Phytochemical and Biosynthetic Studies of Lignans, with a Focus on Indonesian Medicinal Plants
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Chapter 6

Reduced coniferin and enhanced 6-methoxy podophyllotoxin production in *Linum flavum* cell suspension cultures after treatment with Na$_2$EDTA

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Submitted
Abstract

Treatment of cell suspension cultures of *Linum flavum* L. with Na$_2$EDTA reduced the coniferin and enhanced the 6-methoxypodophyllotoxin (6-MPT) production in a concentration-dependent way, in a range of 0.1 - 5 mM. On day 14 after treatment with Na$_2$EDTA, an inhibition of the coniferin production up to 88% was found (content in control cultures 120.7 mg g$^{-1}$ DW). The maximum enhancement of the 6-MPT production was 320% on day 7 after treatment with 5 mM Na$_2$EDTA (control value 0.16 mg g$^{-1}$ DW).

The reduction in coniferin accumulation in the suspension cultures was correlated with an inhibition of coniferyl alcohol glucosyltransferase (CAGT) activity as determined in cell homogenates. On day 14 after treatment with 2 and 5 mM Na$_2$EDTA the CAGT activity was inhibited up to > 89% (control value 8.8 μkat g$^{-1}$). The inhibitory effect of Na$_2$EDTA on CAGT was also shown in a partially purified enzyme preparation. Several metal ions such as Fe$^{3+}$, Cu$^{2+}$, Zn$^{2+}$, Li$^+$, and the elicitors nigeran and salicylic acid had no significant effect on the production of coniferin and 6-MPT.
Introduction

Podophyllotoxin and podophyllotoxin-derived lignans possess cytotoxic and antiviral activities. Teniposide and etoposide are semi-synthetic derivatives of podophyllotoxin that are clinically used as anticancer drugs (Moraes et al., 2002). Podophyllotoxin is also the starting compound for the rheumatoid arthritis drug CPH 82 (Reumacon) (Svensson and Pettersson, 2003). For the production of semi-synthetic podophyllotoxin derivatives on an industrial scale, podophyllotoxin is isolated from the rhizomes of *Podophyllum* plants from wild habitats, which are counted as endangered species (Smollny et al., 1998).

The use of biotechnological approaches to improve the production of podophyllotoxin or related lignans with plant cell and organ cultures, including the biotransformation of suitable precursors and the modification of biosynthetic pathways, is considered to be suitable and economically attractive (Giri and Narasu, 2000). Several investigations to enhance the production of podophyllotoxin-derived lignans by manipulation of cell and organ cultures have been carried out (Van Uden et al., 1990a, 1991a, Woerdenbag et al., 1990, Berim et al., 2005, Petersen and Alfermann, 2001). The production of podophyllotoxin, 6-methoxypodophyllotoxin (6-MPT) and its glucoside could be enhanced in cell cultures of *Podophyllum hexandrum* Royle (Woerdenbag et al., 1990), *Linum flavum* L. (Van Uden et al., 1990a, 1992, Molog et al., 2001), *Callitris drummondii* F. Mueller (Van Uden et al., 1990b), *Linum album* Kotschy (Seidel et al., 2002), and *Linum nodiflorum* (Berim et al., 2005).

Based on the close chemical resemblance with podophyllotoxin (see Fig. 1), 6-MPT is considered also as an interesting starting compound for the preparation of new semi-synthetic derivatives with antitumor properties. Cell cultures of *L. flavum* produce 6-MPT and its glucoside. The cytotoxicity of 6-MPT *in vitro* against tumor cell lines was comparable with that of podophyllotoxin (Middel et al., 1995).

Coniferyl alcohol is an early precursor of both lignins and lignans. The glucosylation of coniferyl alcohol yields coniferin that is accumulated endogenously in *L. flavum* cultures up to 12 % on a dry weight basis (Van Uden et al., 1991b). This reaction is catalysed by coniferyl alcohol glucosyltransferase (CAGT, EC number: 2.4.1.111). Lignans are formed through radical-mediated dimerisation of two coniferyl alcohol units. Blocking the branch leading to the formation of coniferin by inhibiting CAGT could result in an enhanced production of lignans, such as 6-MPT in the cell suspension cultures of *L. flavum*. High coniferin contents in the cell suspension culture correspond with low 6-MPT levels, as was demonstrated in feeding experiment with cell cultures of *P. hexandrum* (Van Uden et al., 1990a). It is known that Na$_2$EDTA (Ibrahim and Grisebach, 1976) and metal ions such as Cu$^{2+}$, Zn$^{2+}$, Li$^+$ and Fe$^{2+}$ (Schmid and Grisebach, 1982, Wunder and William, 1999, Reed et al., 1993) are able to inhibit the glucosyltransferase activity. Addition of one of these compounds may interfere with CAGT.

The aim of this paper is to explore the effect of Na$_2$EDTA and several inorganic salts on the production of coniferin and 6-MPT in cell suspension cultures of *L. flavum*. This effect is compared to that of the elicitors salicylic acid and nigeran. These compounds enhance various biosynthetic pathways, but so far there is little evidence of their effect on the lignan biosynthesis (Guan and Scandalios, 1995, Chong et al., 1999).
Materials and methods

Plant material and culture conditions

Cell suspensions of *Linum flavum* L. (Linaceae) leaves have been initiated and are maintained at the Department of Pharmaceutical Biology, University of Groningen. The cell suspensions are cultured routinely every two weeks by transferring 100 mL of fully grown suspension aseptically into 200 mL of fresh liquid medium. The medium is a MS (Murashige and Skoog 1962) containing 0.2 mg of indole-3-acetic acid (IAA) and 0.2 mg of 6-benzylaminopurine (BAP), purchased from Duchefa (Haarlem, the Netherlands). The cell suspensions were incubated on a rotary shaker (175 rpm) at 26°C under a day/night regime (16/8 h: 3,000 lux, day light L 36W/10, OSRAM, Germany).

Treatment of cell suspensions

Disodium ethylenediamine tetra acetate (Na$_2$EDTA) was purchased from Duchefa, copper (II) sulphate pentahydrate, zinc chloride, and lithium chloride from Merck (Darmstadt, Germany), iron (II) sulphate pentahydrate, salicylic acid from Sigma-Aldrich (Zwijndrecht, the Netherlands), and nigeran from Sigma, St. Louis (USA).

These compounds were added to the culture media used for the cell suspension cultures of *L. flavum* yielding the following final concentrations: Na$_2$EDTA : 0.01, 0.5, 1, 2 and 5 mM, Cu$^{2+}$, Zn$^{2+}$ and Li$^+$ : 0.1, 1 and 5 mM, Fe$^{2+}$ : 0.01, 0.1 and 1 mM, salicylic acid: 0.1, 0.5, 1 and 5 mM; nigeran 20 mg L$^{-1}$. Suspension-grown cells were harvested each 2 days during the growth cycle of 14 days. Samples of about 10 mL were taken aseptically and transferred into a calibrated conical tube and centrifuged for 5 min at 1,500 g. In order to monitor the viability and growth of the cell cultures, the medium pH and the conductance were routinely measured in the supernatant. The cells were filtered using Buchner funnel. Fresh weight (FW) was determined and the cells put overnight in the freezer and subsequently freeze dried. Dry weight (DW) was also determined. Coniferin and 6-MPT contents were subsequently analyzed by HPLC.

Extraction

About 100 mg, accurately weighed of freeze dried and powdered cell material were extracted by ultrasonification in 2 mL methanol (80%; v/v) for 1 h. Dichloromethane (4.0 mL) and water (4.0 mL) were added. The mixture was vortexed and centrifuged (5 min; 1,500 g). For the determination of the 6-MPT concentration, 2.0 mL of the dichloromethane phase were taken and evaporated to dryness. The residue was redissolved in 1.0 mL methanol and centrifuged. For the determination of coniferin 50 µL water phase were diluted with water until 1.0 mL and centrifuged (2 min; 10,000 g).

Treatment of aqueous phase with β-glucosidase

To confirm the coniferin production, the water phase was submitted to enzymatic hydrolysis. A 3.5% (w/v) solution of β-glucosidase (Sigma G-0395) was prepared in 0.1 M phosphate buffer, pH 5.0. To 2.0 mL samples of the water phase 0.5 mL was added, followed by incubation for 5 h at 37°C. The aglucone formed was extracted with 2.0 mL dichloromethane. Of the dichloromethane phase 1.5 mL were taken and evaporated to dryness. The residue was redissolved in 1.0 mL methanol and centrifuged (2 min; 10,000 g). Coniferyl alcohol was determined by HPLC.
Protein purification

Cells were harvested on day 1 after subculturing and stored at -20°C overnight. Frozen cells were suspended in an equal volume of the homogenisation buffer that consisted of 0.2 M Tris-HCl, pH 7.5, 5% polyvinylpyrrolidone, 0.2 % DOWEX® - 1*2 – 100, 0.1 % DTT (w/w) and 10% ethylene glycol. The mixture was homogenised using an ultraturrax (Janke & Kunkel, IKA-WERK, Staufen, Germany). The homogenate was filtered through Miracloth® and clarified by centrifugation for 20 min at 20,000 g. Proteins dissolved in the supernatant were then fractionated by (NH₄)₂SO₄ precipitation. The fraction obtained between 40 and 80% saturation was desalted on a HiPrep 26/10 desalting column (Amersham Biosciences, Uppsala, Sweden) previously equilibrated with 0.02 M Tris-HCl buffer, pH 7.5. The protein eluting from the desalting column was applied to a HiTrap DEAE FF column (Amersham Biosciences, Uppsala, Sweden) which had been equilibrated with 0.02 M Tris-HCl buffer, pH 7.5. The protein was eluted first with 100 mL of 0.02 M Tris-HCl buffer, followed by a linear gradient from 0.02 to 0.2 M Tris-HCl buffer and finally with 100 mL of 0.4 M Tris-HCl buffer, all at pH 7.5. Fractions of 5 mL each were collected at a flow rate of 1 mL min⁻¹ and assayed directly for CAGT activity. Fractions containing the highest activity were combined and concentrated by vivaspin 6 mL concentrator (Vivasciences, Hannover, Germany). The combined fractions with the highest CAGT activity resulting from the desalting column (the third step of the purification procedure, see Table 2) were exposed to Na₂EDTA. The Na₂EDTA was added to 5 mL of the partially purified CAGT yielding final concentrations of 0.1, 0.5, 1, 2 and 5 mM. The mixtures were incubated at 4°C for one day. 200 µL (3x) of the mixture were taken and submitted for CAGT assay.

CAGT assay

The enzyme assay for CAGT was developed from the methods used by Ibrahim et al. (1976) and Schmid et al. (1982). Cells were treated with Na₂EDTA in the range of 0.1-5 mM and harvested at different time points during the growth cycle and stored at -20°C overnight. Frozen cells, 2-3 g, were suspended in homogenisation buffer and the mixture was homogenised using an ultraturrax. The homogenate was centrifuged (3,000 g; 25 min, 2°C). The supernatant was separated from the pellet. The assay buffer was prepared containing 0.2 M Tris-HCl pH 7.5, DTT 0.1 %. The standard assay mixture consisted of 0.32 µmol coniferyl alcohol in 40 µL ethyleneglycol monomethylether, 0.32 µmol uridine diphosphate (UDP)-glucose in 40 µL assay buffer, 200 µL protein homogenate or partially purified CAGT and assay buffer in a total volume of 320 µL. The reaction was started by the addition of protein and vortexed for 5 s immediately followed by incubation for 30 min at 30°C. The reaction was stopped by adding 2.0 mL dichloromethane followed by vortexing the mixture for 20 s and centrifugation (5 min; 1,500 g). The dichloromethane and water layers were used for HPLC analysis of coniferyl alcohol and coniferin, respectively. Protein was determined using the Bradford assay (Bradford, 1976).

Analysis of coniferin, coniferyl alcohol and 6-MPT

Coniferin, coniferyl alcohol and 6-MPT were analyzed by HPLC. The HPLC system consisted of an ISCO Model 2350 pump, a Shimadzu photodiode array detector (Shimadzu, ’s-Hertogenbosch, the Netherlands), UV absorbance at 230 and 290 nm and LiChrocart RP-18 column (250 x 4.6 mm i.d.) (Merck, Darmstadt, Germany). The mobile phase for coniferyl alcohol and 6-MPT analysis was acetonitrile (LAB-SCAN Analytical-sciences, Dublin, Ireland) / water (40:60 v/v; 0.1% phosphoric acid) and for coniferin, methanol/water (30:70; 0.1 % phosphoric acid). Calibration curves were made using coniferyl alcohol (Sigma), coniferin and 6-MPT, which were isolated from L. flavum cell suspension cultures as published previously (van Uden et al., 1991b, 1992). For the statistical evaluation of the data the Sudent’s t-test was used. A P-value <0.05 was considered as significant.
Fig. 1. Chemical structures of podophyllotoxin (1), 6-methoxypodophyllotoxin (2), coniferyl alcohol (3) and coniferin (4).

Results and discussion

The effect of Na₂EDTA on the growth of *L. flavum* cell suspension cultures was determined on the basis of dry weight accumulation as shown in Fig. 2. The growth period of the cultures was 14 days. From day 1 after inoculation the cells grew until day 8. The stationary phase was reached between day 8 and 12. At the end of the period (day 14) the cell suspension was refreshed. There was no significant effect of Na₂EDTA on the growth of the cell suspensions or on the viability parameters at concentrations of 0.1, 0.5 and 1 mM (dry weight and conductivity). At a concentration of 2 and 5 mM, Na₂EDTA inhibited cell growth up to 22% and 59% respectively (maximal effect) on day 8.

In Fig. 3 the effect of treatment with Na₂EDTA on the coniferin production in *L. flavum* cells is shown. Untreated cells (control) contained up to 12.0% coniferin on a dry weight basis on day 14 of the growth cycle. After treatment with Na₂EDTA, the coniferin production was reduced in a concentration dependent way by 18-88% (Fig. 5) on day 14, although it should be noted that the higher concentrations of Na₂EDTA (2 and 5 mM) also inhibited the cell growth.

To confirm the coniferin production, the water phase that contained coniferin was submitted to enzymatic hydrolysis using β-glucosidase. This enzyme catalyses the hydrolysis of monolignol glucosidases, that lead to the release of the corresponding alcohols (Dharmawardhana et al., 1995). The coniferyl alcohol formed fully correlated with the coniferin content as found after hydrolysis.

The accumulation of 6-MPT was enhanced 1.2, 1.9 and 3.2 fold at a concentration of Na₂EDTA of 1, 2 and 5 mM respectively on day 7 in the cell suspensions of *L. flavum* (Fig. 4, Fig. 5). Adding 0.1 mM and 0.5 mM Na₂EDTA did not enhance the 6-MPT production.
Fig. 2. Growth of *L. flavum* cell suspension cultures after treatment with Na$_2$EDTA 5 mM (■), 2 mM (□), 1 mM (●), 0.5 mM (○), 0.1 mM (▲) and without Na$_2$EDTA as a control (Δ). Individual values expressed in g L$^{-1}$ are averages of three independent experiments as means ± standard deviation.

Fig. 3. Coniferin production in *L. flavum* cell suspension cultures after treatment with Na$_2$EDTA 5 mM (■), 2 mM (□), 1 mM (●), 0.5 mM (○), 0.1 mM (▲) and without Na$_2$EDTA as a control (Δ). Individual values expressed in mg g$^{-1}$ of dry weight are averages of three independent experiments as means ± standard deviation.
Fig. 4. 6-MPT production in L. flavum cell suspension cultures after treatment with Na$_2$EDTA 5 mM (■), 2 mM (□), 1 mM (●), 0.5 mM (○), 0.1 mM (▲) and without Na$_2$EDTA as a control (△). Individual values expressed in mg g$^{-1}$ of dry weight are averages of three independent experiments as means ± standard deviation.

Fig. 5. Inhibition of the coniferin production on day 14 (control = 120.7 mg g$^{-1}$ dry weight) (□) and enhancement of the 6-MPT production on the day 7 (control = 0.16 mg g$^{-1}$ dry weight) (■) in L. flavum cell suspension cultures after treatment with Na$_2$EDTA.

In the concentrations used, none of the metal ions (Cu$^{2+}$, Zn$^{2+}$, Li$^+$ and Fe$^{3+}$) inhibited the coniferin or enhanced the 6-MPT production in the cell suspension cultures of L. flavum. In contrast, these salts inhibited the growth of the cultures. Nigeran had no effect on the growth of the cell cultures, neither on the production of 6-MPT nor coniferin. Salicylic acid was lethal to the cell cultures at
concentrations of 2 and 5 mM. The concentrations of 0.1 and 0.5 mM salicylic acid had no effect on the growth of the cell cultures and neither on the production of 6-MPT nor coniferin.

The highest CAGT activity in cell suspension cultures was found on day 1 after inoculation. Untreated cells (control) had an activity 13.7 µkat g\(^{-1}\). The activity was reduced by 30–57 % 1 day after inoculation with 0.1–5 mM Na\(_2\)EDTA. On day 6 the control cells had a CAGT activity of 2.5 µkat g\(^{-1}\) and the concurrent inhibition of Na\(_2\)EDTA 0.1–5 mM was 12–52%. A significant decrease of the enzyme activity was found on day 13 and 14 after inoculation. Enzyme activity of untreated cell was 6.6 (day 13) and 8.8 µkat g\(^{-1}\) (day 14) and the inhibition by Na\(_2\)EDTA 0.1–5 mM was 4-79% and 14-89%, respectively (Table 1).

<table>
<thead>
<tr>
<th>Na(_2)EDTA (mM)</th>
<th>CAGT activity (µkat g(^{-1})) ± (SD) and % inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td></td>
<td>Activity (µkat g(^{-1}))</td>
</tr>
<tr>
<td>0</td>
<td>13.7 (±1.3)</td>
</tr>
<tr>
<td>0.1</td>
<td>9.6 (±2.4)</td>
</tr>
<tr>
<td>0.5</td>
<td>10.8 (±2.5)</td>
</tr>
<tr>
<td>1</td>
<td>7.5 (±1.6)</td>
</tr>
<tr>
<td>2</td>
<td>5.2 (±1.3)</td>
</tr>
<tr>
<td>5</td>
<td>5.9 (±1.0)</td>
</tr>
</tbody>
</table>

For CAGT purification, the enzyme was extracted from 1 day-old cell suspension cultures (when the highest CAGT activity was found). The purification procedure, summarized in Table 2, ultimately resulted in a 41.2-fold enhancement of the CAGT activity, 13.1 % recovery of total activity and a product with a specific activity of 256 µkat g\(^{-1}\) of protein. The amount of protein obtained however, was insufficient to carry out incubation experiments with Na\(_2\)EDTA. Therefore the fraction originating from the desalting step (see Table 2; no. 3, 11.4-fold purified) was used. 5 mM of Na\(_2\)EDTA inhibited the CAGT activity in this partially purified enzyme preparation, up to 53%.

Table 2. Subsequent preparation steps of the partially purified CAGT preparation.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Total activity (µkat)</th>
<th>Specific activity (µkat g(^{-1}) protein)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Crude extract</td>
<td>302.4</td>
<td>1.52</td>
<td>5</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>Ammonium sulphate precipitation</td>
<td>28</td>
<td>1.12</td>
<td>40</td>
<td>8</td>
<td>73</td>
</tr>
<tr>
<td>3</td>
<td>Desalting</td>
<td>4.4</td>
<td>0.25</td>
<td>56.8</td>
<td>11.4</td>
<td>16.4</td>
</tr>
<tr>
<td>4</td>
<td>HiTrap DEAE FF</td>
<td>0.8</td>
<td>0.20</td>
<td>256</td>
<td>41.2</td>
<td>13.1</td>
</tr>
</tbody>
</table>

CAGT is a glucosyltransferase that converts coniferyl alcohol into coniferin. In order to enhance the 6-MPT production in *L. flavum* cell suspension cultures, the formation of coniferin was blocked by inhibition of CAGT using several potential glucosyltransferase inhibitors. Na\(_2\)EDTA inhibited the production of coniferin in the cell suspension cultures of *L. flavum* in a concentration-dependent way, in a range of 0.1-5 mM. The results were confirmed by hydrolysis of the coniferin-containing water layer.
of the cell extract by enzymatic hydrolysis using β-glucosidase. Coniferin was completely converted into coniferyl alcohol and the concentration of the formed coniferyl alcohol related to the original coniferin concentration.

There was a correlation between the coniferin and the 6-MPT production in *L. flavum* cell suspension cultures. Higher coniferin contents corresponded with lower 6-MPT levels. This supports our hypothesis that blocking the branch leading to the formation of lignins by inhibition of glucosyltransferase may result in an enhanced production of lignans. By inhibiting the coniferin production, coniferyl alcohol accumulates and is available as a substrate to produce of 6-MPT and other lignans.

The high 6-MPT content on day 6-9 of the growth cycle (Fig. 4), correlates with the low CAGT content as measured on day 6 (Table 1). This is in agreement with earlier observations (Van Uden et al. 1991b), showing that a maximum coniferyl alcohol content in *L. flavum* cell suspension cultures was preceded by a maximal activity of the enzyme β-glucosidase.

At the beginning of the growth cycle of the control cell suspension cultures no clear relationship existed between CAGT activity and coniferin content. However, after day 6 it appeared that a low activity of CAGT related to a low coniferin content. CAGT activity then increased until day 14, with a simultaneous increase of the coniferin accumulation.

The highest CAGT activity in cell suspensions was found on day 1. Then it declined to a lower level on day 6 and re-increased on day 13 and 14. This is probably affected by β-glucosidase that converts coniferin into coniferyl alcohol. The reaction catalyzed by β-glucosidase is opposite to that of CAGT. In *L. flavum* cell suspension cultures, β-glucosidase activity increased to a maximal value on day 4 of the growth cycle and declined to lowest activity on day 14 (Van Uden et al., 1991b). A high CAGT activity apparently relates to a low β-glucosidase activity.

Reduction of the CAGT activity correlated with a reduction of the coniferin production in the cell suspensions. The coniferin production and CAGT activity were reduced to >88% at the end of a growth cycle after treatment with 5 mM Na₂EDTA, while control values of the coniferin production were at their maximum at this time point. These results strongly suggest that Na₂EDTA inhibits CAGT activity thereby inhibiting the conversion of coniferyl alcohol into coniferin in *L. flavum* cell suspension cultures. Our hypothesis that Na₂EDTA (0.5 mM) is an inhibitor of CAGT activity is further supported by the inhibitory effect on CAGT activity in an 11.4-fold purified enzyme preparation. The effect, in terms of percentage inhibition, however, is less pronounced than in the cell suspensions. This difference may be due to a toxic effect of Na₂EDTA on the cell suspension cultures.

In conclusion, Na₂EDTA appears to be an inhibitor of CAGT activity both *in vivo* and *in situ*. Because of a lack of information about the structure and the function of the CAGT it is not yet clear which mechanism underlies the inhibition by EDTA. If CAGT needs a metal ion as a co-factor for its activity, it can be understood that the Na₂EDTA complexes with the metal ion, thereby reducing the enzyme activity. Further studies directed to the purification, structure and function determination of the enzyme are in progress.

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