On the Mechanism of the Laccase–Mediator System in the Oxidation of Lignin

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Abstract: In an effort to elucidate the role of phenolic and non-phenolic lignin subunits in a laccase mediator (LM) system, vanillyl alcohol was oxidized with laccase in the presence and absence of the mediator 1-hydroxybenzotriazol (HBT). Furthermore, the role of phenolic, aliphatic hydroxyl, and carboxylic acid moieties in lignin degradation was elucidated by selectively blocking them. The modified samples were then subjected to laccase and laccase–HBT treatments. On the basis of this data it was possible to establish the role of this mediator. HBT mediates the oxidation of lignin by inducing side-chain oxidation and oxygen-addition products rather than oxidative coupling reactions.

Keywords: bond energy · laccase · laccase–mediator system · lignin · oxidation

Introduction

Laccase, benzenediol:oxygen oxidoreductase 1.10.3.2, is a multicopper oxidase that performs the reduction of oxygen to water. The enzyme contains four copper centers, one type 1 Cu (T1), one type 2 Cu (T2) and a coupled binuclear type 3 (T3) Cu center.[1, 2] The T2 and T3 sites form a trinuclear Cu cluster onto which O_2 is reduced.[3] The T1 Cu atom oxidizes the reducing substrate and transfers electron to the T2 and T3 Cu atoms.[1, 2]

The typical substrates for laccase are phenolic systems. Their oxidation proceeds through an outer-sphere electron-transfer process that generates a radical cation, which after fast proton abstraction generates a phenoxyl radical.[4]

This enzyme shows a high thermal resistance (stable at 60 °C)[5] and low substrate specificity by being able to oxidize a number of different aromatic substrates. Its lack of substrate inhibition coupled with its high oxidation rates (10–100-fold higher than those of lignin peroxidase or manganese peroxidase) make laccase an ideal candidate for the development of enzymatic pulping processes.[5–7]

The enzyme displays different redox potentials depending on the fungal species that have been used for its production. Such potentials range from 780 to 785 mV versus NHE.[5] In any case the oxidation of non-phenolic substrates is prevented by their high redox potential.

However, laccase itself has a poor effect on pulps, while when used in the presence of radical mediators such as 1-hydroxybenzotriazole (HBT) it has been shown to effectively demethylate kraft pulps.[8] Since a universal increase in laccase reactivity in the presence of radical mediators has been demonstrated, extensive efforts have been made to understand the mechanism of the laccase–mediator (LM) system. Several reports have appeared in the literature attempting to deal with the LM oxidation of non-phenolic compounds; the increased delignification of the LM systems being ultimately ascribed to their ability to effect the oxidation of non-phenolic lignin subunits.[8–17] More specifically in the presence of the LM system it has been shown that low amounts of oxidation of non-phenolic B-O-4 aryl ether model compounds can occur.[10]

Despite the large body of information that exists on the reaction of laccase with several substrates and lignins, to date a comprehensive understanding of the reaction mechanism of the LM system is lacking. The rate-limiting step of laccase-catalyzed N–OH oxidation involves an electron-transfer process from the substrate to the T1 Cu site in laccase.[16] It has been shown that laccase in the presence of HBT generates the oxybenzotriazolyl radical, HBT, a long-lived radical species that can oxidize both phenolic and some non-phenolic lignin models.[19]

Even though radicals are soft species and their reactivity is under the control of the frontier orbitals, reactivity and rate predictions of the HBT mediator cannot be done without a clear detailed insight of the operating reaction mechanism.[16]

Recently it was shown that HBT, as opposed to laccase itself, reacts with non-phenolic lignin models by a radical

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mechanism involving of hydrogen atom abstraction rather than an electron-transfer process.[20, 21] However, since the early finding that laccases in the presence of radical mediators are able to bring about the oxidation of some non-phenolic model compounds, little or no attention has been devoted to their behavior toward phenolic lignin subunits.[22]

Within the context of our efforts to further comprehend the mechanism of activity of the radical mediators ABTS and HBT on laccase oxidations,[23] we have selected an array of model compounds resembling the fundamental bonding patterns of residual kraft lignin. The experiments were carried out on diphenylmethane, 4-O-5′ and 5-5′ coupling products ■■ok? ■■, and stilbene phenolic model compounds; structures that constitute the bulk of residual kraft lignin.[24–27] Such models represent the lignin subunits most recalcitrant to oxidative degradation. Reactions with laccase and laccase + HBT or laccase + ABTS (2,2′-azinobis-(3-ethyl-benzthiazoline-6-sulfonate)) showed that in the presence of HBT or ABTS only side chain oxidation reactions and demethoxylations occurred.[23] Alternatively the oxidation of the corresponding non-phenolic compounds did not occur. On the basis of these data we suggested that the role of radical mediators in the presence of condensed lignin substructures is to act as diffusible lignin oxidizing agents of phenolic systems, since such compounds can access the inner lignin structure with greater facility than the enzyme itself.[23]

In our efforts to further elucidate the role of phenolic or non-phenolic lignin subunits in the LM system we designed a set of experiments aimed at clarifying this issue. More specifically vanillyl alcohol was used as monomeric model compound. It was oxidized with laccase in the presence and in the absence of HBT. The role of phenolic, aliphatic OH, and COOH moieties in lignin degradation was elucidated by selectively blocking each of them by diazomethane methylation, acetylation, and acetylation followed by alkaline hydrolysis, and the modified samples were then submitted to laccase and laccase–HBT treatment.

The modifications induced on the lignin polymer were determined by means of quantitative ^{31}P NMR spectroscopy of lignin samples suitably phosphitylated with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane.[28–33] These experiments provided a clear insight to the actual structure structural modifications of lignin and permitted the clarification of the role of HBT during laccase the oxidation of residual kraft lignin. The LM system was able to modify the content and distribution of phenolic and aliphatic hydroxyl groups only when they were not etherified. On the basis of this data it is now possible to state that the role of HBT in laccase oxidation is to allow radical mediated reactions that involve the oxidation of the side chains and oxygen addition in lignin. In addition, the presence of HBT reduces the probability of formation of oxidative coupling reactions

Results and Discussion

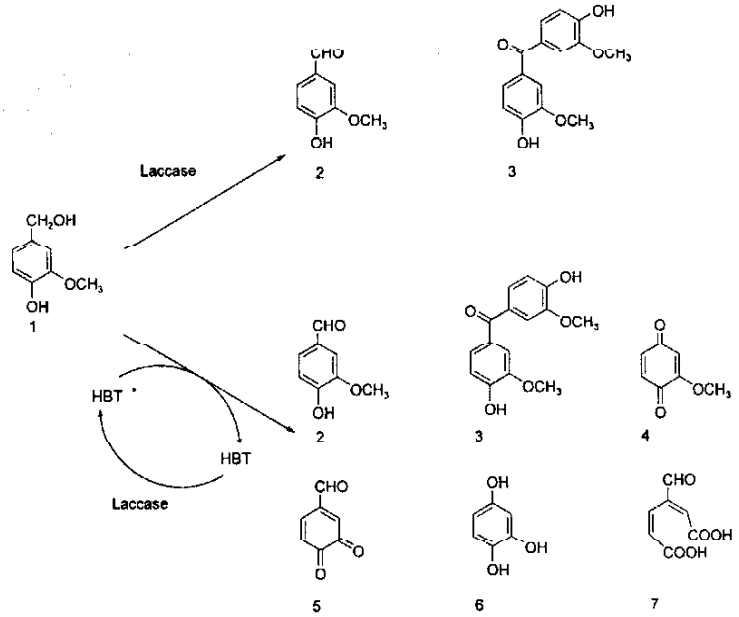
Oxidation of vanillyl alcohol: Vanillyl alcohol **1**, a phenolic substrate, was submitted to laccase oxidation in the presence and in the absence of HBT. After ten minutes, the reaction mixtures were acidified, extracted with ethyl acetate, and the residues were analyzed by quantitative GC-MS analyses in the presence of a suitable amount of 2,4-dimethoxy toluene as an internal standard.

The % conversions of vanillyl alcohol under different reaction conditions, are shown in Table 1 and Scheme 1. It is evident that the presence of HBT did not significantly affect

Table 1. Product identity and yields detected after laccase and laccase + HBT treatments of vanillyl alcohol.[a]

Product	Laccase (% of the starting product)	Laccase + HBT
1	14.8	12.4
2	trace	trace
3	0.8	1.3
4	–	trace
5	–	4.0
6	–	4.0
7	–	6.9

[a] The products missing from the mass balance have been tentatively assigned as radical oxidative-coupling polymeric products and/or volatile one-carbon fragments.



Scheme 1. Conversion of vanillyl alcohol under different reaction conditions.

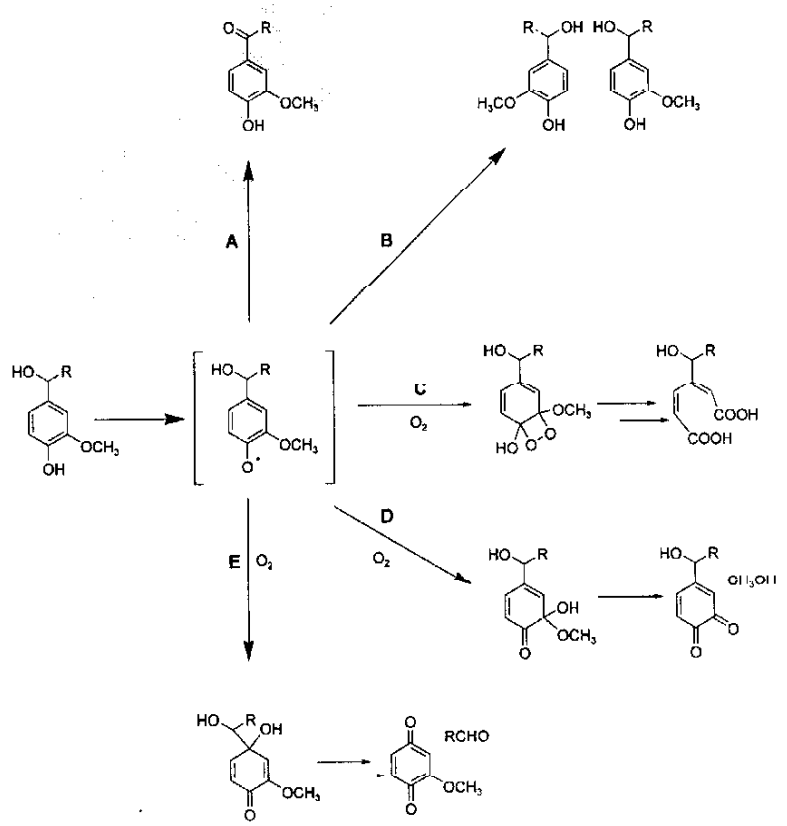
the conversion amount. However, the distribution of the oxidation products produced under the two conditions is fundamentally important for the interpretation of the operating mechanisms. Unfortunately, under our experimental conditions the bulk of reaction products were not identified due to their high molecular weight (> 550). In fact, in a control experiment all the reaction mixtures were freeze dried and the residue was submitted to silylation and GC-MS analyses.

Our experiments showed that no further low-molecular-weight reaction products were lost during the reaction workup, with the exception of volatile one carbon fragments such as methanol.

In the presence of laccase alone, only traces of vanillin **2**, a product of side-chain oxidation, and a low amount of **3**, an α-1-diphenylmethane compound arising probably from oxidative coupling, were detected. In contrast, when the LM system was used in the oxidation of **1**, the reaction pathway seems to be more complex. The presence of HBT in the reaction mixture induced a major change in the mechanism, yielding *p*-quinone **4**, *o*-quinone **5**, phenol **6**, and muconic acid **7** (Scheme 1).

It is assumed that laccase catalyzes the reduction of oxygen to water with the generation of a phenoxyl radical from a phenolic substrate by a one-electron oxidation process and loss of a proton. Phenoxyl radicals are long-lived species with half-life times of hours.[4] The phenoxyl radicals generated by the laccase-mediated oxidation of phenolic lignin subunits, in its early steps, can in principle undergo several different reactions. More specifically, disproportionation reactions yield side-chain oxidation products (Scheme 2, route A); radical coupling reactions yield condensed products (Scheme 2, route B); further oxidation/oxygen addition in the C-3 position yields *o*-quinones and methanol (Scheme 2, route D) with the eventual aromatic ring cleavage via dioxetane intermediates (Scheme 2, route C).[7] Oxidation in C-1 yields alkyl–aryl cleavage with *p*-quinone formation (Scheme 2, route E).[7] However, since phenoxyl radicals do not react with molecular oxygen to a noticeable extent[34, 35] ($k < 10^{-2}\,M^{-1}s^{-1}$) the observed formation of quinones and aromatic-ring cleavage products should be brought about by superoxide anion radicals reacting with phenoxyl radicals.[36] In the presence of laccase and HBT it is assumed that the HBT radical is generated.[19] One may hypothesize, by analogy to the oxidation mechanism of non-phenolic models, that HBT· can react with phenolic lignin subunits by hydrogen abstraction regenerating HBT.

Hydrogen abstraction from vanillyl alcohol is believed to occur either at the phenolic hydroxyl group or at the benzylic carbon atom. To further clarify this issue we carried out a series of computations using the B3LYP/6-31 + G(d,p)// B3LYP/6-31G(d) method in order to estimate the bond dissociation energies (ΔH_{DBE}) for the mentioned abstraction sites. We computed the total electronic energies of vanillyl alcohol (ε_{0VA}), its benzylic carbon-centered radical (ε_{0BR}), its phenoxyl radical (ε_{0PR}), and hydrogen atom (ε_{0H}). The energy change ($\Delta\varepsilon_0$) for the benzylic hydrogen abstraction was calculated as $\Delta\varepsilon_{0BHA} = \varepsilon_{0BR} + \varepsilon_{0H} - \varepsilon_{0VA} = 88.7$ kcal mol^{-1} and the energy change for the phenolic hydrogen abstraction as $\Delta\varepsilon_{0PHA} = \varepsilon_{0PR} + \varepsilon_{0H} - \varepsilon_{0VA} = 89.4$ kcal mol^{-1}. The values $\Delta\varepsilon_{0BHA}$ and $\Delta\varepsilon_{0PHA}$ represent biased estimates of the bond dissociation energies since no corrections (e.g., for temperature) were included. In an effort to obtain more representative values we constructed a calibration graph, including experimental ΔH_{DBE} values,[37] of six compounds and $\Delta\varepsilon_0$ values computed for these compounds as described above (Figure 1).



Scheme 2. Reactions of the phenoxyl radicals generated by the laccase-mediated oxidation of phenolic lignin subunits.

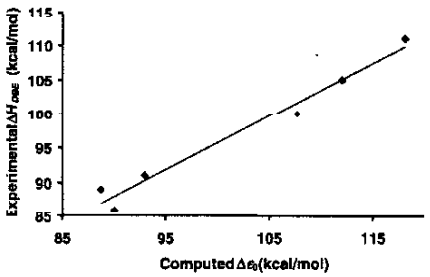


Figure 1. Calibration line for the ΔH_{DBE} values. The experimental ΔH_{DBE} values (from the bottom up) are those of phenol (O-H), methanethiol (S-H), hydrogen sulfide, propane(*n*-propyl), methane, and benzene. The $\Delta\varepsilon_0$ values were computed by means of the B3LYP/6-31+G(d,p)//B3LYP/6-31G(d) method.

The equation of the calibration line is given in Equation (1) ($r^2 = 0.97$; standard error = 1.8 kcal mol^{-1}):

$$\Delta H_{DBE}\ [\text{kcal mol}^{-1}] = \Delta\varepsilon_0\ [\text{kcal mol}^{-1}]\,0.777 + 18.1 \quad (1)$$

By using this equation and the computed $\Delta\varepsilon_0$ values, we obtained a ΔH_{DBE} value of 87.5 kcal mol^{-1} for the benzylic C–H bond of vanillyl alcohol, while for the phenolic O–H bond the value of ΔH_{DBE} was 87.0 kcal mol^{-1}. Apparently, the difference is not significant suggesting that hydrogen abstractions are thermodynamically similar both at the phenolic O–H and at the benzylic C–H bonds.

Our computations also revealed information about charge distribution and spin densities on the examined species. Figure 2 shows orbital plots obtained from the semi-empirical PM3 computations. These plots illustrate the large differences between the carbon-centered (**B**) and the oxygen-centered (**C**) vanillyl alcohol radicals. Their spin densities show approximately complementary patterns.

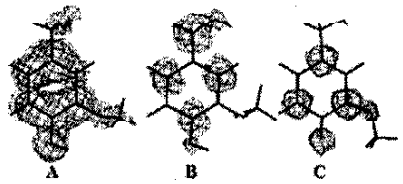


Figure 2. Plots of the highest occupied molecular orbital (HOMO) of vanillyl alcohol (**A**) and total positive spin densities of the benzylic (**B**) and phenoxyl (**C**) radicals.

Table 2 lists quantitative data about spin densities of the radicals, obtained by the B3LYP/6-31+G(d,p)//B3LYP/6-31G(d) method, in close agreement with the semi-empirical plots. Total spin density values indicate the sites at which reactions with other radicals are likely to occur. The phenoxyl radical shows high-spin densities at the phenolic oxygen and at C1 and C3 atoms. This spin distribution is in complete agreement with the observed formation of *o*-, *p*-quinones, 1,2,4-trihydroxybenzene, and the muconic acid derivative. The benzylic radical shows by far the highest spin density at the benzylic carbon, at which the reactions leading to the formation of vanillin and of dimer **3** take place. Furthermore, Table 2 lists charges on all heavy atoms and energies of the highest occupied molecular orbitals, signifying substantial differences among the compounds at some parts of their molecular structure.

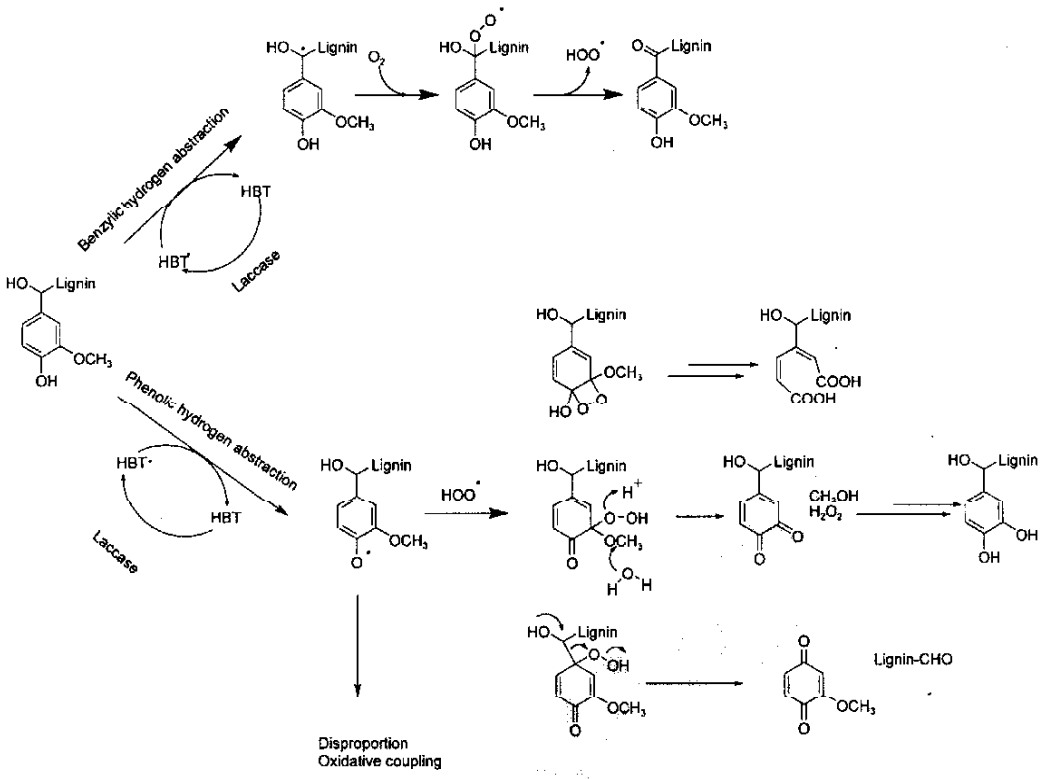
Table 2. Computed electronic properties of vanillyl alcohol (VanAlc) and its benzylic (C$^\cdot$) and phenoxyl (O$^\cdot$) radicals, by means of the B3LYP/6-31+G(d,p)//B3LYP/6-31G(d) method.

	Charge (H included)			Total spin density	
Atom	VanAlc	VanAlc C$^\cdot$	VanAlc O$^\cdot$	VanAlc C$^\cdot$	VanAlc O$^\cdot$
C1	−0.20	0.13	0.31	−0.21	0.31
C2	0.46	0.77	−0.09	0.30	−0.18
C3	0.31	0.01	0.33	−0.12	0.31
C4	−0.36	−0.33	0.11	0.25	−0.03
C5	0.61	0.62	0.33	−0.15	0.20
C6	−0.55	−0.81	−0.50	0.22	−0.09
O_{Phe}	−0.15	−0.15	−0.52	0.04	0.37
O_{MeOH}	−0.45	−0.45	−0.36	0.00	0.09
C_{MeOH}	0.34	0.33	0.34	0.00	0.00
Cα	0.15	−0.05	0.19	0.65	−0.02
Oα	−0.16	−0.07	−0.15	0.07	0.00
Orbital	Eigenvalue [Hartrees]				
HOMO	−0.22				
SOMO		−0.15	−0.22		

In principle, hydrogen abstraction can occur either at the phenolic or at the benzylic position. In the former case a phenoxyl radical would be formed. As such HBT$^\cdot$ would simply act as a diffusible radical mediator. If hydrogen abstraction occurs at the benzylic position a benzylic radical would be formed. This latter intermediate is likely to undergo rapid oxygen addition and after loss of superoxide radical anion, to yield the side-chain oxidation products shown in Scheme 3. It has been shown that benzylic radicals are reactive toward oxygen addition at rates of $10^{10}\,M^{-1}s^{-1}$.[38] The superoxide radical anions formed during this step would in turn readily react with phenoxyl radicals allowing the formation of further oxidation products.

Attack at the C3-position would cause the formation of *o*-quinones and methanol, while addition at the C4-position ultimately would cause the formation of aromatic-ring cleavage products (muconic acids). Finally addition at the C1-position, followed by aryl–alkyl cleavage, would result in the formation of *p*-quinones (Scheme 3). This reaction mechanism could explain the formation of products **3**–**6** from the LM system on vanillyl alcohol. This rationale is in accord with previously reported laccase-mediated oxidations of lignins in which the formation of condensed units, quinones, methanol, aryl–alkyl cleavage, side-chain oxidation, and aromatic-ring oxidation products were reported.[7, 8, 11, 39, 40]

Oxidation of residual kraft lignin: Residual kraft lignin (RKL) was oxidized in the presence of either laccase or laccase + HBT. The lignin samples isolated after the treatments were phosphitylated and then subjected to quantitative ^{31}P NMR analyses in the presence of a suitable amount of cyclohexanol as an internal standard. The assignment of the different signals was carried out on the basis of earlier work.[31, 32, 41]



Scheme 3. Reactions of the benzylic radicals.

All experiments were carried out in triplicate and the quantitative data collected is shown in Table 3. The analysis of the amount and distribution of aliphatic OH, phenolic OH, and COOH groups showed a different oxidation pathway in the presence and in the absence of HBT. Irrespective of the presence or absence of HBT the aliphatic OH and the guaiacyl phenolic OH groups were found in lower amounts than in the original RKL, despite the fact that the decrease was more remarkable in the presence of HBT. This is in agreement with the previously hypothesized role of HBT in lignin degradation. The COOH content was found to increase by 20%, (from 0.34 to 0.41 mmol g^{-1}), upon laccase treatment.

Table 3. Phenolic, aliphatic, and carboxylic OH groups (mmol g^{-1}) present on residual kraft lignin before and after treatment with laccase and laccase + HBT determined by quantitative ^{31}P NMR analysis.[a]

Functional group	RKL[b] [mmol g^{-1}]	RKL + laccase [mmol g^{-1}]	RKL + laccase + HBT [mmol g^{-1}]
aliphatic OH	2.50	2.36	2.10
diphenylmethane OH	0.23	0.31	0.22
4-O-5′ condensed OH	0.36	0.63	0.35
5-5′ Condensed OH	0.66	0.68	0.59
guaiacyl OH	1.25	1.00	0.85
COOH	0.34	0.41	0.34

[a] Errors applicable to these measurements are as those reported in ref. [33]. [b] Residual kraft lignin.

When HBT was present in the reaction mixture no variation in COOH content was evident. The corresponding decrease of guaiacyl OH, condensed phenolic OH, and aliphatic OH groups could be due to the loss of small oxidized fragments.

The distribution of the condensed phenolic units present in residual kraft lignin under different reaction conditions was significantly modified. The diphenylmethane substructures (^{31}P NMR range 144.3–142.8 ppm)[42] were found to increase slightly upon laccase treatment. This could be due to either a reaction with phenolate units or formation of one-carbon fragments. In contrast, when HBT was present in the reaction medium the amount of diphenylmethane did not significantly change. This is indicative of oxidative reactions occurring by a different pathway. In the same fashion the abundance of 4-O-5′ subunits (^{31}P NMR range 142.75–141.75 ppm), which are formed by radical coupling reactions, increased by 75%, (from 0.36 to 0.63 mmol g^{-1}), after the laccase treatment. In the presence of HBT, however, their abundance did not change. Condensed 5-5′ units (^{31}P NMR range 141.75–137.4 ppm), that is, radical coupling products, were also found to be somewhat increased upon laccase treatment, and significantly decreased in the presence of HBT (Table 3).

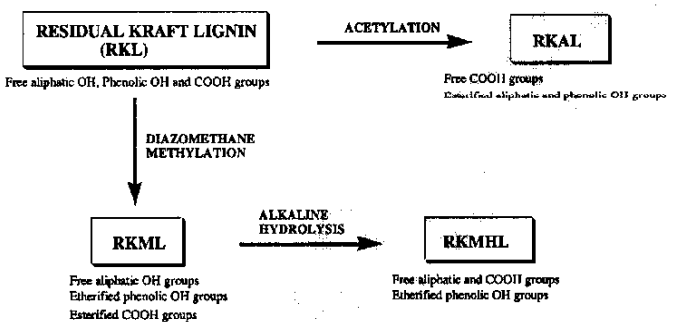
From these data a clear trend becomes apparent: the laccase and the laccase–mediator oxidation of residual kraft lignin (RKL) proceed through different reaction pathways. In the presence of HBT, side-chain oxidation reactions are favored as opposed to radical-coupling and condensation

reactions. When the treatment was carried out in the absence of HBT, the formation of condensed units occurred as the main reaction.

It is likely that HBT could act as a radical mediator by readily diffusing into the lignin's inner structure. Consequently HBT radicals generated by laccase oxidation could drive the overall reaction toward the Cα oxidation pathway by hydrogen-atom abstraction (HAT) reactions. The superoxide radical anions formed in this step (Scheme 3) would be responsible for aromatic-ring cleavage and quinone formation reactions.[43]

Oxidation of lignin with selectively protected moieties: In an effort to establish the role of non-phenolic units during the laccase-HBT catalyzed oxidation of RKL, a selective protection scheme for the various functional groups containing labile protons was devised (Scheme 4). More specifically RKL was submitted to exhaustive diazomethane methylation; this allowed the selective methylation of the phenolic OH and COOH groups, while leaving free the aliphatic OH groups. The quantitative ^{31}P NMR spectrum of the diazomethylated residual kraft lignin (RKML) after phosphitylation showed the effective presence of aliphatic OH groups only.

Diazomethylated residual kraft lignin was treated with laccase and laccase + HBT. The ^{31}P NMR spectra of the



Scheme 4. Selective protection of phenolic, aliphatic and carboxylic OH groups.

recovered samples after phosphitylation were found to be unaltered. In particular, neither condensed phenolic or guaiacyl OH groups were formed. In the presence of laccase alone, an increase in COOH units was apparent, most probably arising from residual acetate buffer. Neither laccase nor laccase + HBT were able to modify the distribution or the amount of aliphatic OH, phenolic OH, and COOH groups in RKML.

The role of COOH groups was studied by exposing diazomethylated RKL to alkaline hydrolysis (RKMHL) (Scheme 4). This scheme liberated the COOH and aliphatic OH groups, while the phenolic OH groups were still protected as confirmed by P NMR spectroscopy. Accordingly, RKMHL was submitted to treatment with laccase and laccase + HBT and was then examined by quantitative ^{31}P NMR spectroscopy. Once again, no degradation of the lignin polymer with respect to the control experiment was apparent. Analogous behavior was found when RKL was submitted to extensive acetylation (RKAL). Under these conditions only COOH groups were free. Neither laccase, nor laccase + HBT were able to modify the amount and/or distribution of the carboxylic acid groups.

These data when coupled with our earlier studies on RKL model compounds,[23] imply that the activity of the laccase–mediator system does not depend on the nature of the non-phenolic lignin subunits. In actual fact, LM system reactions with lignin-containing protected or unprotected phenolic, aliphatic, and carboxylic acid groups, did not result in any appreciable lignin degradation. On the contrary, when the LM system was employed on RKL, HBT showed the ability to drive the degradation pathway toward side-chain oxidation products, and suppressed the formation of condensed structures.

Our data indicates that in the presence of HBT the side-chain oxidation pathway is strongly favored over oxidative coupling reactions.[38] Previous reports describe that the depolymerization of lignin occurs only when treated with laccase and HBT. Earlier accounts indicate that when lignin is treated with the LM system an increase in carbonyl and quinone content is apparent, while in the absence of HBT no depolymerization occurs.[19, 39, 44] These accounts are in complete agreement with the present effort. The fact that the LM system induces the formation of darker pulps that are easily bleached with hydrogen peroxide, than those obtained from treatments in the absence of a mediator, is most likely due to an increased quinone content.

Conclusion

Experiments with vanillyl alcohol showed that in the presence of laccase only side-chain oxidation and oxidative coupling reactions occur. However, in the presence of the LM system the formation of *o*- and *p*-quinones, demethylation, aromatic-ring cleavage, and oxidative coupling reactions were apparent. Estimates of the bond dissociation energies for the hydrogen abstraction from vanillyl alcohol at the phenolic hydroxyl group or at the benzylic carbon atom were found to be thermodynamically equivalent. The LM system does not operating when the phenolic OH groups of residual kraft lignin are protected. Extensive experiments carried out on residual kraft lignins showed the occurrence of coupling reactions during laccase oxidation. In the presence of the LM system, however, the reaction pathway was driven toward side-chain oxidation and oxygen addition products, while the formation of condensed structures was suppressed.

One may argue that HBT does not act as a mediator in the oxidation of non-phenolic lignin subunits. The principal sites of oxidative attack in the laccase/HBT system are the free phenolic groups in lignin.[39] In our view HBT· is able to

promote the oxidative reactivity of laccase toward side-chain oxidation processes. A possible mechanism is the benzylic hydrogen-abstraction reaction. The benzylic radical is a species with a high degree of reactivity toward oxygen. Such an intermediate reacts by oxygen addition yielding a side-chain oxidation product with the simultaneous generation of superoxide radical anion (Scheme 3). In turn the superoxide radical anion reacts with the phenoxyl radical by adding in positions 1, 3, or 4 of the aromatic ring. Thus the formation of side-chain oxidation, oxygen addition, and aromatic-ring cleavage products or substructures are favored, while and 5 5′ and/or 4 O 5′ coupling reactions are depressed.

Table 4. Mass spectrometric data.

Product	Derivative[a]	MS (m/z) data (%)
2	–	152 (100) $[M]^+$, 151 (95), 109 (65), 81 (97)
3	$-Si(CH_3)_3$	418 (4) $[M]^+$, 403 (2), 387 (3), 343 (5), 281 (5), 207 (23), 147 (6), 96 (11), 75 (26), 73 (100)
4	–	138 (70) $[M]^+$, 123 (11), 110 (68), 108 (100), 95 (75) 82 (63), 69 (98), 55 (46), 54 (69)
5	–	136 (45) $[M]^+$, 121 (34), 104 (41), 79 (30), 75 (100)
6	$-Si(CH_3)_3$	342 (5) $[M]^+$, 145 (13), 132 (30), 129 (19), 117 (68) 83 (11), 75 (67), 73 (100)
7	$-Si(CH_3)_3$	315 (6) $[M+1]^+$, 314 (20) $[M]^+$, 207 (17), 131 (24), 129 (16), 117 (80), 111 (18), 106 (11), 97 (13), 85 (14), 82 (20), 75 (93), 73 (100)

[a] –: underivatized; $-Si(CH_3)_3$: trimethylsilylated with *N,O*-bis(trimethylsilyl)acetamide.

Experimental Section

Quantitative ^{31}P NMR spectra were obtained on a Varian XL-300 spectrometer by using methods identical to those described by Argyropoulos et al.[28–33] The chemical shifts were referenced to phosphoric acid. The ^{31}P NMR data reported in this effort are averages of three phosphitylation experiments followed by quantitative ^{31}P NMR acquisitions. The maximum standard deviation of the reported data was 2×10^{-2} mmol g^{-1}, while the maximum standard error was 1.10^{-2} mmol g^{-1}.

Derivatization of the lignin samples with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane(II)[29, 31, 32, 42] were performed as previously described. Samples of lignin (30 mg), accurately weighed, were dissolved in a solvent mixture composed of pyridine and deuterated chloroform, 1.6:1 v/v ratio (0.5 mL). The phospholane (100 μL) was then added, followed by the internal standard and the relaxation reagent solution (100 μL each).

Isolation of residual kraft lignin: Residual kraft lignin was isolated from kraft pulp (*picea mariana*, kappa no. 31.5) by using a slightly modified acidolysis procedure.[45, 46] The yield was 38%, and the purity was confirmed by UV and klason lignin content measurements.

Lignin methylation: Lignin (200 mg) was suspended in diethyl ether (5 mL) and then treated with an excess of diazomethane for 24 h in the dark at room temperature. The treatment was repeated three times. The mixture was centrifuged, washed with ethyl ether, and centrifuged again. The residue was dried under reduced pressure.[29]

Lignin hydrolysis: Lignin (100 mg) was dissolved in NaOH (2M, 10 mL) and stirred under a nitrogen atmosphere at 25 °C for 48 h. HCl (4M) was then added to obtain pH 3, and the mixture centrifuged, washed with water, centrifuged again, and freeze-dried.[29]

Lignin acetylation: Acetylation was carried out with pyridine/acetic anhydride (1:1) at 25 °C for 48 h. HCl (4M) was then added to obtain pH 3, and the mixture was stirred 12 h. The residue was centrifuged, washed with water, centrifuged again, and freeze-dried.[29]

Enzyme assays: Laccase activity was determined by oxidation of ABTS. The assay mixture contained ABTS (0.5 mM), sodium acetate (0.1M), pH 5.0, and a suitable amount of enzyme. Oxidation of ABTS was followed by absorbance increase at 420 nm ($\varepsilon_{420} = 3.6 \times 10^4$ M^{-1} cm^{-1}). Enzyme activity was expressed in units (U = mmol of ABTS oxidized per minute).[47]

Characterization of metabolites : Gas chromatography and gas chromatography/mass spectrometry of the reaction products were performed by using a DB1 column (30 m × 0.25 mm and 0.25 mm film thickness), and an isothermal temperature profile of 80 °C for the first 2 min, followed by a 5 °C min^{-1} temperature gradient to 200 °C and a 15 °C min^{-1} gradient to 280 °C, and finally an isothermal period at 280 °C for 10 min. The injector temperature was 250 °C. Chromatography grade helium was used as the carrier gas. The fragmentation patterns were compared to those of authentic samples. The GC-MS fragmentation patterns are reported in Table 4.

Laccase treatments of vanillyl alcohol 1: The oxidation was performed in a dioxane/water 1:3 (v/v) solvent mixture. The substrate (2 mM) was dissolved in dioxane: acetate buffer pH 5 (0.05M, 4 mL, 1:3 v/v), in the presence or absence of HBT (1 mM), and treated with purified laccase (0.5 U mL^{-1}) from *Trametes versicolor* at 40 °C. The reaction mixtures were kept in open vials under vigorous stirring in order to ensure constant oxygen saturation of the solutions throughout the experiment. The reactions were conducted in the dark in order to avoid possible photochemical oxidation. After 10 min the reactions were stopped by acidification with hydrochloric acid; a suitable amount of 2,4-dimethoxy toluene was then added to act as an internal standard (2 mM), dissolved in dioxane. The reaction mixtures were extracted with ethyl acetate. The solutions were dried over anhydrous $MgSO_4$ and filtered. The organic solvent was evaporated under reduced pressure. In order to analyze the reaction products the residues were dissolved in pyridine (1 mL) and silylated with *N,O*-bis(trimethylsilyl)acetamide. After 30 min the mixtures were subjected to gas chromatography (GC) and gas chromatography/mass spectrometric (GC-MS) analyses.

Laccase treatments of residual kraft lignin: Lignin (100 mg) was suspended in an acetate buffer pH = 5 (0.05M, 10 mL) in the presence or absence of HBT (1 mM) and treated at 40 °C with purified laccase (10 U mL^{-1}) from *Trametes versicolor*. The reaction vessel was purged with oxygen. Oxygen was then applied until a positive pressure was obtained. After 24 h the mixture was cooled, acidified at pH 3 with acetic acid, and centrifuged. The residue was washed with water three times, and then freeze-dried.

Computational methods: The initial structures of all compounds were drawn by using a model builder (HyperChem 5.11 Professional, HyperCube, Inc.). The structures were then subjected to preliminary geometry optimization with the MM+ molecular mechanics[48] force field (to root mean square (RMS) gradient 0.06 kcal mol^{-1} Å^{-1}). A short (10 cycles) semi-empirical PM3 (3rd parametrization method[49, 50]) geometry optimization was then performed to assign partial charges on all atoms. The charges permitted the use of an improved MM+ force field (HyperChem), based on non-bonded electrostatic interactions. Using this force field, we generated the starting structures by simulated annealing (molecular dynamics under increasing and then decreasing temperature). The starting geometries were then optimized with MM+ and finally with the PM3 semi-empirical method with self-consistent-field convergence set to 0.01 and the optimization convergence to RMS gradient 0.06 kcal mol^{-1} Å^{-1}. The unrestricted Hartree–Fock (UHF) algorithm was used for the PM3 calculations.

The PM3 geometries of the compounds served as starting structures for the higher-level geometry optimizations by using the B3LYP/6-31G(d) method as implemented by Gaussian 98.[51] B3LYP is a density functional method created by Becke[52] and based on a Lee, Yang, and Parr[53] method. The optimization was followed by a single-point computation of the electronic energies using the B3LYP/6-31 + G(d,p) method. Thus, by the conventional notation, the method can be described as B3LYP/6-31 + G(d,p)//B3LYP/6-31G(d).

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