Lignin Degradation by 
*Phanerochaete chrysosporium*

The study of lignin biodegradation entered the realm of biochemistry in 1983 with the first reports of a lignin-degrading enzyme, termed ligninase or lignin peroxidase. The powerful peroxidase was discovered in the basidiomycete *Phanerochaete chrysosporium*, the most studied ligninolytic organism. Since then much has been learned about ligninase and about other enzymes and metabolites involved in degrading the complex lignin polymer. The key reaction is ligninase-catalyzed one-electron oxidation of aromatic rings to cation radicals. These degrade via many reactions, including some that cleave intermonomer linkages and others that cleave aromatic nuclei. Thus the fungal degradation of lignin, like its biosynthesis, involves spontaneous reactions of free radicals. The questions remaining about the ligninolytic system are being addressed via biochemical and molecular biological approaches.

**BACKGROUND**

The first lignin-degrading enzyme was discovered in 1983. That discovery culminated a decade of accelerating research that began with the development of the first unequivocal assay for biodegradation, based on 14C-lignins. With the assay, scientists showed that biodegradation is an aerobic process, that basidiomycetous and ascomycetous fungi that decay woody (lignified) tissues are efficient degraders of the complex aromatic polymer, and that bacteria probably play only a secondary role. Because of several experimental advantages, the basidiomycete *Phanerochaete chrysosporium* was selected in the mid-1970s for detailed study and has since become the best understood ligninolytic microbe. By the late 1970s, cultures of *P. chrysosporium* had been optimized for oxidation of 14C-lignin to 14CO2 in chemically defined media, and some of the intriguing physiological features associated with the optimized cultures—most notably that ligninolysis is associated with secondary metabolism—had been described. Specific reactions of lignin degradation by the cultures were elucidated over the next 2 to 3 years through the use of synthesized “dimeric” model compounds representing specific lignin substructures. One of those reactions, an oxidative carbon-carbon cleavage, was used to probe the extracellular culture fluid for the responsible enzyme, resulting in the discovery of ligninase (1,2). This article summarizes the rapid research progress made since that discovery. Recent reviews provide further insight (3,4).

**CURRENT STATUS**

Since its discovery, ligninase has been studied extensively in a number of laboratories. It is a peroxidase and appears to play a major role in the initial depolymerization of lignin by *P. chrysosporium*. In 1984 a second peroxidase, termed manganese peroxidase, was also discovered in the extracellular broth of ligninolytic cultures of *P. chrysosporium*. Manganese peroxidase might have a role similar to that of ligninase, but its function is still being clarified. Both enzymes require extracellular hydrogen peroxide, meaning that the enzyme system supplying that cofactor is also a key component of the polymer-degrading system. Several H2O2-producing enzymes have been implicated. The following discussion deals in turn with ligninase, Mn peroxidase, and H2O2-producing enzymes of *P. chrysosporium*.

### Ligninase

The ligninase isolated originally was shown to be monomeric, to have a molecular weight of about 42 000 and to be H2O2-dependent (1,2). Further study has clarified much about its production and regulation, molecular biology, catalytic cycle, and substrate oxidation.

#### Production and regulation

At the time of its discovery, ligninase activity was associated with secondary metabolism (1,2). Early studies surprisingly had shown that during lignin degradation by *P. chrysosporium*, oxidation to CO2 occurs only during secondary metabolism [see 3,4]. In *P. chrysosporium* this stage of culture development is triggered by nitrogen or carbon limitation and, less efficiently, by sulfur limitation (3,4). The secondary metabolite veratryl (3,4-dimethoxybenzyl) alcohol appears shortly before ligninase activity is detected, and it has been postulated to induce the ligninolytic system and to play additional roles as well (3,4). Regulation of
Ligninase activity is at the transcriptional level (5,6; R. L. Farrell, unpublished). Because several distinct secondary metabolic events have now been defined in *P. chrysosporium* (4), this organism might be useful for studying regulation of secondary metabolism at the molecular level.

Lignin production by *P. chrysosporium* has been increased substantially through strain improvement and optimization of culture parameters. Activities of enzymes from early cultures were low (about 0.3 µkat L⁻¹). Activities up to 75 µkat L⁻¹ were reported recently for cultures of a mutant strain grown in an optimized medium (7), and further increases can be expected. Part of the problem in producing higher levels of ligninase has been that the cultures are limited by nitrogen (or carbon) in the amount of protein they can synthesize. Recently, however, Kuwahara and Asada (8) developed a mutant strain that produces ligninase under conditions of nutrient luxury. The maximum amount of extracellular protein that can be produced by *P. chrysosporium* under such conditions is not yet known.

**Molecular biology** Ligninase exists as multiple isoenzymes. Up to 15 having been reported in strain BKM-F-1767 (9). Published complementary DNA (cDNA) sequences show that at least three closely related structural genes for ligninase exist in strain BKM-F-1767 (6,10); unpublished results point to more (R. L. Farrell, personal communication; D. Cullen, personal communication). Some isoenzymes might represent posttranslational modification of single gene products. Six isoenzymes were found to have somewhat different kinetic parameters but basically the same catalytic properties (11).

The three published cDNA sequences for ligninase genes in *P. chrysosporium* strain BKM 1767 show homology in excess of 71%. The sequences indicate mature polypeptides of 344, 344, and 345 amino acids, with molecular weights of approximately 37 000, and homologies in excess of 68%. The native, glycosylated enzymes have molecular weights of about 42 000. The structural genes are preceded by leaders of 27 or 28 amino acids, consistent with the fact that ligninase is secreted. Ligninase expressed by *Escherichia coli* is not catalytically active, but Farrell (11) was able to reconstitute active enzyme by inserting the protoheme IX moiety in vitro.

Smith et al. (12) recently cloned and sequenced a native ligninase gene from BKM-F-1767. They found the structural gene to be interrupted by eight introns ranging in size from 49 to 69 base pairs. The sequence of the coding region was identical to that reported by Tien and Tu (6) except for a GC instead of a CG pair at positions 591-592.

**Catalytic cycle** In early studies of the reactions of ligninase with H₂O₂, O₂ and lignin model compounds, O₂ was found to be incorporated into certain C-C cleavage products, causing the enzyme to be referred to as an H₂O₂-requiring oxygenase. Subsequent studies, however, showed that ligninase is a peroxidase. The fifth ligand is histidine, as it is in other peroxidases (13). Tien and Tu (6) showed that certain amino acid residues thought to be essential for peroxidase activity can be identified in ligninase and that these sequences show homology with those of other peroxidases, including horseradish peroxidase (HRP).

The catalytic cycle deduced for ligninase (Fig. 1) is based on its spectral changes during catalysis (compared with those of HRP) and on the reactions catalyzed (13,14)

Although ligninase resembles HRP in its mode of catalysis, it has a higher oxidation potential (15), enabling it to oxidize more substrates, including the nonphenolic nits of lignin. In the catalytic cycle, the resting enzyme (Fe³⁺) is first oxidized by H₂O₂ to Compound I (FeIV=O and the π cation radical of the porphyrin). Compound I is reduced by one electron by a suitable substrate, giving Compound II (FeIV=O) and a substrate cation radical. Alternatively, Compound I can apparently be reduced by two electrons by some substrates, such as veratryl alcohol, giving resting enzyme and a two-electron-oxidized product. Renganathan and Gold (16) also showed that ligninase, like HRP, can be reversibly reduced to the Fe²⁺ form, which is not catalytically active. They showed further that resting enzyme and Compound I can be oxidized by excess H₂O₂ to Compound II (FeIII0²'), which self-destructs (bleaches). Tonon and Odier (17) showed recently that destruction by H₂O₂ is a principal reason for loss of ligninase activity in cultures over time, and that destruction can be prevented by veratryl alcohol. This protection probably is due to the isoprenoid superoxide (O²⁻) by veratryl alcohol. Inverting Compound III back to resting enzyme (M. H. Old, personal communication). Fine-tuning H₂O₂ production and consumption and maintaining active ligninase are obviously critical factors, and they are under investigation in several laboratories.

**Substrate oxidation** Kersten et al. (18) and Hammel et al. (19) established the basic simplicity of ligninase oxidation: susceptible aromatic nuclae are oxidized by one electron, producing unstable cation radicals that undergo a variety of nonenzymatic reactions. Kersten et al. (18) proposed this basic mechanism, accounting for the various reactions, based on direct electron spin resonance (ESR) spectroscopic observations of the cation radicals produced by purified ligninase. At about the same time, Schoemaker et al. (20) suspected a cation radical mech-
anism based on some of the reactions catalyzed. Hammel et al. (19) proved the one-electron mechanism of ligninase action by showing that radical coupling dimers are produced stoichiometrically on anaerobic ligninase cleavage of special model compounds, that carbon-centered radical products can be trapped under anaerobic cleavage, and that peroxy radicals are produced by \( \text{O}_2 \) addition to carbon-centered radicals in aerobic ligninase reactions.

Whether an aromatic ring is a substrate for ligninase depends in part on its oxidation potential. Strong electron-withdrawing substituents such as \( \text{C}_\alpha \)-carbonyl groups inactivate aromatic nuclei to ligninase, whereas alkoxyl groups activate them. The positions of the latter also affect oxidizability by ligninase. In lignin, the positions of the alkoxyl groups are set at positions 3, 4, and 5 (Fig. 2), but the number varies from one to three (Fig. 2); the oxidation rates are expected to be in the order 3 >2>1.

Many reactions follow the initial oxidation to the aryl cation radical (3,4,21). Included are nucleophilic attack by water or internal hydroxyl group, loss of the acidic proton at \( \text{C}_\alpha \), \( \text{C}_\alpha \)-C\( _\beta \) cleavage, addition of molecular oxygen to carbon-centered radicals, one-electron oxidation or reduction, and (in the absence of \( \text{O}_2 \), which scavenges radicals) radical-radical coupling. The reactions that the cation radicals undergo lead to a variety of products, which explains the surprisingly large number of degradation intermediates formed from lignin in wood as it is degraded by white-rot fungi (22).

The nonspecific oxidation of lignin, leading to a variety of subsequent reactions and products, led to the suggestion (4) that the process is really an enzymatic “combustion.” The process differs fundamentally from the enzymatic processes that degrade low molecular weight aromatics and other biopolymers (except perhaps condensed tannins and melanins, which resemble lignin, but whose biodegradations have received little attention).

The diversity of reactions and products can be illustrated with model compounds of the \( \beta-O-4 \) type, which is the dominant lignin substructure (Fig. 3). Ligninase can oxidize Ring A and Ring B. In lignin the two rings might have the same substitution and be equally susceptible; the more accessible ring would be expected to be oxidized first. The arrows in Fig. 3 are labeled A or B, denoting which ring is oxidized to give the indicated reaction sequence. The reactions of Fig. 3 are discussed in references 3, 4, and 21, which provide citations to the original papers. Most of these reaction sequences would fragment the lignin polymer.

Important to note are the aromatic ring cleavages, leading to the products at the bottom of Fig. 3. Early studies based on chemical and physical analyses of the partially biodegraded lignin polymer isolated from decayed wood showed that many aromatic rings are oxidatively cleaved while still bound in the polymer (22). The mechanisms of ring cleavage following lignin oxidation of selected model compounds by ligninase have been deduced by T. Higuchi’s group (e.g., the mechanism shown in Fig. 4) (23). Those mechanisms involve addition of molecular oxygen to carbon-centered radicals in the aromatic nuclei. The mechanisms of ligninase cleavage of aromatic nuclei differ fundamentally from dioxygenase-catalyzed cleavages.

The sequence of reactions at the top of Fig. 3 liberates a phenolic unit. Such phenols, as well as the approximately 10% of the aromatic rings in the unattacked lignin polymer that are already phenolic, have lower oxidation potentials than the nonphenolic rings and would be expected to be preferentially oxidized. The resulting phenoxy radicals are expected to undergo primarily coupling/polymerization reactions: studies conducted to date have indicated that polymerization is the major consequence of ligninase action on lignin in vitro. Whether such polymerization is prevented in vivo by other enzymes or reactive metabolites, or whether it simply goes to completion and is ultimately overcome by depolymerizing reactions, is not yet known. Two enzyme systems, cellobiose-quinone oxidoreductase + cellobiose, and glucose-1-oxidase + glucose, have been suggested to reduce phenoxy radicals and thereby prevent polymerization, but Odier et al. (24) showed very recently by ESR experiments that this is not the case.

**Manganese Peroxidase**

A peroxidase activity different from ligninase’s was discovered by M. H. Gold’s group in 1984, and by R. L. Crawford’s group shortly afterwards, in extracellular growth fluid of ligninolytic cultures of *P. chrysosporium*. Both groups (25,26) later purified and characterized the enzymes as manganese peroxidases. Like ligninase, Mn peroxidase requires \( \text{H}_2\text{O}_2 \), contains a single protoheme IX with high-spin ferric ion, and is a glycoprotein. The 46 000 glycoprotein oxidizes Mn\( ^{IV} \) to Mn\( ^{III} \), which in turn oxidizes phenols to phenoxy radicals. Activity depends in the presence of various acids, which chelate and stabilize the Mn\( ^{III} \); lactic and tartaric acids have been used primarily. Leisola et al. (9) separated six Mn peroxidase isoenzymes from the extracellular fluid of *P. chrysosporium*. The DNA and polypeptide sequences have not yet been reported.

Gold’s group showed that Mn peroxidase has essentially the same catalytic cycle as ligninase and HRP, involving three oxidation states: resting state (Fe\( ^{III} \)), Compound I \( \text{Fe}^{IV=O} + \text{porphyrin} \pi \text{cation radical} \), and Compound II (Fe\( ^{III}=\text{O} \)) (26). Like the other two peroxidases, Mn

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**Figure 2** Lignin is a polymer of phenylpropanoid units joined by C-C and C-O-C linkages. It is formed by the oxidative free radical polymerization of \( p \)-hydroxycinnamyl alcohols. This structure illustrates the major substitution patterns and the commonly used designations of the carbon atoms.
Figure 3  Products of oxidation of \( \beta-O-4 \) lignin model compounds by ligninase/H\(_2\)O\(_2\). Oxidation can be in Ring A or Ring B, as noted by the arrows. The scheme is based on results from several laboratories.

Peroxidase can be reduced to the Fe\(^{II}\) state and converted to the Compound III (Fe\(^{III}\)O\(_2^-\)) state. The catalytic cycle involves oxidation of resting enzyme to Compound I by H\(_2\)O\(_2\), then two sequential reductions by Mn\(^{II}\), first to Compound II and then to resting enzyme. Interestingly, Compound I can also be reduced to Compound II by phenols, but Compound II cannot be reduced to resting enzyme by phenols (26). Thus the enzyme requires Mn\(^{II}\) to complete its cycle.

The possible role of Mn peroxidase in lignin degradation is not yet clear, despite the attention it has received. Its oxidation of phenols to phenoxy radicals has the potential to lead to limited degradation of lignin (21), but such reactions are unlikely to offset polymerization reactions. Mn peroxidase has a second catalytic activity, which, perhaps significantly, might have a role in generating extracellular H\(_2\)O\(_2\), as described in the following section.

**H\(_2\)O\(_2\)-Producing Enzymes**

Four intracellular and two extracellular enzymes have been implicated in H\(_2\)O\(_2\) production by ligninolytic cultures of *P. chrysosporium*. The four intracellular enzymes are glucose-1-oxidase (27) and glucose-2-oxidase (28), both of which are associated with secondary metabolism: fatty acyl coenzyme A oxidase (29); and methanol oxidase (8,30). As mentioned previously, one of the extracellular enzymes is Mn peroxidase, which was shown by Paszczynski et al. (25) to oxidize various reduced substrates, including glutathione, reduced nicotinamide adenine dinucleotide phosphate (NADPH), and dihydroxymaleic acid, with the coupled reduction of O\(_2\) to H\(_2\)O\(_2\). The second extracellular activity is a recently discovered secondary metabolic enzyme, glyoxal oxidase, which couples oxidation of \( \alpha \)-hydroxycarbonyl and \( \alpha \)-dicarbonyl metabolites with reduction of O\(_2\) (31). In summary, extracellular H\(_2\)O\(_2\) might be supplied by several different oxidases, although secretion of H\(_2\)O\(_2\) produced intracellularly has not been demonstrated.

**FUTURE DIRECTIONS**

Research is bringing the key features of the ligninolytic system of *P. chrysosporium* into ever sharper focus, but important questions remain. An obvious question is how to reconstitute a system in vitro that duplicates the in vivo depolymerization of lignin. Other questions concern the possible roles of the Mn peroxidase system. Further
research is needed to identify the physiological \( \text{H}_2\text{O}_2 \)-producing system or systems. Intriguing questions surround the possible roles of veratryl alcohol, which has been suggested to be a mediator of ligninase oxidation (32) in addition to its apparent roles as ligninase inducer and ligninase protector. Considerable research emphasis in the near future will be on the molecular biology of lignin degradation. Such studies will provide insight into the synthesis of the extracellular hemeproteins, and into regulation of the ligninolytic system. Specific gene inactivations can be used to clarify the relative importance of the various enzymes. Other research needs have been summarized recently (4).

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**REFERENCES**


22. Chen C-L, Chang H-m, Chemistry of lignin biodegradation.