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Oxidation of non-phenolic substrates

An expanded role for laccase in lignin biodegradation

Robert Bourbonnais and Michael G. Paice

Pulp and Paper Research Institute of Canada, 570 St. John's Blvd, Pointe Claire, Que. H9R 3J9, Canada

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In the presence of substrates such as Remazol Blue and 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonate) (ABTS), laccases *Coriolus (Trametes) versicolor* can also oxidize non-phenolic lignin model compounds Veratryl alcohol (**I**) and 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)-propane-1,3-diol (**III**) were oxidized by laccase and mediator to give the α-carbonyl derivatives The β-1 lignin model dimer, 1-(3,4-dimethoxyphenyl)-2-phenoxy-ethane-1,2-diol (**II**) was cleaved by laccase in the presence of ABTS to give veratraldehyde and benzaldehyde. On the basis of these observations, we propose that laccase is capable of oxidizing both phenolic and non-phenolic moieties of lignin but that the latter is dependent on the co-presence of primary laccase substrates

Oxidation, Laccase; Phenol; Dimer; Lignin

1. INTRODUCTION

Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) is produced abundantly by certain trees and fungi. For many years the true function of this multicopper oxidase has remained a mystery [1–3]. Lately, it has been found that fungal laccase may play at least a limited role in lignin biodegradation [4]. Laccase oxidizes phenols and polyphenols by one-electron abstraction giving radicals which can subsequently polymerize [5,6]. However, phenolic subunits are relatively infrequent in lignin structures, representing, for example, only about one in three C-9 units in spruce milled wood lignin [7]. If oxidation is limited to phenolic subunits, then bulk delignification, which requires that many bonds of the three-dimensional polymer be broken [8], is highly unlikely.

Lignin peroxidase, which was first recognized in the white-rot fungus, *Phanerochaete chrysosporium* [9,10], has subsequently been identified in laccase-producing fungi such as *Coriolus (Trametes) versicolor* [11,12], although under most culture conditions the peroxidase is far less abundant than in *Phanerochaete*. Lignin peroxidase oxidizes both phenolic and non-phenolic substructures of lignin and therefore appears to be a more effective delignifying agent than laccase. We now propose that laccase from *C. versicolor*, in the presence of appropriate substrates, is capable of oxidation of both phenolic and non-phenolic substructures of lignin, and thereby could play a role in depolymerization which is equally as significant as that of lignin peroxidase.

Correspondence address: R. Bourbonnais, Pulp and Paper Research Institute of Canada, 570 St. John's Blvd, Pointe Claire, Que., Canada H9R 3J9

2. MATERIALS AND METHODS

2.1. *Enzyme production and isolation*

Laccase was isolated from *C. versicolor* (ATCC 20869) liquid cultures grown for 7 days on mycological broth. Laccase in culture supernatant was concentrated (Amicon YM-10), and chromatographed on DEAE-Bio-Gel A (pH 6.0), followed by adsorption of non-laccase protein on hydroxyapatite (pH 7.0), gel permeation on Sephacryl S-200 and finally ion-exchange on Mono Q HR (pH 7.0).

2.2. *Electrophoresis*

SDS/polyacrylamide gel electrophoresis was performed on the Phast System (Pharmacia) with a Phast Gel gradient of 10–15% acrylamide. Protein M_r standards (Pharmacia) were α-lactalbumin, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin and phosphorylase *b*. Protein bands were stained with Coomassie blue.

2.3. *Enzyme assay*

Laccase activity was determined by oxidation of 2,2'-azinobis-(3-ethyl benzthiazoline-6-sulphonate) (ABTS) [13]. The reaction mixture contained 0.5 mM ABTS, 0.1 M sodium acetate buffer, pH 5.0, and a suitable amount of enzyme. Oxidation of ABTS was followed by absorbance increase at 420 nm ($\epsilon_{420} = 3.6 \times 10^4\ M^{-1} \cdot cm^{-1}$). Enzyme activity was expressed in units (U = μmol/min).

2.4. *Oxidation of non-phenolic lignin model compounds*

Each reaction mixture contained 3 mM non-phenolic test substrate, 0.1 U/ml of purified laccase **I**, 0.1 M sodium acetate buffer, pH 5.0, and a mediator as specified in section 3. The reactions were left overnight at room temperature, and samples of 0.5 ml were acidified with HCl and filtered through a 0.45 μm pore size filter for HPLC analysis.

The oxidation products were analyzed by reverse-phase HPLC, using a Microbondapak C-18 column (Waters Assoc.) as described

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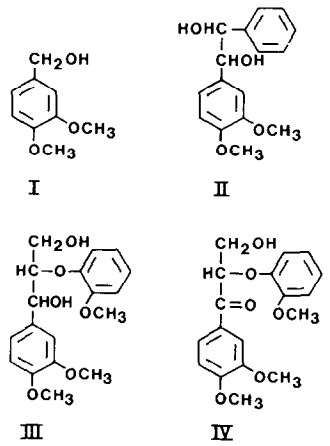

Fig. 1. Structures of non-phenolic lignin model compounds.

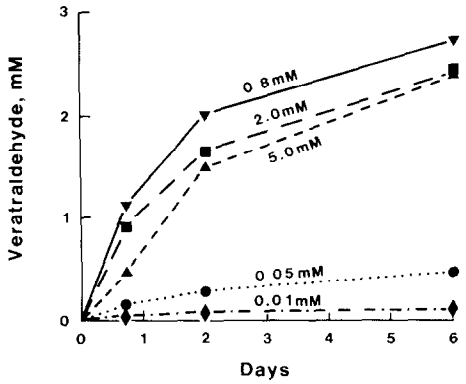

Fig. 2. Oxidation of veratryl alcohol (3 mM) to veratraldehyde by laccase I at different concentrations of ABTS. The ABTS concentrations (mM) are shown on each curve.

previously [14]. The UV spectra of the eluted compounds were obtained with a diode array detector (Hewlett-Packard, 1040A).

2.5. *Chemicals*

All monomeric non-phenolic lignin model compounds and Remazol Brilliant Blue R were obtained from Aldrich Chemical Co. The structures of lignin model dimers are shown in Fig. 1. Dimer **II**, [1-(3,4-dimethoxyphenyl)-2-phenylethanediol] and dimer **III** [1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)-propane-1,3-diol] were synthesized by N.G. Lewis (Virginia Polytechnic Institute and State University). Dimer **IV** [1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)-3-hydroxy-1-propanone] was kindly provided by T. Fukuzumi, Department of Forest Products, Tokyo University of Agriculture.

3. RESULTS

3.1. *Enzyme purification*

After 7 days growth in mycological broth, *C. versicolor* produced up to 11 U/ml of extracellular laccase. Lignin peroxidase activity, determined by oxidation of veratryl alcohol in the presence of H_2O_2 [15], could not be detected under these conditions. DEAE ion-exchange chromatography separated the enzymes into 3 distinct peaks of laccase activity. The first peak (laccase **I**), which contained more than 90% of the total enzyme activity, was further purified. Purified laccase **I** had a specific activity towards ABTS of around 20 U/mg protein. The visible spectrum of the enzyme showed a maximum absorption at 600 nm. SDS/polyacrylamide gel electrophoresis of the purified enzyme gave one band (M_r = 70000).

3.2. *Veratryl alcohol oxidation by laccase and mediators*

Purified laccase **I** alone could not oxidize veratryl alcohol, or other non-phenolic lignin dimer substrates (**II–IV**). However, known laccase-oxidizable compounds such as ABTS and Remazol Brilliant Blue, mediated the oxidation of veratryl alcohol in the presence of laccase. The oxidation rate of veratryl alcohol depended on the nature and the concentration of the mediator. With ABTS as mediator (Fig. 2), maximum veratraldehyde formation was obtained with 0.8 mM ABTS; higher concentrations were inhibitory. Oxidation of veratryl alcohol was slow but was eventually almost quantitative (>90% conversion). With Remazol Brilliant Blue, a similar effect was observed; for example, approx. 50% of veratryl alcohol was oxidized over 4 days in the presence of 3 mM Remazol Brilliant Blue.

The oxidation of ABTS by laccase produces the stable dark green cation radical, $ABTS^{\cdot+}$ [13]. The possibility that this radical alone is able to oxidize veratryl alcohol was tested. ABTS was first oxidized by the laccase and the oxidized $ABTS^{\cdot+}$ was then separated from the enzyme by ultrafiltration (Millipore, CX-10). Table I shows the level of veratraldehyde produced when veratryl alcohol was exposed to $ABTS^{\cdot+}$, ultrafiltered laccase alone, or the complete system. These results strongly suggest that the oxidation of veratryl alcohol will occur only when the enzyme and the mediator are both present.

Samples of laccase **II** and **III**, named on the basis of their elution order from DEAE anion exchange gel, were also found to oxidize veratryl alcohol provided that ABTS was present (data not shown). The rates of oxidation were similar to laccase **I**.

Table I

Effect of $ABTS^{\cdot+}$ and laccase on oxidation of veratryl alcohol

Reaction mixture	Veratraldehyde (mM) after 18 h
Laccase + ABTS + veratryl alcohol	0.99
$ABTS^{\cdot+}$ + veratryl alcohol	0.02
Laccase + veratryl alcohol	0.04

Initial veratryl alcohol concentration = 3 mM, ABTS = 1 mM, laccase = 0.1 U/ml

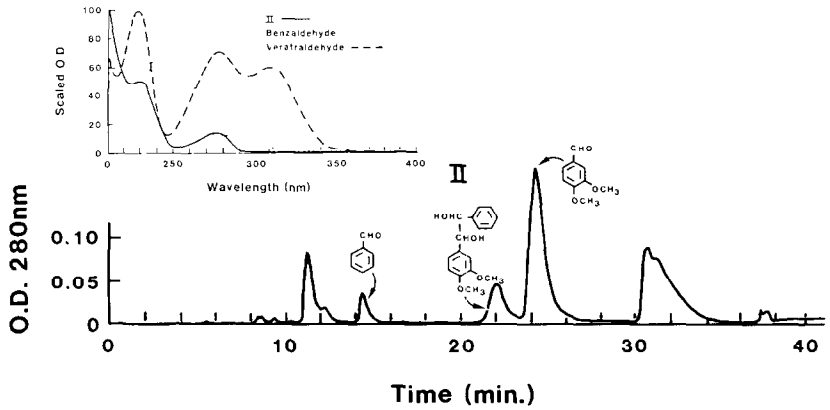

Fig. 3. High-performance liquid chromatogram and UV/visible spectra of reaction products formed during 19 h oxidation of dimer **II** (3 mM) by laccase and ABTS (1 mM).

3.3. *Oxidation and cleavage of non-phenolic lignin model dimers*

The oxidation products of the non-phenolic arylalkyl dimer **II** and the β-aryl ether dimer **III** by laccase and ABTS were analyzed by HPLC and identified by comparing their retention times and UV/visible spectra to known compounds. The chromatogram of dimer **II** oxidation and the UV absorption spectra (Fig. 3) show clearly the formation of veratraldehyde (24.2 min) and benzaldehyde (14.4 min) with a corresponding decrease in the dimer **II** (22 min). The concentrations of these compounds after 19 h incubation were 0.5 mM for benzaldehyde, 0.6 mM for veratraldehyde and 0.6 mM for dimer **II**. The other peaks which appear on the chromatogram were found to be oxidation products of ABTS by laccase since they also appeared in controls. HPLC analysis of the oxidation products of dimer **III** by laccase and ABTS showed that after 3 days incubation about 12% of the dimer **III** was oxidized to form the α-carbonyl derivative **IV**, identified by its retention time and UV/visible absorption spectra relative to authentic **IV**.

4. DISCUSSION

It is generally accepted that laccase-catalyzed oxidation of lignin is limited to phenolic subunits [5]. We have shown here for the first time that, under certain conditions, namely in the presence of recognized primary substrates, laccase is able to oxidatively cleave a Cα–Cβ linkage in a non-phenolic β-1 lignin model dimer, and to oxidize veratryl alcohol to veratraldehyde. Since phenols are likely to be present during wood degradation, we propose that laccase plays an important role in lignin depolymerization and that this role is not limited to phenolic subunits.

In contrast to the β-1 dimer **II** cleavage, veratrylglycerol-β-guaiacyl ether (dimer **III**) in the presence of laccase and ABTS produced mainly the Cα oxidation product, which has previously been identified when dimer **III** was incubated with *Polyporus versicolor*, *Stereum frustulatum* or laccase in the presence of spruce milled wood lignin [16]. Kirk et al. [16] ascribe the Cα oxidation to initial free radical formation in the wood. Although the involvement of an ABTS free radical appears to be likely for the observed effect of laccase and ABTS on non-phenolic structures, we found that laccase-generated ABTS radical cation, in the absence of laccase, was ineffective for non-phenolic oxidations. Either another, more transient radical form of ABTS is the electron-transfer reagent, or the laccase enzyme plays some further role in catalysis.

Morohoshi et al. [4] have shown that one of the laccases from *C. versicolor* (laccase **III**) depolymerizes a water-soluble lignin preparation, while the other laccases cause polymerization. We tested all 3 laccases isolated by ion-exchange chromatography for their ability to oxidize veratryl alcohol. Like laccase **I**, neither laccase **II** nor **III** oxidized veratryl alcohol unless a primary substrate such as ABTS was present. In the presence of ABTS, the reaction rates with all 3 laccases were similar. We are presently investigating the mechanism of non-phenolic oxidation, and the effect of primary substrates on laccase-catalyzed oxidation of macromolecules such as kraft lignin.

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