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Glucosylation of cyclodextrin-complexed podophyllotoxin by cell cultures of *Linum flavum* L.

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Abstract

The glucosylation of the cytotoxic lignan podophyllotoxin by cell cultures derived from *Linum flavum* was investigated. Four cyclodextrins: β-cyclodextrin, γ-cyclodextrin, dimethyl-β-cyclodextrin and hydroxypropyl-β-cyclodextrin were used to improve the solubility of podophyllotoxin by complexation. Dimethyl-β-cyclodextrin met our needs the best and the solubility of podophyllotoxin could be enhanced from 0.15 to 1.92 mM, using a podophyllotoxin/cyclodextrin ratio of 1:1. Growth parameters of the cell suspensions were not affected neither by the addition of cyclodextrins alone, nor when complexed podophyllotoxin was dissolved in the medium.

The complexed lignan disappeared rapidly from the culture medium, within 24 h, under all experimental conditions. Almost simultaneously, between 73 and 100% of detectable podophyllotoxin was bioconverted into podophyllotoxin-β-D-glucoside. A maximal bioconversion rate of 0.51 mmol l^{-1} suspension day^{-1} was calculated for the *L. flavum* cells growing in a medium which included the podophyllotoxin/dimethyl-β-cyclodextrin complex at a final concentration of 1.35 mM.

Introduction

Higher plants are known to produce a great variety of glycosides including many useful, biologically active, compounds (Tabata et al. 1984). Therefore, studies on glycosylation of natural or synthetic compounds by plant cell enzymes can contribute to the production of glycosides of pharmaceutical interest. Several plant cell cultures have been shown to be capable to glucosylate exogenously supplied compounds including cardenolides (Alfermann et al. 1980; Döller et al. 1977), flavonoids (Lewinson et al. 1989), phenolics (Mizukami et al. 1983, 1986; Scholten et al. 1991; Tabata et al. 1976, 1984, 1988), steroids (Furuya 1978) and terpenoids (Furuya 1978).

At present, glucosylation seems one of the most interesting bioconversions. It occurs readily in plant cells, but only with difficulty in microorganisms and the organic chemist has problems with this reaction as well (Pras 1992). This makes the glucosylation of cytotoxic lignans by plant cells a scientifically and biotechnologically interesting bioconversion.

Glucosyltransferases are able to perform the glucosylation reaction under strict stereochemical control (Hösel 1981).

From a pharmaceutical point of view, bioconversions such as glucosylations are not only

suitable for the production of new drugs, but also for the improvement of existing drugs with respect to their stability, solubility, biological availability and biological activity.

Plant cells as well as plant enzymes require an aqueous environment for an optimal performance of bioconversions. When apolar substrates (such as podophyllotoxin) are chosen, problems arise with dissolving these compounds in the cell culture medium. Solvents that are toxic for plant cells and enzymes have to be used in order to improve the availability of such substrates for the bioconversion.

Cyclodextrins are cyclic oligosaccharides able to form water-soluble inclusion complexes with a whole range of apolar ligands (Duchêne & Wouessidjewe 1990a, 1990b). These clathrating agents have been used to create smooth bioconversion circumstances in plant biotechnology. They combine the advantage of apolar systems (higher solubility of the lipophilic substrate) with aqueous systems (compatibility with plant cells with respect to their viability)(Woerdenbag et al. 1990a, 1990b).

Cell cultures of *Linum flavum* (yellow flax) are able to synthesize several podophyllotoxin-related lignans including 5-methoxypodophyllotoxin and its glucoside (Berlin et al. 1986, 1988; Van Uden et al. 1990, 1991a, 1991b, 1992; Wichers et al. 1990, 1991).

In the present study, the cytotoxic lignan podophyllotoxin which is poorly water soluble, was chosen as the substrate to be glucosylated by cell suspensions of *L. flavum*. The glucosylation reaction scheme is depicted in Fig. 1. Four cyclodextrins were applied to prepare water-soluble podophyllotoxin/cyclodextrin complexes.

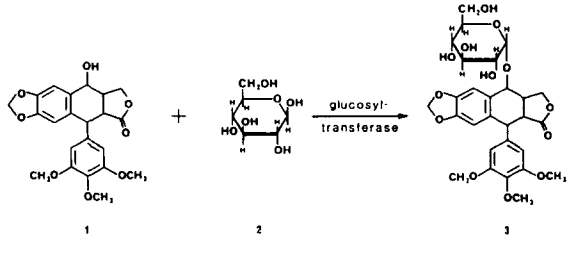

Fig. 1. Glucosylation reaction; podophyllotoxin (1) and (UDP)-glucose (2) are coupled by a glucosyltransferase to yield podophyllotoxin-β-D-glucoside (3).

Materials and methods

Culture methods and growth parameters

Suspension cultures of *Linum flavum* L. (Linaceae) were obtained and grown as described previously and maintained for more than 4 years in our laboratory (Van Uden et al. 1991a). Cells were harvested regularly during the growth cycle. The packed cell volume (PCV) was determined after transfering ca. 10 ml suspension to a calibrated conical tube followed by centrifugation for 5 min at 1,500 *g*. The medium pH and conductance were routinely measured in the resulting supernatant. Cell fresh weight (FW) and dry weight (DW) were determined by a method as described previously (Van Uden et al. 1991a).

Preparation of cyclodextrin-complexed podophyllotoxin

β-Cyclodextrin, γ-cyclodextrin and dimethyl-β-cyclodextrin were obtained from Avebe, Veendam, The Netherlands and hydroxypropyl-β-cyclodextrin (degree of substitution 0.40–0.45) a gift from Roquette, Lille, France.

To determine the solubility of podophyllotoxin complexed with the cyclodextrins, 10 ml of 2 mM solutions of β-cyclodextrin, γ-cyclodextrin, dimenthyl-β-cyclodextrin and hydroxypropyl-β-cyclodextrin in water were prepared.

Pure podophyllotoxin was then added to each solution at an equimolar concentration. To determine the solubility in water without cyclodextrins, an excess of podophyllotoxin was suspended in water.

Complexation was achieved by shaking the suspensions during 24 h at 140 rpm and 25 °C. They were subsequently centrifuged during 5 min at 1,500 *g*. In order to determine the amount of dissolved podophyllotoxin 1.0 ml of the supernatant was vortexed with 4.0 ml of dichloromethane and again centrifuged during 5 min at 1,500 *g*. Of the dichloromethane 1.0 ml was evaporated to dryness and the residue was redissolved in 2.0 ml methanol, followed by HPLC-analysis.

A second method to obtain a podophyllotoxin/cyclodextrin complex was autoclaving 2 mM of

suspended podophyllotoxin in the 2 mM cyclodextrin solutions during 25 min at 120 °C. After cooling to room temperature the extraction procedure as described above after complexation by shaking was followed.

Solubility of podophyllotoxin/β-cyclodextrin complex

To determine the amount of podophyllotoxin that could be dissolved in water using dimethyl-β-cyclodextrin, several ratios of podophyllotoxin and cyclodextrin were tested. The following ratios were chosen: podophyllotoxin/dimethyl-β-cyclodextrin (mM/mM): 2:1; 2:2; 2:5; 2:10; 2:20; 5:1; 5:2; 5:5; 5:10; and 5:20.

Samples of 10 ml were prepared and shaken for 4 days at 140 rpm and 25 °C followed by the same treatment as described under 'Preparation of cyclodextrin-complexed podophyllotoxin'.

Bioconversion experiments

Podophyllotoxin, 145 mg (=2 mM at the final medium volume of 175 ml), was dissolved in 4 ml of methanol and aseptically transferred to a 500 ml bottle. The methanol was then evaporated in a laminar air flow cabinet. Culture media were supplemented with one of the four cyclodextrins, each at 2 mM, and autoclaved during 25 min at 121 °C. The media (175 ml) were added to the bottles containing podophyllotoxin, resulting in a final concentration of 2 mM for this lignan and shaken during 4 days at 140 rpm and 25 °C. The media were decantated and transferred to 500 ml Erlenmeyer flasks and subsequently inoculated with 75 ml of a two-week-old cell suspension of *Linum flavum*. The same experiments were performed with media containing cyclodextrins but without the podophyllotoxin. In control experiments, the medium contained neither cyclodextrins nor podophyllotoxin.

Extraction of podophyllotoxin and podophyllotoxin-β-D-glucoside

To measure intracellular podophyllotoxin, the material resulting form the determination of the cell dry weight (DW) was powdered in a mortar. Hundred mg of dry mass was extracted by ultrasonification for 1 h in 2.0 ml of 80% methanol. Four ml of dichloromethane and 4.0 ml of water were added and the mixture was vortexed. After centrifugation during 5 min at 1,500 *g*, 2.0 ml of the dichloromethane were evaporated to dryness and the residue was redissolved in 1.0 ml methanol and used for HPLC-analysis.

For the determination of podophyllotoxin-β-D-glucoside, the water phase of the above mentioned extraction was submitted to enzymatic hydrolysis. A 2.5% (w/v) solution of β-glucosidase (Sigma G-0395) was prepared in 0.5 M sodium phosphate buffer, pH 5.0. To 4.0 ml of the water phase, 1.0 ml of the enzyme solution was added, followed by an incubation period of 20 h at 37 °C. Then, the mixture was treated as described above for the extraction of dry mass.

For the determination of podophyllotoxin in the medium, the procedure was as follows: after the determination of the PCV, to 1.0 ml of the supernatant obtained as described under 'Culture methods and growth parameters', 4.0 ml of dichloromethane was added and further treated as described above for the extraction of dry mass. To determine the podophyllotoxin-β-D-glucoside in the spent medium, 1.0 ml of the β-glucosidase solution was added to 4.0 ml of the supernatant obtained as described under culture methods and growth parameters. The enzymatic hydrolysis and subsequent extraction of liberated podophyllotoxin was performed as described above.

The different extracts prepared to analyse podophyllotoxin and podophyllotoxin-β-D-glucoside were also screened for the presence of 5-methoxypodophyllotoxin and 5-methoxypodophyllotoxin-β-D-glucoside.

HPLC-analysis

Podophyllotoxin (Sigma P-4405), 5-methoxypodophyllotoxin (isolated from cell cultures of *L. flavum* at our laboratory), 5-methoxypodophyllotoxin-β-D-glucoside (TNO-Zeist, The Netherlands) and picropodophyllin (prepared at the Dept. of Organic Chemistry, University of Groningen, The Netherlands) were analysed and detected using the HPLC systems

as reported previously by Van Uden et al. 1989, 1990).

Results and discussions

Before bioconversion experiments directed to the glycosylation of podophyllotoxin by *L. flavum* cell suspensions were started, the possible toxicity of cyclodextrins to the plant cells was investigated first. It appeared that the growth characteristics of *Linum flavum* cell suspensions were not affected at all, when using these clathrating agents at a concentration of 2 mM. For the control as well as for the cyclodextrin-containing cultures, the packed cell volume (PCV) increased form 20 to 70%, the dry weight (DW) from 6 to 21 $g\,l^{-1}$, the fresh weight (FW) from 125 to 350 $g\,l^{-1}$, while the conductance decreased from 4 to 0.7 mS within one growth cycle. Recently, Woerdenbag et al. (1990b) already demonstrated that β-cyclodextrin did not influence culture characteristics of cells of *Podophyllum hexandrum* Royle. In that study it was also found that the β-cyclodextrin concentration in the culture medium remained unchanged during the growth cycle, indicating that the oligosaccharide was neither metabolized nor taken up by the plant cells.

The preparation of the podophyllotoxin/cyclodextrin complex was achieved by autoclaving or by shaking the suspensions. The high temperature during the sterilisation process appeared to be disadvantageous because this method resulted in ca. 15% decomposition of podophyllotoxin. The main decomposition product most probably was picropodophyllin, based on the comparison of its HPLC-retention time behaviour with that of a sample of authentic picropodophyllin. It is known that podophyllotoxin is rapidly converted into picropodophyllin, its more stable cis-form by epimerization of the trans-lactone ring (Buchardt et al. 1986; Forsey et al. 1989). Therefore, it was concluded that the most convenient method to prepare cyclodextrin-complexed podophyllotoxin is by shaking, where no decomposition was found.

The solubility of podophyllotoxin could be increased by the complexation with all four applied cyclodextrins. Using guest and host molecule in a ratio of 1:1 and at a concentration of 2 mM, γ-cyclodextrin yielded a maximal solubility of 0.20 mM, hydroxypropyl-β-cyclodextrin of 0.68 mM, β-cyclodextrin of 0.91 mM, dimethyl-β-cyclodextrin of 1.92 mM, whereas the control (podophyllotoxin without cyclodextrin) only yielded a maximum of 0.15 mM dissolved podophyllotoxin. From these results it was concluded that dimethyl-β-cyclodextrin met our needs the best in terms of substrate availability. The solubility of podophyllotoxin could thus be increased by a factor 12.8 using this cyclodextrin. Changing the ratio of podophyllotoxin/cyclodextrin using 2 mM and 5 mM concentrations of podophyllotoxin, it was found that even a concentration of 5 mM dissolved podophyllotoxin could be reached.

The solubility of podophyllotoxin in water supplemented with dimethyl-β-cyclodextrin is depicted in Fig. 2.

In the bioconversion experiments five podophyllotoxin-containing media (without cyclodextrin, with 2 mM β-cyclodextrin, γ-cyclodextrin, dimethyl-β-cyclodextrin or hydroxypropyl-β-cyclodextrin) were incubated with *L. flavum* cell suspensions. A standard-grown culture was used as a control.

Compared with the control, growth characteristics were hardly affected, although the cells

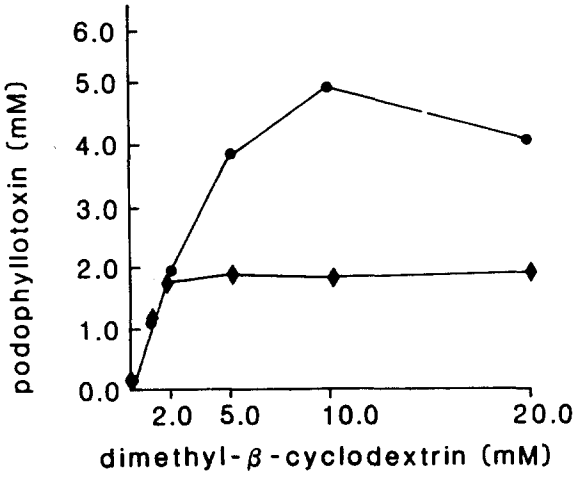

Fig. 2. Solubility of podophyllotoxin at a concentration of 2 mM (◆) or 5 mM (●) in medium supplemented with different amounts of dimethyl-β-cyclodextrin.

grew in the presence of a rather high concentration of the cytotoxic lignan. Generally, the parameters PCV, FW and DW reached the same values as found for cultures growing without podophyllotoxin. In the bioconversion experiments however, maximal values of these parameters were reached two days later. Podophyllotoxin very rapidly vanished from the culture media (Fig. 3). Already one day after incubation, podophyllotoxin could not be detected in the culture media any more, except in the case when podophyllotoxin was complexed with dimethyl-β-cyclodextrin. Under these conditions still ca. 0.025 mg ml^{-1}, being only 4% of added compound, was present after one day. After three days podophyllotoxin was also undetectable (< 0.001 mg ml^{-1}) in this culture medium. Quite remarkably, endogenously only small amounts were found. Between 0 and 8 h after the incubation, ca. 0.2% podophyllotoxin (DW), corresponding with 16 mg podophyllotoxin l^{-1} suspension, was found in the cells, for all tested cyclodextrin/lignan-containing media. In the case of the dimethyl-β-cyclodextrin-complexed podophyllotoxin this implicated that only 3% of the added podophyllotoxin was detectable at that time.

The glucosylation of podophyllotoxin started quickly. Already 8 h after incubation podophyllotoxin-β-D-glucoside was detectable (> 0.001% DW) in the cells.

The addition of the highly cytotoxic podophyllotoxin to the growth medium of *L. flavum* cell suspensions, probably stimulated the cells to detoxify the lignan. Bioconversion to the more polar podophyllotoxin-β-D-glucoside may enable storage in vacuoles and in that way decreases toxicity.

The time course of endogenously accumulated podophyllotoxin-β-D-glucoside is depicted in Fig. 4. It can be seen that podophyllotoxin was bioconverted rapidly to podophyllotoxin-β-D-glucoside. Nearly under all conditions maximal levels of podophyllotoxin-β-D-glucoside were found after one day. Only with the medium containing the highest concentration of podophyllotoxin, i.e. complexed with dimethyl-β-cyclodextrin, the highest level of glucoside was found after three days. No lignan glucoside could be detected in the spent culture medium.

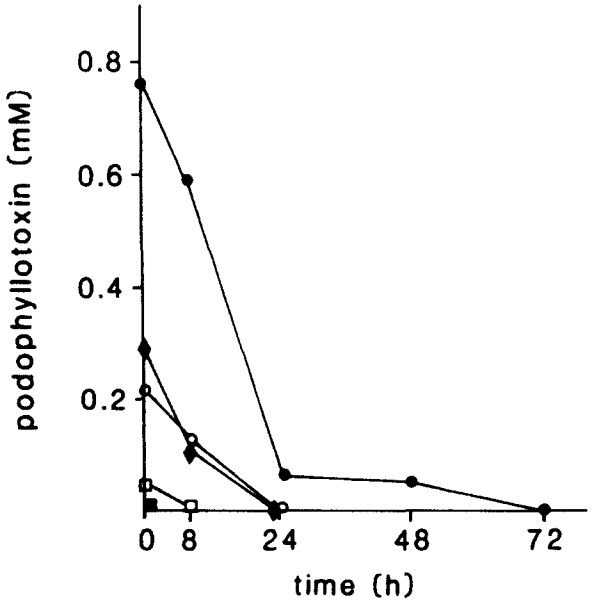

Fig. 3. Time course of the podophyllotoxin concentration in the spent medium of a *L. flavum* cell suspension fed with podophyllotoxin (■) solely, or with β- (◆), γ- (□), dimethyl-β- (●) and hydroxypropyl-β-cyclodextrin (○) complexed podophyllotoxin.

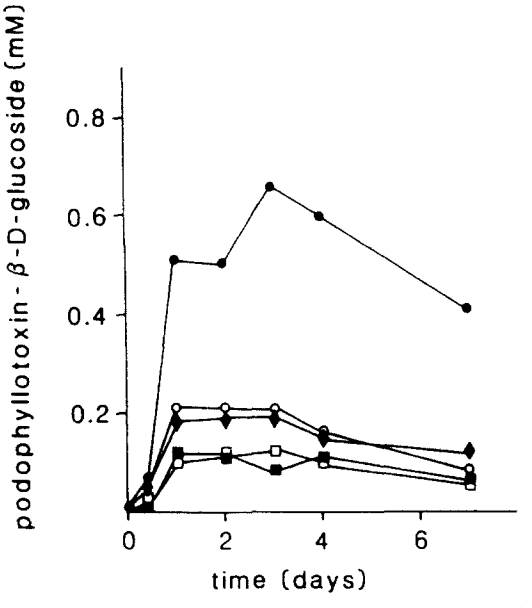

Fig. 4. Time course of the accumulation of podophyllotoxin-β-D-glucoside in *L. flavum* cells after feeding of podophyllotoxin, non-complexed (■), or complexed with β- (◆), γ- (□), dimethyl-β- (●) and hydroxypropyl-β-cyclodextrin (○).

The highest bioconversion rate was found for this latter culture condition and was calculated to be 6.6 μmol g^{-1} FW day^{-1}, corresponding with 0.51 mmol l^{-1} suspension day^{-1} (see Table 1). If the solubility of podophyllotoxin was not the limiting factor, more could have been bioconverted into its glucoside. Non-complexed podophyllotoxin was converted at a rate of 0.11 mmol l^{-1} suspension day^{-1}. From Table 1 it can be seen that the highest bioconversion percentage after one day was found for the culture growing with uncomplexed podophyllotoxin. However, the absolute amount of converted podophyllotoxin was maximal (0.51 mM) in the medium that contained dimethyl-β-cyclodextrin.

So far, several other glucosylation reactions carried out by plant cell cultures have been reported in the literature. An example of a glucosylation of another complex substrate has been reported by Kreis et al. (1986). Cell cultures of *Digitalis lanata* were able to convert the cardenolide digitoxin into purpurea-glycoside at a rate of 1.6 μmol g^{-1} FW day^{-1}.

L. flavum cell cultures predominantly accumulate 5-methoxypodophyllotoxin and its glucoside (Berlin et al. 1986, 1988; Van Uden et al. 1990, 1991a, 1991b, 1992; Wichers et al. 1990, 1991). The cell line used in the present study accumulated only traces (< 0.001% DW) of 5-methoxypodophyllotoxin and its glucoside under standard conditions as well as in the bioconversion experiments.

Recently, traces of podophyllotoxin have been found in undifferentiated cell suspensions of *L. flavum* (Wichers et al. 1991). Roots of *L. flavum* plants have been reported to contain traces of podophyllotoxin-β-D-glucoside (Broomhead & Dewick 1990). From these reports it is difficult to say whether podophyllotoxin is a naturally occurring substrate in *L. flavum* cells that is recognized and converted by its own glucosyltransferase. In the standard-grown cell line of *L. flavum* used in this study, no podophyllotoxin nor its glucoside could be detected. After bioconversion, the cell cultures used in this study accumulated maximally 2.9% (DW) podophyllotoxin as its glucoside when the substrate was complexed with dimethyl-β-cyclodextrin. Free podophyllotoxin, or podophyllotoxin complexed with β-, γ-, or hydroxypropyl-β-cyclodextrin yielded maximally 0.6%, 0.9%, 0.5% and 1.2% podophyllotoxin (present as -β-D-glucoside), respectively. *De novo* synthesis of podophyllotoxin glucosyltransferase is possible, but less obvious because of the rapid induction of the bioconversion.

It is also possible that the added podophyllotoxin is rapidly glucosylated by the transferase that normally uses 5-methoxypodophyllotoxin as a substrate.

The isolation and characterization of glucosyltransferase from *L. flavum* cell suspensions is under current investigation.

To our knowledge, this is the first example of an exogenously supplied lignan, containing a non-phenolic hydroxyl moiety, that could be bioconverted into its corresponding glucoside by a plant cell culture.

Table 1. Solubility of podophyllotoxin in culture medium, noncomplexed and as a cyclodextrin complex, and the corresponding bioconversion percentages.

Cyclodextrin	PT[1] in suspension (mM)	Bioconversion after 1 day	
		(mM)	(%)
none	0.11	0.11	100
β-CD[2]	0.64	0.18	28
β-DMCD[3]	1.34	0.51	38
β-HPCD[4]	0.48	0.23	47
γ-CD[5]	0.14	0.10	72

[1] podophyllotoxin
[2] β-cyclodextrin
[3] dimethyl-β-cyclodextrin
[4] hydroxypropyl-βcyclodextrin
[5] γ-cyclodextrin

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