Exploring the potential of fungal manganese-containing lipoxygenase for pitch control and pulp delignification

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ABSTRACT

The potential of the lipoxygenase from *Gaeumannomyces graminis* to remove lipophilic extractives from eucalypt and flax pulps was investigated. Pulp treatments were performed with the lipoxygenase both in the presence and absence of linoleic acid, and were followed by a peroxide bleaching stage. The main lipophilic extractives from eucalypt pulp such as conjugated and free sterols decreased up to 40% and 7%, respectively, by the lipoxygenase treatment in the presence of linoleic acid. Different degradation patterns were observed among the lipophilic compounds present in flax pulp, although a high removal of all the extractives classes, including alkanes (21–55%), fatty alcohols (42–61%), and free (16–55%) and glycosylated (45–71%) sterols, was attained in all the lipoxygenase treatments. Reactions of the lipoxygenase with model lipid mixtures were carried out to better understand the degradation patterns observed in pulps. Finally, pulp delignification by the lipoxygenase treatments was also evaluated.

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1. Introduction

The non-polar extractable fraction from wood and nonwood fibers, commonly referred to as lipophilic extractives, includes fatty and resin acids, fatty alcohols, alkanes, sterols, sterol esters and triglycerides. These lipophilic compounds are the precursors of the so-called pitch deposits within the pulp and paper manufacturing processes (Back and Allen, 2000). Pitch deposition results in low quality pulp, and can cause the shutdown of pulp mill operations. Specific issues related to pulps with high extractives content include runnability problems, spots and holes in the paper, and sheet breaks.

In addition to physicochemical methods, biological methods including both enzymes and microorganisms (Gutiérrez et al., 2001a, 2009), have been investigated to control pitch problems in the pulp and paper industry. Lipases, which hydrolyze triglycerides, are applied with success in softwood (mainly pine) mechanical pulping at mill scale (Fujita et al., 1992) and find widespread use nowadays. However, pitch problems in most of the chemical and mechanical processes using other raw materials have not yet been solved. Other compounds, such as free and esterified sterols, resin acids, fatty alcohols and alkanes, are responsible for pitch problems in these processes (Gutiérrez et al., 2009). In addition to lipases, the use of sterol esterases has also been suggested (Barfod, 2000) 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, the modification of some lipophilic extractives by the use of oxidative enzymes such as laccases has been reported (Buchert et al., 2002; Molina et al., 2008; Zhang et al., 2005). Moreover, the use of laccases in the presence of redox mediators that have been extensively studied for pulp delignification (Bourbonnais and Paice, 1990; Call, 1994; Camarero et al., 2004; Fillat et al., 2010; Moldes et al., 2008) has recently been described for the removal of the lipophilic extractives responsible for pitch deposition (Gutiérrez et al., 2006a,b, 2007; Molina et al., 2008; Valls et al., 2009).

In addition to laccases, other oxidative enzymes, such as soybean lipoxygenases have been tested for the degradation of extractives in softwood thermo-mechanical pulp (Zhang et al., 2007). Earlier work had also suggested the use of lipoxygenases to reduce model wood “pitch” mixtures in pulp and paper (Borch et al., 2003). Lipoxygenases are dioxygenases which catalyze the oxidation of polyunsaturated fatty acids to hydroperoxides. Despite extensive studies on this group of enzymes (Brash, 1999; Hamberg et al., 1998; Prigge et al., 1997; Su and Oliw, 1998), there is a lack of utilization of these natural abundant enzymes in industrial processing. The specific activity of lipoxygenases to degrade linoleic acid leads to a potential application in papermaking processes to degrade wood extractives that have adverse effects on pulp and paper properties. In the present work, we study the capability of the lipoxygenase from the fungus *Gaeumannomyces graminis* to

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remove lipophilic extractives from hardwood (eucalypt) and non-woody (flax) pulps. This enzyme is a manganese-containing lipoxygenase with several exceptional features different from other lipoxygenases (Hamberg et al., 1998; Su and Oliw, 1998). It has a broad pH range (being active between pH 5 and 11), and good heat stability (being active at temperatures up to 60 °C) (Su and Oliw, 1998) which confer great potential of use under mill process conditions. Since the oxidation of polyunsaturated fatty acids by lipoxygenase leads to the formation of reactive fatty acid hydroperoxides and lipid radicals (Prigge et al., 1997) that can co-oxidize lignin in addition to lipids (Kapich et al., 2010), delignification of this pulp mediated by lipoxygenase treatment in the presence of linoleic acid was also investigated.

2. Methods

2.1. Pulp samples

Unbleached kraft pulp from eucalypt (*Eucalyptus globulus*) wood, with 44% ISO brightness and 13.5 kappa number was obtained from ENCE (Pontevedra, Spain). Unbleached soda/anthraquinone (AQ) pulp from flax (*Linum usitatissimum*) with 34% ISO brightness and 11 kappa number was provided by CELESA (Tortosa, Spain).

2.2. Model lipids

Linoleic acid, cholesteryl linoleate, nonacosane and octacosanol (from Sigma–Aldrich) and sitosterol (from Calbiochem) were used.

2.3. Lipoxygenase

The *G. graminis* lipoxygenase (GLOX) preparation was provided by Novozymes (Bagsvaerd, Denmark). GLOX is a recombinant enzyme, and the preparation used had a purity of 50% based on the sodium dodecyl sulfate gel including very low levels of protease, amylase and lipase activities. The other proteins present derived from GLOX proteolysis. GLOX activity was 130,000 units per mg, where one unit will cause an absorbance increase at 234 nm of 0.001 units per min, at pH 7.0 and 30 °C, when linoleic acid is used as substrate in a reaction volume of 1.0 ml (light path 1 cm).

2.4. Enzymatic treatments of paper pulps

Pulp treatments with GLOX (10 mg/g eucalypt pulp and 20 mg/g flax pulp) were carried out using 5 g pulp (dry weight) at 1% consistency (w:w) in 100 mM monosodium phosphate (pH 7), 30 °C, with oxygen bubbling, in a thermostatic shaker at 170 rev/min for 4 h. Pulp treatments were performed in the presence and absence of linoleic acid (0.1 mg/g pulp). In a subsequent step, pulps at 5% consistency (w:w) were submitted to a bleaching stage using 3% (w:w) H₂O₂ and 1.5% (w:w) NaOH, both referred to pulp dry weight, at 90 °C for 2 h. Controls without GLOX were performed. Treated pulp samples were Soxhlet extracted with acetone (8 h) and the extracts were evaporated to dryness and redissolved in chloroform for gas chromatography/mass spectrometry (GC–MS) and GC analyses. When required, bis(trimethylsilyl)trifluoroacetamide (BSTFA, from Supelco) in the presence of pyridine was used to prepare trimethylsilyl derivatives.

2.5. Enzymatic reactions with model lipids

Enzymatic reactions with mixtures of model compounds listed in Section 2.2 (1 mg) were performed using GLOX (0.1 mg/mg lipid), and Tween 20 as dispersant (1% v/v) in 100 mM monosodium

phosphate (pH 7), 30 °C, with oxygen bubbling, in a thermostatic shaker at 100 rev/min for 2 h. Controls without GLOX were performed.

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 GC and GC–MS analyses. When required, BSTFA in the presence of pyridine was used to prepare trimethylsilyl derivatives.

2.6. GC and GC–MS analyses of lipids

The GC analyses of lipids from both the enzymatic treatments of pulps and enzymatic reactions with model compounds were performed in an Agilent 6890 N 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. The temperature program was started at 100 °C with 1 min hold, and 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 and 350 °C, respectively. Helium was used as carrier gas, and the injection was performed in splitless mode (Gutiérrez et al., 1998). Peaks were quantified by area, and data from replicates were averaged. In all cases the standard deviations were below 7% 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 (1 min) to 380 °C at 10 °C/min, and held for 5 min. In all the GC–MS analyses, the transfer line was kept at 300 °C, the injector was programmed from 120 (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 fragmentation, and by comparing their mass spectra with those of the Wiley and NIST libraries and standards.

2.7. Pulp evaluation

Pulp brightness, kappa number and intrinsic viscosity were analyzed following ISO 3688:1999, ISO 302:1981 and ISO 5351/1:1981 standard methods, respectively (International Organisation for Standardization Documentation and Information (ISO), 2003). Data from replicates were averaged. In all cases the standard deviations were below 2% of the mean values.

3. Results and discussion

3.1. Composition of lipophilic extractives in eucalypt and flax pulps

The lipophilic extractives from eucalypt and flax pulps were analyzed by GC and GC–MS. The main lipophilic compounds present in eucalypt pulp included sterols (predominantly sitosterol) in free and conjugated form, both as glycosides and esters, as well as fatty acids, mainly palmitic acid. The detailed composition of lipophilic extractives from eucalypt pulp has been published elsewhere (Gutiérrez and del Río, 2001; Gutiérrez et al., 2001b). Among these compounds, free and esterified sterols have been reported to be the main responsible for pitch deposition during the manufacturing of eucalypt pulp (Gutiérrez et al., 2009). On the other hand, fatty alcohols including hexacosanol, octacosanol, and triacontanol, free sterols with sitosterol predominating, and fatty acids, predominantly palmitic acid, linoleic acid, oleic acid and stearic acid were the main lipophilic extractives identified in flax pulp. Minor amounts of alkanes such as nonacosane and sterol

glycosides were also present. The detailed characterization of lipophilic extractives from flax pulp has already been reported (Marques et al., 2010). Fatty alcohols and alkanes have been shown as the main responsible for pitch problems during manufacturing of nonwoody soda pulps (Gutiérrez and del Río, 2005).

From the analysis of pulp extractives it would not be expected to see a significant change in their total content after treatment with GLOX given that the amount of extractives with the 1-*cis*,4-*cis*-pentadiene structure (i.e. linoleic acid) is quite scarce. Still it was chosen to pursue testing of GLOX on both pulps and model substrates since other studies (Gutiérrez et al., 1999) had shown the presence of linoleic acid esterified with sitosterol as the main sterol ester among eucalypt extractives and, at the same time, other studies had surprisingly described an ability of lipoxygenase to significantly impact other components in unbleached eucalypt kraft pulp (Borch et al., 2003).

3.2. Removal of pulp lipophilic extractives by lipoxygenase treatment

The eucalypt and flax pulps were treated with the lipoxygenase from *G. graminis* (GLOX) to evaluate the potential of this enzyme to remove lipophilic extractives from these pulps. Additional assays adding linoleic acid to the enzymatic reactions were also performed to study the mediating effect of peroxidation products on the lipid removal. After the enzymatic treatments, the pulps were subsequently submitted to a hydrogen peroxide bleaching stage, which is a common stage in the bleaching of these alkaline pulps. The composition of the lipophilic extractives in pulps after the treatment with GLOX was analyzed by GC and GC–MS and compared with that of the control pulp treated under the same conditions but without enzyme addition.

3.2.1. Eucalypt pulp treatments

Table 1 shows the percentage of degradation of the main eucalypt pulp extractives after the enzymatic treatment with GLOX. GLOX treatment produced a removal of 20% and 22% of the sterol esters and sterol glycosides, respectively, but the amount of free sterols remained unchanged. A similar lack of degradation of free sterols was also observed after TMP pulp treatment with soybean lipoxygenase (Zhang et al., 2007).

When the GLOX treatments were performed in the presence of linoleic acid, a higher decrease of sterol esters and glycosides (up to 29% and 24%, respectively) was observed but the content of free sterols was not modified. On the other hand, in the GLOX treatments followed by a hydrogen peroxide stage, the addition of linoleic acid to the enzymatic reaction caused a higher removal of sterol esters (from 27% to 40%) and sterol glycosides (from 20% to 39%). It appears that the effect of the lipoxygenase treatment can extend well beyond the primary substrates of the enzyme via the fatty acid hydroperoxides. A slight decrease of free sterols (from 0% to 7%) was also observed. Higher reductions in the content of free and conjugated sterols have been reported in treatments of eucalypt pulp with laccases in the presence of redox mediators (Gutiérrez et al., 2006a,b, 2007).

Table 1

Removal (percentage of reduction) of the main lipophilic extractives in eucalypt pulp after treatment with the *G. graminis* lipoxygenase (GLOX) in the absence and presence of linoleic acid (LA), without and with a subsequent H₂O₂ stage (P).

	Without H ₂ O ₂		With H ₂ O ₂	
	GLOX	GLOX/LA	GLOX-P	GLOX/LA-P
Free sterols	0	0	0	7
Sterol glycosides	22	24	20	39
Sterol esters	20	29	27	40

To get further insight into these enzymatic reactions, GLOX reactions with model compounds (Fig. 1) representative for the main lipophilic extractives in eucalypt pulp, including free and esterified sterols (sitosterol and cholesteryl linoleate, respectively), and free fatty acids (linoleic acid) were carried out. The reactivity of the different lipids was studied by GC and GC–MS. The content of cholesteryl linoleate decreased about 20% while the content of free sitosterol remained unchanged by the GLOX treatment. In addition, linoleic acid was completely degraded, as expected. When the GLOX reactions were performed with a mixture of the three model compounds, reductions of 83% and 25% of cholesteryl linoleate and sitosterol, respectively, were observed in the reactions of lipoxygenase, suggesting that peroxidation reactions could mediate the co-oxidation of these model lipids. The co-oxidation of sitosterol observed in the reaction of GLOX with the model mixture was not observed in the eucalypt pulp treatment. One potential reason for the limited effect of the pulp treatment on the free sterols may be the predominant localization of these compounds inside the pulp elements (Speranza et al., 2002).

In the reaction of lipoxygenase with mixtures of the three model lipids, oxidative derivatives of cholesteryl linoleate and free sitosterol were observed. The reaction products of cholesteryl linoleate were cholesta-3,5-dien-7-one and the cholesteryl ester core aldehyde. Oxidized derivatives of free sitosterol, namely 7 β -hydroxysitosterol (and traces of 7 α -hydroxysitosterol) and 7-ketositosterol were also observed. The chemical structures of the main oxidation products identified are shown in Fig. 2. These oxidized derivatives were also identified in the enzymatic reactions of these model lipids with laccase in the presence of 1-hydroxybenzotriazole (HBT) as redox mediator (Molina et al., 2008).

Fig. 1. Chemical structures of the model compounds representative for main paper pulp lipophilic extractives used in the enzymatic reactions: (a) linoleic acid; (b) sitosterol; (c) cholesteryl linoleate; (d) nonacosane and (e) octacosanol.

Fig. 2. Chemical structures of the main oxidized derivatives identified after the lipoxygenase reactions with free sterols (sitosterol) and sterol esters (cholesteryl linoleate): (f) 7-ketositosterol; (g) 7 β -hydroxysitosterol; (h) cholesta-3,5-dien-7-one and (i) cholesteryl 9-oxononanoate.

3.2.2. Flax pulp treatments

The percentage of removal of the lipophilic extractives from flax pulp by the GLOX treatment is shown in Table 2. All these lipophilic compounds decreased significantly in the several GLOX treatments. A high removal of alkanes (up to 48%), fatty alcohols (upto 61%) and sterol glycosides (up to 65%) was observed. Surprisingly, the content of free sterols in flax pulp decreased up to 55% after the GLOX treatments, contrasting with the lack of removal in eucalypt pulp where no effect on free sterols was observed. This can be due to a different distribution of sterols in both pulps. The sterols present in flax are likely distributed throughout the surface layers of the fibers alongside the other lipophilic components (Morrison and Akin, 2001) and may be partially co-solubilized with them, following the reaction with the lipoxygenase on the *cis-cis*-pentadiene structures present in flax pulp.

The above reductions in the lipophilic extractives contents by the GLOX treatment are similar to those reported for laccase treatments of flax pulp in the presence of HBT (Gutiérrez et al., 2006b). The GLOX treatment has the advantage that no mediator was added.

The addition of linoleic acid to these enzymatic reactions caused different effects upon the different lipophilic compounds. A higher removal of alkanes was observed by the addition of linoleic acid whereas the contrary happened with fatty alcohols, free sterols

and sterol glycosides. In the GLOX treatments of flax pulp followed by a hydrogen peroxide stage, the addition of linoleic caused an increase in the removal of all the lipophilic extractives.

Reactions of GLOX with mixtures of four model compounds (Fig. 1) representative for the main lipophilic extractives in flax pulp, including alkanes (nonacosane), fatty alcohols (octacosanol), free sterols (sitosterol), and free fatty acids (linoleic acid) were also carried out. The GC and GC–MS analyses showed reductions of 100%, 51%, 65% and 55% of linoleic acid, nonacosane, octacosanol and sitosterol, respectively, after the GLOX treatment in agreement with the results observed in pulps. These results show that peroxidation reactions could mediate the co-oxidation of these lipids. Similar findings were reported in the removal of alkanes and fatty alcohols from flax pulps with laccase in the presence of HBT as mediator (Molina et al., 2008). The fact that lipid radicals generated from peroxidation of unsaturated lipids such as linoleic acid participate in the oxidation of the less reactive lipophilic compounds suggests that GLOX can be used to remove lipophilic extractives from pulps where linoleic acid is present.

3.3. Pulp properties after lipoxygenase treatment

In addition to the enzymatic removal of lipophilic extractives from pulps, the effect of the lipoxygenase treatment on some selected properties of the pulps were assessed, including kappa number (a rough estimation of the lignin content in pulp), brightness and intrinsic viscosity (an estimation of cellulose integrity). Table 3 shows the results for eucalypt pulp. Pulps with lower kappa number (1.2 points decrease) and increased brightness (2.6 and 3.4 points increase, in the absence and presence of linoleic acid, respectively) were obtained after treatment with GLOX and subsequent peroxide stage. No improvement of pulp brightness (and only a decrease of 0.7–0.8 points in kappa number) was observed when the enzymatic treatment was not followed by a hydrogen peroxide stage, revealing the need of an alkaline peroxide stage after the lipoxygenase treatment to improve pulp properties. Pulp viscosity was maintained after the enzymatic treatment although decreased after the peroxide stage.

Table 2

Removal (percentage of the reduction) of the main lipophilic extractives in flax pulp after treatment with the *G. graminis* lipoxygenase (GLOX) in the absence and presence of linoleic acid (LA) without and with a subsequent H₂O₂ stage (P).

	Without H ₂ O ₂		With H ₂ O ₂	
	GLOX	GLOX/LA	GLOX-P	GLOX/LA-P
Alkane (C27)	26	39	21	46
Alkane (C29)	48	54	35	55
Fatty alcohol (C26)	48	44	45	50
Fatty alcohol (C28)	55	44	42	49
Fatty alcohol (C30)	61	43	52	52
Free sterols	55	30	16	34
Sterol glycosides	65	51	45	71

Table 3

Properties of eucalypt pulp treated with the *G. graminis* lipoxygenase (GLOX) in the absence and presence of linoleic acid (LA) before and after a H₂O₂ stage (P), and control without enzyme.

	Control		GLOX		GLOX/LA	
	Initial	P	Initial	P	Initial	P
Kappa number	13.5	10.7	12.8	9.5	12.9	9.5
Brightness (% ISO)	44.0	55.9	43.8	58.5	43.9	59.3
Intrinsic viscosity (mL/g)	1140	925	1148	800	1143	767

Table 4

Properties of flax pulp treated with the *G. graminis* lipoxygenase (GLOX) in the absence and presence of linoleic acid (LA) before and after a H₂O₂ stage (P), and control without enzyme.

	Control		GLOX		GLOX/LA	
	Initial	P	Initial	P	Initial	P
Kappa number	9.3	5.1	9.1	5.2	8.6	5.5
Brightness (% ISO)	35.1	61.2	35.1	62	35.3	62.4
Intrinsic viscosity (mL/g)	787	535	762	648	779	602

Although higher increases in ISO brightness and decreases in kappa number than those shown here (Table 3) have been reported for some laccase treatments in the presence of redox mediators (especially synthetic mediators such as HBT or violuric acid), it should be mentioned that large differences in these values are observed in the different studies that are highly dependent on the operating conditions (Gutiérrez et al., 2006a, 2007; Ibarra et al., 2006; Moldes et al., 2008).

In the case of flax pulp (Table 4), an increase of 0.8 and 1.2 points of ISO brightness was achieved after the enzymatic treatment followed by the hydrogen peroxide stage, in the absence and presence of linoleic acid, respectively (no increase of brightness was observed before the hydrogen peroxide stage). No significant decrease of kappa number (lower than 1 point) was observed after the enzymatic treatment of flax pulp (before the peroxide stage). After peroxide, the kappa number did not decrease and the viscosity increased. Higher enhancement in ISO brightness and decrease in kappa number have been attained by laccase treatment of flax pulp in the presence of mediators (Camarero et al., 2004; Fillat et al., 2010).

The better delignification values observed in the GLOX treatment of eucalypt pulp compared with those of flax pulp treatment can be related with the different composition and structure of lignin in these two types of pulp. The lignin from eucalypt pulp is characterized by a high abundance of syringyl units (Ibarra et al., 2005) and, therefore, it is easier to delignify than lignin from flax pulp that is mainly constituted by guaiacyl units (Camarero et al., 2004).

4. Conclusions

The potential of GLOX to remove lipophilic extractives from eucalypt and flax pulps was studied. A high removal of esterified and glycosylated sterols was achieved by the GLOX treatment of eucalypt pulps, while only a slight decrease of free sterols was observed. Higher decreases of lipophilic extractives in flax pulp were produced, including free sterols. In addition, the GLOX treatment produced a significant increase of brightness and decrease of kappa number in eucalypt pulp, although only limited brightness enhancement in flax pulp. Given the significant alkaline activity of lipoxygenases, it is suggested that these may have potential use in brown stock treatment as a means for reducing down-

stream deposition problems as well as reducing the amount of chemicals in the subsequent bleaching stages.

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