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ENZYMATIC SYNTHESIS OF STERYL 6′-*O*-ACYL-β-D-GLUCOSIDES IN *SINAPIS ALBA* AND SOME OTHER PLANTS

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Key Word Index—*Sinapis alba*; Cruciferae; steryl glucosides; acylated steryl glucosides; biosynthesis; enzymatic transacylation.

Abstract—An acyltransferase which catalyses the acylation of [4-^{14}C]cholesteryl glucoside to form monoacyl steryl glucoside was found in particulate fractions of white mustard seedlings. Solubilized, relatively stable preparations of the enzyme were obtained and partially purified by gel filtration of Sephadex G-150. The acyltransferase has MW 130 000 and pH optimum 8.0. The solubilized preparations have an absolute requirement for exogenous acyl lipids as fatty acid sources for acylation. Galactosylglycerides (both MGDG and DGDG) and, at somewhat lower rate, phosphoglycerides (PE, PC and PS) are active in this process. The utilization of galactosyl- and phosphoglycerides is most probably catalysed by the same enzyme protein. Acyltransferase preparations from wheat roots, carrot roots and broad bean leaves obtained by the same method and assayed under identical incubation conditions show similar specificity patterns with respect to various acyl donors.

INTRODUCTION

Since the discovery of 6′-*O*-acyl derivatives of steryl β-D-glucosides (acylated steryl glucosides, ASG) in potato tubers, in 1964 [1], the occurrence of these steryl conjugates has been demonstrated in various tissues of numerous higher plants [2, 3] as well as in green [4], red and blue-green [5] algae. Their physiological role is unknown but apparently ASG, along with steryl glucosides (SG) and free sterols, are membrane components and, therefore, may be important in membrane functions [3, 6].

It has been found that various subcellular fractions from various plant organs convert labelled SG into ASG using endogenous acyl donors [4, 6–12]. In some cases a distinct stimulation of ASG formation in the presence of exogenous acyl lipids was demonstrated. This suggested that these acyl lipids were directly utilized for the acylation of SG as fatty acid sources. Such a mechanism of ASG biosynthesis was confirmed using some phospho- [6, 7] or galactosylglycerides [8, 9] labelled in the acyl groups. However, the data on the specificity of acyltransferases from different plants are contradictory. The enzyme from wheat roots [10, 11] and *Calendula officinalis* seedlings [7] catalysed acyl transfer from some phosphoglycerides, mainly phosphatidylethanolamine, but was unable to utilize fatty acids from galactosylglycerides. On the other hand, acyltransferase from broad bean leaves [8] utilized only galactosylglycerides while enzyme preparation from carrot roots [9] preferentially utilized galactosylglycerides but, to some extent, also phosphoglycerides. Contradictory results were also presented on some other properties of the acyltransferase from different sources, such as subcellular localization, optimum pH or effect of detergents on its activity. This led to suggestions [3, 8] that at least two types of the acyltransferase catalysing ASG formation are present in higher plants: a cytosol enzyme specific for galactosylglycerides and a particulate enzyme specific for phosphoglycerides. However, it cannot be excluded that the differences in the properties of the enzyme reported by various authors may be due to uncomparability of the conditions of enzyme isolation and activity assays.

We now report the isolation of solubilized and partly purified enzyme preparations from white mustard seedlings. The specificity of this acyltransferase for various acyl lipids as acyl donors was compared under identical incubation conditions with specificities of enzyme preparations obtained by the same method from broad bean leaves, carrot roots and wheat roots.

RESULTS AND DISCUSSION

When crude homogenates of white mustard seedlings in alkaline Tris–HCl buffers were incubated with [4-^{14}C]cholesteryl glucoside (labelled SG) the radioactivity was incorporated into a metabolite which migrated with the natural 6′-*O*-acyl steryl glucoside (ASG) fraction on TLC in several different solvent systems. Furthermore, when this metabolite was subjected to alkaline saponification all the radioactivity was recovered in the form of cholesteryl glucoside. After acid hydrolysis labelled cholesterol was quantitatively released.

The synthesis of ASG took place over a wide pH range 6.0–9.2 (0.1 M phosphate or Tris–HCl buffers). Below pH 7.0, a considerable hydrolysis of labelled SG was observed. This is consistent with our previous results [13] indicating that white mustard seedlings contain a glucosidase which can hydrolyse SG and which has pH optimum 5.2–5.6. Over pH 7.5 the sole labelled product of the reaction was ASG. At this pH the incorporation of SG into ASG was linear with time up to 4.5 hr. After this time SG was acylated in a yield of *ca* 25%. The enzyme is present mainly in cotyledons. In cotyledons of 5–9-day-old seedlings specific activity of ASG formation (per mg protein)

was 8–9 times higher than in roots. For 11- or 14-day-old seedlings the specific activity in cotyledons was, respectively, 23 or 68 times higher than in roots.

Successive centrifugation of the homogenate of cotyledons of 7-day-old plants at 300, 15 000 and 105 000 *g* demonstrated that almost all activity was present in particulate fractions. Only traces of the activity were found in cytosol (105 000 *g* supernatant). *Ca* 65–80% of total activity was present in the 15 000 *g* pellet and, subsequently, this fraction was used for attempts to solubilize the enzyme. It should be mentioned that in the experiments with crude subcellular fractions the synthesis of ASG took place in the absence of any exogenous acyl donors. The presence of an additional source of acyl groups in the incubation mixture (various phospho- or galactosylglycerides, as indicated in Table 1) failed to stimulate ASG formation.

Table 1. Effect of some acyl lipids on the acylation of [4-^{14}C]cholesteryl glucoside by acyltransferase from *S. alba* cotyledons

Acyl donor*	ASG formation (dpm × 10^{-3})
None	0.34
S. alba lipids:	
Crude lipid fraction	1.72
Neutral lipid fraction	0.40
Polar lipid fraction	2.88
Individual acyl lipids:	
Monogalactosyldiacylglycerol (bean leaves)	7.76
Monogalactosyldiacylglycerol†	0.22
Digalactosyldiacylglycerol (bean leaves)	5.06
Phosphatidylethanolamine (*E. coli*)	2.26
Phosphatidylethanolamine (dipalmitoyl-)	2.32
Phosphatidylcholine (egg yolk)	1.51
Phosphatidylserine (bovine brain)	1.37

*The following amounts of lipids were added: 40 μg/sample (lipid fractions) or 25 nmol/sample (individual acyl lipids).

†Boiled enzyme control.

Further experiments have shown that delipidation of the 300–15 000 *g* pellet with a large excess of cold acetone (see Experimental) with subsequent extraction of the residue with 0.1 M Tris–HCl buffer (pH 8.0) resulted in a large part of the activity (40–65% in parallel experiments) being found in a soluble form and not sedimenting at 105 000 *g* for 1 hr. In contrast to the acetone precipitated enzyme, which could be stored as dry powder, at −15°, for at least 2–3 weeks without appreciable loss of the activity, the solubilized enzyme was much less stable. It lost *ca* 60% of the activity during 24 hr. Both acetone precipitated enzyme and solubilized enzyme showed an absolute requirement for exogenous acyl lipids as acyl donors (see below).

The solubilized acyltransferase was considerably purified from other proteins present in the extract by gel filtration on Sephadex G-150 (Fig. 1). The enzyme was eluted as a single peak distinctly after the void volume. In order to determine the MW of the enzyme, the column was calibrated with blue dextran, rabbit aldolase (MW 147 000), bovine serum albumin (MW 67 000), egg albumin (MW 45 000) and cytochrome *c* (MW 12 400). For the acyltransferase a MW of *ca* 130 000 was obtained by interpolation. This value is about twice as high as the MW reported for acyltransferase from carrot roots [9], the only enzyme for which MW has been determined previously. Using the identical procedure as described in this paper for the isolation of acyltransferase from white mustard we obtained the solubilized preparation from carrot roots. Chromatography of this preparation on Sephadex G-150 gave an almost identical elution profile of acyltransferase activity. It seems, therefore, that white mustard cotyledons and carrot roots contain acyltransferases with similar molecular properties. It is possible that the peak with MW 130 000 observed for carrot and white mustard enzymes when our procedure of enzyme isolation is used, represents an active dimer of the enzyme with MW of *ca* 60 000 which was reported earlier for carrot roots [9].

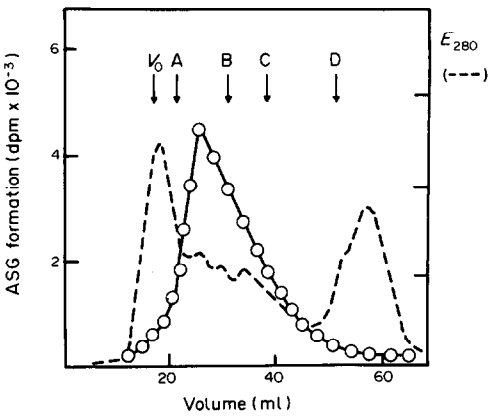

Fig. 1. Gel filtration of *S. alba* acyltransferase (○) on Sephadex G-150. Activity was assayed as described in the Experimental using crude polar lipids from *S. alba* (40 μg per sample) as acyl donor. V_0, void volume; A, B, C, D, elution volumes of rabbit aldolase, bovine serum albumin, egg albumin and cytochrome *c*, respectively.

The solubilized enzyme from white mustard showed optimum activity at pH 8.0. Low concentrations of Triton X-100 (up to 0.1%) somewhat stimulated the activity (15% at 0.05% concentration) but higher concentrations were strongly inhibitory. Ethanol, at concentrations up to 6%, had no effect on the rate of ASG formation and, therefore, both cholesteryl glucoside and acyl lipids were added to the incubation mixtures as solutions in ethanol.

As it has been pointed out earlier in studies with a solubilized enzyme preparation an addition of exogenous sources of acyl groups was necessary. The stimulation of ASG synthesis by various acyl lipids is shown in Table 1. The acylation of SG could be restored by the addition of crude lipids from white mustard seedlings (whole extract with chloroform–methanol, 2:1). The separation of these crude lipids into the neutral fraction, containing mainly tri- and diacylglycerols as well as wax and steryl esters (TLC analysis) and the polar fraction, containing mainly galactosyl- and phosphoglycerides, demonstrated that only the polar lipids are active in this process. The acylation of SG was also observed in the presence of some individual, pure lipids. The best results were obtained using monogalactosyl- and digalactosyldiacylglycerol

(MGDG and DGDG) but some phosphoglycerides also stimulated ASG formation. Among phosphoglycerides, phosphatidylethanolamine (PE) was definitely the most active. Phosphatidylcholine (PC) and phosphatidylserine were less efficient. Phosphatidic acid, 2-lyso-PE and 2-lyso-PC were completely inactive (data not shown). It is noteworthy that synthetic dipalmitoyl-PE and natural PE (*E. coli*) containing both saturated and unsaturated fatty acids stimulated ASG synthesis at similar rates. This suggests that the fatty acid composition is less important than the general structure of the acyl donor.

As pointed out in the introduction two different types of acyltransferase catalysing ASG synthesis have been described so far: (1) a cytosolic enzyme specific for galactosylglycerides as acyl donors; and (2) a particulate enzyme specific for phosphoglycerides (mainly PE). The former enzyme was isolated from broad bean leaves [8] and carrot roots [9], the latter from cotton fibers [6], wheat roots [10, 11] and *Calendula officinalis* seedlings [7]. In order to check if the ability of the enzyme preparation from white mustard cotyledons to catalyse the acylation of SG with utilization of both galactosyl- and phosphoglycerides was due to the presence of two different acyltransferases, the experiment was repeated employing gel chromatography on Sephadex G-150 using a longer (90 cm) column (Fig. 2). The acylation of labelled SG was measured in each fraction with the use of MGDG and PE as acyl donors. Overlapping peaks of enzymic activity were obtained. The activity ratio with MGDG and PE in individual fractions was constant and very similar to that shown by the enzyme preparation applied to the column. It appears, therefore, that the synthesis of ASG with both galactosyl- and phosphoglycerides as acyl donors is catalysed by the same enzyme protein.

Using the method described for the isolation of solubilized acyltransferase from white mustard cotyledons we were able to obtain active enzyme preparations from a number of other plants, among them from carrot roots, broad bean leaves and wheat roots. In all cases a very similar distribution of the enzyme activity between crude subcellular fractions was observed when compared with white mustard cotyledons. Almost all the activity was found in particulate fractions, especially in the 300–15 000 *g* pellet. The specificity of these preparations for acyl donors was compared with the specificity of the white mustard enzyme. The results of this experiment (Table 2) clearly indicate that all these preparations can utilize both galactosyl- and phosphoglycerides for SG acylation. In all cases the rate of ASG formation decreased in the following sequence: MGDG > DGDG > PE > PC.

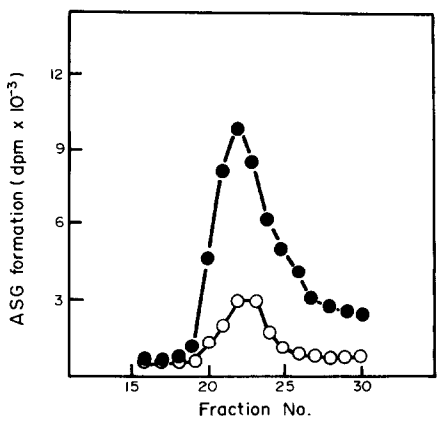

Fig. 2. Acylation of [4-^{14}C]cholesteryl glucoside in the presence of monogalactosyldiacylglycerol (●) or phosphatidylethanolamine (○) as acyl donor. Solubilized enzyme preparation was chromatographed on Sephadex G-150 column (90 × 1 cm). Each fraction was incubated with MGDG or PE (25 nmol/sample). Incubation time was 1 hr.

Table 2. Specificity of solubilized acyltransferase preparations from different plants for acyl donor

Source of the enzyme	Relative rates of ASG formation (%)*			
	MGDG	DGDG	PE	PC
White mustard (cotyledons)	100 (157.9)†	65	29	19
Wheat (roots)	100 (63.2)	57	24	12
Carrot (roots)	100 (351.6)	54	28	15
Broad bean (leaves)	100 (111.7)	61	32	10

*The rate of ASG formation with MGDG was taken as 100%.
†The reaction rates in nmol/mg protein·hr are given in parentheses. Sources of acyl lipids tested as indicated in Table 1. For other details see Experimental.

Our results do not confirm earlier suggestions [3, 8] that two types of acyltransferase catalysing ASG synthesis occur in high plants which differ in subcellular localization and specificity for acyl source. We were unable to detect a cytosolic form of the enzyme in any of the plants tested. Using our procedure for isolation of subcellular structures practically all the enzyme activity was found in particulate fractions. However, the enzyme seems to be bound rather weakly as it can be easily solubilized by acetone treatment and buffer extraction. It is possible that, under the slightly different conditions of homogenization used by other authors (different pH or different ionic strength of the buffer, presence of metal ions or sulphydryl reagents), a part of the enzyme may be washed from the cell structures.

Our results suggest that earlier controversy regarding the specificity of acyltransferases from various plants for galactosyl- or phosphoglycerides as acyl sources may be explained by the lack of comparable experimental conditions used by various authors for activity assays (different pH, different origin and purity of acyl lipids tested, different physical form of these lipids added to the incubation mixtures, etc.). Our results indicate that, at least in white mustard cotyledons, the acylation of SG in the presence of both galactosyl- and phosphoglycerides is catalysed by a single enzyme with MW 130 000.

EXPERIMENTAL

Plant material. 7-Day-old white mustard (*Sinapis alba*) seedlings were used in most expts. Seedlings were obtained by

germinating seeds on wet filter paper, at 20°, under illumination (16 hr/day). Some expts were also made with enzyme preparations from roots of 15-day-old wheat (*Triticum vulgare*) seedlings, leaves of 3-week-old broad bean (*Vicia faba*) or roots of mature carrot plants (*Daucus carrota*).

Enzyme preparations. Fresh plant material (30 g) was homogenized in a blender with 0.1 M Tris–HCl buffer, pH 7.3 (90 ml). After filtration through cheese-cloth the homogenate was successively centrifuged at 300 *g* (5 min) and 15000 *g* (20 min). The second pellet was suspended in a small amount of buffer (as above) and the suspension was added dropwise, with stirring, to a 20-fold amount of cold (−15°) Me_2CO. The ppt was collected by centrifugation (4000 *g*, 5 min), washed several times with cold, dry Me_2CO and dried *in vacuo*. Crude Me_2CO ppted enzyme was stable, at −15°, for at least 2 weeks. This preparation (60 mg) was suspended in 10 ml 0.1 M Tris–HCl, pH 8.0. The suspension was stirred at 4° for 30 min and then centrifuged at 10000 *g* for 30 min. The supernatant was applied to a Sephadex G-150 column (1 × 60 cm). Elution was carried out with the same buffer containing additionally 0.5 M NaCl. All operations were performed at 0–4°.

Acyltransferase assay. Incubations were carried out at 30°, usually for 30 min with the following assay mixtures: 1.0 ml enzyme preparation (0.05–0.2 mg protein) in 0.1 M Tris–HCl buffer, pH 8.0; [4-^{14}C]cholesterylglucoside (1.5 nmol, 10^5 dpm) and various acyl lipids as acyl donors (usually 25 nmol). Steryl glucoside and acyl lipids were added as solns in EtOH (0.045 ml). The reaction was stopped by the addition of 1 ml MeOH and 4 ml *n*-BuOH. The organic phase was washed with H_2O (3 × 4 ml) and evaporated to dryness. Radioactive ASG was separated from labelled SG by TLC on Si gel in $CHCl_3$–MeOH (9:1, R_f values for SG and ASG: 0.28 and 0.59, respectively). Autoradiography was used for localization of labelled compounds on the plates. The ASG spots were scraped off and eluted with $CHCl_3$–MeOH (1:4) directly into scintillation vials. Radioactivity was assayed as described previously [13].

Labelled SG. [4-^{14}C]Cholesteryl β-D-glucoside was prepared enzymatically by a modification of an analytical procedure described previously [14]. Crude Me_2CO ppted enzyme preparation containing UDPG: sterol glucosyltransferase was obtained from the 15000 *g* fraction of *Physarum polycephalum* microplasmodia according to ref. [14]. The incubation mixture contained in a total vol. of 35 ml: enzyme preparation (250 mg); 2.5 mmol Tris–HCl, pH 7.3; [4-^{14}C]cholesterol (100 μCi, sp. act. 47 mCi/mmol); UDPG diNa salt (70 mg); egg phosphatidylcholine (0.6 mg) and EtOH (1.15 ml). Cholesterol and phosphatidylcholine were added in EtOH. Incubations were carried out at 30° for 4 hr. After 2 hr a second portion of UDPG (70 mg) was added. Radioactive SG was extracted with $CHCl_3$–MeOH (2:1) and purified by repeated TLC on Si gel with $CHCl_3$–MeOH (9:1) as the solvent. Yield after TLC purification was 34%.

Other methods. SG and ASG, as reference substances, were obtained from *Calendula officinalis* leaves as described previously [7]. Galactosylglycerides were prepared from bean leaves according to Kates [15]. Lipids from white mustard seedlings were isolated and fractionated according to Lepage [1]. Protein was measured by the Lowry method [16] with bovine serum albumin as standard.

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