Biochemical Approaches to Wood Preservation

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Abstract

Much progress has been made in the past several years concerning the biochemistry and molecular genetics of fungal wood decay. Aspects of this knowledge were obtained with the intent of using the fungi and their enzymes for selective and beneficial purposes; e.g., in biopulping and biobleaching of wood fiber, in bioremediation of pollutants, and in biomass conversions for fuel and chemical feedstocks. Accordingly, enzymes for lignin, cellulose and xylan degradation have been identified, Although there are obvious gaps in our knowledge of the decay process, details of discrete steps should provide opportunities to develop new wood preservatives that selectively disrupt the metabolic pathways of wood decay. The biochemistry of lignin biodegradation by *Phancrochaete chrysosporium* is summarized with specific examples of enzyme inhibitors and discussion of potential metabolic markers that may be useful in developing wood preservatives.

Introduction

Increasing concerns over the environmental impact of conventional wood preservatives have provided impetus to look for alternatives. A prime reason for the environmental concern is the broad-spectrum toxicity of the preservatives; i.e., they are toxic to many organisms, not just the wood-decay organisms. Furthermore, the disposal of treated wood presents complications that may extend far into the future.

A potential strategy in the search for alternative preservatives against fungal decay is to target the extracellular wood-decay process itself, rather than the decay organisms. In this way, a preservative may make wood indigestible to fungi, not toxic. Possible advantages of such an approach include 1) the preservative is fungistatic, as opposed to fungicidal and therefore would be expected not to have broad-spectrum toxicity, 2) extracellular fungal metablism presents novel targets that would provide selectivity in the mechanism of preservation, and 3) extracellular targets would circumvent the need for preservatives to cross fungal cell membranes and therefore provide greater latitude in the design of a preservative.

This paper discusses a few considerations in the search for alternative preservatives directed at the extracellular decay process. Because such a biochemical strategy to wood preservation necessarily targets metabolic pathways, knowledge of the enzymes, non-enzymic catalysts, metabolites, and regulatory mechanisms is necessary for a rationale approach. The major polymers of wood are cellulose, xylan and lignin, and therefore the disruptions of their metabolism are potential strategies for preservation. The fungal biodeteriation of lignin is discussed as an example.

Biochemistry of Ligninolysis

Lignin Degradation by *P. chrysosporium*. The *most* extensively studied lignindegrading system is that of the white-rot fungus *Phanerochaete chrysosporium*. Important culture parameters required by *Phanerochaete* for lignin degradation to CO_2 and H_2O have been determined using ^{14}C-lignin as substrate in defined media. Ligninolysis is triggered by nutrient limitation; cultures starved for carbon, nitrogen, or sulfur are able to oxidize lignin to CO_2 (10,16,18,29). There is also great influence by O_2 partial pressure (18,29), metal ion balance (10) and pH (18). The fungus is typically grown in stationary liquid medium with glucose as the carbon source and NH_4^+ as the limiting nitrogen source to induce the ligninolytic system. Culture conditions have also been determined with shaken cultures to scale up production for enzyme purifications (9). Extracellular enzymes of the lignin-degrading system so far discovered in defined glucose media include lignin peroxidase (LiP), manganese peroxidase (MnP) and the H_2O_2-producing enzyme glyoxal oxidase (GLOX). Peroxide generated by GLOX is coupled to the peroxidase reactions (Figure 1). The biochemistry and molecular genetics of lignocellulose biodegradation by *Phanerochaete* have been reviewed (2). Specifics of enzymes secreted by this

fungus are briefly outlined below to set the context for discussion.

Lignin Peroxidase. Growing evidence indicates that the LiP of the fungus *P. chrysosporium* plays a central role in the initial degradation of the complex lignin polymer by this white-rot fungus. LiP was first discovered based on the H_2O_2-dependent C_α-C_β cleavage of lignin model compounds and subsequently shown to catalyze the partial depolymerization of methylated lignin in vitro (5,6,34,35). There are several isozymes of lignin proxidase, all of which are glycoproteins of molecular weights estimated at 38-46 kDa (17,22,31). It is clear from N-terminal and genetic analyses that there are multiple LiP isozymes that are separate gene products.

LiPs are true peroxidases (19,30,36) and the kinetics of enzyme intermediates have been studied in detail (1,24,39). The underlying principle behind the array of reactions catalyzed by LiP is explained by the ability of the peroxidase to oxidize the aromatic nuclei of substrates by one electron; the resulting aryl cation radicals degrade spontaneously via many reactions dependent on the structure of the substrate and on the presence of reactants (Figure 2). Evidence that LiP oxidizes substrates to cation radical intermediates was demonstrated with methoxybenzenes as substrates by electron spin resonance (e.s.r.) (15), The results with one of these substrates, 1,3,5-trimethoxybenzene, is discussed below as an enzyme inactivator. Detailed reviews on the radical chemistry of LiP-catalyzed reactions have been provided (8,32).

Manganese Peroxidase. Another heme peroxidase found in the extracellular fluid of ligninolytic cultures of *P. chrysosporium* is MnP (21,27). MnP oxidizes Mn^{2+} to Mn^{3+} using $H2O_2$ as oxidant, Activity of the enzyme is stimulated by simple organic acids which stabilize the Mn^{3+} and allow it to oxidize organic compounds including phenolic lignin model compounds (3,4). As with LiP, the prosthetic group is iron protoporphryn IX and several isozymes are detected (22,25,28,37). MnP exhibits enzyme intermediates analogous to other peroxidase (37,38). The biomimetic oxidation of lignin model compounds by Mn^{3+} suggests that it may play a role in oxidizing both phenolic and nonphenolic residues of lignin (7).

Glyoxal oxidase. Our efforts have been concentrated on GLOX as a source of H_2O_2 required by the peroxidases (11,14). A number of simple aldelhyde-, alpha-hydroxycarbonyl-, and alpha-dicarbonyl compounds are substrates for GLOX. Purified GLOX shows a marked stimulation in activity when incubated with Cu^{2+}; no activation is observed with the divalents Mg^{2+}, Zn^{2+}, Fe^{2+}, or Mn^{2+}, Nevertheless, Mn^{2+} does appear to be crucial in expression of GLOX in the overproducing strain PSBL-1 (26).

We have recently cloned and sequenced the cDNA encoding GLOX (12). The molecular weight of 57 kDa (537 amino acids) for the predicted mature pep tide is in reasonable agreement with the experimentally determined molecular weight of 68 kDa. The sequence of *glx-1c* has confirmed our earlier suspicions that GLOX is different than other oxidases so far sequenced; computer searches of databanks indicate no striking homology with any other recorded gene / protein or copper-binding domains. Northern analysis shows that GLOX is regulated on the transcriptional level and coordinate with LiP. GLOX transcript is expressed under both nutrient nitrogen and carbon limitation (33).

Irreversible Inactivation

A method to disrupt a metabolic pathway is to irreversibly inactivate an enzyme in the pathway. Desirable characteristics of a preservative would be

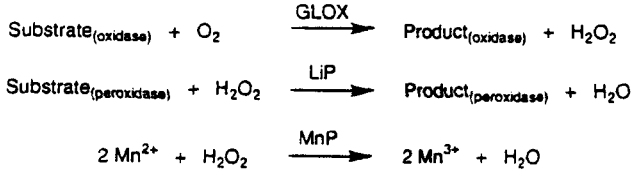

Figure 1. *Secreted oxidase and peroxidase of P. chrysosporium. The peroxide generated by GLOX is coupled to the oxidations catalyzed by LiP and MnP. Nonphenolic aromatics are substrates for LiP. MnP oxidizes Mn^{2+} to Mn^{3+} which in turn may oxidize phenolic compounds.*

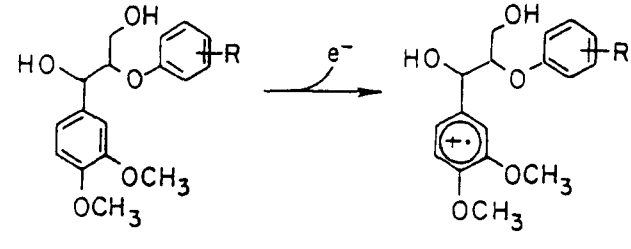

Figure 2. *One-electron oxidation of lignin. Oxidations of nonphenolic aromatics by LiP generate cation radical intermediates. This is depicted here with a generalized β-O-4 lignin substructure.*

specific inactivation, at the time of incipient decay, and under ambient conditions. A possible strategy is to use the specificity of the enzyme to supply the specificity of inactivation, and the structure of the substrate to supply the necessary chemistry for irreversible inactivation. Such irreversible inactivators are sometimes referred to as suicide substrates or mechanism-based inhibitors.

A possible example of a suicide substrate was described in a study on the oxidation of methoxybenzenes by LiP (13), The methoxybenzenes, of which there are twelve congeners, were particularly useful as substrates in elucidating the cation radical mechanism of the peroxidase. The half-wave potentials of the methoxybenzenes range from 0.81 V to 1.76 V vs. a saturated calomel electrode. This allows relationships to be examined between the redox potentials of the substrates and the ability of enzymes to oxidize them by one electron. LiP from *P. chrysosporium,* horseradish peroxidase (HRP), and laccase from *Trametes versicolor* were chosen for comparison. LiP oxidized the ten congeners with the lowest half-wave potentials, whereas HRP oxidized the four lowest and laccase oxidized only the lowest. It was concluded that the three enzymes affect their substrates similarly, and that whether a non-phenolic aromatic compound is a substrate depends in large part on its redox potential. Notably the oxidation of l,3,5-trimethoxybenzene by LiP was unusual because LiP was rapidly inactivated during catalysis.

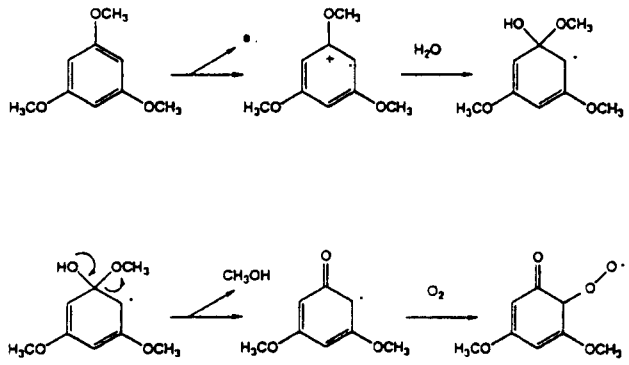

Figure 3. *One-electron oxidation of 1,3,5-trimethoxybenzene. A mechanism for the oxidation of 1,3,5-trimethoxybenzene by LiP/H_2O_2 via a cation radical intermediate is presented. The peroxyl radical formed in the presence of oxygen may lead to the inactivation of the enzyme.*

The oxidation of 1,3,5-trimethoxybenzene with LiP was also unusual because it was the only oxidation in the series for which a radical species could be easily detected by e.s.r. spectroscopy using 5,5-dimethyl-1-pyrroline N-oxide as spin-trap. The e.s.r spectra were consistent with trapping of a carbon-centered radical in the absence of O_2 and an O_2-centered radical in air. It is conceivable that inactivation of LiP in air is linked to the production of peroxyl radicals, as has been suggested for HRP (23). A plausible mechanisim for carbon-centered radical formation and peroxyl radical formation with 1,3,5-trimethoxybenzene is shown in Figure 3. 1,3,5-Trimethoxybenzene has a relatively high redox potential in the series (E1/2 = 1.49) and was not oxidized by HRP/H_2O_2 or laccase/O_2.

Reversible Inactivation

Regulation of metabolism is a critical aspect in any biological system for the control of metabolize concentration and flux. Perhaps the property of GLOX of most interest and of considerable physiological significance is that, in the absence of a peroxidase system, the oxidase is reversibly inactivated (11). The enzyme is reactivated, however, by reconstituting the complete peroxidase system, including both LiP and LiP substrate (Figure 4). These results suggest that there is an extracellular regulatory mechanism for the supply of H_2O_2 by GLOX.

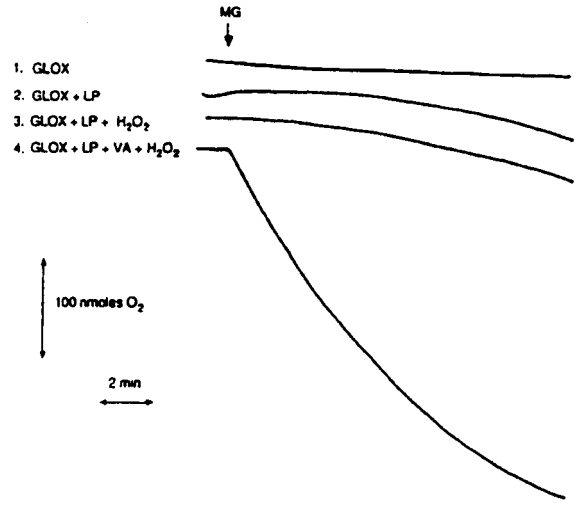

Figure 4. *Inactivation and activation of GLOX. Purified GLOX would be expected to rapidly consume oxygen in* the *presence of its substrate methylglyoxal (Figure 1). This is not the case. Shown are oxygen uptake experiments using where indicated 60 mU GLOX, 114mU lignin peroxidase (LP), 1 mM veratryl alcohol (VA; a lignin peroxidase substrate) and 5 mM methylgiyoxal (MG). Activity is observed only if all the components for the coupled-peroxidase reaction are included. Figure is from prior publication (11).*

To characterize the modulation of this extracellular oxidase activity, we have studied effects of pH, peroxide concentration, peroxidase source (fungal vs plant), and peroxidase substrate with recombinant GLOX (rGLOX) (20). Our results show that a peroxidase system is not required for rGLOX activity. However, the activity is transient and the enzyme is partly and reversibly inactivated by the produced peroxide. Both LiP and HRP maybe used to activate rGLOX with methoxybenzenes as peroxidase substrates, provided the methoxybenzene is a good substrate for the particular peroxidase. In contrast, inhibitory effects are observed with other phenolic compounds, even though they are good substrates for the peroxidase. The mechanism for this reversible switch that turns GLOX activity on and off is not known. However, the sensitivity of this "switch" to subtle changes, and the inter-relatedness of GLOX activity with that of the fungal peroxidase, presents it as a reasonable target for disruption.

Concluding Remarks

Two examples of potential targets (LiP and GLOX) for the disruption of wood decay are presented here. In the case of LiP, a mechanism of inactivation was discussed in which specificity (via redox potential) and reactivity (via a radical mechanism) were conferred with a substrate/inactivator. In the case of GLOX, specific details of the reversible inactivation and reactivation is not known but answers are approachable and theories are not lacking. There are many other potential targets (e.g., the hydrolytic enzymes of extracellular carbohydrate metabolism) and possible mechanisms of inactivation. Whether these present suitable targets for disruption and provide the basis for the development of new wood preservatives is yet to be determined. Considerations of efficacy, wide applicability (e.g., against white-rot and brown-rot fungi), economics, and environmental compatibility are not trivial and must be met before bringing theory to practice.

Acknowledgements

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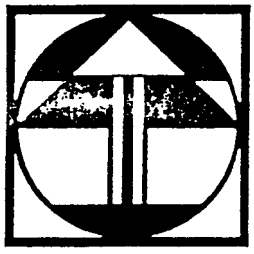

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