STERYL GLYCOSIDE ACYLTRANSFERASE FROM CARROTS

W. EICHENBERGER and H. P. SIEGRIST
Department of Organic Chemistry, University of Bern, Länggasstr. 7, Bern, Switzerland

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1. Introduction

Acylated steryl glycoside (ASG) of higher plants [1–4,8] is synthesized in two steps: a) glycosylation of a sterol and b) subsequent acylation of the steryl glycoside (SG) [5,6]. Enzymes catalyzing the two reactions in vitro have been found in seeds, seedlings and storage tissue of different plants [2,5,7]. The formation of ASG is worth investigating, because the acyl transfer is closely related to the lipid metabolism of the cell. In most cases, the enzyme activity was found to be associated with microsomal structures. A soluble enzyme was obtained in the high-speed supernatant from carrots [7] homogenized with water. A similar activity was found in wheat seedlings homogenized with 0.5% Triton X-100 [6].

The present work reports on the purification and characterization of the acyltransferase from carrots, which we call steryl glycoside acyltransferase (SGAT).

2. Materials and methods

2.1. *Enzyme preparation*

Fresh plants from the garden or from the local market were washed, peeled and homogenized for 2 min in a blender under N_2 at 4°C. After filtration through cheese-cloth the homogenate was precipitated with 20% and 60% saturated ammonium sulfate. The second precipitate was solubilized in 0.1 M Tris–HCl buffer, pH 8.2, and chromatographed on a Sephadex G-100 (3 × 62 cm) column. Protein was estimated in clear solutions according to Lowry [13], and in membraneous fractions by the Kjeldahl procedure [14].

2.2. *Enzyme assay*

The standard assay contained 250 µl 0.1 M citrate–phosphate buffer, 100 nM tritiated SG (^{3}SG), and 100 nM digalactosyl diglyceride (DG) (both solubilized in buffer by sonication) in a total vol of 700 µl; incubation was for 15 min at 25°C. The reaction was stopped by addition of 1 ml of chloroform–methanol 2:1 (v/v). After addition of 2 mg of leaf lipids as a carrier the lipids were extracted with diethyl ether and chromatographed on Silicagel G plates with chloroform–methanol 6:1 (v/v). The ASG spots were transferred to a scintillation vial, mixed with 4 ml methanol and 10 ml toluene, containing 0.7% PPO, and counted on a Packard Scintillation Counter.

2.3. *Tritiated steryl glycoside*

SG was isolated from dried potato tubers by extraction with chloroform-methanol 2:1 (v/v) and chromatography on silicic acid [15]; 57 mg were tritiated with 300 mCi $^3H_2$ (2.5 Ci/M) in 10 ml dioxane with Pd as a catalyst for 18 hours at 40°C. $^3H_2$ reduces the Δ^5-double bond of the sterol, which consist mainly of sitosterol [8]. The product, purified by TLC on Silicagel G with chloroform–methanol 6:1 (v/v) had a specific radioactivity of 138 Ci/M.

^{14}C-labeled Chlorella lipids were prepared by TLC on Silicagel G with chloroform-methanol-7 N ammonia 65:30:4 (v/v) [16]. The random distribution of label within the molecule was verified by saponification and by counting the label in the isolated fatty acids.

3. Results

In order to find out what plant is best suited for

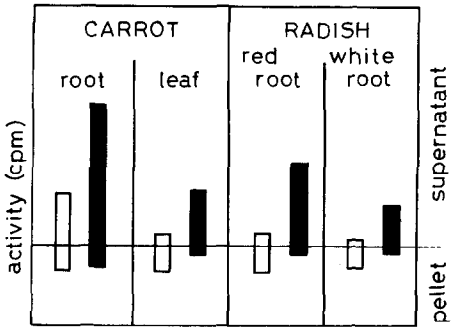

Fig.1. Steryl glycoside acyltransferase (SGAT) in different plants and plant organs. Homogenization in 0.1 M Tris–HCl, pH 8.2, with (■) and without (□) Triton X-100 (0.5%). Assay: 8 nM ^{3}SG (1.3 × 10^5 dpm), 100 nM DG, 0.1 M Tris–HCl, pH 8.2, total vol 700 µl, 15 min at 25°C.

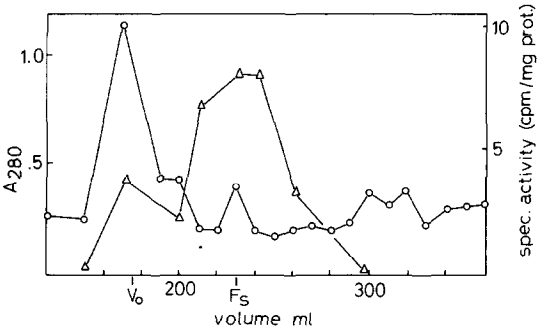

Fig.2. Gel filtration of SGAT on Sephadex G-100. Protein (○), specific activity (△), V_o = void volume.

the purification of the enzyme, the activity of acyltransferase has been estimated in different storage organs, since such tissues usually contain large amounts of ASG [1]. Roots of a red species of radish (*Raphanus sativus* L. ssp. radicula (Pers.) D.C. var. rotundus D.C.), a white species (*Raphanus sativus* L. ssp. niger (Mill.) D.C. var. albus D.C.) and carrot roots and leaves have been checked for enzyme activity. Equal amounts of fresh material were homogenized with 0.1 M Tris–HCl, pH 8.2, with and without the addition of Triton X-100. The homogenate was centrifuged for 60 min at 100 000 *g*. Supernatant and pellet were incubated with ^{3}SG and cold DG as an acyl donor. The radioactivity incorporated into ASG was measured.

Fig.1 shows the total activity for the species investigated. The activities of the two radish species and of the carrot leaves are located predominantely in the pellet. In carrot roots the activity is twice as high as in the other materials tested and concentrated in the supernatant, showing a differential distribution of the carrot enzyme from organ to organ. The addition of Triton to the medium generally increases the activity in the supernatant and lowers that in the pellet. The increase of total activity may be due to a better accessibility of the solubilized enzyme molecules to the substrate. Solubilization can also be accomplished by freezing and thawing. These preparations, however, show a rapid decline of activity with time. The enzyme was further purified by precipitating the homogenate with ammonium sulfate at 20 and 60% saturation. The second precipitate (F_A) was dissolved in Tris-buffer and fractionated by gel filtration.

Fig.2 shows, that the eluate contains one big protein peak eluting with the void volume and several minor peaks. However, assay of the different fractions showed, that the peak eluting at 230 ml (fraction F_S) had the highest specific activity. In order to determine the mol. wt, the protein was filtered on Sephadex G-100 calibrated with blue dextrane, bovine serum albumin, and trypsinogen. For the acyltransferase a molecular weight of approx. 60 000 was obtained by interpolation.

Fraction F_S produces two protein bands when subjected to electrophoresis on polyacrylamide gel. The enzyme activity was found mainly in the minor band (fraction 1), as shown in fig.3.

The influence of pH on the activity has been investigated by incubation of fraction F_S in citrate–phosphate buffer for the region of pH 2 to 7.6, and Tris HCl buffer for the region of pH 7.2 to 9. As shown

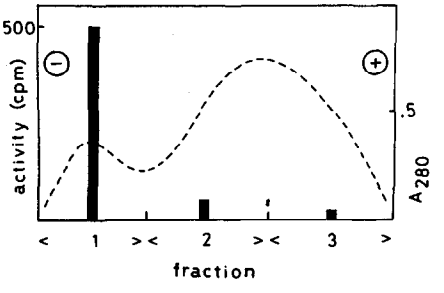

Fig.3. Gel electrophoresis of SGAT. 5% acrylamide, 0.1 M Tris–glycine, pH 8.2, 3 mA, 120 min. A_{280} (– – – –), cpm incorporated (■).

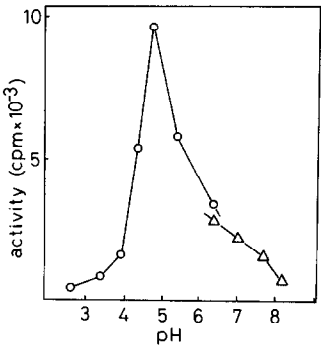

Fig.4. Influence of pH on the activity of SGAT. 0.1 M citrate–phosphate (○), 0.1 M Tris–HCl (△).

in fig.4, the pH optimum for enzyme activity is at pH 4.8.

The values of apparent Michaelis constants were determined as $1.43 \cdot 10^{-5}$ M^{-1} for SG and $1.92 \cdot 10^{-5}$ M^{-1} for DG (fig.5).

As to the origin of the acyl donor, it has been observed earlier [7], that glyco- and phosphoglycerides stimulate the acylation reaction in vitro. From this we concluded, that these lipids act as the acyl donors, although there was no direct proof of an acyl transfer. Therefore, the purified enzyme was incubated with a mixture of ^{3}SG and ^{14}C-labeled glycosyl glycerides (MG,DG) or phosphoglycerides (mixture of PC, PE and PG)* to produce doubly-labeled ASG. Based on

* *Abbreviations:* MG = monogalactosyl diglyceride; PC = phosphatidyl ethanolamine; PG = phosphatidyl glycerol.

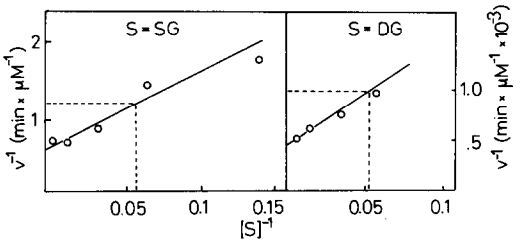

Fig.5. Apparent Michaelis constants of SGAT.

the specific radioactivity of substrates and on the label of both ^{3}H and ^{14}C in the product, the molar ratio of glucoside residue/acyl residue of the ASG could be calculated.

Table 1 shows, that experimental values are very close to the expected value of 1.0. Therefore, the acyl residues transferred to SG originate exclusively from the glycerides. As to the efficiency of different glycerides, MG is 8 times, the mixture of phospholipids 14 times less effective than DG.

In order to get further information on the mechanism of acyl transfer, the influence of CoA and ATP as cofactors was investigated. A dialyzed high-speed supernatant of the homogenate was used for incubation.

From table 2 we conclude that some formation of ASG occurs already with DG only. CoA stimulates the reaction only in presence of ATP. Palmitoyl-CoA has the same effect as DG, CoA and ATP in combination. In this case, addition of ATP has no effect. Free fatty acids are not accepted for the acylation.

Table 1
Incorporation in ASG of ^{3}SG and ^{14}C-labeled acyl groups from different glycerides* by purified SGAT from carrots. 200 nM ^{3}SG (0.03 mCi/mM), 200 nM acyl donor (DG, MG or phosphoglycerides), citrate–phosphate buffer 0.1 M, pH 4.8, total volume 700 μl, 15 min, 25°C

Acyl donor	Specific radioactivity mCi/mM acyl-	Radioactivity of ASG cpm ^{3}H	^{14}C	nM incorporated in ASG SG	acyl-	Molar ratio SG/acyl- exp.	theoret.	Efficiency of acyl donors DG = 1.0
^{14}C-DG	0.0052	2544	573	88.3	81.6	0.92	1.0	1.0
^{14}C-MG	0.022	374	386	12.9	10.4	0.80	1.0	0.12
^{14}C-phospho-glycerides	0.014	192	185	6.6	6.1	1.06	1.0	0.07

* Randomly labeled. Specific radioactivity of acyl residues calculated from stoichiometric composition.

Table 2
Influence of CoA and ATP on the activity of SGAT (standard assay)

	cpm incorporated
– DG	350
+ DG	1400
+ DG + CoA	1350
+ DG + CoA + ATP	6130
+ Palmitoyl-CoA	6650
+ Palmitoyl-CoA + ATP	5970
+ Palmitate	120
+ Palmitate + CoA	125
+ Palmitate + CoA + ATP	95

4. Discussion

The SGAT obtained from carrots is the first soluble enzyme of this kind which has been purified and characterized. Since it catalyzes the in vitro transfer of fatty acids from glycosyl and phosphoglycerides to SG, but neither the formation of acylated glycosylglycerides [9] nor sterol esters [17], we call it steryl glycoside acyltransferase (SGAT). We suggest that this enzyme is involved in the synthesis of ASG in vivo as well.

For the purified enzyme, a mol. wt of approx. 60 000 has been found. Its pH-optimum in citrate–phosphate buffer is 4.8, close to pH 4.5, the pH value of carrot homogenates.

Since the carrot lipids contain 63% neutral lipids, 21% glycosyl glycerides and 16% phosphoglycerides [10], several compounds may act as in vivo acyl donors. From a comparison of the fatty acid pattern of ASG to that of the total lipid, it was concluded, that acyl residues of ASG do not originate from galactosyl diglycerides, which are typical for chromatophores [11]. Our experiments, however, revealed DG to be the most effective acyl donor in vitro. On the other hand, Peaud-Lenoel et al. [6] found PE to be an efficient in vitro acyl donor for a similar enzyme from wheat seedling. Since these authors did not investigate the efficiency of galactolipids, their results can not directly be compared to ours. They also found that fatty acids were transferred more rapidly from an unsaturated lipid than from a saturated one [6]. We do not yet know whether the enzyme from carrots behaves similarly. In any case, its in vitro preference for DG can not be explained by a fatty acid specificity, since MG-being even more unsaturated [12] should accordingly be more effective than DG.

Since CoA and ATP stimulate the acyl transfer from DG and since acyl-CoA acts as an acyl donor, CoA-esters are suggested to be intermediates in the reaction. The acylation of SG by dialyzed enzyme preparations with DG alone, however, makes possible also a direct transfer, as observed to occur with lecithin-cholesterol-acyltransferase [17]. The mechanism of acyl transfer is being further investigated.

Acknowledgements

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