table 1 also shows the extent of formation of the individual very long chain $(n-9)$ *cis*-monounsaturated fatty acids upon incubation of the subcellular fractions from the developing seeds with 18:1-CoA. It is evident that, under the conditions used, the particulate fractions from *S. alba* synthesize predominantly 20:1, whereas the corresponding fractions from both *L. annua* and *T. majus* synthesize, in addition to 20:1, substantial amounts of 22:1 and 24:1.

Table 2 records the total elongase activity in the 15000 × *g* particulate fraction as well as the composition of the very long chain fatty acids formed upon incubation with either [1-^{14}C]18:1-CoA or [2-^{14}C]malonyl-CoA as radioactive substrate. The results for *S. alba* show a distinctly higher rate of elongation with ^{14}C-labeled malonyl-CoA together

Table 2

Elongation of [1-^{14}C]oleoyl-CoA and [2-^{14}C]malonyl-CoA by the 15000 × *g* particulate fraction from developing seeds

Species	Substrates	Elongation activity (nmol · h^{-1} · assay^{-1})	Elongation products (% of total ^{14}C-labeled fatty acids)		
			20:1	22:1	24:1
S. alba	[1-^{14}C]18:1-CoA + malonyl-CoA	2.9	22.7	1.7	0
	[2-^{14}C]malonyl-CoA + 18:1-CoA	4.8	9.4	90.6	0
L. annua	[2-^{14}C]malonyl-CoA + 18:1-CoA	3.8	9.2	41.6	46.2
	[2-^{14}C]malonyl-CoA	3.0	23.6	55.6	20.8

with unlabeled 18:1-CoA than with the radioactive 18:1-CoA in conjunction with unlabeled malonyl-CoA. Moreover, the data in table 2 show that radioactive malonyl-CoA yields overwhelmingly 22:1 and 24:1, whereas from radioactive 18:1-CoA predominantly 20:1 is formed. These findings are attributed to the availability of the primers, i.e. endogenous vs exogenous acyl-CoA derivatives, at the reactive site of the elongase(s).

The composition of the acyl-CoA fraction in the products formed by incubation of the 15000 × *g* particulate fraction with [2-^{14}C]malonyl-CoA revealed the presence of CoA derivatives of 20:1 and 22:1 (for both *S. alba* and *L. annua*) and that of 24:1 (for *L. annua*). These findings further support the view that the elongation of the (*n* − 9) *cis*-monounsaturated fatty acids occurs by condensation at the level of their CoA derivatives.

The rate of elongation by the 15000 × *g* particulate fraction from *L. annua* using [2-^{14}C]malonyl-CoA as substrate is somewhat higher when unlabeled 18:1-CoA is added than without this exogenous primer (table 2). In the absence of exogenous 18:1-CoA, the endogenous pool of acyl-CoA derivatives (or the acyl-CoA derivatives formed by the thiokinase from the endogenous pool of fatty acids) are condensed with radioactive malonyl-CoA to yield the very long chain (*n* − 9) *cis*-monounsaturated fatty acids; however, the composition of the elongation products varies, depending on whether or not exogenous 18:1-CoA is used as primer (table 2). These findings can also be explained by the differences in availability of the acyl-CoA primers at the reactive site of the elongase(s).

Further studies on characterization, solubilization and purification of the elongases involved in the biosynthesis of very long chain (*n* − 9) *cis*-monounsaturated fatty acids are in progress.

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