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Inhibitory effects of Scots pine heartwood extractives on enzymatic holocellulose hydrolysis by wood decaying fungi

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Abstract

The heartwood of Scots pine contains extractives that protect it against wood decaying fungi. Pine extractives such as pinosylvins are fungicidal compounds, but they may also have other mechanisms of action. This experiment investigated whether pinosylvins and other heartwood extractives can act as inhibitors of holocellulose hydrolysis, similarly to many other biomass-derived phenolic compounds. The inhibitory properties of extractives were studied against enzymes secreted by a brown rot (*Coniophora puteana*) and a white rot fungus (*Trametes versicolor*), as well as against a commercial *Trichoderma reesei* enzyme cocktail. The extractives were studied as wood-free extracts and extractives-containing wood powders. In all experiments, the behaviour of the white rot differed from that of the other two. The white rot hydrolases were strongly inhibited and deactivated by extractives, particularly pinosylvins, whereas the others showed only mild or moderate inhibition and no deactivation. The white rot enzymes seemed to modify the pinosylvins, with further studies suggesting that the modified pinosylvins may form complexes with enzymes and cause their deactivation. These results suggest that pine heartwood extractives have potential to contribute to decay resistance as hydrolase inhibitors but only when the fungus produces enzymes capable of modifying the extractives.

Keywords: cellulase, heartwood, inhibition, pinosylvins, Scots pine, xylanase

1. Introduction

Wood extractives are biologically active secondary metabolites produced by trees. Extractives often accumulate in the heartwood (HW), where they increase the resistance of the wood material to decay and other forms of fungal and insect attack. Due to the great economic significance of decay resistance in wood products, the formation and properties of HW and its extractives have been extensively studied (Hillis. 1987, Taylor et al. 2002).

In Scots pine (*Pinus sylvestris*), the decay resistance of HW is typically classified as either moderate or slight, making the HW more durable than the sapwood or the woods of other common local trees such as spruce or birch (EN 350, Jebrane et al. 2014, Plaschkies et al. 2014,

Van Acker et al. 2003). The HW extractives of Scots pine consist primarily of resin acids and pinosylvins (Willför et al. 2003, Fang et al. 2013), both of which have been implicated in the decay resistance of pine HW (Harju et al. 2002, 2003, Venäläinen et al. 2003, 2004). While there are conflicting results on the significance of resin acids, all of these studies have demonstrated that pinosylvins play a role in decay resistance. Pinosylvins are fungicidal to a range of wood decaying fungi (Hart and Shrimpton. 1979, Seppänen et al. 2004), but their mechanism of action and other possible means of decay prevention are poorly characterised. Pinosylvins may, for example, contribute to decay resistance as antioxidants, by interfering with the oxidative wood degradation mechanisms of fungi (Belt et al. 2017). Pinosylvins may also be capable of inhibiting the enzymatic hydrolysis of holocellulose to digestible sugars, but this potential mechanism has not yet been explored.

While pinosylvins have not been studied as inhibitors of carbohydrate hydrolases, other biomass-derived aromatics such as lignins and low molecular weight phenolics have been the topic of extensive study. Lignins block the access of enzymes to holocellulose and cause the unproductive adsorption of enzymes onto their surface, resulting in reduced hydrolysis (Palonen et al. 2004, Rahikainen et al. 2011, Rollin et al. 2011). Smaller phenolics, whether lignin derived or not, can also inhibit cellulases and hemicellulases. The smaller phenolics have been shown to form soluble and insoluble complexes with enzymes, causing enzyme deactivation and inhibition of hydrolysis (Boukari et al. 2011, Sharma et al. 1985, Tejirian and Xu. 2011, Ximenes et al. 2011). As phenolic compounds the pinosylvins may have similar effects, although the possibility of other extractives taking part in hydrolase inhibition cannot be excluded. Resin acids, for example, have previously been found to inhibit cellulose hydrolysis (Leskinen et al. 2015).

In this study, Scots pine HW extractives were tested for their ability to inhibit the enzymatic hydrolysis of wood polysaccharides. The effects of the extractives were tested against extracellular enzymes secreted by cultures of brown rot (*Coniophora puteana*) and white rot (*Trametes versicolor*) fungi, which differ in their mechanisms of wood degradation and may therefore differ in their susceptibility to inhibition by extractives. White rot fungi degrade wood by means of a full cellulase system and lignin-degrading enzymes such as peroxidases and laccases, whereas brown rots employ a non-enzymatic degradation system at the initial stages of decay, followed by enzymatic attack using hemicellulases and typically an incomplete cellulase system (Riley et al. 2014, Zhang et al. 2016). The effects of extractives were also tested against a commercial *Trichoderma reesei* cellulase preparation. Extractives were studied as inhibitors and deactivators of holocellulose hydrolysis and their mechanisms of action investigated. In all experiments, the extractives were studied as wood-free extracts and as extractives-containing wood powders, to determine whether the inhibitory effects are significant also when the extractives are associated with their natural wood matrix.

2. Materials and methods

2.1 Wood material and extract processing

The wood material used in this experiment was obtained from a freshly felled 70 year old Scots pine tree that was stored frozen until use. Discs were sawn from a section of the trunk at approx. 1 m height, and HW material was cut from the discs. The HW material was ground to a fine powder in a Wiley mill (0.5 mm mesh) and freeze-dried. A portion of the powder was then Soxhlet extracted (6 h, 300 mL solvent and approx. 10 g powder) with either acetone or

sequentially with n-hexane and MeOH. Small aliquots of each extract were taken for composition analysis by GC and GC-MS. The remaining acetone and MeOH extracts were evaporated to dryness and redissolved in EtOH, while residual solvents were evaporated from the wood powders. The acetone (AE) and methanol (ME) extracts and the unextracted (UEW), hexane extracted (HEW), and hexane + methanol extracted (HMEW) wood powders were used to study the inhibitory properties of HW extractives.

2.2 Enzyme production

The brown rot fungus *Coniophora puteana* (strain BAM Ebw. 15, Federal institute for materials research and testing, Germany) and the white rot fungus *Trametes versicolor* (strain PRL 572, University of Helsinki culture collection, Finland) were maintained on 2% malt extract agar. For enzyme production, five pieces of agar (approx. 1 mm² in size) from the growing edges of mycelium were used to inoculate 50 mL batches of culture medium, which contained (per L) 3 g yeast extract, 3 g tryptone, 0.8 g NaH₂PO₄, 0.5 g MgSO₄ x 7H₂O, 0.4 g K₂HPO₄ x 3H₂O, and 50 g acetone-extracted Scots pine sapwood flour. The pH of the medium was set to 5.5 before autoclaving. The fungi were grown stationary in the dark for 11 days at 28°C, after which the culture medium was separated by filtration and concentrated in spin columns (10 kDa MW cut-off). Celluclast 1.5L, a *Trichoderma reesei* cellulase preparation, was purchased from Sigma Aldrich and used as received.

2.3 Inhibition and deactivation experiments

All hydrolyses were conducted in 2 mL capped tubes in an inverting mixer. In a total volume of 1 mL, each hydrolysis reaction contained 50 mM acetate buffer (pH 5.0), 0.1 mg of enzyme, and 10 mg of bleached softwood Kraft pulp as substrate. Hydrolyses with *C. puteana* and *T. versicolor* enzymes were performed at 30°C, whereas hydrolyses with Celluclast were performed at 30 and 50°C to determine whether an increase in hydrolysis temperature changes the effects of extractives. Each hydrolysis was run for 24 h, after which the release of monosaccharides was determined by high performance anion exchange chromatography (HPAEC). All hydrolyses were performed in triplicate.

Inhibition of hydrolysis by extractives was studied by combining extractives, enzyme, and substrate immediately at the beginning of hydrolysis. Extracts were studied at a concentration of 2.5 mg mL⁻¹, and due to their hydrophobicity, they were added from an EtOH solution (final EtOH concentration 2.5%, v v⁻¹) to produce a stable suspension. The wood powders were added at a concentration of 25 mg mL⁻¹, because the extractives content of the UEW was approx. 10%. The sugar yields of extract hydrolyses were compared to a reference hydrolysis containing an equal volume of pure EtOH in place of extract, while the yields of wood powder hydrolyses were compared to a reference containing 25 mg mL⁻¹ of HMEW.

Deactivation of enzymes by extractives was studied by incubating the enzymes with extractives prior to hydrolysis. Enzymes and extractives were combined at the same concentrations as used in hydrolysis and incubated at hydrolysis temperature for 24 h. After incubation, the enzyme-extractives mixtures were combined with substrate and hydrolysis commenced. Samples with pure EtOH and HMEW, also incubated at hydrolysis temperature for 24 h, served as references.

2.4 Enzyme distribution experiments

To study whether extractives bind or precipitate enzymes, the extractives and enzymes were combined without substrate at the concentrations specified in section 2.3 and incubated at hydrolysis temperature for 24 h. Two different kinds of enzyme distribution experiments were performed: one studying protein content and the other studying hydrolytic activity. In the protein content study, the samples were centrifuged at the end of the incubation period and the supernatants collected. The supernatants were extracted with 1% (w v⁻¹) of polyvinylpolypyrrolidone to remove solubilised phenolics and then filtered through 0.2 µm syringe filters. The protein contents of the supernatants were measured and compared to that of a similarly treated reference containing pure EtOH or HMEW.

In the activity experiment, the samples were again centrifuged after incubation and separated into supernatant and solids fractions. The supernatants were extracted with 1% polyvinylpolypyrrolidone and filtered, while the solids were resuspended in fresh buffer. The extract solids were resuspended by first adding EtOH to the final 2.5% (v v⁻¹) concentration, followed by the addition of buffer and manual disruption with a small spatula to disperse the remaining solids. The extracted supernatants and the resuspended solids were then used to hydrolyse the pulp substrate. Samples with pure EtOH and HMEW, also incubated at hydrolysis temperature for 24 h, served as references. All enzyme distribution experiments were performed in triplicate.

2.5 Modification of pinosylvins

The modification of pinosylvins by the enzyme preparations was studied in substrate-free incubations using the same enzyme and extractives concentrations as in section 2.3. The incubations were conducted at hydrolysis temperature, with samples withdrawn after 4, 8, and 24 hours of incubation. The ME reactions were conducted in a volume of 1 mL, and 250 µL aliquots were withdrawn at the indicated times and directly processed for analysis by GC. The UEW reactions were also conducted in a volume of 1 mL but in individual tubes. At each time point, a set of tubes was withdrawn, centrifuged, and the supernatants discarded. The solid powders were washed once with 1 mL of water, and then extracted twice with 1 mL of MeOH in a sonicator (45°C, 30 min per extraction). The two MeOH extracts were collected and combined, then 1 mL portions of the combined extracts were processed for analysis by GC. The modification incubations were conducted in triplicate.

2.6 Protein analyses and enzyme activities

Protein contents were determined using the Bio-Rad protein assay reagent according to the manufacturer's instructions, with bovine serum albumin as standard.

Total cellulase activity was measured according to Ghose (1987) using filter paper as substrate. Endoglucanase and xylanase activities were measured with 1% CMC (low viscosity, DS 0.7) and xylan (birchwood) as substrates, respectively, using the xylanase assay procedure of Bailey et al. (1992). The release of reducing sugars was determined with the dinitrosalicylic acid reagent against glucose and xylose standard curves. β-glucosidase and β-xylosidase activities were measured with p-nitrophenyl β-D-glucopyranoside and p-nitrophenyl β-D-xylopyranoside as substrates, respectively, following the β-glucosidase assay procedure of Li et al. (2012). The

buffer used in all assays was 50 mM acetate (pH 5.0). *C. puteana* and *T. versicolor* activities were measured at 30°C, while the activities of Celluclast were measured at 50°C.

Laccase activity was measured with 5 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) as substrate in 50 mM citrate buffer (pH 3.5) at 25°C. Appropriately diluted enzyme was mixed with the substrate solution, and the increase in absorbance was monitored at 420 nm ($\epsilon = 36 \text{ mM}^{-1} \text{ cm}^{-1}$) for 5 min.

2.7 Sugar analyses

The monosaccharide content of the pulp hydrolysates was determined with HPAEC. The hydrolysates were diluted with MilliQ water and an aliquot of the internal standard (fucose) was added. The samples were analysed with a Dionex ICS-3000 instrument equipped with pulsed amperometric detection and a CarboPac PA20 column. Water was used as the eluent (0.37 mL min^{-1}), and pure monosaccharides were used as standards for quantification.

2.8 Extractives analyses

The composition of extractives in the Soxhlet extracts and degraded samples was determined with GC and GC-MS. An aliquot of each extractives solution and the internal standard (heneicosanoic acid) were evaporated to dryness under vacuum and then redissolved in 700 μL of pyridine. The extractives were silylated at 70°C for 20 min after addition of 300 μL of N,O-bis(trimethylsilyl)trifluoroacetamide with 5% chlorotrimethylsilane. The extractives were quantified by GC-FID (Shimadzu GC 2010 Plus) with a HP-5 column (30 m x 0.23 mm i.d., 0.25 μm film thickness). The oven temperature program was 2 min at 100°C, 6°C min^{-1} to 280°C, and 5 min at 280°C. Helium (1 mL min^{-1}) was used as the carrier gas. The extractives were identified by GC-MS (Trace 1300 GC/Thermo Scientific ISQ single quadrupole MS), using the same type of column and the same oven temperature programme as with GC. Mass spectra (50-700 m/z) were recorded at 70 eV.

3. Results and discussion

3.1 Characterisation of extractives and enzyme preparations

Table 1 shows a summary of the chemical composition of the HW extracts and wood powders. The analysis was limited to main component groups, but detailed descriptions of the composition of Scots pine HW extractives are available elsewhere (Fang et al. 2013, Willför et al. 2003). The acetone extract (AE), which can be considered a total HW extract, consisted mostly of resin acids. Pinosylvins were the second most abundant component in the AE, while fatty acids accounted for only 3.1% of the extract. In the MeOH extract (ME), the proportion of pinosylvins was greatly increased due to the removal of resin acids and fatty acids in the hexane extraction step. The unextracted wood powder (UEW) contained 9.8% extractives removable by sequential extraction with hexane and MeOH. The relative amounts of the different extractives were very similar to those in the AE, which shows that the AE is a good representation of the total HW extractives. The hexane extracted wood powder (HEW) contained only 2.1% of MeOH soluble extractives, but it had retained most of the pinosylvins

present in the UEW. The slight loss of pinosylvins seen in the HEW is due to the partial solubility of pinosylvins monomethyl ether in hexane.

Table 1. Composition of extractives in the acetone (AE) and methanol (ME) extracts (% g g⁻¹ of extract) and the unextracted (UEW) and hexane extracted (HEW) wood powders (mg g⁻¹ of oven-dry wood)

	AE	ME	UEW ^a	HEW
	%	%	mg g ⁻¹	mg g ⁻¹
Gravimetric yield	-	-	98.0	20.8
Pinosylvins	15.0	64.4	15.4	13.4
Resin acids	75.2	5.8	71.8	1.3
Fatty acids	3.1	0.0	2.9	0.0

^aSum of the hexane and MeOH extractions

The protein contents and hydrolase activities of the brown rot, white rot, and commercial enzyme preparations are presented in Table 2. Celluclast, the commercial *T. reesei* cellulase cocktail, showed much higher specific cellulase activities than the *C. puteana* and *T. versicolor* secretomes, particularly in the case of total cellulase and endoglucanase activity. The cellulase activities of *T. versicolor* were lower than those of *C. puteana*, which is in agreement with earlier results (Irbe et al. 2014). All of the enzyme preparations also had significant xylanolytic activity, with *C. puteana* showing the highest and *T. versicolor* the lowest activity. The different activity profiles of the enzymes were also reflected in hydrolysis monosaccharide yields, which are given in Table 3 for the two reference hydrolyses (pulp + EtOH for extracts, pulp + HMEW for wood powders). As expected, Celluclast showed the highest hydrolytic efficiency, producing mostly glucose from the pulp or pulp-wood mixture and a proportionally small amount of xylose. *C. puteana* produced significantly more glucose than *T. versicolor*, which is likely to be due to the low hydrolase activity of the white rot enzyme preparation (Table 1). The natural preference of white rots for hardwood substrates is unlikely to be a significant factor, as the preference is believed to be mostly related to lignin (Faix et al. 1985, Highley 1982). In addition to glucose, the two wood decaying fungi produced proportionally large amounts of xylose and other hemicellulosic sugars, mostly mannose.

Table 2. Protein contents (mg mL⁻¹) and hydrolase activities (U mg⁻¹ protein) of the *C. puteana* (Cp), *T. versicolor* (Tv), and Celluclast (Cell.) enzyme preparations

		Cp	Tv	Cell.
Protein content	mg mL ⁻¹	3.8	4.5	44.6
Total cellulase	U mg ⁻¹	0.10	0.05	0.63
Endoglucanase	U mg ⁻¹	6.2	0.7	26.8
Xylanase	U mg ⁻¹	22.6	2.2	9.5
β-glucosidase	U mg ⁻¹	0.41	0.75	0.80
β-xylosidase	U mg ⁻¹	0.14	0.11	0.35

Table 3. Monosaccharide yields (mg L⁻¹) in pulp reference hydrolyses by the *C. puteana* (Cp), *T. versicolor* (Tv), and Celluclast (Cell.) enzyme preparations. Pulp + 2.5% EtOH is used as reference for hydrolyses containing extracts, pulp + 2.5% HMEW for those containing wood powders

		Cp	Tv	Cell. 30°C	Cell. 50°C
1% pulp + 2.5% EtOH	Glc	384.5	227.8	2933.8	3774.6
	Xyl	91.0	113.3	360.8	386.2
	Other	99.5	53.4	40.5	30.8
1% pulp + 2.5% HMEW	Glc	545.8	264.5	3098.9	4045.2
	Xyl	113.6	123.0	549.3	560.7
	Other	175.5	125.8	46.9	57.2

3.2 Inhibition

To study the effects of extractives on holocellulose hydrolysis, pulp was hydrolysed by the three enzyme preparations in the presence of extracts and wood powders. The sugar yields were then compared to the reference yields given in Table 3. As shown in Fig. 1, all enzyme preparations showed some degree of inhibition by extractives. Glucose and xylose (and mannose in the case of *C. puteana* and *T. versicolor*, data not shown) generally showed similar levels of inhibition by each extractives preparation, although the glucose yields tended to be slightly more affected than the yields of hemicellulosic sugars.

Figure 1. Inhibition of enzymatic glucose (a) and xylose (b) release from pulp by extractives. AE, acetone extract; ME, methanol extract; UEW, unextracted wood powder; HEW, hexane extracted wood powder; Cp, *C. puteana*; Tv, *T. versicolor*; Cell., Celluclast. *statistically significant difference to reference ($p < 0.05$, Tukey's range test)

The release of glucose by the brown rot fungus *C. puteana* was mildly inhibited by both the extracts and wood powders. The pinosylvin-rich ME was more inhibitory than the AE, while the two wood powders did not differ in their effects, suggesting that the inhibition is primarily

due to pinosylvins. The extent of inhibition was similar for the extracts and wood powders, which shows that extractives are capable of interacting with enzymes even in wood and can thus be of relevance in wood decay. However, the release of xylose (and mannose, data not shown) by *C. puteana* was not substantially inhibited by extractives. As brown rot fungi such as *C. puteana* tend to preferentially degrade hemicelluloses (Highley. 1987, Irbe et al. 2006), the slight inhibition of glucose release from wood is unlikely to be a significant factor in preventing wood decay.

The hydrolases of the white rot fungus *T. versicolor* were also inhibited by both the extracts and wood powders. The wood powders had only a small effect, with the release of glucose slightly inhibited and the release of xylose unaffected. The pinosylvins-rich ME, however, was found to have a strong influence on *T. versicolor* hydrolases. The release of glucose was reduced to 35% of the reference, and even xylose release was affected, with the ME-containing hydrolysis releasing 43% of the reference xylose.

Celluclast, the commercial cellulase preparation, showed very little inhibition by extractives when the hydrolyses were conducted at 30°C. At 50°C the AE became moderately inhibitory, with the glucose and xylose yields reduced to approx. 65% of the reference. The effects of the other extractives preparations on sugar release remained minor, which shows that hydrophobic compounds are more inhibitory than pinosylvins at a higher hydrolysis temperature, at least in the case of *T. reesei* hydrolases. The inhibiting component is likely to be the resin acids, which accounted for 75% of the AE (Table 1). Cellulase inhibition by abietic acid, the most abundant Scots pine HW resin acid, has been previously documented (Leskinen et al. 2015). However, the inhibitory action of the hydrophobic fraction appears to require a wood-free extract: the UEW was less inhibitory than the AE, even though both hydrolysis reactions contained approximately the same amount of extractives in the same proportions (Table 1).

3.3 Deactivation

In addition to inhibition, the ability of extractives to deactivate enzymes was investigated. The ME and UEW were selected for deactivation studies, as they represent the most inhibitory and the most natural extractives preparations used in this experiment. The enzymes were first incubated with extractives for 24 h, then the enzyme-extractives mixtures were used to hydrolyse pulp. The protein contents of the incubation supernatants were also tested, to determine whether the loss of hydrolytic activity is due to unproductive binding or precipitation of enzyme protein, which are common deactivation mechanism with lignins and many simple and oligomeric phenolics (Palonen et al. 2004, Rahikainen et al. 2011, Tejirian and Xu. 2011). The effects of ME and UEW incubation on sugar yields and protein content are presented in Figs. 2 and 3, respectively.

In the case of *C. puteana* and Celluclast, a 24 h pre-incubation of the enzymes with extractives caused only small changes in sugar yield compared to the inhibition experiment (Fig. 1). The ME caused moderate inhibition of glucose release by *C. puteana* (61% of reference glucose), but apart from that, *C. puteana* and Celluclast remained only slightly or not at all affected by extractives. The protein content determinations also showed that little to no supernatant protein was lost during the 24h incubation period (Fig. 3). Thus, it appears that extractives do not deactivate the *C. puteana* and Celluclast hydrolases and that the inhibition of sugar release seen in their presence arises by some mechanism other than precipitation or unproductive binding of enzymes.

Figure 2. Deactivation of enzymatic glucose (a) and xylose (b) release from pulp by extractives. AE, acetone extract; ME, methanol extract; UEW, unextracted wood powder; HEW, hexane extracted wood powder; Cp, *C. puteana*; Tv, *T. versicolor*; Cell., Celluclast. *statistically significant difference to reference ($p < 0.05$, Tukey's range test)

Figure 3. Loss of supernatant protein due to incubation with the methanol extract (ME) or the unextracted wood powder (UEW). Cp, *C. puteana*; Tv, *T. versicolor*; Cell., Celluclast. *statistically significant difference to reference ($p < 0.05$, Tukey's range test)

Unlike *C. puteana* and Celluclast, *T. versicolor* showed almost complete deactivation. With pre-incubation, the glucose yield of the ME-containing reaction dropped to 14% of the reference, compared to the 35% obtained without pre-incubation (Fig. 1). The UEW had also become significantly more inhibitory with pre-incubation, with the glucose yield decreasing from 79% (Fig. 1) to 55% of the reference. Xylose yields were also reduced compared to hydrolyses without pre-incubation. Finally, protein content measurements (Fig. 3) showed a loss in supernatant protein following incubation with the ME and UEW, suggesting that the inhibitory and deactivating effects of the extractives may be due to complex formation between enzymes and extractives.

The deactivation of only the *T. versicolor* hydrolases suggests that the white rot enzyme preparation differs from the other two in some key way. While differences in the nature of the hydrolases are possible, another explanation is that the *T. versicolor* secretome contains enzymes that are capable of modifying the extractives to more inhibitory compounds. Supporting this modification hypothesis is the finding that the white rot enzyme preparation caused a gradual change in the colour of extractives-containing samples. Pinosylvins are likely

to be the modified extractives component: the pinosylvins-rich ME showed the strongest inhibition of *T. versicolor* (Fig. 1) and also the strongest colour change (from light brown to pink to orange over the course of several hours). *T. versicolor* produces a number of enzymes that act on phenolic substrates such as pinosylvins, most notably laccases and peroxidases (Moldes et al. 2004, Schlosser et al. 1997). Laccases in particular are known to act on a wide variety of compounds (Baldrian. 2006), including resveratrol and its derivatives, which are structural analogues of the pinosylvins (Nicotra et al. 2004, Ponzoni et al. 2007, Schouten et al. 2002). The *T. versicolor* enzyme preparation used in this experiment had 4.6 U mg⁻¹ of laccase activity, while the *C. puteana* and Celluclast preparations showed no activity at all.

3.4 Mechanism of *T. versicolor* inhibition and deactivation

To determine whether *T. versicolor* causes modification of pinosylvins, the enzyme preparation was incubated with the ME and the UEW and samples collected at different time points for GC analysis. *C. puteana* and Celluclast were tested as well but were found to cause no changes in the concentration of extractives. The results of *T. versicolor* incubation are given in Fig. 4, which shows the changes in the pinosylvins content of the samples. The two primary HW pinosylvins, pinosylvins and pinosylvins monomethyl ether, were monitored separately. During the course of 24 hours, *T. versicolor* was able to cause significant degradation of both compounds. Pinosylvins were removed from both the extract and the wood powder, with the degradation of pinosylvins proceeding more rapidly than that of the methyl ether derivative.

The laccase-catalysed oxidation of phenolic compounds typically leads to the formation of oligomeric and polymeric products (Mita et al. 2003, Sun et al. 2013). A number of different oligomers can be produced from resveratrol by laccase oxidation (Nicotra et al. 2004, Ponzoni et al. 2007, Schouten et al. 2002), and given the structural similarity of resveratrol and pinosylvins, the formation of analogous oligomers from pinosylvins by the *T. versicolor* enzyme seems possible. No oligomers or other reaction products were detected in this experiment, but their lack may be due to the unsuitability of the employed GC method for the detection of oligomeric products.

Figure 4. Disappearance of pinosylvins from the methanol extract (ME) or the unextracted wood powder (UEW) during incubation with the *T. versicolor* enzyme preparation. PS, pinosylvins; PSM, pinosylvins monomethyl ether

The results presented in Figs. 1, 2, and 4 suggest that the strong inhibition and deactivation of *T. versicolor* hydrolases by pine HW extractives is due to the modification of pinosylvins by *T. versicolor* enzymes, possibly laccases. Taken together with the protein content results (Fig. 3),

the modified pinosylvins then potentially complex and deactivate enzymes. However, the simple protein content measurements do not provide information on the type of enzymes that have been complexed, nor do they indicate whether the enzymes have been deactivated. Therefore, to better understand the mechanism of inhibition, the loss and distribution of *T. versicolor* enzyme activities in the ME and UEW samples was investigated in more detail.

The *T. versicolor* enzyme preparation was first incubated with the ME and the UEW and then centrifuged and separated into supernatant and solid fractions. Both fractions were used to hydrolyse pulp to determine whether any hydrolytic activity had been lost from the supernatant and to see whether any activity remained in the solids. The glucose and xylose yields of the fractionated hydrolyses are presented in Fig. 5, which shows a clear loss of hydrolytic activity in the ME and UEW samples. Cellulase activity was lost from both supernatants, with the ME supernatant releasing less than 10% and the UEW 38% of the reference glucose. The loss of cellulase activity in the supernatants may be due to complex formation between enzymes and extractives, as suggested by the reduction in supernatant protein content (Fig. 3), although irreversible deactivation without complexation is also possible. Very little cellulase activity was found in the ME and UEW solids, suggesting that if cellulases are present, they have been almost completely deactivated. Xylanase activity was also lost from the supernatants, but unlike the cellulases, a notable portion of the xylanase activity could be recovered from the solids. The presence of active xylanase in the solid fraction could explain why xylose release appears to be less affected by extractives than the release of glucose, at least in case of *T. versicolor* and *C. puteana* (Figs. 1 and 2).

Figure 5. Residual glucose and xylose releasing enzyme activity in the supernatant and solid fractions of *T. versicolor* after a 24 h incubation with the methanol extract (ME) or the unextracted wood powder (UEW)

The results shown in Fig. 5 therefore demonstrate that the loss of protein seen in Fig. 3 is accompanied by a loss of hydrolytic activity. Hydrolytic activity is lost from solution and cannot be fully recovered from the solids, supporting the earlier hypothesis that the inhibition of *T. versicolor* hydrolases is due to complex formation between enzymes and modified pinosylvins. The enzymes may be complexed by the hypothetical pinosylvins oligomers, resulting in enzyme deactivation. Alternatively, the enzymes may be attacked by the radical intermediates of pinosylvins: experiments with resveratrol have shown that enzymatic oxidation of the 3,5-OH structure produces an unstabilised m-semiquinone radical that can attack and deactivate enzymes, becoming non-covalently incorporated into the enzyme in the process (Szewczuk et al. 2004, 2005). In this experiment only the white rot fungus was capable of modifying pinosylvins, but assuming that the modification is catalysed by laccase, the ability

will not be limited to just white rot fungi. Many brown rot fungi produce laccases, including some strains of *C. puteana* (D'souza et al. 1996, Lee et al. 2004).

4. Conclusions

This experiment investigated the ability of Scots pine HW extractives to inhibit the enzymatic hydrolysis of holocellulose. The hydrolases of the commercial cellulase preparation and the brown rot fungus *C. puteana* were only slightly affected by the extractives, whereas the enzymes of the white rot fungus *T. versicolor* showed strong inhibition and deactivation, particularly by the pinosylvins-rich ME. Investigation of the causes of inhibition indicated that the *T. versicolor* enzyme preparation was capable of modifying pinosylvins: the modified pinosylvins might have complexed the enzymes and caused their deactivation. Modification of pinosylvins appeared to be required for significant hydrolase inhibition by the HW extractives, suggesting that hydrolase inhibition is unlikely to be effective against fungi that do not secrete enzymes capable of modifying pinosylvins. Against fungi that do secrete such enzymes, hydrolase inhibition may be an additional means of decay prevention by extractives.

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