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STERYL GLUCOSIDE BIOSYNTHESIS IN THE ALGA *PROTOTHECA ZOPFII*

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Key Word Index—*Prototheca zopfii*; Chlorophyta; algae; biosynthesis; steryl glucosides; UDP-Glc:sterol glucosyltransferase; Golgi apparatus.

Abstract—A particulate enzyme fraction from the Chlorophyta *Prototheca zopfii* catalysed the transfer of glucose-[U-^{14}C]from UDP-Glc-[U-^{14}C] to endogenous sterol acceptors and the esterification of steryl glucosides with fatty acids from an endogenous acyl donor. Glucose was the only sugar present, and it appeared to have the β-configuration. In the acylated derivatives the glucose-acyl linkage appeared in the C-6 position of glucose, as indicated by periodate oxidation. UDP-Glc:sterol glucosyltransferase was solubilized with detergent and purified 34-fold. The solubilized enzyme showed no specificity for the sterol but a high affinity for the sugar nucleotide UDP-Glc. Time-course incorporation into steryl glucoside (SG) and the acylderivative (ASG) indicated that SG was the precursor of ASG and that phosphatidyl ethanolamine stimulated the formation of the latter compound, presumably acting as acyl donor. A high sterol glucosylating activity was found in the Golgirich fraction. All this evidence indicates that steryl glucosides and their acylated derivatives were synthesized by algae. The early assumption that these compounds were not present in algae must be revised.

INTRODUCTION

Steryl glycosides (SG) and their acyl derivatives (ASG) have been isolated from many higher plant tissues [1], and they occur probably in all plants. Glucose is the sugar most often found attached to the 3β-hydroxyl position of the sterol, and the carbohydrate moiety can be esterified at the C-6 position with longchain fatty acids. Two pathways have been proposed for the synthesis of these compounds. The first poses the following sequence of reactions: free sterol → SG → ASG [2–6]. The second pathway would produce ASG through an unidentified steroid component that appears to be dominant in the *in vivo* tobacco system [7, 8]. The glucose donor for both pathways is UDP-Glc. The intracellular localization of the glucosyltransferase involved in steryl glucoside biosynthesis consistently indicates the Golgi apparatus as the site of synthesis [3, 9, 10].

It has been reported that one of the biochemical differences between algae and higher plants is that SG and ASG are present only in the latter [11]. In this paper we report the synthesis of SG and ASG by a cell-free system of the Chlorophyta *Prototheca zopfii*, the partial purification of the UDP-Glc:sterol glucosyltransferase and its intracellular localization.

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RESULTS AND DISCUSSION

Characterization

Membranes from *P. zopfii* were incubated with UDP-Glc-[U-^{14}C] and the glucolipids formed were extracted with *n*-BuOH. Several butanolic extracts were pooled and chromatographed on a DEAE-cellulose ($AcOO^-$ form) column equilibrated with $CHCl_3$ and eluted by increasing stepwise MeOH in $CHCl_3$ [12]. A single radioactive peak eluted with $CHCl_3$–MeOH (95:5) as do steryl glucosides. This material was submitted to TLC on Si gel G developed with solvent A, and two radioactive compounds were separated (R_f: 0.65 and 0.85). Both glucolipids gave a positive Liebermann–Burchard reaction indicating the presence of sterol. After alkaline treatment (1 hr, 0.1 N NaOH in $CHCl_3$–MeOH, 2:1, 37°) only one compound was detected with an R_f of 0.65. This indicated that a fatty acid had been removed, and the compound with R_f 0.85 was an acylated steryl glucoside.

When submitted to acid hydrolysis (20 hr, N H_2SO_4, 100°) radioactivity became water soluble. PC of the water phase in solvents E and G showed that glucose was the only radioactive compound. In order to determine whether glucose was linked in an α- or β-configuration, SG purified by TLC was incubated with α- and β-glucosidases. β-Glucosidase hydrolysed 69% of the SG but only 5% was cleaved by α-glucosidase, indicating that glucose was linked in a β-configuration. Another result supporting this assumption was provided by alkaline treatment of the SG (N NaOH in BuOH 99%, 65–68°, 90 min). Under these conditions 92% of the radioactivity became water soluble. PC of the water phase with solvent E showed that the product was a compound with identical mobility to 1,6-anhydroglucosan. The identity

Table 1. Periodate oxidation of SG and ASG

Products	Samples	
	SG cpm	ASG cpm
Original sample	200000	200 000
Glycerol	97000	95000
Erythritol	135	107
Sorbitol	89	93
R_f before cleavage	0.1	0.2
R_f after cleavage	0 2	0.5

Samples were submitted to periodate treatment, reduced, hydrolysed, and the products separated by PC with solvent F. The areas corresponding to the alditols were cut out and counted. Following periodate treatment aliquots were submitted to TLC on Si gel G with solvent B. The positions of radioactive bands were determined by counting Si gel sections.

of the glucosan was confirmed by acid hydrolysis (N HCl, 100°, 3 hr in sealed tube): it produced only radioactive glucose. This confirmed that glucose was linked in a β-configuration since α-derivatives do not form anhydroglucosan under the conditions employed [13].

In order to determine the position of the sugar-acyl linkage, samples of both SG and ASG were cleaved by periodate oxidation. Both samples were checked by TLC with solvent B before and after cleavage, in order to detect possible deacylation by the periodate treatment. In all cases the radioactivity migrated as a single band. As can be seen in Table 1, the acyl moiety was still intact after periodate cleavage, since if it had been lost both SG and ASG products would have had identical mobilities. Glycerol was the only radioactive product obtained by periodate oxidation (Table 1) of ASG, indicating that the C-2 or C-6 positions were the sites of acylation. Since the radioactive yield approached 50% rather than 100%, the data were consistent with the formation of one mole of glycerol per mole of glucose. This suggests that acylation occured at the C-6 position. However, the localization of the C-6 position rather than in the C-2 position might still be open to discussion.

Partial purification of the glucosyltransferase

UDP-Glc:sterol glucosyltransferase was solubilized from crude membranes by 0.6% Triton X-100. The solubilized enzyme was purified 34-fold by DEAE-cellulose and Sephadex G-100 column chromatography (Table 2).

Table 3. Incorporation of [^{14}C] sugars from different nucleotides into SG and ASG

Nucleotides	SG + ASG (pmol)
UDP-Glc-[U-^{14}C]	23.34
ADP-Glc-[U-^{14}C]	0.37
GDP-Glc-[U-^{14}C]	0.90
UDP-Ara-[U-^{14}C]	2
UDP-Gal-[U-^{14}C]	7.88
UDP-GlcNAc-[U-^{14}C]	0.5
UDP-Xil-[U-^{14}C]	0.19
GDP-Man-[U-^{14}C]	0.38

Incubations were carried out with the standard conditions, but different nucleotides were added in the place of UDP-Glc-[U-^{14}C]. Butanolic phases were analysed by ion exchange paper chromatography.

The incorporation rate of glucose from UDP-Glc into steryl glucosides was linear to about 2×10^{-5} M UDP-Glc, and showed a pH optimum of 6; similar results have been obtained with *Phaseolus aureus* [9]. Triton X-100 stimulated the transferase activity even in the absence of exogenous sterols; the optimum concentration was 0.6%. Several sugar nucleotides were tested for their ability to act as sugar donors for the SG synthesis. The results shown in Table 3 indicate that UDP-Glc was the best donor, as found in the higher plant systems [1]. 95% inhibition in SG formation was produced by 2 mM UDP.

The endogenous sterol was purified as described in the Experimental, and it was assayed for glucosylation as well as exogenous sterols (sitosterol and cholesterol). Fig. 1 shows that the enzyme was not specific for the sterol acceptor; nevertheless, when compared on an equimolecular basis, the native sterol seems a better substrate. This differential specificity for the sugar nucleotide and for the sterol seems to be a general feature in plants, since a similar situation occurs with cotton fibres [3].

Time-course formation of SG and ASG

Fig. 2 shows the time-course incorporation of glucose from UDP-Glc-[U-^{14}C] into SG and ASG. As shown in the inset, incorporation of radioactivity into total glucolipids was almost complete within 25–30 min. During the last 30 min a steady state in the formation of the SG was reached, while incorporation into ASG increased after an initial lag. This indicated a first incorporation of glucose-[U-^{14}C] into SG and subsequent conversion into ASG. In order to confirm that ASG was formed from SG, SG-[U-^{14}C] was purified by TLC and

Table 2. Purification of UDP-Glc:sterol glucosyltransferase from algae

Fraction	Vol. (ml)	Protein (mg)	Total activity (pkat)	Specific activity (pkat/mg)	Purification
Crude membranes	5	560	140	0.25	1
Triton X-100	5	68	31	0.45	1.8
DEAE-cellulose	5	7.2	6.7	0.93	3.7
$(NH_4)_2SO_4$ 20–60%	1	0.4	2.4	6	24
Sephadex G-100	1	0.2	1.7	8 5	34

Assays were carried out in standard conditions with sitosterol as substrate.

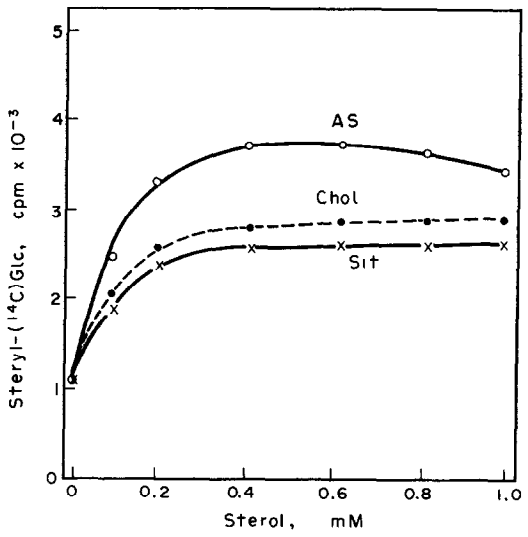

Fig. 1. Formation of SG from different sterols. Incubations were carried out as described using UDP-Glc-[U-^{14}C] as glucosyl donor and different sterols. AS: algal sterol; Chol: cholesterol; Sit: sitosterol. Protein concentration: 210 μg/tube. SG was separated from polar glucolipids by ion exchange PC.

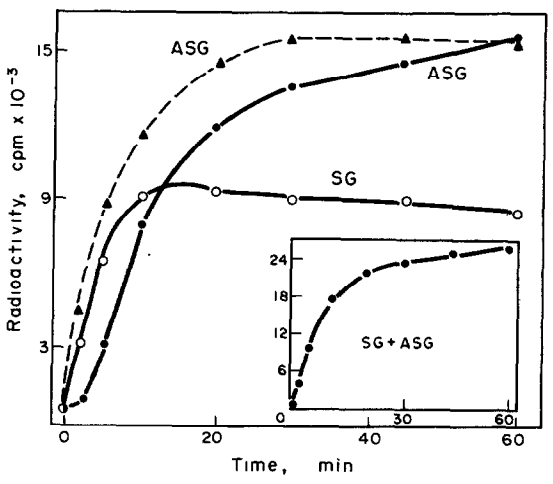

Fig. 2. Time-course formation of SG and ASG. Glucose was incorporated into SG (○——○) and ASG (●——●) from UDP-Glc-[U^{14}C] by particulate enzymes from algae. Incubations were carried out with the standard conditions using the endogenous sterols as glucose acceptors. SG-[U-^{14}C] was incorporated into ASG (▲- - -▲) when purified SG-[U-^{14}C] was the radioactive precursor. SG and ASG were separated by TLC and counted.

used as substrate. The results shown in Fig. 2 indicate that no lag period in the formation of ASG occurred when SG-[U^{14}C] was used as substrate.

It has been shown that lecithin stimulates the formation of $CHCl_3$–MeOH soluble products in cotton fibres [3]. Lecithin from soya was fractionated into different components by DEAE-cellulose column chromatography. The different fractions were assayed fror their ability to stimulate the formation of SG and ASG. Only the fraction eluted with $CHCl_3$–MeOH (3:2) stimulated the formation of glucolipids, the principal component of this fraction was found to be phosphatidyl ethanolamine (PE) as judged by TLC with solvent E. When the formation of both SG and ASG was assayed in the presence and absence of PE, it was found that the formation of SG was not changed, but the incorporation into ASG was stimulated 2.7-fold. These results are in agreement with those found in higher plants [3,4] indicating that the following reactions occur:

UDP-Glc + sterol → UDP + 3-steryl-β-D-glucoside (SG)

3-steryl-β-D-glucoside + PE → lyso PE + 3-steryl-(6-acyl-D-glucoside) (ASG)

Subcellular fractionation

Different membrane fractions from *P. zopfii* were separated by differential and discontinuous sucrose-gradient centrifugation. Table 4 shows the specific activities of the marker enzymes in different fractions. The highest specific activity for UDP-Glc:sterol glucosyltransferase was found in the Golgi-rich fraction. Similar results have been obtained for higher plants, such as cotton [3], mung bean [9], onion stem and *Calendula officinalis* [10].

From the results presented in this paper it can be

Table 4. Specific activities of marker enzymes in different subcellular fractions and synthesis of steryl glucosides

Fraction	Ubiquinone (mmol)	5'-Nucleotidase (pkat/mg)	Glc-6-Pase (nkat/mg)	Thiamine pyrophosphatase (nkat/mg)	UDP-Glc:sterol glucosyltransferase (pkat/mg)
Mitochondria	407	178.3	0.17	0.14	0.033
Endoplasmic reticulum	16	343.3	15.48	1.14	0.156
Plasma membrane	98	1336.6	0.43	0.47	0.24
Golgi apparatus	30	ND	0.19	3.36	0.606

Fractions were separated by differential and discontinuous sucrose-gradient centrifugation. Enzyme assays were made with 0.1 ml samples of different fractions except for steryl glucoside synthesis which was made with 30 μl samples and 0.5 mM sitosterol. Ubiquinone as detected by UV absorbance at 275 nm. ND: not detected.

concluded that *Prototheca zopfii* synthesizes SG and ASG. These compounds are similar to those found in higher plants and have the same sequence of biosynthetic reactions and site of synthesis. Even if we cannot say that SG and ASG are normal components of algal membranes, surely the earlier assumption that these compounds were not present in algae was not correct [11], and presumably this was due to insufficient observations.

EXPERIMENTAL

Chemicals. UDP-Glc-[U-^{14}C] (263 Ci/mol) and UDP-Gal-[U-^{14}C] (268 Ci/mol) were obtained from the Instituto de Investigaciones Bioquímicas, Fundación Campomar, Buenos Aires, Argentina. ADP-Glc-[U-^{14}C] (189 Ci/mol), GDP-Man-[U-^{14}C] (221 Ci/mol), UDP-GlcNAc-[U-^{14}C] (269 Ci/mol) (Amersham/Searle Corp.), GDP-Glc-[U-^{14}C] (203 Ci/mol (ICN), UDP-Ara-[U-^{14}C] (208 Ci/mol), UDP-Xil-[U-^{14}C] (213 Ci/mol) (NEN) were obtained commercially. Lecithin from soya (Sigma) was submitted to DEAE-cellulose column chromatography [12] and different fractions were collected and purified by TLC with solvent D. These fractions were assayed for their ability to stimulate the formation of SG and ASG. SG-[U-^{14}C] and ASG-[U-^{14}C] synthesized by the algae were purified by DEAE-cellulose column chromatography, followed by TLC with solvents C and B. Algal sterols were extracted with $CHCl_3$–MeOH (2:1) [14], saponified, precipitated with digitonin [15] and extracted with hexane.

Enzyme preparation and incubations. Crude membrane preparations of *Prototheca zopfii* strain PR-5 (ATCC 16533) were made as described [16] using a French press cell for disruption. The pellet obtained at 100000 *g* was used for glucosylation assays. UDP-Glc:sterol glucosyltransferase was solubilized from this 100000 *g* pellet with 0.6% Triton X-100, 50 mM Tris–HCl (pH 7.5), 5 mM β-mercaptoethanol and 1 mM EDTA, and centrifuged. The supernatant was passed through a DEAE-cellulose column (1.2 × 35 cm) and eluted with 50 ml 0.4M KCl. The active fractions were pooled, dialysed and submitted to $(NH_4)_2SO_4$ fractionation between 20 and 60% satn. The resuspended and dialysed ppt. was passed through a Sephadex G-100 (1 × 60 cm) column using the same buffer. Incubations were carried out at 25° for 30 min with the following mixture: 2.5 μmol HEPES (pH 6), 0.5 μmol β-mercaptoethanol, 0.4 nmol UDP-Glc-[U-^{14}C] (200000 cpm), 100–300 μg of protein in a final vol. of 50 μl. When exogenous sterols were added 0.6% Triton X-100 was incorporated into the incubation mixture. The reaction was stopped by the addition of 0.1 ml *n*-BuOH and the organic phase was submitted to PC on Whatman DE-20 paper with *n*-BuOH in order to separate the polar and neutral glucolipids. Radioactive SG and ASG were separated by TLC with solvent C. Subcellular fractions from algae were prepared as described [17]. The purity of different fractions was determined using marker enzymes. Plasma membrane was controlled by 5′-nucleotidase activity (EC 3.1.3.5), glucose-6-phosphatase (EC 3.1.3.9) was used as a marker for endoplasmic reticulum and thiamine pyrophosphatase (EC 3.1.4.-) was a marker for Golgi apparatus. These enzymes were measured as described [18]. Mitochondria were associated with ubiquinone content, detected by its UV spectrum at 275 nm [19]. SG-[U-^{14}C] purified by TLC was suspended in 0.1% Triton X-100 and shaken vigorously Then the following components were added: 10 μmol Tris–HCl (pH 6.8) and 100 μg α-glucosidase from yeast (EC 3.2.1.1) in a final vol. of 0.1 ml, and incubated 5 hr at 37°. When β-glucosidase from almonds (EC 3.2.1.4) was used, the same procedure was followed except for the buffer: 10 μmol Tris–AcOH (pH 5). The reaction was stopped by the addition of 0.1 ml *n*-BuOH and the phases were separated, washed and counted.

Analytical methods. The Liebermann–Burchard test was performed as described [20]; phosphate was determined according to ref. [21] and protein was measured by the Lowry method [22] with BSA as standard. Periodate oxidation was performed according to ref. [3] and the alditols were separated by PC with solvent F. Sterol was estimated as described [23]. Purification of lipids and glucolipids by DEAE-cellulose column chromatography (1.2 × 60 cm) was performed as described [12]. TLC was performed on Si gel G developed with the following solvents: solvent A: $CHCl_3$–MeOH–H_2O (85:25:4); solvent B: $CHCl_3$–MeOH–H_2O (95:5:0.2); solvent C: $CHCl_3$–MeOH–H_2O (65:25:4), and solvent D: $CHCl_3$–MeOH–AcOOH–H_2O (170:30:20:7). PC was carried out on Whatman No. 1 paper developed with: solvent E: *n*-BuOH–Py–H_2O (6:4:3); solvent F: *n*-BuOH–Py–N HCl (5:3:2); solvent G: *iso*-PrOH–AcOH–H_2O 29:4:3

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