Solubilization of Acyl-CoA Elongases from Developing Rapeseed (*Brassica napus* L.)

Anne Créach and **René Lessire***
Institut de Biochimie Cellulaire, CNRS, 33077 Bordeaux Cedex, France

Acyl-CoA elongases are important in producing high-erucic acid rapeseed. The effects of Triton X-100, *N*-octyl-β-D-glucopyranoside and deoxycholate on the C18:1-CoA and C20:1-CoA elongase(s) have been studied by using a 15,000 × *g* pellet from developing rapeseed. The synthesis of very long chain monounsaturated fatty acids (VLCMFA) and, in particular, that of erucic acid were stimulated by Triton X-100, whatever the substrate used. In the presence of *N*-octyl-β-D-glucopyranoside, the elongase activity was practically unchanged, whereas deoxycholate strongly inhibited VLCMFA synthesis. Triton X-100 was chosen for the solubilization, at an optimal Triton X-100/protein (w/w) ratio of 2.5. Acyl-CoAs were the major products synthesized by the solubilized acyl-CoA elongase(s). The analysis of the reaction intermediates showed that the entire elongation complex has been solubilized and was still functional.

KEY WORDS: Acyl-CoA elongase, *Brassica napus*, erucic acid, very long chain monounsaturated fatty acid biosynthesis.

Triacylglycerols of developing rapeseed (*Brassica napus* L.) contain a large amount of very long chain monounsaturated fatty acids (VLCMFA): eicosenoic acid (C20:1), erucic acid (C22:1) and nervonic acid (C24:1) (1). Erucic acid has many applications in oleochemical industries as a valuable starting material for the production of plasticizers and lubricants (2). For this reason, it would be interesting to enhance erucic acid production in rapeseed. Conventional breeding methods have been unsuccessful in producing rapeseed varieties with a high-erucic acid content, and, thus, the necessity for genetic engineering methods is becoming increasingly important. Acyl-CoA elongases are key factors in obtaining high-erucic acid rapeseed.

Previous studies of cruciferous seeds strongly suggested that VLCMFA could be formed from C18:1-CoA by successive condensations with malonyl-CoA (3–8) carried out by a membrane-bound elongation system (4–8). In *Lunaria annua*, it has been demonstrated that the elongation products are acyl-CoAs, and the metabolic intermediates of the reaction have been characterized (9). It has been proved that, as in animal systems (10), four intermediate reactions are involved in the elongation system of plants: (i) the condensation of oleoyl-CoA with malonyl-CoA yielding a β-ketoacyl-CoA, (ii) the reduction of the β-ketoacyl-CoA to β-hydroxyacyl-CoA, (iii) the dehydration of β-hydroxyacyl-CoA to *trans* 2-enoyl-CoA and, finally, (iv) the reduction of *trans* 2-enoyl-CoA to long-chain acyl-CoA (9).

In *Allium porrum*, the elongation system of saturated acyl-CoAs has been well characterized (11–13), where it was solubilized by Triton X-100 and then partially purified. The intermediate reactions have been well characterized, and a mechanism for the elongation reaction was proposed (13). Despite these results, the structure of the acyl-CoA elongase remains unknown. The elongation system of unsaturated acyl-CoAs has been solubilized from *L. annua* and *Sinapis alba* seeds by Triton X-100 (14), and the partial purification of acyl-CoA elongase from *L. annua* has been reported by Fehling *et al.* (15). This paper reports the effect of detergents on the elongation system in a particulate fraction (15,000 × *g*) from developing rapeseed (*B. napus* L.) and the successful solubilization of the acyl-CoA elongase.

EXPERIMENTAL PROCEDURES

Materials. Plants of rape (*B. napus* L.), enriched in erucic acid, were grown outdoors at INRA (Le Rheu, France). Developing seeds were collected between 6 or 8 wk after flowering and frozen at −80°C. [2-^{14}C] malonyl-CoA (1.8 GBq/mmol) came from (Amersham; Les Ellis, France). All other chemicals were purchased from Sigma (St. Louis, MO).

Preparation of the 15,000 × g fraction. Developing seeds were gently ground in a mortar with a 0.08M HEPES (pH 7.2) buffer containing 0.32M sucrose, 10 mM β-mercaptoethanol and 50 mg.mL^{-1} of polyvinyl/polypyrrolidone (PVP) (Buffer A). The homogenate was filtered through two layers of Miracloth and centrifuged successively at 300 × *g* (5 min) and 15,000 × *g* (25 min). The pellet from the latter centrifugation was then washed and re-suspended in buffer A without PVP (Buffer B). Protein was determined by the method of Smith *et al.* (16), with serum albumin as the standard.

Solubilization of proteins. About 0.5 mg of proteins from the 15,000 × *g* pellet were incubated with Triton X-100 for two hours at 4°C, at different Triton X-100/protein ratios (w/w) or at the optimal ratio of 2.5. The resulting suspension was centrifuged 15 min at 150,000 × *g* in a Hitachi CS 100 Micro Ultracentrifuge (Hitachi Koki Co., Ltd.; Tokyo, Japan), which is equivalent to a centrifugation of 1 h at 100,000 × *g* in a conventional ultracentrifuge, and the supernatant was used as the solubilized fraction. In some experiments, the pellet was re-suspended in Buffer B to measure the remaining activity.

C18:1-CoA and C20:1-CoA elongation. About 50 μg protein was incubated 1 h at 30°C with 19.6 μM [2-^{14}C] malonyl-CoA (1.8 GBq/mmol), 9.2 μM oleoyl-CoA or eicosenoyl-CoA or stearoyl-CoA, 0.5 mM NADH, 0.5 mM NADPH, 1 mM $MgCl_2$ and 2 mM DTT, in a final volume of 0.1 mL in Buffer B. The reaction was stopped by the addition of 100 μL of 5N KOH in H_2O/CH_3OH (9:1, vol/vol). The mixture was saponified for 1 h at 80°C and then acidified with 100 μL of 10N H_2SO_4 containing 10% of malonic acid. The fatty acids were extracted with 2 mL chloroform and washed with water (3 × 2 mL). After evaporation of the chloroform, the radioactivity was measured directly with a Packard Tri Carb 2000 CA liquid scintillation spectrometer (Downers Grove, IL) in order to measure the overall condensation reaction.

Analysis of intermediates. The different intermediates of the elongation reaction (β-hydroxyacyl-CoA, nonadecanone, free fatty acids) were separated on Silica Gel 60F254 (Merck, Darmstadt, Germany) plates (10 × 10 cm) eluted with hexane/diethylether/acetic acid (70:30:1, vol/vol/vol) (10). The radioactivity was localized by

*To whom correspondence should be addressed at Institut de Biochimie Cellulaire, CNRS and Université de Bordeaux 2. 1, rue Camille Saint-Saëns, 33077 Bordeaux Cedex, France.

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autoradiography and measured by scanning the autoradiograms in a Camag 76510 photodensitometer (Muttenz, Switzerland) coupled to a Spectra-Physics integrator (San Jose, CA) (17) or/and by scraping the labelled lipids from the plate (17).

Analysis of the reaction products. The incubation was stopped by adding 600 μL of chloroform/methanol (1:1, vol/vol). Thin-layer chromatography (TLC) of the resulting homogeneous phase was carried out on Silica Gel 60F254 (Merck) plates (10 × 10 cm). The solvent for the first migration (4.5 cm) was butanol/acetic acid/water (5:2:3, vol/vol/vol). Under these conditions, acyl-CoAs, phospholipids and free fatty acids with neutral lipids were separated (17). A second migration (7.5 cm) with hexane/diethyl ether/acetic acid (70:30:1, vol/vol/vol) as eluant allowed the separation of the different neutral lipids from free fatty acids. Neutral lipids and free fatty acids were revealed with iodine vapors, and the acyl-CoAs were visualized at 254 nm. The quantitation of each lipid class was performed as described above (17).

Radio-gas-liquid chromatography (GLC) analysis. The fatty acid methyl esters were analyzed with a Packard 429 GLC equipped with a flame-ionization detector and a column packed with 3% OV-1 on chromosorb W-P 80/100 (2 mm × 2 m) coupled with a Packard 894 gas proportional counter. The flow rate of the carrier gas (argon) was 60 mL/min and the temperature was programmed from 180 to 290°C at 8°C/min.

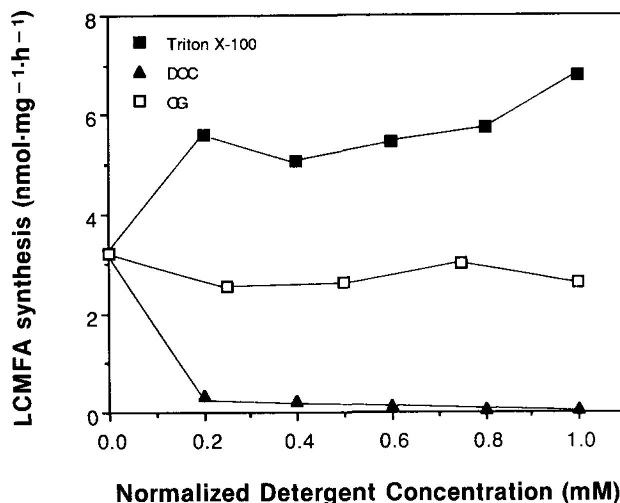

FIG. 1. Effect of detergents on C18:1-CoA elongation. Incubations were performed with [2-^{14}C] malonyl-CoA (19.6 μM) and 9.2 μM C18:1-CoA, 0.5 mM NADH, 0.5 mM NADPH, 2 mM DTT, 1 mM $MgCl_2$ in the presence of different quantities of detergents. The maximal concentrations for Triton X-100, OG and DOC studied were 0.3, 2.5 and 1 mM, respectively, and were normalized to 1. VLCMFA, very long-chain monounsaturated fatty acids; Doc, oleoxycholate; OG, *N*-octyl-β-D-glucopyranoside.

RESULTS AND DISCUSSION

Effects of detergents on the C18:1-CoA and C20:1-CoA elongation. The effects of different detergents on the elongation of C18:1-CoA and C20:1-CoA by [2-^{14}C] malonyl-CoA were studied with the 15,000 × *g* fraction (about 100 μg protein) as enzyme source. To determine the appropriate detergent for the further solubilization of the elongase(s), Triton X-100, *N*-octyl-β-D-glucopyranoside (OG) and deoxycholate (DOC) were used, at concentrations below their critical micellar concentrations (0.3 M, 0.9 M and 25 mM, respectively). Under these conditions, only the effect of the monomeric form of each detergent is studied. The results reported in Figure 1 show that the elongation of C18:1-CoA was stimulated by increasing concentrations of Triton X-100. Total VLCMFA synthesis was multiplied by two at a detergent concentration of 0.3 mM, compared to the control in the absence of Triton X-100. The VLCMFA synthesis was practically unchanged in the presence of OG and was inhibited by DOC, even at low concentrations (0.2 mM) (Fig. 1). When C18:1-CoA was replaced by C20:1-CoA, the VLCMFA synthesis was stimulated by 1.87-fold (Fig. 2), whereas OG had no effect and DOC caused the total inactivation of the acyl-CoA elongase.

The distribution of the radioactivity among the different fatty acids formed by C18:1-CoA elongation was determined by radio-GLC and is reported in Table 1. In the absence of detergent, similar amounts of C20:1 and C22:1 were synthesized: 49% (1.58 nmol/mg/h) and 51% (1.65 nmol/mg/h), respectively. In the presence of Triton X-100, C22:1 synthesis was greatly increased from 1.65 to 4.28 nmoles/mg/h, whereas the amount of C20:1 was only increased from 1.58 to 2.5 nmol/mg/h. The distribution of the label among the different fatty acids at 0.3 mM of detergent was 37% (2.51 nmol/mg/h) for C20:1 and 63% (4.28 nmol/mg/h) for C22:1.

Similar results have been obtained with microsomal preparations from *A. porrum* (11). Triton X-100 stimulated the saturated very long chain fatty acid (VLCFA) and preferentially that of C22:0. A smaller stimulation of VLCMFA synthesis by concentrations of Triton X-100 of 4.5 mM has been reported in *S. alba* and *L. annua* (14).

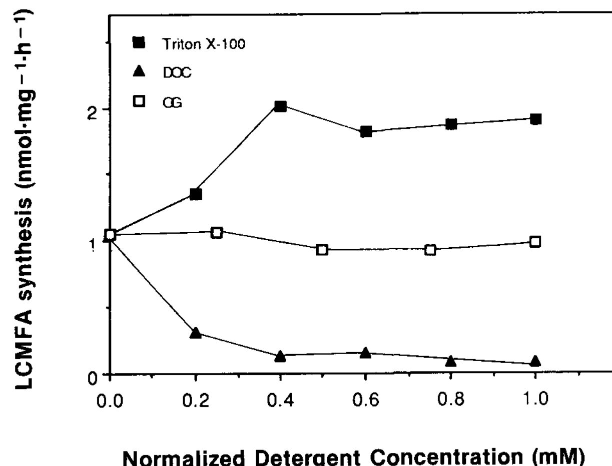

FIG. 2. Effect of detergents on C20:1-CoA elongation. Incubations were performed with [2-^{14}C] malonyl-CoA (19.6 μM) and 9.2 μM C20:1-CoA, 0.5 mM NADH 0.5 mM NADPH, 2 mM DTT, 1 mM $MgCl_2$ in the presence of different quantities of detergents. The detergent concentrations were normalized as indicated in Figure 1. Abbreviations as in Figure 1.

TABLE 1

Effect of Triton X-100, Glucopyranoside (OG) and Deoxycholate (DOC) on the Elongation of Oleoyl-CoA by [2-^{14}C] Malonyl-CoA by the 15,000 × *g* Pellet of Developing Rapeseed[a]

	C20:1 (nmol/mg/h)	C22:1 (nmol/mg/h)	Total VLCMFA (nmol/mg/h)
Untreated conditions	1.58 (49%)	1.65 (51%)	3.23
Triton X-100			
0.06 mM	2.58 (46%)	3.02 (54%)	5.60
0.12 mM	2.54 (50%)	2.54 (50%)	5.07
0.18 mM	2.29 (42%)	3.16 (58%)	5.46
0.24 mM	2.35 (41%)	3.37 (59%)	5.72
0.30 mM	2.51 (37%)	4.28 (63%)	6.79
OG			
5 mM	1.38 (54%)	1.18 (46%)	2.56
10 mM	1.33 (51%)	1.28 (49%)	2.61
15 mM	1.52 (51%)	1.47 (49%)	2.99
20 mM	1.23 (47%)	1.39 (53%)	2.62
DOC			
0.2 mM	n.d.	n.d.	0.340
0.4 mM	n.d.	n.d.	0.250
0.6 mM	n.d.	n.d.	0.150
0.8 mM	n.d.	n.d.	0.066
1 mM	n.d.	n.d.	0.064

[a]The complete incubation mixture contained 19.6 μM [2-^{14}C] malonyl-CoA, 0.5 mM NADH, 0.5 mM NADPH, 2 mM DTT, 1 mM $MgCl_2$, 9.2 μM oleoyl-CoA, 0.08 M HEPES buffer, pH 7.2, containing β-mercaptoethanol (10 mM), 100 μg of protein and variable quantities of detergent, in a final volume of 0.1 mL. The incubation medium was incubated for 1 h at 30°C, and the very long chain monounsaturated fatty acids (VLCMFA) were extracted and analyzed by radio-gas-liquid chromatography.

TABLE 2

Effect of Triton X-100, Glucopyranoside (OG) and Deoxycholate (DOC) on the Elongation of Eicosenoyl-CoA by [2-^{14}C] Malonyl-CoA with the 15,000 × *g* Pellet[a]

	C22:1 Synthesis (% of control)
Triton X-100	
0.05 mM	129
0.12 mM	191
0.18 mM	172
0.24 mM	177
0.30 mM	181
OG	
5 mM	101
10 mM	89
15 mM	88
20 mM	93
DOC	
0.2	30
0.4	12
0.6	15
0.8	8
1.0	7

[a]The complete incubation mixture contained 19.6 μM [2-^{14}C] malonyl-CoA, 0.5 mM NADH, 0.5 mM NADPH, 2 mM DTT, 1 mM $MgCl_2$, 9.2 μM eicosenoyl-CoA, 0.08 M HEPES buffer pH 7.2 containing β-mercaptoethanol (10 mM), 100 μg of protein and variable quantities of detergent, in a final volume of 0.1 mL. The incubation medium was incubated for 1 h at 30°C and the long-chain monounsaturated were extracted and analyzed by radio-gas-liquid chromatography. The results are expressed as percentages of the untreated membrane activity (control), which was 1.05 $nmol \cdot mg^{-1} h^{-1}$.

The OG did not significantly modify the distribution of the radioactivity among the different fatty acids (Table 1). These results are quite different from those reported for *A. porrum*, where OG (30 mM) stimulated about 1.5-fold the VLCFA in microsomal preparations but did not change the label distribution among the fatty acids (11, 12). On the other hand, VLCMFA synthesis in *S. alba* and *L. annua* was inhibited by OG, even at low concentrations (14). In the presence of DOC, the VLCMFA synthesis level was too low and rendered the radio-GLC impossible.

The effect of Triton X-100 on the C20:1-CoA elongation by [2-^{14}C] malonyl-CoA was also studied (Table 2). In contrast with the leek system (11), Triton X-100 did not allow the synthesis of longer fatty acids (C24 and C26). However, in our system, C22:1 synthesis was stimulated 1.8-fold by a detergent concentration of 0.3 mM (Table 2). Nevertheless, the stimulation was lower than in the case of the C18:1-CoA elongation. These results are not surprising, considering the specificity of the elongase toward the acyl-CoAs shown previously (18). The C22:1 fatty acid synthesis was unchanged in the presence of OG, whereas it was drastically inhibited by DOC, even at low concentration.

These results show that, unlike DOC, monomers of Triton X-100 and OG did not damage the elongation activity and Triton X-100 even stimulated VLCMFA synthesis. For these reasons, Triton X-100 was chosen for solubilizing the acyl-CoA elongase.

Optimization of the C18:1-CoA and C20:1-CoA solubilization. Attempts to solubilize the elongation system from the 15,000 × *g* fraction were carried out at different concentrations of Triton X-100 in the presence of variable quantities of protein. After two hours of solubilization at 4°C, the treated fractions were centrifuged at 150,000 × *g* for 10 min in a Micro Ultracentrifuge. The elongase activities were evaluated in the supernatants and their corresponding pellets. Under these conditions, the specific activities of the elongation (Table 3), even with the nontreated 15,000 × *g* fraction, were globally lower than in the experiments reported above (Figs. 1 and 2). This could be due to a loss of elongase activity at 4°C, as previously shown in *A. porrum* (13).

The best results were obtained at a detergent:protein ratio of between 2 and 3; about 60% of the 15,000 × *g* proteins were recovered in the solubilized fraction (Fig. 3), and the activity was 1.1-fold that in the 15,000 × *g* fraction. Under these conditions, the recoveries of the total and the specific activities in the solubilized fraction were about 65 and 109% for the C18:1-CoA elongase and about 80 and 135% for the C20:1-CoA elongase, respectively (Table 3). Although representing about 40% of the proteins issued from the treated fraction, the pellet of the treated fraction was devoid of elongase activity, whatever the acyl-CoA as substrate (Table 3). These data indicated that the remaining membrane particles were devoid of elongation activity and strongly suggested that the acyl-CoA elongase was largely solubilized. A Triton X-100/protein ratio of 2.5 (w/w) was used for the solubilization of the elongation system of *L. annua* (15), but, in contrast with the situation for *B. napus*, the solubilization significantly reduced the VLCMFA synthesis.

Higher concentrations of Triton X-100 did not improve the solubilization of rapeseed membrane proteins and even inhibited the elongase(s) activities (Fig. 3). Similarly, the

TABLE 3

Solubilization of C18:1-CoA and C20:1-CoA Elongase[a]

	Specific activity		Total activity		
	18:1-CoA (nmol/mg/h)	20:1-CoA (nmol/mg/h)	18:1-CoA (nmol/h)	20:1-CoA (nmol/h)	Total proteins μg
15,000 × *g*	1.83	1.21	0.82	0.54	448
Solubilized fraction	1.99	1.63	0.53	0.43	264
Pellet	0.28	0.18	0.05	0.03	184

[a]About 0.5 mg of protein of the 15,000 × *g* pellet was incubated with Triton X-100 at the optimal ratio of 2.5. The treated fraction was centrifuged 15 min at 150,000 × *g* (Micro Ultracentrifuge; Hitachi; Tokyo, Japan), and the supernatant was used as the solubilized fraction. C18:1-CoA and C20:1-CoA Elongation activities were evaluated as described in Materials and Methods.

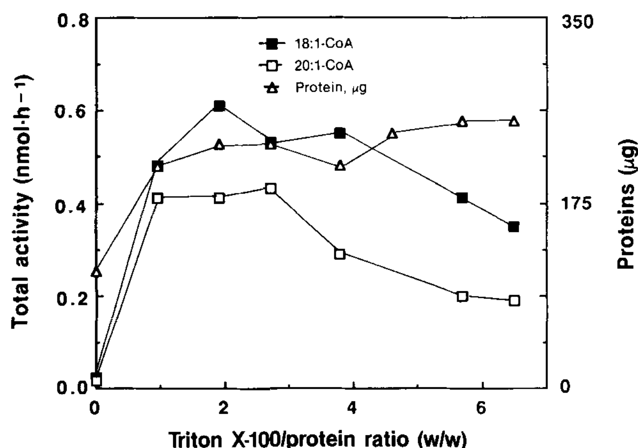

FIG. 3. Effect of the detergent/protein ratio on the solubilization of proteins, C18:1-CoA elongase and C20:1-CoA elongase. The 15,000 × *g* pellet (about 0.5 mg) was incubated for two hours at 4°C with different quantities of Triton X-100. The treated fraction was centrifuged 15 min at 150,000 × *g* (Micro Ultracentrifuge; Hitachi; Tokyo, Japan). The supernatant was used as the solubilized fraction.

total elongation in *S. alba* (14) was strongly inhibited for concentrations of Triton X-100 up to 0.3% (wt/vol).

Nature of the reaction products of C18:1-CoA, C20:1-CoA and C18:0-CoA elongation by the solubilized fractions. To check whether the solubilized elongation system remains functional after Triton X-100 solubilization, the proteins were incubated for 1 h with [2-^{14}C] malonyl-CoA in the presence of C18:1-CoA, C20:1-CoA or C18:0-CoA. The different lipid classes were analyzed, and the results are reported in Table 4. Acyl-CoAs were synthesized as major products by the solubilized acyl-CoA elongase(s). The percentages were 45, 38 and 44% of very long-chain acyl-CoAs when the substrates were C18:1-CoA, C20:1-CoA and C18:0-CoA, respectively, compared to 68% of the total products in the nontreated 15,000 × *g* fraction when C18:1-CoA was the primer.

However, in the solubilized fraction, more than 30% of the radioactivity was associated with free fatty acids, indicating that thioesterase(s) were still active in the treated fraction and were also solubilized. Very long-chain acyl-CoAs were incorporated to a lesser extent (about 7%) into triacylglycerols. At the difference observed for untreated membranes, labeled phosphatidylcholine was detected (about 17%) in our reaction mixture. These data indicated that the acyltransferase(s) were also functional in the solubilized fraction, even when no acceptor was added to our reaction mixture. Moreover, the absence of labeled phosphatidylcholine, from untreated membranes as enzyme source, could be interpreted as less accessibility of the substrates to the acyl-CoA transferase(s), due to competing enzymes also using acyl-CoA or to membrane fluidity.

These results show that elongase activities were preserved during the solubilization procedure. The presence of functional acyltransferases and thioesterases was demonstrated and could eventually decrease the elongation by substrate competition and, hence, reduce erucic acid synthesis.

TABLE 4

Nature of the Reaction Products of the C18:1-CoA, C20:1-CoA and C18:0-CoA Elongations by the Solubilized Fraction[a]

	Treated membranes substrates			Untreated membranes
Products	18:1-CoA (nmol/mg/h)	20:1-CoA (nmol/mg/h)	18:0-CoA (nmol/mg/h)	18:1-CoA (nmol/mg/h)
Acyl-CoAs	0.84 (45%)	0.39 (38%)	0.36 (44%)	68%
Phosphatidylcholine	0.32 (17%)	0.18 (17%)	0.11 (14%)	n.d.
Free fatty acids	0.60 (31%)	0.39 (38%)	0.29 (36%)	23%
Triacylglycerols	0.13 (7%)	0.08 (7%)	0.05 (6%)	9%

[a]C18:1-CoA, C20:1-CoA and C18:0-CoA Elongation activities were evaluated as described in Materials and Methods. The reaction products were separated by the method of Juguelin and Cassagne (17).

TABLE 5

Analysis of the Reaction Intermediates of C18:1-CoA Elongation by the Solubilized Fraction[a]

Product	15,000 × *g* fraction (nmol/mg/h)	Solubilized fraction (nmol/mg/h)
β-Hydroxy fatty acids	0.15	0.92
Fatty acids	0.99	0.70
Nonadecanone	0.00	0.09
Total (overall reaction)	1.14	1.71

[a]The C18:1-CoA elongation was evaluated as described in Materials and Methods. The reaction intermediates were separated by thin-layer chromatography with hexane/diethyl ether/acetic acid (70:30:1, vol/vol/vol).

Analysis of the reaction intermediates of the solubilized acyl-CoA elongase(s). It was important to know if the activity of the different reactions involved in the VLCFA synthesis (condensation, first reduction, dehydration and second reduction) was modified during the solubilization. For this, analysis of the intermediate reaction products of the elongation was undertaken. The method used to isolate the reaction intermediates of the elongation allowed the separation of the nonadecanone (*i.e.*, β-ketoacyl intermediate), β-hydroxyacyl-CoA and the very long chain fatty acids after saponification. Nonadecanone was the result of a nonenzymatic decarboxylation of β-ketoacyl-CoA during the saponification process. The incubation of C18:1-CoA with [2-^{14}C] malonyl-CoA in the presence of proteins from the 15,000 × *g* fraction led to the synthesis of 0.99 nmol/mg/h of VLCMFA and 0.15 nmol/mg/h of β-hydroxy-acyl-CoA. The synthesis of nonadecanone was not detected (Table 5), indicating that, under these conditions, the condensing enzyme and the first reductase were fully active. However, the detection of β-hydroxyacyl-CoA indicated that the dehydratase activity is a limiting factor of the elongation process. The solubilization by Triton X-100 led to a stimulation of the acyl-CoA elongase activity (1.71 nmol/mg/h compared to 1.14 for the untreated membranes), but the distribution of the label among the intermediates was changed (Table 5). The synthesis of fatty acids represented 71% of that observed with native membranes. Nonadecanone, which was undetectable in the control, represented 5% of the total radioactivity, and the β-hydroxyacyl-CoA level was increased 6-fold compared to the control. Due to the difficulty to evaluate the part of the enzyme complex preferentially solubilized or preferentially stimulated by Triton X-100, no clear interpretation of the label distribution changes could be advanced. The reaction intermediates were also checked during the solubilization of the acyl-CoA elongase(s) of *L. annua* (15); The β-ketoacyl-CoA synthase activity was stimulated, but in contrast with our system, the overall reaction leading to the VLCFA synthesis was inhibited.

The results reported here show that the optimal conditions for the solubilization of the elongation complex of developing rapeseed have been well defined. Triton X-100 was the most efficient detergent. At the optimal Triton X-100/protein ratio of 2.5, the solubilization led to an enrichment of the C18:1-CoA and C20:1-CoA elongase activities. Acyl-CoAs were synthesized as major products. Analysis of the reaction intermediates showed that solubilization induced changes in product ratios, suggesting that some intermediate enzyme activities have been modified. Nevertheless, the presence of VLCM acyl-CoAs demonstrated that the entire elongation system was solubilized and was still functional.

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