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Degradation of nonphenolic lignin by the laccase/1-hydroxybenzotriazole system

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Abstract

Phenolic and nonphenolic (permethylated) synthetic [^{14}C]lignins were depolymerized by *Trametes villosa* laccase in the presence of a radical mediator, 1-hydroxybenzotriazole (HOBT). Gel permeation chromatography of the treated lignins showed that ~10% of their substructures were cleaved. The system also cleaved a β-*O*-4-linked model compound, 1-(4-ethoxy-3-methoxy-*ring*-[^{14}C]phenyl)-2-(2-methoxyphenoxy)-propane-1,3-diol, and a β-1-linked model, 1,2-bis-(3-methoxy-4-[^{14}C]methoxyphenyl)-propane-1,3-diol, that represent nonphenolic substructures in lignin. High performance liquid chromatography of products from the oxidized models showed that they were produced in sufficient yields to account for the ability of laccase/HOBT to depolymerize nonphenolic lignin. Published by Elsevier Science B.V.

Keywords: Laccase; 1-Hydroxybenzotriazole; Radical mediator; Lignin; Delignification; Kraft pulp

1. Introduction

Laccases are copper-containing oxidases that catalyze the oxidation of electron-rich substrates such as phenols. They are secreted by most of the basidiomycetes that cause white rot of wood (Hatakka, 1994), and may play a role in ligninolysis by these fungi (Kirk et al., 1968; Kawai et al., 1988). There is little evidence that laccases by themselves can catalyze ligninolysis, but they are able to depolymerize synthetic lignin (Kawai et al., 1999) and delignify wood pulps (Bourbonnais et al., 1997; Call and Mücke, 1997) when they are combined with various low molecular weight electron transfer agents. These so-called laccase/mediator systems are potentially applicable for industrial pulp bleaching.

One of the most intensively researched mediators is 1-hydroxybenzotriazole (HOBT), which is oxidized to its nitroxide radical by laccase (Bourbonnais et al., 1998; Potthast et al., 1999b). The

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HOBT nitroxide has been shown to oxidize a variety of aromatic compounds (Aurich et al., 1977), and probably acts as the proximal oxidant of lignin when HOBT is used with laccase (Potthast et al., 1999b). Although the nitroxide undergoes significant side reactions that prevent its use as a truly recyclable mediator (Li et al., 1998; Sealey and Ragauskas, 1998a; Potthast et al., 1999b), the laccase/HOBT system has given good results in pulp bleaching trials (Bourbonnais et al., 1997; Sealey and Ragauskas, 1998b; Chakar and Ragauskas, 1999; Poppius-Levlin et al., 1999). It probably promotes delignification in part because, unlike laccase, the HOBT nitroxide is small enough to access occluded lignin in pulps.

The chemistry of delignification by laccase/HOBT is not well understood. The system evidently oxidizes and cleaves phenolic lignin units, which become depleted in bleached pulps (Sealey and Ragauskas, 1998b; Poppius-Levlin et al., 1999; Potthast et al., 1999a). In addition, work with lignin model compounds has shown that the laccase/HOBT system oxidizes the nonphenolic β-*O*-4-linked units that predominate in native lignin. Most of the resulting products are uncleaved ketones, whose presence may enhance bleaching because they are susceptible to cleavage by alkali in subsequent treatments (Bourbonnais et al., 1997). However, nonphenolic lignin models are also cleaved directly by laccase/HOBT (Srebotnik et al., 1998; Kawai et al., 1999). To determine whether these cleavage reactions could contribute significantly to ligninolysis, we compared the extents to which laccase/HOBT degraded a phenolic synthetic lignin, a nonphenolic synthetic lignin, and two nonphenolic lignin model dimers.

2. Materials and methods

2.1. Reagents

Guaiacyl β-^{14}C-labeled synthetic lignin (0.01 mCi mmol^{-1} of phenylpropane substructures) was prepared and a portion of it was permethylated as described previously (Kirk and Brunow, 1988; Hammel et al., 1993). Analysis of the permethylated lignin by UV difference spectrophotometry (Hammel et al., 1993) showed that its phenolic content was less than 1%. The lignins were fractionated by gel permeation chromatography (GPC) as described (Hammel et al., 1993) to obtain high molecular weight fractions for use in laccase/HOBT reactions.

The β-*O*-4-linked model 1-(4-ethoxy-3-methoxy-*ring*-[^{14}C]phenyl)-2-(2-methoxyphenoxy)-propane-1,3-diol (**1**, see Fig. 1 for chemical structures) was prepared by reducing 1-(4-ethoxy-3-methoxy-*ring*-[^{14}C]phenyl)-1-oxo-2-(2-methoxyph-

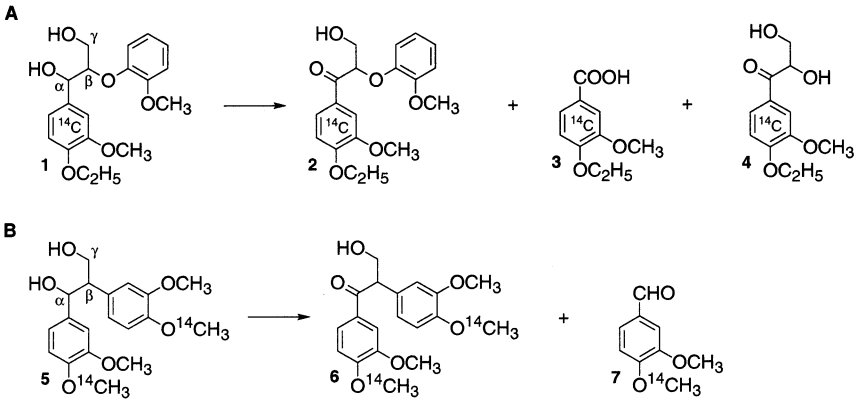

Fig. 1. (A) Structure of lignin model **1** and of oxidation products (**2**–**4**) derived from it. (B) Structure of lignin model **5** and of oxidation products (**6**, **7**) derived from it.

enoxy)-propan-3-ol (**2**, 0.74 mCi $mmol^{-1}$) with $NaBH_4$ in 95% ethanol at room temperature (Furniss et al., 1989). Model **1** was purified to greater than 99% chemical and radiochemical purity by preparative high performance liquid chromatography (HPLC) before use.

Compound **2** was prepared from *ring*-[^{14}C]-acetovanillone by methods described previously (Landucci et al., 1981). *ring*-[^{14}C]-Acetovanillone was custom synthesized by New England Nuclear.

4-Ethoxy-3-methoxybenzoic acid (**3**) was prepared by alkylating vanillic acid with ethyl iodide, and the methyl ester of **3** was prepared by treating **3** with diazomethane (Furniss et al., 1989). 1-(4-Ethoxy-3-methoxyphenyl)-1-oxo-propane-2,3-diol (**4**) was kindly provided by Shingo Kawai (Gifu University, Japan).

The β-1-linked model 1,2-bis-(3-methoxy-4-[^{14}C]methoxyphenyl)-propane-1,3-diol (**5**, 6.2 mCi $mmol^{-1}$) was obtained from T.K. Kirk of this laboratory. HPLC analysis showed that model **5** was more than 99% chemically and radiochemically pure.

A chromatographic standard of the β-1-linked ketone 1,2-bis-(3-methoxy-4-[^{14}C]methoxyphenyl)-1-oxo-propan-3-ol (**6**) was prepared by oxidizing model **5** with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (Furniss et al., 1989). 3,4-Dimethoxybenzaldehyde (**7**), 3,4-dimethoxybenzyl alcohol, and the other chemicals used were reagent grade materials from Aldrich.

2.2. Enzyme

Crude recombinant *Trametes villosa* laccase (form 1) (Yaver et al., 1996) was a generous contribution from Alan Klotz (Novo Nordisk Biotech. Corp., Davis, CA). The enzyme was purified by ion-exchange chromatography on a column of DEAE Biogel A, using a linear gradient of sodium acetate (20–330 mM) at pH 6.0. Fractions containing laccase activity were pooled and further purified by GPC on Sephacryl S-200 in 330 mM acetate buffer (pH 6.0). The active fractions were dialyzed against 30 mM acetate buffer (pH 6.0), concentrated by ultrafiltration, and frozen. The purified laccase had an A_{275}/A_{615} ratio of 15.4. The corresponding ratio for the pure recombinant enzyme was reported to be 15.1 (Yaver et al., 1996).

Laccase activity was determined by monitoring the oxidation of 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) to its cation radical at 420 nm ($\varepsilon = 36\ mM^{-1}\ cm^{-1}$) (Bourbonnais and Paice, 1990). The assay contained 0.5 mM ABTS and 50 mM sodium acetate (pH 4.0) at ambient temperature. One unit of activity was defined as the amount of enzyme that formed 1 μmol of ABTS cation radical per min.

2.3. Depolymerization of lignin

The reactions (30 ml) contained phenolic or permethylated synthetic [^{14}C]lignin (200 μg, 2.1×10^4 dpm, added as a stock solution in 30 μl of *N*,*N*-dimethylformamide), HOBT (0.9 mM), Tween 20 (0.25%), laccase (0.5 U ml^{-1}), and sodium acetate (20 mM, pH 4.0). The mixtures were shaken in foil-covered 125-ml Erlenmeyer flasks for 6 or 24 h at 130 rpm and 30°C, after which they were concentrated by rotary vacuum evaporation and centrifuged to remove insoluble material. The resulting samples (5 ml) contained approximately 85% of the ^{14}C originally present and were subjected to GPC on a 1.85×37 cm column of Sephadex LH-60 in *N*,*N*-dimethylformamide that contained 0.1 M LiCl. Fractions (1.5 ml) were collected and assayed for ^{14}C by scintillation counting. The GPC column was calibrated with polystyrene molecular weight standards, and nominal number-average molecular weights (M_n) and weight-average molecular weights (M_w) for the lignin samples were calculated with the standard equations (Yau et al., 1979). The average number of scissions incurred per polymer was calculated from M_n values as described (Hammel et al., 1993).

2.4. Oxidation of model *1*

The reactions (2.0 ml) contained **1** (0.50 mM), HOBT (1.0 mM), laccase (2 U ml^{-1}) and sodium acetate (50 mM, pH 4.0). The mixtures were shaken in loosely capped vials at 150 rpm and 30°C. Samples (350 μl) were removed and frozen in a dry ice/ethanol bath at 4, 8 and 24 h.

To identify products, individual samples were thawed rapidly and filtered through a 0.45 μm pore size nylon membrane, after which 300 μl of the filtrate was immediately fractionated by reversed phase HPLC on a phenylhexyl column (Phenomenex Luna, 5 μm particle size, 150 × 4.6 mm). The column was eluted at 1.0 ml min^{-1} and ambient temperature with the following linear gradient: 0 min: water/methanol/formic acid 80:20:0.1. 49 min: water/methanol/formic acid 25:75:0.1. Fractions (0.5 ml) were collected and assayed for ^{14}C by scintillation counting.

Preliminary identifications were made by comparing the HPLC retention times of products **2**–**4** with those of authentic standards. The identification of cleavage product **3** was then confirmed by collecting it from the HPLC column, treating it with diazomethane, and subjecting it again to HPLC and scintillation counting to determine whether the radiocarbon then ran with a standard of 4-ethoxy-3-methoxybenzoic acid methyl ester.

In addition, the identification of cleavage products **3** and **4** was checked by gas chromatography/mass spectrometry. The products from a 10-ml, 25-h reaction were fractionated by HPLC on a preparative column, and each product was concentrated to dryness by rotary vacuum evaporation. Each dried fraction was redissolved in 50 μl of bis(trimethylsilyl)acetamide/*N*,*N*-dimethylformamide, 1:1, and heated briefly. Several microliters of each silylated product was then analyzed on a Hewlett–Packard gas chromatograph/mass spectrometer that was equipped with a 30-m DB-5 (nonpolar silicone polymer) column. Standards of compounds **3** and **4** were analyzed by the same procedure.

2.5. Oxidation of model *5*

The experiments were done as described above for model **1**, but with the following modifications: the reactions (1.0 ml) contained 0.14 mM **5** and 0.34 mM HOBT. HPLC analysis of the reactions was done on the phenylhexyl column with the following elution program: 0–10 min, isocratic at water/acetonitrile/formic acid 90:10:0.1; 10–11 min, linear gradient to water/acetonitrile/formic acid 85:15:0.1; 11–40 min, isocratic at water/acetonitrile/formic acid 85:15:0.1; 40–50 min, linear gradient to acetonitrile/formic acid 100:0.1. Products **6** and **7** were tentatively identified by comparing their retention times with those of authentic standards.

The identification of the cleavage product **7** was confirmed by collecting it from the HPLC column, treating it with $NaBH_4$, and subjecting it again to HPLC and scintillation counting to determine whether the radiocarbon then ran with a standard of 3,4-dimethoxybenzyl alcohol. The quantity of model **5** available for these experiments was too low to attempt analysis of the reaction products by gas chromatography/mass spectrometry.

3. Results

3.1. Depolymerization of synthetic lignins

In the absence of HOBT, *T. villosa* laccase polymerized a synthetic guaiacyl lignin (Table, 1, Fig. 2A). The extent of polymerization could not be quantified, because much of the radiolabeled lignin was excluded from the GPC resin. The reaction also produced a negligible quantity of material with a molecular weight lower than that of the initial polymer.

When HOBT was included in the reaction, the lignin was depolymerized and no material larger than the initial polymer was detectable (Table 1, Fig. 2A). The nominal M_n of the lignin decreased from 4800 to roughly one fifth of this value after 24 h of oxidation, i.e. the average polymer was cleaved about four times. In a repetition of this experiment (data not shown), the average polymer was cleaved between two and three times.

No molecular weight changes were detectable in the permethylated (nonphenolic) synthetic lignin when it was treated with laccase alone. However, when HOBT was included in the reaction, the lignin was depolymerized at nearly the same rate that phenolic lignin was (Table 1, Fig. 2B). The nominal M_n of an average polymer decreased from 4300 to a little over one fourth of this value in 24 h, i.e. the average polymer was cleaved between two and three times. A repetition of this

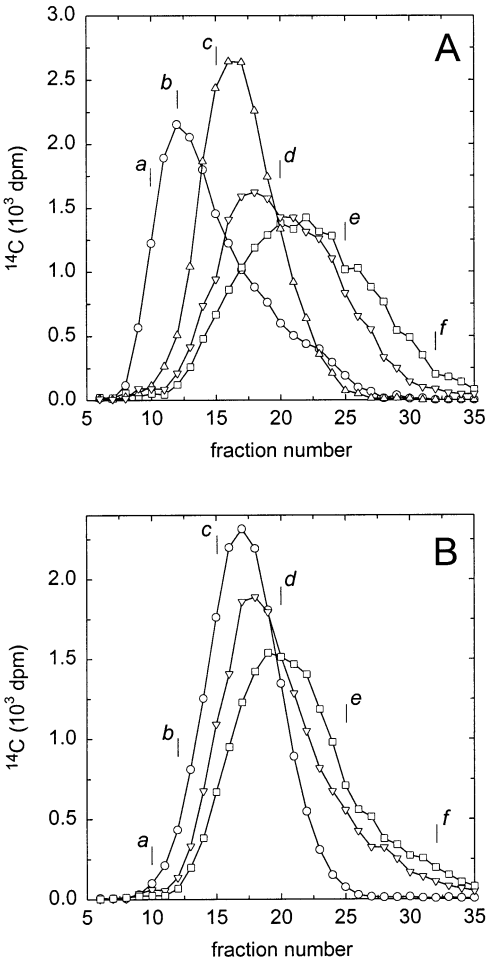

Fig. 2. (A) GPC analyses of phenolic synthetic [^{14}C]lignin after 6 h of treatment (▽) and after 24 h of treatment (□) with laccase/HOBT. A 24-h control reaction that lacked laccase had no effect on the lignin (△), whereas a 24-h control reaction that lacked HOBT (○) polymerized it. (B) GPC analyses of nonphenolic (permethylated) synthetic [^{14}C]lignin after 6 h of treatment (▽) and after 24 h of treatment (□) with laccase/HOBT. A 24-h control reaction that lacked HOBT (○) had no effect on the lignin. Labels (a–e) correspond to the elution positions of polystyrene standards with the following molecular weight values: (a) 34 500 (excluded from gel); (b) 22 000; (c) 10 000; (d) 3100; (e) 1050. Standard (f) was veratraldehyde (166). See Table 1 for nominal M_n and M_w values of the lignins.

experiment (data not shown) gave the same result. The degree of depolymerization obtainable was no greater in an additional 90-h experiment in which the laccase was added continuously with a pump (data not shown).

3.2. Cleavage of nonphenolic lignin model dimers

When the β-*O*-4-linked model **1** was oxidized by laccase/HOBT, HPLC analysis with radiochemical detection showed that the major product was the uncleaved ketone **2** (Fig. 1, Table 2), as reported earlier for a similar guaiacyl β-*O*-4-linked dimer (Bourbonnais et al., 1997; Li et al., 1999). However, **1** was also oxidized to more polar compounds in 14–15% yield after 24 h. Although most of the polar material consisted of numerous minor products that were poorly resolved from each other on the HPLC column, two distinct radiocarbon peaks had retention times that indicated they were the cleavage products **3** (4–5% yield) and **4** (1–2% yield). No oxidation occurred if laccase or HOBT was omitted from the reaction (data not shown).

The structures of both **3** and **4** were confirmed by gas chromatography/mass spectrometry analysis of the collected HPLC fractions (Table 2). Moreover, when the HPLC fraction that contained product **3** was collected, methylated with diazomethane, and subjected again to HPLC analysis, all of the ^{14}C then ran with 4-ethoxy-3-methoxybenzoic acid methyl ester (data not shown). These structure confirmations allow us to estimate that the proportion of β-*O*-4 model cleaved was at least 5–7% (the range of yields we obtained for the known cleavage products **3** and **4)**, but probably no more than 14–15% (the range of yields for all polar products).

When the β-1-linked model **5** was oxidized by laccase/HOBT, the major product was, again, an uncleaved ketone (**6**). However, polar compounds were also produced in 20–22% yield after 24 h (Fig. 1, Table 3). Most of this polar material co-eluted with a standard of cleavage product **7** (12–15% yield). Model **5** was not oxidized if laccase or HOBT was omitted from the reaction (data not shown).

When the HPLC fraction that contained product **7** was collected, reduced with $NaBH_4$, and subjected again to HPLC analysis, all of the ^{14}C then ran with 3,4-dimethoxybenzyl alcohol (data not shown). This confirmation of the

product **7** structure allows us to estimate that laccase/HOBT cleaved at least 12–15% of the β–1 structure.

4. Discussion

Past work has shown that the laccase/HOBT system attacks phenolic structures preferentially when it depolymerizes lignin in kraft pulps (Sealey and Ragauskas, 1998b; Poppius-Levlin et al., 1999; Potthast et al., 1999a). It also degrades phenolic model compounds that represent condensed kraft lignin structures such as biphenyls, stilbenes, and diphenylmethanes (Xu et al., 1997; Crestini and Argyropoulos, 1998). These reactions are undoubtedly important, because the phenolic substructure content of residual softwood kraft lignin is generally 25–50% (Francis et al., 1991; Lachenal et al., 1999).

However, softwood kraft pulps also contain 6–7% of the β-*O*-4 structure that predominates in native lignin (Gellerstedt et al., 1999). Most of these residual β-*O*-4 structures are probably nonphenolic, because phenolic β-*O*-4 structures react quickly under kraft pulping conditions (Gierer, 1985). In addition, some minor nonphenolic lignin structures, for example β-1-linked units, may be enriched in kraft lignin because they resist nucleophilic attack by hydrosulfide (Gierer, 1985). It would be advantageous if laccase/HOBT could degrade these structures.

To determine whether this was so, we first compared the system's ability to depolymerize phenolic and nonphenolic ^{14}C-labeled synthetic lignins. Although these polymers are obviously not kraft lignins, they are useful high molecular weight models that contain some of the relevant structures. We took this approach because it allowed radiochemical rather than spectrophotometric quantitation of the degraded polymer fractions. Molecular weight comparisons based on spectrophotometric detection can lead to significant errors if oxidation of the lignin introduces new conjugated structures such as benzylic carbonyls and carboxylates that have high UV absorptivities.

The results showed that laccase/HOBT depolymerized nonphenolic lignin approximately as well as it did the phenolic polymer. One possible explanation is that the system attacks both types of polymer at the same initial rate, but this is unlikely because laccase/mediator systems cleave nonphenolic structures inefficiently (Srebotnik et al., 1998; Kawai et al., 1999), whereas typical turnover numbers for laccases with lignin-related phenols are high (Xu, 1996). Instead, the data probably indicate that the phenolic groups in lignin somewhat retard ligninolysis by laccase/HOBT, despite their high reactivity, because they can act as sites for transient polymerization of the lignin via phenoxy radical intermediates. These polymerization reactions will decrease the phenolic content of the lignin, in accord with the observations of Poppius-Levlin et al. (1999), but depolymerization will not be impeded because laccase/HOBT can cleave some of the nonphenolic structures.

It is not possible to calculate precisely the proportion of intermonomer linkages in nonphenolic

Table 1
Calculated molecular weight distributions for synthetic [^{14}C]lignins treated with laccase/HOBT

Reaction conditions	Phenolic lignin		Nonphenolic lignin	
	M_n	M_w	M_n	M_w
Complete, 6 h	1560	5010	1580	5000
Complete, 24 h	940	3840	1120	3820
Minus HOBT, 24 h	>6500	>15 200	4340[a]	7560[a]
Minus laccase, 24 h	4830[a]	8090[a]	–[b]	–[b]

[a] M_n and M_w values for these samples were essentially the same as those obtained for the original, untreated lignins.
[b] Not determined.

Table 2
HPLC and GC/MS data on oxidation products derived from model **1**

Compound	HPLC peak retention time (min)	Yield[a] (% of total soluble ^{14}C)				Principal ions in mass spectrum[b] (m/z [relative intensity])
		Experiment 1			Experiment 2	
		4 h	8 h	24 h	24 h	
1	39	54.4	43.2	29.9	36.4	–[c]
2	46	34.1	42.8	53.3	45.8	–[c]
3	26	2.9	3.6	4.2	4.7	268 [M^+, 81]; 253 [100]; 225 [81]; 209 [97]; 181 [64]; 179 [53]; 151 [73]
4	13	0.6	0.7	1.1	1.9	384 [M^+, 7]; 369 [7]; 354 [7]; 268 [76]; 253 [16]; 205 [33]; 179 [100]
All products eluting before **1**		9.4	11.7	14.2	14.8	–[c]

[a] About 85% of the ^{14}C originally supplied was typically recovered as soluble material for HPLC analysis.
[b] Of trimethylsilyl derivatives.
[c] Not determined.

Table 3
HPLC data on oxidation products derived from model **5**

Compound	HPLC peak retention time (min)	Yield[a] (% of total soluble ^{14}C)			
		Experiment 1			Experiment 2
		4 h	8 h	24 h	24 h
5	44	80.2	72.7	57.9	54.0
6	50	12.0	15.3	21.8	24.1
7	39	4.4	7.2	12.4	14.6
All products eluting before **5**		7.3	11.8	20.0	22.0

[a] About 85% of the ^{14}C originally supplied was typically recovered as soluble material for HPLC analysis.

lignin that was cleaved in these experiments, because calibration of a GPC column with polystyrene molecular weight standards does not yield absolute molecular weight distributions for lignins that are analyzed on the column. Moreover, we have observed that the peak elution position of a given lignin sample from Sephadex LH-60 can vary by as much as three percent of the total elution volume. When this variability is introduced into the standard equations for calculation of the number of polymer scissions, the resulting error is as large as $\pm 50\%$ (data not shown).

Therefore, we can estimate the cleavage yields in nonphenolic lignin only very roughly. A nonphenolic guaiacyl lignin with $M_n = 4300$ contains about 22 lignin substructures. Given that each nonphenolic polymer was cleaved between two and three times, the proportion of structures cleaved was in the region of 2/22-3/22, i.e. $\sim 10\%$. The yields of known cleavage products we obtained with the nonphenolic lignin model dimers **1** and **5** agree reasonably with this estimate.

Finally, we note a singularity in the results obtained with dimeric models. Both the β-*O*-4-linked model **1** and the β-1-linked model **5** underwent C_α–C_β cleavage, but the benzylic product was an acid (**3**) in the former case and an aldehyde (**7**) in the latter. Previous studies of the laccase/HOBT system with β-*O*-4-linked models also found benzoic acids as C_α–C_β cleavage products (Srebotnik et al., 1998: Kawai et al., 1999), and it has been observed that the residual lignins in laccase/HOBT-bleached pulps are enriched in carboxylic acid groups (Chakar and Ragauskas, 1999; Poppius-Levlin et al., 1999). β-1-linked models have apparently not been investigated in the laccase/HOBT system until now, but earlier work did show that it cleaves hydrobenzoin dimers between C_α and C_β to give benzaldehydes (Xu et al., 1997; Bourbonnais et al., 1997).

So far, we cannot propose a satisfactory mechanism to account for this disparity. The oxidation of β-*O*-4- and β-1-linked lignin dimers to their cation radicals is well known to yield benzaldehydes as C_α–C_β cleavage products (Hammel et al., 1986; Kirk et al., 1986), but this route appears unlikely for the laccase/HOBT system because 4-ethoxy-3-methoxybenzaldehyde was not produced in significant quantity when **1** was oxidized. In theory, the benzoic acid **3** that we found instead could have been produced from 4-ethoxy-3-methoxybenzaldehyde or from the ketone **2**, but this idea must be rejected as well because we found that neither of the latter compounds was oxidized by laccase/HOBT (data not shown). The data, incomplete though they are, suggest that laccase/HOBT does not cleave nonphenolic lignin structures by the cation radical mechanism that fungal lignin peroxidases employ.

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References

Aurich, H.G., Bach, G., Hahn, K., Küttner, G., Weiss, W., 1977. Aminyloxide (nitroxide). XXV. Reaktionen von benzotriazolyloxid-radikalen mit aromaten. J. Chem. Res. (M) 15, 44–66.

Bourbonnais, R., Paice, M., 1990. Oxidation of non-phenolic substrates: an expanded role for laccase in lignin biodegradation. FEBS Lett. 267, 99–102.

Bourbonnais, R., Paice, M.G., Freiermuth, B., Bodie, E., Borneman, S., 1997. Reactivities of various mediators and laccases with kraft pulp and lignin model compounds. Appl. Environ. Microbiol. 63, 4627–4632.

Bourbonnais, R., Leech, D., Paice, M.G., 1998. Electrochemical analysis of the interactions of laccase mediators with lignin model compounds. Biochim. Biophys. Acta 1379, 381–390.

Call, H.-P., Mücke, I., 1997. History, overview and applications of mediated lignolytic systems, especially laccase-mediator-systems (lignozym(*R*)-process). J. Biotechnol. 53, 163–202.

Chakar, F.S., Ragauskas, A.J., 1999. Fundamental investigation of laccase mediator delignification on high lignin content kraft pulps. In: Proceedings of the 10th International Symposium on Wood and Pulping Chemistry, Yokohama, Japan, vol. 1, pp. 566–570.

Crestini, C., Argyropoulos, D.S., 1998. The early oxidative biodegradation steps of residual kraft lignin models with laccase. Bioorg. Med. Chem. 6, 2161–2169.

Francis, R.C., Lai, Y.-Z., Dence, C.W., Alexander, T.C., 1991. Estimating the concentration of phenolic hydroxyl groups in wood pulps. Tappi J. 74, 219–224.

Furniss, B.S., Hannaford, A.J., Smith, P.W.G., Tatchell, A.R. (Eds.), 1989. Vogel's Textbook of Practical Organic Chemistry, 5th edn., Wiley, New York.

Gellerstedt, G., Wafa Al-Dajani, W., Zhang, L., 1999. On the structure of residual lignins in alkaline pulps. In: Proceedings of the 10th International Symposium on Wood and Pulping Chemistry, Yokohama, Japan, vol. 1, pp. 346–349.

Gierer, J., 1985. Chemistry of delignification. Part 1: general concept and reactions during pulping. Wood Sci. Technol. 19, 289–312.

Hammel, K.E., Kalyanaraman, B., Kirk, T.K., 1986. Substrate free radicals are intermediates in ligninase catalysis. Proc. Natl. Acad. Sci. USA 83, 3708–3712.

Hammel, K.E., Jensen, K.A. Jr, Mozuch, M.D., Landucci, L.L., Tien, M., Pease, E.A., 1993. Ligninolysis by a purified lignin peroxidase. J. Biol. Chem. 268, 12274–12281.

Hatakka, A., 1994. Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. FEMS Microbiol. Rev. 13, 125–135.

Kawai, S., Umezawa, T., Higuchi, T., 1988. Degradation mechanisms of phenolic β-1 lignin substructure model compounds by laccase of *Coriolus versicolor*. Arch. Biochem. Biophys. 262, 99–110.

Kawai, S., Asukai, M., Ohya, N., Okita, K., Ito, T., Ohashi, H., 1999. Degradation of a non-phenolic β-*O*-4 substructure and of polymeric lignin model compounds by laccase of *Coriolus versicolor* in the presence of 1-hydroxybenzotriazole. FEMS Microbiol. Lett. 170, 51–57.

Kirk, T.K., Brunow, G., 1988. Synthetic ^{14}C-labeled lignins. Methods Enzymol. 161, 65–73.

Kirk, T.K., Harkin, J.M., Cowling, E.B., 1968. Degradation of the lignin model compound syringylglycol-β-guaiacyl ether by *Polyporus versicolor* and *Stereum frustulatum*. Biochim. Biophys. Acta 165, 145–163.

Kirk, T.K., Tien, M., Kersten, P.J., Mozuch, M.D., Kalyanaraman, B., 1986. Ligninase of *Phanerochaete chrysosporium*. Mechanism of its degradation of the non-phenolic arylglycerol β-aryl ether substructure of lignin. Biochem. J. 236, 279–287.

Lachenal, D., Sevillano, R.M., George, J., Chirat, C., 1999. Understanding the structure of residual lignin. A key to progress in pulping and bleaching. In: Proceedings of the 10th International Symposium on Wood and Pulping Chemistry, Yokohama, Japan, vol. 1, pp. 354–357.

Landucci, L.L., Geddes, S.A., Kirk, T.K., 1981. Synthesis of ^{14}C-labeled 3-methoxy-4-hydroxy-α-(2-methoxyphenoxy)-β-hydroxypropiophenone, a lignin model compound. Holzforschung 35, 66–69.

Li, K.-C., Helm, R.F., Eriksson, K.-E.L., 1998. Mechanistic studies of the oxidation of a non-phenolic lignin model compound by the laccase/*N*-hydroxybenzotriazole redox system. Biotechnol. Appl. Biochem. 27, 239–243.

Li, K.-C., Xu, F., Eriksson, K.-E.L., 1999. Comparison of fungal laccases and redox mediators in oxidation of a nonphenolic lignin model compound. Appl. Environ. Microbiol. 65, 2654–2660.

Poppius-Levlin, K., Wang, M., Tamminen, T., Hortling, B., Viikari, L., Niku-Paavola, M.L., 1999. Effects of laccase/HBT treatment on pulp and lignin structures. J. Pulp Paper Sci. 25, 90–94.

Potthast, A., Rosenau, T., Koch, H., Fischer, K., 1999a. The reaction of phenolic model compounds in the laccase-mediator system (LMS) — investigations by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Holzforschung 53, 175–180.

Potthast, A., Rosenau, T., Kosma, P., Fischer, K., 1999b. Chemistry and kinetics of the laccase-mediator system. In: Proceedings of the 10th International Symposium on Wood and Pulping Chemistry, Yokohama, Japan, vol. 1, pp. 596–601.

Sealey, J., Ragauskas, A.J., 1998a. Investigation of laccase/*N*-hydroxybenzotriazole delignification of kraft pulp. J. Wood Chem. Technol. 18, 403–416.

Sealey, J., Ragauskas, A.J., 1998b. Residual lignin studies of laccase-delignified kraft pulps. Enzyme Microb. Technol. 23, 422–426.

Srebotnik, E., Jensen, K.A., Hammel, K.E., 1998. Cleavage of nonphenolic lignin structures by laccase in the presence of 1-hydroxybenzotriazole. In: Proceedings of the 7th International Conference on Biotechnology in the Pulp and Paper Industry, Vancouver, Canada, pp. B195–B197.

Xu, F., 1996. Oxidation of phenols, anilines, and benzenethiols by fungal laccases: correlation between activity and redox potentials as well as halide inhibition. Biochemistry 35, 7608–7614.

Xu, H., Lai, Y.-Z., Slomczynski, D., Nakas, J.P., Tanenbaum, S.W., 1997. Mediator-assisted selective oxidation of lignin model compounds by laccase from *Botrytis cinerea*. Biotechnol. Lett. 19, 957–960.

Yau, W.W., Kirkland, J.J., Bly, D.D., 1979. Modern Size-Exclusion Liquid Chromatography. Wiley, New York.

Yaver, D.S., Xu, F., Golightly, E.J., Brown, K.M., Brown, S.H., Rey, M.W., Schneider, P., Halkier, T., Mondorf, K., Dalbøge, H., 1996. Purification, characterization, molecular cloning, and expression of two laccase genes from the white rot basidiomycete *Trametes villosa*. Appl. Environ. Microbiol. 62, 834–841.