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Oxidative modification of paper pulp lipophilic extractives by the laccase-mediator system

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Abstract

Recently, we have shown the effectiveness of the laccase-mediator system in removing lipophilic extractives from pulp regardless the pulping process and the raw material used. This paper summarizes our research findings on the chemistry of the reactions of the main lipophilic extractives present in paper pulps with the laccase-mediator system. The results attained in reactions of several model lipids - including alkanes, fatty alcohols, fatty acids, resin acids, free sterols, sterol esters and triglycerides - with a fungal laccase in the presence of HBT as mediator, are discussed in the context of enzymatic control of pitch deposits, to explain the removal of complex lipid mixtures during laccase-mediator treatment of different pulp types, including eucalypt pulp.

Keywords: lipophilic extractives; pitch deposits; laccase-mediator system; eucalypt pulp.

Introduction

Lipophilic extractives, such as fatty and resin acids, fatty alcohols, alkanes, steroids and triglycerides cause pitch deposits along the pulp and paper manufacturing processes [1]. Besides physicochemical methods, enzymes and microorganisms have been investigated to solve pitch problems [2,3]. Lipases, which hydrolyze triglycerides, are successfully applied in softwood (mainly pine) mechanical pulping at mill scale [4]. However, pitch problems in most of the chemical and mechanical processes using other raw materials have not been solved yet. Other compounds, such as free and esterified sterols, resin acids, fatty alcohols and alkanes, are responsible for pitch problems in these processes [3]. Particularly, free and conjugated sterols are the main responsible for pitch problems during manufacturing of eucalypt pulp [3]. In contrast to lipases, laccases are oxidative enzymes whose action is directed toward phenols, anilines and related compounds. The interest on laccases as industrial biocatalysts has, however, increased after discovering the effect of some synthetic compounds [5,6] expanding the action of laccases to non-phenolic aromatic substrates and, therefore, increasing their potential in degradation of lignin and other recalcitrant compounds. Moreover, the use of laccases in the presence of redox mediators has recently been described for the removal of lipophilic extractives responsible for pitch deposits from wood and nonwood paper pulps [7,8]. Further investigations on the chemistry of the reactions of the laccase-mediator system with several model compounds representative for the main lipophilic extractives from different pulp types (including eucalypt pulp), have been carried out to better understand the degradation patterns observed in pulps [9], which are summarized here.

Experimental

Model lipophilic compounds

Alkanes (octadecane), fatty alcohols (1-hexadecanol), fatty acids (palmitic, oleic and linoleic acids), resin acids (abietic acid), free sterols (sitosterol), sterol esters (cholesteryl palmitate, cholesteryl oleate and cholesteryl linoleate) and triglycerides (triheptadecanoin and trilinolein), were used.

Laccase and mediator

The laccase used was from *Pycnoporus cinnabarinus*. Laccase activity was measured during oxidation of 5 mM 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) to its cation radical (ε_{436} 29300 M^{-1} cm^{-1}) in 0.1 M sodium acetate (pH 5) at 24ºC. One activity unit was defined as the amount of enzyme transforming 1 µmol of ABTS per min. 1-Hydroxybenzotriazole (HBT) was used as mediator.

Enzymatic reactions with model compounds

The enzymatic treatments (five replicates) of the different model lipids (1 mg) were performed using laccase (0.5 U/mg lipid), HBT (1 mg/mg lipid), and Tween 20 as dispersant (1% v/v) at pH 4, 50ºC, and different reactions times (5, 15, and 30 min, 1, 2, and 8 h). Oxygen was bubbled through the reaction flasks. In control experiments, lipids were treated under the same conditions but without laccase and mediator. Additional controls including laccase alone and boiled laccase were also performed. Mixtures of the saturated lipids (octadecane, hexadecanol, palmitic acid or triheptadecanoin) with linoleic acid or cholesteryl linoleate were treated (2 h) with laccase-HBT under the same conditions described above. After the enzymatic treatments, the lipid dispersions were immediately evaporated, and the reaction products recovered with chloroform:methanol (1:1), dried and redissolved in chloroform for gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analyses. Bis(trimethylsilyl)trifluoroacetamide in the presence of pyridine was used to prepare trimethylsilyl derivatives, before and after sodium borohydride reduction.

GC and GC-MS analyses of lipids

The GC and GC-MS analyses were performed as previously described [10]. Peaks were quantified by area, and data from replicates were averaged. In all cases the standard deviations were below 5% of the mean values. Longer columns (30 m) were also used when necessary.

Results and Discussion

Reactivity of different model lipids with laccase-HBT and laccase alone

Twelve model lipids (Figure 1) representative for the main lipophilic extractives from different woody and nonwoody pulps were treated with the high redox-potential laccase from *P. cinnabarinus* in the presence and absence of HBT as mediator, to get further insight into the chemistry of these reactions.

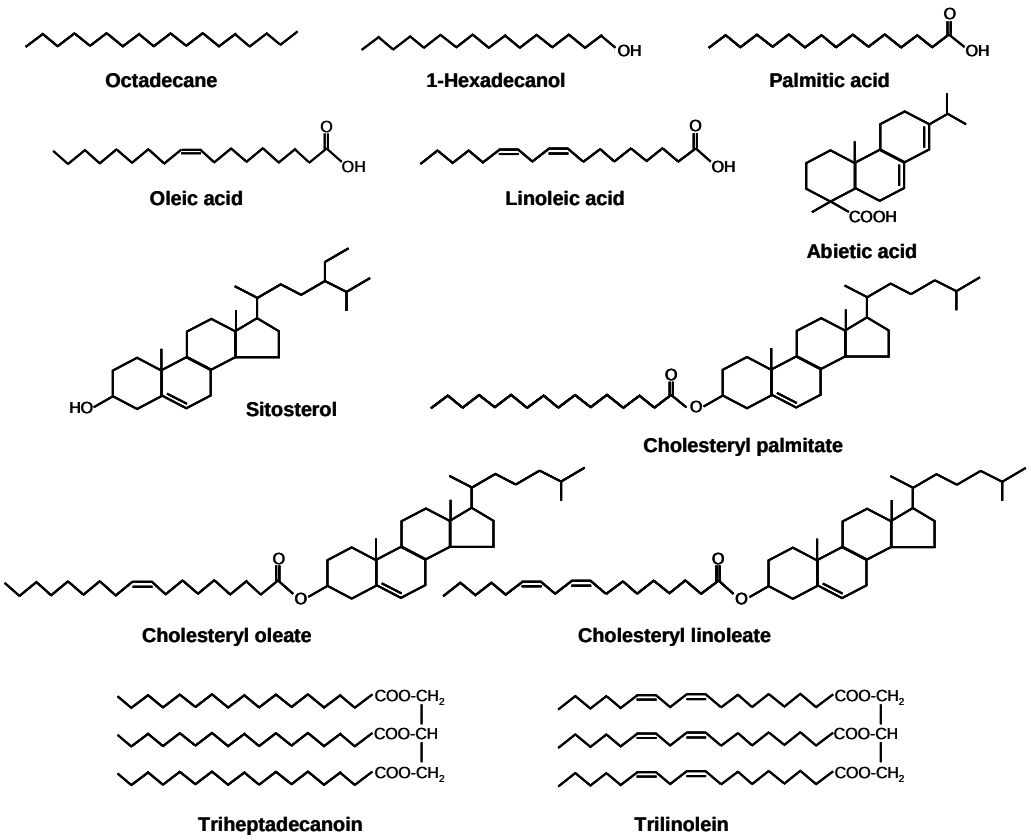

Figure 1. Chemical structures of model lipids representative for main paper pulp lipophilic extractives used in the enzymatic reactions

Two-hour laccase reactions were carried out with all the model lipids. The chromatographic analyses evidenced that octadecane, hexadecanol, palmitic acid and triheptadecanoin were not modified after 2-h treatment neither with laccase alone nor with laccase-HBT. No decrease in the amount of these saturated compounds was observed even when longer (8 h) reactions were carried out (data not shown). In contrast, 60-100% decrease of the initial amount of unsaturated compounds (Figure 2) such as abietic acid, trilinolein, linoleic and oleic acids, sitosterol, cholesteryl palmitate, oleate and linoleate, was found at the end of 2-h laccase-HBT treatment. Likewise, a decrease of 20-40% of these unsaturated lipids was observed after treatment with laccase alone except in the cases of abietic acid that decreased 95%, and cholesteryl palmitate and sitosterol that were not affected (data not shown).

When shorter laccase-HBT treatments (60, 30, 15 and 5 min) of the latter lipids were analyzed, different reactivities were observed (Figure 2). Thirty minutes of laccase-HBT treatment were enough to attain 40-100% reduction of all these unsaturated lipids. Abietic acid and trilinolein were already completely oxidized after only 5-min enzymatic treatment. High reduction (≥95%) of cholesteryl linoleate also required only 5-min treatment, whereas 30 min were required for cholesteryl oleate and linoleic acid. On the other hand, up to 75% reduction of sitosterol was attained after 1-h treatment, and complete transformation of this sterol was attained after 2 h. Therefore, sitosterol, together with cholesteryl palmitate and oleic acid, was the most refractory towards the laccase-HBT treatment among the different unsaturated lipids assayed.

Reaction products from the enzymatic treatment of unsaturated model lipids

The formation of oxidized products during 2-h treatment of eight model lipids by laccase-HBT is shown in Figure 2**,** together with the decrease of the initial compounds. Although abietic acid, trilinolein, and linoleic acid were completely or strongly transformed after 5 and 15 min of laccase-HBT treatment, respectively, new products were not observed.

In contrast, several oxidized compounds were identified after laccase-HBT treatment of oleic acid including one epoxy-fatty acid (*cis* and *trans* forms of 9,10-epoxyoctadecanoic acid), and four hydroxy-fatty acids (8-hydroxy-9-octadecenoic acid, 9-hydroxy-10-octadecenoic acid, 10-hydroxy-8-octadecenoic acid and 11-hydroxy-9-octadecenoic acid) quantified together, whose presence suggests the formation of four allylic hydroperoxides (with -OOH groups on carbons 8, 9, 10 or 11). The epoxy-fatty acid formed could be produced by hydroperoxide reaction with oleic acid [11].

During free sitosterol treatment with laccase-HBT the main new compound identified was 7-ketositosterol together with minor amounts of stigmasta-3,5-dien-7-one. Traces of 7α- and 7β-hydroxysitosterol were also detected. By observing the oxysterol pattern formed it could be inferred that the oxidation would be a free radical reaction with formation of 7-hydroperoxide, whose degradation forms the 7α- and 7β-hydroxysitosterol and 7-ketositosterol. On the other hand, the laccase-HBT system exhibited different degradation behaviors on the different sterol esters assayed. Cholesteryl palmitate was mainly oxidized during the first hour of treatment, and 40% of the initial ester remained at the end of the reaction. The most prominent compound formed was tentatively identified as 7-ketocholesteryl palmitate. In addition, minor amounts of cholesta-3,5-dien-7-one and traces of 7α- and 7β-hydroxycholesteryl palmitate were also identified. On the other hand, cholesteryl oleate and linoleate exhibited relatively similar degradation patterns after the enzymatic treatment since both compounds were completely transformed by laccase-HBT after 15-30 min reaction. The above cholesta-3,5-dien-7-one was produced from both sterol esters, being the main product at the end of the reactions. 7-Ketocholesteryl oleate was also detected from cholesteryl oleate. Additional products were identified during the first 15 min of reaction, including free oleic acid from cholesteryl oleate, and several new compounds from cholesteryl linoleate. These included cholesterol or 7-ketosterol esters containing fatty-acid chains of different lengths, including those tentatively identified as cholesteryl and 7-ketocholesteryl ester core aldehydes. Traces of free cholesterol and 7-ketocholesterol were also found during the initial stages of cholesteryl linoleate transformation by laccase-HBT. By observing the oxidation products from the above cholesterol esters it could be deduced that the oxysterols formed depend on the fatty-acid moiety.

Finally, it should be mentioned that none of the oxidized compounds mentioned above was detected when the reactions were performed with laccase alone.

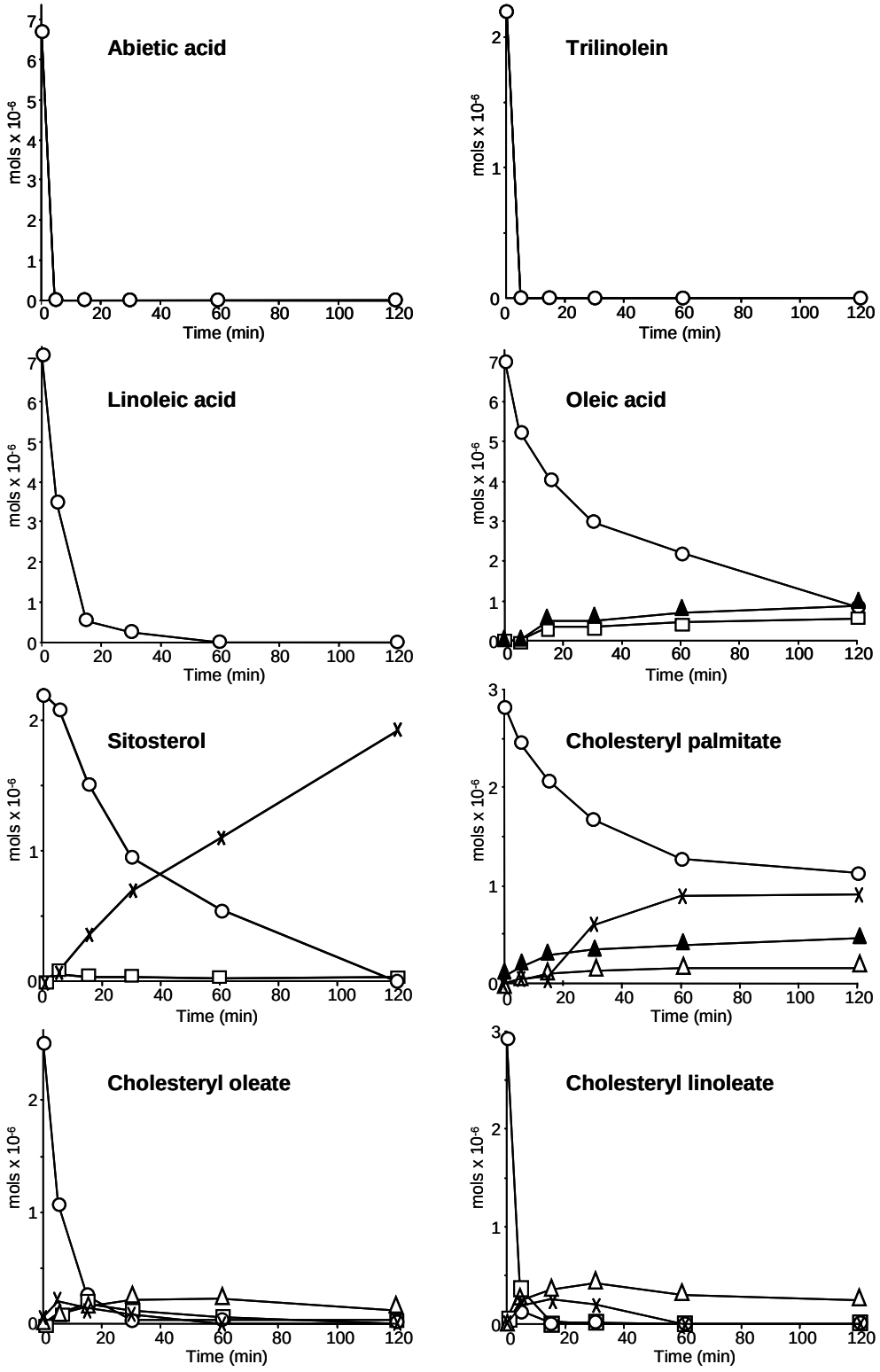

Figure 2. Oxidation of eight model lipids (○) and formation of oxidation products during laccase-HBT reactions: abietic acid; trilinolein; linoleic acid; oleic acid (□, epoxy-fatty acids; and ▲, hydroxy-fatty acids); sitosterol (□, stigmasta,3-5-dien-7-one; and x, 7-ketositosterol); cholesteryl palmitate (▲, palmitic acid; x, 7-ketocholesteryl palmitate; and Δ, cholesta-3,5-dien-7-one); cholesteryl oleate (□, 7-ketocholesteryl oleate; x, oleic acid; Δ, cholesta-3,5-dien-7-one); and cholesteryl linoleate (□, cholesteryl core aldehydes; x, 7-ketocholesteryl core aldehydes; Δ, cholesta-3,5-dien-7-one). Adapted from Molina et al. (2008) [9]

Laccase-HBT action on model lipid mixtures

During treatment of individual lipids, it was found that octadecane, 1-hexadecanol, palmitic acid and triheptadecanoin, were not modified by laccase-HBT. However, the corresponding lipid classes were extensively removed during the laccase-HBT treatment of pulps [8]. With the aim of studying whether the transformation of saturated lipids in pulps could be influenced by the presence of other lipid compounds, 2-h reactions of saturated lipids with laccase-HBT were carried out in the presence of unsaturated ones such as linoleic acid and cholesteryl linoleate. It could be observed that 1-hexadecanol decreased near 25% with laccase-HBT in the presence of the unsaturated lipids. Moreover, octadecane decreased 52% and 26% with laccase-HBT in the presence of linoleic acid or cholesteryl linoleate, respectively. This confirmed that the presence of unsaturated lipids in paper pulp facilitates the modification of the saturated ones, suggesting that lipid radicals generated from peroxidation of unsaturated lipids by laccase-HBT participate in the oxidation of the less reactive saturated lipids. More modest decreases were observed in reactions with laccase alone. In contrast, triheptadecanoin was not modified, and only a slight decrease of palmitic acid was attained. Hexadecanoic acid was formed after the reaction of laccase-HBT with 1-hexadecanol, in the presence of either linoleic acid or cholesteryl linoleate. In contrast, no reaction product was found after laccase-HBT reaction with octadecane in the presence of the latter unsaturated lipids.

Conclusions

We report the reaction of several model lipids with laccase in the presence and absence of HBT. Different reactivities were observed being correlated with the number of double bonds in their structures. The search for reaction products yielded variable results. No evidence for reaction products could be obtained for the most reactive compounds (such as abietic acid, linoleic acid and trilinolein). In contrast, oxygenated compounds appeared, and subsequently decreased, in the reactions of lipids with an intermediate reactivity degree (such as cholesteryl linoleate and oleate). With the less reactive lipids (such as oleic acid, sitosterol and cholesteryl palmitate) accumulation of reaction products was observed. The nature and abundance of the above oxidation products provided some clues on the mechanisms of lipid oxidation by laccase-HBT. Finally, some compounds (namely octadecane, 1-hexadecanol, palmitic acid and triheptadecanoin) did not show any reactivity with the laccase-HBT, although they could be modified in mixtures with unsaturated lipids. These results help to understand those obtained during laccase-HBT treatment of pulps, and can contribute to the development of enzymatic methods for pitch control in pulp and paper manufacturing and other biotechnological applications of the laccase-mediator system.

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