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Phytochemistry 69 (2008) 374–381
PHYTOCHEMISTRY
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UDP-glucose:(6-methoxy)podophyllotoxin 7-*O*-glucosyltransferase from suspension cultures of *Linum nodiflorum*

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Received 8 June 2007; received in revised form 30 July 2007
Available online 17 September 2007

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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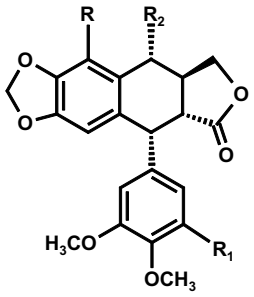
doi:10.1016/j.phytochem.2007.07.030



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