The role of fungal lignin-degrading enzymes in xenobiotic degradation

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Recent studies have reported the first pathways for the degradation of important xenobiotics by lignin-degrading fungi, and have elucidated the role that lignin peroxidases and manganese-dependent peroxidases play both in these pathways and in the decolorization of pulp bleach plant effluents.

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Introduction

Lignin peroxidases (LiP), manganese-dependent peroxidases (MnP) and laccases have been investigated intensively to elucidate their roles as the primary catalytic agents involved in the oxidative depolymerization of lignin by lignin-degrading fungi (LDF). The extracellular lignin-degrading systems of a variety of LDF have been shown to be composed of combinations of multiple isozymes of laccase and one or both of the peroxidases [1]. A notable exception is the most studied LDF, Phanerochaete chrysosporium, which produces LiP and MnP, but not laccase. The role of peroxidases and laccases in lignin depolymerization was unclear until recently because attempts to demonstrate in vitro depolymerization of lignin by crude enzyme mixtures or by purified preparations of peroxidases and/or laccases gave conflicting results. Depolymerization of high molecular weight lignin was usually accompanied by repolymerization of the resulting low molecular weight fractions. However, recent work has now established a role for these enzymes in lignin depolymerization by demonstrating the in vitro depolymerization of lignin by crude enzyme mixtures or by purified preparations of peroxidases and/or laccases giving conflicting results. Depolymerization of high molecular weight lignin was usually accompanied by repolymerization of the resulting low molecular weight fractions. However, recent work has now established a role for these enzymes in lignin depolymerization by demonstrating the in vitro depolymerization of synthetic lignins by crude LiPs [2•] and a purified MnP [3•], both from Phanerochaete chrysosporium, and of Hevea lignins by a mixture of MnPs and laccases from Rigidoporus lignosus [4•].

LDF are also able to degrade a variety of important aromatic and chlorinated aromatic xenobiotic compounds, and to decolorize darkly-colored pulp bleach plant effluents (BPE), which contain lignin-derived chromophores. Because xenobiotic mineralization and decolorization of BPE takes place under ligninolytic conditions, and because fungal peroxidases and laccases have been shown to catalyze the oxidation of a variety of xenobiotics, these enzymes may also play important roles in xenobiotic mineralization and BPE decolorization by LDF. This review will describe recent reports that have verified and extended our understanding of the role of fungal peroxidases and laccases in the degradation of xenobiotic compounds and in the decolorization of BPEs.

Xenobiotic degradation

The results of numerous investigations have demonstrated the ability of lignin-degrading fungi to mineralize a number of important xenobiotic compounds under ligninolytic conditions. Until recently, however, the involvement of peroxidases and laccases in degradation pathways and the identification of intermediates had only been addressed in a few cases. Recent investigations give evidence of the direct involvement of P. chrysosporium LiP in the metabolism of anthracene [5••], and of both Li and MnP from P. chrysosporium in the metabolism of 2,4-dichlorophenol (DCP) [6••], and 2,4-dinitrotoluene (DNT) [7••]. Anthracene was oxidized to 9,10-anthraquinone (AQ) in vitro by purified LiP and mineralized in vivo by ligninolytic cultures of P. chrysosporium [5••]. Direct involvement of LiP in anthracene metabolism was demonstrated in isotope dilution experiments by identification of the ring fission product phthalic acid, from both anthracene and AQ in fungal cultures [5••]. These results are consistent with the pathway anthracene→AQ→phthalate. Phthalic acid was also identified as a metabolite in the LiP catalyzed oxidation of 2-hydroxy-1,4-naphthoquinone (HNQ) in the presence of veratryl alcohol (VA) [8•]. VA is thought to act as a cofactor for LiP, functioning as an oxidative mediator or electron transfer agent. These results suggest that LiP and VA may also be involved in the in vivo oxidation of AQ and quinonoid metabolites of xenobiotic compounds other than anthracene.

Abbreviations

AA–anisyl alcohol; AQ–9,10-anthraquinone; BPE–bleach plant effluent; DCP–2,4-dichlorophenol; DNT–2,4-dinitrotoluene; HNQ–2-hydroxy-1,4-naphthoquinone; LDF–lignin-degrading fungus; LiP–lignin peroxidase; MnP–manganese-dependent peroxidase; VA–veratryl alcohol.
Both LiP and MnP from *P. chrysosporium* have been shown to catalyze the in vitro oxidation of DCP and several of its metabolites found in fungal cultures [6••]. Valli et al. have proposed a pathway for the degradation of DCP that includes several oxidations that are thought to be catalyzed by LiP and/or MnP (Fig. 1) [6••]. LiP and MnP from *P. chrysosporium* also catalyze the in vitro oxidation of several DNT metabolites found in ligninolytic cultures of the fungus [7••] and the proposed pathway for the degradation of DNT by *P. chrysosporium* includes several oxidations thought to be catalyzed by LiP and/or MnP [7••] (Fig. 2). The pathway is similar to that proposed for the degradation of DCP in that it involves several oxidations (peroxidase-catalyzed), reductions and methylations to yield 1,2,4-trihydroxybenzene which, like 1,2,4,5-tetrahydroxybenzene can be ring cleaved. A significant difference, however, is that peroxidase activity in DNT metabolism must be preceded by reduction of one of the nitro groups to an amine [7••].

Involvement of extracellular LiP and MnP in the metabolism of DCP or DNT by *P. chrysosporium* both in peroxidase-catalyzed oxidations in the extracellular medium and intracellular reductions and methylations that regenerate peroxidase substrates would require metabolites to shuttle across the fungal plasmalemma. Almost nothing is known about such processes, or about the absorption and intracellular metabolism of the myriad lignin fragments produced by lignin depolymerization. It is possible, however, that peroxidases similar to LiP and MnP, and other oxidases, are produced intracellularly for metabolism of lignin fragments and that these same enzymes could be involved in the intracellular metabolism of DCP and DNT by the proposed or similar pathways.

*P. chrysosporium* has also been shown recently to mineralize a non-aromatic xenobiotic, cyanide [9•]. The N-tert-butyl-α-phenylnitro-cyanyl radical adduct was produced by the reaction of LiP isozyme H2 with sodium cyanide in the presence of peroxide. This suggests that LiP is involved in the initial oxidation of cyanide that leads to its mineralization.

Recent evidence [10, 11] confirms previous results that suggested that LiP and MnP are involved in the degradation of azo dyes by *P. chrysosporium*. Crude extracellular medium from *P. chrysosporium* cultures converted methylene blue (3,7-bis(dimethylamino)phenothiazin-5-ium chloride) to the tri-demethylated derivative Azure C, in the presence of peroxide [10]. The requirement for peroxide indicates that the reaction is catalyzed by a peroxidase. The fact that the reaction was not stimulated by the addition of Mn²⁺, had an optimum pH of 3.5, and was inhibited by high concentrations of peroxide, indicates that LiP, and not MnP, is involved in the N-demethylation reaction(s). Sulfanilic acid, acid yellow 9 (4-amino-1,1'-azobenzene-3,4'-disulfonic acid), and two dyes synthesized by attaching guaicol to either sulfanilic acid or acid yellow 9 through an azo linkage, were all degraded by ligninolytic cultures of *P. chrysosporium* [11]. The synthesized dyes, which were substrates for in vitro oxidation by MnP but not by LiP, were almost completely (93–94%) depleted in fungal cultures. Sulfanilic acid and acid yellow 9, which were substrates for in vitro oxidation by LiP but not by MnP, were depleted by the fungus to a lesser extent (68–79%). Differences in the degree of depletion can be attributed to the extent of oxidation of the synthesized dyes by MnP, compared with that of sulfanilic acid and acid yellow 9 by LiP.

As would be expected, substrate specific differences that are observed between Lip and MnP for lignin model compounds are also evident for xenobiotic com-
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Fig. 2. The proposed pathway for the degradation of 2,4-dinitrotoluene by *Phanerochaete chrysosporium*, including several oxidations thought to be catalyzed by lignin peroxidase (LiP) and/or manganese-dependent peroxidase (MnP).

Compounds, for example the aforementioned azo dyes, and their metabolites. LiP has been shown to be capable of oxidizing methoxybenzene congeners with high oxidation potentials, whereas MnP or chelated Mn$^{3+}$ can only oxidize low potential congeners [12]. LiP, but not MnP, was capable of catalyzing the in vitro oxidation of nitrodimethoxybenzenes [7••] and chlorodimethoxybenzenes [6••] whereas MnP, but not LiP, catalyzed the in vitro oxidation of the DNT metabolites 2-amino-4-nitrotoluene and 4-nitrocatechol [7••]. The existence of substrate specific differences between LiP and MnP for DCP and DNT metabolites suggests that the peroxidases may work in conjunction with the oxidative degradation of these and other xenobiotic compounds.

Laccases have also been evaluated for their role in xenobiotic oxidation by LDF and for their ability to oxidize selected xenobiotics. Laccases from *Trametes versicolor* catalyzed the oxidation of a number of chlorophenols and chloroguaicols [13••]. The extent of oxidation of substrates such as pentachlorophenol which are difficult to oxidize, was greater in the presence of more easily oxidized chlorophenols than when the compounds were oxidized alone. This result indicates that the presence of the more easily oxidized chlorophenols in some way facilitated pentachlorophenol oxidation by laccase.

Role of VA in LiP-catalyzed oxidations

VA is a secondary metabolite produced by *P. chrysosporium* and a number of other LDFs. The presence of VA has been shown to be necessary for the in vitro depolymerization of synthetic lignin by crude LiP from *P. chrysosporium* [2•], and for the oxidation of several xenobiotic compounds by purified LiP from *P. chrysosporium* [8•,14•].

Several mechanisms have been proposed for the involvement of VA in the enhancement of LiP-catalyzedoxidations.
oxidations that are relevant to the LiP-catalyzed oxidation of recalcitrant xenobiotic compounds. One hypothesis, which is supported by recent results, is that VA acts as a cation radical mediator between LiP and less readily oxidized substrates [15•]. Recent investigations aimed at demonstrating the mediator role have given conflicting results. Both VA and 1,4-dimethoxybenzene were found to be ineffective mediators in the in vitro oxidation of anisyl alcohol (AA) by the biomimetic Lip-model catalyst iron meso-tetra (2,6-dichloro-3-sulfonatophenyl) porphyrin chloride [15•]. In contrast, indirect support for a mediator role for VA and several methoxybenzenes was obtained by enhancement of the in vitro oxidation of AA and 4-methoxymandelic acid by purified LiP from *P. chrysosporium* when either 1,2-dimethoxybenzene, 1,4-dimethoxybenzene or VA were present in the reaction mixture [16•]. Interestingly, the enhancement produced by the dimethoxybenzenes was greater than that caused by VA. The in vitro oxidation of vanillyl alcohol (AA) by the biomimetic Lip-model catalyst ferrous tetra (3,5-dichloro-2-sulfonicphenyl) porphyrin chloride [15•] was shown to be proportional to increasing VA. The results of these investigations do not provide direct evidence for a cation radical mediator role for VA or the methoxybenzenes; nor do they conflict with other popular hypotheses that suggest that VA acts to prevent or to rescue LiP from inactivation by peroxide.

In the presence of excess peroxide at pH 3, native LiP and its more highly oxidized states (LiPI, LiPII and LiPIII) are reported to be converted to a bleached and inactive form designated LiPIII* [19••]. Addition of excess VA to LiPIII* results in its rapid reversion to the native ferric state (LiP) with the release of stoichiometric amounts of the superoxide anion radical, without conversion of VA to veratraldehyde [19••]. The rescue mechanism would appear to be supported by the results of several investigations, in which appreciable oxidation of VA by LiP did not occur until after complete oxidation of a third less easily oxidized substrate such as an azo dye [14••]. HNQ [8,18] or vanillyl alcohol [17].

Enhanced oxidation of HNQ in the presence of VA was inhibited by the superoxide anion radical (HOO•) scavenger Mn(II) [18]. This result led the authors to hypothesize that HOO• is involved in HNQ oxidation and that, in the presence of HNQ, VA simply functions to rescue LiP by releasing HOO• from LiPIII* until HNQ is completely degraded. However, although purified LiP from *P. chrysosporium* was able to catalyze the in vitro oxidation of several azo dyes only in the presence of VA, in the absence of VA, LiPII but not LiPIII or LiPIII*, accumulated after short incubations with several azo dyes [14••]. When VA was present only native LiP was observed. It was concluded that only LiPI could catalyze the oxidation of azo dyes and that VA functioned to enhance oxidation of the dyes by completing the catalytic cycle (i.e. by recycling LiPII to native LiP) [14••]. These results suggest that LiPIII and LiPIII* only occur in vitro where the ratio of peroxide to LiP can be artificially manipulated to be greater than would be expected to occur in vivo. Evidence for the existence of LiPIII* is based on the observation of a Soret maximum at 419 nm [19••]. However, the existence of LiPIII* has been disputed and the 419 nm maximum may be explained by the presence of both LiPII and LiPIII in solution [20•].

Laccase catalytic activity is enhanced in the presence of 2,2′ azino di-(3-ethylbenzthiazoline) sulfonate, in a similar manner to the enhancement of LiP activity by VA. For example, the oxidation of pentachlorophenol by laccase from *Trametes versicolor* was significantly enhanced in the presence of this compound [13••].

It is clear that further studies are needed to elucidate the mechanism(s) of enhanced LiP-catalyzed substrate oxidation by VA and other compounds. The practical implication is that enhanced oxidation of recalcitrant xenobiotics by Lip-producing fungi or by crude or purified LiP preparations may be possible through the use of such 'mediators.' Also, it would be interesting to determine if pathway intermediates, such as the methoxybenzenes that have been identified as intermediates in the degradation of DCP [6••] and DNT [7••] by *P. chrysosporium*, are able to facilitate the oxidation of the parent compounds.

### Bleach plant effluent decolorization

Wood pulps made by chemical processes are bleached by chlorination and the subsequent alkaline extraction of the residual lignin. The effluent from the first alkaline extraction is highly colored due to the presence of high molecular weight, oxidized, and chlorinated lignin (chlorolignin). Many LDFs are able to decolorize these effluents [21], but until recently the mechanism of decolorization and the involvement of fungal peroxidases or laccases had not been demonstrated. Two independent investigations [22••,23••] have now demonstrated that the extracellular peroxidases of *P. chrysosporium*, are responsible for BPE decolorization, and that decolorization accompanies depolymerization of chlorolignin, primarily by MnP. The involvement of the peroxidases was demonstrated by the observation that little or no decolorization of BPE occurred when *P. chrysosporium* was grown under nitrogen-sufficient conditions, which suppress expression of the lignolytic system of this fungus, or when a mutant of *P. chrysosporium*, per which lacks the ability to produce MnP and LiP, was grown under nitrogen-limiting conditions [22••]. The fact that MnP plays a dominant role in the decolorization process, relative to that of LiP, was demonstrated by the temporal correlation between the rate of decolorization and MnP, but not LiP, activity, the negligible effect on the rate of decolorization when LiP activity was suppressed, the significant decrease in the decolorization rate when MnP activity was suppressed and, finally, by the fact that the rate and extent of BPE decolorization by a *P. chrysosporium* lip mutant, which produces MnP but not LiP, was only slightly less than that of the wild type
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[22••]. Direct evidence for involvement of MnP in de-colorization was provided by the in vitro depolymerization of high molecular weight chlorolignin by MnP in the presence of Mn²⁺ and peroxide [23••]. Further insight into the mechanism of chlorolignin depolymerization came from the demonstration that chlorolignin, which was physically separated from MnP by dialysis tubing, could be depolymerized, and that chemically generated Mn³⁺, chelated with lactate, could also cause depolymerization [23••]. These results show that direct contact between chlorolignin and the MnP is not necessary for depolymerization and that Mn³⁺ is the catalytic agent responsible for depolymerization.

**Conclusion**

Our understanding of the role played by fungal peroxidases and laccases in xenobiotic degradation by LDF was extended significantly in 1991. The most salient findings were the first reports of the pathways of xenobiotic degradation in the LDF *P. chrysosporium*. These studies demonstrated involvement of peroxidases, and elucidated the mechanism of BPE decolorization, including the identification of the dominant role played by MnP in this process. Reports of the pathways for the degradation of DCP and DNT by *P. chrysosporium* filled a large void in the application of LDF in the treatment of xenobiotics and provide a focus for determining how other pollutants are degraded by this organism and other LDFs. Identification of the important role of MnP in these degradations could lead to remediation technologies based on LDF that produce this enzyme, on isolated MnP or on Mn³⁺ itself.

**References and recommended reading**

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest


Reports the first demonstration of *in vitro* depolymerization of a dilute dispersion of synthetic lignin by crude LIP from *P. chrysosporium* in the presence of the secondary metabolite, VA.

A significant role for MnP in the depolymerization of lignin was demonstrated by *in vitro* partial depolymerization of feed different synthetic lignin preparations by purified MnP from *P. chrysosporium*.

Demonstrates that MnP and laccase from *Rigidoporus lignosus* act synergistically to catalyse the depolymerization of *Herba lignosus*. Depolymerization was greater in the presence of glucose/glucose oxidase than when peroxide was added exogenously.

Demonstrates that the major pathway of anthraquinone degradation in *P. chrysosporium* proceeds from anthraquinone to phthalic acid and CO₂ and that it is probably mediated by LIP and other enzymes involved in lignin metabolism.

A pathway for the degradation of DCP by *P. chrysosporium* is presented in which both chlorine atoms are removed by peroxidase-catalyzed dechlorination prior to ring cleavage. Evidence is presented for involvement of MnP and LIP in the oxidation of chlorinated phenols and catechols and LIP in the oxidation of chlorinated methoxybenzenes that are metabolites of DCP.

A pathway for the degradation of DNT by *P. chrysosporium* is proposed in which both nitro groups are removed prior to ring cleavage. Evidence is presented for involvement of MnP in oxidative deamination and of both MnP and LIP in the denitrification of pathway intermediates.

Demonstrates the complete oxidation of HQQ by LIP in the presence of peroxide and VA to yield phthalic acid.

Reports the mineralization of cyanide by *P. chrysosporium* and gives evidence for involvement of LIP' in the initial reaction.

Laccases from *Trametes versicolor* were shown to partially decolorize a variety of polychlorinated phenols and guaiacols. The rate and extent of decolorization were significantly affected by substrate and enzyme concentration and the presence of multiple substrates.

Demonstrated the ability of VA to facilitate the oxidation of several azo dyes by LIP from *P. chrysosporium*. The hypothesis that for compounds that are only oxidized by LIP, VA acts to reduce LIP II back to the resting state for completion of the catalytic cycle is presented.
Demonstrates that VA and 1,4-dimethoxybenzene are ineffective mediators for the oxidation of AA by a LiP biomimetic catalyst but that VA could mediate the electrochemical oxidation of the polymeric dye Poly B-411.

The ability of di-, tri- and tetra-methoxybenzenes to facilitate the LiP-catalyzed oxidation of AA and 4-methoxymandelic acid is demonstrated.


The authors propose the existence of a novel oxidation state of LiP designated LiPIII, that is bleached and inactivated in the presence of excess peroxide. They demonstrate that addition of VA to LiPIII results in the release of stoichiometric amounts of superoxide and rapid reversion of LiPIII to the native enzyme.

Reviews the current knowledge of the kinetic and structural characteristics of the active site of LiP from P. chrysosporium.


A key role for the two extracellular peroxidases of P. chrysosporium in decolorization of pulp BPEs is demonstrated. In addition, evidence for a major role for MnP compared to a minor role for LiP in the process is presented.

Depolymerization of chloro lignin by MnP in the presence of Mn²⁺ and peroxide, and by Mn²⁺-lactate is demonstrated. Also shows that the catalytic agent involved in depolymerization of chloro lignin by P. chrysosporium is enzymatically (MnP) generated Mn³⁺.

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