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Coniferyl alcohol metabolism in conifers — I. Glucosidic turnover of cinnamyl aldehydes by UDPG: coniferyl alcohol glucosyltransferase from pine cambium

Valerie Steeves^a, Hartmut Förster^{b,1}, Ulrich Pommer^b, Rodney Savidge^{a,*}

^aFaculty of Forestry and Environmental Management, University of New Brunswick, Fredericton, NB, E3B 6C2, Canada ^bFSU Jena, Institut für Allgemeine Botanik, Bereich Pflanzenbiochemie, Kühnhäuser Str. 101, 99189 Erfurt-Kühnhäusen, Germany

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Abstract

UDPG: coniferyl alcohol glucosyltransferase (CAGT; EC 2.4.1.11) isolated from cambial tissues of *Pinus strobus* was able to convert cinnamyl aldehydes as well as dihydroconiferyl alcohol into their corresponding 4- $O\beta$ -D-glucosides in vitro. Cinnamyl aldehydes were glucosylated with comparable efficiency to coniferyl alcohol, the physiological substrate for CAGT. Seasonal patterns of CAGT activity for aldehydes were similar to those of coniferyl alcohol. Formation of cinnamyl aldehyde and additional monolignol glucosides indicates that precursor flux and availability for lignification is likely greater than previously recognized. © 2001 Elsevier Science Ltd. All rights reserved.

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1. Introduction

The 4-O-β-D-glucopyranosides of *trans*-cinnamyl alcohols, i.e. E-coniferin and E-syringin, frequently accumulate in the cambial region of woody plants, and they have long been considered as participants in lignification, conceivably as either storage reserves (see, for example, Terazawa and Miyake, 1984) or transported forms of monolignols (Freudenberg and Torres-Serres, 1967). While coniferin is known to accumulate in conifer cambial tissues (earlier examples include: Freudenberg and Harkin, 1963; Marcinowski and Grisebach, 1977; Schmid and Grisebach, 1982; Terazawa and Miyake, 1984; Terazawa et al., 1984a,b; Savidge, 1988, 1989, 1991), it has also been isolated from cambial tissues of various angiosperms (Terazawa et al., 1984a,b), needles of Douglas fir (Morris and Morris, 1990) and Norway spruce (Slimestad and Hostettmann, 1996), cell

E-mail address: savidge@unb.ca (R. Savidge).

¹ Joint first author

suspensions of Linum flavum (van Uden et al., 1990), Paulownia tomentosa bark (Sticher and Lahloub, 1982), flue-cured tobacco leaves (Ito et al., 2000), Daphne oleides stems (Ullah et al., 1999) and leaves and stems of Viscum album ssp. (Deliorman et al., 1999). However, its accumulation evidently is greatest in conifers, such as Pinus banksiana (Leinhos and Savidge, 1993; Leinhos et al., 1994; Förster and Savidge, 1995), Larix laricina (Savidge, 1991) and Picea abies (Schmid and Grisebach, 1982), where the contents of endogenous coniferin were reported to be as high as 10 mmol/g_{fw}. In isolated cambial protoplasts the concentration of coniferin reached 1.6 mM and accounted fully for the content found in tissue extracts (Leinhos and Savidge, 1993; Leinhos et al., 1994), indicating that very little if any was present in walls of the primary-walled radially expanded cambial derivatives from which the protoplasts were obtained.

In springtime, the cinnamyl alcohol glucosides accumulate in cambial derivatives of conifers in the complete absence of lignification (Savidge, 1988, 1989, 1991; Förster and Savidge, 1995; Fukushima et al., 1997; Rowland and Arora, 1997; Savidge et al., 1998); however, there is ample evidence that once lignification is

^{*} Corresponding author. Tel.: +1-506-453-4919; fax: +1-506-453-3538.

underway they can contribute to that process (Förster et al., 1999, 2000), as earlier surmised by Freudenberg and Harkin (1963). In order for coniferin to accumulate to high levels, it must either be compartmentally shielded or produced many times more rapidly than it turns over (Marcinowski and Grisebach (1977) report a half-life of 60-120 h for coniferin) or is utilised in support of lignification. Freudenberg (1964) reported that when the coniferyl alcohol moiety of coniferin was radiolabelled, the radioactivity was readily incorporated into spruce lignin. The incorporation of isotopically labelled monolignol glucosides into lignin has been further investigated in autoradiographic studies by Terashima et al. (1986, 1993, 1995, 1997), Terashima and Fukushima (1988) and Eglinton et al. (2000). Monolignol glucosides enriched with carbon-13 at specific side chain carbons were administered to growing tree stems resulting in newly formed lignin shown to be carbon-13 enriched at the side chain carbons corresponding to those of the glucosides (Terashima et al., 1995; Xie and Terashima, 1991; Xie et al., 1994).

In agreement with the concept that the activity of coniferin-hydrolysing β -glucosidase may influence the rate of lignification (Grima-Pettenati and Goffner, 1999), a β -glucosidase specific to coniferin and of much lower abundance and/or activity than numerous other β glucosidases of wood and cambial tissues was isolated, primarily on the basis of its unique ability to hydrolyse authentic coniferin to E-coniferyl alcohol, as confirmed by GC/MS (Leinhos et al., 1994). It remains unclear whether that coniferin-specific β -glucosidase is identical to the enzyme investigated by Dharmawardhana et al. (1995, 1999), the N-terminal sequences of the two being notably distinct although both evidently have similar pI values, or to the enzyme isolated from inner secondary cell walls in young spruce seedlings by Marcinowski et al. (1979), or that localized to xylem epi-, endo- and exodermis in stems and roots of chick pea seedlings by Burmeister and Hösel (1981). A problem may exist, however, in using antibodies for localisation; supposedly specific antibodies have been repeatedly observed to bind non-specifically with lignified secondary cell walls (R.A. Savidge, unpublished data).

Coniferin has been found present in the cambial zone and its derivatives throughout the period of cambial growth, but it becomes undetectable at the onset of cambial dormancy in autumn, before the last cambial derivatives have completed their maturation into latewood (Savidge, 1988, 1989, 1991; Förster and Savidge, 1995; Savidge et al., 1998). Coniferin has never been detected in stems during cambial dormancy (Freudenberg and Harkin, 1963; Terazawa and Miyake, 1984; Savidge, 1988, 1989, 1991; Förster and Savidge, 1995; Savidge et al., 1998). Quantitative investigations of endogenous indol-3-ylacetic acid, sucrose and coniferin in *Larix laricina* cambium revealed that, of the three, only coniferin content varied parallel to seasonal cambial activity (Savidge, 1991), and biochemical investigations revealed that the presence or absence of UDPG: coniferyl alcohol glucosyltransferase (CAGT; EC 2.4.1.111) activity is the explanation for the qualitative seasonal variation of coniferin, indicating that seasonal variation in coniferin has its explanation in seasonal change in gene expression (Förster and Savidge, 1995; Savidge and Förster, 1998; Förster et al., 2000).

Ibrahim and Grisebach (1976) were first to report purification and characterization of a CAGT, isolated from suspensions of Paul's scarlet rose. The enzyme catalysed the transfer of glucose from UDPG to coniferyl alcohol and had the highest substrate specificity reported to date for a CAGT ($K_m = 3.3 \times 10^{-9}$ M for coniferyl alcohol; $K_m = 2 \times 10^{-5}$ M for UDPG). In 1977, Ibrahim confirmed the substrate specificity using CAGT isolated from young lignifying stems of *Forsythia ovata*, demonstrating it to be distinct from other glucosyltransferases, which are capable of nonspecifically glucosylating hydroxy phenols, flavonols, anthocyanidins and phenolic acids.

Schmid and Grisebach (1982) purified and characterized CAGT from Picea abies, and found coniferin to inhibit CAGT in a noncompetitive manner, with product inhibition starting at approximately 10 mmol/g. Schmid et al. (1982) also immunolocalized CAGT to the epidermis, subepidermis and vascular bundles of young P. abies seedlings. Intracellularly, it was located in the parietal cytoplasmic layer, indicating that CAGT is a soluble enzyme of the cytoplasm, in agreement with Ibrahim (1977). Förster and Savidge (1995) reported that coniferin below 10 mmol/g_{tissue} showed no inhibitory effect on activity of CAGT of Pinus banksiana, but it did inhibit CAGT above 10 mmol/g. The catalytic activity of the coniferin-specific β-glucosidase isolated from lignifying tissues was not found to reside in CAGT obtained from the same tissues, at the same developmental stage (Savidge and Förster, 1998). Recently, Förster et al. (1999, 2000) confirmed the pronounced specificity of CAGT for UDPG; however, they unexpectedly found that CAGT from P. strobus also expressed high activity when UDP-galactose was supplied to the enzyme.

E-Coniferaldehyde has been detected in the cambial sap of conifers (Freudenberg and Neish, 1968), bamboo (Tanaka et al., 1998) and oak (Fernààndez de Simòn et al., 1996). Despite the generally low endogenous coniferylaldehyde levels in plants and the efficient reduction of cinnamyl aldehydes to cinnamyl alcohols, coniferyl aldehyde glucosides have nevertheless been isolated from bark of *Syringa velutina* (Park et al., 1999) and *Kolopanax pictus* (Sano et al., 1991).

Cinnamyl aldehydes have also been reported as suitable substrates for CAGT (Ibrahim and Grisebach, 1976; Schmid and Grisebach, 1982; Förster et al., 1999, 2000). Preliminary investigations indicated that CAGT catalysed the conversion of cinnamyl aldehydes to their corresponding 4-O-β-D-glucosides in vitro (Förster et al., 1999, 2000). CAGT from Pinus strobus appeared to produce higher amounts of cinnamyl aldehyde glucosides than monolignol glucosides; however, the specific turnover rate (i.e. ratio between administered substrate and obtained product) was approximately 8 times lower (Förster et al., 2000). In seasonal investigations of CAGT activity, Förster et al. (2000) presented evidence for in vitro generation of coniferyl aldehyde glucoside to parallel similar seasonal patterns to that observed for coniferin. In the present study, we confirm, for the first time, by proton NMR spectroscopy, the enzymatic glucosidic turnover of cinnamyl aldehydes catalysed by CAGT isolated from pine cambial tissue. Seasonal patterns of CAGT activity for monolignols and cinnamyl aldehydes are provided and their $K_{\rm m}$ values reported for the first time. This is the first report of in vitro biosynthesis of dihydroconiferyl alcohol glucoside.

2. Results and discussion

Molecular structures of the predicted enzymatic products were confirmed by ¹H NMR spectroscopic analyses. Two-dimensional COSY and HMQC NMR spectra were obtained to aid in structure elucidation. For both semi-preparative and analytical HPLC, an assay was developed to effectively separate the cofactor uridine 5'-diphosophoglucose (UDPG), the monolignol substrates and the corresponding product glucosides, allowing exact quantitation of the catalysed reaction. Based on this assay, enzymatic products were separated using semi-preparative HPLC techniques, collected and lyophilized, resulting in mg quantities of the desired compounds.

The monolignol-specific UDPG was extracted and partially purified (Savidge and Förster, 1998) from approximately 400 g of differentiating xylem from *Pinus strobus* harvested in late May. IEF and native PAGE indicated purification to homogeneity, however, SDS– PAGE and N-terminal sequencing of the resulting major band revealed the presence of two proteins, one without any evident homology to known proteins and the other yielding perfect homology to the internal sequence of a glucosyltransferase precursor. Because of the almost identical physico-chemical features (p*I* 5.0 and molecular weight 43 kDa) of both proteins, complete purification could not yet be achieved (Förster et al., 2000).

All substrates tested in the assay were successfully converted to their 4-O- β -D-glucosides, as evidenced by the chemical shifts of the glucosyl moieties (not reported) in the ¹H NMR spectra. The glucosyl moiety did not bond to the alcoholic hydroxyl group of the phenylpropane side chain of the monolignol substrates (i.e. as in *iso*-coniferin), which is in agreement with Schmid and Grisebach's (1982) findings for *Picea abies*. Although Ibrahim (1977) obtained small amounts of *iso*-coniferin (i.e. *trans*-coniferyl alcohol-1-O- β -D-gluco-side) from *Forsythia ovata*, this isomer of coniferin to our knowledge has not yet been reported in conifers.

¹H NMR data for the enzymatic products, along with that of coniferyl alcohol, are presented in Table 1, while corresponding molecular structures are found in Fig. 1. Carbon 9 (Table 1) refers to either the primary alcoholic carbon atom (i.e. as in coniferin (1), syringin (2), dihydroconiferyl alcohol glucoside (5) and coniferyl alcohol) or the aldehydic carbon atom (i.e. as in coniferyl aldehyde glucoside (3) and sinapyl aldehyde glucoside (4)). As evidenced by large coupling constants for the 7-H/8-H of the double bond (i.e. J=15.8, 15.8, 16.6 and 14.8 Hz for 1–4, respectively), products 1–4 maintained a *trans* orientation. Chemical shifts for glucosyl hydrogens are not reported, but were observed ranging from $\delta = 3.1-3.8$ ppm.

Chemical shifts of 9.61 and 9.63 ppm, corresponding to the aldehyde hydrogens for **3** and **4**, respectively, clearly demonstrate that the glucosyl moiety must have attached at the 4'-position of the aromatic ring, as opposed to the primary alcohol of the phenylpropane side chain.

When comparing the ¹H NMR spectra of coniferin and coniferyl alcohol, a larger up-field shift (i.e. up to 0.38 ppm) is observed for the aromatic hydrogens compared to the aliphatic ones. This suggests the glucose attached to the phenol oxygen, and not that of the primary alcohol. Since the structures and chemical shifts for 2 and 5 are similar to 1, the same conclusions were obtained.

As previously mentioned, *E*-coniferin formation has been reported in various plants throughout the plant kingdom; however, by way of contrast, American beech exceptionally accumulates *cis*-monolignols. Yamamoto et al. (1990) reported a glucosyltransferase in bark of *Fagus grandifolia*, which showed an unusual preference for *Z*-coniferyl alcohol catalysing the formation of *Z*coniferin. The glucosyltransferase isolated from cambial tissues of *Pinus strobus* did not accept *cis*-coniferyl alcohol and a mixture of *cis* and *trans* substrates always resulted in formation of *E*-coniferin, as evidenced by HPLC separation (results not shown). Plausibly, the maintenance of a *trans* orientation during catalysis is an essential requirement for converting suitable substrates to $4-O-\beta$ -D-glucosides in the cambium of *Pinus strobus*.

The catalytic activity of UDPG: CAGT in the formation of monolignol glucosides increased more than 3fold in early June compared to CAGT isolated in mid-May (Fig. 2). The data agree with results obtained from cambial tissue of *Pinus banksiana* (Savidge and Förster, 1998); however, considerable differences in CAGT activity observed between cambium and developing xylem of *Pinus banksiana* did not appear in corresponding tissues of *Pinus strobus* (data not shown). Seasonal fluctuations in CAGT activity and levels of monolignol

Table 1
¹ H NMR spectral data for the monolignol glucosides and coniferyl alcohol

			δ^{1} H (ppm) [int, mult, J (Hz)]			
С	Coniferyl aldehyde glucoside	Sinapyl aldehyde glucoside	Dihydroconiferyl alcohol glucoside	Coniferin	Coniferyl alcohol	Syringin
2'	7.32	7.03	6.86	7.06	7.00	6.74
	[1H, <i>d</i> , 1.6]	[1 of 2H, <i>s</i>]	[1H, <i>d</i> , 2.2]	[1H, <i>d</i> , 1.9]	[1H, <i>d</i> , 1.9]	[1 of 2H, <i>s</i>]
5'	7.21 [1H, <i>d</i> , 8.3]	a	7.07 [1H, <i>d</i> , 8.2]	7.10 [1H, <i>d</i> , 8.3]	6.72 [1H, <i>d</i> , 8.0]	_a
6'	7.25	7.03	6.74	6.94	6.84	6.74
	[1H, <i>d</i> , 8.3]	[1 of 2H, s]	[1H, <i>dd</i> , 8.2, 2.1]	[1H, <i>dd</i> , 8.3, 1.9]	[1H, <i>dd</i> , 8.1, 1.6]	[1 of 2H, <i>s</i>]
7	7.61	7.61	2.63	6.54	6.50	6.54
	[1H, <i>d</i> , 16.0]	[1H, <i>d</i> , 16.0]	[2H, <i>t</i> , 7.6]	[1H, br d, 15.8]	[1H, br d, 15.9]	[1H, br d, 15.9]
8	6.71	6.78	1.82	6.27	6.19	6.32
	[1H, <i>dd</i> , 16.6, 7.7]	[1H, <i>dd</i> , 14.8, 7.0]	[2H, <i>tt</i> , 7.7, 6.3]	[1H, <i>dt</i> , 15.8, 5.6]	[1H, <i>dt</i> , 15.6, 5.9]	[1H, <i>dt</i> , 15.8, 5.6]
9	9.61	9.63	3.55	4.20	4.19	4.21
	[1H, <i>d</i> , 8.0]	[1H, <i>d</i> , 7.7]	[2H, <i>t</i> , 6.5]	[2H, <i>dd</i> , 5.8, 1.4]	[2H, <i>dd</i> , 6.0, 1.1]	[2H, <i>dd</i> , 5.6, 1.0]
OCH ₃	3.91	3.86	3.85	3.87	3.85	3.85
	[3H, <i>s</i>]	[6H, <i>s</i>]	[3H, <i>s</i>]	[3H, <i>s</i>]	[3H, <i>s</i>]	[6H, s]

^a Both C3' and C5' methoxyls yield equivalent proton shifts.





1. Coniferin: $R_1 = CH_2OH$, $R_2 = OCH_3$, $R_3 = H$

5. Dihydroconiferyl alcohol glucoside: $R_1 = CH_2OH$, $R_2 = OCH_3$

2. Syringin: $R_1 = CH_2OH$, $R_2 = OCH_3$, $R_3 = OCH_3$

3. Coniferaldehyde glucoside: $R_1 = CHO$, $R_2 = OCH_3$, $R_3 = H$

4. Sinapaldehyde glucoside: $R_1 = CHO$, $R_2 = OCH_3$, $R_3 = OCH_3$

Fig. 1. Structure of monolignol glucosides.

glucosides in cambial tissue parallel each other (Fig. 2, Savidge et al., 1998) with comparable efficiencies in the formation of aldehydic and alcoholic glucosides.

In addition, dihydroconiferyl alcohol (DHCA) was accepted as a suitable substrate for the pine CAGT and converted to its corresponding 4-O- β -D-glucoside (Fig. 1, Table 1). The 4-O- β -D-glucoside has been reported in needles of *Pinus contorta* (Higuchi et al., 1977). Ralph et al. (1997) recently reported dihydroconiferyl alcohol as a main constituent of lignin in a mutant loblolly pine. The involvement of dihydrophenylpropanoids in the biosynthesis of plant pigments (Schmitt and Schneider, 1999) suggests, in general, that dihydro-compounds play a role in secondary metabolism. The effective turnover of both cinnamyl alcohols and cinnamyl aldehydes by CAGT during the growing season was confirmed by the determination of the apparent $K_{\rm m}$ values of the various substrates (Table 2). The apparent $K_{\rm m}$ values were obtained from Lineweaver–Burk plots at saturating concentrations of UDPG. For coniferyl alcohol, when assayed in phosphate buffer at pH 7.6, a $K_{\rm m}$ of 120 µM was obtained. Sinapyl alcohol, although not synthesized in pines, also served as a substrate with an apparent $K_{\rm m}$ of 154 µM. The data confirm former results for *Pinus banksiana*, where sinapyl alcohol reached 96% of the relative activity of coniferyl alcohol (Savidge and Förster, 1998). A comparable $K_{\rm m}$ value (250 µM) was reported for spruce (Schmid and Grisebach, 1982).



Fig. 2. Seasonal activity pattern of UDPG:CAGT in developing xylem of Pinus strobus as determined by in vitro experiments.

Table 2 Enzymatic parameters of the partially purified UDPG:CAGT from cambium of *Pinus strobus*

Substrate	Enzymatic product	$K_{\rm m}~(\mu{ m M})$	V_{\max} (nM×mg _{protein} ×min ⁻¹)
Coniferyl alcohol	Coniferin	119.6	1.17
Sinapyl alcohol	Syringin	153.5	1.54
Coniferaldehyde	Coniferaldehyde-4-	317.4	1.85
Sinapaldehyde	O - β -D-glucopyranoside Sinapaldehyde-4- O - β -D-glucopyranoside	241.7	1.38

Coniferyl aldehyde and sinapyl aldehyde glucosylation resulted in $K_{\rm m}$ values of 317 and 242 µM, respectively, thus working in close proximity to the order of cinnamyl alcohols. Nevertheless, existing differences in the enzymatic turnover of cinnamyl alcohols and aldehydes, evident by the reported $K_{\rm m}$ values, underline that the hydroxyl group of the phenylpropane side-chain constitutes a more efficient substrate configuration. As reported before (Förster et al., 2000), cinnamyl alcohols and cinnamyl aldehydes compete with each other for CAGT when provided as dual substrates, with alcohols being more efficient in inhibiting the formation of cinnamyl aldehyde glucosides.

Cinnamyl aldehydes have been previously reported as appropriate substrates; however, structure and physiological relevance was not investigated. The amount of aldehydic glucosides obtained in investigations of the substrate specificity of CAGT in *Picea abies* (Schmid and Grisebach, 1982) and suspension cultures of Paul's Scarlet Rose (Ibrahim and Grisebach, 1976) was expressed as relative activity in comparison to coniferyl alcohol. Because of the lack of authentic compounds for co-chromatography, the structure of the presumed glucosidic products could not be unequivocally elucidated. Hence, the data in this report based on HPLC separation and NMR investigation (Table 2) confirm, to our knowledge for the first time the real structure of the enzymatic products (Fig. 1). In contrast to the glucosyltransferase purified from Forsythia ovata (Ibrahim, 1977) and in agreement with the glucosyltransferase purified from Picea abies (Schmid and Grisebach, 1982) the CAGT from *Pinus strobus* expressed almost no activity with cinnamic acids (Förster et al., 2000). The glucosylation of cinnamic acids (Nurmann and Strack, 1980; Lorenzen et al., 1996; Fons et al., 1998; Wang and Ellis, 1998) has been demonstrated previously, with the glucosyl moiety attached to the carboxyl group of the side-chain.

The observed seasonal fluctuations of both cinnamyl alcohol and aldehyde glucoside formation (Fig. 2) coincide with events of wood formation, i.e. formation of primary walled radially enlarged cells and the first appearence of differentiated tracheids undergoing secondary-wall formation and lignification (Savidge and Förster, 1998). Preliminary examination revealed both high activities of the glucosyltransferase and developmental processes during June and July (Fig. 2) in the cambial zone, thus concentrating the characterization and purification of the CAGT to this time of the season. Lignification is not developmentally correlated to the formation of monolignol glucosides as shown previously (Leinhos and Savidge, 1994; Savidge and Förster, 1998). However, some observed metabolic connections to wood formation and lignification once the glucosides are produced in abundance needs further clarification.

CAGT, in catalyzing the formation of cinnamyl alcohol glucosides, evidently competes with coniferyl alcohol dehydrogenase (CAD) and monolignol oxidising enzymes for coniferyl alcohol. Our data (Fig. 2, Table 2) indicate that the glucosyltransferase must also compete with CAD for available cinnamyl aldehydes by forming the corresponding aldehydic glucosides (Fig. 1).

Results obtained in the past few years have prompted a reconsideration of the established lignin biosynthetic pathway. It is clear now that inter-conversions of lignin precursors may occur at varied points along the lignification pathway, and not necessarily solely at the phenylpropanoid acid stage. In addition, the ratio of guaiacyl to syringyl units can be modified at the level of CoA esters (Grimmig et al., 1999), cinnamyl aldehydes (Osakabe et al., 1999), cinnamyl alcohols (Chen et al., 1999a,b) and monolignol glucosides (Matsui et al., 1996).

Taken together, the questions of if and how CAGT (i.e. alone or in a regulatory manner with the coniferinspecific β -glucosidase) may participate in controlling availability of cinnamyl alcohols and/or cinnamyl aldehydes for lignin formation deserves further efforts.

3. Experimental

3.1. General experimental

Coniferyl alcohol, sinapyl alcohol, coniferyl aldehyde and sinapyl aldehyde were purchased from Aldrich. Econiferin was isolated from *P. strobus* (Savidge, 1989) and dihydroconiferyl alcohol was obtained as previously described by Savidge (1987). Before processing and HPLC analysis, handcut sections of cambium and developing xylem were examined by microscopy as previously described by Savidge (1988).

3.2. Trees

Healthy *Pinus strobus* between 15 and 18 years old growing in the University of New Brunswick Woodlot, Fredericton, NB, Canada, were selected. Sufficient amounts of protein for this study were isolated for purification from a number of trees harvested in late May 1997. Individual trees were harvested during the growing season (April–September) for investigations into seasonal CAGT activity patterns. On each date, stem segments from the main axis were removed with a handsaw and brought directly to the laboratory for processing. The bark was peeled from the wood and the exposed inner bark surface (i.e. cambial zone) and wood surface (i.e. developing xylem) were scraped individually into cryogenic vials (Savidge et al., 1982). The tissue was then stored in liquid nitrogen until preparation for CAGT analysis.

3.3. Enzyme isolation

Buffer systems used include: extraction buffer (0.2 M phosphate (Na₂HPO₄, NaH₂PO₄), 10% polyvinylpyrroli done, 7.5% glycerol, 15 mM aminocaproic acid, 10 mM dithiothreitol, 14 mM β-mercaptoethanol, pH 7.6), equilibration buffer (0.1 M phosphate (Na₂HPO₄, NaH₂PO₄), 14 mM β-mercaptoethanol, pH 7.6), sodium phosphate buffer (Na₂HPO₄, NaH₂PO₄, 100 mM, pH 7.6). Cambial tissue was pulverized to a homogeneous powder and extracted for 1 h in extraction buffer. The suspension was filtered and the filtrate centrifuged for 20 min $(4^{\circ}C)$ at 15,500×g. The supernatant was centrifuged (1 h, 4° C) at 100,000×g to remove microsomes. Protein in the supernatant was precipitated with $(NH_4)_2SO_4$. Protein precipitated between 40 and 85% (NH₄)₂SO₄ was collected by centrifugation $(23,300 \times g, 20 \text{ min}, 4^{\circ}\text{C})$. The resulting pellet was resuspended in phosphate buffer (2.5 ml), then applied to Sephadex G-25 (20 mm i.d.×100 mm, previously equilibrated in equilibration buffer). One ml fractions of soluble protein were collected. Fractions showing transferase activity were pooled and concentrated by centrifugation at $2455 \times g$ using a Centricon YM-10 concentrator. All subsequent isolation and purification of UDPG: coniferyl alcohol glucosyltransferase (CAGT) was carried out according to Förster and Savidge (1995) and Savidge and Förster (1998).

3.4. Protein determination

Protein concentrations were determined using the method developed by Lowry et al. (1951), modifying final volumes to 40% of the original assay. The UDPG: CAGT was then applied in a 40-fold dilution, using bovine serum albumin as a standard, and measured at 578 nm.

3.5. Semi-preparative HPLC and product separation

Reversed-phase HPLC of the enzyme assay aliquots was performed according to Savidge (1989) on a Milton Roy system with a semi-preparative C18 ODS Ultrasphere column (Beckman, 5 μ m spheres, 150×10 mm i.d.). Assays were carried out on a larger scale (i.e. total volume 2 ml) in order to obtain sufficient product for analytical investigations. The enzyme incubation mixture consisted of 100 mM phosphate buffer (pH 7.6), 2 mM UDPG, 1mM of the substrates in question and approximately 800 µg protein. Enzyme assay incubation time was 60 min at 36°C. Product separation was achieved with a linear gradient over 30 min (5–100% MeOH, adjusted to pH 3.0 with acetic acid) with a flow rate of 5 ml/min and UV detection at 260 nm: R_t coniferin 8.0 min, 266 nm: R_t syringin 8.7 min, 330 nm: R_t coniferyl aldehyde glucoside 9.0 min, 320 nm: R_t sinapyl aldehyde glucoside 9.8 min, 262 nm: R_t p-coumaryl alcohol glucoside 8.7 min, 276 nm: R_t dihydroconiferyl alcohol glucoside 8.3 min., 266 nm. Enzymatic products were collected from semi-preparative HPLC eluant and freeze dried. The compounds were characterised by GC–MS and ¹H NMR and used as standards in analytical HPLC.

3.6. Ultraviolet-visible spectra

Ultraviolet-visible spectra of the purified enzymatic products were obtained using a computer-controlled UV/vis spectrophotometer (Shimazu, UV-2102-PC). Spectra were recorded from 195 to 450 nm and compared to spectra obtained in analytical HPLC work-up.

Coniferin (1) λ_{max} (MeOH) nm (log ε): 260 (1,3), 214, λ_{min} (MeOH) nm: 238, $\lambda_{shoulder}$ (MeOH) nm: 296; syringin (2) λ_{max} (MeOH) nm (log ε): 266, (1,23), 221, λ_{min} (MeOH) nm: 240; coniferyl aldehyde glucoside (3) λ_{max} (MeOH) nm (log ε): 330 (1,16), 236, λ_{min} (MeOH) nm: 266, $\lambda_{shoulder}$ (MeOH) nm: 306; sinapyl aldehyde glucoside (4) λ_{max} (MeOH) nm (log ε): 320 (0,55), 236, λ_{min} (MeOH) nm: 268; dihydroconiferyl alcohol glucoside (5) λ_{max} (MeOH) nm: 276, 224, λ_{min} (MeOH) nm: 248.

3.7. Nuclear magnetic resonance

Approximately 1–2 mg of coniferin, coniferyl aldehyde glucoside, syringin and sinapyl aldehyde glucoside were each dissolved in 600–700 µl deuterated methanol and placed in individual 5 mm NMR tubes. Only microgram quantities of dihydroconiferyl alcohol glucoside were available, which was subsequently dissolved in approximately 600 µl deuterated methanol, and placed in a 5 mm NMR tube. ¹H spectra were recorded on a 400 MHz, Unity 400 NMR spectrometer, at 25°C. The residual solvent peak, CHD₂OD, (3.30 ppm) was used as an internal standard. Two-dimensional COSY spectra and one-dimensional spectra were obtained.

3.8. Analytical HPLC and glucosyltransferase assay

Analytical HPLC was performed on a computer-controlled Jasco system consisting of an autosampler (AS950-10), multiwavelength detector (MD 910), ternary gradient unit (LG 980-02) and a 3-line-degasser (DG 980-50) on a Peltier-thermostated Sepsil C18 column (Jasco, 5 μ m spheres, 125×3 mm i.d.). Cinnamyl alcohol glucosides were eluted in low pressure gradient mode in H₂O: MeOH (containing 1% HCO₂H) over 55 min (2–39% MeOH for 35 min, 39–100% MeOH for 15 min), and cinnamyl aldehyde glucosides over 70 min (2– 60% MeOH for 50 min, 60–100% MeOH for 20 min) with a flow rate of 0.5 ml/min. Column temperature was adjusted to 26° C. Data analysis was accomplished using Borwin PDA (version 1.0) and chromatography (version 1.22.03 B) software.

The enzyme incubation mixture (i.e. total volume 200 μ l) contained 200 nM UDPG in 10 μ l H₂O, various amounts of substrate in 10 μ l MeOH, 80 μ l sodium phosphate buffer and approximately 100 μ g protein dissolved in sodium phosphate buffer. The various substrates were incubated for 10 min at 36°C, then terminated using 20 μ l HOAc (50%). K_m values were calculated at fixed concentration of UDPG (1 mM) by linear regression of 1/v vs. 1/s (Lineweaver-Burk plot) with Grafit (version 4.0.1). All experiments were performed in triplicate and compared to a control containing all constituents but terminated prior to catalytic activity by HOAc precipitation of the enzyme.

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