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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Oxidative degradation of model lipids representative for main paper pulp lipophilic extractives by the laccase–mediator system

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Abstract Different model lipids—alkanes, fatty alcohols, fatty acids, resin acids, free sterols, sterol esters, and triglycerides—were treated with *Pycnoporus cinnabarinus* laccase in the presence of 1-hydroxybenzotriazole as mediator, and the products were analyzed by gas chromatography. The laccase alone decreased the concentration of some unsaturated lipids. However, the most extensive lipid modification was obtained with the laccase–mediator system. Unsaturated lipids were largely oxidized and the dominant products detected were epoxy and hydroxy fatty acids from fatty acids and free and esterified 7-ketosterols and steroid ketones from sterols and sterol esters. The former compounds suggested unsaturated lipid attack *via* the corresponding hydroperoxides. The enzymatic reaction on sterol esters largely depended on the nature of the fatty acyl moiety, i.e., oxidation of saturated fatty acid esters started at the sterol moiety, whereas the initial attack of unsaturated fatty acid esters was produced on the fatty acid double bonds. In contrast, saturated lipids were not modified, although some of them decreased when the laccase–mediator reactions were carried out in the presence of unsaturated lipids suggesting participation of lipid peroxidation radicals. These results are discussed in the context of enzymatic control of pitch to explain the removal of lipid mixtures during laccase–mediator treatment of different pulp types.

Keywords Laccase–mediator system · Fungal enzymes · Model lipids · Reaction mechanisms · Paper pulps · Pitch deposits

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Introduction

The nonpolar extractable fraction from wood and other lignocellulosic materials, commonly referred to as lipophilic extractives, includes fatty and resin acids, fatty alcohols, alkanes, steroids, and triglycerides. These lipophilic compounds cause the so-called pitch deposits along the pulp and paper-manufacturing processes (Back and Allen 2000). Pitch deposition results in low quality of pulp and can cause the shutdown of pulp mill operations. In addition, pulps contaminated with lipophilic extractives are at the origin of the pitch problems in paper machine operation, including spots and holes in the paper, sheet breaks, and technical shutdowns in paper mills.

In addition to physicochemical methods, biological methods including both enzymes (Fischer and Messner 1992; Fischer et al. 1993; Fujita et al. 1992) and microorganisms (Behrendt and Blanchette 1997; Farrell et al. 1993; Gutiérrez et al. 1999, 2001) have been investigated to solve pitch problems in the pulp and paper industry. Lipases, which hydrolyze triglycerides, are successfully applied in softwood (mainly pine) mechanical pulping at mill scale (Fujita et al. 1992). However, pitch problems in

most of the chemical and mechanical processes using other raw materials have not been solved yet. Indeed, other compounds, such as free and esterified sterols, resin acids, fatty alcohols, and alkanes, are responsible for pitch problems in these processes (del Río et al. 1999, 2000; Gutiérrez and del Río 2005). In addition to lipases, the use of sterol esterases has also been suggested (Calero-Rueda et al. 2004; Kontkanen et al. 2004) because sterol esters are often at the origin of pitch deposits owing to their high tackiness and resistance to kraft cooking. However, free sterols released by the action of these esterases are as problematic as sterol esters.

On the other hand, modification of lignans, and other colloidal substances in process waters and pulps from softwood pulping, by laccase treatment has been reported (Buchert et al. 2002; Zhang et al. 2000, 2005). In contrast to lipases and sterol esterases, laccases are oxidative enzymes whose action is directed toward phenolic compounds. The interest on laccases as industrial biocatalysts has, however, increased after discovering the effect of some synthetic compounds (Bourbonnais and Paice 1990; Call 1994) expanding the action of laccases to nonphenolic substrates and, therefore, increasing their potential in degradation of lignin and other aromatic compounds (Bourbonnais and Paice 1996; Camarero et al. 2004; Ibarra et al. 2006; Nelson et al. 1998; Poppius-Levlin et al. 1999; Sealey et al. 1999; Widsten and Kandelbauer 2008). Moreover, the use of enzymes of the group of laccases in the presence of redox mediators has very recently been described for the removal of the lipophilic extractives responsible for pitch deposition, from wood and nonwood paper pulps (Gutiérrez et al. 2006a, b). In these previous works, different patterns of lipid removal were observed in the different pulp types. In the present work, further investigations on the chemistry of the reactions of the laccase–mediator system with several model compounds representative for the main lipophilic extractives present in wood (hardwood and softwood) and nonwoody paper pulps are carried out to better understand the degradation patterns observed in pulps.

Materials and methods

Model lipophilic compounds

Several model lipids, including 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. Sitosterol was purchased from Calbiochem, and all the other lipids were obtained from Sigma-Aldrich.

Laccase and mediator

The laccase used was provided by Beldem (Andenne, Belgium). It was obtained from a laccase-hyperproducing strain of the fungus *Pycnoporus cinnabarinus* (Herpoël et al. 2000) and showed a unique protein band after sodium dodecyl sulfate-polyacrylamide gel electrophoresis, which corresponded to laccase as revealed by activity staining in zymograms. Laccase activity was measured as initial velocity taking linear increments during oxidation of 5 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, from Roche) to its cation radical (ε_{436} 29,300 M^{-1} cm^{-1}) in 0.1 M sodium acetate (pH 5) at 24°C. One activity unit was defined as the amount of enzyme transforming 1 µmol of ABTS per minute. 1-Hydroxybenzotriazole (HBT), used as laccase mediator, was obtained from Sigma-Aldrich.

Enzymatic treatments of model compounds

The enzymatic treatments (five replicates) of the above 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, 50°C, and different reactions times (5, 15, 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 without mediator and boiled laccase were also performed.

Mixtures of the saturated lipids (namely 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. When required, bis(trimethylsilyl)trifluoroacetamide (from Supelco) in the presence of pyridine was used to prepare trimethylsilyl derivatives, before and after sodium borohydride reduction.

Enzymatic treatments of paper pulps

Unbleached kraft pulp from eucalypt (*Eucalyptus globulus*) was obtained from ENCE (Spain), thermomechanical pulping (TMP) pulp after the primary refiner from spruce (*Picea abies*) was provided by UPM-Kymmene (Finland), and unbleached soda–anthraquinone (AQ) pulp from flax (*Linum usitatissimum*) was supplied by CELESA (Spain).

Pulp (200 g) treatments with laccase (20 U/g pulp) and HBT (15 mg/g pulp) were carried out at pH 4, 50°C, and 10% consistency, under oxygen atmosphere, for 2 h (Gutiérrez et al. 2006b). Controls without laccase and/or HBT were performed. Treated pulp samples (8 g) were extracted with acetone (8 h), and the extracts obtained were evaporated and redissolved in chloroform for GC and GC–MS analyses.

GC and GC–MS analyses of lipids

The GC analyses of lipids during the enzymatic treatments of model compounds and pulps were performed in an Agilent 6890N Network GC system using a short-fused silica DB-5HT capillary column (5 m×0.25 mm internal diameter, 0.1-μm film thickness) from J&W Scientific, enabling simultaneous elution of the different lipid classes (Gutiérrez et al. 1998). The temperature program was started at 100°C with 1-min hold, then raised to 350°C at 15°C/min, and held for 3 min. The injector and flame ionization detector (FID) temperatures were set at 300°C and 350°C, respectively. Helium (5 ml/min) was used as carrier gas, and the injection was performed in splitless mode. Peaks were quantified by area, and data from replicates were averaged. In all cases, the standard deviations were below 5% of the mean values.

The GC–MS analyses were performed with a Varian 3800 chromatograph coupled to an ion trap detector (Varian 4000) using a medium-length (12 m) capillary column of the same characteristics described above for GC–FID. The oven was heated from 120°C (1 min) to 380°C at 10°C/min and held for 5 min. Longer columns (30 m) in a Varian model Star 3400 GC equipped with an ion trap detector (Varian model Saturn 2000) were also used when necessary. In this case, the oven was heated from 50°C to 110°C (at 30°C/min) and then to 320°C (at 6°C/min). In all GC–MS analyses, the transfer line was kept at 300°C; the injector was programmed from 120°C (0.1 min) to 380°C at 200°C/min and held until the end of the analysis, and helium was used as carrier gas at a rate of 2 ml/min. Compounds were identified by mass fragmentography and by comparing their mass spectra with those of the Wiley and NIST libraries and standards.

Results

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

Twelve model lipids (Fig. 1) representative for the main lipophilic extractives present in different hardwood, softwood, and nonwoody pulps were treated with the high redox potential fungal laccase from *P. cinnabarinus* in the presence of HBT as mediator, to get further insight into the chemistry of these reactions. The reactivity of the different lipids was studied by GC and GC–MS.

Firstly, 2-h laccase reactions were carried out with all the model lipids in the presence and absence of mediator. The chromatographic analyses evidenced different extents of lipid modification by laccase–HBT and by laccase alone (Table 1). It was observed that octadecane (a), hexadecanol (b), palmitic acid (c), and triheptadecanoin (k) 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) enzymatic treatments were carried out (data not shown). In contrast, 60–100% decrease of the initial amount of oleic (d), linoleic (e) and abietic (f) acids, sitosterol (g), cholesteryl palmitate (h), oleate (i) and linoleate (j), and trilinolein (l) was found after 2-h laccase–HBT treatment. Likewise, a decrease of 20–40% of these unsaturated lipids was observed after treatment with laccase (without HBT) except in the cases of abietic acid that decreased 95% and cholesteryl palmitate and sitosterol that were not affected by the laccase alone.

Then, shorter laccase–HBT treatments (60, 30, 15, and 5 min) were attempted with the latter lipids, whose amount was extensively reduced in the 2-h treatments, and different reactivities were observed (Table 1). 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, is 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 lipid reaction products after different treatment times with laccase–HBT and laccase alone were analyzed by GC and GC–MS using short- and medium-length capillary columns. Longer columns, with a different temperature program, were also used to investigate low-molecular-mass compounds. The chemical structures of the different reaction products identified from fatty acids, sterols, and sterol esters are shown in Fig. 2, including epoxy (m) and hydroxy fatty acids (n–q), oxosteroids (r–t), and sterol

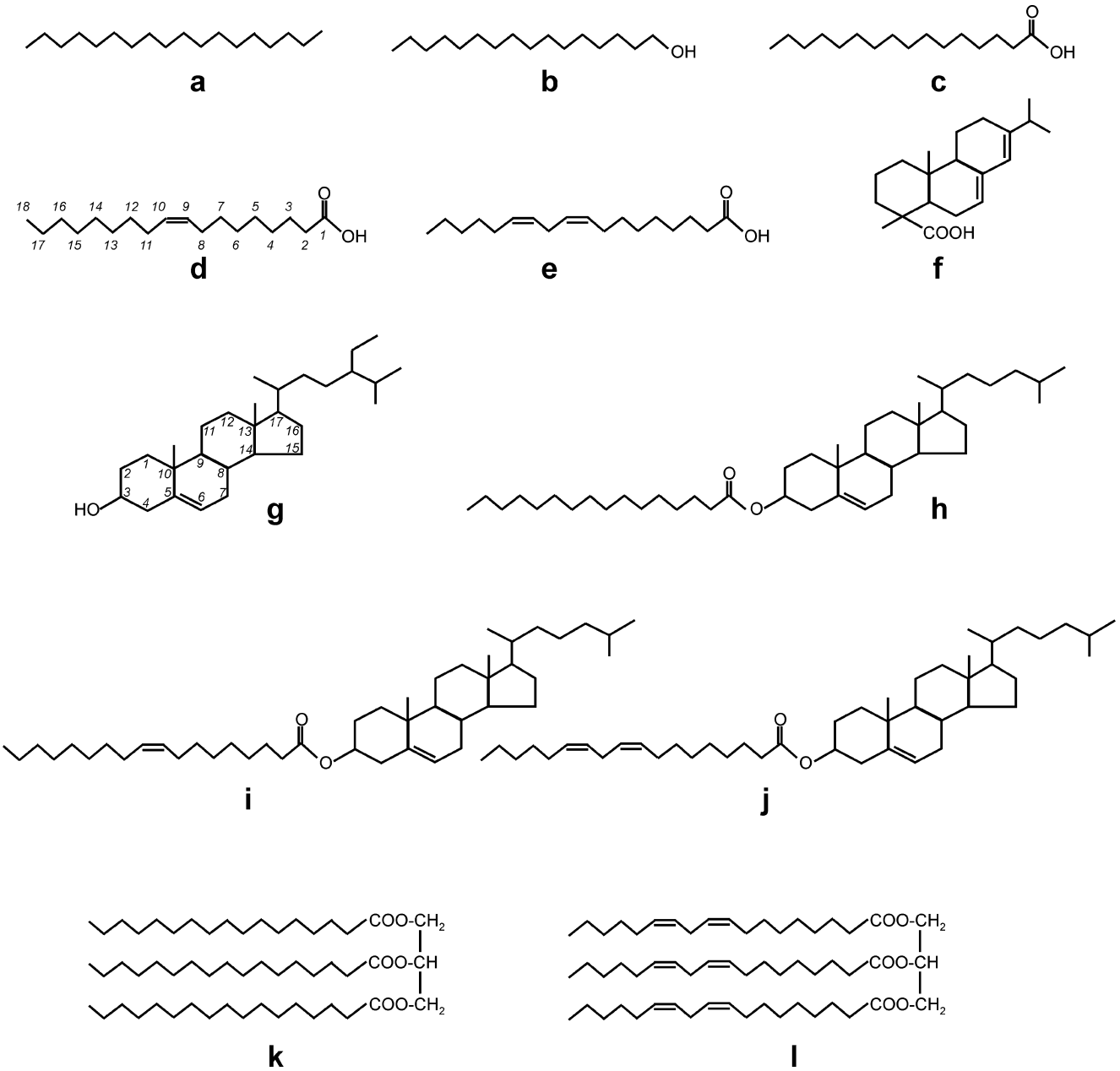

Fig. 1 Chemical structures of model lipids representative for main paper pulp lipophilic extractives used in the enzymatic reactions: **a** octadecane; **b** 1-hexadecanol; **c** palmitic acid; **d** oleic acid; **e** linoleic acid; **f** abietic acid; **g** sitosterol; **h** cholesteryl palmitate; **i** cholesteryl oleate; **j** cholesteryl linoleate; **k** triheptadecanoin; and **l** trilinolein

esters oxidized at the sterol (u and v) or the fatty acid (w) or in both moieties simultaneously (x). The decrease of the initial compounds and the formation of new oxidized products during 2-h treatment of six selected model lipids by laccase–HBT are shown in Fig. 3.

Abietic (f) and linoleic (e) acids were completely or strongly (over 90%) transformed, after 5 and 15 min of laccase–HBT treatment, respectively, but new compounds were not observed (Fig. 3a). The same happened after treating trilinolein (l) since no reaction products could be detected, despite complete disappearance of this unsaturated triglyceride was already observed after 5 min. Likewise, no peaks were detected in the reactions of these lipids with laccase alone.

In contrast, several oxidized compounds were identified after laccase–HBT treatment of oleic acid (d; Fig. 3b). These oxidized compounds, which were identified as trimethylsilyl ether derivatives after borohydride reduction, included one epoxy fatty acid (*cis* and *trans* forms of 9, 10-epoxyoctadecanoic acid; m) and four hydroxy fatty

Table 1 Effects of the laccase–mediator system on 12 model lipids (structures a–l in Fig. 1) after different reaction times (percentages of reduction in concentration)

	Laccase	Laccase–HBT				
	2 h	5 min	15 min	30 min	1 h	2 h
Alkanes:						
Octadecane (a)	0	0	0	0	0	0
Fatty alcohols:						
1-Hexadecanol (b)	0	0	0	0	0	0
Fatty acids:						
Palmitic acid (c)	0	0	0	0	0	0
Oleic acid (d)	25	25	38	58	69	88
Linoleic acid (e)	22	51	93	97	100	100
Resin acids:						
Abietic acid (f)	95	100	100	100	100	100
Free sterols:						
Sitosterol (g)	0	4	31	54	75	100
Sterol esters:						
Cholesteryl palmitate (h)	0	16	18	39	55	60
Cholesteryl oleate (i)	32	57	90	100	100	100
Cholesteryl linoleate (j)	38	95	99	100	100	100
Triglycerides:						
Triheptadecanoin (k)	0	0	0	0	0	0
Trilinolein (l)	37	100	100	100	100	100

acids (8-hydroxy-9-octadecenoic acid, 9-hydroxy-10-octadecenoic acid, 10-hydroxy-8-octadecenoic acid, and 11-hydroxy-9-octadecenoic acid; n–q) quantified together, whose final concentration was similar to that of remaining oleic acid. None of these oxidized compounds was detected when the reactions were performed with laccase alone.

During free sitosterol (g) treatment with laccase–HBT (Fig. 3c), the main new compound identified was 7-ketositosterol (r) together with minor amounts of stigmasta-3,5-dien-7-one (s). Whereas the amount of the latter compound scarcely varied along the reaction time, the amount of 7-ketositosterol progressively increased (up to 85% of the initial sterol) paralleling the decrease of sitosterol, which completely disappeared at the end of the reaction. Traces of 7α- and 7β-hydroxysitosterol were also detected. None of the above oxidized compounds were detected when the reaction was performed with laccase alone.

Interestingly, the laccase–HBT system exhibited different degradation behaviors on the different sterol esters assayed, resulting in different chromatographic profiles, as illustrated in Fig. 4 for two of them after different reaction times. Cholesteryl palmitate (h) was mainly oxidized during the first hour of treatment, and 40% of the initial ester remained at the end of the reaction (Fig. 3d). The most prominent compound formed was tentatively identified as 7-ketocholesteryl palmitate (u; Fig. 4c, e). In addition, minor amounts of cholesta-3,5-dien-7-one (t) and traces of 7α- and 7β-hydroxycholesteryl palmitate were also identified. On the other hand, cholesteryl oleate (i) and linoleate (j) exhibited relatively similar chromatographic patterns after the enzymatic treatment since both compounds were completely transformed by laccase–HBT after 15–30-min reaction (Fig. 3e,f). The above cholesta-3,5-dien-7-one (t) was produced from both sterol esters, being the main product at the end of the reactions, as shown in Fig. 4f for cholesteryl linoleate. 7-Ketocholesteryl oleate (v) was also detected from cholesteryl oleate. Additional products were identified during the first 15 min of reaction, including free oleic acid (d) from cholesteryl oleate and several new compounds from cholesteryl linoleate (Fig. 4d). These included cholesterol or 7-ketosterol esters containing fatty acid chains of different lengths, including those tentatively identified as cholesteryl (w) and 7-ketocholesteryl ester core aldehydes (x). Traces of free cholesterol and 7-ketocholesterol were also found during the initial stages of cholesteryl linoleate transformation by laccase–HBT. The small amounts of cholesta-3,5-diene and cholesta-3,5,7-triene (asterisks in Fig. 4) could be formed by dehydration reactions at the GC injector. In contrast, no reaction products were observed after treating the cholesteryl oleate and linoleate with laccase alone, in spite that up to 38% removal was obtained after 2-h treatment.

Laccase–HBT action on complex lipid mixtures in paper pulps and laboratory preparations

Lipids in paper pulps appear as complex mixtures whose composition varies in function of the raw material used and the pulping process applied. Three paper pulps characterized

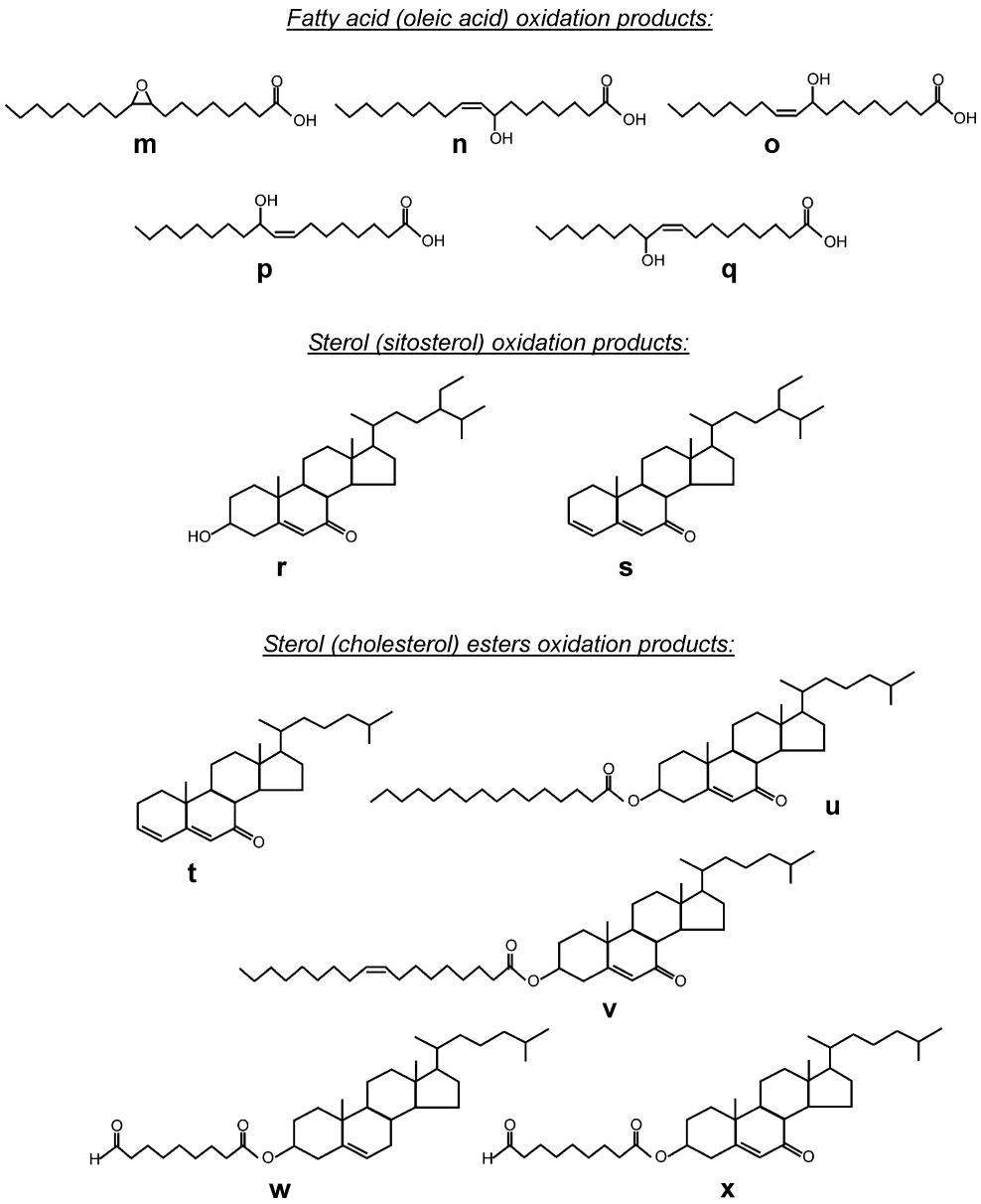

Fig. 2 Chemical structures of the main lipid oxidation products identified after laccase–HBT reactions with fatty acids, sterols, and sterol esters: **m** *cis*-9,10-epoxyoctadecanoic acid; **n** 8-hydroxy-9-octadecenoic acid; **o** 9-hydroxy-10-octadecenoic acid; **p** 10-hydroxy-8-octadecenoic acid; **q** 11-hydroxy-9-octadecenoic acid; **r** 7-ketositosterol; **s** stigmasta-3,5-dien-7-one; **t** cholesta-3,5-dien-7-one; **u** 7-ketocholesteryl palmitate; **v** 7-ketocholesteryl oleate; **w** cholesteryl 9-oxononanoate; and **x** 7-ketocholesteryl 9-oxononanoate

by the very different composition of their lipophilic extractives, namely unbleached eucalypt kraft pulp (Fig. 5a), TMP spruce pulp (Fig. 5c), and flax soda–AQ unbleached pulp (Fig. 5e), were treated with laccase–HBT and the lipids present were analyzed by GC and GC–MS. The chromatographic analysis of the lipids extracted from the enzymatically treated eucalypt (Fig. 5b), spruce (Fig. 5d), and flax (Fig. 5f) pulps, compared with the untreated pulps, revealed that the laccase–HBT treatment completely (or greatly) removed most of the lipids present including: (1) free sitosterol (peak 12) and sitosterol esters and glycosides (peaks 16 and 18) in eucalypt pulp; (2) triglycerides (peak 19), sterol esters (peak 18), and resin acids (peak 2) in spruce pulp; and (3) fatty alcohols (peaks 4, 5, 7, 9, and 14) and sitosterol (peak 12) in the flax pulp. Some amounts of 7-ketositosterol (peak 15) and steroid ketones (peaks 11 and 13) were found from the oxidation of pulp steroids, in agreement with the results described above for pure sitosterol and cholesterol esters.

During treatment of individual standards, it had been found that saturated lipids such as octadecane, 1-hexadecanol, palmitic acid, and triheptadecanoin were not modified by laccase–HBT. However, the corresponding lipid classes—including fatty alcohols, alkanes, and free and esterified saturated fatty acids—were extensively removed during the laccase–HBT treatment of pulps. With the aim of studying whether the transformation of saturated lipids in pulps could be influenced by the presence of other lipid compounds,

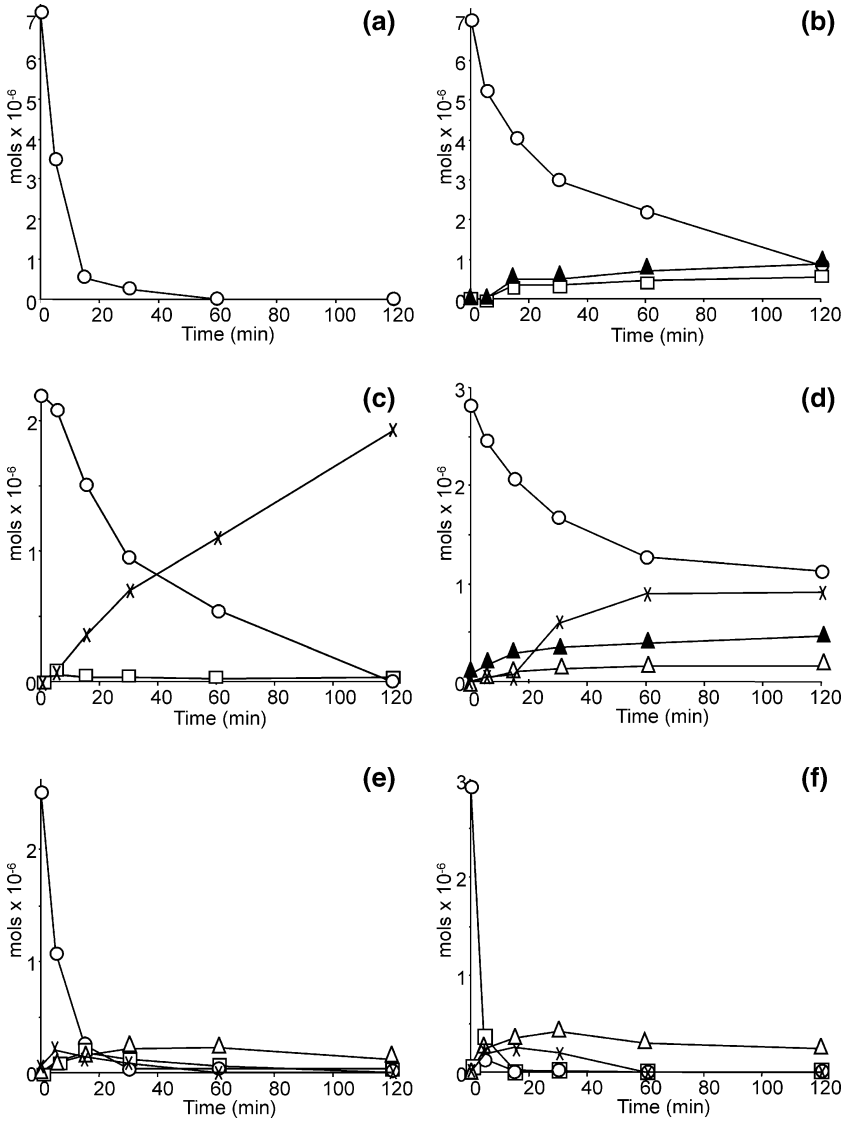

Fig. 3 Removal of six model lipids (*circles*) and formation of oxidation products during laccase–HBT reactions: **a** linoleic acid; **b** oleic acid (*squares*, epoxy fatty acids; and *triangles*, hydroxy fatty acids); **c** sitosterol (*squares*, stigmasta-3,5-dien-7-one; and *multiplication signs*, 7-ketositosterol); **d** cholesteryl palmitate (*filled triangles*, palmitic acid; *multiplication signs*, 7-ketocholesteryl palmitate; and *triangles*, cholesta-3,5-dien-7-one); **e** cholesteryl oleate (*squares*, 7-ketocholesteryl oleate; *multiplication signs*, oleic acid; *triangles*, cholesta-3,5-dien-7-one); and **f** cholesteryl linoleate (*squares*, cholesteryl core aldehydes; *multiplication signs*, 7-ketocholesteryl core aldehydes; *triangles*, cholesta-3,5-dien-7-one)

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, and the results are shown in Table 2. 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. 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. The decrease of palmitic acid was variable due to its limited solubility. 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.

Discussion

Laccase modification of lipophilic extractives

Lipophilic extractives exert a negative impact in pulp and paper manufacture causing the so-called pitch problems that affect both product quality and paper machine runnability and cause deposits at the mills (Back and Allen 2000). Using model compounds, we showed that laccase alone was able to modify some unsaturated lipids, such as linoleic, oleic, and abietic acids, cholesteryl oleate and linoleate, and trilinolein. In contrast, it was unable to act on free sitosterol and cholesteryl palmitate. It is conceivable that laccase, which is able to bind aromatic substrates, could also bind the unsaturated moieties of different lipids and that the oxidation reaction could be favored by the

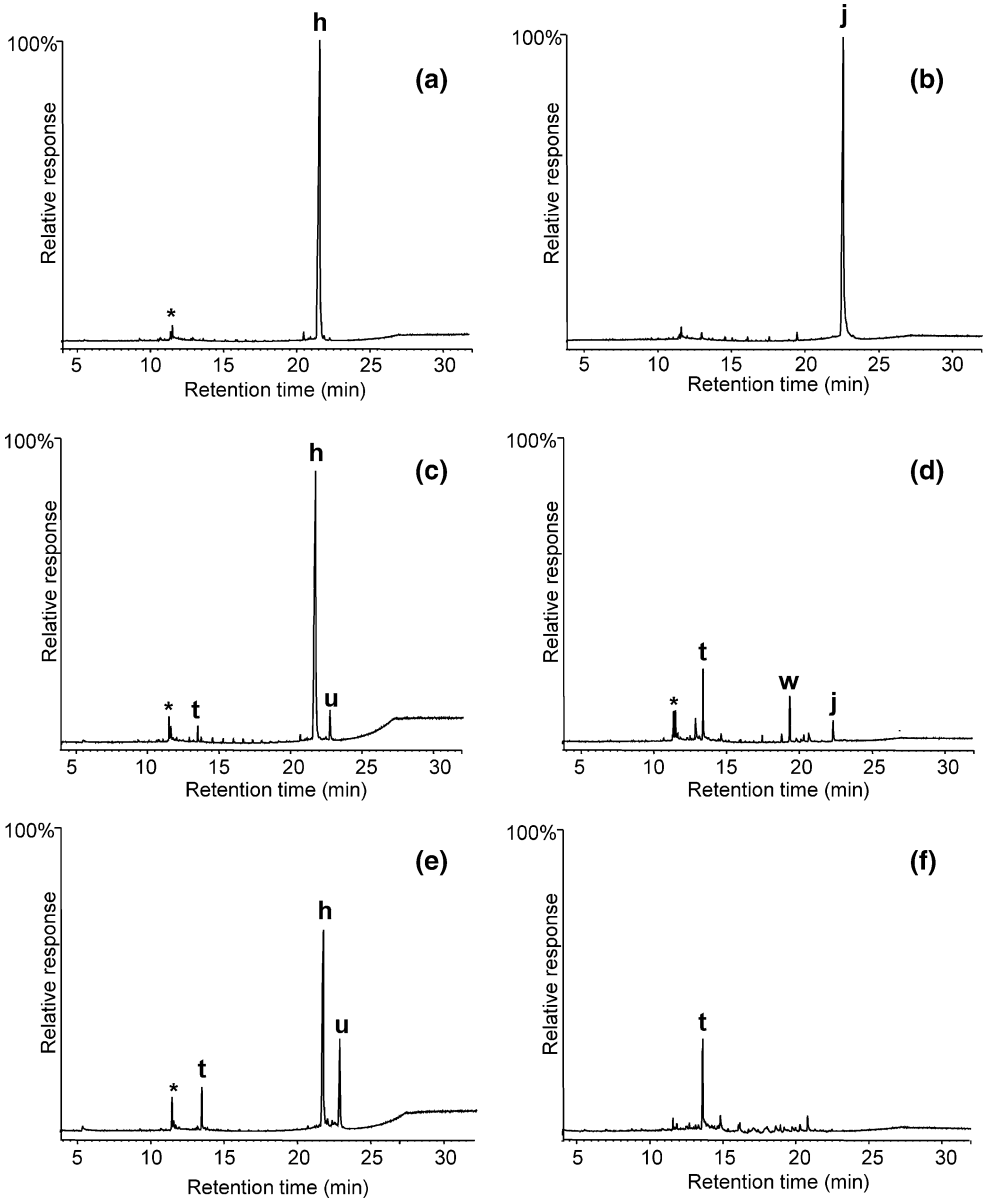

Fig. 4. GC–MS analyses after different times, 0 min (**a** and **b**), 5 min (**c** and **d**), and 30 min (**e** and **f**), during treatment of cholesteryl palmitate (*left*) and cholesteryl linoleate (*right*) with laccase–HBT, revealing different transformation patterns and formation of oxidation products. Peak identification: *letters* correspond to structures in Figs. 1 and 2; and the *asterisks* correspond to cholesta-3,5-diene and cholesta-3,5,7-triene peaks

unsaturation degree of lipids increasing electron density. The partial reactivity of laccases with unsaturated fatty acids, conjugated resin acids, and trilinolein had been already reported, including hydroperoxide and epoxide formation from the latter compound (Karlsson et al. 2001; Zhang et al. 2002). In contrast, other authors reported no significant peroxidation of linoleic acid by laccase (Srebotnik and Boisson 2005). No evidence for oxidation products was obtained here, but a decrease of unsaturated lipids by laccase was produced.

The results from pulp treatment with the laccase–mediator showed that this oxidative system removes to a large extent (often completely) the main lipid classes present in paper pulp (including free and conjugated sterols, triglycerides, resin and fatty acids, and fatty alcohols) as reported for the first time by Gutiérrez et al. (2006a, b). Moreover, using model compounds, we demonstrated that the laccase–HBT system is able to oxidize, to a much larger extent than laccase alone, a variety of unsaturated lipids, such as linoleic, oleic, and abietic acids, free sitosterol, cholesteryl palmitate, oleate and linoleate, and trilinolein. Some oxidized derivatives were produced in most cases, whose formation is congruent with the oxidative nature of the laccase attack that is a copper-enzyme-catalyzed oxygen reaction. Formation of these oxidized derivatives in the reactions of laccase–HBT with model lipids, with special emphasis on sterols, fatty acids, and sterol esters, as well as with lipophilic extractives present in paper pulps, is discussed below.

Laccase–mediator oxidation of free fatty acids and sterols

Hydroperoxide- and epoxide-type compounds are the predominant products from oxidation of unsaturated fatty

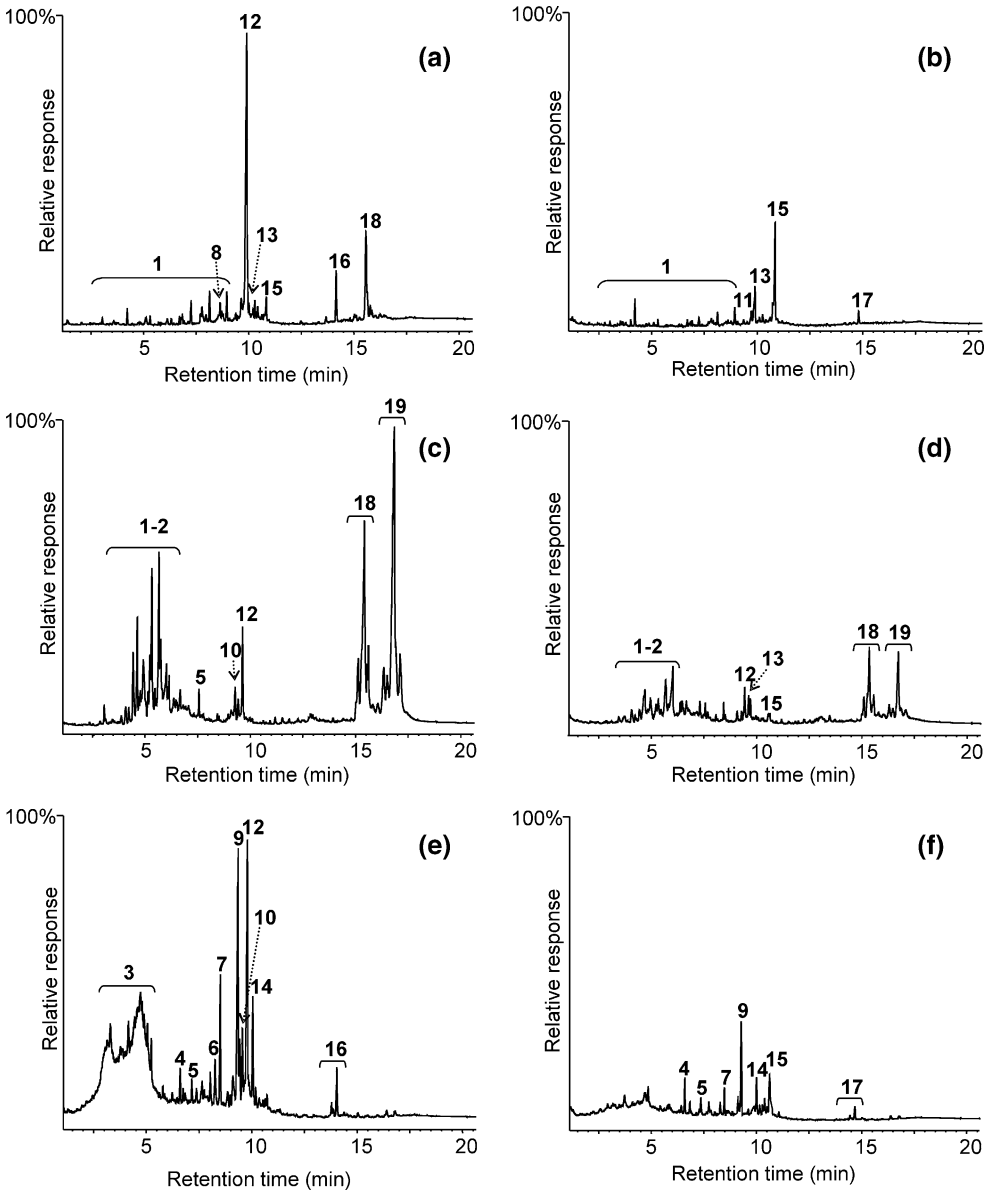

Fig. 5 GC analyses of lipophilic compounds in eucalypt kraft pulp (**a** and **b**), spruce TMP pulp (**c** and **d**), and flax soda–AQ pulp (**e** and **f**) before (**a**, **c**, and **e**) and after (**b**, **d**, and **f**) laccase–HBT treatment. Peak identification: *1*, fatty acids; *2*, resin acids; *3*, defoamer; *4*, docosanol; *5*, tetracosanol; *6*, nonacosane; *7*, hexacosanol; *8*, steroid hydrocarbons; *9*, octacosanol; *10*, campesterol; *11*, stigmastan-3-one; *12*, sitosterol; *13*, stigmasta-3,5-dien-7-one; *14*, triacontanol; *15*, 7-ketositosterol; *16*, sterol glycosides; *17*, 7-ketosterol glycosides; *18*, sterol esters; and *19*, triglycerides

acids (Frankel 2005). Free radical autoxidation of oleic acid involves hydrogen abstraction at the allylic carbons 8 or 11 producing two delocalized three-carbon allylic radicals. Oxygen attack on these intermediates produces a mixture of four allylic hydroperoxides (on carbons 8, 9, 10, or 11). The corresponding four hydroxy fatty acids were identified during laccase–HBT reaction with oleic acid suggesting the same reaction mechanism. In addition, 9,10-epoxyoctadecanoic acid was also found, which could be produced by hydroperoxide reaction with oleic acid (Frankel 2005). In contrast, no degradation products were found after the enzymatic treatment of linoleic acid, even when longer GC columns and lower temperature programs were used. This seems related to the high reactivity of linoleic acid that would lead to rapid decomposition of the hydroperoxides formed.

In the reaction of laccase–HBT with sitosterol, the main compound identified was 7-ketositosterol with only traces of 7α- and 7β-hydroxysitosterol. Small amounts of stigmasta-3,5-dien-7-one were also detected. By observing the oxysterol pattern formed, it could be inferred that the oxidation would be a free radical reaction starting with the abstraction of a reactive allylic hydrogen at carbon 7 followed by oxygen attack to form the 7-peroxy radical. Hydrogen addition to this radical gives the 7-hydroperoxide, whose degradation forms the 7α- and 7β-hydroxysitosterol and 7-ketositosterol (Johnsson 2004).

Laccase–mediator oxidation of sterol esters

When sterol esters were oxidized by laccase–HBT, the oxysterols formed depended on the fatty acid moiety.

Table 2 Effects of the laccase–mediator system on five saturated model lipid (structures a–c and k in Fig. 1) in mixtures with linoleic acid or cholesteryl linoleate (percentages of reduction in concentration after 2-h treatment)

	Laccase	Laccase–HBT
Mixtures with linoleic acid:		
Octadecane (a)	3	52
1-Hexadecanol (b)	7	25
Palmitic acid (c)	0–3	0–8
Triheptadecanoin (k)	0	0
Mixtures with cholesteryl linoleate:		
Octadecane (a)	9	26
1-Hexadecanol (b)	9	24
Palmitic acid (c)	0–5	0–14
Triheptadecanoin (k)	0	0

Cholesteryl palmitate was the most resistant towards the enzymatic attack followed by cholesteryl oleate, whereas cholesteryl linoleate was the most susceptible to oxidation. Therefore, the rate of oxidation was proportional to the degree of unsaturation of the fatty acyl moiety, as found in other studies (Brown et al. 1996; Frankel 2005).

Several products were detected after the reaction of cholesteryl palmitate with laccase–HBT, such as 7-ketocholesteryl palmitate, cholesta-3,5-dien-7-one, and palmitic acid. In contrast, the amount of oxidation products from cholesteryl oleate and linoleate was much lower, and a fast removal was achieved. In the cholesteryl linoleate reaction, the first products observed were a pool of cholesteryl and 7-ketocholesteryl esters with shortened fatty acyl chains. Similar cholesteryl ester core aldehydes (oxoalkanoyl esters of cholesterol and 7-ketocholesterol) were described during copper-catalyzed peroxidation of plasma lipoproteins (Kamido et al. 1995). The concentration of core aldehydes then decreased with a concomitant increase of cholesta-3,5-dien-7-one that was the only reaction product at the end of the reaction. As mentioned above, 7-ketocholesteryl palmitate and 7-ketocholesteryl oleate appeared after the enzymatic reaction with cholesteryl palmitate and oleate, respectively. In contrast, no 7-ketocholesteryl linoleate was detected at any stage of oxidation. A similar result was reported during copper oxidation of plasma lipoproteins (Brown et al. 1996).

By observing the oxidation products from the above cholesterol 3β-acyl esters, it could be deduced that initial radical formation on saturated fatty acyl esters occurs at the 3β-acyl carbonyl, after which the epimeric 7-hydroperoxide 3β-esters are formed. The 7-ketocholesterol ester detected is an oxidation product of the latter compound, and the cholesta-3,5-dien-7-one indicated subsequent fatty acyl ester elimination (Smith 1996). In contrast, oxidation of unsaturated fatty acyl esters of cholesterol is initiated by carbon-centered radical formation at the acyl allylic sites, with formation of hydroperoxides of the same types formed by the parent fatty acid. Finally, oxidation may also involve abstraction of a 7-hydrogen atom of the sterol moiety by the initially formed acyl carbon-centered radical, yielding 7-oxygenated 3-acyl derivatives (Smith 1996). In agreement with the above mechanisms, three types of oxidized sterol esters were observed after laccase–mediator treatment: esterified oxysterols, sterol esters of oxidized fatty acyl moieties, and oxysterol esters of oxidized fatty acids moieties.

Enzymatic removal of pulp lipids

Laccase–mediator treatment of paper pulps resulted in the removal of the main lipid classes present in the different pulp types assayed, including free and conjugated sterols and fatty acids, among others. The enzymatic removal of these pulp lipids also resulted in the formation of some oxidized derivatives that were absent or in low abundances in the initial pulps. The most prominent oxidation products observed included oxidized steroids, such as 7-ketositosterol and stigmasta-3,5-dien-7-one, that appeared or increased significantly after the laccase–HBT treatment of pulps containing free and/or esterified sterols, in agreement with previous studies (Gutiérrez et al. 2006a, b). After studying the enzymatic reactions with individual model lipids, it could be deduced that the 7-ketositosterol found in pulps were mainly formed by the laccase–HBT oxidation of free sitosterol, while the stigmasta-3,5-dien-7-one found was formed by the enzymatic oxidation of esterified sterols.

Laccase was unable to modify some saturated lipids (such as octadecane, hexadecanol, palmitic acid, and triheptadecanoin) even in the presence of HBT. However, it was observed that the amount of some of these compounds was partially decreased during the laccase–HBT treatment of pulps, in agreement with previous results (Gutiérrez et al. 2006b). This could be due to the presence of other unsaturated lipids in the pulp that could participate in peroxidation reactions leading to the partial modification of the saturated lipids. In an attempt to verify this hypothesis, reactions of these compounds with laccase–HBT (and laccase alone) in the presence of linoleic acid or cholesteryl linoleate were performed. It was observed that the amount of most of the saturated lipids decreased (up to 50%) when the reactions with laccase–HBT were performed in the presence of linoleic acid or cholesteryl linoleate. 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. Laccase oxidation of polycyclic aromatic hydrocarbons in the presence of HBT and unsaturated lipids, *via* peroxidation reactions, has been already reported (Bohmer et al. 1998).

Concluding remarks

We report for the first time the rapid 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 the model compounds. 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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