Solubilization of C18-CoA and C20-CoA elongases from *Allium porrum* L. epidermal cell microsomes

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The effects of *n*-octyl-β-D-glucopyranoside, Triton X-100 and deoxycholate on acyl-CoA elongation by *Allium porrum* L. epidermal cell microsomes showed that the Triton X-100 specifically stimulated the synthesis of C22–C26 acids using C18-CoA as primer, whereas the fatty acid elongation products of C20-CoA remained essentially unchanged. *n*-Octyl-β-D-glucopyranoside increased the C20 and C22 fatty acid syntheses to the same extent and deoxycholate inhibited C18-CoA and C20-CoA elongation. The presence of two different elongation systems, as suggested by these results, has been demonstrated. After solubilization by Triton X-100, the C18-CoA and C20-CoA elongases were separated by sucrose density centrifugation. The fractions corresponding to sucrose concentrations of 0.51 and 0.62 M presented the maximal activities for C18-CoA and C20-CoA elongases, respectively. In addition, by gel filtration on a Sephacryl S-300 column, the C20-CoA and the C18-CoA elongases have estimated apparent molecular masses under detergent conditions of 600 and 350 kDa, respectively.

Acyl-CoA elongation *Very long chain fatty acid* *Detergent* Allium porrum *L.*
Microsomal enzyme solubilization

1. INTRODUCTION

The synthesis of VLCFA has been demonstrated in cell-free preparations from germinating pea cotyledons [1], excised epidermis of pea and leek leaves [2,3] and animals [4–7]. Such studies showed that elongation required malonyl-CoA and NADPH as elongation substrate and reductant, respectively. The well-documented systems [7,8] suggest the existence of 2 classes of elongating activities; the first one, ATP-dependent, using endogenous precursors, and the second one capable of elongating exogenous acyl-CoAs in the absence of ATP. In *Allium porrum* L. etiolated seedling microsomes, it has been demonstrated that stearoyl-CoA and arachidoyl-CoA are the best substrates for the exogenous acyl-CoA elongation system [9]. C18-CoA elongation took place in the endoplasmic reticulum membrane [10] and the reaction products were released as acyl-CoAs [11]. However progress in elucidating the nature, number, partial reactions and effect of lipids on the activity of the elongases is limited by the complexity of the membrane fractions used as the enzyme sources [12] and will depend on the possibility of purifying the acyl-CoA elongases. No solubilization of any elongase, whatever its origin, has so far been reported, though, in one case, the partial solubilization and purification of an enzyme involved in the elongation process was achieved [12]. As a prerequisite step for a further purification of the acyl-CoAs elongases, the effect of various detergents on the elongation was studied and attempts to solubilize the acyl-CoA elongases were carried out.

Abbreviations: DDT, dithiothreitol; VLCFA, very long chain fatty acids; BSA, bovine serum albumin

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2. MATERIALS AND METHODS

2.1. *Materials*

All chemicals were from Sigma, Sephacryl S-300 from Pharmacia and [2-^{14}C]malonyl-CoA (58 Ci/mol) was purchased from Amersham.

2.2. *Microsome preparation*

Leeks (*A. porrum* L.) were obtained from a local market and microsomes were prepared as described [8]. 7–9 g of peeled epidermis were mortar-ground with sand, in 0.08 M Hepes buffer (pH 7.2), containing 0.32 M sucrose and 10 mM β-mercaptoethanol. After filtration through 4 layers of Miracloth, the filtrate was centrifuged for 5 min at 3000 × *g* and the resulting supernatant was centrifuged for 15 min at 13000 × *g*. The resulting supernatant was centrifuged again for 1 h at 150000 × *g* and the resulting pellet was washed with 14 ml of 0.08 M Hepes buffer (pH 6.8), containing 10 mM β-mercaptoethanol, and resedimented at 150000 × *g* for 1 h. The resulting microsomal pellet was resuspended in 1 ml of the above buffer (pH 6.8) and used as the enzyme source.

2.3. *Acyl-CoA elongation activity measurements*

Routinely, stearoyl-CoA and arachidoyl-CoA were used at a concentration of 9 μM in the presence of [2-^{14}C]malonyl-CoA (58 Ci/mol) (17 μM), NADPH (0.5 mM), NADH (0.5 mM), $MgCl_2$ (1 mM), DTT (2 mM), various concentrations of detergent (see table and figure legends) and 60 μg of protein in 0.08 M Hepes buffer (pH 6.8), containing 10 mM β-mercaptoethanol. The final volume was 0.1 ml and the incubation was carried out for 1 h at 30°C. The reaction was stopped by addition of 0.1 ml of 5 N KOH in methanol. Fatty acids were extracted and the label was determined as described [8]. The distribution of label among the fatty acids was determined by radio-GLC under the conditions described in [11].

2.4. *Solubilization of microsomal proteins*

Usually, 0.5 mg of microsomal proteins were incubated for 1 h at 4°C, with shaking in a (5 × 20 mm) centrifuge tube with detergent, using a Triton X-100/protein ratio of 1, in 0.08 M Hepes buffer (pH 6.8) containing 10 mM β-mercaptoethanol. The reaction mixture was then centrifuged for 15 min at 27 lb/inch^2 (150000 × *g*) using a Beckman airfuge centrifuge. The supernatant was used as the solubilized enzyme source.

2.5. *Ultracentrifugation studies*

The sedimentation experiments were performed according to Schweizer et al. [13]. 400 μg of solubilized proteins were layered on a 0.44–1.1 M linear sucrose gradient of 3.8 ml and centrifuged for 16 h at 100000 × *g*. 170 μl fractions (10 drops) were collected and C18-CoA and C20-CoA elongation activities were measured using 45 μl of each fraction and 2 h incubation time.

2.6. *Sephacryl S-300 chromatography*

2.5 mg of solubilized proteins were loaded on a Sephacryl S-300 column (63 × 1.6 cm), previously equilibrated with 0.08 M Hepes (pH 7.2) buffer, containing 0.02% (w/v) Triton, 2% (v/v) glycerol and 10 mM β-mercaptoethanol, and eluted with the same buffer at a flow rate of 5 ml/h. 1 ml fractions were collected (80 drops) and elongase activities were determined using 45 μl of each sample and 4 h incubation time.

Before each chromatographic run, the column was calibrated using marker proteins; the elution volumes were measured by weighing. Under these conditions, the void volume of the column was 36.4 ml and the elution volumes for thyroglobulin (670 kDa), ferritin (440 kDa), catalase (232 kDa), BSA dimer (128 kDa), BSA monomer (64 kDa) and cytochrome *c* (13.5 kDa) were 37.4, 47.5, 54.6, 64.7, 70.8 and 101.0 ml, respectively.

Proteins were estimated by the method of Bradford [14] using BSA as the standard and sucrose concentration by refractometry.

3. RESULTS

3.1. *Effects of detergents on C18-CoA and C20-CoA elongation*

The elongation of stearoyl-CoA by [2-^{14}C]malonyl-CoA, in the presence of NADPH and NADH, was tested at different concentrations of Triton X-100, *n*-octyl-β-D-glucopyranoside and deoxycholate. Each detergent was used at concentrations below its critical micellar concentration so that only the effect of the monomeric form of each detergent was studied. The critical micellar concentrations being respectively 25, 0.9 and 0.3 mM for

n-octyl-β-D-glucopyranoside, deoxycholate and Triton X-100 [15], the concentration ranges studied were 5–25 mM for *n*-octyl-β-D-glucopyranoside, 0.2–1 mM for deoxycholate and 0.06–0.3 mM for Triton X-100.

After elongation of C18-CoA by [2-^{14}C]malonyl-CoA, the labelling of fatty acids was determined by radio-GLC. The distribution of the radioactivity is reported in table 1. In the absence of detergent, C20 was the major component and represented 82.4% of the total radioactivity. The addition of *n*-octyl-β-D-glucopyranoside increased the total radioactivity but, whatever the concentration used, did not change the label distribution in the fatty acids. Consequently, each fatty acid synthesis was stimulated in the same manner. In the presence of Triton X-100, the label of C20 decreased by about 28% compared to that observed in the absence of detergent. However, the synthesis of longer fatty acids was largely stimulated and, in particular, those of C24 and C26, undetectable in the absence of detergent, reached 14.9 and 4.6%, respectively, of the total radioactivity in the fatty acids, at a concentration of 0.3 mM Triton X-100. Whatever the concentration of deoxycholate used, the radioactivity was only localized in C20. These results indicate that deoxycholate inhibited the synthesis of C22 and C24. The data reported in table 2 support this hypothesis. The distribution of label in the fatty acids resulting from the elongation of C20-CoA showed that the C22 acid was the major product in the absence of detergent, but that C24 and C26 acids were also synthesized. In the presence of deoxycholate, practically no label was found in the fatty acids, so their analysis was impossible. The addition of *n*-octyl-β-D-glucopyra-

Table 1

Direct effects of detergents on C18-CoA elongation products

Conditions	Synthesis of fatty acids (nmol/mg per h)			
	C20	C22	C24	C26
Normal untreated	0.86 (82.4)	0.18 (17.5)	t	–
Triton X-100				
0.06 mM	1.79 (75.7)	0.45 (18.9)	0.09 (3.7)	0.04 (1.7)
0.12 mM	1.64 (74.4)	0.41 (18.6)	0.11 (5.0)	0.04 (2.0)
0.18 mM	1.68 (67.1)	0.47 (18.8)	0.29 (11.5)	0.07 (2.6)
0.24 mM	1.97 (60.7)	0.78 (23.9)	0.40 (12.4)	0.10 (3.1)
0.30 mM	1.74 (54.9)	0.81 (25.7)	0.47 (14.9)	0.15 (4.6)
n-Octyl-β-D-glucopyranoside				
5 mM	1.21 (83.3)	0.24 (16.7)	–	–
15 mM	1.88 (83.0)	0.39 (17.0)	–	–
20 mM	1.94 (82.2)	0.42 (17.8)	–	–
25 mM	1.28 (83.2)	0.26 (16.8)	–	–
Deoxycholate				
0.2 mM	0.93 (100)	–	–	–
0.4 mM	0.94 (100)	–	–	–
0.6 mM	1.00 (100)	–	–	–
0.8 mM	0.65 (100)	–	–	–
1.0 mM	0.55 (100)	–	–	–

The incubation mixture contained 60 μg microsomal proteins/assay. All other conditions are as described in section 2. The numbers in brackets represent the percentage of radioactivity; t, traces

Table 2

Direct effects of detergents on C20-CoA elongation products

Conditions	Fatty acids synthesized (nmol/mg per h)		
	C22	C24	C26
Normal untreated	0.36 (51.4)	0.23 (32.6)	0.11 (16.0)
Triton X-100			
0.06 mM	0.49 (46.9)	0.42 (40.0)	0.14 (13.1)
0.12 mM	0.72 (51.7)	0.47 (33.3)	0.21 (15.0)
0.18 mM	0.72 (49.4)	0.56 (38.5)	0.18 (12.1)
0.24 mM	0.89 (49.1)	0.73 (40.1)	0.20 (10.8)
0.30 mM	0.76 (47.7)	0.67 (42.1)	0.16 (10.2)
n-Octyl-β-D-glucopyranoside			
5 mM	0.33 (57.7)	0.15 (25.9)	0.09 (16.3)
10 mM	0.37 (82.7)	0.08 (17.3)	t (0)
15 mM	0.41 (81.6)	0.09 (18.4)	t (0)
20 mM	0.48 (80.6)	0.11 (19.4)	t (0)
25 mM	0.55 (79.1)	0.09 (12.8)	0.06 (8.1)
Deoxycholate	not analyzed		

60 μg of proteins were incubated for 1 h under the conditions defined in section 2. The numbers in brackets represent the percentage of radioactivity; t, traces

noside resulted in an increase of the proportion of label in C22 acids, reaching about 80% and, consequently, the proportion of label in C24 and C26 acids decreased. In the presence of Triton X-100, the synthesis of the different fatty acids was stimulated in the same manner and no really significant change of the label distribution was observed.

All these data, and particularly the effect of deoxycholate, are strongly in favour of the existence of 2 different elongases.

3.2. *Solubilization of C18-CoA and C20-CoA elongases*

To check the presence of 2 different elongases, the solubilization of the C18-CoA and C20-CoA elongation activities was undertaken. Assays using *n*-octyl-β-D-glucopyranoside and Triton X-100 were carried out and the recovery of activities was measured at different concentrations of detergent with variable quantities of microsomes. After centrifugation of the reaction mixture at 150000 × *g* (section 2) the recoveries of total and specific activities were 22 and 45%, respectively, for the C18-CoA elongase, and 15 and 48% for the C20-CoA elongase, using *n*-octyl-β-D-glucopyranoside as detergent. The best results were obtained using a Triton X-100/protein ratio of 1. Under these optimal conditions, after 1 h incubation at 4°C, 65% of the total microsomal proteins were recovered in the 150000 × *g* supernatant fraction, corresponding to 42 and 59% of the total activities of C18-CoA and C20-CoA microsomal elongation activities, respectively, and 51 and 91% of the specific activities measured using untreated microsomes. The reaction products were checked by radio-GLC and as expected, C18-CoA and C20-CoA primers led uniquely to the formation of C20 and C22 saturated fatty acids, respectively (fig.1), indicating that the Triton X-100 solubilizing treatment did not change the enzymatic reactions studied.

3.3. *Ultracentrifugation studies*

The solubilized proteins were subjected to sedimentation on a sucrose gradient: after centrifugation, fractions were collected and assayed for elongation activities. The sucrose density gradient sedimentation profiles of the C18-CoA and C20-CoA elongation activities are reported in fig.2. The C18-CoA elongation activity profile was composed of a sharp peak, corresponding to the maximal activity, at 0.51 M sucrose, followed by a shoulder in the 0.6–0.7 M sucrose region. The C20-CoA elongase activity profile presented a broad peak with a maximum situated at 0.62 M sucrose. These results showed the presence of 2 different elongases, migrating to 0.51 and 0.62 M sucrose, respectively, and suggested that the C20-CoA elongase presents a lower specificity towards the acyl-CoA primer than does the C18-CoA elongase.

3.4. *Sephacryl S-300 column chromatography*

The microsomal proteins solubilized by Triton X-100 treatment were loaded on a Sephacryl S-300 column. The elution profile (fig.3) showed that no activity was found in the void volume and that C18-CoA and C20-CoA elongation activities were separated. In contrast to fig.2 the C18-CoA elongase activity was drastically diminished although C20-CoA elongase activity was better preserved. The elution volumes were 39.5 and 47.7 ml for the C20-CoA and C18-CoA elongases, respectively, corresponding to apparent molecular masses of 600 and 350 kDa.

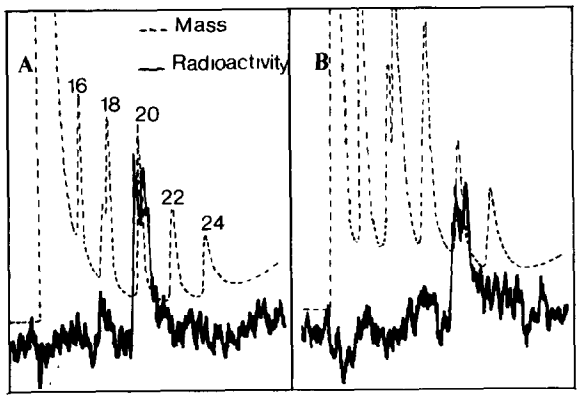

Fig.1. Radio-GLC of C18-CoA and C20-CoA elongation by solubilized proteins. 130 μg of solubilized proteins were incubated at 4°C for 1 h as described in section 2.

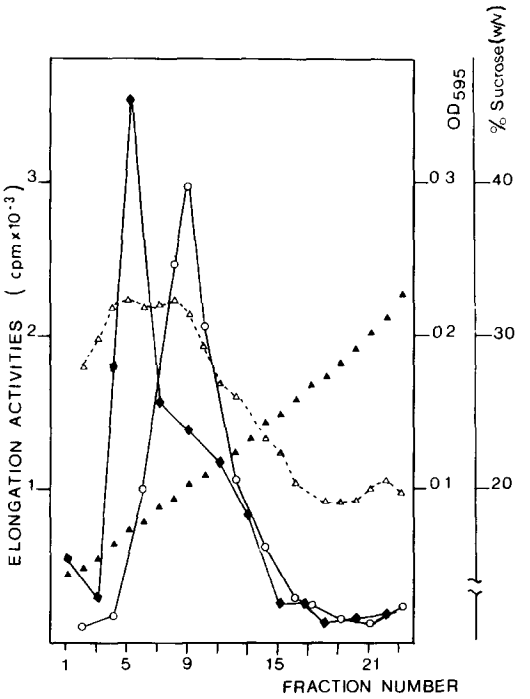

Fig.2. Sucrose density gradient centrifugation: distribution of C18-CoA and C20-CoA elongation activities. Each fraction was examined for C18-CoA elongation (◆—◆) and C20-CoA elongation (○—○) activities. Proteins (△---△) and sucrose molarity (▲) were also measured using 50 and 10 μl of each fraction, respectively.

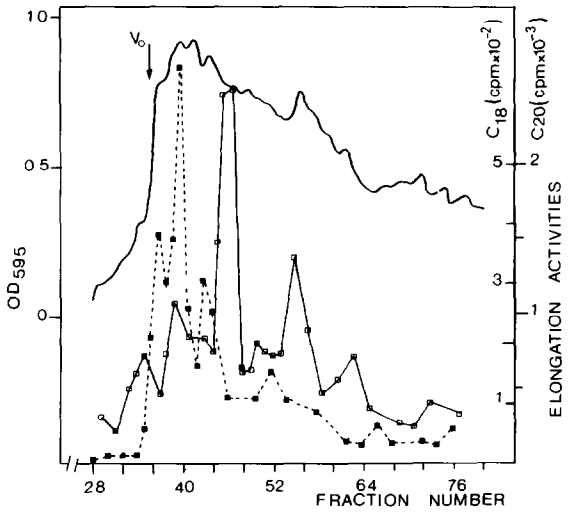

Fig.3. Sephacryl S-300 column chromatography. Each fraction was analyzed for C18-CoA (□—□) and C20-CoA elongation (■---■) activities. Proteins (——) were measured by Bradford's method using 100 μl of each fraction.

4. DISCUSSION

Very few studies have so far been devoted to the solubilization of the microsomal acyl-CoA elongases, and no data are as yet available on plant elongases. Bernert and Sprecher [12] reported that the addition of deoxycholate to microsomes from rat liver resulted in the inactivation of the elongases. It was postulated that separate (multiple) enzymes rather than a single multifunctional enzyme were operative in rat microsomes. The further partial purification of β-hydroxyacyl-CoA dehydrase [12], as well as other indirect evidence [16], supported this view. All attempts to determine the complete sequence of acyl-CoA elongation after deoxycholate treatment have failed. In our hands, the use of deoxycholate, even at low concentrations where the detergent acts as a membrane perturbant rather than as a solubilizing agent, led to a drastic decrease of the elongating activity, whatever the substrate (C18-CoA or C20-CoA). However, non-ionic detergents such as Triton X-100 and *n*-octyl-β-D-glucopyranoside, used below or in the region of the critical micellar concentration, not only did not inhibit the elongation, but also stimulated the activity. This was particularly clear in the case of Triton X-100 which was therefore used for the solubilization of the elongases. The solubilization was maximal for a 1:1 detergent to protein ratio. The products of labelled malonyl-CoA incorporation in the presence of primer acyl-CoAs were saturated fatty acids, demonstrating that the whole elongation process took place in the supernatant after Triton X-100 treatment. This result suggested that the elongases were probably not dissociated into discrete multiple enzymes. This assumption was greatly supported by the further gel filtration and/or centrifugation on sucrose gradient of the solubilized proteins. Besides being the first demonstration that elongases are really solubilized, this study revealed that the apparent molecular masses of the elongases are high. However, the data concerning the molecular mass should be analyzed with care and do not account for the true value of the elongase, because of the presence of detergents and the existence of mixed micelles. With these restrictions in mind, the high molecular masses observed here are compatible with a complex containing all the activities required for elongation, and which is able to synthesize saturated VLCFA from C18-CoA or C20-CoA. These molecular masses differ markedly from those reported for the plant fatty acid synthetases (87 kDa and about 60–90 kDa for barley and spinach, respectively [17,18]) and suggest that the membrane acyl-CoA elongases differ greatly from the FAS, in size, localization and function.

The most interesting result of this study is the demonstration of the solubilization of 2 acyl-CoA elongases, the existence of which had been suggested from multiple, but indirect, observations [8,9].

For instance, the addition of ATP, or ACP, drastically affected C22-C30 acid formation, whereas C20 acid synthesis was not affected [10]. More recently, studies using cerulenin, a well-known inhibitor of the fatty acid synthetases, showed that at low concentrations (40 μM) C18 and C20 acid syntheses were increased, while the formation of C22–C26 acid was strongly diminished [8]. The role of these different elongases in the cell requires further examination. Whether a lipid requirement exists for both elongases is unknown. No exogenous lipid was added for measuring the activity, but it is likely that some tightly bound lipid remained associated with the solubilized elongases. To study the elongation process further, as well as its eventual modulation by membrane lipids, attempts to purify the solubilized acyl-CoA elongases will be undertaken in our laboratory.

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