The production of podophyllotoxin and related cytotoxic lignans by plant cell cultures

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CHAPTER 5

IMPROVEMENT OF THE PODOPHYLLOTOXIN PRODUCTION BY FEEDING OF PHENYLPROPANOIDS TO CELL CULTURES OF *PODOPHYLLUM HEXANDRUM* ROYLE.

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ABSTRACT

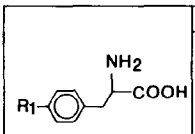
In order to improve the production of the cytotoxic lignan podophyllotoxin, seven phenylpropanoids from the phenylpropanoid biosynthesis routing and one related compound were fed to cell suspension cultures derived from the roots of *Podophyllum hexandrum* Royle. Only upon addition of coniferin, a stimulation of the podophyllotoxin accumulation was observed. Permeabilization using isopropanol, in combination with coniferin as a substrate, did not result in an additional increase in podophyllotoxin accumulation. Concentrations of isopropanol exceeding 0.5% (v/v) were found to be toxic for suspension-grown cells of *P. hexandrum*. When coniferin was fed in the presence of such isopropanol concentrations, β-glucosidase activity was still present, resulting in the formation of the aglucone coniferyl alcohol. In addition, podophyllotoxin was released into the medium under these permeabilization conditions.
Entrapment of *P. hexandrum* cells in calcium-alginate as such or in combination with the feeding of biosynthetic precursors, did not improve the podophyllotoxin production. Cell-free medium from suspension cultures at later growth stages incubated with coniferin, resulted in the synthesis of the lignan pinoresinol.
In parallel experiments, the use of β-cyclodextrin in feeding the poorly water-soluble precursor coniferyl alcohol to these cultures was studied. By complexation with β-cyclodextrin, a solution of 3 mM coniferyl alcohol could be fed, resulting in a 6-fold enhanced podophyllotoxin accumulation. The same concentration of non-complexed, suspended coniferyl alcohol had a lesser effect on the podophyllotoxin accumulation. β-Cyclodextrin itself was proven to be non-toxic for the cells. It did not influence the podophyllotoxin content and it was not metabolized or used as a carbon source by the cells. For comparison, coniferin, the water-soluble β-D-glucoside of coniferyl alcohol, was also fed in the same concentration. The effect of coniferin on the podophyllotoxin accumulation was ca. 5-fold stronger than that of coniferyl alcohol complexed with β-cyclodextrin.

INTRODUCTION

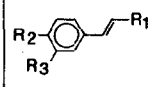
Etoposide (VP-16-213) and teniposide (VM-26) are two clinically applied semi-synthetic cytostatics, chemically prepared from podophyllotoxin (Holthuis 1988; Van Maanen et al. 1988). Podophyllotoxin belongs to the chemical group of lignans, which are dimerization products of phenylpropane (C6-C3) units, coupled by way of the β-carbons of their side chains (MacRae and Towers 1984; Pelter 1986). Podophyllotoxin is isolated from the rhizomes of *Podophyllum peltatum* or *Podophyllum hexandrum* (Berberidaceae)

(Jackson and Dewick 1984c). The availability of the plants is limited, principally because of a long juvenile phase and poor reproduction capacities (Rust and Roth 1981; Chuang and Chang 1987). Therefore, the biotechnological production of podophyllotoxin by means of plant cell tissue cultures, may be an attractive alternative.

Recently, we successfully initiated cell suspension cultures of *P. hexandrum*. Podophyllotoxin was accumulated up to 0.1% on a dry weight basis (Van Uden et al. 1989; Chapter 2). To our knowledge, precursor feeding studies using cell suspension with the aim to improve podophyllotoxin accumulation have not been performed so far. In the present study, we explored the potential of these cultures in feeding experiments. The phenylpropanoids involved were intermediates from the phenylpropanoid routing: L-phenylalanine, L-tyrosine, *trans*-cinnamic acid, *trans*-caffeic acid, *trans*-coumaric acid, *trans*-ferulic acid and coniferin. One structurally related compound, *trans*-3,4-methylenedioxycinnamic acid, was also tested. The structural formulas of these compounds are depicted in Fig. 1.



R_1		
H		L-phenylalanine
OH		L-tyrosine



R_1	R_2	R_3	
COOH	H	H	trans-cinnamic acid
COOH	OH	H	trans-coumaric acid
COOH	OH	OCH_3	trans-ferulic acid
COOH	OH	OH	trans-caffeic acid
COOH	$-OCH_2O-$		trans-3,4-methylenedioxy-cinnamic acid
CH_2OH	OGluc	OCH_3	coniferin

Fig. 1. Structural formulas of the precursors of podophyllotoxin used in this study.

The use of several organic solvents in order to accomplish permeabilization of plant cells has been reported (Rhodes 1985). Coniferin-feeding was combined with isopropanol with the aim to increase the formation of podophyllotoxin by freely suspended cells.

In addition, the cell suspension was immobilized in order to stimulate the production of podophyllotoxin after substrate feeding. Several successful attempts have been reported on the production of valuable compounds by immobilized plant cells (Brodelius and

Mosbach 1982; Wichers et al. 1983; Hall et al. 1988; Pras et al. 1988). The cells of *P. hexandrum* were entrapped in calcium alginate and a number of precursors were tested. Since freely suspended cells can release enzymes into the medium, dissolved substrates may be bioconverted extracellularly. Cell-free media were therefore checked on possible enzyme activities.

Several precursors are poorly soluble in aqueous media and consequently poorly water-soluble precursors have often been applied in two-phase systems. However, many of the plant cells used hardly converted precursors in the presence of organic phases, often due to a dramatic decrease of cell vitality (Beiderbeck and Knoop 1988). Another approach to solve this problem is to combine the advantages of apolar systems (higher solubility) and aqueous systems (compatibility with plant cells with respect to vitality) by carrying out bioconversions in the presence of clathrating agents, such as cyclodextrins (Woerdenbag et al. 1990).

In a parallel study therefore, coniferyl alcohol (Fig. 1), a key-precursor of the biosynthetic pathway of podophyllotoxin (Jackson and Dewick 1984b), has been chosen as a model substrate because of its very poor water-solubility.

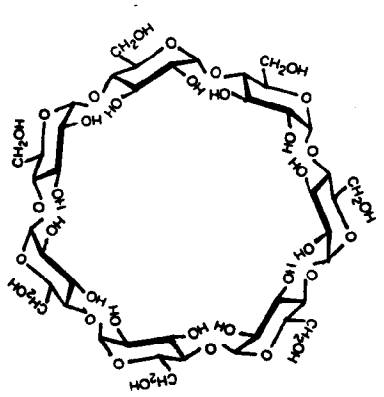


Fig. 2. Structural formula of β-cyclodextrin.

We investigated podophyllotoxin contents in *P. hexandrum* cell cultures, after feeding coniferyl alcohol, complexed with β-cyclodextrin (Fig. 2). For comparison, we fed the cultures with non-complexed, suspended coniferyl alcohol as well as with coniferin, the water-soluble β-D-glucoside of coniferyl alcohol. In addition, the influence of β-cyclodextrin on cell viability was monitored.

MATERIALS AND METHODS

CULTURE CONDITIONS AND CHARACTERISTICS

Cell suspensions of *Podophyllum hexandrum* Royle (Berberidaceae) derived from the roots, were grown as described previously (Van Uden et al. 1989; Chapter 2), here subculturing was done after a 3-week growth period. In the present study, the dark-grown cultures were used. The packed cell volume (PCV), pH, conductance and dry weight (DW) were routinely determined.

ENTRAPMENT

Freely suspended cells were immobilized in calcium alginate according to the procedure of Wichers et al. (1983), resulting in beads with a cell loading of 33% (w/v) and with an average volume of 84.9 µl.

PRECURSOR FEEDING

Bioconversions were carried out aseptically in Erlenmeyer flasks on a rotary shaker at 150 rpm at 26 °C. The precursors L-phenylalanine, L-tyrosine, *trans*-cinnamic acid, *trans*-caffeic acid, *trans*-ferulic acid, *trans*-coumaric acid and *trans*-3,4-methylene-dioxycinnamic acid were all purchased from Janssen Chimica. Coniferin, which is not commercially available, was a generous gift from Prof. Dr. K. Weinges, Heidelberg and Dr. J. Berlin, Braunschweig, both Germany.
All these compounds were added to the media in a standard concentration of 2.5 mM, prior to autoclaving. Cells in the stationary phase were washed once with medium and then inoculated at a packed cell volume of ca. 16%, resulting in final substrate concentrations of 2.1 mM. Media including 0.6, 1.0, 2.5, 4.8 and 9.5 mM coniferin as a substrate were tested. Immobilization experiments were carried out in Erlenmeyer flasks with 20 g beads in 100 ml medium.
In feeding experiments using calcium alginate-entrapped cells, the precursors L-phenylalanine, L-tyrosine, caffeic acid, ferulic acid and coniferin were tested.
Samples of ca. 10 ml were taken from the suspension-grown or immobilized cultures for a 3-4 week period.
The amount of substrate converted was calculated from the maximal difference in podophyllotoxin content between the control and the substrate-fed cultures. The bioconversion percentage was calculated as follows: substrate converted (mM)/ initial substrate concentration (mM) x 100%.
In order to determine conversion of coniferin in the medium, 2.0 ml of cell-free medium were combined with 2.0 ml of an aqueous 2.5 mM coniferin solution. In the control assay, fresh medium was used in stead of cell-free medium.

PRECURSOR FEEDING USING β-CYCLODEXTRIN

β-Cyclodextrin (AVEBE, Veendam, The Netherlands) is a cyclic oligosaccharide consisting of 7 glucose units, linked by α(1→4) glycosidic bonds (Fig. 2). Complex formation of coniferyl alcohol (Fluka, no. 27740) with β-cyclodextrin was achieved by autoclaving (20 min, 120 °C) these compounds in equimolar amounts, simultaneously with the cell culture media, resulting in complete dissolution of the coniferyl alcohol.
The increase in water-solubility of coniferyl alcohol, as a complex with β-cyclodextrin, was determined as described earlier (Higuchi and Connors 1965; Woerdenbag et al. 1990). The complex stability constant, K_C, was determined according to the method described by Selvidge and Eftink (1986). Sodium 4-(4-hydroxy-1-naphthylazo)-1-naphthalene sulfonate (a generous gift from Dr. Matsui, Shimane University, Japan) was used as the chromophoric ligand.

Each experiment was initiated by incubating 10 ml of packed cells of a 3-week-old cell suspension culture in 100 ml fresh medium. Coniferyl alcohol, as a β-cyclodextrin complex, was added in a concentration of 3 mM at the beginning of the growth cycle (day 0). For comparison, 3 mM coniferyl alcohol without β-cyclodextrin as well as 3 mM coniferin (coniferyl alcohol-β-D-glucoside) were added in analogous experiments. Control experiments were performed with standard-grown cell suspensions, either without or with 3 mM β-cyclodextrin.
During the three-week experiments, 10 ml samples of cell suspension were taken at regular intervals.
β-Cyclodextrin concentrations in the culture medium and in the aqueous extract of the dried and powdered cell material were determined according to Vikmon (1982). Briefly, samples were 30-fold diluted with water. To 1.0 ml sample, 1.0 ml alkaline phenolphthalein solution (phenolphthalein 5 x 10^{-5} M, sodium carbonate 0.01 M in water) was added and the sample was mixed. The UV-absorption at 550 nm was measured. The difference in absorption as compared with reference sample (water) was determined. The β-cyclodextrin concentration was calculated from a calibration curve prepared from samples with known β-cyclodextrin concentrations.

PERMEABILIZATION

Permeabilization of freely suspended cells was carried out using the organic solvent isopropanol (2-propanol, Merck). The isopropanol was added aseptically via a 0.2 μm filter (Millipore) after autoclaving the media. Concentrations of 0.5, 1.0, 2.5, 5.0 and 10% (v/v) were used.

RESPIRATION ACTIVITY

The respiration activity of calcium alginate-entrapped cells was determined by means of measuring the oxygen consumption, in reference to freely suspended cells. Oxygen was measured with an oxygen electrode (O_2-meter CG 867, Schott). The consumption of 2.0 g beads or 1.0 g freely suspended cells in oxygen-saturated growth medium in a 23.0 ml vessel was monitored under stirring at 20 °C during 30 min. The oxygen consumption is expressed as mg O_2 consumed per h per g fresh weight cells (mg O_2 $h^{-1}g^{-1}$FW).

β-GLUCOSIDASE ACTIVITY (E.C. 3.2.1.21)

Portions of ca. 0.3 g, fresh weight, of *P. hexandrum* cells were ground with quartz in liquid nitrogen and transferred to 2.0 ml 0.5 M sodium phosphate buffer, pH 5.0, containing ca. 100 mg Dowex (Sigma No. 1x2-100). The extract was centrifuged at 17,000 g for 10 min at 4 °C. The supernatant, designated as the soluble protein fraction, was directly used for activity measurement, while the pellet (cell fractions) was resuspended in 2.0 ml 0.5 M phosphate buffer, pH 5.0. One ml of the supernatant or resuspended pellet was added to 4.0 ml of an aqueous 2.5 mM solution of coniferin. To measure β-glucosidase activity in cell-free medium, a cell suspension sample was centrifuged at 17,000 g for 10 min at 4 °C. One ml of supernatant plus 1.0 ml 0.5 M phosphate buffer, pH 5.0, were added to 4.0 ml 2.5 mM aqueous coniferin solution. Standard incubations were done for 1 h at 40 °C. For determination of the coniferyl alcohol content in the enzyme fractions, samples were taken at t=0 min. The reaction was stopped by adding 2.0 ml of the assay mixture to 4.0 ml dichloromethane, followed by immediate vortexing in order to extract the liberated coniferyl alcohol. Two ml of the dichloromethane phase were evaporated to dryness and the remaining residue was redissolved in 1.0 ml methanol. These extracts were analyzed by means of HPLC-UV (see below). The β-glucosidase activity is expressed as mmol coniferyl alcohol formed per s per kg protein (mkat kg^{-1} protein) in medium and supernatant or mmol coniferyl alcohol formed per h per kg fresh weight (mmol h^{-1} kg^{-1} FW) in cell fractions.

PEROXIDASE ACTIVITY (E.C. 1.11.1.7)

Peroxidase activity was measured in the soluble protein and pellet fraction of *P. hexandrum* cells, as well as in cell-free medium. These samples were obtained according to the procedure described under the section β-GLUCOSIDASE ACTIVITY. Here, a 0.1 M phosphate buffer, pH 7.0, was used. The activity was determined spectrophotometrically according to the method of Maehly and Chance (1954).
Peroxidase activity is expressed as mmol guaiacol consumed per s per kg protein (mkat kg^{-1} protein) in medium or supernatant, or mmol guaiacol consumed per h per kg fresh weight (mmol h^{-1} kg^{-1} FW) in cell fractions.

PROTEIN CONTENT

The protein content was determined according to the method of Bradford (1976) using bovine serum albumin (BSA, Sigma no. A7638) as the standard protein.

EXTRACTION PROCEDURE

Crude extracts to be used for the several methods of analysis were prepared to a described procedure (Van Uden et al. 1989; Chapter 2), immobilized cells were dried by means of lyophilization.

ANALYSIS BY MEANS OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Podophyllotoxin was analyzed and detected using the HPLC-UV system as reported previously (Van Uden et al. 1989; Chapter 2). Production rates were calculated as described in Chapter 2. Coniferyl alcohol was measured in the same extracts that were used to determine podophyllotoxin, at 290 nm using a Lichrosorb RP-18 column at a flow rate of 0.3 ml min^{-1}. A reference solution of 0.1 mg ml^{-1} coniferyl alcohol (Fluka, no. 27740) in methanol was used.
Analysis of coniferin was performed using a Chrompack Nucleosil 5C-18 column (100 x 3 mm i.d.). The water phase of the extraction and the spent medium were directly used for the analyis of coniferin. The mobile phase consisted of methanol/water (3:7), the flow rate was 0.15 ml min^{-1} and the detection was at 230 nm. A coniferin solution of 0.01 mg ml^{-1} in water was used as the standard.

MASS SPECTROMETRY

Mass spectrometric analysis (direct sampling) of cell-free medium extracts were performed under the conditions as described (Van Uden et al. 1989; Chapter 2) and revealed the presence of pinoresinol. The following values were found: m/z, relative intensities (%): [M^+] 358 (7), 205 (9), 163 (26), 152 (35), 151 (100), 137 (77).
In addition, a chemical ionization using OH^- was carried out. The samples were introduced into the mass spectrometer by means of direct sampling and identified at 100 eV. For pinoresinol an $[M-H]^-$ value of 357 was found.

RESULTS AND DISCUSSION

PRECURSOR FEEDING TO FREELY SUSPENDED CELLS

Seven intermediates from the phenylpropanoid pathway and one related compound were tested as substrates with freely suspended cells of *Podophyllum hexandrum* Royle. It appeared that 2.1 mM concentrations of cinnamic acid, coumaric acid and 3,4-methylenedioxycinnamic acid were too toxic for the cells, as determined on basis of packed cell volume, dry weight, pH and conductance. None of these precursors resulted in enhanced podophyllotoxin levels. The intermediates L-phenylalanine, L-tyrosine, ferulic acid, caffeic acid and coniferin did not result in any effect on these culture characteristics. Phenylalanine, tyrosine, ferulic acid and caffeic acid administration resulted even in a decrease of the podophyllotoxin content.

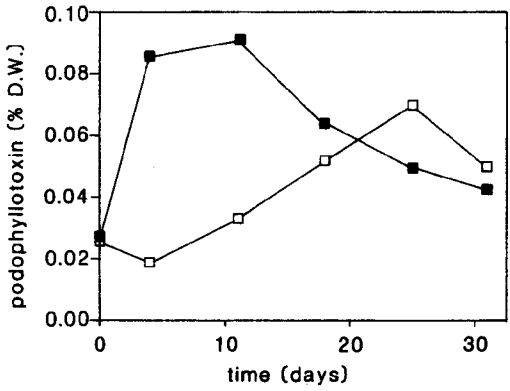


Fig. 3. Time course of the podophyllotoxin content on a dry weight basis of a suspension culture of *P. hexandrum*; standard-grown (□) and after feeding of 2.1 mM coniferin (■).

From the precursors tested, only coniferin was found to exhibit a positive and reproducible effect on the podophyllotoxin accumulation. The highest calculated bioconversion percentage was 1.88% at a 2.1 mM coniferin concentration. In Fig. 3 it is shown that in coniferin-fed cultures of *P. hexandrum* a 4.5 times higher podophyllotoxin content was found at day 4, compared to untreated cultures, respectively 0.085% and 0.018% on a dry weight basis. A high increase factor does not imply a high absolute podophyllotoxin content. For the coniferin-fed culture a production rate of 1.2 (11 days) could be calculated, while for the standard culture a value of 0.5 mg podophyllotoxin l^{-1} day^{-1} (25 days) was found. The podophyllotoxin contents (production rates) found for standard-grown cells in this study were lower than those published earlier (Van Uden et al. 1989; Chapter 2). Apparently this cell line is subjected to fluctuations in accumulation levels. Remarkable was that the highest contents in standard-grown cultures were at the stationary phase of the growth cycle, while coniferin-fed cultures on the contrary, reached

maximal values already in the early lineair phase. The addition of coniferin mainly resulted in gain of time, which is also reflected in the production rates, the final podophyllotoxin contents were not markedly increased. Coniferin was not detectable in standard-grown cells and together with this rapid production of podophyllotoxin, this might point to an intracellular substrate limitation.

As is shown in Fig. 4, the optimal substrate concentrations lie in the range of 0.8 to 2.1 mM coniferin. Only at concentrations exceeding 4.0 mM, growth inhibitory effects occurred, indicating that coniferin is a rather non-toxic substrate.

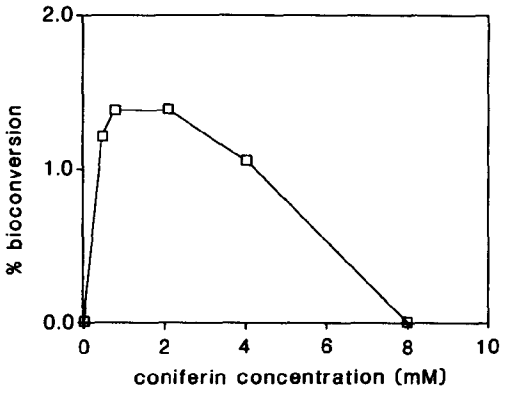


Fig. 4. Relationship between the coniferin concentration and its bioconversion percentage, after administration to suspension-grown cultures of *P. hexandrum*.

The coniferin administered to the cell suspensions disappeared rapidly from the medium, 55% of the substrate vanished from the medium within 2 h. After 24 h only 1% was still present extracellularly. However, maximally only 1.8% of originally administered coniferin was endogenously found at that moment. Since the aglucone of this substrate, coniferyl alcohol, could not be detected intra- or extracellularly, it can be suggested that 98% was converted into unknown products. From the three alcohols, known to be incorporated into lignin, the constituent of cell walls of various types of plants, coniferyl alcohol is most efficiently used (Freudenberg and Harkin 1963; Hahlbrock 1977; Luckner 1986). Lignin might be a possible 'catcher' of supplied coniferin (via coniferyl alcohol) in bioconversion experiments, as has also been proposed by Stöckigt and Klishies (1977).

PERMEABILIZATION OF FREELY SUSPENDED CELLS

In order to improve the availability of coniferin for bioconversion by freely suspended cells, this substrate was supplied in combination with isopropanol as a permeabilizing agent. All isopropanol concentrations exceeding 0.5% (v/v) inhibited growth and caused an increase in conductivity of the cell-free medium, suggesting cell lysis, as is shown in

Fig. 5. It has been reported, that even mild treatments with the strongly related organic solvent n-propanol, resulted in severe growth inhibition (Berlin et al. 1989). The combination of coniferin-feeding and cell-permeabilization did not lead to an additional increase of podophyllotoxin accumulation. At very high isopropanol concentrations, 5 or 10%, coniferyl alcohol was found in the medium, indicating the hydrolysis of coniferin to its aglucone (Fig. 6). When 10% isopropanol was used, all coniferin was converted into coniferyl alcohol after 48 h, 5% isopropanol resulted in a lower conversion percentage of coniferin.

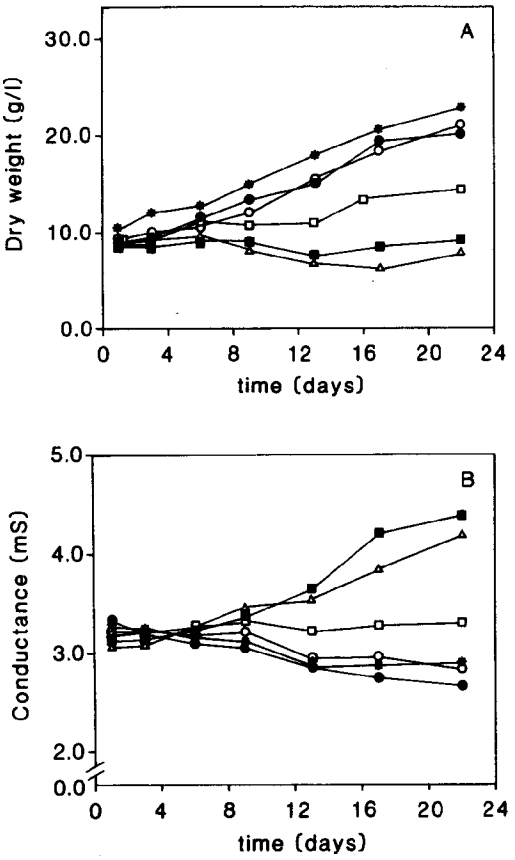


Fig. 5. Dry weight (**A**) and conductance (**B**) changes under various permeabilization conditions. Control (●); 2.5% (v/v) isopropanol (■); 2.1 mM coniferin (✱); 2.1 mM coniferin + 0.5% (v/v) isopropanol (○); 2.1 mM coniferin + 1.0% (v/v) isopropanol (□); 2.1 mM coniferin + 2.5% (v/v) isopropanol (△).

On the contrary, negligible amounts of coniferyl alcohol were detected in the medium of a coniferin-fed and isopropanol-untreated culture.

It seems likely that this hydrolysis of coniferin into the aglucone takes place at the cell wall/plasmalemma: β-glucosidase activity was measured in the cell fraction and not in the

medium, while coniferyl alcohol was detected in the medium and only for trace amounts intracellularly. Isopropanol did not influence the total β-glucosidase activity during the experiment (9-12 mmol h^{-1} kg^{-1} FW), but remarkably was the increase of activity associated with the soluble protein fraction, from ca. 1 to 2.5 mkat kg^{-1} protein and from ca. 1 to 4.5 mkat kg^{-1} protein, for 5% and 10% isopropanol respectively. These results indicate that the hydrolysis of coniferin was due to the increased activity in the soluble fraction under these permeabilization conditions.

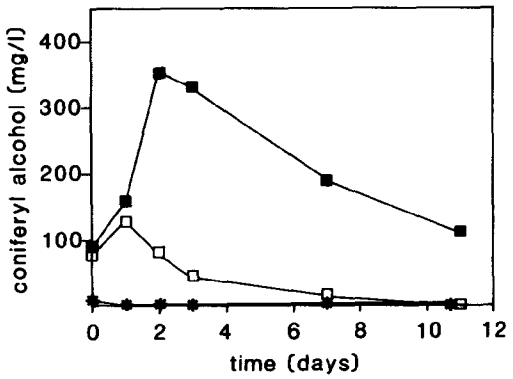


Fig. 6. Coniferyl alcohol in the cell-free medium after feeding 2.1 mM coniferin to suspension cultures of *P. hexandrum*: no permeabilization (✱), permeabilization with 5% (v/v) isopropanol (□), and 10% (v/v) isopropanol (■).

Although cell growth ceased, a β-glucosidase was still able to convert coniferin into its aglucone. Apparently, severely stressed plant cells are able to convert administered intermediates by way of active enzyme systems.

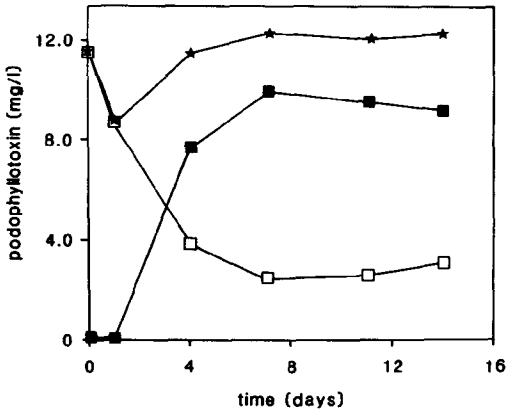


Fig. 7. Time course of extracellular (■), intracellular (□) and total (✱) podophyllotoxin content of a suspension culture of *P. hexandrum* after feeding 2.1 mM coniferin in combination with 5% (v/v) isopropanol.

As an additional effect, the endogeneously present podophyllotoxin was released into the medium after isopropanol treatment. For example, when 5% isopropanol was used, 50% of podophyllotoxin was released after 3 days, the release being complete after 7 days (Fig.

7). This release might be a helpful tool for the isolation of intracellularly stored podophyllotoxin, although the method is cell-destructive.

PRECURSOR FEEDING TO ENTRAPPED CELLS

In some studies it has been observed that precursor feeding to immobilized cells resulted in an increased production of secondary products when compared to freely suspended cells under the same conditions (Brodelius and Mosbach 1982; Wichers et al. 1983; Rhodes 1985; Hall et al. 1988; Pras et al. 1988). In a recent study, calcium alginate proved to be a suitable matrix for cell entrapment in combination with precursor feeding (Pras et al. 1989). Therefore, the bioconversion experiments were carried out using calcium alginate-entrapped cells of *P. hexandrum*. Only precursors that were non-toxic for freely suspended cells were tested: L-phenylalanine, L-tyrosine, caffeic acid, ferulic acid and coniferin. The immobilization procedure itself did not lead to an improved podophyllotoxin accumulation, the synthesis of podophyllotoxin even ceased. Metabolic behaviour of cells may be altered after entrapment. Analogously, for cells of *Mucuna pruriens* it has been reported that the endogenous L-DOPA production stopped completely upon immobilization (Wichers et al. 1983).
The oxygen consumption of freely suspended cells of *P. hexandrum* was 0.30 mg O_2 h^{-1} g^{-1} FW. The respiration activity decreased immediately after the entrapment to a value of 0.16 mg O_2 h^{-1} g^{-1} FW, which remained stable during the experiments. Possibly, the entrapped state is a stress environment for the cells, in which they are not able to synthesize podophyllotoxin anymore. Feeding of L-phenylalanine, L-tyrosine, ferulic acid and caffeic acid did not result in an enhanced production of podophyllotoxin. Also with coniferin, in contrast with freely suspended cells, no podophyllotoxin increase was measured. This substrate disappeared completely from the medium within 7 days, which is much slower than measured for freely suspended cells.

EXTRACELLULAR ENZYME ACTIVITIES

Since the release of enzymes from plant cells into the medium can not be excluded (Sticher et al. 1981; Rhodes 1985), coniferin was added to cell-free medium in order to study its possible extracellular bioconversion. Mass spectrometric analysis of cell-free medium extracts revealed the presence of the lignan pinoresinol (Fig. 8), which showed the same fragmentation pattern as was found by Duffield (1967). This extracellularly formed product was only present in later growth stages. Theoretically, the synthesis of pinoresinol in cell-free medium can only be achieved when a β-glucosidase activity and a peroxidase activity are present. Glucosidase activity is required, since glucosides are not substrates for peroxidase (Hösel and Todenhagen 1980). Peroxidase activity is essential

for the formation of coniferyl alcohol radicals, needed for further biosynthesis into lignans (Stöckigt and Klishies 1977). Activities of both enzymes could indeed be measured in the cell-free medium at the stationary phase, and fluctuated around 4.2 mkat kg^{-1} protein for peroxidase and around 0.6 mkat kg^{-1} protein for β-glucosidase activity.

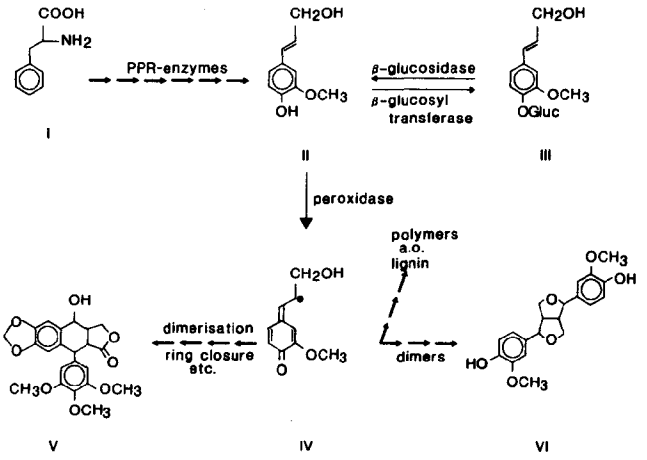


Fig. 8. A simplified scheme of the biosynthesis of podophyllotoxin. From phenylalanine (I), coniferyl alcohol (II) is synthesized by the action of phenylpropanoid (PPR) enzymes. This can be converted into coniferin (III) and vice-versa. A peroxidase converts (II) to coniferyl alcohol radicals (IV), which are coupled and synthesized further to lignans such as podophyllotoxin (V) or pinoresinol (VI).

In presence of these enzymes, the formation of pinoresinol from coniferyl alcohol, via coniferin, has been reported several times (Freudenberg and Rasenack 1953; Erdtman 1955; Freudenberg and Harkin 1963; Harborne 1980; Luckner 1986). Thus, in order to avoid unwanted extracellular bioconversion of coniferin, this substrate has to be added to *P. hexandrum* cells in the early growth stage.

PRECURSOR FEEDING USING β-CYCLODEXTRIN

Cyclodextrins are cyclic oligosaccharides that are able to form inclusion complexes with a variety of apolar ligands. Through complexation the physical-chemical properties of the ligands are changed, including their solubility in aqueous solution (Szejtli 1982). At 26 °C the water-solubility of coniferyl alcohol increased from 0.15 mM without, to a maximum of about 3.4 mM with β-cyclodextrin, in a molar ratio of 1:1. A stable complex was formed, with a K_c of 1360 M^{-1}. Cells of *P. hexandrum*, endogenously accumulated podophyllotoxin in concentrations ranging from 0.001 to 0.002% (production rate 0.02 mg l^{-1} day^{-1}, 13 days), calculated on a dry weight, during their growth cycle (Fig. 9). The podophyllotoxin concentrations, as measured in the control experiments, are somewhat

lower than those published earlier from the same undifferentiated cell line (Van Uden et al. 1989; Chapter 2). Apparently, the cell line is subject to fluctuations and not stable for a longer period in this respect, which is also demonstrated in terms of production rates. During maintenance of these cultures, the rates decreased from 1.3, via 0.5 to 0.02 mg podophyllotoxin l^{-1} day^{-1}.

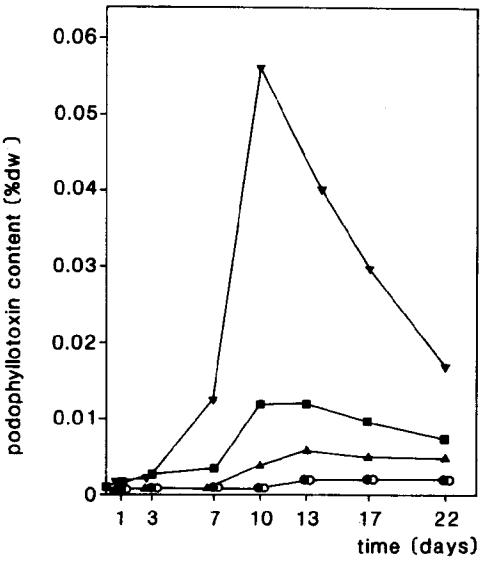


Fig. 9. Time course of the podophyllotoxin content, expressed as percentage of dry weight, in suspension-grown cultures of *P. hexandrum*. Control (●); 3 mM β-cyclodextrin (○); 3 mM coniferyl alcohol/β-cyclodextrin complex (■); 3 mM coniferyl alcohol (▲); 3 mM coniferin (▼).

The precursor-feeding experiments were performed with the highest possible coniferyl alcohol/β-cyclodextrin concentration. Feeding of 3 mM coniferyl alcohol, dissolved in the culture medium as a β-cyclodextrin complex, resulted in enhanced podophyllotoxin accumulation, with a maximum of 0.012% on day 10 of the growth cycle (production rate 0.2 mg podophyllotoxin l^{-1} day^{-1}, 10 days). Non-complexed coniferyl alcohol, suspended in the medium in a concentration of 3 mM, also enhanced the podophyllotoxin production, but only to a maximum of 0.006% (production rate 0.1 mg l^{-1} day^{-1}, 13 days). Feeding 3 mM coniferin caused the largest increase, with a maximum of 0.056% on day 10 (Fig. 9), and a corresponding production rate of 0.8 mg podophyllotoxin l^{-1} day^{-1}. This was in agreement with the former results, found in parallel studies, in which only water-soluble precursors were tested.

The accumulation pattern of podophyllotoxin, after adding only β-cyclodextrin to the culture medium, did not differ from the control conditions. The culture characteristics, *i.e.* cell growth in terms of dry weight, pH and conductivity, were not altered by any of the additions. The β-cyclodextrin concentration in the culture medium remained unchanged during the whole growth cycle and no β-cyclodextrin could be detected intracellularly.

This indicates that the oligosaccharide was not metabolized by plant cell enzymes or used as a carbon source. Moreover, it did not penetrate the plant cells.

The lignan biosynthesis proceeds via a radical mediated dimerization reaction of two phenylpropane units, such as coniferyl alcohol (Pelter 1986). Coniferyl alcohol originates from coniferin, which looses glucose by specific β-glucosidase activity in the cultures (Freudenberg 1965). From the two precursors used, only coniferyl alcohol is commercially available.

It was found that the effect of coniferin was more pronounced than that of the coniferyl alcohol/β-cyclodextrin complex. Apparently, it is not advantageous in this case to feed a precursor, more closely related to the radical coupling reaction (coniferyl alcohol) than a substrate that first has to undergo cleavage of its sugar moiety (coniferin). In this respect, it has been postulated that intermediates of the lignan and lignin biosynthesis are carried in a hydrophilic, soluble form to the site of dimerization and polymerization, respectively (Hahlbrock 1977).

No podophyllotoxin could be detected in the culture medium, indicating that it was only present intracellularly and not excreted into the medium. Under control conditions, cells contained only 2 μg coniferyl alcohol per g dry weight. After feeding the β-cyclodextrin/substrate complex, an increased coniferyl alcohol content was found, 35 and 13 μg g^{-1}, respectively on day 1 and 3 of the growth cycle. In later stages of the growth cycle the low control levels were measured again. From the culture medium coniferyl alcohol had vanished within 1 day, despite this rapid disappearance, no direct podophyllotoxin formation was measured.

The direct benefit, with respect to a higher podophyllotoxin production, was probably obtained from the fact that coniferyl alcohol is delivered to the cells in a dissolved state, due to complexation with β-cyclodextrin. This may lead to an increased uptake of the precursor in the cells, finally resulting in an increased bioconversion, as compared with the non-complexed precursor. This observation seems to be confirmed by the relatively efficient incorporation of the readily soluble analog coniferin. At present, a lack of knowledge exists on the underlaying mass transfer processes that take place near the cell envelope. Recently, we successfully applied β-cyclodextrin to solubilize the steroid hormone 17β-estradiol, that subsequently was *ortho*-hydroxylated efficiently by a phenoloxidase from *Mucuna pruriens* cell cultures (Woerdenbag et al. 1990). In contrast with this one step bioconversion, the lignan routing is much more complex and several enzymatic steps and interconversions are involved, probably resulting in lower product yields.

FINAL CONCLUSIONS

From the results presented in this study, it may be concluded that the use of coniferin as a precursor increases the podophyllotoxin production of freely suspended cells of *P. hexandrum*. This conclusion fits in the biosynthesis scheme of podophyllotoxin, which has been based upon previous reports (Erdtman 1955; Jackson and Dewick 1984b; Pelter 1986) (Fig. 8). On the other hand, since no labelling experiments were performed, an indirect effect of coniferin can not be excluded. Improvement of the podophyllotoxin production could not be achieved by permeabilization or entrapment.

Precursors other than coniferin did not result in increased podophyllotoxin contents in our undifferentiated cell cultures, but on the other hand it is known that they can be incorporated into podophyllotoxin using intact plants (Ayres 1969; Ayres et al. 1981; Jackson and Dewick 1984a).

In addition, feeding lipophilic precursors of the lignan biosynthesis as a β-cyclodextrin complex, offers a possibility to enhance the podophyllotoxin content in cell cultures of *P. hexandrum*. In order to solubilize organic compounds, cyclodextrins offer perspectives in plant cell biotechnology. By their application very smooth bioconversion conditions are created with respect to cell viability. They may be used to facilitate precursor-feeding of poorly water-soluble intermediates or not naturally occurring compounds. Moreover, they open up perspectives to feed labelled lipophilic compounds, to be incorporated in secondary metabolites, in order to study a biosynthetic pathway.

Despite the positive results on the production of podophyllotoxin by feeding of coniferin and its aglucone, the biotechnological production of this lignan forms no commercial alternative yet. Since the podophyllotoxin accumulation in *P. hexandrum* cell suspensions appeared to be unstable and low contents were measured after a longer period of maintenance, it was decided to emphasize the further studies on the production of 5-methoxypodophyllotoxin by cell cultures of *L. flavum*.

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