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SEPARATION AND ANALYSIS OF ACYLTHIOESTERS FROM HIGHER PLANTS

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Abstract—The fatty acid composition of acylthioesters, acyl-CoAs and acyl-ACPs, of four plant tissues has been determined. This composition is very different to that presented for the endogenous lipids in each tissue. The composition of acylthioesters in relation to the role of acyl carriers in lipid metabolism is discussed.

INTRODUCTION

An important aspect of lipid metabolism is that related to acyl carrier molecules: coenzyme A (CoA) and acyl carrier protein (ACP). Since the bibliography on this subject is abundant, we will only say that both carriers bind to acyl groups through a thioester bond. This union is of great physiological significance as it changes the insoluble and unreactive fatty acids into new soluble and reactive molecules.

The type of carrier is specific for each metabolic route. It is well known that acyl-CoA is the substrate in the breakdown of fatty acids (β-oxidation), whilst in biosynthetic reactions acyl-ACP is the main protagonist [1]. There is some evidence, likewise, that acylation of different positions of lipids involves specific carriers [2].

It is obvious, therefore, that knowledge about fatty acid composition of acylthioesters of a biological system is of great value for the comprehension of lipid metabolism in that system. That is why several methods have been developed for the determination of acylthioesters in biological samples. Two of them are sensitive enough to permit the quantitative analysis of acylthioesters. The first is the classic method of Barron and Mooney [3] based on the selective reduction of acylthioesters to the corresponding alcohols, in the presence of acyl oxygen esters, using $NaBH_4$. The alcohols are readily analysed by GLC, directly or as TMSi derivatives. The method is quick and elegant but has two disadvantages; that is, it does not permit the differentiation of acyl-CoAs and acyl-ACPs and, as has been demonstrated [4], the reactivity of oxygen esters toward $NaBH_4$ depends on the polarity of the particular lipid. Thus, although triacylglycerols and free fatty acids are stable, the more polar lipids, such as phospholipids and galactosyl diglycerides, are in part reduced to the corresponding alcohols. The second method is that proposed by Mancha *et al.* [5] that overcomes these limitations. The method is based on the initial extraction of free fatty acids and oxygen esters into petrol from aqueous isopropanol; the precipitation of acyl-ACPs in the presence of ammonium sulphate and chloroform–methanol; and the selective adsorption of acyl-CoAs on neutral alumina gel. The 3 fractions can be recovered for further utilization or analysed for fatty acid composition by GLC as methyl esters.

In this work, the acylthioesters of 4 plant tissues (developing soybean, spinach leaves, potato tubers and olive exocarp) have been separated by the method of Mancha *et al.* and their fatty acid composition determined.

RESULTS AND DISCUSSION

The results clearly show that fatty acid composition of acylthioesters is very different to that of corresponding lipids. Thus, in the olive (Table 1), oleic acid, that represents 50–75% of lipids, polar lipids (PL) and triacylglycerols (TG), only reaches half of these values in acylthioesters. On the other hand, palmitic acid, that is poorly represented in the lipids, is an important component of acylthioesters, being the major fatty acid in one fraction. However, the differences in potato, spinach and soybean are more remarkable. In the potato (Table 2), linoleic acid represents *ca* 50% of the lipids, whereas in the acylthioesters it does not reach 10%. The second quantitatively important acid in potato lipids, linolenic, is absent from acylthioesters. In spinach (Table 3), linolenic acid, the dominant acid in lipids, is not represented at all in acylthioesters, these being represented by oleic and palmitic as their major acids. Finally, in soybean (Table 4), unsaturated acids account for *ca* 85% in lipids, whereas acylthioesters are poorly represented.

From the analytical viewpoint, the marked difference in fatty acid composition between lipids (oxygen esters) and acylthioesters gives experimental support to the efficiency of the separation method. Specially, the absence in acylthioesters of acids that are important in lipids (linolenic in spinach, linoleic in soybean) indicates that the separation has been achieved without contamination.

The composition of both types of acylthioesters

Table 1. Fatty acid composition of acylthioesters and lipids of olives (*Olea europea*) in two maturation steps: 60 and 135 days after floration (DAF)

Fatty acids	60 DAF				135 DAF			
	Acyl-CoAs	Acyl-ACPs	PL	TG	Acyl-CoAs	Acyl-ACPs	LP	TG
12:0	0.8	1.3	1.4	*t*	1.1	0.5	0.2	—
14:0	3.1	5.5	1.1	*t*	3.3	1.9	0.3	—
15:0	1.0	2.7	—	—	1.4	0.8	—	—
16:0	27.7	38.9	16.6	15.6	26.9	29.4	12.0	13.8
16:1	2.0	5.9	1.3	1.5	3.9	2.1	0.7	—
17:0	0.7	1.2	—	—	0.6	0.6	0.7	—
17:1	0.3	1.0	—	—	*t*	*t*	—	—
18:0	10.8	12.8	3.8	1.5	7.6	7.8	2.2	2.0
18:1	36.0	26.4	49.5	74.0	42.6	35.8	66.3	74.8
18:2	15.7	2.9	10.7	5.5	8.5	18.5	15.5	9.4
20:0	1.0	0.6	—	—	1.1	1.1	*t*	—
20:1	0.9	0.7	—	—	2.2	0.8	—	—
18:3	—	—	13.6	1.9	—	—	1.6	0.9

Results are expressed as percentage of total fatty acids. *t* = trace.

from the same source is very similar. However, there are great differences in composition between acylthioesters from different sources, in the same way that we find differences in the composition of lipids from different plant tissues.

In all acyl-ACPs are represented the intermediaries of what Shine *et al.* [6] named ACP track, namely, the '*de novo*' synthesis, the elongation to stearic acid and the desaturation to oleic acid. So, in the acyl-ACPs are present the series of saturated fatty acids from 12:0 to 18:0, together with some unusual odd-numbered, and oleic acid. On the other hand, the presence of oleic and saturated acids in the acyl-CoAs is also coherent with the scheme proposed by Shine *et al.* [6] in which the change of carrier occurs by means of an acylthioesterase, that liberates fatty acids from acyl-ACPs, and an acylthiokinase, that binds the fatty acids to the CoA. Both enzymes, working in succession, constitute what they call the switching system between the ACP and the CoA tracks. With regard to odd and very long chain fatty acids, they behave according to the same general scheme, being coherent in their existence in both types of acylthioesters.

The presence of linoleyl-CoA in 3 of the tissues examined requires a more extensive discussion. The evidence accumulated about the synthesis of linoleate from oleate is contradictory in relation to substrate and product of reaction. So, Gurr *et al.* [7] and Gurr [8] suggested that the true substrate of the desaturation reaction in *Chlorella vulgaris* is 18:1-PC and the final product 18:2-PC. The same conclusion was supported by Slack *et al.* [9] working with microsomes from maize leaves; Wharfe and Harwood [10] with

Table 2. Fatty acid composition of acylthioesters and lipids of aged potato tuber (*Solanum tuberosum*)

Fatty acids	Acyl-CoAs	Acyl-ACPs	PL	TG
12:0	0.7	0.7	*t*	0.2
14:0	2.3	2.1	0.3	0.7
15:0	0.8	0.8	0.2	0.2
16:0	20.9	18.9	18.9	12.4
16:1	3.9	5.8	*t*	0.6
17:0	0.7	0.3	*t*	*t*
17:1	0.7	0.5	—	—
18:0	5.1	4.5	3.2	2.6
18:1	53.6	56.9	1.9	6.1
18:2	8.3	7.1	52.7	49.2
20:0	0.4	*t*	—	—
18:3	—	—	22.7	27.9
20:1	1.4	0.8	—	—
20:2	1.2	1.6	—	—

Results are expressed as percentage of total fatty acids. *t* = trace.

Table 3. Fatty acid composition of acylthioesters and lipids of spinach leaves (*Spinacea oleracea*)

Fatty acids	Acyl-CoAs	Acyl-ACPs	Lipids
12:0	1.8	1.1	0.1
12:1	—	*t*	—
12:2	*t*	*t*	—
14:0	4.6	3.1	0.3
14:1	0.3	0.4	—
14:2	—	0.3	—
15:0	1.7	0.9	*t*
15:1	*t*	*t*	—
16:0	25.9	21.2	14.2
16:1	3.9	4.2	0.4
17:0	1.3	0.5	*t*
16:2	0.8	0.7	*t*
17:1	*t*	*t*	—
18:0	10.3	7.8	0.7
18:1	38.4	47.7	6.7
16:3	—	—	4.2
18:2	7.7	9.7	12.7
20:0	1.2	0.2	*t*
20:1	3.8	1.8	—
18:3	—	—	56.2
22:0	*t*	—	—

Results are expressed as percentage of total fatty acids. *t* = trace.

Table 4. Fatty acid composition of acylthioesters and lipids of developing soybean (*Glycine max*)

Fatty acids	Acyl-CoAs	Acyl-ACPs	Lipids
12:0	3.6	1.8	—
13:0	0.4	0.6	—
14:0	9.8	7.8	0.1
15:0	3.3	3.1	—
16:0	63.7	55.1	10.8
16:1	—	—	0.2
17:0	1.7	1.5	0.2
18:0	18.3	23.4	4.5
18:1	1.0	7.2	25.9
18:2	—	—	49.6
20:0	—	—	0.4
18:3	—	—	8.3

Results are expressed as percentage of total fatty acids.

leaves of barley, wheat and pea; Slack *et al.* [11] using cotyledons of soybean, linseed and safflower; and Stymne and Appelqvist [12] with microsomes from developing safflower seeds. According to this, the existence of 18:2-CoA would demand the presence of a phospholipase that liberates linoleic acid from PC and a thiokinase that binds it to CoA. Both enzymes are widely distributed in plant tissues.

However, Vijay and Stumpf [13, 14] using the microsomal fraction from developing safflower seeds found that oleyl-CoA was the specific substrate of oleate desaturase, and linoleyl-CoA the product of the reaction. This would justify by itself the existence of 18:2-CoA.

The fact that oleate desaturase is a membrane-bound enzyme makes its purification difficult; this, and the high activity of oleyl-CoA and linoleyl-CoA transferase, explains the confusion on this point.

What is really surprising is the presence of 18:2 in the acyl-ACPs. To justify this, one of these alternatives would have to be demonstrated: (a) a completely unusual desaturation in which the substrate were 18:1-ACP and the product 18:2-ACP; (b) starting with 18:2-CoA, the action of a linoleyl-CoA:ACP transacylase, analogous to that working on acetyl-CoA and malonyl-CoA, that has not been found, or a thioesterase–thiokinase system similar to that proposed by Shine *et al.* [6] but working back: 18:2-CoA → 18:2 → 18:2-ACP; (c) assuming that the true product of desaturation is a 18:2-PL, the pass of 18:2 to ACP would implicate the intervention of a phospholipase and an ACP specific thiokinase, acyl-ACP synthetase, similar to that demonstrated by Ray and Cronan [15] in *E. coli*.

Another interesting point is the absence of linolenic acid in both acylthioesters. The synthesis of this acid, the main component of membrane lipids of chloroplasts, has been the object of discussion for several years. Jacobson *et al.* [16] and Kannangara *et al.* [17] presented evidence suggesting that linolenic acid of photosynthetic tissues does not proceed from linoleic acid, and they proposed that the desaturations occur at the level of C_{12} fatty acids to give 12:3, which in turn is elongated to 18:3. On the other hand, Cherif *et al.* [18] demonstrated that the synthesis of 18:3 in leaf tissues occurs by successive desaturations at the level of C_{18} fatty acids. However, none of these papers gives evidence about the carrier or the substrate implicated. Our results suggest two possibilities: (a) 18:2-PC is desaturated to give 18:3-PC; (b) a linoleylthioester is desaturated to give the corresponding linolenylthioester which has a short life being transferred very fast to the lipids.

In summary, although analytical data about fatty acid composition of acylthioesters of a tissue do not permit definitive conclusions about the role of carriers in fatty acids metabolism, they constitute a basis on which the results of other experiments can be interpreted. Any proposed mechanism must be in agreement with that type of analytical data. On the other hand, the method of Mancha *et al.* [5] can be used as a preparative method to isolate and purify acyl-CoAs and acyl-ACPs to be used as substrate.

EXPERIMENTAL

Spinach leaves and potato tubers were from the local market. Soybean seeds were picked 30 days after flowering. Olives were picked at two different stages of maturation, 60 and 135 days after flowering. Young spinach leaves, which show the higher lipid synthesis, were selected. Thin slices (1 × 10 mm) excised from potato tubers were rinsed and aged by shaking at 25° in 0.1 mM $CaSO_4$ for 16 hr. Olives were used after the stones were removed. Soybean seeds were blended without previous treatment.

Separation of acylthioesters. Separation of acylthioesters was achieved by the method of ref. [5] modified according to the mass of biological material. The starting material (300–1000 g) was blended in the same vol. (spinach, potato), or double (soybean, olive), of mixture *iso*-PrOH–K-Pi buffer (0.1 M, pH 7.2) (1:1). The homogenate was filtered through cheesecloth and then centrifuged. The supernatant was extracted ×3 with *iso*-PrOH–H_2O satd petrol. The residue was extracted with $CHCl_3$–MeOH (2:1) as described in ref. [19]. Both extracts were mixed and named the oxygen ester fraction. Subsequently, 12.5 μl/ml of 18 M HOAc, 25 μl/ml of satd $(NH_4)_2SO_4$ soln and 4 ml/ml of $CHCl_3$–MeOH (1:2) were added to the defatted aq. phase, mixed and set aside for 20 min at room temp., and then centrifuged to pellet the salt–protein ppt. The acyl-ACPs were collected in the pellet, whilst the supernatant contained the acyl-CoAs contaminated with some of the more polar lipids. The supernatant was poured over a column containing 100 g neutral Al_2O_3 per l. of defatted soln. The column was rinsed with $CHCl_3$–MeOH (1:2) and, finally, the acyl-CoAs were eluted with 5 ml of mixture MeOH–K-Pi buffer (50 mM, pH 7.2) (1:1) per g of adsorbent.

Preparation of Me esters. The pellet containing the acyl-ACPs was redissolved in 1 vol. of 150 ml *iso*-PrOH–K-Pi buffer (50 mM, pH 7.2) (1:2) per l. of original defatted soln. After adding 40 ml KOH per 100 ml, the soln was saponified by heating for 30 min at 80°. Then it was cooled and acidified with 6 N HCl and free fatty acids extracted into petrol. To the aq. soln containing the acyl-CoAs, 25 ml 40% KOH per 100 ml were added, saponified and the free fatty acids extracted as before. The fatty acids so obtained, after the solvent was removed, were converted into the corresponding Me esters by heating at 80° for 5 min, in the presence of an excess of 4% HCl in MeOH. The Me esters were extracted into petrol, after dilution with H_2O and analysed by GLC.

Separation of oxygen esters. Esters from olives and potato were chromatographed on Si gel G TLC plates developing with petrol–Et_2O (9:1). Tri acylglycerols and polar lipids (origin) were viewed by exposing the edge to I_2 vapor, scraped off and methylated with 0.2 N Na methylate in MeOH and 4% HCl in MeOH successively, by heating at 80° for 5 min each time. Total oxygen esters from soybean and spinach were methylated as before, without previous separation. All Me esters were extracted into petrol, evapd and analysed by GLC in a glass column (0.27 × 200 cm) packed with 5% EGS isothermally at 170° using dual FID.

Chromatography on Si gel G–$AgNO_3$. An aliquot of Me/esters from each fraction was chromatographed on Si gel plates impregnated with 10% $AgNO_3$, developing with petrol–Et_2O (23:2). The bands, corresponding to Me esters of fatty acids of 0, 1, 2 and 3 double bonds, were viewed under UV after spraying with fluorescein, scraped off and eluted with Et_2O. All these fractions were analysed by GLC as previously described. This technique allows the separation of homologous series; thereby checking the nature of fatty acids. It also solves critical pairs such as 16:3–18:1 and 18:3–20:1 that cannot be separated by GLC on EGS columns.

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