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PARTIAL PURIFICATION AND SPECIFICITY OF TRIACYLGLYCEROL: STEROL ACYLTRANSFERASE FROM *SINAPIS ALBA*

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Key Word Index—*Sinapsis alba*; Cruciferae; steryl esters; triacylglycerol: sterol acyltransferase; enzymatic acyl transfer.

Abstract—Triacylglycerol:sterol acyltransferase is present in roots of *Sinapis alba* seedlings. The enzyme is located predominantly in the cell membrane structures sedimenting at 300–16 000 *g* but can be solubilized by acetone treatment and buffer extraction. During gel filtration on Sephadex G-100 the acyltransferase activity was separated into two peaks corresponding to MW 1.8×10^4 and MW $\geq 10^5$, respectively. A number of natural 3β-hydroxysterols can be esterified by the solubilized acyltransferase. The rate of esterification is much higher for sterols containing a planar ring system. The number and position of double bonds, as well as the structure of the side chain at C-17 of the sterol molecule, are of secondary importance. Triacylglycerols containing fatty acids C_6–C_{22} can be utilized as acyl donors. Among triacylglycerols containing saturated fatty acids, tripalmitoylglycerol ($C_{16:0}$) is the best acyl donor. For triacylglycerols containing C_{18}-fatty acids the following sequence was observed: trioleoylglycerol ($C_{18:1}$) > trilinoleoylglycerol ($C_{18:2}$) > trilinolenoylglycerol ($C_{18:3}$) > tristearoylglycerol ($C_{18:0}$).

INTRODUCTION

Part of the sterols present in plant cells occurs in the form of esters with long-chain fatty acids. The sterol and fatty acid composition of these esters may vary with the plant species, vegetation period [1, 2], and the kind of tissue [2–4] and subcellular structures from which steryl esters originate [3–5]. In some cases considerable quantitative differences in sterol composition between free and esterified sterol fractions [1, 2, 6–8], and essential differences in the fatty acid composition as compared with other acyl lipids isolated from the same tissue have been reported [6, 7]. These differences have been suggested to result possibly from the substrate specificity of the enzyme system catalysing sterol esterification [7].

At present it is assumed that the mechanism of steryl ester synthesis in higher plants differs from that operating in animals or fungi. *In vitro* experiments have shown that in plant tissues various acylglycerols can be efficiently utilized as acyl donors for sterol esterification [9–11].

In a previous paper [11] we have reported that crude cell-free preparations from *Sinapis alba* roots catalysed the acyl transfer from triacylglycerols to sterols. The present study was aimed at partial purification of this acyltransferase and closer characterization of the enzyme, in particular an examination of its substrate specificity.

RESULTS AND DISCUSSION

Partial purification of acyltransferase

In a previous paper [11] we have shown that the cell membrane fraction 300–16 000 *g* obtained from the roots of 7-day-old *Sinapis alba* seedlings contains an acyltransferase which catalyses sterol esterification utilizing triacylglycerols (to a lesser extent also di- and monoacylglycerols) as acyl sources. Repeated extraction of the crude membrane fraction with cold acetone afforded acyltransferase preparations ("acetone powders") almost completely free from endogenous acyl donors and acceptors, i.e. exhibiting the steryl ester synthesis ability only upon addition of exogenous sterol as acceptor and of acylglycerol as acyl donor.

In continuation of these studies we found that extraction of the acetone powder with 0.05 M Tris–maleate containing 0.5 M NaCl (see Experimental) affords solubilized acyltransferase preparation. As a result of extraction about 75% of the enzyme activity originally present in the acetone powder was obtained in a form not sedimenting at 105 000 *g* during 1 hr. The solubilized preparation showed fairly high stability; storage at 4° for 24 hr resulted in only a small (~10%) drop in the ability to catalyse sterol esterification.

During chromatography of the solubilized preparation on Sephadex G-100 (Fig. 1) the activity of triacylglycerol:sterol acyltransferase was eluted in two peaks, the first of them being eluted in the void volume of the column (MW $\geq 10^5$). For determination of the MW of the protein corresponding to the second peak, the column was calibrated with standard proteins: ribonuclease A, chymotrypsinogen, egg albumin and bovine albumin. Interpolation afforded a MW of 1.8×10^4 for this peak. Specific activity (per mg protein) of the pooled fractions corresponding to the peak with lower MW was about 12 times higher than that of the preparation applied onto the Sephadex G-100 column. The interrelations between both activity peaks are not clear. However, in some experiments involving molecular filtration on Sephadex it was observed that the relative intensities of both peaks were somewhat different from those shown in Fig. 1. This suggests that the fraction with higher MW may consist of the aggregated form of the enzyme with MW 1.8×10^4.

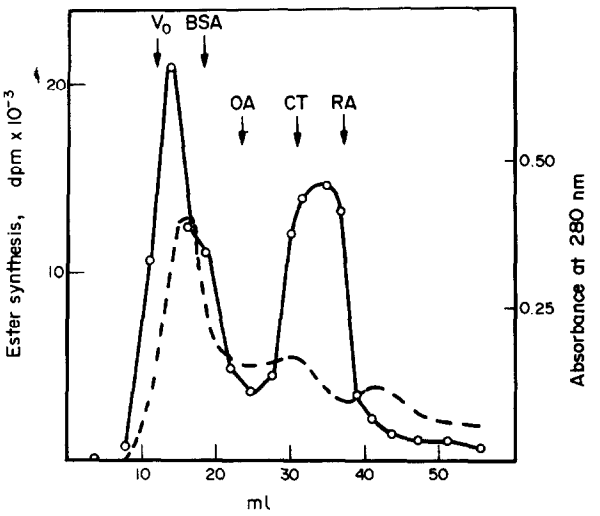

Fig. 1. Gel filtration of solubilized acyltransferase from *S. alba* roots on Sephadex G-100. Fractions of 2 ml were collected and assayed for protein content (—) and acyltransferase activity (○) with [4-^{14}C]cholesterol as acceptor and triglycerides from olive oil as acyl source. Arrows show elution volumes of blue dextran (V_0); bovine serum albumin (BSA); egg white albumin (OA); chymotrypsinogen (CT) and ribonuclease A (RA) used for column calibration.

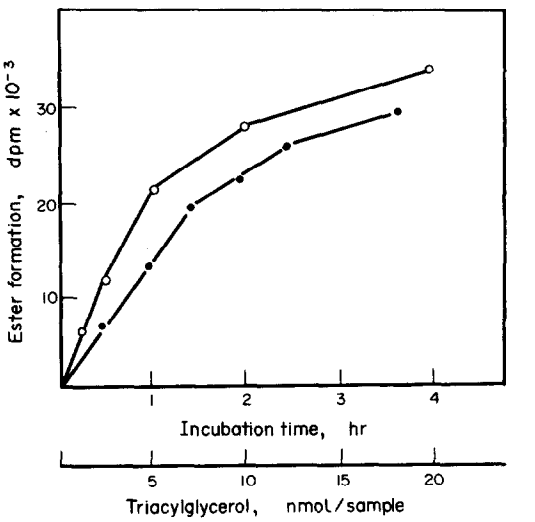

Fig. 2. Effect of incubation time (○) or triacylglycerol concentration (●) on [4-^{14}C]cholesterol esterification by solubilized enzyme preparation from *S. alba* roots. A mixture of triacylglycerols from olive oil (12.4 nmol/sample) was used as acyl donor. The incubations in the presence of increasing amounts of triacylglycerol were carried out for 30 min. For other details see Experimental.

Some properties of solubilized acyltransferase

The optimal conditions of the acyltransferase action *in vitro* were studied using the solubilized enzyme preparation. Incubations were carried out with [4-^{14}C]-cholesterol as acceptor and with a mixture of triacylglycerols from olive oil as acyl donor. It is stressed that in the absence of triacylglycerols the incorporation of labelled cholesterol into the material with polarity of steryl esters was very low (no more than 230 dpm per sample) and only slightly exceeded the control values obtained for complete incubation mixtures containing thermally inactivated enzyme preparation.

Acyltransferase exhibited high activity within a fairly wide pH range of 5.0–6.8 (0.05 M Tris–maleate buffer) with a slightly expressed activity maximum at pH 5.8. Optimal temperature was 52° (for incubations not exceeding 1 hr). Fig. 2 illustrates esterification of cholesterol as a function of the incubation time or triacylglycerol concentration in the incubation mixture. Under the experimental conditions used, the formation of cholesteryl ester was linear up to 1.5 hr. A linear increase in the esterification rate occurred also upon addition to the incubation mixtures of increasing amounts of the enzyme preparation up to about 0.1 mg protein per sample (Fig. 3). At higher concentrations of the enzyme preparation the reaction rate showed a distinct reduction.

Since both cholesterol and triacylglycerol were added to the incubation mixture as solutions in ethanol (or acetone), the effect of these solvents on the sterol esterification rate was studied (Fig. 4). The investigated acyltransferase exhibited relatively high tolerance with respect to both solvents. Esterification was maximal when the ethanol or acetone concentrations in the incubated sample amounted to 25 or 20%, respectively. The reason for the stimulation of sterol esterification by a relatively high concentration of these solvents is not clear. Possibly the solvents facilitate dispersion of the substrates in the incubation medium.

Reagents containing free –SH groups (2-mercaptoethanol, dithiothreitol) and divalent metals Mg^{2+} or Ca^{2+} had no stimulatory effect on the acyltransferase activity. Likewise, the presence of ATP and CoA hardly affected the esterification rate.

Specificity for fatty acids

The acyltransferase specificity for the transferred fatty acid was determined by incubation of the solubilized enzyme with [4-^{14}C]cholesterol and with various

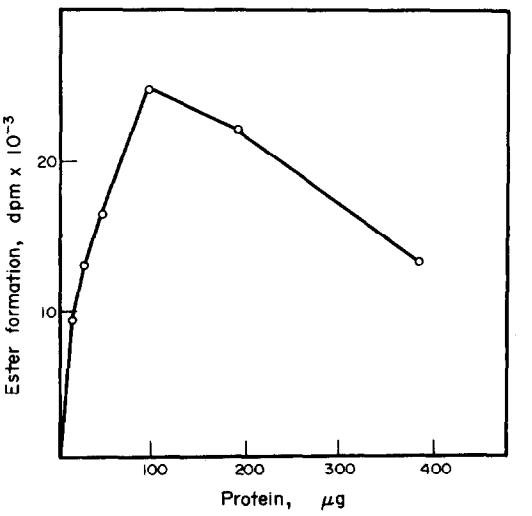

Fig. 3. Effect of enzyme concentration on [4-^{14}C]cholesterol esterification. Incubation time was 1 hr. A mixture of triacylglycerols from olive oil (12.4 nmol/sample) was used as acyl donor.

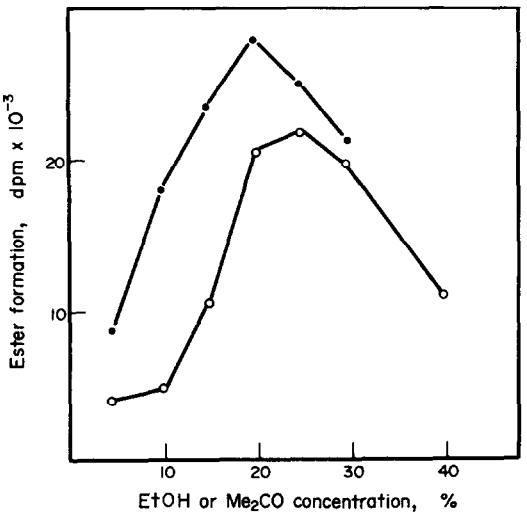

Fig. 4. Effect of ethanol (○) or acetone (●) concentration on the rate of [4-^{14}C]cholesterol esterification by solubilized enzyme from *S. alba* roots. Incubation conditions were as described in the legend to Fig. 2.

triacylglycerols containing fatty acids of different chain length or varying in the degree of unsaturation. Triacylglycerols with uniform acyl composition were used.

Table 1 shows the cholesterol esterification rate in the presence of triacylglycerols containing saturated fatty acids C_2–C_{22}. Tripalmitoylglycerol ($C_{16:0}$) was utilized at the highest rate. Both shortening and elongation of the carbon chains of fatty acids distinctly reduced the utilization rate of the respective triacylglycerols. Triacetylglycerol ($C_{2:0}$) and tribehenoylglycerol ($C_{22:0}$) were utilized at very low rate. It is stressed that in these experiments cholesterol esterification in the absence of triacylglycerol did not exceed 140 dpm per sample.

Table 2 summarizes the results of a similar experiment with triacylglycerols containing unsaturated fatty acids. It is evident that all these acylglycerols were utilized much

Table 1. Specificity of acyltransferase from *S. alba* roots for fatty acids of different chain length

Triacylglycerol added (12.4 nmol)	Esterification of [4-^{14}C]-cholesterol (dpm × 10^{-3})	(%)
Triacetylglycerol ($C_{2:0}$)	0.3	2
Tricaproylglycerol ($C_{6:0}$)	4.8	31
Tricaprylylglycerol ($C_{8:0}$)	7.1	46
Tricaprylglycerol ($C_{10:0}$)	8.1	52
Trilauroylglycerol ($C_{12:0}$)	8.4	54
Trimyristoylglycerol ($C_{14:0}$)	11.8	76
Tripalmitoylglycerol ($C_{16:0}$)	15.5	100
Trimargaroylglycerol ($C_{17:0}$)	11.2	72
Tristearoylglycerol ($C_{18:0}$)	5.6	36
Triarachidoylglycerol ($C_{20:0}$)	4.5	29
Tribehenoylglycerol ($C_{22:0}$)	1.4	9

Incubations were carried out for 30 min. Enzyme concentration 0.12 mg protein/sample. For other details see Experimental.

Table 2. Specificity of acyltransferase from *S. alba* roots for unsaturated fatty acids

Triacylglycerol added (12.4 nmol)	Esterification of [4-^{14}C]-cholesterol (dpm × 10^{-3})	(%)*
Tripalmitoleoylglycerol ($C_{16:1}$; Δ^9 *cis*)	23.2	150
Trioleoylglycerol ($C_{18:1}$; Δ^9 *cis*)	27.0	482
Trielaidoylglycerol ($C_{18:1}$; Δ^9 *trans*)	18.3	327
Trilinoleoylglycerol ($C_{18:2}$; Δ^9 *cis*, Δ^{12} *cis*)	12.5	224
Trilinolenoylglycerol ($C_{18:3}$; Δ^9 *cis*, Δ^{12} *cis*, Δ^{15} *cis*)	10.6	189
Trieicosenoylglycerol ($C_{20:1}$; Δ^{11} *cis*)	21.5	478
Trierucoylglycerol ($C_{22:1}$; Δ^{13} *cis*)	16.8	1200

Experimental conditions were as in Table 1.

*Esterification rate with triacylglycerol containing saturated fatty acid of the same chain length is taken as 100%.

more efficiently than triacylglycerols containing saturated fatty acids with the same chain length. This effect was particularly evident in the case of triacylglycerols containing acids with longer chains. For example, triacylglycerol containing erucic acid ($C_{22:1}$) was utilized at a greater rate (*ca* 12 times higher) than that containing behenic acid ($C_{22:0}$). For the series of triacylglycerols containing C_{18}-fatty acids with 1–3 double bonds, the order of utilization was: trioleoylglycerol ($C_{18:1}$) > trilinoleoylglycerol ($C_{18:2}$) > trilinolenoylglycerol ($C_{18:3}$). Configuration around the double bond (*cis* or *trans*) also seemed to influence significantly the reaction rate (cf. trioleoylglycerol and trielaidoylglycerol).

Radioactive esters obtained by incubation of the enzyme preparation with labelled cholesterol and with various triacylglycerols were additionally analysed by TLC on silica gel–silver nitrate under conditions permitting separation of the esters differing in the number of double bonds (see Experimental). Chromatograms were analysed by autoradiography. As expected, the labelled esters obtained by incubation with triacylglycerols containing fatty acids with different numbers of double bonds differed in their chromatographic mobility. For example, cholesteryl esters obtained by incubation with triacylglycerols containing stearic ($C_{18:0}$), oleic ($C_{18:1}$), linoleic ($C_{18:2}$) or linolenic ($C_{18:3}$) acid exhibited R_f values of 0.51, 0.36, 0.28 or 0.12, respectively, and they co-chromatographed with the appropriate synthetic cholesteryl esters. This proves that the acyl moiety undergoes no chemical changes during enzymic transfer from triacylglycerol to sterol.

Specificity for sterols

Acyltransferase specificity towards the acyl acceptor was determined with the use of another incubation variant, i.e. incubation with acyl-labelled tripalmitoylglycerol and with various unlabelled sterols or sterol-like substances as acceptors (Table 3). It is evident that when comparing the four fully saturated isomeric C_{27}-sterols

Table 3. Specificity of acyltransferase from *S. alba* roots towards sterol acceptors

Sterol added (25 nmol)	Formation of sterol-[$^{1\text{-}14}$C]palmitate (dpm × 10^{-3})	(%)
5α-Cholestan-3β-ol (cholestanol)	19.5	155
5α-Cholestan-3α-ol (epicholestanol)	0.0	0
5β-Cholestan-3β-ol (coprostanol)	3.0	24
5β-Cholestan-3α-ol (epicoprostanol)	0.0	0
Cholest-5-en-3β-ol (cholesterol)	12.6	100
24*R*-Methylcholest-5-en-3β-ol (campesterol)	15.0	119
Stigmast-5-en-3β-ol (sitosterol)	12.4	99
Stigmasta-5,22-dien-3β-ol (stigmasterol)	13.6	108
Cholesta-5,7-dien-3β-ol (7-dehydrocholesterol)	2.1	17
5α-Cholest-7-en-3β-ol (Δ^7-cholestanol)	0.7	5
Ergosta-5,7,22-trien-3β-ol (ergosterol)	1.1	9
5α-Androstan-3β-ol (androstanol)	18.4	146

Incubation mixtures contained solubilized enzyme (0.09 mg protein) and tri-[1-^{14}C]-palmitoylglycerol as acyl donor. Incubation was carried out for 30 min. For other details see Experimental.

(cholestanol, epicholestanol, coprostanol and epicoprostanol), differing in configuration of the hydroxyl group at C-3 and/or in the coupling of rings A and B (*cis* or *trans*), cholestanol (3β-OH, A/B *trans*) was utilized at the highest rate. Coprostanol (3β-OH, A/B *cis*) was utilized about 6 times slower, whereas epicholestanol (3α-OH, A/B *trans*) and epicoprostanol (3α-OH, A/B *cis*) failed to be esterified. These results clearly indicate that the β-configuration of the hydroxyl group at C-3 is absolutely required for the action of acyltransferase. Moreover, a distinct preference for sterols with a planar ring system (A/B *trans* or double bond at C-5) was observed. The presence and position of double bonds in the ring system to some extent influenced the esterification rate. This effect is evident when comparing the data for C_{27} sterols differing in the number (or position) of double bonds. These sterols were utilized in the following sequence: C_{27}, $\Delta^0 > C_{27}$, $\Delta^5 \gg C_{27}$, $\Delta^{5,7} > C_{27}$, Δ^7. The presence of a double bond at C-22 of the side chain only slightly changed the esterification rate (cf. sitosterol and stigmasterol). Likewise, the presence of additional alkyl groups in the side chain at C-24 had no important effect (cf. cholesterol, campesterol and sitosterol). The side chain does not seem to play any important role because androstanol, which does not contain a side chain, was utilized at a similar rate to cholestanol.

The present results clearly suggest that acyltransferase present in *S. alba* roots exhibits group specificity for natural sterols. It is well known that all plant sterols (except some of their metabolites) are 3β-hydroxysterols containing a planar ring system and only differ in the number (or position) of double bonds or in the structure of side chain. Moreover, we showed that triterpenes structurally resembling sterols such as lanosterol or β-amyrin failed to be esterified under the applied incubation conditions. In this light the investigated enzyme can be regarded as a specific triacylglycerol: sterol acyltransferase.

EXPERIMENTAL

Solubilized enzyme preparation. Roots of 7-day-old *S. alba* seedlings were used as plant material. The preparation of crude Me_2CO-precipitated enzyme from the 300–16000 *g* membrane fraction has been described in detail earlier [11]. Crude Me_2CO-precipitated enzyme (50 mg) was suspended in 0.05 M Tris–maleate buffer, pH 5.8, containing 0.5 M NaCl (100 ml). The suspension was stirred at 4° for 30 min and then centrifuged at 105000 *g* for 1 hr. The supernatant solubilized enzyme preparation contained about 75% of the triacylglycerol:sterol acyltransferase activity originally present in the crude Me_2CO-precipitated enzyme preparation. All operations were carried at 0–4°.

Gel filtration on Sephadex G-100. The solubilized enzyme preparation (2 ml, 0.8 mg protein) was applied to a Sephadex G-100 column (60 × 1 cm) equilibrated with 0.05 M Tris–maleate, pH 5.8, containing 0.5 M NaCl. Elution was carried out with the same buffer. Fractions of 2.0 ml were collected. Blue dextran; bovine ribonuclease A, MW 13800; bovine chymotrypsinogen, MW 25000; egg albumin, MW 45000 and bovine albumin, MW 67000 were used for column calibration.

Triacylglycerol:sterol acyltransferase assays. Variant A: The standard reaction mixture (total vol. 1.25 ml) contained 1.0 ml of solubilized enzyme preparation (usually 0.1 mg protein) in 0.05 M Tris–maleate, pH 5.8; [4-^{14}C]cholesterol (0.1 μCi, sp. act. 47 Ci/mol) and various unlabelled triacylglycerols (12.4 nmol). Cholesterol and triacylglycerol were added as EtOH soln (0.25 ml). Variant B: The incubation mixture was as above but various unlabelled sterols were added instead of labelled cholesterol, and unlabelled triacylglycerol was replaced by tri-[1-^{14}C]-palmitoylglycerol (0.4 μCi, sp. act. 21.3 Ci/mol). Incubations were carried out at 40°, usually for 15–60 min. The reaction was stopped by boiling with 1 ml MeOH. Radioactive steryl esters were extracted with $CHCl_3$, purified by TLC on Si gel in *n*-hexane–C_6H_6 (3:2) and the radioactivity measured as previously described [11].

Other methods. For characterization of [4-^{14}C]cholesterol esters formed in the presence of triacylglycerols containing fatty acids with different number of double bonds, TLC on Si gel–$AgNO_3$ (10%) was applied. Chromatograms were run in *n*-hexane–C_6H_6 (1:1). Protein was determined by the Lowry method [12].

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