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UDP-glucose:digitoxin 16′-O-glucosyltransferase from suspension-cultured *Digitalis lanata* cells

Wolfgang Kreis, Ursula May, and Ernst Reinhard

Pharmazeutisches Institut (Pharmazeutische Biologie) der Universität Tübingen, Auf der Morgenstelle 8, D-7400 Tübingen, FRG

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ABSTRACT

Suspension cultures from several cell lines of Digitalis lanata, as well as cultures from 6 other plant species were checked for their ability to form purpurea-glycoside A from digitoxin. An in-vitro assay for the UDP-glucose: digitoxin 16′-O-glucosyltransferase (DGT, EC 2.4.1.-) has been established based on an HPLC method. The enzyme is located in the soluble fraction. Its pH optimum is at 7.4. No enzyme activity was found in either purified vacuole preparations or lysed vacuoles. Ascorbate (10 mM) increased the transferase activity about 4-fold. Of the sugar nucleotides tested, only UDP-glucose served as a glucosyl donor. Digitoxin, digoxin, α-acetyldigitoxin, and α-acetyldigoxin are substrates for the glucosyltransferase. The role of the DGT during the biotransformation of cardenolides in Digitalis lanata cell suspension cultures is discussed.

INTRODUCTION

Cell cultures of Digitalis lanata modify exogenous cardenolides (Reinhard et al. 1975, Heins et al. 1978, Döller and Reinhard 1979). Upon the addition of various cardiac glycosides to the culture medium different reactions occur, including glucosylation, acetylation, deacetylation, and 12ß-hydroxylation. Among these reactions, the 12ß-hydroxylation of A-glycosides to the corresponding C-glycosides is of special interest, since these 12ß-hydroxylated cardenolides (e.g. digoxin) are of greater pharmaceutical relevance than the respective deoxy compounds.

However, attempts to use digitoxin (produced during the technical isolation of digoxin from Digitalis lanata plants) as the substrate for hydroxylation have failed so far. Most of the digitoxin administered to suspension-cultured foxglove cells is rapidly glucosylated at the 16′-position of the sugar chain (fig. 1) and purpurea-glycoside A is formed. Upon prolonged incubation, all of the digitoxin administered to the cultured cells is biotransformed to a series of primary glycosides, such as the lanatosides A and C, purpurea-glycoside A, and deacetyl-lanatoside C. Obviously, glucosylation is the most prominent biotransformation reaction and may be regarded as a competitive reaction to the hydroxylation.

As a continuation of our attempts to optimize the 12ß-hydroxylation process with cultured Digitalis cells we decided to characterize and localize the formation of purpurea-glycoside A from digitoxin. We here report that the glucosylation of secondary glycosides is catalysed by a UDP-glucose-requiring glucosyltransferase (DGT, EC 2.4.1.-). In addition we present evidence that this reaction takes place in the cytosol, rather than in the vacuolar space of cultured Digitalis lanata cells.

SECONDARY GLYCOSIDES	R^1	R^2	R^3
Digitoxin	H	H	H
Digoxin	H	H	OH
α-Acetyldigitoxin	H	$COCH_3$	H
α-Acetyldigoxin	H	$COCH_3$	OH

PRIMARY GLYCOSIDES	R^1	R^2	R^3
Purpurea-glycoside A	Glucosyl	H	H
Deacetyl-lanatoside C	Glucosyl	H	OH
Lanatoside A	Glucosyl	$COCH_3$	H
Lanatoside C	Glucosyl	$COCH_3$	OH

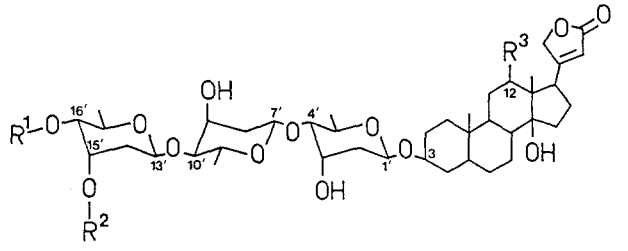

Fig.1. Conversion of secondary cardenolides to their corresponding primary cardenolides in D. lanata cell suspension cultures.

MATERIALS AND METHODS

Cell suspension cultures: Suspension cultures were established from D. lanata callus (Heins 1978) and routinely subcultured every 10.5 d in a modified Murashige and Skoog (1962) medium as described (Kreis and Reinhard 1985a). the cultures were grown under either dark or light conditions (continuous fluorescent light Osram L 18W/77 Fluora, approx. 2000 lx).

Protoplasts and vacuoles: Protoplasts were prepared from cultured cells by incubation for 1.5 h in a modified Murashige and Skoog medium containing 0.5 M mannitol, 1% desalted Cellulase RS Onozuka (Yakult Honsha Co., Japan) and 0.3% Rhozyme HP 150 (Rhom and Haas Co., USA). Vacuoles were released from the protoplasts by osmotic shock treatment at pH 8 in the presence of EDTA. The vacuoles were then purified by floatation through a three-step Ficoll gradient. Details of protoplast and vacuole isol-

Offprint requests to: W. Kreis

ation have been described in a previous publication (Kreis and Reinhard 1985a).

In-vivo assay for glucosyltransferase activity: 2 g (fresh weight) of cells in 20 ml conditioned culture medium were incubated with digitoxin (100 mg/l) under culture conditions (24°C, 120rpm). After 6 h the cells were separated from the medium by filtration through paper. The wet cells were suctioned dry after which 400 mg of the dried material were mixed with 800 µl of methanol, sonically lysed and extracted for 5 min at 40°C (Bandelin Sonorex RK 510). After vortexing (30 s) the cell residues were pelleted by centrifugation (15000 x g, 15 min) and the supernatant analysed by HPLC for its cardenolide content.

In-vitro assay for the glucosyltransferase: Cells were frozen with liquid nitrogen and then extracted with 2 volumes of 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM ascorbate. The enzyme activity was assayed in a final volume of 500 µl containing 18 mM UDP-glucose and 780 µM digitoxin (stock solution 10 mg/ml in DMSO). After 3h of incubation at 37°C the reaction was stopped by adding 500 µl of methanol. The precipitate was spun down and the supernatant analysed by HPLC for the purpurea-glycoside A formed.

Protein was determined according to Bradford (1976) with bovine serum albumin as the standard.

High performance liquid chromatography (HPLC): The cell extracts (or the cell-free incubation mixtures) were analysed for their cardenolide content with a Hewlett Packard 1084 B Liquid Chromatograph using a combination of a 40 x 4.6 mm column and a 250 x 4.6 mm column, both filled with Nucleosil 5 C 18 (Macherey and Nagel, F R G) as the stationary phase. The cardenolides were eluted with a three-step acetonitrile-water gradient (fig.2). The products of the glucosyl transfer were identified by their retention times and quantified on the basis of their extinction coefficients at 220 nm. In addition their identity was checked by thin layer chromatography on silica gel (chloroform: methanol: water = 80: 18: 2).

Lanatoside A was purchased from Serva AG, FRG. ß-Methyldigoxin was produced via hydroxylation of ß-methyldigitoxin in fermenter cultures of Digitalis lanata (Heins et al. 1978). Purpurea-glycoside A was prepared by biotransformation of digitoxin with the D. lanata cell line K 1 OHL (Details will be published elsewhere). All other cardiac glycosides were generous gifts from Boehringer Mannheim AG, FRG.

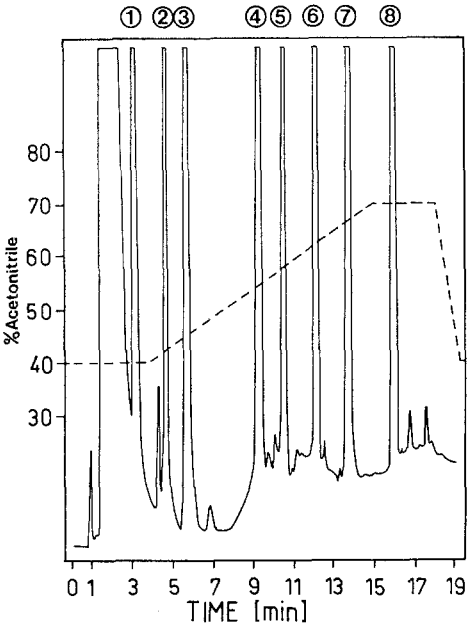

Fig.2. HPLC separation of substrates and products of the UDP-glucose:digitoxin 16'-O-glucosyltransferase. (1) deacetyl-lanatoside C, (2) lanatoside C, (3) digoxin, (4) ∝-acetyldigoxin, (5) purpurea-glycoside A, (6) lanatoside A, (7) digitoxin, (8) ∝-acetyldigitoxin. The dotted line indicates the percentage of acetonitrile (84%) in the liquid phase. HPLC parameters: flow rate 2 ml / min, oven temp. 40°C, temp. solvent A (water) 50°C, temp. solvent B (acetonitrile) 30°C. Detection at 220 nm, injection volume 40 µl. Computing integrator: Hewlett Packard 79850 B LC Terminal.

RESULTS

1. Glucosylation in-vivo

In a first set of experiments we checked several D. lanata cell lines for their ability to convert digitoxin into purpurea-glycoside A (tab. 1). We found considerable variation among them, not only in terms of their glucosylation capacity but also with regard to their 12 ß-hydroxylation rate. Lines with low hydroxylation capacity showed the highest glucosylation rates in-vivo.

In addition, culture strains derived from 6 other plant species were tested for their ability to glucosylate digitoxin to purpurea-glycoside A (tab. 1). As expected, the cultured Berberis and Hyoscyamus cells were not able to perform this biotransformation step. Cell cultures of Thevetia neriifolia, which is a cardenolide-producing plant, also failed to form purpurea-glycoside A from digitoxin. The 3

Tab.1. In-vivo 16'-O-glucosylation and 12ß-hydroxylation rates of various cell suspension cultures.

Cell culture	Glucosylation[a]	Hydroxylation[b]
D. lanata line		
K 1 HD	65	0
K 1 OHD	63	0
K 1 OHL	29	0
K 2 HD	4	16
K 2 OHD	13	11
K 2 OHL	3	1
K 3 HD	3	18
K 3 OHD	2	11
K 3 OHL	3	14
K 4 HD	4	10
K 4 OHD	4	7
K 4 OHL	24	6
D. heywoodii	22	n.d.[c]
D. purpurea	16	n.d.
D. lutea	8	n.d.
Berberis wilsonae	0	n.d.
Hyoscyamus muticus	0	n.d.
Thevetia neriifolia	0	n.d.

[a] Glucosylation rates are expressed in terms of µmol purpurea-glycoside A · kg^{-1} fresh weight · h^{-1} formed in the standard in-vivo assay.

[b] Hydroxylation rates were determined by incubating 15 g cells (fresh weight) with ß-methyldigitoxin (40 mg/l) for 48 h. The culture medium was extracted and analysed for its ß-methyldigoxin content. The activities are calculated as µmol ß-methyldigoxin · kg^{-1} fresh weight · h^{-1}.

[c] n.d. = not determined.

Digitalis species, on the other hand, showed considerable glucosylation rates in the respective in-vivo experiments.

The changes during a cell-culture cycle of in-vivo glucosylation of digitoxin were examined in 2 different cell strains, cultivated either under dim light or in the dark (fig. 3). In the case of the strain which glucosylated well, glucosyl transfer was optimal during the logarithmic growth phase (i.e. Days 4-8 of subculture). No such clear optimum was found for the cell strain with low glucosylation capacity. Significant differences were found between dark or light-grown cell lines of common origin (i.e. derived from the same culture strain) in terms of their glucosyltransferase activity in-vivo.

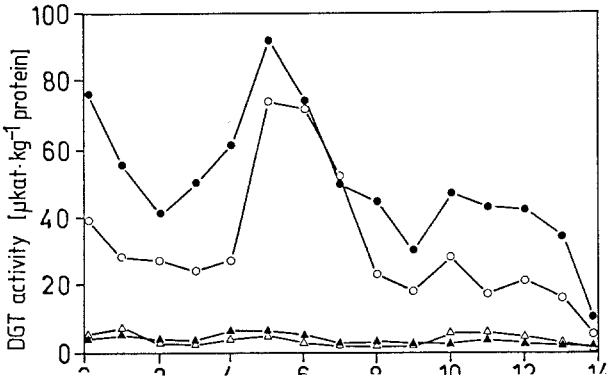

Fig.3. Time course of in-vivo glucosyltransferase activity. 2 different D. lanata cell strains were monitored (●-●/o-o strain K 1, ▲-▲/△-△ strain K 3). Light symbols indicate the light-grown cell lines (K 1 OHL, K 3 OHL), shaded symbols the dark-grown lines (K 1 OHD, K 3 OHD).

2. Glucosyltransferase activity in-vitro

The glucosyltransferase activity could be recovered in 15000 x g supernatants of extracts from cultured D. lanata cells. As expected, the extractable specific enzyme activity was much higher in the non-hydroxylating cell lines than in those hydroxylating well (fig. 4). Cell line K 1 OHL (cultured under light) showed the highest activity in the in-vitro assay. Therefore this line was used in further investigations into the properties of the glucosyltransferase.

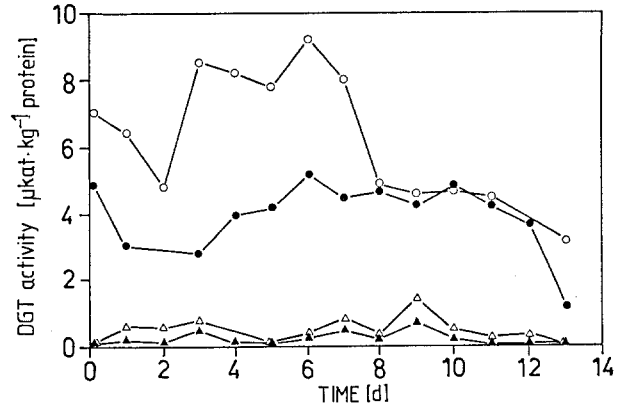

Fig.4. Time course of UDP-glucose:digitoxin 16'-O-glucosyltransferase activity in-vitro. The cell lines K 1 OHL, K 1 OHD, K 3 OHL, and K 3 OHD were monitored. (Symbols are explained in the legend of fig.3).

It should be added that, with the assay described, DGT activity was also detected in cell-free extracts of other Digitalis cell cultures, such as D. heywoodii, D. purpurea, and D. lutea (data not shown).

The glucosyltransferase described requires two substrates, a secondary cardiac glycoside and a sugar nucleotide. Of the five sugar nucleotides tested only UDP-glucose served as a glucosyl donor, whereas CDP-glucose, GDP-glucose, ADP-glucose, and TDP-glucose, as well as glucose-6-phosphate failed to show any activity. The K_M value for UDP-glucose, as calculated from a double reciprocal plot, was 5 mM.

The glucosylation rate in-vitro was linear up to 3 hours (at 37°C) and then decreased rapidly. No loss of activity was observed when storing the crude extracts in the presence or absence of 10% glycerol for 4 weeks at -20°C.

Optimal transferase activity occured at pH 7.4, with half maximal activities at pH 7.1 and 7.7, respectively. The addition of ascorbate or other reducing agents to the extraction buffer resulted in a considerable increase in the transferase activity. The effects, alone or in combination, of ascorbate, mercaptoethanol, and dithiothreitol are summarized in tab. 2.

Of the cardenolides tested digitoxin was the best substrate for the glucosyltransferase (tab.3) which we therefore propose to be referred to as a UDP-glucose:digitoxin 16'-O-glucosyltransferase (EC 2.4.1.-; DGT). The relative activities for the respective substrates as well as their K_M values, which were taken from Lineweaver-Burk plots, are summarized in tab.3.

Tab.2. The effects of ascorbate, mercaptoethanol, and dithiothreitol on the glucosyltransferase activity in-vitro.

Compound	Concentration (mM)	Relative DGT activity (%)
Control	–	100
Dithiothreitol	1	182
	10	264
Mercaptoethanol	1	209
	10	295
Ascorbate	2	254
	10	455
	50	659
Ascorbate (10 mM) + dithiothreitol	1	623
	10	669

Tab.3. K_M values for substrates of UDP-glucose:digitoxin glucosyltransferase.

Substrate	Relative activity[a] (%)	K_M (µM)
Digitoxin	100	110
Digoxin	71	410
α-Acetyldigitoxin	21	540
α-Acetyldigoxin	18	810

[a] The amount (in terms of nM) of purpurea-glycoside A formed in the standard assay was set to equal 100 %. The relative DGT activities for the other substrates were calculated on the basis of the amounts (nM) of the corresponding primary glycosides formed in a standard assay with the appropriate substrate. The K_M values were taken from Lineweaver-Burk plots.

3. Subcellular localization of the DGT.

Only a little DGT activity was found in the 15000 x g pellet. Upon centrifugating the 15000 x g supernatant for 1 h (100000 x g) the DGT activity was recovered in the supernatant and no enzyme activity could be detected in the microsomal pellet.

Protoplasts as well as crude protoplast lysates containing intact vacuoles showed considerable DGT activity, whereas no activity was detected in purified vacuole preparations or lysed vacuoles even after prolonged incubation (up to 10 h) with digitoxin and UDP-glucose. Thus we concluded that, in suspension-cultured D. lanata cells, the DGT is a cytosolic enzyme.

DISCUSSION

The biotransformation of cardenolides by suspension-cultured D. lanata cells has been studied for many years. However, nothing was known about the enzymes involved in these reactions. Recently, Petersen and Seitz (1985) reported on a cytochrome P-450 dependent monooxygenase which catalyses the 12 ß-hydroxylation of methyldigitoxin to methyldigoxin in cell-free extracts of D. lanata cell cultures.

The UDP-glucose:digitoxin 16'-O-glucosyltransferase described in our present report catalyses the formation of primary cardiac glycosides from secondary ones. The properties of the DGT found in the extracts of D. lanata cells are at some variance with those of a UDP-glucose-dependent digitoxin glucosyltransferase in the leaf homogenates of D. purpurea (Franz and Meier 1969). The pH optimum was at pH 6.8 and the K_M for digitoxin 240 uM (as calculated from the data presented). This discrepancy with our data may be partially due to the fact that, in the case of D. purpurea leaf extracts, high levels of digipurpidase are present (Stoll et al. 1935). This specific glucosidase hydrolyses primary glycosides to their corresponding secondary glycosides. Although quite a bit of unspecific ß-glucosidase is present in suspension-cultured foxglove cells (Kreis and Reinhard 1985b), a specific glucosidase ("digilanidase") was detected neither in-vivo nor in-vitro (unpublished observations).

Franz and Meier (1969) found the DGT activity associated mainly with a particulate protein fraction. In the case of D. lanata suspension cultures the DGT was located predominantly in the soluble fraction. Maybe the enzyme is solubilized more easily in non-differentiated cells than in mature leaf cells. Löffelhardt and Kopp (1981) found a similar effect for a cardenolide glucosyltransferase in the leaves of Convallaria majalis. Two weeks after the onset of flowering, the enzyme activity was located mainly in the soluble fraction, whereas later on glucosylation was performed by both a soluble and a particulate protein fraction.

It should be noted that the sterol:UDP-glucose glucosyltransferase purified from D. purpurea leaves and callus (Yoshikawa and Furuya 1979) also glucosylates digitoxin to some extent.

Since the isolated, purified vacuoles prepared via protoplasts from the cultured foxglove cells were not able to form purpurea-glycoside A from digitoxin, we inferred that the DGT is located in the cytosol and neither associated with the tonoplast nor located in the vacuolar sap, although the possibility of dissociation from the tonoplast during the vacuole isolation cannot be ruled out. On the other hand, we found that after 2 d of incubation with digitoxin the purpurea-glycoside A formed is almost exclusively located in the vacuoles of cultured Digitalis cells (Kreis and Reinhard 1985c). This type of spatial organisation, i.e. glucosylation of a given compound in the cytosol and subsequent storage of the glycoside formed in the vacuole, seems to be a common principle of natural product accumulation and/or detoxification and has been demonstrated in many cases (see Matile 1984, for a review). In this context it has to be mentioned, however, that Löffelhardt and Kopp (1981), when investigating the enzymatic glucosylation of convallatoxin to convalloside in Convallaria found the glucosyltransferase activity associated with a very light membrane fraction which was thought to represent vacuolar membranes. As a consequence the authors proposed that the cardenolide glucosylation takes place at the tonoplast.

In suspension-cultured Digitalis lanata cells the high DGT activity, together with the lack of specific glucosidase, is most likely responsible for the fact that the in-vivo 12 ß-hydroxylation of digitoxin to its corresponding C-glycoside, namely digoxin, has not succeeded up to now. As pointed out, the DGT plays a key role in the storage of cardenolides in cultured foxglove cells. A knowledge of the properties and the localization of the DGT may lead to a better understanding of the cellular processes involved in the biotransformation of cardenolides in D. lanata cells. This in turn may result in the establishment of a cell culture system with a high capacity for 12 ß-hydroxylation of digitoxin to digoxin or other cardenolides of the C-series.

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References

Alfermann AW, Bergmann W, Figur C, Helmbold U, Schwantag D, Schuller I, Reinhard E (1983) In: Mantell SH, Smith H (eds) Society for Experimental Botany, Seminar Series 18, Plant Biotechnology, Cambridge University Press, pp 67-74
Bradford MM (1976) Anal Biochem 72:248-254
Döller PC, Reinhard E (1979) Planta Med 37:277-288
Franz G, Meier H (1969) Planta Med 27:396-400
Heins M (1978) doctoral thesis, Universität Tübingen
Heins M, Wahl J, Kaiser F, Reinhard E (1978) Planta Med 33:57-62
Kreis W, Reinhard E (1985a) J Plant Physiol 121:385-390
Kreis W, Reinhard E (1985b) Acta Agronom Suppl 34:16
Kreis W, Reinhard E (1985c) Pharm Zeitung 130:2315-2316
Löffelhardt W, Kopp B (1981) Phytochem 20:1219-1222
Matile, Ph (1984) Naturwiss 71:18-24
Murashige T, Skoog F (1962) Physiol Plant 15:473-497
Petersen M, Seitz HU (1985) FEBS Lett 188:11-14
Reinhard E, Boy HM, Kaiser F (1975) Planta Med Suppl 33:163-168
Stoll A, Hoffmann A, Kreis W (1935) Hoppe-Seyler's Z Physiol Chem 235:249-260
Yoshikawa T, Furuya T (1979) Phytochem 18:239-241

ABBREVIATION : DGT, UDP-glucose:digitoxin 16'--C-glucosyltransferase