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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.

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, 2016, 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). The phenolic 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

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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 mm^2 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 NaH_2PO_4, 0.5 g $MgSO_4$ x $7H_2O$, 0.4 g K_2HPO_4 x $3H_2O$, 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 h 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.