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SPECIFICITY OF STEROL-GLUCOSYLATING ENZYMES FROM *SINAPIS ALBA* AND *PHYSARUM POLYCEPHALUM*

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Summary

1. *Sinapis alba* L. seedlings contain glycosyltransferase catalyzing the synthesis of sterol glucosides in the presence of UDPglucose as sugar donor. The major activity occurs in the membranous fraction sedimenting at 300—9000 × *g*. Successive treatment of the particulate enzyme fraction with acetone and Triton X-100 affords a soluble glucosyltransferase preparation which can be partly purified by gel filtration on Sephadex G-150. Molecular weight of the glucosyltransferase is $1.4 \cdot 10^5$. Apparent K_m values for UDPglucose and sitosterol are $8.0 \cdot 10^{-5}$ M and $5.0 \cdot 10^{-6}$ M, respectively.

2. Comparison was made of the *S. alba* glucosyltransferase with a similar sterol-glucosylating enzyme isolated from non-photosynthesizing organism *Physarum polycephalum* (Myxomycetes). UDPglucose was the most efficient glucose donor in both cases but the enzyme from *Ph. polycephalum* can also utilize CDPglucose and TDPglucose. Glucose acceptors are, in case of both enzymes, sterols containing a β-OH group at C-3 and a planar ring system (5α-H or double bond at C-5). The number and position of double bonds in the ring system and in the side chain, as well as the presence of additional alkyl groups in the side chain at C-24 are of secondary importance.

3. The present results indicate that both enzymes can be regarded as specific UDPglucose:sterol glucosyltransferases. Certain differences in their specificity towards donors and acceptors of the glucosyl moiety suggest, however, a different structure of the active sites in both enzymes.

Introduction

Steryl β-D-monoglucosides, along with free sterols, are common constituents of the cell membranes of higher plants [1—3]. Biosynthesis of steryl glucosides in the presence of UDPglucose as a source of sugar has been observed in crude cell-free preparations from several higher plants [4—8]. It has been found that glucosyltransferase is tightly bound to cell membranes, occurring mainly in plasmalemma [9] and Golgi membranes [10,11]. Although the possibility of extraction of this enzyme with Triton X-100 from membrane fractions of cotton seeds [4]. *Calendula officinalis* seedlings [6] and wheat roots [5] has been reported, many molecular and catalytic properties of the enzyme have not yet been determined. Insufficient insight has been gained with respect to enzyme specificity, especially towards the glucosyl acceptor. It has been reported that sitosterol, stigmasterol, cholesterol and some other natural plant sterols can be glucosylated [4—6]. However, no systemic studies designed to determine the structural features of the acceptor, required for the enzymic action, have so far been carried out. Thus it remains unclear, whether it is justified to refer to the enzyme as specific UDPglucose:sterol glucosyltransferase, as it is done by some authors.

Recently we have reported [12] that cell-free preparations from non-photosynthesizing microorganism *Physarum polycephalum* (Myxomycetes) catalyze formation of steryl glucosides. Glucosyltransferase with a molecular weight of about $7 \cdot 10^4$ has been obtained in soluble form and was purified about 28 times. Many properties of this enzyme differ from the glucosyltransferase isolated from higher plants [4,6,8]. Among others glucosyltransferase from *Ph. polycephalum* is strongly inhibited by Triton X-100, shows no stimulation in the presence of Mg^{2+} or Ca^{2+} and is weakly membrane bound [12].

In this paper we report isolation and some properties of a solubilized glucosyltransferase from white mustard seedlings, as well as the results of detailed comparative studies on the specificity of this enzyme and of the glucosyltransferase isolated from *Ph. polycephalum*.

Materials and Methods

Nucleoside diphosphate derivatives of glucose were obtained from Calbiochem, glucose 1-phosphate from BDH, Triton X-100 from Serva.

The following sterols were used: coprostanol, epicoprostanol and campesterol were from Serva, epicholestanol from Steraloids, 7-dehydrocholesterol from BDH. Poriferasterol and fucosterol were kindly supplied by Dr. L.J. Goad, University of Liverpool. α-Spinasterol was isolated from a plant source as described earlier [13]. Other sterols were from Koch-Light. The purity of all sterols used in this work was at least 96% as demonstrated by GLC on SE-30 and OV-17. The commercial preparation of sitosterol contained about 8% impurities (mainly campesterol).

UDP[U-^{14}C]glucose, spec. act. 328 Ci/mol was obtained from The Radiochemical Centre, Amersham, and [4-^{14}C]cholesterol, spec. act. 47 Ci/mol, from W/O Isotop (U.S.S.R.).

Methods

Glucosyltransferase from Sinapis alba. Whole 7-day-old seedlings of white mustard (*S. alba* L.) were homogenized with 0.1 M Tris-HCl buffer, pH 7.3 (5 ml/1 g of plant material). The homogenate was squeezed through a cheesecloth and successively centrifuged at 300 × *g* (5 min) and 9000 × *g* (10 min). Organelles from the 9000 × *g* pellet were suspended in a small amount of buffer and added dropwise, with stirring, to a 20-fold amount of cold (−15° C) acetone. The precipitating protein was collected by centrifugation, washed twice with acetone and dried in a vacuum. Crude acetone precipitated enzyme (100 mg) was suspended in 0.1 M Tris-HCl buffer, pH 7.3, containing 0.3% Triton X-100 (5 ml). The suspension was stirred at 4° C for 1 h and then centrifuged at 105 000 × *g* for 1 h. The supernatant (3.6 mg protein) was applied on a Sephadex G-150 column (90 × 1 cm) equilibrated with 0.3% Triton X-100 in Tris-HCl buffer. Elution was carried out with 0.1 M Tris-HCl, pH 7.3, containing 0.3% Triton X-100 and 0.5 M NaCl.

Glucosyltransferase from Ph. polycephalum. Solubilization and partial purification of the glucosyltransferase from *Ph. polycephalum*, strain M_3C IV, microplasmodia has been described earlier [12].

Enzyme assay with labelled UPDglucose. The incubation mixtures contained in a total volume of 0.545 ml: enzyme preparation (0.5 ml); Tris-HCl, pH 7.3 (50 μmol); UDP[U-^{14}C]glucose (152 pmol, $1.1 \cdot 10^5$ dpm); sterol (25 nmol) and ethanol (40 μl). Sterol was added as a solution in ethanol. Incubation was carried out at 30° C usually for 30 min. The reaction was terminated by addition of 1 ml methanol and boiling for 3 min. Subsequently 4 ml of *n*-butanol was added and the butanol extract was washed with water (five times 3 ml). Aliquots of the butanol extract containing the radioactive steryl glucoside were taken for radioactivity measurement in a liquid scintillation spectrometer using PPO (3%) and POPOP (0.3%) in toluene as the scintillation fluid.

Enzyme assay with labelled cholesterol. The incubated samples contained in a total volume 1.14 ml: enzyme preparation (1 ml); Tris-HCl, pH 7.3 (100 μmol); nucleoside disphosphate derivative of glucose (164 nmol); [4-^{14}C]-cholesterol (10.6 nmol, $1.1 \cdot 10^6$ dpm) and ethanol (40 μl). After 1 h incubation at 30° C samples were extracted with *n*-butanol as above and the radioactive cholesteryl glucoside was separated by TLC on silica gel using chloroform/methanol (9 : 1, v/v) as the solvent. The separation was checked by autoradiography, steryl glucoside bands were scraped off and eluted with methanol, and the radioactivity was assayed as above.

Results and Discussion

Some properties of sterol-glucosylating enzyme from S. alba

Crude homogenate of white mustard seedlings catalyzes the incorporation of glucose from UDP[U-^{14}C]glucose into a substance with chromatographic properties of steryl monoglucoside. An addition of sitosterol to the incubation mixture increased labelling of this substance by about 30%. A radioactive product with identical chromatographic mobility was formed when the homogenate was incubated with [4-^{14}C]cholesterol in the presence of unabelled UDPglucose.

During differential centrifugation of the homogenate 85% of the investigated enzymic activity sedimented within the 300—9000 × *g* pellet. Most probably this pellet contains not only chloroplasts and mitochondria but also a wide variety of fragments derived from intact vesicles. The specific activity of this fraction was 6.5 times higher than that of the crude homogenate. Extraction of the 300—9000 × *g* pellet with cold acetone afforded a relatively stable enzymic preparation which could be stored in dry form for about 1 week, without any marked activity loss. This enzyme preparation with labelled UDPglucose and exogenous sitosterol showed enhanced synthesis of steryl glucoside, up to 12 times the synthesis with endogenous acceptors only. Glucosyltransferase was solubilized by extraction of the acetone powder with buffer containing 0.3% Triton X-100. A 1 h extraction gave about 65% of the activity in a form not sedimenting at 105 000 × *g* for 1 h.

Chromatography of the solubilized enzyme preparation on Sephadex G-150 afforded a single peak of glucosyltransferase activity, corresponding to molecular weight of approx. $1.40 \cdot 10^5$ (Fig. 1). This glucosyltransferase peak was free of endogenous acceptors and was completely dependent on an addition of sterol acceptor. The soluble preparation was unstable (a 60% loss of activity during 12 h).

Apparent Michaelis constants as deduced from Lineweaver-Burk plots were $8.0 \cdot 10^{-5}$ for UDPglucose and $5.0 \cdot 10^{-6}$ for sitosterol. The measurements were made as described in Table I with an exception of the substrate concentrations. Sitosterol, 46 μM and variable concentrations of UDPglucose (0.1—50 μM) were used for determination of the enzyme activity towards UDPglucose. The affinity for sitosterol was studied using 0.3 mM UDPglucose and variable con-

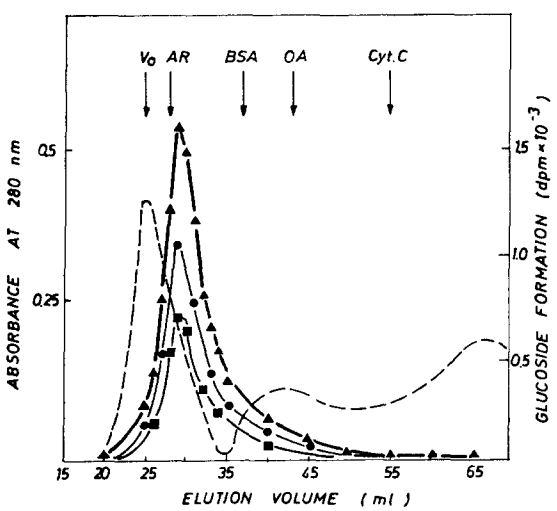

Fig. 1. Gel filtration of solubilized glucosyltransferase from *S. alba* seedlings on Sephadex G-150, 1 ml fractions were collected and assayed for protein content (- - - - - -) and glucosyltransferase activity with UDP[U-^{14}C]glucose as the sugar donor and sitosterol (▲), pregnenolone (●) or androstenolone (■) as the acceptors (for details see Materials and Methods). Arrows show elution volumes of blue dextran (V_0); rabbit aldolase (AR); bovine serum albumin (BSA); egg white albumin (OA) and cytochrome *c* (Cyt. *c*) used for column calibration.

centrations of sitosterol (0.2—10 μM). In both cases UDPglucose was labelled.

In contrast to the particulate enzyme preparations from pea seedlings [8] or wheat roots [5], the partially purified glucosyltransferase from white mustard failed to be stimulated by Mg^{2+} or Ca^{2+} within the concentration range of 10^{-2}—10^{-6} M. Zn^{2+} and Hg^{2+} exerted a strong inhibitory effect, 50% inhibition at $4 \cdot 10^{-4}$ M and $6 \cdot 10^{-6}$ M, respectively.

Comparison of the specificity of glucosyltransferase from S. alba and Ph. polycephalum

Previously [12] we have described isolation of solubilized, partially purified glucosyltransferase from non-photosynthesizing microorganism *Ph. polycephalum* (Myxomycetes) catalyzing glucosylation of cholesterol or sitosterol.

For comparison of the specificity of *Ph. polycephalum* and white mustard glucosyltransferase towards glucose donor solubilized preparations of both enzymes (0.12 or 0.19 mg protein, respectively) were incubated with various unlabelled glucose donors and [4-^{14}C]cholesterol as described in Materials and Methods. Glucosyltransferase from *S. alba* showed absolute specificity towards UDPglucose whereas the enzyme from *Ph. polycephalum* also utilized CDP-glucose and TDPglucose, though at an about six times slower rate. Glucose 1-phosphate, GDPglucose and ADPglucose were inactive with both enzymes.

For determination of the specificity with respect to the glucosyl moiety acceptor, incubations were carried out in the presence of UDP[U-^{14}C]glucose and appropriate unlabelled acceptors (Table I). Under incubation conditions described in Table I the formation of glucoside with sitosterol, cholesterol and pregnenolone as acceptors was proportional with time up to 45—60 min. Of the four fully saturated isomeric C_{27} sterols (cholestanol, epicholestanol, coprostanol and epicoprostanol), differing in configuration in the -OH group at C-3 and/or in the coupling of rings A and B (*trans* or *cis*), only cholestanol (5α-cholestan-3β-ol (A/B *trans*)) showed glucosylation by both enzymes. All 3β-OH sterols containing a double bond in position 5 are glucosylated. Since both the *trans* coupling of rings A and B (5α-sterols) and the presence of a double bond at C-5 are prerequisites for the planeness of the sterol ring system, it can be assumed that planar structure of the acceptor molecule is an indispensable condition for enzyme action. Thus the enzyme from *S. alba* and that from *Ph. polycephalum* can be regarded as specific sterol glucosyltransferases. All known natural sterols, except for some of their metabolites, are 3β-OH sterols with a planar ring system.

Both glucosyltransferases seem to exhibit a rather broad specificity pattern if a planar ring system and a 3β configuration of the -OH group are fulfilled. The number and position of double bonds, both in the ring system and in the side chain, as well as the presence of additional alkyl groups at C-24 exert an effect on the glucosylation. Of the C_{27} sterols the Δ^5 sterol (cholesterol) is most highly glucosylated by both enzymes. The $\Delta^{8,14}$ sterol was less highly glucosylated and the $\Delta^{5,7}$ conjugated diene (7-dehydrocholesterol) was least glucosylated. The Δ^5 and Δ^7 sterols showed the same specificity with *S. alba* glucosyltransferase but the enzyme from *Ph. polycephalum* showed a higher specificity for the Δ^5 sterol. The presence of an additional double bond in the side chain at C-22 (cf. sitosterol and stigmasterol) has no effect on the *S. alba*

TABLE I

SPECIFICITY OF THE PARTIALLY PURIFIED ENZYMES FROM *S. ALBA* AND *PH. POLYCEPHALUM* FOR STEROL ACCEPTORS

Complete assay system: UDP [U-^{14}C]glucose (152 pmol, $1.1 \cdot 10^5$ dpm); sterol acceptor (25 nmol); Tris-HCl (pH 7.3, 50 μmol); solubilized enzyme preparation from *S. albra* or *Ph polycephalum* (0.09 mg or 0.06 mg protein, respectively). Sterols were added in ethanol (0.04 ml). Total volume 0.5 ml; incubation 30 min at 30°C. Samples incubated with *S. alba* enzyme contained additionally Triton X-100 (final concentration 0.3%). Trivial names of sterols used are given in parentheses.

Sterol added	Glucoside formed (pmol/1 h)	
	S. alba	*Ph. polycephalum*
5α-Cholestan-3 β-ol (cholestanol)	11.6	2.7
5α-Cholestan-3 α-ol (epicholestanol)	0.0	0.0
5β-Cholestan-3 β-ol (coprostanol)	0.0	0.0
5β-Cholestan-3 α-ol (epicoprostanol)	0.0	0.0
Cholest-5-en-3β-ol (cholesterol)	28.4	10.8
5α-Cholest-7-en-3β-ol	27.0	4.5
5α-Cholesta-5,7-dien-3β-ol (7-dehydrocholesterol)	3.4	4.5
5α-Cholesta-8,14-dien-3β-ol	7.2	9.0
(24-*R*)-24-Methylcholest-5-en-3β-ol (campesterol)	71.7	39.6
Stigmast-5-en-3β-ol (sitosterol)	48.2	90.0
Stigmasta-5,22-dien-3β-ol (stigmasterol)	47.7	9.0
(24-R)-24-Ethylcholesta-5,22-dien-3β-ol (poriferasterol)	51.0	32.4
5α-Stigmasta-7,22-dien-3β-ol (α-spinasterol)	42.4	26.1
Stigmasta-5,*E*-24 (28)-dien-3β-ol (fucosterol)	38.6	69.3
Ergosta-5,7-22-trien-3β ol (ergosterol)	41.5	9.9
5α-Androstan-3β-ol	17.3	1.6
Androst-5-en-3β-ol-17-one (androstenolone)	20.7	0.0
Pregn-5-en-3β-ol-20-one (pregnenolone)	31.3	0.9

enzyme but lowers glucosylation by 90% with *Ph. polycephalum* preparation. A double bond at C-24(28) (cf. sitosterol and fucosterol) reduces the glucosylation almost equally with both enzyme preparations.

The effect of an alkyl group at C-24 can best be observed when cholesterol, campesterol and sitosterol are compared. Campesterol, a C_{28} sterol, was the best substrate for the glucosyltransferase from *S. alba*, however, the *Ph. polycephalum* enzyme prefered sitosterol. In general both enzymes best utilize the Δ^5 sterols alkylated at C-24. This may be interpreted as an adaptation of the enzyme to its natural substrates. *S. alba* mainly contain sitosterol and campesterol as well as small amounts of cholesterol and other sterols [14]. *Ph. polycephalum* contains a mixture of Δ^5 C_{27}—C_{29} sterols with a predominance of C_{29} sterols [15] mainly isomers of sitosterol and stigmasterol with an opposite configuration of the ethyl group at C-24, i.e. clionasterol and poriferasterol, respectively [16]. Poriferasterol is glucosylated by the enzyme from *Ph. polycephalum* 3.5 times more rapidly than its 24-epimer(stigmasterol), but no difference was found with the enzyme from *S. alba*.

Glucosylation of derivatives androstane and pregnane is catalyzed by the enzyme from *S. alba* and it appears to be the same glucosyltransferase (Fig. 1). Note the overlapping of the peaks of enzymic activity as measured with sitosterol, pregnenolone and androstenolone as glucose acceptors. The 3β-OH derivatives of androstane and pregnane are natural metabolites in a number of

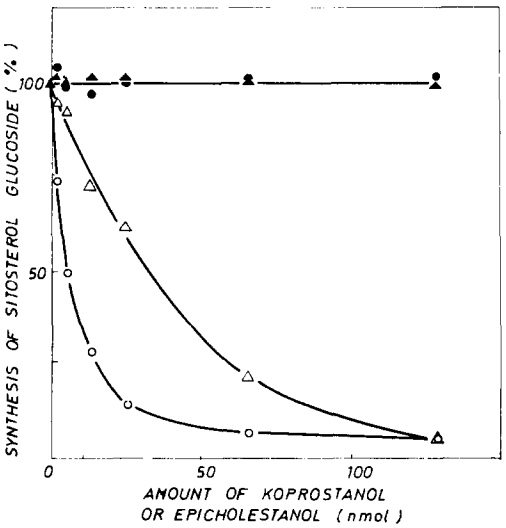

Fig. 2. Effect of coprostanol and epicholestanol on the glucosylation of sitosterol by solubilized glucosyltransferases from *S. alba* and *Ph. polycephalum*. Enzyme preparations were incubated with UDP[U-^{14}C]-glucose and sitosterol as described in Table I in the presence of increasing amounts of coprostanol or epicholestanol (0—130 nmol/sample). Enzyme from *S. alba:* ●, incubation with coprostanol; ▲, incubation with epicholestanol. Enzyme from *Ph. polycephalum:* ○, incubation with coprostanol; △, incubation with epicholestanol.

higher plants and in some cases they occur as glucoside [17]. Pregnenolone glucoside has been isolated from *Nerium odoratum* [18] and possibly the same glucosyltransferase catalyzes the glucosylation of sterols and 3β-OH pregnane and androstane derivatives.

The glucosyltransferase preparations from *S. alba* and *Ph. polycephalum* have many similarities, however they also show differences in their specificity pattern. Fig. 2 shows the effect of epicholestanol and coprostanol on the glucosylation of sitosterol in case of both glucosyltransferases. Coprostanol and epicholestanol undergo no glucosylation (Table I) but are potent inhibitors of sitosterol glucosylation by the enzyme from *Ph. polycephalum*, whereas they do not exert an effect on the reaction catalyzed by the enzyme from *S. alba*.

In summary our results clearly indicate that, irrespective of the possible differences in the structure of the active center, both *S. alba* and *Ph. polycephalum*, contain enzymes which can be classified as UDPglucose:sterol glucosyltransferases with a broad specificity pattern with respect to natural sterols.

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