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A fungal metabolite mediates degradation of non-phenolic lignin structures and synthetic lignin by laccase

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Abstract Lignin peroxidase is generally considered to be a primary catalyst for oxidative depolymerization of lignin by white-rot fungi. However, some white-rot fungi lack lignin peroxidase. Instead, many produce laccase, even though the redox potentials of known laccases are too low to directly oxidize the non-phenolic components of lignin. *Pycnoporus cinnabarinus* is one example of a laccase-producing fungus that degrades lignin very efficiently. To overcome the redox potential barrier, *P. cinnabarinus* produces a metabolite, 3-hydroxyanthranilate that can mediate the oxidation of non-phenolic substrates by laccase. This is the first description of how laccase might function in a biological system for the complete depolymerization of lignin.

Key words: Lignin degradation; White-rot; Cinnabarinic acid; 3-Hydroxyanthranilate; Redox mediator; *Pycnoporus cinnabarinus*

1. Introduction

The biodegradation of lignin occupies a significant position in the global carbon cycle since lignin constitutes the second largest sink for fixed carbon after cellulose. White-rot fungi are the only organisms with a demonstrated capacity to mineralize lignin; consequently, the enzyme mechanisms they employ for lignin breakdown have attracted considerable research interest [1,2]. *Phanerochaete chrysosporium* has long been the organism of choice for studies of lignin degradation, and much of this work has focused on two peroxidases, lignin peroxidase (LiP) [3–6] and manganese peroxidase (MnP) [7–9], secreted by this fungus under ligninolytic conditions. LiP has generally been considered the key component of this ligninolytic system, at least in part because its high redox potential makes it capable of oxidizing the non-phenolic aromatic moieties that can comprise up to 85% of the lignin polymer [3,10]. However, many white-rot fungi, including a number of aggressive lignin degraders, seem to operate without expressing LiP activity [11–13]. Laccase (EC 1.10.3.2) is another phenoloxidase that white-rot fungi often produce in conjunction with LiP, MnP or both [14,15]. However, the role played by laccase in lignin degradation has remained obscure since the low redox potential of this enzyme appeared to make it incapable of oxidizing non-phenolic lignin constituents [16].

Through an extensive screening program, *Pycnoporus cinnabarinus* PB, a basidiomycete obtained from decaying pine in Queensland, Australia, was identified as a good candidate for studying the role played by laccases in lignin degradation. Under ligninolytic conditions, laccase was the predominant phenoloxidase secreted by *P. cinnabarinus* and neither LiP nor MnP were produced [17]. Despite the absence of LiP or MnP, *P. cinnabarinus* appears to degrade lignin just as rapidly, and to the same extent, as *P. chrysosporium* [18]. Thus, *P. cinnabarinus* provided an ideal model system in which to study alternative strategies for lignin biodegradation.

2. Materials and methods

2.1. Organism and reagents

P. cinnabarinus, strain PB, was maintained on malt agar slants and used for laccase preparation as described previously [17]. Laccase was purified to apparent homogeneity from liquid cultures of *P. cinnabarinus* grown from conidiospore inocula. LiP isozyme H8 was isolated from a crude enzyme solution of *P. chrysosporium* (Tienzyme Inc., PA) [19]. 3-Hydroxyanthranilate (3-HAA) and 2,2′-azino-bis-(3-ethylthiazoline-6-sulfonate) (ABTS) were purchased from Sigma. Lignin model compounds (Table 1) were provided by Dr. Masahiro Samejima (University of Tokyo), and a ^{14}C-ring-labeled dehydrogenative polymer of coniferyl alcohol was prepared by the 'Zutropfverfahren' (Odier and Heckman, INRA, France) and previously characterized as described in [2]. Cinnabarinic acid was synthesized according to [20].

2.2. Oxidation of lignin model compounds I-III

Oxidation of the three dimeric lignin model compounds was monitored photospectroscopically at 30°C in 50 mM sodium tartrate buffer with 3 μg *P. cinnabarinus* laccase at pH 4.0 or 2.5 μg *P. chrysosporium* LiP at pH 3.5, respectively. For LiP reactions, hydrogen peroxide was included to a final concentration of 100 μM. To these reaction mixtures each model compound was added to give a final concentration of 125 μM using 25 mg/ml stock solutions of the compounds dissolved in dimethylformamide. Spectral scans were taken between 230 and 400 nm at 10 min intervals for a total of 3 h.

2.3. Mediated oxidation of lignin model compound III and veratryl alcohol by laccase

Lignin model compound III (100 μM) was incubated with *P. cinnabarinus* laccase (3 μg/ml final concentration) in the presence of 3-hydroxyanthranilate (1.5 mM) in a total volume of 1 ml of 50 mM sodium tartrate (pH 4.0). After 48 h incubation at 25°C, reaction mixtures were acidified with phosphoric acid, centrifuged (10 min at 7000×*g*) and analyzed by reversed-phase HPLC using a C-18 column (Partisphere (4.6 mm×250 mm), Whatman, OR). Reaction products were eluted using a linear gradient of 5–100% methanol in 0.086% (v/v) phosphoric acid at a flow rate of 1 ml/min and their identity initially ascertained by comparison of their retention times with reference compounds. Fractions containing eluted compounds were collected and the identification of reaction products was further verified by comparison of their absorbance spectra with those of reference compounds. If not stated otherwise, reported values represent the mean of three independent experiments with an average sample mean deviation of less than 7%.

For ABTS-mediated oxidation of veratryl alcohol by *P. cinnabarinus* laccase (3 μg/ml reaction mixture), final concentrations were 0.8

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Abbreviations: ABTS, 2,2′-azino-bis-(3-ethylthiazoline-6-sulfonate); 3-HAA, 3-hydroxyanthranilate; CA, cinnabarinic acid; DHP, dehydropolymerizate; LiP, lignin peroxidase; MnP, manganese-dependent peroxidase

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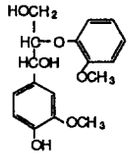
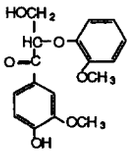
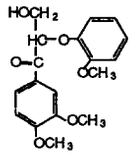
mM for ABTS and 3 mM for veratryl alcohol. Concentrations of 0.2–1.0 mM for ABTS, and 20–250 μM for compound III were tested for ABTS-mediated oxidation of lignin model compound III. For 3-HAA-mediated oxidation of veratryl alcohol, concentrations of 0.2–1.5 mM for 3-HAA, and 0.5–3.0 mM for veratryl alcohol were tested. All reactions were carried out in 1 ml total volume of 50 mM sodium tartrate (pH 4.0). Reaction products were analyzed by HPLC after 24 h and 48 h of incubation at 25°C as described above.

2.4. Depolymerization of ^{14}C-ring-labeled DHP using laccase and 3-HAA

To examine the effect of the laccase/3-HAA couple on polymeric lignin, a synthetic dehydrogenative polymer (DHP) of ^{14}C-ring-labeled coniferyl alcohol was tested using an apparatus designed to keep the synthetic lignin separate from laccase. A dialysis membrane cassette (Slide-A-Lyzer Dialysis Cassette, 10 kDa cutoff; Pierce, Rockford, IL) was clamped between two reaction chambers, and 500 μl of 10% (v/v) *N,N*-dimethylformamide in 50 mM sodium acetate (pH 4.0) containing ^{14}C-ring-labeled DHP (100 kdpm) were injected into the cassette (Fig. 1). Prior to incubation with the laccase/3-HAA couple, low M_r components of the DHP preparation were allowed to diffuse out of the cassette by repeated dialysis against 50 mM sodium acetate (pH 4.0). This washing step resulted in the release of approximately 58% of the DHP through the membrane. After 72 h, when no further release of low M_r material was detected, purified laccase (3 μg/ml) and 0.5 mM 3-HAA in 25 ml of filter-sterilized 50 mM sodium acetate (pH 4.0) were added to each reaction chamber (50 ml total volume per reaction). In control reactions, either laccase, 3-HAA, or both were omitted. All reactions were run over 4 days in duplicate at room temperature. Samples (2 ml) were collected from each reaction chamber at regular intervals for scintillation counting (Tri-Carb 1900 CA liquid scintillation analyzer, Packard Instr. Company, Downers Grove, IL), and the reaction volume was kept constant by replacing the sample volume with 50 mM sodium acetate (pH 4.0). After the reactions were stopped, the integrity of the ultrafiltration membrane was verified by injecting a solution of Blue Dextran into the cassette, and making sure no dye diffused from the cassette. To compare the M_r distribution of the reaction products with that of the starting material from inside the cassette, aliquots were fractionated on a gel permeation column (Sephadex LH 60/LH 20 (1:1 (wt/wt), Pharmacia LKB, Biotechnology Inc., 8×200 mm) in *N,N*-dimethylformamide/0.1 M $LiCl_2$. Fractions (0.5 ml) were collected at a flow rate of ca. 5 ml/h and analyzed by scintillation counting. The column was calibrated using polystyrene standards having M_r values of 9100 and 2700, and veratrylalcohol (M_r=166), respectively.

Table 1
Oxidation of three dimeric lignin model compounds by lignin peroxidase, laccase, and laccase plus redox mediator, 3-hydroxyanthranilate

	I	II	III
			
LiP[a]	+	+	+
Laccase[a]	+	±	−
Laccase +3-HAA[b]	n.d.	n.d.	+[b]

Structures of lignin model compounds: I: guaiacyl glycerol-β-guaiacyl ether (α-alcoholic form); II: guaiacyl glycerol-β-guaiacyl ether (α-carbonyl form); III: veratryl glycerol-β-guaiacyl ether (α-carbonyl form). +, oxidation; ±, slow oxidation; −, no oxidation; n.d., not determined.

[a]Oxidations were monitored photospectroscopically at 30°C in 50 mM sodium tartrate buffer with 3 μg *P. cinnabarinus* laccase (pH 4.0) and 2.5 μg *P. chrysosporium* lignin peroxidase (LiP) (pH 3.5), respectively. For LiP reactions hydrogen peroxide was included at a final concentration of 100 μM. To these reaction mixtures, each model compound was added to a final concentration of 125 μM.

[b]Reaction products were analyzed after 48 h incubation at 25°C by reversed-phase chromatography.

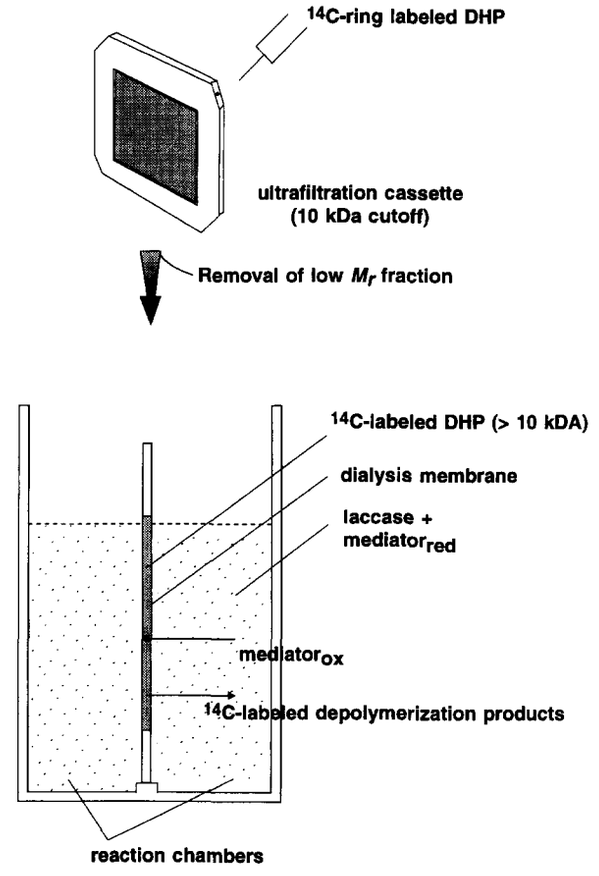


Fig. 1. Picture detailing an apparatus for testing the ability of ligninolytic systems based on redox mediators to fragment high M_r polymeric substrates. Radio-labeled polymer is injected into a commercially available dialysis membrane cassette which is sandwiched between two reaction chambers. Various combinations of enzymes, mediators, substrates or reductants may be added to each chamber, and fragmentation of the polymer can be followed by quantitating the low M_r material passing through the membrane.

3. Results and discussion

Laccases, isolated from different plant and fungal sources have been shown to display significant differences in their redox potentials [21,22]. The possibility that a fungus lacking LiP might produce a laccase having an unusually high redox potential was tested by comparing the oxidative activity of purified *P. cinnabarinus* laccase with that of LiP purified from *P. chrysosporium*. Because of the complexity of the lignin polymer, synthetic substrates representing lignin substructures are commonly used to assess ligninolytic activities [2]. Three such model compounds (Table 1, I–III), representing both phenolic and non-phenolic moieties commonly found in lignin, were used to test the oxidative ability of the *P. cinnabarinus* laccase. The exceptionally high redox potential of LiP was illustrated by its ability to catalyze the oxidation of lignin model compounds II and III (Table 1). In contrast, the oxidation of compound II by laccase was much slower owing to the

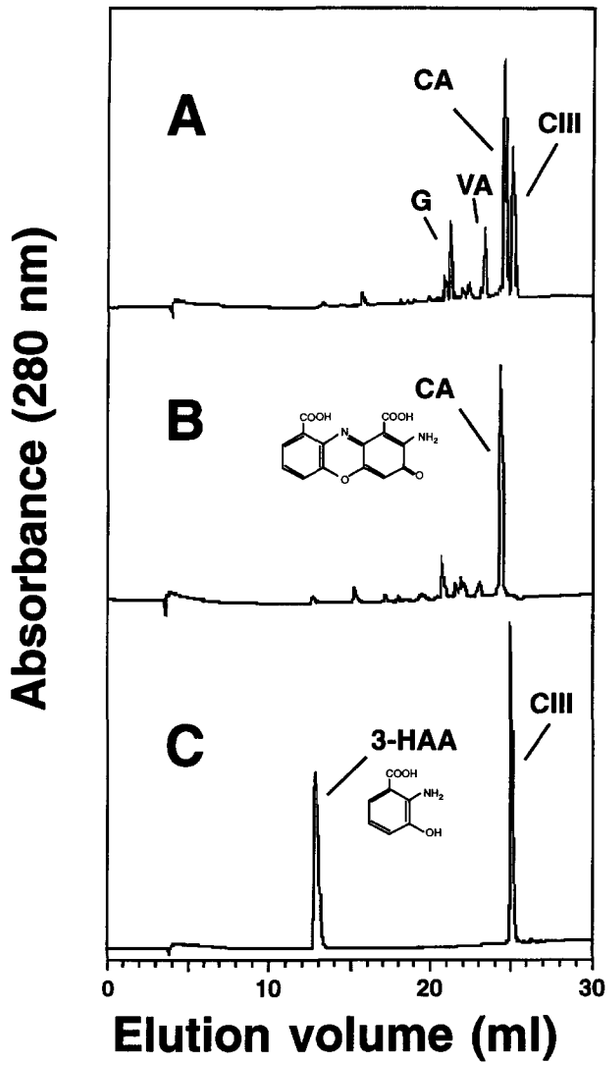


Fig. 2. HPLC analysis of model compound V (CIII) oxidation in the presence of laccase and 3-HAA. Oxidation and cleavage of compound V by laccase plus 3-HAA resulted in two peaks identified as guaiacol (G) and veratric acid (VA) (panel A). Omitting of model compound V resulted in the production of cinnabarinic acid (CA) only from 3-HAA (panel B), while omission of laccase showed that 3-HAA and lignin model compound III were stable for at least 72 h (panel C).

lower redox potential of this enzyme, and compound III, the non-phenolic lignin model dimer, was not oxidized at all. Typical for a fungal laccase, only lignin model compounds I and II, which contained phenolic hydroxyl groups, were oxidized by the *P. cinnabarinus* enzyme. Thus, for *P. cinnabarinus* to completely mineralize lignin using only laccase, an indirect mode of action would be required for attack on the non-phenolic portions of the polymer. Bourbonnais and Paice [23,24] recently demonstrated that the synthetic laccase substrate, ABTS (2,2′-azino-bis[3-ethylthiazoline-6-sulfonate]), could act as a redox-mediator enabling laccase indirectly to oxidize non-phenolic lignin model compounds and partially delignify wood pulp. This suggested that *P. cinnabarinus* might produce its own redox-mediator to facilitate laccase-mediated lignin degradation. The *P. cinnabarinus* basidiocarp is bright orange in color, and the pigments responsible for this coloration are produced when the fungus is cultured on either solid or liquid media. The pigments have previously been identified as the phenoxazinone derivatives, cinnabarinic acid (Fig. 2) and cinnabarin [25]. The phenoxazinone ring structure is also found in the actinomycin class of antibiotics synthesized by the *Streptomycetes* where it is formed through the oxidation of anthranilate derivatives by a blue copper oxidase, phenoxazinone synthase [26,27]. In a similar manner, the *P. cinnabarinus* laccase was shown to catalyze the formation of cinnabarinic acid from 3-hydroxyanthranilate (3-HAA) in vitro (Fig. 2), and 3-HAA was readily detectable in *P. cinnabarinus* cultures [28]. The 6-electron oxidation leading to formation of the phenoxazinone ring from anthranilate precursors has been suggested to follow any one of several different reaction sequences [25,29], all of which proceed through highly reactive intermediates, such as quinone imines. The indirect oxidation of non-phenolic lignin model dimers catalyzed by laccase in the presence of ABTS was suggested to result from the interaction of a highly reactive intermediate with the model compound [23]. Thus, there was potential for a similar reaction to proceed from the 3-HAA oxidation products.

To test this hypothesis, lignin model compound III was incubated with the *P. cinnabarinus* laccase and 3-HAA for 48 h, after which time 58% of model compound III was oxidized. The decrease in compound III coincided with the formation of two peaks, identified as veratric acid (VA) and guaiacol (G), from oxidative cleavage of compound III (Fig. 2A). A major peak corresponding to cinnabarinic acid (CA), as well as several minor peaks, representing oxidation products of 3-HAA, were also resolved by HPLC. These same peaks were also produced when laccase was incubated with 3-HAA in the absence of compound III (Fig. 2B). In the absence of laccase, 3-HAA and the lignin dimer did not react and were stable for at least 72 h (Fig. 2C).

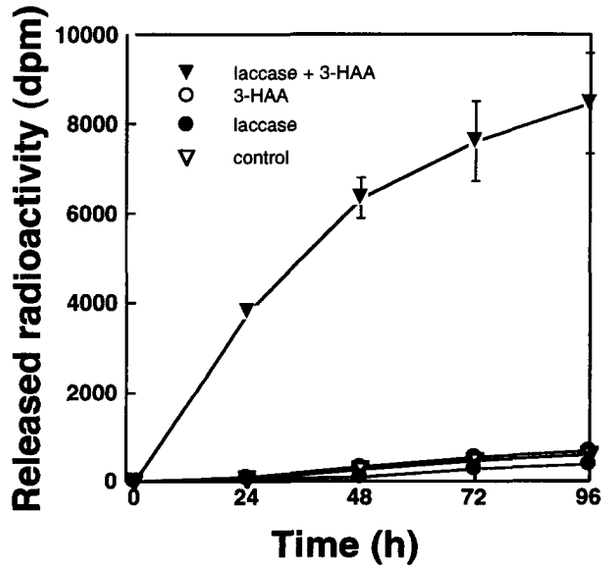


Fig. 3. Release rate for low M_r ^{14}C-labeled fragments produced from guaiacyl DHP through the action of the laccase/3-HAA couple. Released counts passing through a dialysis membrane were quantified at regular intervals. Control reactions omitting laccase, 3-HAA or both showed little or no fragmentation of DHP.

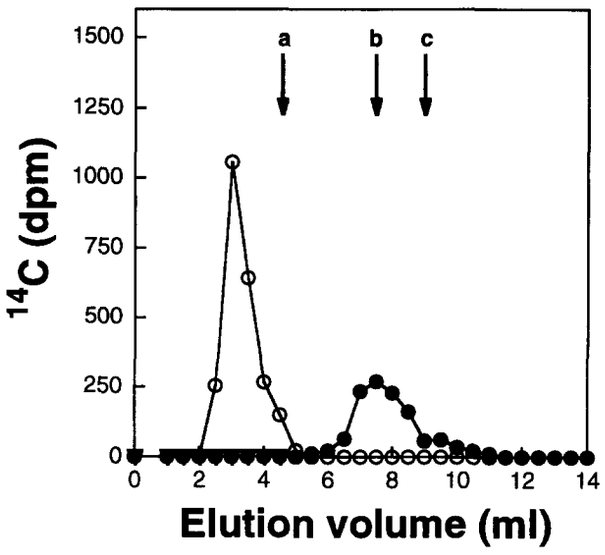


Fig. 4. Gel permeation chromatography of untreated DHPs and fragments released through the action of the laccase/3-HAA couple. The DHP starting material (20 μl, ○) eluted with a mean M_r >9100, while the fragments (150 μl, ●) passing through the dialysis membrane had a mean M_r of ≈3000. Arrows denote the elution points for polystyrene standards having M_r of (a) 9100 and (b) 2700, and (c) vertrylaldehyde (M_r = 166), respectively.

Degradation of compound III in the presence of laccase and varying amounts of 3-HAA was examined to assess whether the ratio of mediator to model compound was important for optimizing the reaction. In fact, an unexpectedly sharp optimal concentration ratio of about 15:1 was determined for this mediator/model compound pair. This was the concentration ratio at which 58% of model compound III was degraded after 48 h (Fig. 2). In contrast, only 5% of compound III was oxidized when the mediator/model compound ratio was increased to 100:1, and less than 5% cleavage was observed for ratios below 2:1. This result suggests that regulation of 3-HAA biosynthesis and secretion could provide a further regulatory option through which the fungus could modulate lignin degradation. Although the optimal pH for oxidation of 3-HAA by *P. cinnabarinus* laccase was 4.0, the pH optimum for mediated oxidation of model compound III was about pH 3.5. A similar result was reported for mediated oxidation of veratryl alcohol (3,4-dimethoxybenzene) by *Trametes versicolor* laccase and ABTS [30]. It is possible that the increased capacity for mediated oxidation at lower pH stems from increased stability of the reactive products of 3-HAA oxidation.

ABTS and 3-HAA displayed clear differences with respect to their efficiency and specificity for oxidizing different substrates. Whereas the 3-HAA/laccase couple could oxidize nearly 60% of model compound III within 48 h under optimal conditions, less than 10% was oxidized using the ABTS/laccase couple. In accordance with previous studies using the *T. versicolor* laccase, veratryl alcohol was not oxidized by the *P. cinnabarinus* laccase alone, but was completely oxidized to veratrylaldehyde within 24 h of ABTS addition. In contrast, only 15% of veratryl alcohol was converted to veratrylaldehyde after 48 h in the presence of 3-HAA and laccase. The mechanism underlying these laccase/mediator reactions is as yet unclear.

The ability of the laccase/3-HAA couple to fragment polymeric material more closely resembling lignin was tested using a synthetic polymer (DHP) prepared from ^{14}C-ring-labeled coniferyl alcohol. In order to test whether polymer fragmentation resulted from interaction with a diffusable redox mediator, rather than through direct oxidation by laccase, a membrane apparatus was used to keep the DHP preparation and laccase separated. Repolymerization reactions are a common feature of in vitro systems for lignin degradation, which have consequently presented a problem for demonstrating ligninolysis of high molecular weight substrates by LiP and other phenoloxidases. By using a low-volume membrane cassette to contain the high molecular weight substrate, fragments can rapidly diffuse away from the polymer, thereby avoiding repolymerization. Thus, high M_r ^{14}C-ring-labeled DHP was placed inside a dialysis membrane cassette having a nominal cutoff of ca. 10 kDa, while *P. cinnabarinus* laccase (M_r ca. 81 000 [17]) was added to a reaction chamber outside the membrane (Fig. 1). The high M_r DHP in the cassette was incubated with a 25 ml reaction volume containing 3 μg/ml purified *P. cinnabarinus* laccase and 500 μM 3-HAA in 50 mM sodium acetate (pH 4.0) in the outer reaction chamber (50 ml total volume per reaction). Radioactive material diffused into the outer reaction chambers when both laccase and 3-HAA were present in the reaction mixture; after 96 h approximately 19% of the high M_r material was recovered as low M_r, soluble compounds when both laccase and 3-HAA were present (Fig. 3). Gel permeation chromatography of the reaction products resulted in a broad peak of material ranging from M_r ≈4000 to monomeric phenolic compounds (Fig. 4). In control incubations lacking enzyme, 3-HAA, or both, no significant release of low M_r radio-labeled material was detected, and gel permeation chromatography of the material retained within the cassette showed that the DHP had a M_r of >9100 (Fig. 4). This experiment clearly showed that treatment with the laccase/3-HAA couple led to fragmentation of the DHP. Although the exact reactive species that mediate laccase-catalyzed oxidation of lignin remain unidentified, it is clear that diffusable and reactive low molecular weight compounds are responsible for the attack on the lignin polymer.

The products resulting from 3-HAA oxidation show striking similarities to the synthetic mediators recently investigated for pulp bleaching. Unlike the fungal mediator, 3-HAA, the synthetic mediators are heterocyclic compounds belonging to the general classes of phenoxazinones, phenothiazines [31] or phenoxybenzothiazoles [32]. However, not all phenoxazinones can act as redox mediators for lignin depolymerization, since cinnabarinic acid could not serve this function. Thus, we anticipate that the study of naturally occurring mediators will help to uncover compounds that can serve more efficiently in biotechnological processes.

Because the enzymes involved in lignin oxidation are too large to penetrate the unaltered wood cell wall [33], the use of low molecular weight, diffusable compounds to oxidize the polymer makes sense. For this reason, LiP has been proposed to use the veratryl cation radical as a mediator [34,35], while MnP has been suggested to produce Mn(III)-acid complexes for similar reasons [36,37]. The identification of 3-HAA as a naturally occurring redox mediator for laccase is the first evi-

dence to support this ligninolytic system as having equivalent potential to ligninolytic systems based on LiP or MnP.

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