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INCORPORATION OF FREE FATTY ACIDS INTO ACYLTHIOESTERS AND LIPIDS OF DEVELOPING SUNFLOWER SEEDS

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Abstract—The synthesis of lipids from radioactive fatty acids in developing sunflower seeds has been examined. Lauric, palmitic, stearic and oleic acids were used as precursors. In both the intact tissue and a cell-free extract, an active triacylglycerol synthetase activity was found. The analysis of the acylthioesters showed that only lauric acid was bound to the acyl carrier protein but all of them were activated to esters of Coenzyme A. Lauric acid was elongated to stearic acid. Oleic acid was desaturated to linoleic acid. All the fatty acids tested were incorporated into polar lipids, diacylglycerols and triacylglycerols

INTRODUCTION

In contrast to the large amount of data that have been published on the lipid composition of oil-rich seeds, only minor attention has been devoted to the mechanisms by which the lipids are synthesized and accumulated [1].

In most of the studies on lipid metabolism in developing seeds the intact or sliced tissue is incubated with labelled acetate or glycerol [2, 3]. When subcellular fractions are used, labelled acyl-CoAs are the preferred substrates [4]. The simplest substrates such as acetate, malonate or their CoA derivatives, although actively incorporated into different glycerolipids, give little information about the intermediate acylthioesters since the corresponding fractions are usually poorly labelled. The use of free fatty acids (FFAs) as substrates, however, gives good incorporations into acyl-CoAs and, depending on the nature of the fatty acid, into acyl-ACPs.

In this paper, we have directed our attention to an oil seed almost unknown in studies on lipid biosynthesis: the sunflower. In a cell-free homogenate, we have studied the activation of FFAs to acyl-ACPs and acyl-CoAs, the fatty acids that are modified and the glycerolipids into which they are incorporated.

RESULTS AND DISCUSSION

Composition of the acylthioesters and lipids occurring in the seeds

The fatty acid composition of the acylthioesters and lipids occurring in sunflower seeds are shown in Table 1. As in other tissues [5], most of the fatty acids present in the lipids were present also as acyl-ACPs and acyl-CoAs. In the acyl-ACPs were found the products of the *de novo* synthesis, the elongation and the first desaturation, i.e. saturated up to 16:0, 18:0 and 18:1, respectively. Surprisingly, 18:2 was also present. In the acyl-CoAs were found all these acids and also 18:3 which was not detected in other tissues. For an extensive discussion see ref. [5].

Incorporation of oleate by intact tissue

In a preliminary experiment, we studied the capacity of intact developing sunflower seeds to incorporate [^{14}C]oleate into different lipid classes. The results are shown in Table 2. Most of the radioactivity was found in triacylglycerols, which was in accord with the physiological conditions of the seeds, i.e. at the stage of triacylglycerols accumulation. Radio-GLC of fatty acids from the different glycerolipids showed a remarkably high oleate desaturase activity as well as a preferential accumulation of linoleate in triacylglycerols.

Table 1. Fatty acid composition of acylthioesters and lipids of developing sunflower seeds

Fatty acids	Acyl-ACPs	Acyl-CoAs	Polar lipids	Triacyl-glycerols
14:0	7.5	4.1	0.8	0.2
15:0	—	—	3.0	—
16:0	35.3	43.4	27.8	12.3
16:1	8.1	—	—	—
17:0	4.0	—	0.6	—
18:0	13.8	7.4	8.4	10.9
18:1	21.6	11.5	22.3	41.6
18:2	9.6	26.1	34.3	32.2
18:3	—	2.5	2.8	2.8

Results are expressed as percentage of total fatty acids.

After a single incubation time of 20 hr (this period was used to ensure a reasonable incorporation of radioactivity) no conclusions can be drawn about the mechanisms by which the FFAs were incorporated into lipids. However, this experiment shows the situation at an advanced step of the metabolism and the products that the seeds are capable of synthesizing *in vivo*.

Incorporation of labelled fatty acids into lipids by a cell-free homogenate

In experiments with cell-free homogenates the biosynthetic reactions can be stimulated by the addition of cofactors. We were interested in studying the

Table 2. Incorporation of ^{14}C-labelled oleate into acyl-CoAs and different lipid classes of developing sunflower seeds

Fraction	cpm (%)	18:1 (%)	18:2 (%)
Acyl-CoAs	0.8	—	—
Triacylglycerols	57.8	47	53
Diacylglycerols	13.3	77	23
Polar lipids	22.1	81	19
Free fatty acids	6.0	—	—

incorporation of FFAs into acylthioesters and lipids of developing sunflower seeds in the presence of ACP and CoA. A homogenate that contained the soluble and microsomal fractions was used to study the fate of ^{14}C-labelled lauric, palmitic, stearic and oleic acids. The results obtained from these experiments are shown in Table 3. All the precursors were actively incorporated into acylthioesters and the different lipid classes. The percentage of incorporation is a measure of acyl-thiokinase activity, since fatty acids have to be activated to acylthioesters before entering the metabolic pathways. The high percentage of oleate incorporation indicated a high oleylthiokinase activity. The irregularities observed in the experiments where ACP was present were attributed to the presence of a mass of inert protein in our ACP preparation. Independently of the nature of the fatty acid used as substrate, triacylglycerols, as in intact tissue, were more heavily labelled than any other glycerolipids. This suggests that our extract contained at least a major part of triacylglycerol synthetase activity. The incorporation of radioactivity into acyl-ACPs was very small when 16:0, 18:0 and 18:1 were used as substrates, even in the presence of ACP. On the other hand, the incorporation of 12:0 was appreciable when assayed without exogenous ACP and was increased markedly when ACP was present. The incorporation into acyl-CoAs was very high for all the substrates tested, which indicated a very high acyl-CoA synthetase activity that allows FFA to enter the CoA track [6]. As a result, all fatty acids were transferred into polar lipids, diacylglycerols and, especially, triacylglycerols.

The distribution of radioactivity between the α and β positions of the glycerol molecule (Table 4) showed that saturated fatty acids were located preferentially in the α positions. On the contrary, oleic acid was almost evenly distributed in both positions.

The fatty acid compositions of the acylthioesters and lipids from the incubations are given in Table 5. These data suggest that 12:0 was activated to 12:0–ACP, which then entered the ACP track [6] and was elongated to 14:0, 16:0 and 18:0. The fatty acid composition of the acyl-ACP fraction supports this hypothesis since the major fatty acids are 16:0 (when only endogenous ACP was present) or 16:0 and 18:0 (when exogenous ACP was added). These results explain the early observation of James [7] that only free acids up to 14:0 are elongated and desaturated by plant systems and support the hypothesis that acyl-ACP synthetase is active with fatty acids up to 14:0 but its activity falls dramatically above this chain length [8]. The fatty acid composition of the acyl-CoA fractions, obtained after incubation with 12:0, indicated that the fatty acids formed by elongation of the precursor were transferred to the CoA, which established that the switching system between the ACP track and the CoA track [6] was functioning correctly. 16:0 and 18:0 were also activated to acyl-CoAs and then transferred into lipids but were not elongated or desaturated. The acyl-CoAs from the incubation with 18:1 contained 15% of 18:2-CoA. A similar degree of desaturation was found in the polar lipids and triacylglycerols from the same origin, indicating that an equilibrium was reached probably as a result of the combined action of lipases, thiokinases and acyltransferases.

In summary, developing sunflower seeds showed a remarkable capacity for metabolizing preformed fatty acids. Thiokinase, *de novo* synthetase, elongase, oleate desaturase and polar lipids and triacylglycerol synthetase were retained in the cell-free homogenate. The results obtained demonstrate that developing sunflower seeds constitute an excellent material for the study of these activities.

EXPERIMENTAL

Materials. Developing sunflower seeds were harvested just after the endosperm became solid and the petals were still fresh and yellow. Spinach ACP was obtained and purified by a standard method [10]. Pancreatic lipase was prepared from pig

Table 3. Incorporation of ^{14}C-labelled fatty acids into acylthioesters and different lipid classes by a homogenate from developing sunflower seeds

	Labelled precursor*					
Fraction	12:0	16:0	18:0	18:1	12:0 (ACP)†	16:0 (ACP)†
Acyl-ACPs	0.9 ± 0.7	0.3 ± 0.1	0.3 ± 0.0	0.1 ± 0.0	10.2 ± 6.6	1.1 ± 0.7
Acyl-CoAs	7.7 ± 7.5	14.7 ± 1.8	14.0 ± 1.8	3.0 ± 1.1	13.3 ± 8.4	19.7 ± 7.0
Triacylglycerols	62.0 ± 4.1	39.3 ± 3.8	45.9 ± 5.3	39.0 ± 6.7	44.1 ± 8.1	34.0 ± 16.0
Diacylglycerols	6.9 ± 1.6	13.2 ± 4.0	15.9 ± 6.6	12.5 ± 4.4	9.2 ± 2.0	31.8 ± 0.8
Polar lipids	22.5 ± 4.0	32.6 ± 8.1	23.7 ± 0.5	42.3 ± 12.3	23.1 ± 8.9	13.4 ± 9.1
% incorporation	37 ± 4	38 ± 9	26 ± 15	92 ± 3	19 ± 10	21 ± 4

*Results are expressed as percentage of cpm. Mean ± s.d. (3 expts).
†Spinach ACP included in the incubation mixture.

Table 4. Positional distribution of radioactivity after incubation of a homogenate from developing sunflower seeds with labelled fatty acids

Fraction	Position	Labelled precursor* 16:0		18:0		18:1	
Triacylglycerols	α	87	(±1)	83	(±1)	60	(±1)
Triacylglycerols	β	13		17		40	
Diacylglycerols	α	86	(±1)	72	(±3)	52	(±5)
Diacylglycerols	β	14		28		48	
Polar lipids	α	78	(±6)	64	(±1)	55	(±1)
Polar lipids	β	22		36		45	

*Results are expressed as percentage of cpm. Mean ± s.d. (2 expts).

Table 5. Fatty acid composition of acylthioesters and lipids after incubation of a homogenate from developing sunflower seeds with labelled fatty acids

Fraction	Labelled precursor	Fatty acid composition (%) 12:0	14:0	16:0	18:0	18:1	18:2
Acyl-ACPs	12:0	14	12	74	—	—	—
	12:0 (ACP)*	4	3	41	52	—	—
Acyl-CoAs	12:0	70	4	16	10	—	—
	12:0 (ACP)*	63	3	12	22	—	—
	16:0	—	—	100	—	—	—
	18:0	—	—	—	100	—	—
	18:1	—	—	—	—	85	15
Triacylglycerols	18:1	—	—	—	—	80	20
Polar lipids	18:1	—	—	—	—	85	15

*Spinach ACP included in the incubation mixture.

pancreas [9]. ^{14}C-labelled fatty acids were purchased from The Radiochemical Centre, Amersham, U.K.

Separation and analysis of acylthioesters and lipids from sunflower seeds. The separation of acylthioesters was achieved by the method of ref. [11] suitably modified for the extraction of a mass of 300 g [5]. The separation of polar lipids and triacylglycerols and the preparation of Me esters was also achieved according to ref. [5]. Me esters were analysed by GLC in a glass column (0.27 × 200 cm) packed with 8% BDS, isothermally at 185°C and using a FID.

Preparation of sunflower seeds homogenate. Peeled seeds (7.5 g) were homogenized with 15 ml of 0.1 M KPi buffer, pH 7.2, in an Omni-Mixer homogenizer at 0–4°. The homogenate was centrifuged at 20000 g for 10 min. The supernatant was then filtered through filter paper to remove the floating fat layer and used immediately. The protein content was 9 mg/ml [12].

Incubations. Peeled seeds (1 g) were incubated in 2 ml of 0.1 M KPi buffer, pH 7.2, containing 3 μCi of ^{14}C-labelled oleate, in 50-ml vessels with air as the gas phase, at 30° and constant shaking for 20 hr. Incubations of cell-free homogenate were carried out in glass tubes, in a total volume of 2 ml, at 30° and with constant shaking for 2 hr. The standard assay mixture contained per ml: 4 μmol ATP, 2 μmol CoA, 2 μmol NADH, 10 μmol $MgCl_2$, 2 μCi of labelled precursor as its ammonium salt and 1.5 ml of homogenate. Where indicated, 0.5 ml of spinach ACP solution (14 mg protein/ml) and 2 μmol NADPH were added.

Separation of acylthioesters and lipids after incubation with labelled substrates. The intact tissue, at the end of the incubation, was rinsed with H_2O and blended in 3 ml *iso*-PrOH and 3 ml 0.1 M KPi buffer, pH 7.2. Then the mixture was treated according to [11] in order to separate lipids, acyl-CoAs and acyl-ACPs. When the homogenate was assayed, the reaction was stopped by adding 2 ml *iso*-PrOH and 0.1 ml 18 M HOAc and treated as before. The isolated acyl-ACPs and acyl-CoAs were saponified and methylated. The lipids were separated by TLC on Si gel developed with petrol–Et_2O–CO_2H (50:15:1). Triacylglycerols, FFAs, diacylglycerols and polar lipids (origin) were localized by exposing the edge of the plate, where a standard mixture was run simultaneously, to I_2 vapour. The bands were then scraped off and eluted with $CHCl_3$–MeOH (2:1). Aliquots of all fractions from acylthioesters and lipids were added to 10 ml toluene, containing 3.5 g/l PPO and 0.1 g/l diMe-POPOP and assayed for radioactivity in a liquid scintillation spectrometer. Samples of triacylglycerols and polar lipids were methylated by heating (80°) with 0.2 N Na in MeOH for 5 min followed by 4% HCl in MeOH for 5 min. The Me esters were then extracted in petrol.

Positional analysis. The remaining diacyl- and triacylglycerols were submitted to enzymatic hydrolysis with pancreatic lipase according to ref. [13]. The reaction products were extracted into Et_2O and separated by prep. TLC on Si gel developed with petrol–Et_2O–HCO_2H (50:25:1). FFAs and 2-monoacylglycerols were scraped off and methylated directly on the gel with Na

methoxide and HCl–MeOH as described. The polar lipids were treated with phospholipase A [14]. The reaction products, namely FFAs and 1-lyso polar lipids, were separated by TLC under the same conditions as described above, scraped off and methylated. A sample of pure phosphatidylcholine was completely hydrolysed under these conditions. Aliquots of all these fractions were assayed for radioactivity.

Analysis of radioactive Me *esters.* Radioactive Me esters were analysed by radio-GLC in a glass column (0.27 × 200 cm) packed with 5% EGS, isothermally at 170°.

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