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PURIFICATION AND PROPERTIES OF STEROL: UDPG GLUCOSYLTRANSFERASE IN CELL CULTURE OF *DIGITALIS PURPUREA**

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Key Word Index—*Digitalis purpurea*; Scrophulariaceae; cell culture; sterol: UDPG glucosyltransferase; glucosylation; particulate enzyme; phytosterol; epiandrosterone; sterol-glucoside.

Abstract—Sterol: UDPG glucosyltransferase was isolated for the first time from cell culture. *Digitalis purpurea* cultured cells had 2–5 times higher activity than that of the original plant. The enzyme in the particulate fraction was purified 70.2-fold from cell culture and 76-fold from the plant by cellular fractionation and column chromatography. The properties of purified enzyme from cultured cells were similar to those of the enzyme from the intact plant. The substrate specificity was the highest for a phytosterol.

INTRODUCTION

It was shown in a previous paper that *Digitalis purpurea* and *D. lanata* cell cultures were unable to biosynthesize the cardenolides which are produced by the original plant [1–3]. In order to investigate cardenolide production by the cell culture, we have examined the ability of *Digitalis purpurea* cell cultures to metabolize some intermediates of the cardenolide biosynthetic pathways [4, 5]. The results indicated that some cardenolide intermediates undergo stereospecific reduction of the C-3 and/or C-20 ketone to give either α- or β- hydroxyl groups and in the ring to 5α-H. This is followed by the glucosylation of each hydroxyl group or esterification with palmitic acid. The glucosides were formed in high yield and not metabolized by the following step in cardenolide biosynthesis. We now report on enzymological investigation of the glucosylation reaction using *Digitalis* cell culture and compare the results with those obtained with the original plant.

RESULTS AND DISCUSSION

The growth of the *Digitalis* cell culture reached the maximum on the third week, giving up to an 8–10 fold yield of the innoculum. The glucosyltransferase activity also increased from 5 to 8 fold in the same time and thereafter declined sharply. In each strain of the cell cultures, the enzyme activity was 2–5 times higher than that found in the original plant leaves.

The enzyme was stabilized by the addition of mercaptoethanol, EDTA and sucrose in a high concentration of Tris–HCl buffer. After homogenizing in the presence of these stabilizing reagents, the enzyme was isolated and purified by a cellular fractionation method. The enzyme of *Digitalis* leaves was distributed 58.5% in the sedimenting microsomal fraction at 105000 *g*; this is similar to the enzyme found in soybean seeds [6] and wheat root [7]. In the *Digitalis* cultured cells, the enzyme was not localized in a specific fraction, but it was found in the fraction obtained between 10000 *g* and 105000 *g* with cells grown under intenser illumination (up to 30000 lx). The most active fractions were collected, lyophilized and stored in a freezer. Each 3 *g* was collected from 1815 *g* (fr. wt) of the illuminated cell cultures and from 500 *g* (fr. wt) of the plant leaves. The enzyme activity (57–60%) was solubilized by 0.1% DOC (Na-deoxycholate) treatment in 0.1 M Tris–HCl buffer (pH 7.5) containing 10^{-2} M EDTA, 10^{-3} M mercaptoethanol and 0.25 M sucrose. After centrifuging at 105000 *g* for 1 hr, the supernatant, containing the solubilized enzyme, was concentrated with a collodion bag and subjected to the purification steps described in the Experimental. The procedure and yield in the purification of sterol: UDPG glucosyltransferase are summarized in Table 1.

By means of Sephadex G-100, DEAE-Sephadex A-50 and Sepharose 6B column chromatography, the enzymes were purified 70.2-fold from the cell cultures and 76.0-fold from the original plant leaves. Both the purified enzymes showed a single band on polyacrylamide disc electrophoresis. The purified glucosyltransferase had a pH optimum of *ca* pH 7.5 in both cultured cells and plant leaves, and was activated by EDTA, DIECA and mercaptoethanol and inhibited by NH_4^+, Zn^{2+} and Mn^{2+}.

The purified enzyme was incubated for 2 hr with [4-^{14}C]-epiandrosterone (1) as the glucose acceptor and cold UDPG as the glucose donor. The reaction product was identified as the epiandrosterone 3β-glucoside by comparison with an authentic sample by TLC on Si gel.

A number of sterol compounds were compared with epiandrosterone to determine the relative specificity of the sterol: UDPG glucosyltransferase. As shown in Table 2, the sterols having a double bond in the B-ring,

* Part 31 in the series "Studies on Plant Tissue Cultures". For Part 30, see Furuya, T., Nakano, M. and Yoshikawa, T. (1977) *Phytochemistry* **17**, 891. Part of this work was presented in the 5th Symposium for Plant Tissue Culture at Sendai, Japan (July 9, 1976).

Table 1. Purification of sterol: UDPG glucosyltransferase from *Digitalis* cell cultures and plant leaves

Purification step	Callus (fr. wt 1815 g)			Plant (fr. wt 500 g)		
	Total vol. (ml)	Protein (mg/ml)	Specific activity (units/mg)*	Total vol. (ml)	Protein (mg/ml)	Specific activity (units/mg)
250 *g* sup.	1159	3941	8.6	2280	7524	4.1
10000–105000 *g* fraction	(3 *g*)	51	125.9	(3 *g*)	370	37.8
Solubilized fraction	93	15	293.4	97	43.4	53.6
Sephadex G-100	40	5.3	513.7	35	13.4	110.4
DEAE-Sephadex	60	1.9	445.4	45	3.5	284.4
Sepharose 6B	20	0.9	601.1	25	2.2	313.3

* One enzyme unit is defined as the enzyme quantity which catalyses the formation of 1 nmol product per hr at 30° in the enzyme assay.

such as cholesterol, stigmasterol, sitosterol and pregnenolone (**2**), gave high specificity to the enzyme. Furthermore, the sterols having an A/B *trans* juncture, such as epiandrosterone (**1**) and 3β-hydroxy-5α-pregnan-20-one (**4**), showed higher specificity than those with an A/B *cis* juncture, such as 3β-hydroxy-5β-pregnan-20-one (**3**) and 3α-hydroxy-5β-pregnan-20-one (**5**). In the A/B *trans* sterols, epiandrosterone (**1**) with a 3β-hydroxyl group, showed higher affinity than that of androsterone (**6**) with a 3α-hydroxyl group, while in the A/B *cis* steroids, that with a 3α-hydroxyl group showed higher affinity than that having a 3β-hydroxyl group. The structure of digitoxigenin (**7**) in the A/B rings is the same as that of 3β-hydroxy-5β-pregnan-20-one (**3**), whose affinity was very low. Therefore, this fact suggests that the glucoside of digitoxigenin is not found in the *Digitalis* plant. The results on the substrate specificity of the various sterols corresponded well with the biotransformations observed in our earlier experiments [4, 5], except in the case of (**8**) [2]. Though the ability of *Digitalis* cell culture to biotransform epiandrosterone was not examined, the high activity of the enzyme may be presumed from the high biotransformation ratio observed in tobacco cell culture [8]. As expected, it was now demonstrated that the enzyme activity in *Digitalis* cell culture was in fact 8 times higher than that found in tobacco.

It was demonstrated that the enzymes of both *Digitalis* cell cultures and leaves were very similar in their properties. Therefore, it does not necessarily follow that the glucosyltransferase itself inhibits directly many steps on the biosynthetic pathway to cardenolides in *Digitalis* cell cultures. This could be due to incomplete compartmentation in the cultured cells rather than the inhibition by the glucosyltransferase. This was indicated by the fact that shoot redifferentiated from the cultured cells, had restored ability to biosynthesize a cardenolide [9].

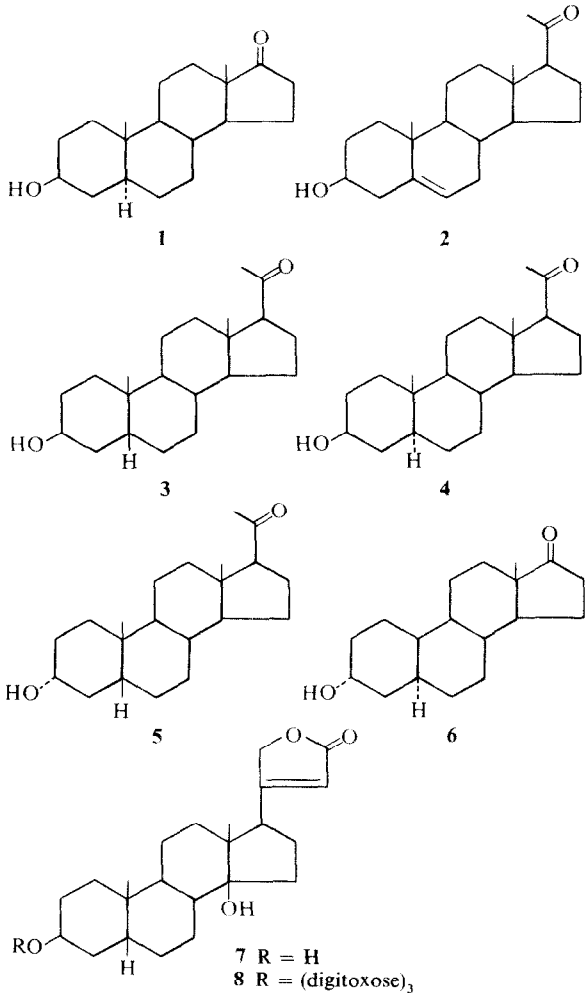

Table 2. Substrate specificity of sterol: UDPG glucosyltransferase from *Digitalis* cell cultures and plant leaves

Substrate	Callus	Plant	Substrate	Callus	Plant
Cholesterol	103.7	91.1	3β-Hydroxy-5β-pregnan-20-one (**3**)	3.3	1.0
Stigmasterol	122.4	108.4	3α-Hydroxy-5β-pregnan-20-one (**5**)	19.0	17.1
Sitosterol	88.2	87.4	3β-Hydroxy-5α-pregnan-20-one (**4**)	57.3	53.4
Pregnenolone (**2**)	57.4	72.6	Digitoxigenin (**2**)	2.2	2.6
Epiandrosterone (**1**)	100	100	Digitoxin (**8**)	5.6	3.2
Androsterone (**6**)	1.8	0.2	Testosterone	2.5	10.4

Values indicated the relative enzyme activity related to 100 for epiandrosterone as glucose acceptor. Reaction mixtures contained 0.2 μmol of UDPglucose U-[^{14}C] (0.005 μCi), 0.34 mmol of sterol substrate in 50 μl of 8% Triton X-100, and 90 μg protein of enzyme in callus and 220 μg in plant, respectively, in 100 μl of 0.1 M Tris–HCl buffer (pH 7.5).

EXPERIMENTAL

Cell cultures and plant materials. *Digitalis purpurea* cell culture was derived from plant leaves and subcultured for *ca* 2 yr. The callus was grown on the modified Murashige and Skoog's tobacco medium containing 1 ppm 2,4-D as auxin and 0.1 ppm kinetin as cytokinin and subcultured every 3 weeks. They were cultured in the dark and with illumination of *ca* 10000, 20000 and 30000 lx for 16 hr/day, respectively, at 26°. *Digitalis* plant leaves were collected at 6–10 month old and immediately frozen until used.

Enzyme preparation. 1815 *g* of the cultured cells and 500 g of plant leaves were suspended in 1815 ml or 1000 ml respectively of 0.1 M Tris–HCl buffer (pH 7.5) containing 10^{-3} M mercaptoethanol, 2×10^{-2} M EDTA and 0.25 M sucrose and homogenized with a Teflon homogenizer for 5 min. The homogenates were subjected to cell fractionation by centrifugation from 10000 *g* to 105000 *g*, and the particulate fractions obtained were lyophilized. Each 3 *g* was resuspended in the same buffer as above and then solubilized with 0.1% DOC for 1 hr. After centrifuging at 105000 *g* for 1 hr, the supernatant was concd with a collodion bag (Sartorius-membrane filter GmbH) to 5 ml and CC on Sephadex G-100 was carried out. The buffer used contained 10^{-3} M mercaptoethanol, 2×10^{-2} M EDTA and 0.25 M sucrose and all steps were at 4°. After dialysing against 0.02 M Tris–HCl buffer (pH 7.5), the fraction obtained from the Sephadex G-100 column was chromatographed with a discontinuous gradient of Tris–HCl buffer (pH 7.5) on a column of DEAE-Sephadex A-50 equilibrated with 0.02 M Tris–HCl buffer; 80 ml of fraction eluted with 0.5 M Tris–HCl buffer (pH 7.5) were concd with a collodion bag to 5 ml. The soln was then applied to a column of Sepharose 6B equilibrated with 0.1 M Tris–HCl buffer (pH 7.5). The fractions eluted subsequent to the first peak of protein were concd as above, dialysed against 0.05 M Tris–HCl buffer (pH 7.5) and finally lyophilized and stored at −20°.

Enzyme assay. Reaction mixtures contained 10 μl 23.8 mCi/mmol [U-^{14}C]-UDPG (0.001 μCi), 3.4 mmol epiandrosterone in 50 μl 8% Triton X-100, 50 μl enzyme soln and 100 μl 0.1 M Tris–HCl buffer (pH 7.5) containing 10^{-3} M mercaptoethanol and 10^{-2} M EDTA, in a total vol. of 210 μl. The reaction was run at 30° and stopped after 2 hr by the addition of 2 ml $CHCl_3$–MeOH (4:1). The organic extracts obtained were evapd under red. pres. to dryness in vials and 10 ml scintillation fluid was added. Then the radioactivity was counted by liquid scintillation spectrometer. The scintillation fluid consisted of 4 *g* DPO (2.5-diphenyloxazole) and 0.5 *g* of POPOP (1,4-bis 2′-(5′-phenyloxazolyl)-benzene) per l. of toluene. It was confirmed that 95% of [4-^{14}C]-epiandrosterone-3β-glucoside was recovered by this extraction method.

Identification of reaction product. After the enzyme was incubated for 2 hr with [4-^{14}C]-epiandrosterone and UDPG, the reaction mixture was extracted with $CHCl_3$–MeOH (4:1). The reaction product was identified by comparison with an authentic sample by TLC on Si gel with 2 solvent systems of C_6H_6–Me_2CO (4:1) R_f = 0.01 and $CHCl_3$–MeOH (7:1) R_f = 0.51. The radioactivity on TLC was measured by a TLC scanner.

Polyacrylamide gel disc electrophoresis. Disc electrophoresis on polyacrylamide gel was carried out according to Ornstein and Davis's method [10].

Protein measurement. Protein contents were measured by the Lowry method [11] or by absorption at 280 nm.

Chemicals. UDP [U-^{14}C]-glucose (238 mCi/mmol) was purchased from New England Nuclear. Unlabelled UDP glucose (Na-salt) was from Sigma. [4-^{14}C]-Epiandrosterone-3β-glucoside was obtained by the biotransformation from [4-^{14}C]-testosterone (Daiichi Pure Chemicals) using tobacco callus by Furuya's procedure [8]. [4-^{14}C]-Epiandrosterone was obtained by acid hydrolysis with 1 N HCl of the glucoside. The other steroids were obtained from the commercial products.

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