Oxidation of a Tetrameric Non-phenolic Lignin Model Compound by Lignin Peroxidase†

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The present study maps the active site of lignin peroxidase in respect to substrate size using either fungal or recombinant wild type as well as mutated recombinant lignin peroxidases. A nonphenolic tetrameric lignin model was synthesized which contains \( \beta \)-O-4 linkages. The fungal and recombinant wild type lignin peroxidase both oxidized the tetrameric model forming four products. The four products were identified by mass spectral analyses and compared to synthetic standards. They were identified as tetrameric, trimeric, dimeric and monomeric carbonyl compounds. All four of these products were also formed from single turnover experiments. This indicates that lignin peroxidase is able to attack any of the \( C_\alpha-C_\beta \) linkages in the tetrameric compound and that the substrate-binding site is well exposed. Mutation of the recombinant lignin peroxidase (isozyme H8) in the heme access channel, which is relatively restricted and was previously proposed to be the veratryl alcohol binding site (E146S), had little effect on the oxidation of the tetramer. In contrast, mutation of a Trp residue (W171S) in the alternate proposed substrate-binding site completely inhibited the oxidation of the tetrameric model. These results are consistent with lignin peroxidase having an exposed active site capable of directly interacting with the lignin polymer without the advent of low molecular weight mediators.
INTRODUCTION

White-rot fungi degrade the aromatic polymer lignin with extracellular oxidative enzymes. These enzymes include peroxidases and/or laccases (1,2). The role of each enzyme in this complicated process is an active area of research and debate. The most studied white rot fungus, *Phanerochaete chrysosporium* produces two types of peroxidases, Mn peroxidase (MnP)$^1$, and lignin peroxidase (LP) (3,4) but no detectable laccase. The heme-containing peroxidases undergo the classical catalytic cycle, where H$_2$O$_2$ first oxidizes the native (ferric) enzyme, forming compound I (5). Two reducing substrates then donate two electrons, one each, to complete the catalytic cycle via the formation of a compound II intermediate (5).

MnP and LP are similar in structure (6-8) but distinguished and unique in the nature of their reducing substrates. MnP can oxidize phenolic substrates but compound II exhibits specificity for complexed Mn$^{2+}$, oxidizing it to Mn$^{3+}$ (9). Mn$^{3+}$, in turn, is able to oxidize a wide range of phenolic substrates including phenolic lignin (9). In contrast, LP oxidizes a variety of phenolic and nonphenolic aromatic compounds (10,11). How these two enzymes interact with their ultimate substrate, lignin, has been intensely investigated. Several studies have shown that the pore size of the plant cell wall does not allow for direct contact between the peroxidases and lignin (12-14). For MnP, trivalent Mn is proposed to deliver the oxidizing equivalents of the heme active site to the aromatic substrate (9). For LP, the secondary metabolite, veratryl (3,4-dimethoxybenzyl) alcohol has been proposed to play a similar role (15). This alcohol has been shown to facilitate the oxidation of many compounds in vitro (16-18). This observation led these investigators to speculate that the veratryl alcohol cation radical is the low molecular weight mediator in the case of LP. However, the life span of veratryl alcohol cation radical is too short to permit diffusion to distal sites (19). Therefore, LP may be involved in the oxidation of the soluble, partially degraded lignin fragments. However, the possibility that LP can directly oxidize lignin in the partially-degraded cell wall cannot be excluded.

The present study maps the site of electron transfer for LP in respect to substrate size. Two likely substrate-binding sites have been proposed. One is the so-called heme access channel (8), which allows for direct interaction between the substrate and the heme. Residues Ile85, Val184, Gln222, Phe148, His82, Glu146 and Asp183 are located in this channel. This channel is sterically restricted and would not allow access to large bulky (lignin) substrates. The other site, Trp171 is
located at the enzyme surface and would only allow for long-range electron transfer (20). We have
designed a tetrameric nonphenolic lignin model compound, which does not fit into the heme access
channel (Fig. 1) and determined the initial products of its oxidation. The product profile indicates
that the site of electron transfer of LP is very exposed. Site-directed mutagenesis studies where
Glu146 and Trp171 were altered, indicate that the site of electron transfer is Trp171.

MATERIALS AND METHODS

**Chemicals.** Ammonium cerium(IV) nitrate and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone
(DHQ) were purchased from Aldrich. The oligonucleotides for site directed mutagenesis were
synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). \( \text{H}_2\text{O}_2 \) was purchased from
VWR and prepared fresh daily; the concentration was determined spectrophotometrically at 240 nm
using an extinction coefficient of 39.4 M\(^{-1}\) cm\(^{-1}\) (21). The tetrameric lignin model and its carbonyl
degradation products (Fig. 2) were synthesized and purified as described by (22). The tetrameric
compound is a mixture of two diastereomeric compounds (ratio 1:1) because of the presence of R
and S configurations in the terminal \( \alpha \)-position. All other chemicals were commercially available
and used without further purification.

**Enzymatic and chemical oxidation of the tetramer.** Incubations of the tetramer (50 \( \mu \)M)
with fungal LP isozyme H2 and wild type or mutated recombinant LP H8 (1 \( \mu \)M) were performed
in 50 mM sodium tartrate buffer (pH 3.5). The reaction was initiated by the addition of \( \text{H}_2\text{O}_2 \) to
yield a final concentration of 0.2 mM. Products were also analyzed over a longer time period. In
these experiments, the reaction mixture volume was 15 ml and initially contained 110 \( \mu \)M tetramer
and 1 \( \mu \)M LP isozyme H2 in 50 mM sodium tartrate buffer (pH 3.5). The reaction was initiated
with the addition of 10 \( \mu \)l \( \text{H}_2\text{O}_2 \) yielding a final concentration of 0.05 mM. After initiation of the
reaction, an additional aliquot of enzyme (0.5 nmol) and \( \text{H}_2\text{O}_2 \) (150 nmol) were added in every two
min. to yield a final concentration of 1.5 \( \mu \)M and 0.14 mM, respectively. Aliquots (1 ml) were
removed at different time points from the reaction mixture and added to 2 ml of acetonitrile and
analyzed by HPLC. When the concentrations of the products were determined, results were
corrected by the dilution factors of the reaction mixture due to the continuous addition of enzyme
and \( \text{H}_2\text{O}_2 \).
Oxidation of the tetramer (100 µM) by 10 µM DDQ was performed in 1 ml dioxane containing one drop of methanol as described by Fenn and Kirk (23). Oxidation of 100 µM tetramer by Ce\(^{4+}\) (15 µM) was carried out in 1 ml distilled water which was acidified by a few drops of concentrated sulfuric acid.

**Identification of the oxidation products of the tetramer.** As described above, reaction mixtures were sampled and the reaction was quenched by the addition of one volume of acetonitrile. This aliquot was injected onto a reverse phase Discovery C18 column (15 cm x 4.6 mm) with 5 µm of pore size (Supelco, Bellefonte, PA). Samples were eluted at 0.8 ml/min with a 0-95 % step gradient of acetonitrile in water over 40 min. Products were monitored at 280 nm and identified by comparison to retention times of authentic standards and by mass spectrometry.

Liquid chromatography-mass spectrometry (LC/MS) was performed using a Model 1100 series HPLC (Hewlett Packard, Palo Alto, CA) coupled to a Mariner orthogonal acceleration/time-of-flight mass spectrometer (Perseptive Biosystems, Framingham, MA) equipped with an atmospheric pressure chemical ionization (APCI) source. The HPLC system was equipped with a column with stationary phase identical to that described above. Mass spectra were acquired in positive ion mode using a nebulizer temperature of 350 C.

**Mutagenesis, heterologuous expression and enzyme purification.** The mutagenesis (E146S) of the pET21aH8(+) was described previously by Ambert-Balay et al. (24). Mutagenesis to obtain W171S was performed by a QuickChange Site-Directed Mutagenesis Kit and protocol provided by Stratagene (LaJolla, Ca). The following oligonucleotides were used where the mutated sequences are underlined; 5’-CTCGAGCTTGTCTCGATGCTCTCC-3’,
5’-GGAGAGCATCGAGACAAGCTCGAG-3’

The mutations were confirmed by DNA sequencing. The plasmid encoding the mutated LP was transformed into *E. coli* strain BL21(DE3)pLysS. The recombinant wild type and mutant peroxidases were refolded and purified as previously described (24).

Wild type fungal LP isozymes H2 and H8 were purified as previously described (25). The concentrations of the peroxidases were determined spectrophotometrically at 409 nm using an extinction coefficient of 169 mM\(^{-1}\) cm\(^{-1}\) and 168 mM\(^{-1}\) cm\(^{-1}\) for LP isozymes H2 and H8, respectively (26). The purified preparations exhibited an RZ ratio (A409/A280) of least 3.
Identification of the tetramer oxidation products from single turnover experiments. The stopped flow apparatus was purchased from KinTek Instruments (State College, PA). All reactions were run at 28°C. To generate compound I, 30 µM LP isozyme H2 in 150 mM sodium tartrate pH (3.5) in one syringe was mixed with 28 µM H2O2 in the second syringe. The reaction mix was aged for 1.5 s. This freshly-prepared compound I was then mixed with the tetrameric model compound which was in the third syringe at 300 µM. The reaction mixture (2 ml) was complete within 1 minute as monitored by changes in Soret absorbance. The sample was then collected and analyzed by HPLC immediately.

RESULTS

Identification of oxidation products. Incubation of the tetramer with fungal LP isozyme H2 and H2O2 resulted in cleavage of the tetramer without the addition of the putative mediator, veratryl alcohol. Previous work (10) had shown that the predominant products from LP-catalyzed oxidation of lignin models are that of Cα-Cβ cleavage. The predicted products from such cleavage are shown in Fig. 2. Incubation under multiple turnover conditions resulted in formation of four products as detected by HPLC (Fig. 3A), which were identified by LC/MS. The mass spectra and the corresponding retention times of the oxidation products were compared to those of the authentic standards (Table 1). The proposed products from Cα-Cβ cleavage are also intermediates in the synthesis of the tetramer (22) and consequently were available as known standards. As shown in Table 1, three of the four products were identical to the standards and were identified as the trimeric, dimeric and monomeric aldehydes. The fourth predicted product, labeled 2 in Fig. 3 did not readily ionize and thus was not easily identified by LS/MS. Previous work had also shown that oxidation of Cα alcohols to ketones was also a predominant reaction of LP catalyzed reactions (10). We thus suspected that unknown 2 is a products where one of the Cα alcohols was oxidized to the corresponding carbonyls. To test this possibility, we incubated the tetramer (100 µM) with a chemical oxidant specific for oxidizing Cα alcohols to the ketone/aldehyde, DDQ (23). The oxidation was carried out with tenfold less DDQ relative to the tetramer to ensure that only one product was formed. Analysis by HPLC (Fig. 3B) indicated that the product generated by DDQ had an identical retention time as that of the unknown degradation product. This is consistent with unknown 2 as one of the four possible tetrameric carbonyl compounds.
Time course of oxidation. To determine the product/precursor relationship of the four products, the time course of product formation from the tetramer was determined. Our initial data (not shown) indicated that extended incubations where \( \text{H}_2\text{O}_2 \) was in excess of the tetramer did not result in complete oxidation of the tetramer. This is consistent with our previous results showing enzyme inactivation with poor substrates of LP (10). Thus, to extend the time course of tetramer oxidation, the enzyme and \( \text{H}_2\text{O}_2 \) were added to the reaction multiple times in small aliquots every two min. (See Materials and Methods). During the 30 min. incubation, all four products were detected at every time point (Fig. 4A). Because subsequent experiments were performed with recombinant enzyme produced by the cDNA encoding isozyme H8, similar time course experiments were performed with fungal isozyme H8. The same results were obtained (Fig. 4B).

The results also suggest that the oxidation products are substrates for further LP catalysis. We thus determined whether the trimer, dimer and monomeric (aldehyde) models were substrates for LP. These models were incubated under the same conditions as used in the experiment of tetramer oxidations. The products were again identified and quantified by HPLC (Table 2). The trimeric and dimeric aldehydes were further oxidized, while the monomer was not a substrate for isozyme LPH2. The oxidation of the trimeric compound resulted in formation of both dimeric and monomeric products, whereas the dimer yielded only the monomeric aldehyde.

Effect of veratryl alcohol. Several previous studies reported the stimulatory effect of veratryl alcohol on the oxidation of a variety of chemicals with ranging from aromatic monomers to polymeric lignin. Therefore, we also tested the effect of veratryl alcohol on the oxidation of this tetrameric lignin model. Fig. 5A shows the decrease of tetramer in the absence and presence of veratryl alcohol over a four minute time period. The addition of veratryl alcohol enhanced the rate of tetramer degradation. Approximately 70% of the tetramer was degraded in the presence of veratryl alcohol during the first 30 seconds, whereas only 50% of the tetramer disappeared without veratryl alcohol in the same interval. The product profile was similar with or without added veratryl alcohol. The fate of veratryl alcohol was also followed by HPLC during this experiment. All of the veratryl alcohol was oxidized to veratraldehyde within 2 min. (Fig. 5B).

Products formed from single turnover. The time course experiment shown in Fig. 4 suggested that LP equally oxidized all four possible cleavage sites. If LP acts only as an "exo" lignin-degrading enzyme, then the time course would first show the formation of the trimer,
concomitant with formation of the monomer. This would then be followed by formation of dimers. To further investigate whether LP could act as an "endo" enzyme yielding also dimers as the initial product, single turnover experiments were performed. Single turnover conditions were attained in a three-syringe stopped flow apparatus as described previously (27). Slightly less than one equivalent of H$_2$O$_2$ was pre-incubated with LP isozyme H2; this ensured only single turnover. The reaction mixture then aged for 1.5 second to permit complete formation of compound I. The second push of the stop flow then mixed the freshly prepared compound I with the tetramer. This mixture was collected and immediately analyzed by HPLC. As observed under steady state conditions, all four oxidation products were detected (Table 3). The products were roughly similar in concentration.

*Oxidation by Ce$^{4+}$.* Detection of all four possible oxidation products in single turnover experiments would indicate that no specificity existed with LP-catalyzed reactions. To corroborate these results, we also determined the product formed from oxidation of the tetramer with a chemical oxidant. As shown in Table 3, oxidation of the tetramer with sub-stoichiometric amount of Ce$^{4+}$, a single electron oxidant, resulted in the formation of all four possible products.

*Characterization of mutants of LP.* Two substrate binding sites have been suggested for LP, the so-called heme access channel and a more surface exposed site with Trp 171 (8,28). We investigated the nature of the substrate-binding site using the tetrameric model as the substrate. Mutant E146S (heme access channel) and W171S (surface residue) were generated in the recombinant mutant LP (isozyme H8). Their activity with the tetramer was then examined and compared to wild type LP. The product profile from oxidation of the tetramer by the fungal H8 and the recombinant H8 (rH8) was similar to that of fungal isozyme H2. Mutant E146S, located at the heme-access channel was also able to oxidize the tetramer. The product profile was similar to the wild type enzyme (Table 4). In contrast, oxidation of the tetramer was completely inhibited by the mutation of W171S (Table 4). However, as reported by Doyle et al. (28), the oxidation of ABTS was not inhibited by this mutation (data not shown).

**DISCUSSION**

Results from the present study help resolves the issue of whether LP can degrade polymeric lignin without redox mediators and has also identified the site of electron transfer for polymeric
substrates. These results have relevance on the role of LP in lignin degradation, a question still debated after the discovery of LP 17 years ago. This debate is due, in part, to the finding that many lignin-degrading fungi apparently do not contain LP (29) and to the relatively slow rate of this enzyme with the substrate lignin (30). Because LP is proficient in oxidizing low molecular weight models of lignin, some have proposed that the role of LP in lignin degradation is solely for the oxidation of smaller lignin fragments (30), generated by MnP catalysis. The ability of LP to bind to its putative substrate, lignin, has also been questioned. Research from a number of laboratories has shown that the pores of the wood matrix are not large enough to allow access to enzyme (12-14). However, similar to how MnP can degrade lignin through utilizing Mn$^{2+}$/Mn$^{3+}$ as a mediator, Harvey et al. (15) suggesting veratryl alcohol serves as a redox mediator for LP. In this mechanism, the oxidation to the cation radical followed by diffusion into the wood matrix delivers the oxidizing power of the heme active site to the distal substrate lignin. Consistent with this hypothesis, in vitro studies by Hammel et al. (31) showed maximal depolymerization of lignin by LP occurred only in the presence of veratryl alcohol. This hypothesis, however, has been questioned by kinetic studies (18) and by chemical studies showing the chemical instability of the veratryl alcohol cation radical (19).

Relevant to the question of the role of LP in lignin degradation is whether LP can directly interact with the lignin polymer. To date, crystallographic and spectroscopic data have yielded no information on the substrate-binding site. Based on mainly theoretical studies at least three substrate-binding sites have been proposed. Poulos et al. (8) suggested a binding site for veratryl alcohol an access channel analogous to that found in CcP. This channel provides a hydrophobic environment near to the heme. This channel allows for direct interaction between the substrate and the heme. Modeling of substrates into this channel indicates that only monomeric substrates would bind or that larger linear polymers could bind through an end-on manner. The ability of LP to directly interact with lignin would not exclude this site since the polymer could be envisioned to bind through and "exo" mechanism. In addition to the heme access channel, two long-range electron transfer routes have been proposed. Schoemaker and Piontek (32) proposed residues 82-84 (His, Pro, Asn) of isozyme H8, whereas his 82 was proposed also as the part of the heme access channel (8). The other site was proposed by Blodig et al. (20) involving residues Trp171 and Leu172. Both of these long-range sites connect the heme to surface residues thus allowing for
direct contact with lignin. Here, LP could be envisioned to interact in an "endo" manner with lignin.

To gain insight into the nature of the binding site, we have synthesized a larger (tetrameric) model of lignin to map the size of the LP site of electron transfer. We utilized a tetrameric substrate, which is actually larger than a tetramer since a benzyl group rather than a methyl group is attached to the terminal phenolic oxygen. The tetramer was designed such that if the site of electron transfer is the heme access channel, it could only gain access from an “end-on” manner. This is clearly illustrated in Fig. 1 where the size of the tetramer is compared to that of LP and its heme access channel. Our studies clearly indicate, through time course studies and through single turnover studies, that LP is able to oxidize the tetramer to yield the dimer as the first product. In all incubations with the tetramer, the monomeric, dimeric, trimeric, tetrameric aldehydes were detected. Of significance here is the finding that dimeric products are formed from single turnover studies. This clearly maps the site of electron transfer to be relatively unhindered and that LP can oxidize the lignin polymer directly. In accord with this interpretation is our results from the chemical oxidation system. The single-electron oxidant, Ce\(^{4+}\), yielded the same profile of products. Thus, LP can act as an "endo" lignin-degrading enzyme. This is consistent with LP being able to interact with lignin directly and catalyze \(C_\alpha-C_\beta\) cleavage anywhere along the polymer.

Indirect evidence for interaction between LP and lignin (synthetic polymer DHP) was recently obtained with a resonant mirror biosensor Johjima et al. (33). The interaction between the enzyme and DHP was only specific for LP; none was observed with laccase, MnP or horseradish peroxidase. In addition to this spectroscopic evidence, these workers also obtained rate constants between LP compound I and the DHP. However, this study did not reveal the nature of the products or reveal the location of the substrate-binding site. Terminal phenolic groups may have served as the reducing agent for both compounds I and II in this study.

Despite the finding that LP can directly interact with a large polymeric lignin substrate, we also investigated whether veratryl alcohol could enhance the oxidation. As found in the oxidation of many other substrates for LP, veratryl alcohol stimulated the oxidation of the tetrameric model. The stimulation was measured to be approximately 20%, a result, which neither supported nor refuted the mediation hypothesis. Past work has shown that facilitation of oxidation of secondary
substrates can be explained by several mechanisms. Although, our experiments were not designed to address the mediation issue, they are more consistent with veratryl alcohol not acting as a mediator. In incubations where veratryl alcohol acts as a mediator, a lag phase in its oxidation has been observed (18). As shown in Fig. 5, a lag phase of veratraldehyde formation was not observed. On the contrary, both the tetramer and veratryl alcohol were oxidized simultaneously. Thus, it most probably acted to protect LP from inactivation. In the case of poor LP substrates, veratryl alcohol has been demonstrated to promote the completion of the enzymatic catalytic cycle, decreasing the inactivation of LP, and consequently increase the oxidation rate of the other substrate. The mechanism of stimulation was not further investigated. Even if some mediation does occur with veratryl alcohol, these results do not refute our finding that LP is able to directly oxidize large lignin molecules.

Our studies have also identified the location of the electron transfer for polymeric substrates. We have made mutations among the amino acids in the heme excess channel (24). Mutation of E146 at this site was further characterized in the present study. This amino acid residue was proposed to participate in veratryl alcohol binding and involved in pH dependency (8). The enzyme mutated at the heme access channel was able to oxidize the tetrameric lignin model resulting in the same products as those by the wild type. Recently W171 of the surface exposed potential binding site was mutated (28). The involvement of this amino acid in the oxidation of veratryl alcohol was proposed. The W171S mutant, in contrast, was not able to oxidize the tetramer, no products were found. These findings illustrate the significance of this site for electron transfer with veratryl alcohol and the polymeric lignin. The involvement of W171 in veratryl alcohol oxidation is supported by other studies (34,35). The significance of W171 is also suggested from the results on bi-functional MnPs. Certain MnPs from Bjerkandera spp. and Pleurotus oxidize veratryl alcohol (36,37). These enzymes also contain a Trp at the homologous site (37). Moreover, when MnP H4 was mutated to contain a Trp (168W) (34,35), it was able to oxidize veratryl alcohol.

In conclusion, our results support the hypothesis that LP can directly oxidize lignin. Therefore, LP may have important role in the degradation of phenolic and nonphenolic lignin. Site-directed mutagenesis studies suggest that the substrate-binding site is surface exposed and that this site may accommodate both lignin and veratryl alcohol. However, other compounds such as ABTS and DFAD may bind at an alternate site. The presence of more than one binding site is
supported by the fact that the mutation of W171 in LP did not affect the oxidation of certain dyes such as ABTS and DFAD (28).

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1Abbreviations used: MnP: Mn peroxidase; LP: lignin peroxidase; DDQ: 2,3-dichloro-5,6-dicyano-1,4-benzoquinone; DHP: dehydropolymer; ABTS: 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonate); DFAD: 4-[(3,5-difluoro-4-hydroxyphenyl)azo]benzene-sulfonic acid.
Table 1. Mass spectra and HPLC retention times of the various lignin model compounds.\(^a\)

| Compound                | Retention time (minute) | Mass spectrum [m/z] (relative intensity (%))                                                                 |
|-------------------------|-------------------------|-------------------------------------------------------------------------------------------------------------|
| Tetrameric model        | 16.5                    | 899(8, [M+Na]\(^+\)), 329(32), 243(38), 181(55), 163(100)                                                 |
| Trimeric aldehyde       | 22.4                    | 657(22, [M+Na]\(^+\)), 599(93), 509(23), 331(68), 153(100)                                               |
| Dimeric aldehyde        | 25.1                    | 439(5,[M+H]\(^+\)), 421(100), 331(14), 269(31), 153(23)                                                 |
| Monomeric aldehyde      | 35.7                    | 243(100, [M+H]\(^+\)), 153(3), 112(4)                                                                        |

\(^a\)See Materials and Methods for HPLC conditions.

Table 2. Oxidation of the trimeric, dimeric, and monomeric carbonyls by LP Isozyme H2.\(^a\)

| Starting compound (50 µM) | Products Identified (µM) | Tetrameric model | Tetrameric carbonyl | Trimeric aldehyde | Dimeric aldehyde | Monomeric aldehyde |
|---------------------------|--------------------------|------------------|---------------------|-------------------|------------------|--------------------|
| Tetrameric model          | 12.5                     | 9.6              | 10.5                | 13.6              | 12.9             |
| Trimeric aldehyde         | na\(^b\)                | na               | 22.6                | 9.8               | 18.4             |
| Dimeric aldehyde          | na                       | na               | na                  | 34.9              | 16.1             |
| Monomeric aldehyde\(^c\)  | na                       | na               | na                  | na                | 44.0             |

\(^a\)LP (1 µM) was incubated with 50 µM tetramer in 50 mM sodium tartrate buffer, pH 3.5. Reactions were initiated by addition of H\(_2\)O\(_2\) (0.2 mM final).

\(^b\)Not applicable.

\(^c\)No oxidation products were found.
Table 3. Comparison of the identified products of the tetrameric model compound during a single turnover of LP isozyme H2 and with Ce⁴⁺.  

| Oxidant  | Products (µM) | Tetrameric carbonyl | Trimeric aldehyde | Dimeric aldehyde | Monomeric aldehyde |
|----------|---------------|---------------------|-------------------|------------------|--------------------|
| Isozyme H2 | 4.1 6.2 9.9 8.2 |                     |                   |                  |                    |
| Ce⁴⁺     | 8.8³ | 1.7 0.7 2.4 |                     |                  |                    |

³Single turnover experiments were performed in the stopped flow as described in Materials and Methods. LP (30 µM) was first incubated with 28 µM H₂O₂ resulting in the formation of compound I. This was then mixed with the tetramer (final concentration of 100 µM).

³In incubations containing Ce⁴⁺, the tetramer (100 µM) was incubated with 15 µM Ce⁴⁺ in distilled water acidified by sulfuric acid as described in Materials and Methods.

Table 4. Oxidation of the tetrameric lignin model compound by the wild type and mutated LP isozyme H8.  

| Mutation  | Tetrameric model remained | Tetrameric carbonyl | Trimeric aldehyde | Dimeric aldehyde | Monomeric aldehyde |
|-----------|---------------------------|---------------------|-------------------|------------------|--------------------|
| Fungal H8 | 23.8 12.3 9.5 5.1 3.4 |                     |                   |                  |                    |
| rH8       | 20.3 11.6 9.5 7.2 3.9 |                     |                   |                  |                    |
| rH8 E146S | 31.2 7.6 7.8 4.5 3.6 |                     |                   |                  |                    |
| rH8 W171S | 47.1 0.0 0.0 0.0 0.0 |                     |                   |                  |                    |

³Incubation conditions were the same as those described in Table 2.

³The initial concentration of the tetramer was 50 µM.
FIGURE LEGENDS

Fig. 1. Structure of LP Isozyme H8 and the tetramer model. The figure shows the van der Waal surface of LP (blue) and the tetramer (yellow). The left structure shows Glu164 highlighted in green near the heme access channel with the heme shown in red. The right structure shows Trp171 highlighted in green along with the tetrameric model. Trp171 and Glu164 are on opposite sides of the protein. The figure was constructed using WebLab ViewerPro and coordinates of Poulos et al. (8).

Fig. 2. Structure of the tetrameric lignin model compound and its degradation products. The arrows drawn in the structure show location of cleavage. Arrows leading away from these smaller arrows point to products formed from cleavage at that site.

Fig. 3. The HPLC profile of the tetrameric lignin model compound degradation by LP isozyme H2 (A) or with the Cα oxidizer DDQ (B). Peaks labeled in figure correspond to: Peak 1: tetrameric model compound; 2: tetrameric carbonyl; 3: trimeric aldehyde; 4 dimeric aldehyde; and 5: monomeric aldehyde compound. See Materials and Methods for HPLC conditions.

Fig. 4. Time course of the oxidation of the tetrameric lignin model compound by LP isozyme H2 (Panel A) and isozyme H8 (Panel B). Symbols: (■), tetrameric model compound; (▲), tetrameric carbonyl; (●), trimeric aldehyde; (△), dimeric aldehyde; (O), monomeric aldehyde. Products were identified by LC/MS and quantitated by HPLC using standard curves generated from synthetic standards.

Fig. 5. Effect of veratryl alcohol on the oxidation of the tetrameric lignin model. Panel A: Decrease of the tetrameric lignin model in the absence (●) and presence (O) of 10 µM veratryl alcohol. Panel B: decrease in veratryl alcohol (●) and increase in veratraldehyde (O).
Fig. 1. Mester et al.
Fig. 2. Mester et al.
Fig. 3. Mester et al.
Fig. 4. Mester et al.
Fig. 5. Mester et al.
Oxidation of a tetrameric Non-phenolic lignin model compound by lignin peroxidase
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