In: Kuwahara, M.; Shimada, M.; eds. Biotechnology in pulp and paper industry: Proceedings, 5th International conference on biotechnology in pulp and paper industry; 1992 May 27–30; Kyoto, Japan. Tokyo, Japan: Uni Publishers Co., Ltd. 1992. Chapter 83.

83 What Is the Primary Agent of Lignin Degradation in White-rot Fungi?

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A number of enzymes have been proposed to function in fungal ligninolysis, with extracellular lignin peroxidases generally considered the primary agents of this process. However, recent work in three areas suggests that the situation may not be so simple: 1. Several white-rot basidiomycetes have been reported to degrade lignin without producing detectable lignin peroxidase activity. 2. Electron microscopic studies do not clearly show that enzymes are responsible for early stages of wood decay. 3. It has been difficult to demonstrate ligninolysis by isolated lignin peroxidases in vitro. These results indicate that some ligninolytic agent other than lignin peroxidase is produced by white-rot fungi. The available evidence suggests that this agent is enzymatically produced manganese(III), but new data also support some role for lignin peroxidases in fungal lignin degradation.

1. INTRODUCTION

Since their discovery in 1983 (1,2), lignin peroxidases (LiPs) have widely been considered the primary agents of ligninolysis in white-rot fungi. There are good reasons for this view: LiPs catalyze the one-electron oxidation of lignin-related model substrates, and the resulting radical products undergo a variety of nonenzymatic substitution and fission reactions that adequately explain many of the chemical changes that occur when whole fungal cultures degrade macromolecular lignin. In particular, LiPs catalyze the oxidative C_α-C_β cleavage of nonphenolic lignin model dimers, a novel biochemical route for carbon-carbon bond cleavage which corresponds to one of the principal ligninolytic reactions observed in vivo (3,4). In addition, LiP expression at the transcriptional level and the appearance of LiP activity in fungal cultures both correlate with the onset of the organism's ability to degrade lignin (3-5). It would be remarkable if an enzyme possessing these unique features, and seemingly tailor-made for the job at hand, played no role in fungal ligninolysis.

Nevertheless, there are problems with the hypothesis that LiPs catalyze fungal ligninolysis. First, several fungi are now known that can degrade lignin in culture, but produce no detectable LiP activity under these conditions. Second, it appears that LiPs do not penetrate

the lignocellulosic matrix in sound wood. It may be that LiPs can operate alone as ligninolytic catalysts at the surface of the lignin, but it is also possible that some lower molecular weight agent is responsible instead for the initial steps of ligninolysis. And finally, attempts by several groups to depolymerize lignins with LiP in vitro have been unsuccessful. These problems prompt us to take another look at what comprises the ligninolytic system in fungi.

2. FUNGI THAT DEGRADE LIGNIN WITHOUT PRODUCING LiP

There have been several reports in the literature of fungi that can degrade lignin but produce no LiP. Périé and Gold showed that *Dichomitus squalens* is able to mineralize synthetic lignins (dehydrogenative polymers of *p*-hydroxycinnamyl alcohols, = DHPs) in 40-50% yield. This work, which was done with selectively radiolabeled DHPs, showed that *D. squalens* was equally able to degrade the aromatic ring, the propyl side chain, and the ring methoxyl groups of DHP. *D. squalens* produced no detectable LiP activity in ligninolytic cultures, but did exhibit high extracellular manganese-dependent peroxidase (MnP) and laccase activities under these conditions (6). *Phlebia tremellosa,* a white-rotter that causes selective delignification of wood, has also been shown to mineralize a ring-labeled guaiacyl DHP, albeit in low yield (7). This organism had previously been found to produce MnP, but not LiP, under a variety of culture conditions (8). *Rigiduporus lignosus* is another baidiomycete that degrades lignin in the absence of any detectable LiP activity, but produces a MnP and a laccase (9). Results such as these have led to the proposal that MnP and laccase may function as ligninolytic enzymes during white rot.

The idea that phenol-oxidizing enzymes such as MnPs and laccases may catalyze ligninolysis is not a new one, but renewed interest in the subject derives from several recent findings. Both enzymes, although apparently inactive towards nonphenolic lignin substructures, have been shown to catalyze cleavage reactions in phenolic syringyl lignin model dimers (10,11). Laccases do this directly, whereas MnPs oxidize manganese(II) to give manganese(III), which acts as the proximal oxidant at a distance from the enzyme active site (12,13). However, it remains unclear whether these enzymes can cleave phenolic guaiacyl substructures in lignin, as would be required to explain the fact that fungi such as *D. squalens* are capable of degrading gymnosperm lignins. There has been one report, though, that a purified MnP catalyzed the partial depolymerization of several DHPs (14). It is also noteworthy that MnP, and not LiP, was the major peroxidase detected in aspen pulp that was being degraded by *Phanerochaete chrysosporium* (15). All of these results favor a mechanism for fungal ligninolysis that is able to operate in the absence of LiP, but they do not rule out an important role for LiP in certain fungi such as *P. chrysosporium* that produce this enzyme.

3. PENETRATION OF FUNGAL ENZYMES INTO WOOD

There is general agreement in the literature that LiPs cannot penetrate sound wood. The most important evidence comes from LiP immunolabeling of wood decayed by white-rot fungi (16-19). Transmission electron microscopy of such specimens has shown that LiP is secreted by fungi such as *P. chrysosporium* and *Trametes versicolor* in wood. The enzyme is found in association with the fungal hyphae and with partially degraded areas in the wood secondary cell wall. LiP is also found in the middle lamella when this region has been exposed via extensive degradation of the S2 and S1 layers, but is not found in undecayed areas of the wood. In some cases, delignification appears to occur progressively from the cell lumen through the secondary wall, immediately adjacent to fungal hyphae and via the formation of erosion troughs. In this case, LiPs might be responsible for ligninolytic attack at the cell wall surface. Electron micrographs have shown, in agreement with this picture, that LiP immunolabeling is extensive in secondary wall erosion troughs. In other cases, though, delignification appears to involve a diffuse loss of lignin throughout the secondary wall, apparent in electron micrographs as a loss of electron density. LiP penetrates these regions when they are already partially decayed, but whether it initiates the process is open to question. The data do not tell us whether LiP has gradually eroded the lignin surface in these specimens, leaving behind modified secondary wall components that remain apparent in electron micrographs, or whether some smaller oxidant has first penetrated and degraded the wall, leaving the way clear for LiP to enter later. If such a low molecular weight species is indeed required (and this remains unproven), Mn(III) is the most likely candidate. It is noteworthy in this regard that another low molecular weight oxidative system, consisting of heme and *t*-butyl hydroperoxide, has been shown to penetrate and delignify specimens of sound wood (20).

4. EFFECTS OF LiP ON LIGNIN IN VITRO

Experimental evidence that LiPs can depolymerize lignin has been difficult to get. Tien and Kirk (1) found that LiP cleaved radiolabeled fragments from ^{14}C-methylated spruce lignin, but depolymerization of the unmodified polymer was not reported. Umezawa and Higuchi (21) linked a nonphenolic lignin model compound to the terminal regions of a DHP, and reported that LiP catalyzed the release of model compound fragments from the synthetic lignin in low yield. Depolymerization of the DHP was not reported in this experiment. Several groups, by contrast, have reported on the apparent inability of LiP to depolymerize lignin in vitro. Haemmerli et al (22) found that spruce milled wood lignin at 1 mg/ml was polymerized rather than degraded by crude and purified LiPs, and attributed the increase in

molecular weight to bimolecular coupling reactions between LiP-generated phenoxy radicals. Isolated lignins typically have phenolic contents of 10-20%, and the LiP-catalyzed cleavage of β-*O*-4 units in the polymer produces yet more of these substructures. Haemmerli et al concluded that LiPs alone are not sufficient to cleave lignin; some extracellular reductive mechanism must also be required to scavenge lignin phenoxy radicals and prevent their polymerization. Odier et al (23) obtained a similar result with a guaiacyl DHP at a lower concentration of 45 μg/ml, and found that polymerization was not prevented by cellobiose-quinone oxidoreductase, a proposed scavenger of phenoxy radicals. Kern et al (24) found in a third study that a guaiacyl DHP at 1 mg/ml was polymerized by LiP. These workers also reported that no structural changes indicative of degradation were observable in ^{13}C-NMR experiments with LiP-treated lignin. Recently, Sarkanen et al (25) found again that a guaiacyl DHP was polymerized by LiP. In this last study, LiP was shown to polymerize coniferyl alcohol, the natural precursor of guaiacyl lignin in plant cell walls, to give a typical guaiacyl DHP. The authors argued that a peroxidase which can synthesize lignin is unlikely to catalyze its degradation. In fact, the results of this last study have no clear relevance to the bona fide problem of what LiPs actually do: all peroxidases, including LiPs, are able to catalyze the intramolecular coupling of phenols, yet lignin is degraded rather than polymerized in vivo in the presence of these enzymes. Clearly, some factor operates during white rot to prevent the polymerization of lignin phenolic groups by these peroxidases. We do not know what this factor is, but it may simply be that lignin is too insoluble and extensively crosslinked to undergo radical coupling reactions in the solid state. Under circumstances such as these, it is entirely possible that LiPs might catalyze fragmentation of the lignin polymer.

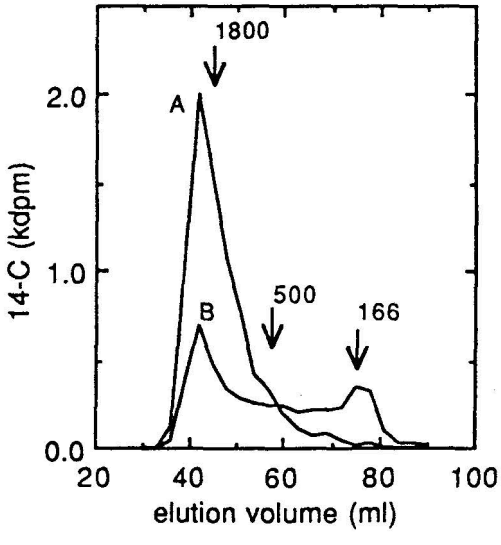

Fig. 1. Gel filtration chromatography on Sephadex LH20 of a synthetic $^{14}C_{\beta}$-labeled syringyl/guaiacyl lignin after treatment with crude LiP. A: Treated with H_2O_2 alone. B: Treated with H_2O_2 and LiP. The arrows indicate molecular weight standards. Data are excerpted from ref. 26.

In recent work, Hammel and Moen (26) obtained promising results when an angiosperm type synthetic lignin was treated in vitro with crude LiP. These investigators reasoned that, since lignin is insoluble in the usual aqueous systems used to assay LiPs, attempts to observe in vitro depolymerization should be performed with finely divided. dilute dispersions of the polymeric substrate. In addition, the oxidant (H_2O_2) should be added continuously over an extended time to minimize bimolecular coupling reactions of lignin phenoxy radicals. Under these conditions, partial depolymerization of a guaiacyl/syringyl DHP was obtainable at a lignin concentration of 2 µg/ml in buffered H_2O/dimethylformamide, with fragments of <200 MW detected as reaction products by gel permeation chromatography (Fig. 1). This study provides the first direct evidence that LiPs may catalyze ligninolysis in vivo. However, the results do not rule out the possibility that some polymerization of the DHP may also have occurred during the reaction. There are also two characteristics of the DHP used in this work that might have made it especially susceptible to depolymerization by LiP: First, the polymer was relatively small, with an average MW around 2000. Second, guaiacyl/syringyl lignins are methoxylated at many of their aromatic 5-positions, and are consequently less condensed and less liable to polymerization than guaiacyl polymers are. These caveats aside, it is now clear for the first time that LiPs are fundamentally capable of ligninolysis.

5. CONCLUSIONS

It is becoming clear that LiPs are not obligatory participants in fungal ligninolysis. However, this does not mean that they do not catalyze lignin depolymerization when they are present. We see two major problems that need to be addressed in future research:

First, LiP-independent mechanisms of ligninolysis need to be identified. The effect of purified MnPs in vitro on the molecular weight of lignin needs to be assessed in gel permeation chromatography systems that can detect both polymerization and depolymerization of the starting material. Purified MnPs or chelated manganese(III) should also be tested in electron microscopic experiments for the ability to delignify wood specimens. Fungi that produce MnP but not LiP need to be examined with regard to the effect they have on the molecular weight distribution of polymeric lignin—it is clear that these organisms can oxidize lignin to CO_2, but it remains possible that they may polymerize lignin at the same time.

Second, improved systems for the treatment of lignin with LiP in vitro are needed. It is difficult, if not impossible, to reproduce in an in vitro reaction the rigid, crosslinked structure of lignin in wood. One experimental approach might be to crosslink radiolabeled lignins to inert carriers, and then to determine whether LiP can catalyze the release of radiolabeled fragments from these carriers. Another possibility might be to increase the accessibility of

lignin to LiP by adding a suitable cosolvent to the reactions—it is likely that natural ligninolysis does not occur in a simple aqueous system, but rather in a more hydrophobic environment due to the presence of extracellular fungal polysaccharides (17). Finally, we need to consider the possibility that LiP might not play a role in the initial stages of ligninolysis, but might function instead in the subsequent metabolism of partially degraded lignins that are made accessible through the action of smaller oxidants.

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