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Role of fungal peroxidases in biological ligninolysis

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The degradation of lignin by filamentous fungi is a major route for the recycling of photosynthetically fixed carbon, and the oxidative mechanisms employed have potential biotechnological applications. The lignin peroxidases (LiPs), manganese peroxidases (MnPs), and closely related enzymes of white rot basidiomycetes are likely contributors to fungal ligninolysis. Many of them cleave lignin model compounds to give products consistent with those found in residual white-rotted lignin, and at least some depolymerize synthetic lignins. However, none has yet been shown to delignify intact lignocellulose *in vitro*. The likely reason is that the peroxidases need to act in concert with small oxidants that can penetrate lignified tissues. Recent progress in the dissolution and NMR spectroscopy of plant cell walls may allow new inferences about the nature of the oxidants involved. Furthermore, increasing knowledge about the genomes of ligninolytic fungi may help us decide whether any of the peroxidases has an essential role.

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Introduction

Almost all terrestrial fixed carbon consists of lignocellulose, the principal structural component of vascular plants, or of humic substances derived from it. The turnover of this large biomass pool, a crucial step in global carbon cycling, is accomplished chiefly by certain filamentous fungi (Figure 1). The biodegradative mechanisms they have developed to meet this challenge are biochemically unusual because lignocellulose has unique properties that make it hard to degrade. Chief among these is the chemical recalcitrance and low porosity of lignin, which coats the energy-rich cellulose and hemicelluloses of vascular plant cell walls. This feature of lignocellulose not only puts a brake on the natural recycling of photosynthetically fixed carbon, but also complicates the industrial usage of vascular plant polysaccharides in paper manufacture and biofuels production. Here, we review evidence for the involvement of fungal peroxidases in the delignification of plant biomass.

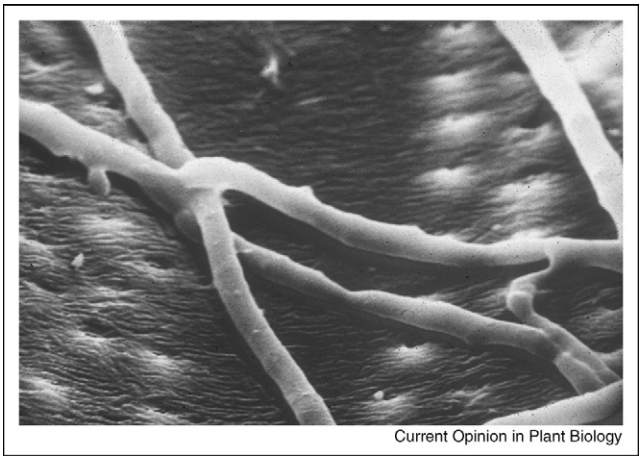
Nature of lignin

Lignin is chemically difficult to degrade because the free radical coupling mechanism responsible for its biosynthesis from phenolic cinnamyl alcohols results in a polymer interconnected through diverse carbon–carbon and ether bonds that are not hydrolyzable under biological conditions [1]. The predominant structures, making up about half the total, are β-*O*-4-linked ethers, followed by phenylcoumarans, resinols, and various minor subunits (Figure 2). It has clearly been shown that lignin is racemic [2], and consequently even a simple β-*O*-4-linked dimer, with two asymmetric carbons, exists as four stereoisomers. Since the number of isomers increases geometrically with the number of subunits, the three-dimensional surface presented by lignin is complex and non-repeating.

This feature of lignin structure has important consequences for biological delignification. Although lignin can be cleaved by strong nucleophiles – the industrial use of alkaline sulfide in Kraft pulping of wood is one well-known example – organisms are constrained to produce analogous species within the active sites of enzymes, where their high reactivity can be harnessed without damaging their hosts. Since a strong nucleophile buried in the active site of an enzyme cannot make close contact with the highly variable surface of lignin, it is unlikely that any organism can degrade the polymer by this mechanism. Despite much interest in the possibility [3•,4], no ligninolytic enzyme with a nucleophilic mechanism has come to light, and it is probable that all biological delignification occurs by less specific oxidative mechanisms.

There is good evidence that lignocellulolytic filamentous fungi oxidize lignin when they degrade lignocellulose. The clearest examples come from white rot basidiomycetes, the most efficient delignifiers of wood, which mineralize lignin to CO_2 while introducing characteristic scissions between C_α and C_β of the aliphatic side chain in the residual polymer. As a result of this reaction, benzoic acid residues accumulate in the lignin, as shown by ^{13}C NMR spectroscopy and other analytical techniques [5]. It has long been known that the C_α–C_β cleavage of alkylaromatic compounds is accomplished by a variety of one-electron oxidants that abstract an electron from the ring to produce an unstable cation radical intermediate [6], and this chemistry became a unifying principle in studies of white rot once researchers realized the connection [7]. Since then,

Figure 1



Scanning electron micrograph showing hyphae of *P. chrysosporium* growing in a conifer tracheid.

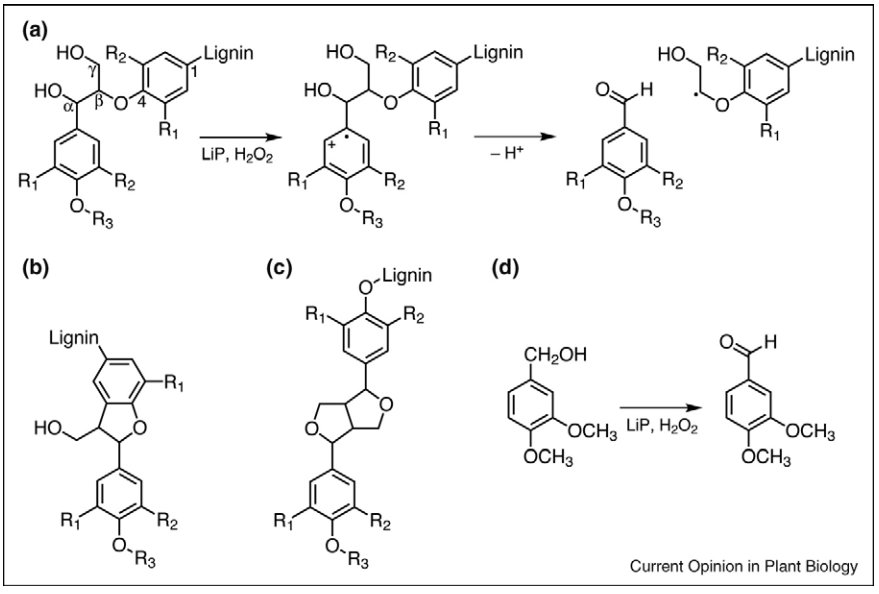
many research groups have joined an ongoing quest to identify the fungal oxidants that cause ligninolysis.

One prerequisite is that these oxidants must be strong enough to attack nonphenolic – that is, ether-linked – lignin structures. Aryl ethers are more difficult to oxidize than phenols, and the phenolic content of lignin is only on the order of 10%. A second requirement is that some of the oxidants must be small species that can penetrate the secondary cell wall in lignified plant biomass. Although some biodegradation during white rot occurs by erosion of the cell wall surface, lignin is also removed from within the wall, and the porosity of intact lignocellulose is too low to admit enzymes [8]. A variety of ligninolytic candidates have been proposed, including laccases that might operate in conjunction with diffusible redox mediators and iron-reducing systems that might use free Fe^{2+} to generate biodegradative hydroxyl radicals. However, the best attested and most intensively researched ligninolytic mechanisms involve secreted fungal peroxidases. These enzymes and a variety of extracellular systems to produce the H_2O_2 they require are widespread among white rot fungi [9].

Lignin peroxidases

Lignin peroxidases (LiPs) were first discovered in the extracellular medium of *Phanerochaete chrysosporium* grown under nitrogen limitation [7]. They are monomeric hemoproteins with molecular masses around 40 kDa, and resemble classical peroxidases such as the enzyme from horseradish, in that their Fe(III) is pentacoordinated to the four heme tetrapyrrole nitrogens and to a histidine residue. Like the classical peroxidases, LiPs are oxidized by H_2O_2 to give a two electron-oxidized intermediate (Compound I) in which the iron is present as Fe(IV) and a free radical resides on the tetrapyrrole ring (or on a nearby amino acid). Compound I then oxidizes a donor substrate

Figure 2



Chemical structures and reactions discussed in the text. **(a)** The principal β-*O*-4 structure of lignin and pathway for its C_α–C_β cleavage by LiP. **(b)** A phenylcoumaran lignin structure. **(c)** A resinol lignin structure. **(d)** LiP-catalyzed oxidation of the fungal metabolite veratryl alcohol. Gymnosperms contain lignins in which most subunits have $R_1 = OCH_3$ and $R_2 = H$. Angiosperm lignins also contain these structures, but have in addition subunits in which $R_1 = OCH_3$ and $R_2 = OCH_3$. Grass lignins contain both types of structures but have in addition some subunits in which $R_1 = H$ and $R_2 = H$. These nonmethoxylated lignin structures are more difficult to oxidize than those that contain one or two methoxyl groups. In the predominating nonphenolic structures of lignin, R_3 = lignin, whereas $R_3 = H$ in the minor phenolic structures.

by one electron, yielding a substrate-free radical and Compound II, in which the iron is still present as Fe(IV) but no radical is present on the tetrapyrrole. Compound II then oxidizes a second molecule of donor substrate, giving another substrate-free radical and the resting state of the peroxidase.

The key functional difference between LiPs and classical peroxidases is that LiPs can oxidize aromatic rings that are only moderately activated by electron-donating substituents, whereas classical peroxidases act only on strongly activated aromatic substrates. Thus, LiPs and horseradish peroxidase both oxidize 1,2,4,5-tetramethoxybenzene, as well as many phenols and anilines, but LiPs are also capable of abstracting an electron from aromatics that carry only two or three ether substituents and thus resemble the major nonphenolic structures of lignin [10]. The initial products of these oxidations are transient cation radical intermediates that spontaneously fragment (Figure 2). Most of the scissions occur between C_α and C_β of the propyl sidechain to give benzaldehydes [11], which are reasonable precursors of the benzoic acid residues that have been observed in lignin decayed by white rot fungi.

Two main attributes of LiPs appear responsible for their unusual activity. First, the iron in the porphyrin ring is more electron-deficient than in classical peroxidases, thus making the LiPs stronger oxidants [12]. Second, an invariant tryptophan residue – trp171 in the isozyme termed LiPA – is present in an exposed region on the enzyme surface, and is thought to participate in long-range electron transfer from aromatic substrates that cannot make direct contact with the oxidized heme [13]. This second feature enables LiPs to oxidize bulky lignin-related substrates directly. For example, *P. chrysosporium* LiPA was shown to oxidize a lignin model tetramer with a molecular mass greater than 1200 but lost this activity when its trp171 was changed to a serine via site-directed mutagenesis [14]. However, the efficiency of LiP-catalyzed oxidation decreases markedly as the size of the targeted lignin structure increases. Recent work showed that the catalytic efficiency (k_{cat}/K_m) for oxidation of a lignin model trimer by a LiP was only about 4% of the value found for oxidation of a monomeric model [15].

When LiPs have been used on yet larger models, that is, synthetic lignins containing about 20 subunits, oxidative cleavage still occurs but requires the presence of veratryl alcohol [16] (Figure 2). This compound is both a LiP substrate and a metabolite secreted by *P. chrysosporium*. One explanation is that the veratryl alcohol cation radical may act as a diffusible mediator that oxidizes lignin at remote locations [17]. This mechanism would circumvent the low efficiency of direct LiP catalysis on large lignin structures and might also allow LiP to oxidize lignin structures within the poorly permeable cell walls of vascular plants. In support of this role, two studies have shown that the veratryl alcohol cation radical has a fairly long half-life near 40 ms under the acidic conditions typical of wood undergoing early white rot [18,19]. However, this hypothesis remains unproven because attempts to observe oxidations mediated by chemically generated veratryl alcohol cation radicals have so far proven unsuccessful [19].

Another explanation for the veratryl alcohol effect on LiP-catalyzed reactions is that this compound prevents the enzyme from remaining oxidized for long periods while it catalyzes the relatively slow cleavage of bulky lignin structures. According to this view, veratryl alcohol acts as an efficient electron donor to rescue LiP from oxidative inactivation by H_2O_2 [20]. Alternatively, the role of veratryl alcohol may be to reduce Compound II of LiP after Compound I has been reduced by nonmethoxylated lignin structures (Figure 2), which are relatively difficult to oxidize because they carry only one electron-donating ether group. This proposal arose because Compound II of LiP is a weaker oxidant than Compound I [21]. If either of these hypotheses is correct, veratryl alcohol has no role as a diffusible mediator, and LiPs might be relegated to eroding the cell wall surface or mopping up lignin oligomers that are released by other oxidative mechanisms.

The recently published genome sequence of a monokaryotic *P. chrysosporium* strain [22,23••] (http://www.jgi.doe.gov/whiterot) has expanded our knowledge of LiPs, regardless of their precise function. The results have confirmed earlier work, which showed the presence of 10 LiP genes designated *lipA* through *lipJ* [24]. All of the enzymes encoded contain the invariant tryptophan residue characteristic of LiPs [22], and most have been shown to oxidize nonphenolic lignin model compounds [25]. It still remains unclear why there are so many LiPs, as none of these enzymes have been shown to differ significantly from each other in reactivity.

The *lip* genes are differentially regulated by culture conditions. For example, Northern blot analyses showed that steady-state levels of *lipD* transcripts are far more abundant than those of *lipA* under carbon starvation, whereas the situation is reversed under nitrogen starvation [26]. Subsequent competitive RT-PCR and nuclease protection assays have shown that differential regulation can exceed five orders of magnitude and that transcript profiles in defined media poorly predict profiles in complex substrates such as wood [27]. Eight of the LiP genes are clustered within 3% recombination, which corresponds to 96 kb [22], but the observed patterns of expression show no clear relationship with genome organization.

Manganese peroxidases

A longstanding problem with the idea of a central ligninolytic role for LiPs is that many white rot fungi apparently lack them. A different group of secreted enzymes, the manganese peroxidases (MnPs) is more widespread and has been extensively researched as a possible alternative [28,29]. MnPs are also strongly oxidizing and undergo a classical peroxidase cycle but do not oxidize nonphenolic lignin-related structures directly because they lack the invariant tryptophan residue required for electron transfer to aromatic substrates. Instead, they have a manganese-binding site that consists of several acidic amino acid residues plus one of the heme propionate groups. Accordingly, one-electron transfer to Compound I of MnP occurs from bound Mn^{2+} [30,31]. The product, Mn^{3+}, is released from the active site if various bidentate chelators are available to stabilize it against disproportionation to Mn^{2+} and insoluble Mn^{4+}. The physiological chelator is thought to be oxalate, an extracellular metabolite of many white rot fungi [32].

The purpose of this reaction is evidently to transfer the oxidizing power of MnP to a small agent – Mn^{3+} – that can diffuse into the lignified cell wall and attack it from within. This is an attractive feature of MnP action, as the low permeability of intact lignocellulose is directly addressed. However, the stability conferred by anionic chelators such as oxalate increases the electron density on the Mn^{3+}, thus making it a considerably weaker oxidant. As a result, the Mn^{3+}-organic acid chelates produced by MnPs are unable to oxidize the predominating nonphenolic structures of lignin by electron transfer. Mn^{3+} chelates can attack the infrequent phenolic structures in lignin, but these units probably occur largely as end groups on the polymer [1], and their oxidation does not result in extensive ligninolysis [16,33].

If MnPs do have a major role in lignin degradation, one possibility is that subsequent reactions of Mn^{3+} may generate other oxidants that can cleave nonphenolic structures. To take one example, Mn^{3+} oxidizes the oxalate that chelates it to generate CO_2 and a formate anion radical, which adds O_2 to give another molecule of CO_2 and superoxide ($O_2^{\bullet-}$) [34]. At the low pH values in wood undergoing white rot, most of this $O_2^{\bullet-}$ will occur in its protonated form as the perhydroxyl radical ($HOO^{\bullet}$), a strong oxidant that can abstract hydrogen atoms from donors such as unsaturated fatty acids [35]. The result of this chemistry would be lipid peroxidation, that is, a radical chain reaction that generates potentially ligninolytic peroxyl radicals.

In agreement with this hypothesis, it has been shown that MnPs catalyze lipid peroxidation in the presence of chelated Mn^{2+} and H_2O_2, that nonphenolic synthetic lignin is cleaved *in vitro* as a result, and that white rot fungi produce extracellular lipids that could provide the necessary peroxidizable substrates in wood [36–38]. A role for peroxyl radicals in these reactions is also suggested by data that showed other peroxyl radical-generating systems to cleave nonphenolic lignin structures [39]. However, the extent of side reactions that oxidize lignin structures without cleaving them is very high in these peroxidation systems, and it remains difficult to reconcile the inefficiency of ligninolysis by this route with the characteristically thorough delignification that some MnP-producing, apparently LiP-negative fungi cause.

Analysis of the *P. chrysosporium* genome has shown that it encodes five MnPs. cDNAs were previously reported for genes *mnp1*, *mnp2*, and *mnp3* [40–43], whereas two new genes, *mnp4* and *mnp5*, were revealed by Blast searches [22]. The five *mnp* sequences are remarkably conserved, as are the number and positions of introns. Recent data show that *mnp4* is actively transcribed when *P. chrysosporium* is grown on wood-containing soil samples [44]. Gene *mnp5* corresponds to the N-terminal amino acid sequence of an MnP purified from *P. chrysosporium*-colonized wood pulp [45]. As with the LiPs, the reason for so much redundancy in MnPs is unclear because they all appear to have similar reactivity.

MnP production in *P. chrysosporium* is stimulated not only by nutrient limitation but also by Mn^{2+}. Putative metal response elements (MREs) have been identified upstream of *mnp1* and *mnp2*, and transcript levels from these genes respond to Mn^{2+} supplements in low nitrogen media. By contrast, *mnp3* lacks MREs, and its transcript levels are not influenced by addition of Mn^{2+} [46,47]. These results suggest a role for MREs in *mnp* regulation, but the question remains open. In recent work based on a green fluorescent protein reporter system, a 48-base pair sequence containing at least one Mn^{2+}-responsive *cis* element was identified upstream of *P. chrysosporium mnp1*. None of the six putative MREs present in *mnp1* is contained in the aforementioned region, and functional evaluation of four of these MREs showed no significant effect on the gene's response to Mn^{2+} [48].

Versatile peroxidases

When an Mn^{2+}-binding site was introduced into a *P. chrysosporium* LiP by site-directed mutagenesis, the resulting enzyme had MnP activity [49]. Conversely, when a tryptophan residue analogous to the essential one in LiPs was introduced into a *P. chrysosporium* MnP, this enzyme acquired LiP activity [50]. These results show that hybrid peroxidases with both activities could occur naturally. Recently, enzymes of this type, now termed versatile peroxidases (VPs), have been found in various *Pleurotus* and *Bjerkandera* species and extensively characterized [51,52]. *P. chrysosporium* apparently

lacks VPs, although its genome encodes a putative extracellular peroxidase (GenBank accession AY727765) related to *Pleurotus* VPs, the crucial catalytic and Mn-binding residues are not conserved [53].

The *P. eryngii* VP termed VPL has the three acidic amino acid residues required for Mn^{2+} binding, and a catalytic efficiency (k_{cat}/K_m) for Mn^{2+} oxidation in the general range exhibited by typical MnPs. In addition, VPL has a tryptophan residue, trp164, analogous to the LiPA trp171 that participates in electron transfer from aromatic donors and consequently enables the enzyme to oxidize nonphenolic lignin-related structures [54•]. However, the catalytic efficiency of *P. eryngii* VPL on veratryl alcohol is relatively low at about $1 \times 10^3\ M^{-1}\ s^{-1}$, as opposed to about $3 \times 10^4\ M^{-1}\ s^{-1}$ when the same reaction is catalyzed by *P. chrysosporium* LiPA [54•,55]. Given the already low efficiency of LiP when it directly oxidizes large lignin model compounds, it will be important to determine how well VPs deal with these and with even larger synthetic lignins.

Conclusions

LiPs are strong oxidants that interact directly with nonphenolic lignin structures to cleave them, but they do so inefficiently, and apparently cannot penetrate the small pores in sound lignocellulose. MnPs produce small, diffusible strong oxidants that can penetrate the substrate, but these cleave the principal structures of lignin in low yields. VPs require more research, and it remains to be seen how efficient they are on lignin oligomers or whether they can generate diffusible ligninolytic oxidants more powerful than Mn^{3+}. The unique properties of these peroxidases strongly suggest that they contribute to ligninolysis, but their relative importance remains unclear, and so far none has been used successfully to delignify intact lignocellulose *in vitro*.

Progress may come on two fronts. First, recent work shows that different ligninolytic oxidants oxidize different stereoisomers of nonphenolic lignin structures with differing selectivities [56•]. Given recent advances in the solubilization and solution-state NMR analysis of plant cell walls [57•,58], it may finally prove possible to infer the nature of the oxidants that fungi employ by analyzing oxidative and stereochemical changes that occur in the lignocellulose during decay. Second, LiP and MnP genes have now been identified in many fungi, and analyses show that they generally fall within clearly defined clades and can be discriminated by certain key residues [59]. Thus, as more fungal genomes are sequenced, it may eventually become clear which peroxidases can be absent without compromising ligninolytic capability.

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- • of special interest
- •• of outstanding interest

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