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UDP-glucose:(6-methoxy)podophyllotoxin 7-*O*-glucosyltransferase from suspension cultures of *Linum nodiflorum*

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Dedicated to Prof. Dr. A. Wilhelm Alfermann on the occasion of his 65th birthday

Abstract

Cell cultures of *Linum* species store 6-methoxypodophyllotoxin (MPTOX), podophyllotoxin (PTOX) and related lignans as *O*-glucosides. UDP-glucose:(M)PTOX 7-*O*-glucosyltransferase has been detected and characterised in protein preparations of suspension-cultured cells of *Linum nodiflorum* L. (Linaceae). The maximal lignan glucoside contents in the cells are preceded by a rapid increase of the specific glucosyltransferase activity on day six of the culture period. MPTOX glucoside is the major lignan with up to 1.18 mg g^{-1} of the cell dry wt which is more than 30-fold of the PTOX glucoside content. Of the three aryltetralin lignans tested as substrates, PTOX and MPTOX display comparable apparent K_m values of 4.7 and 5.4 μM, respectively. 5′-Demethoxy-6-methoxypodophyllotoxin is converted with the highest velocity of 25.2 pkat mg^{-1} while also possessing a higher K_m of 14.7 μM. Two-substrate test series indicate that all three compounds compete for the active site of a single protein. The structurally similar lignan β-peltatin acts as competitive inhibitor as well. However, the 6-*O*-glucosidation is most likely catalysed by a separate enzyme. The (M)PTOX 7-*O*-glucosyltransferase works best at a pH around 9 and a temperature around 35 °C. A 15–30% increase of the reaction rate is effected by the addition of 0.9 mM Mn^{2+}.

Keywords: *Linum nodiflorum* L.; Linaceae; Lignans; Glucosyltransferase; 6-Methoxypodophyllotoxin; Podophyllotoxin; β-Peltatin

1. Introduction

Suspension cultures of selected *Linum* species (Linaceae) accumulate 6-methoxypodophyllotoxin (MPTOX) and podophyllotoxin (PTOX) as glycosides alongside structurally similar congeners (Fig. 1). They have been employed for lignan biosynthesis research for more than a decade now (Petersen and Alfermann, 2001; Fuss, 2003). Several enzyme activities were identified in cell-free protein extracts from *Linum* cells, e.g. deoxypodophyllotoxin 6-hydroxylase and β-peltatin 6-*O*-methyltransferase (Molog et al., 2001; Kuhlmann et al., 2002; Kranz and Petersen, 2003), helping to propose a (tentative) biogenetic pathway. Its last step is the glucosidation at the 7-OH moiety. Glycosidation increases the water solubility and enables vacuolar storage, thus reducing lignan toxicity for the producing cell (Berlin et al., 1988; Middel et al., 1995; Stähelin and von Wartburg, 1991). Once stored away, the hydrophilic glucosides are unable to cross the tonoplast by diffusion (Bowles et al., 2006). Upon damage of the cell compartmentation,

Abbreviations: CAD, cinnamyl alcohol dehydrogenase; GT, glucosyltransferase; G6PDH, glucose-6-phosphate dehydrogenase; MPTOX, 6-methoxypodophyllotoxin; PTOX, podophyllotoxin; 5′-dMPTOX, 5′-demethoxy-6-methoxypodophyllotoxin; UDPG, uridine diphosphoglucose.

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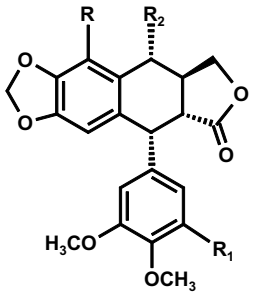

R = H, R_1 = OCH_3, R_2 = OH	PTOX
R = OH, R_1 = OCH_3, R_2 = H	β-peltatin
R = OCH_3, R_1 = OCH_3, R_2 = OH	MPTOX
R = OCH_3, R_1 = H, R_2 = OH	5′-dMPTOX

Fig. 1. Structures of aryltetralin lignans used as substrates and inhibitors.

glucosides are hydrolysed back into the bioactive form by a specific endogenous cytosolic glucosidase present both in *Linum* and *Podophyllum* cells (Berlin et al., 1988; Dayan et al., 2003). Increased glucosidation rates might therefore result in enhanced lignan production. To date there are, to our awareness, no definite reports on the nature of (M)PTOX 7-*O*-glucosyltransferase(s) in either *Podophyllum* or *Linum* species. The knowledge of elementary enzyme properties and assay optimisation provides an indispensable basis for the purification and molecular studies of any protein. We therefore set out to demonstrate the activity and to document some essential features of (M)PTOX 7-*O*-glucosyltransferase from *Linum nodiflorum*.

2. Results and discussion

Glycosidation is the last step of (M)PTOX glucoside formation in producing plants (Kuhlmann et al., 2002). The glucosides are stored in the vacuole as shown for PTOX glucoside in suspension cells of *Linum album* (Henges, 1999). Hitherto, only the glucosidation of PTOX exogenously fed to *Linum flavum* cells has been described (Van Uden et al., 1993). This conversion might as well have occurred as a part of a (xenobiotic) detoxification process and therefore did not explicitly demonstrate the specificity of the enzyme catalysing this reaction. We here report on the *in vitro* detection and primary characterisation of the corresponding specific UDP-glucose-dependent (M)PTOX 7-*O*-glucosyltransferase (E.C. 2.4.1.-) in suspension-cultured cells of *L. nodiflorum*.

2.1. Reaction of (M)PTOX 7-O-glucosyltransferase

Incubation of a crude protein extract from suspension-cultured cells of *L. nodiflorum* with the designated substrates PTOX, MPTOX and 5′-demethoxy-6-methoxypodophyllotoxin (5′-dMPTOX) (see Fig. 1 for structures) resulted in a UDP-glucose-(UDPG) dependent formation of new more hydrophilic substances. The identity of PTOX 7-*O*-glucoside was confirmed by LC-NMR, the structures of the reaction products with MPTOX and 5′-dMPTOX as substrates by LC–MS (see below). The β-glucosidal anomeric configuration of the products could be additionally shown by enzymatic hydrolysis with substrate-unspecific α- and β-glucosidases. Solely β-glucosidase was able to cleave the reaction product. To exclude chemical degradation, controls containing no enzymes were run.

Protein preparations from non-PTOX-producing species (*Melissa officinalis*, *Justitia hyssopifolia*, *Anthoceros agrestis*) could not catalyse the same reaction under identical conditions. As expected, active glucosidation was detected in protein extracts from the cells of two other (M)PTOX-accumulating *Linum* species, *L. album* and *L. flavum* (data not shown).

The acceptance of two potential sugar donors, ADP- and UDP-glucose, was tested showing that adenosine diphosphoglucose participating in starch synthesis *in planta* could not substitute for UDPG. To date, almost exclusively UPD-activated sugars have been reported as sugar donors for small molecule glycosidation (Bowles et al., 2006).

The reaction products were routinely not partitioned into organic solvent, but were analysed after stopping the assay by addition of 40 μl ice-cold acetonitrile and chilling on ice. We, however, probed the EtOAc solubility of the glucosides. It took two subsequent extraction steps with double the volume of EtOAc to recover about 80% of PTOX-glucoside from the aq. phase. In comparison, almost 90% of the more lipophilic MPTOX-glucoside were extracted by the same procedure.

2.2. Structure determination of PTOX 7-O-glucoside

PTOX 7-*O*-glucoside was identified as the reaction product by HPLC-SPE-^{1}H NMR spectroscopy. The earliest hints were provided by the chromatographic behaviour consistent with the glucoside's higher polarity and the similarity of the UV spectrum to that of the substrate. The ^{1}H NMR spectrum of the enzyme product, compared to that of the aglycone, displayed additional signals assignable to a glucose moiety. The coupling constant ($J = 6.7$ Hz) of the anomeric H-1″ revealed the β-configuration of the glucose unit, already suggested by the enzymatic digestion. This also indicated the *O*-glycosidation later confirmed by the NMR data (see Section 4).

Glucosidation of MPTOX and 5′-dMPTOX was proven by LC-MS (see Section 4).

2.3. Biochemical characterization

The activity of (M)PTOX 7-*O*-glucosyltransferase strongly depends on the pH. While being negligible at pH 7, it is half-maximal around pH 8 and highest at pH 9 and above, as measured in 100 mM Tris/HCl at 25 °C (see Supplementary Fig. 1). The preference for a slightly basic milieu is a known feature of many glucosyltransferases (Vogt, 2000). In the case of (M)PTOX this is essential in a

cellular context. The glucosides should be transformed back into the aglyca upon the destruction of the vacuole which also results in acidification. The already reported endogenous glucosidase from *L. flavum* acts only under non-basic conditions and is virtually inactive at pH-values above 8 (Berlin et al., 1988). Similar characteristics were found for the corresponding specific β-glucosidase from *Podophyllum peltatum* (Dayan et al., 2003). Stringent pH-requirements of the antagonistic proteins thus ensure the balance between the toxic aglyca and the sugar conjugates. Several factors had to be considered when choosing the reaction conditions. pH-values above 8.5 are hardly physiological. Moreover, the lactone ring of lignans is significantly unstable at high pH and temp. (Ayres and Loike, 1990). On the other side, the product cleavage by action of the lignan glucosidase(s) has to be prevented. All characterisation assays were therefore conducted at pH 8.5 which is close to the pH-optimum and at 25 °C, both to extend the linear reaction rate time span and to prevent lignan decomposition.

Several buffers (0.1 M glycine/NaOH, 0.1 M K-Pi, 0.2 M Bicin, 0.05 M Tricin and Britton–Robinson buffer) were compared to 250 mM Tris/HCl at the same pH of 8.5. Relative activities in Tricin, the second-best system of those tested, barely exceeded 7% of that in Tris/HCl, followed by Britton-Robinson (ca. 6%) and Bicin (ca. 4%). This pronounced buffer effect was the same for all three used substrates. Assays in Tris/HCl of the same pH but varied Tris concentrations indicated marginal advantage of 250 mM buffer (data not presented), which was therefore mostly used, e.g., for determination of enzyme kinetics and inhibition studies. The temperature optimum was determined to be at 35 °C (see Supplementary Fig. 1).

The glucosyltransferase protein could be concentrated by fractionated $(NH_4)_2SO_4$ precipitation. The specific activity of the fraction precipitating between 50% and 70% saturation of $(NH_4)_2SO_4$ was 1.5–1.8-fold of that measured in the crude extract, the total activity in this fraction corresponded to 33–37% of that in original crude extracts. For comparison, the activity recovery from combined protein precipitating at 0–70% $(NH_4)_2SO_4$ saturation was 54–59% of the original.

Up to four freeze-thaw cycles with intermediate storage at −80 °C did not significantly reduce the enzyme activity in desalted extracts. Once frozen, it maintained >90% of the original activity even after 11 weeks of storage.

The effect of several divalent cations on the reaction rate was tested. Over 80% inhibition was found for 0.9 mM Cu^{2+} and Zn^{2+} as is common for many glycosyltransferases (Vogt, 2000). Surprisingly, Mn^{2+} supplied at 0.45–0.9 mM accelerated the reaction by up to 30%. Only few glucosyltransferases involved in plant secondary metabolism are known to be slightly stimulated by divalent ions (Vogt, 2000; Hasegawa et al., 1997). EDTA (2.25 mM) does not measurably reduce the reaction rates. A distinct Mg^{2+}- or Mn^{2+}-dependence is so far only reported for several macromolecule synthesising GTs (e.g., Konishi et al., 2004; Leonard et al., 2005). Remarkably, most of these enzymes are apparently membrane-bound or -associated. GTs of plant small molecules are predominantly cytosolic (Jones and Vogt, 2001). Judging by the high pH required for catalysis, a vacuolar localisation appears unlikely for (M)PTOX 7-*O*-glucosyltransferase. Microsomes prepared by Mg^{2+}-precipitation also catalysed the reaction. However, the specific activities were only about 10% of those found in the original crude extract and the supernatant left after membrane sedimentation. The activities of typically cytosolic enzymes such as glucose-6-phosphate dehydrogenase (G6PDH) and cinnamyl alcohol dehydrogenase (CAD) showed a similar distribution into the microsomal fraction. Of the specific cytosolic G6PDH- and CAD-activites (10.7 nkat mg^{-1} protein and 188.6 pkat mg^{-1} protein, respectively), roughly 10% were detected in the $MgCl_2$-precipitate. Obviously, the glucosyltransferase activity measured in microsomes merely reflects the grade of contamination with soluble protein.

The presence of Triton X-100 (0.1%) in the extraction and assay buffers was slightly stimulating for the glucosidation rates of MPTOX and 5′-dMPTOX. Higher detergent concentrations severely reduced the turnover of 5′-dMPTOX while affecting the glucosidation of both other substrates to a lesser extent (Table 1). Tested with PTOX as substrate, the addition of Mn^{2+} remains stimulating in the detergent's presence. The differential effect of Triton X-100 upon the turnover rates would so far be the only reason to consider that more than one enzyme might catalyse the reactions with the three above-mentioned lignans. Based on the unequal reaction impairment by non-ionic detergents together with dissimilar inhibitor behaviour Paczkowski et al. (1998) argumented that two different proteins were involved in sterol 3-*O*-glucosidation in eggplant.

2.4. Substrate specificity

Three relevant lignans (PTOX, MPTOX, 5′-dMPTOX; Fig. 1) were used as potential substrates. In particular, we were interested in the substrate preference of the glucosyltransferase. The lowest apparent K_m value of 4.7 ± 1.0 μM was measured for PTOX followed by 5.4 ± 0.9 μM for MPTOX (Table 2). 5′-Demethoxy-6-methoxypodophyllotoxin (5′-dMPTOX) differs from

Table 1
Differential effect of Triton X-100 on the turnover rates of tested substrates

Triton X-100 [%]	Substrate			
	PTOX (%)	PTOX + 0.9 mM Mn^{2+} (%)	MPTOX (%)	5′-dMPTOX (%)
0.1	88.8	110.3	106.6	102.8
0.5	66.8	77.7	87.8	50.5
1.0	55.8	66.1	76.8	32.1

Parallel assays containing 0.9 mM Mn^{2+} were run with PTOX. Means of four independent tests are presented. 100% (no Triton X-100) is 3.5 pkat mg^{-1} (PTOX), 23.6 pkat mg^{-1} (5′-dMPTOX), 16.9 pkat mg^{-1} (MPTOX) and 4.2 pkat mg^{-1} (PTOX + Mn^{2+}).

Table 2
Michaelis–Menten-constants (K_m) and maximal velocities (V_{max}) for the lignan substrates

Substrate	Apparent K_m (μM)	V_{max} (pkat mg^{-1})	V_{max}/K_m
PTOX	4.0 ± 1.0	3.3 ± 0.1	0.71
MPTOX	5.4 ± 0.9	18.2 ± 1.6	3.37
5′-dMPTOX	14.8 ± 2.1	25.2 ± 3.7	1.71

Presented values are means ± s.d. ($n = 5$–7).

both other tested lignans by the substitution pattern of the pendant ring. This appears to be of consequence for the substrate binding as 5′-dMPTOX displays both the highest Michaelis–Menten-constant (14.8 ± 2.1 μM) and the highest reaction velocity. Calculating the V_{max}/K_m ratios reveals MPTOX as the most efficiently transformed lignan. The results indicated by the determination of K_m values were corroborated by two-substrate competition test series. Dixon inhibition diagrams disclosed a behaviour typical of full competitors when MPTOX and PTOX were tested, all plots intercepting at 8–10 μM which is only slightly above the determined K_m for PTOX (Fig. 2). Accordingly, solely

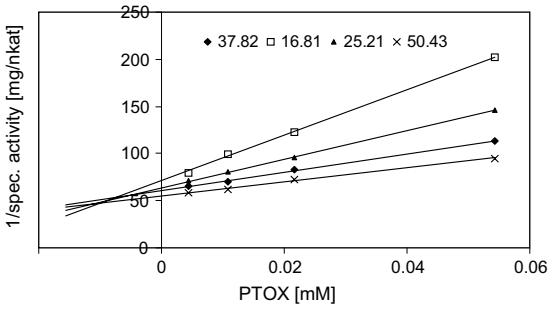

Fig. 2. Inhibition of MPTOX glucosidation by PTOX. MPTOX concentrations [μM] are indicated in the legend. The intercept served for the graphical estimation of a K_i value at 8–10 μM.

Table 3
Relative residual activities in competition tests

Substrate	Inhibitor	Residual activity (%)	Total activity (%)
A			
PTOX [108.7 μM]	MPTOX	47.1 ± 0.04	103.5
5′-dMPTOX [108.7 μM]		16.7 ± 0.01	102.1
MPTOX [108.7 μM]	PTOX	56.4 ± 0.05	
	5′-dMPTOX	85.4 ± 0.01	
B			
MPTOX [50.0 μM]	β-peltatin	95.0 ± 7.2	183.9
5′-dMPTOX [54.4 μM]		65.8 ± 2.9	149.0
PTOX [54.4 μM]		97.9 ± 3.5	184.2
β-peltatin [54.4 μM]	MPTOX	88.8 ± 6.2	
	5′-dMPTOX	83.1 ± 5.0	
	PTOX	86.3 ± 6.4	

Final concentrations are given in square brackets. Presented data are average ± s.d. ($n = 3$).

the distribution of the total turnover but no additive effect was observed when two substrates were supplied simultaneously at saturation concentrations (Table 3A). The corresponding plots for the interference of 5′-dMPTOX and MPTOX implied the same for these substrates. With 5–7 μM, the intercept lay here within the K_m range of MPTOX. From these results it is not possible to define "the genuine" substrate of the enzyme. The obtained kinetic data suggest that only one protein is responsible for the glucosidation of all three compounds. The inhibition of betanidin 5- and 6-glucosidation by flavonoids was studied similarly by Vogt et al. (1997) to consolidate the activity of the glucosyltransferases towards both, betanidin and flavonoids, which was later irrevocably proven by cloning and heterologous expression (Vogt et al., 1999).

The lower enzyme affinity towards the sugar donor UDP-glucose is reflected by its apparent K_m of 231.2 ± 41.9 μM which matches well with presently available data for other glucosyltransferases (Vogt, 2000).

2.5. Inhibition by β-peltatin

β-Peltatin, a postulated intermediate of the biosynthetic route to MPTOX in *Linum* species, served as an alternative inhibitor. Merely the position of the hydroxyl group in position 6 (aromatic ring) instead of 7 (aliphatic ring) makes up the structural difference from PTOX (Fig. 1). β-Peltatin glucoside was found in leaf and stem tissue of *Linum capitatum* (Broomhead and Dewick, 1990). Moreover, Koulman et al. (2003) proposed a transient storage of exogenously fed deoxypodophyllotoxin as β-peltatin glucoside in *L. flavum* cell cultures prior to conversion into MPTOX glucoside. A rapid UDPG- and enzyme-dependent formation of a new compound was measurable in the protein preparations used for PTOX 7-*O*-glucosyltransferase assays when β-peltatin was offered as substrate. Treatment of this compound with β-glucosidase yielded β-peltatin. The rates of both glucosidation reactions at saturating concentrations of two substrates, β-peltatin together with PTOX or MPTOX, were barely affected. The total of residual relative turnovers amounted to as much as 180% of both carried out independently (Table 3B). Predictably, this effect was weaker with 5′-dMPTOX as substrate due to the higher apparent K_m value for this compound. This suggested the presence of at least two distinct enzymes responsible for the 6-*O*- and 7-*O*-glucosidation of β-peltatin- and PTOX-like substrates, respectively. Several other discrepant characteristics confirm this notion. β-Peltatin appears though to suppress the glucosidation of PTOX, MPTOX and 5′-dMPTOX by blocking the active site. Inhibition constants for β-peltatin were derived graphically from Dixon plots using 5′-dMPTOX and MPTOX (Fig. 3) as substrates. The determined values of about 20 μM with the former and 28 μM with the latter are similar, thus lending further support to the hypothesis that the 7-*O*-glucosidation of both substrates is catalysed by one

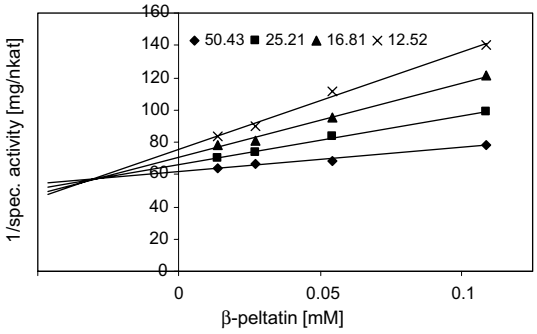

Fig. 3. Dixon plot for the competitive inhibition of MPTOX glucosidation by β-peltatin. MPTOX was supplied at the concentrations indicated in the legend. A K_i value of 28 μM was determined for β-peltatin.

protein. Analogously, MPTOX and 5′-dMPTOX interfere with the conversion of β-peltatin. The interaction of β-peltatin with PTOX-turnover was not kinetically evaluated due to the large difference in reaction rates of 6-*O*- and 7-*O*-glucosidation inevitably leading to a significant decrease of the inhibitor concentration during the assay incubation. In contrast to PTOX and derivatives, the glucosylated hydroxyl group of β-peltatin is phenolic. It is therefore plausible that the cells possess enzymes specific enough to discern between the two substrate types. In fact, numerous studies showed that glucosyltransferases are highly regio- while less core-specific enzymes (Vogt and Jones, 2000; Bowles et al., 2006). The glucosidation of β-peltatin will be characterised separately.

2.6. Enzyme activity and accumulation of lignan glucosides in suspension cultures of L. nodiflorum

The time courses of 6-*O*- and 7-*O*-glucosyltransferase activities during the culture period of *L. nodiflorum* cell cultures were recorded and compared to the lignan glucoside content of the same cells. All three designated substrates were present in cell extracts. MPTOX-glucoside dominated with up to 1.18 mg g^{-1} dry wt whereas only very small amounts of PTOX-glucoside were found. The concentration of 5′-dMPTOX glucoside rose to ca. 0.16 mg g^{-1} dry wt. Solely glucosides were detected in plant material extracted under non-hydrolysing conditions. Smollny et al. (1998) found about 2% of the total PTOX content as aglycon in *Linum album* cell cultures. Such amounts would be below the detection level in our procedure. Generally, this observation stresses the importance of detoxification and compartmentalisation of the cell-toxic lignans after glycosidation for plant cells.

A direct correlation between the specific 7-*O*-glucosyltransferase activity in crude protein extracts and the cellular lignan glucoside concentration is demonstrated in Fig. 4. Upon inoculation, a concomitant initial decrease of the glucosidation rates and (5′-d)MPTOX production is measured. Thereafter, the enzyme activity reaches its maximum shortly before the highest amounts of lignan glycosides are accumulated. The GT activity is rather rapidly

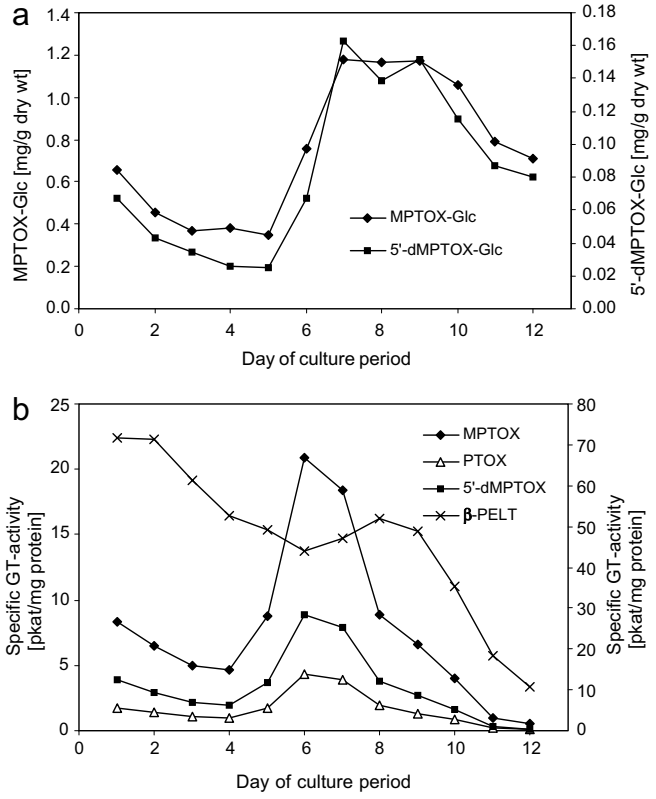

Fig. 4. Time-course of glucosyltransferase activity and lignan glucoside accumulation in suspension cultures of *Linum nodiflorum*. Presented results are means of two culture periods. (a) Accumulation of MPTOX- and 5′-dMPTOX-glucosides. (b) Specific glucosyltransferase activity with PTOX, MPTOX, 5′-dMPTOX and β-peltatin (β-PELT). The reaction rates of β-peltatin and 5′-dMPTOX are scaled on the right *Y*-axis.

lost while the lignan glucosides stay stored in the cells for several days. The turnover rate ratios between 5′-dMPTOX, MPTOX and PTOX remain strictly parallel throughout the monitored time (Fig. 4b). This provides another point in proof of only one enzyme catalysing these conversions. The cells are subcultured weekly, explaining the high starting values on day 1 of the culture period. Only the changes of 5′-dMPTOX- and MPTOX-glucoside amounts are plotted in Fig. 4a. The PTOX-glucoside content followed the general pattern but did not exceed 0.044 mg g^{-1} of the cell dry wt which was reached on day 8 of the monitored time span.

In contrast, the time course of β-peltatin glucosidation rates is distinctly different (Fig. 4a). The graph of β-peltatin glucosyltransferase activity shares but little similarity with 7-*O*-glucosidation curves of (5′dM)PTOX.

3. Conclusion

Our study establishes a standard assay and describes the properties of a specific UDP-glucose:(M)PTOX 7-*O*-glucosyltransferase in cell-free protein extracts of *L. nodiflorum* suspension cultures. This enzymatic activity is expected to be essential for the vacuolar storage of the otherwise toxic

lignans by the living plant cells and is shown to correlate with lignan glucoside accumulation. The kinetic constants reflect the high substrate affinity for PTOX and MPTOX and a lower one for 5′-dMPTOX. There is evidence that all three are glucosylated by the same protein. The reaction is competitively inhibited by β-peltatin. Judging by the observed additive effects, it is proposed that not PTOX 7-*O*-glucosyltransferase primarily catalyses the glycosidation of β-peltatin but a distinct 6-*O*-glucosyltransferase. The unequivocal proof of substrate acceptances and preferences must await the molecular cloning and heterologous expression of the respective glucosyltransferase(s).

4. Experimental

4.1. Chemicals and plant material

(−)-Podophyllotoxin was purchased from Roth (Karlsruhe, Germany), 6-methoxypodophyllotoxin and 5′-demethoxy-6-methoxypodophyllotoxin were purified as described previously (Berim et al., 2005), β-Peltatin was kindly donated by Prof. Medarde, Salamanca, Spain. Uridine- and adenosine-5′-diphosphoglucose were from Fluka (Sigma–Aldrich, Taufkirchen, Germany). All other solvents and chemicals were purchased from Roth (Karlsruhe, Germany) in analytical grade.

Suspension cell cultures of *L. nodiflorum* were maintained as described previously (Kranz and Petersen, 2003). Other cell cultures mentioned were from our laboratory collection.

4.2. Preparation of crude protein extracts

Suspension-cultured cells of *L. nodiflorum* (cell line "nod S") were harvested by suction filtration. For later use they were frozen in liquid nitrogen and stored at −80 °C. For protein extraction, 10% of the cell fr. wt of Polyclar 10 and 1 ml g^{-1} extraction buffer (100 mM Tris/HCl pH 7.5, 1 mM dithiothreitol (DTT)) were added. Homogenisation was performed for 2×20 s with intermediate cooling on ice using an Ultra-Turrax (Janke & Kunkel, Staufen im Breisgau, Germany). The supernatant after a 20 min centrifugation at 10,000*g* and 4 °C was desalted and changed into appropriate buffers by passing through a PD-10 column (Sephadex G-25, Pharmacia, Freiburg, Germany). After monitoring the specific activity of (M)PTOX 7-*O*-glucosyltransferase over 12 days after inoculation, 7-day-old cells were routinely used as plant material for protein extraction.

4.3. Preparation of microsomes

Crude protein extraction was performed as above. Microsomes were then precipitated as described by Petersen and Seitz (1985). The crude extract was adjusted to 50 mM $MgCl_2$ by adding an appropriate volume of 1 M $MgCl_2$ solution, stirred on ice for 20 min and centrifuged at 37,500*g* and 4 °C for 20 min. The precipitated membranes were resuspended in 2.7 ml extraction buffer using a Potter–Elvehjem homogeniser and desalted as above.

4.4. Determination of protein concentrations

Protein concentrations were measured according to Bradford (1976) using bovine serum albumin as standard. Appropriate controls were run when extraction buffers contained varying Triton X-100 amounts.

4.5. Enzyme assays

Standard assays had a final volume of 110 μl containing up to 120 μg protein in 100 μl 250 mM Tris/HCl, 1 mM DTT, pH 8.5, 5 μl 50 mM UDPG (final conc. 2.3 mM), and 5 μl 1.25 mM substrate in 50% aq. MeOH (final conc. 56.8 μM). Standard incubations were performed at 25 °C. Linearity of the reaction rates under assay conditions was verified. Assay mixes were pre-warmed for 5 min prior to start. The reaction was stopped by adding 40 μl ice-cold MeCN and chilling on ice. If not analysed immediately, assays were stored at −20 °C. Prior to HPLC-analysis all samples were centrifuged for 5 min at 16,100*g*.

For the determination of kinetic properties of PTOX, MPTOX and 5′-dMPTOX, UDPG was supplied at a fixed concentration of 4.6 mM while lignan concentrations ranged from 3 to 114 μM. K_m and V_{max} of the sugar donor were measured with 56.8 μM PTOX as substrate and 15–4500 μM UDPG. Reported constants were derived from Lineweaver–Burk plots.

The final volume of competition as well as ion-influence assays was expanded to 115 μl due to the addition of a second substrate/salt solution. To ensure complete saturation, double-concentrated UDPG solution (5 μl 100 mM, final conc. 4.3 mM) was provided when β-peltatin was involved as second substrate.

To measure the partitioning of the glucosides into organic solvent, the reaction was stopped by adding double the volume of EtOAc instead of MeCN and mixing for 30 s. Three subsequent organic phases were collected separately to monitor the extraction progress. For comparison, parallel control assays were run as above.

4.6. Hydrolysis of reaction products with glucosidases

Samples containing only the lignan glucosides were prepared by incubating the test mix with higher amounts of protein for several hours and extracting the reaction products with EtOAc thrice. After evaporation of EtOAc, dry residues were redissolved in 50% aq. MeOH. An aliquot of this soln was diluted for HPLC-analysis. Further aliquots were mixed with 220 μl 100 mM morpholinoethanesulfonate buffer (pH 5.5) or acidified H_2O (pH 5.0 with phosphoric acid) and 0.2 mg β-glucosidase (from almonds, Roth, Karlsruhe, Germany) in 20 μl H_2O and incubated at

35 °C for 35 min or longer. Alternatively, 1 U of α-glucosidase (type IV from yeast, Sigma) and 220 µl 100 mM Tris/HCl pH 6.8 were supplied, the incubation lasted over night. Blind controls contained H_2O instead of the enzymes. Reaction products were again extracted with 3×500 µl EtOAc, the organic solvent evaporated and the dry residues suspended in 50% aq. MeOH to yield the same concentration as the unhydrolysed extracts.

4.7. Structure elucidation of PTOX 7-O-glucoside

Several enzyme assays were extracted with EtOAc, the organic phases evaporated to dryness, redissolved in MeOH, and subjected to reversed phase isocratic HPLC on a LiChrospher 100 RP-18 column using MeCN-H_2O 30:70 with 0.1% TFA as a solvent at a flow rate of 1.5 ml min^{-1}, the monitoring wavelengths were 220 nm and 254 nm. The HPLC system was connected to a Prospekt 2 SPE unit (Spark Holland), which was used for trapping. The post-column eluent flow was diluted with H_2O by a makeup pump before trapping the peak eluting at R_t 6.1 min on a poly(divinylbenzene) SPE cartridge (HySphere resin GP, Spark Holland). The SPE device was coupled to a Bruker Avance 500 MHz spectrometer equipped with a Cryofit™ flow insert (30 µl active volume). The cartridge containing the trapped peak was dried with a stream of N_2 for 30 min. Then the compound was eluted with MeCN-d_3 and directly transferred to the NMR for data acquisition at 300 K. A ^{1}H NMR spectrum was measured in MeCN-d_3.

In order to compare the ^{1}H NMR spectra of the substrate and product directly, PTOX was measured in the same solvent, MeCN-d_3, which was used for LC-SPE-NMR of the enzyme product. The PTOX sample was measured in a conventional 5 mm tube instead of using the Cryofit™ flow insert. In addition to ^{1}H NMR, ^{1}H,^{1}H homocorrelation (COSY) and ^{1}H, ^{13}C heterocorrelation spectra (HMBC, HMQC) of PTOX were measured for unambiguous assignment of proton signals.

PTOX-Glc: ^{1}H NMR (500 MHz; MeCN-d_3): δ 7.32 (1H, *br s*, H-6), 6.46 (1H, *s*, H-3), 6.19 (2H, s, H-2′/6′), 5.96 (1H, *d*, $J = 1.0$ Hz, O–CH_2–O), 5.94 (1H, *d*, $J = 1.0$ Hz, O–CH_2–O), 4.97 (1H, *d*, $J = 9.4$ Hz, H-7), 4.59 (1H, *dd*, $J = 8.8$, 7.0 Hz, H-9a), 4.55 (1H, *d*, $J = 4.6$ Hz, H-7′), 4.38 (1H, *d*, $J = 6.7$ Hz, H-1″), 4.17 (1H, *dd*, $J = 8.8$, 10.1 Hz, H-9b), 3.78 (1H, *dd*, $J = 11.8$, 2.5 Hz, H-6″a), 3.69 (6H, *s*, OCH_3-3′/5′), 3.68 (3H, *s*, OCH_3-4′), 3.64 (1H, *dd*, $J = 11.8$, 5.6 Hz, H-6″b), 3.34–3.16 (4H, *m*, H-2″-H-5″), 3.00 (1H, *dd*, $J = 14.5$, 4.5 Hz, H-8′), 2.92 (1H, *dddd*, $J = 14.5$, 10.1, 9.4, 7.0 Hz, H-8).

PTOX: ^{1}H NMR (500 MHz; MeCN-d_3): δ 7.12 (1H, *d*, $J = 0.9$ Hz, H-6), 6.44 (1H, *s*, H-3), 6.39 (2H, s, H-2′/6′), 5.94 (1H, *d*, $J = 2.0$ Hz, O–CH_2–O), 5.93 (1H, *d*, $J = 2.0$ Hz, O–CH_2–O), 4.67 (1H, *dd*, $J = 9.5$, 7.3 Hz, H-7), 4.51 (1H, *dd*, $J = 8.5$, 7.2 Hz, H-9a), 4.54 (1H, *br d*, $J = 5.2$ Hz, H-7′), 4.08 (1H, *dd*, $J = 8.5$, 10.4 Hz, H-9b), 3.84 (1H, *d*, $J = 7.3$ Hz, OH-7), 3.69 (6H, *s*, OCH_3-3′/5′), 3.67 (3H, *s*, OCH_3-4′), 2.97 (1H, *dd*, $J = 14.4$, 5.2 Hz, H-8′), 2.73 (1H, *dddd*, $J = 14.4$, 10.4, 9.5, 7.2 Hz, H-8).

4.8. Analysis of MPTOX and 5′-dMPTOX glucosides by LC–MS

HPLC-ESI/MS analysis was performed with a Finnigan LCQ Deca XP ion trap mass spectrometer (Thermo Finnigan, Dreieich, Germany) coupled to an Agilent (Agilent, Waldbronn, Germany) 1100 series HPLC system. Separations were achieved on a Eurosphere RP C18 column (250×2 mm, 5 µm) using MeCN–H_2O (containing 0.1% formic acid) for gradient elution (min:%-MeCN: 0:10, 2:10, 35:100, 50:100) at a flow rate of 0.4 ml min^{-1}. The following ESI–MS traces were recorded: (1) positive ions from *m/z* 100 to 1000, (2) wideband MS–MS of the most intense ion from (1), (3) negative ions from *m/z* 100 to 1000, and (4) wideband MS–MS of the most intense ion from (3). These four different modes were switched every second. For the MS–MS spectra, the normalized collision energy was set at 35% according to the manufacturer's specifications. The capillary temperature was set at 285 °C, and the source voltage was 5 kV.

4.9. Extraction of lignan glucosides from L. nodiflorum cells

1.5 ml of 70% aq. EtOH was added to 20 mg powdered lyophilised *L. nodiflorum* suspension cells. Extraction was carried out by 2×10 min sonication at 60–70 °C. Cell debris was sedimented by centrifuging for 10 min at 3500*g*. The resulting extract was diluted with 70% aq. EtOH for HPLC analysis. All samples were prepared in duplicate.

4.10. HPLC analysis

An isocratic protocol with 30% aq. MeCN as mobile phase at 1.5 ml min^{-1} using a HyPurity Elite column (270×4.6 mm, 5 µm) was suitable for the analyses of PTOX, MPTOX, 5′-dMPTOX, β-peltatin and their respective glycosides. For the separation of PTOX- from 5′-dMPTOX-glucoside, a Nucleodur Sphinx RP (125×4 mm, 5 µm) column was used with an MeCN–H_2O gradient (min:%MeCN: 0:22, 10:25, 15:32, 18:32, 19:40, 21:40, 23:22, 27:22). Eluting substances were detected at 220 nm, DAD range was 220–400 nm. The extinction coefficients of the aglyca were utilised for the quantification of their respective glucosides since differences in the molar extinction coefficients between glucosides and aglyca were reported to be marginal (Stoll et al., 1954).

4.11. Measurement of G6PDH and CAD activities

G6PDH was assayed essentially as described by Löhr and Waller (1974). The test volume of 1 ml contained 6.5 mM $MgCl_2$, 1.2 mM glucose-6-phosphate, 0.39 mM NADP and 86 mM Tris/HCl (pH 7.6). The reaction was

started by the addition of up to 15.2 μg microsomal or 10 μg soluble protein.

CAD assays were carried out essentially as described by Wyrambik and Grisebach (1975). Assays consisted of 100 μM coniferyl alcohol, 50 μM NADP and up to 75 μg microsomal or 25 μg soluble protein in a total volume of 1 ml 90 mM Tris/HCl buffer (pH 8.8), coniferyl alcohol was omitted in the reference. Incubation temperature was 30 °C. The turnover rates were monitored spectrophotometrically at 340 (G6PDH) or 400 nm (CAD).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2007.07.030.

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