CLEAVAGE OF NONPHENOLIC LIGNIN STRUCTURES BY LACCASE IN THE PRESENCE OF 1-HYDROXYBENZOTRIAZOLE

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ABSTRACT

The degradation of nonphenolic lignin model compounds by laccase in the presence of a mediator, 1-hydroxybenzotriazole (HBT), was investigated The results show that the laccase/HBT couple is able to accomplish the C_β-*O*-aryl cleavage of a nonphenolic β-*O*-4-linked lignin dimer, presumably via a one-electron oxidation mechanism The α-carbonyl derivative of the β-*O*-4-linked model was also cleaved by laccse/HBT.

INTRODUCTION

White rot fungi produce extracellular oxidative enzymes that enable them to degrade lignin a recalcitrant polymeric constituent of wood [1]. Most of the lignin consists of nonphenolic aromatic structures that are particularly resistant to enzymatic oxidation. One group of extracellular white rot enzymes laccases, oxidizes phenolic lignin structures that are minor constituents in lignin but exhibits no direct activity towards the major nonphenolic structures in the polymer. However, it has been demonstrated that the substrate range of laccase extends to nonphenolic lignin structures when redox mediator compounds such as 1-hydroxybenzotriazole (HBT) are present. Under these conditions, nonphenolic β-*O*-4-linked lignin dimers are known to be oxidized without cleavage to the corresponding dimeric α-carbonyl compounds, but monomeric cleavage products indicative of ligninolysis have not yet been identified [2].

Here we show that the laccase/HBT couple cleaved a nonphenolic β-*O*-4-linked lignin model compound, presumably via a one-electron oxidation mechanism.

MATERIALS AND METHODS

Chemicals and enzymes

α-[^{14}C]-1-(4-ethoxy-3-methoxyphenyl)-2-(4-ethoxyphenoxy)propane-l,3-diol (model **I**), and α-[^{14}C]-1-(4-ethoxy-3-methoxyphenyl)-1-oxo-2-(4-ethoxyphenoxy)-3-hydroxypropane (model **II**) were prepared as published elsewhere [3]. 1-(4-ethoxy-3-methoxy-phenyl)-propane-1,2,3-diol (**III**), and 1-(4-ethoxy-3-methoxyphenyl)-1-oxo-propane-2,3-diol (**IV**) were obtained from T. K. Kirk Foretst Products Laboratory, Madison, WI.

Recombinant *Trametes villosa* laccase (form 1) [4] was obtained from A. Klotz (Novo Nordisk Biotech Davis, CA), and was purified by ion-exchange chromatography on a column of DEAE Biogel A, pre-equilibrated with 20 mM acetate buffer, pH 6.0. Proteins were eluted with a linear gradient (20 mM to 330 mM) of acetate buffer, PH 6.0. Fractuons containing laccase activity were pooled and further purified by gel permeation chromatography on Sephacryl S-200 in 330 mM acetate buffer, pH 6.0. Pooled fractions containing laccase activity were diafiltrated against 30 mM acetate buffer, pH 6.0, and concentrated using a 10 kDa cut-off membrane (Amicon YM10). Laccase activity was determined by monitoring the oxidation of 2,2' -azino-bis-(3-ethylbertzthiazotine-6-sulfonic acid (ABTS) at 420 nm ($\varepsilon = 43.2\ cm^2 \mu mol^{-1}$). The laccase assay contained 0.5 mM ABTS and 50 mM sodium acetate (pH 4.0). 1 U of activity was defined as the amount of enzyme that formed 1 μmol of product per min. 1-hydroxybenzotriazole was from Sigma. All other chemicals were reagent grade.

Oxidation of models I and II

Reactions (0.5 ml) were carried out in loosely capped 4 ml vials at 30°C, and were rotary-shaken for 24 h at 130 rpm. Water and buffer were autoclaved, and all other reagents were filtered through sterile 0.22 μm pore size filters. The reactions contained 0.32 mM **I** (7.0×10^4 dpm) or 0.19 mM **II** (4.1×10^4 dpm). 0.9 mM HBT and 50 mM sodium acetate (pH 4), and were started by adding 0.33 U (model **I**) or 1.4 U (model **III** of laccase.

A portion (0.25 ml) of each reaction was analyzed by high-performance liquid chromatography (HPLC) on a reverse-phase column (10 μm particle size, 4.6 × 250 mm, Vydac 201TP). The column was eluted at 1 ml min^{-1} with methanol-water-formic acid (100:900:5) for 5 min, followed by a linear gradient to methanol-water-formic acid (700:300:5) between 5 and 50 min. Fractions (1.0 ml) were collected and assayed for ^{14}C by scintillation counting. To identify the labeled products, HPLC fractions were pedal extracted, and subjected to gas chromatography-mass spectrometry (GC-MS).

RESULTS AND DISCUSSION

Oxidation of models I and II

Model **I** (Fig. 1), which represents a nonphenolic β-*O*-4-linked lignin structure, was oxidized by laccase in the presence of HBT (Fig. 2). **I** was converted to **II** (25% of total ^{14}C in chromatogram), and a mixture of more polar products (54% of total ^{14}C). A significant amount of **III** which is diagnostic for β-*O*-aryl cleavage of **I,** was found as a mixture of its *threo* and *erythro* isomers (13% of total ^{14}C). **IV,** which could have been formed either by cleavage of **II** or oxidation of **III,** was also identified (8% of total ^{14}C). Model **II** (Fig. 1), which was the major product of model I oxidation was further oxidized and cleaved by laccase/HBT to give significant amounts of IV (26% of total ^{14}C) (Fig. 2B).

The structure of **III** and **IV** were confirmed by GC-MS of the trimethylsilyl derivatives. **III:** *m/z* (relative intensity) 458 (M^+, 1) 255 (5), 253 (100). **IV:** *m/z*

(relative intensity) 384 (M^+, 6), 369 (3), 268 (53), 205 (38), 204 (12), 179 (100), 151 (22).

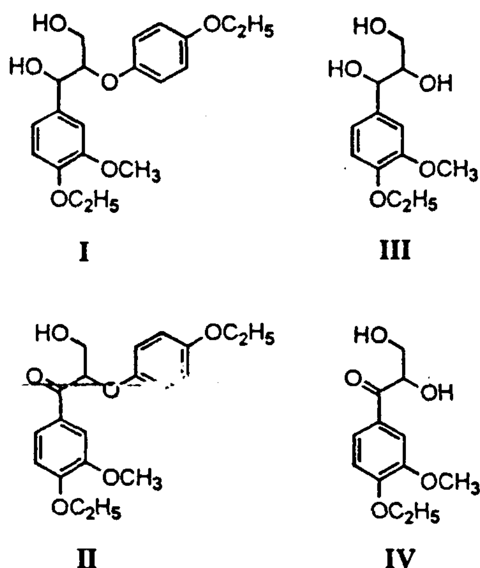

Figure 1. Chemical structures of model compounds and oxidation products.

These results show that the laccase/HBT couple was able to oxidize a nonphenolic β-*O*-4-linked linked lignin model as well as its corresponding a-carbonyl derivative to give monomeric cleavage products. β-*O*-aryl cleavage products accounted for at least 39% of the total ^{14}C-labeled monomeric products, suggesting that one-electron oxidation is a major pathway of model **I** cleavage by laccase/HBT.

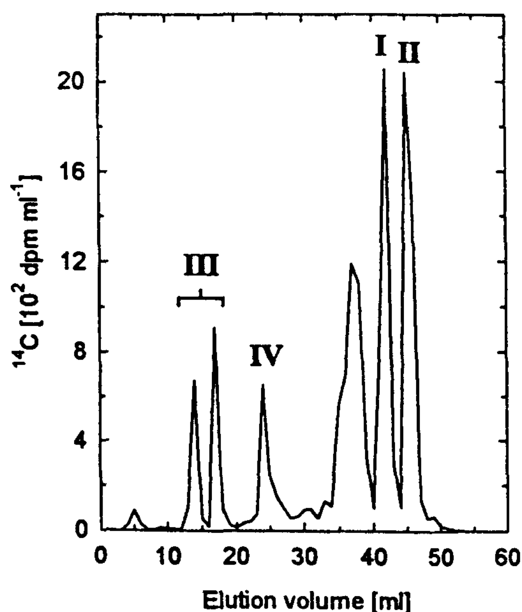

Figure 2. HPLC of products obtained when model I was oxidized by laccase/HBT.

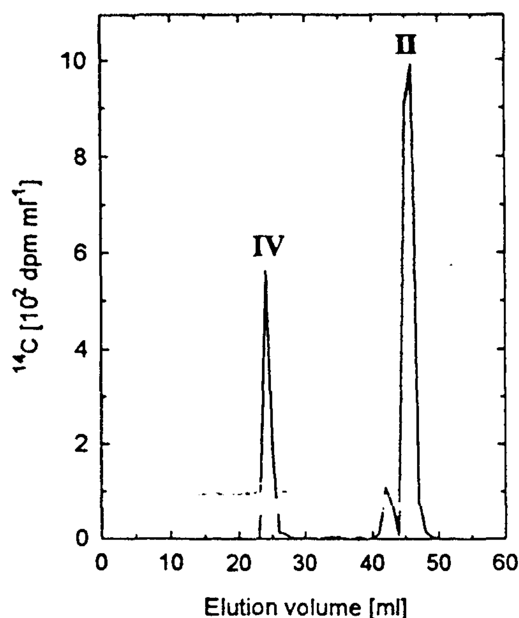

Figure 3. HPLC of products obtained when model II was oxidized by laccase/HBT.

Previous work [2] has shown that, in the presence of another mediator, ABTS, laccase oxidized a similar nonphenolic β-*O*-4-linked model to the corresponding dimeric α-carbonyl compound, but monomeric cleavage products indicative of ligninolysis were not identified, There are two possible reasons for this discrepancy. 1) Our dimer (**I**) may be more reactive than the dimers used by others [2], because it contains a para- rather than an ortho-substituted alkoxyl substituents on its arylglycerol-linked aromatic ring, or 2) HBT may form more reactive intermediates than ABTS does when oxidized by laccase. In fact, the ABTS cation radical is very stable in aqueous solutions [5] whereas the nitroxide radical derived from HBT is not [6], and rapidly reacts with various aromatic compounds [7]. Experiments are under way to distinguish between these two possibilities.

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