PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

Lignin-Degrading Enzymes [and Discussion]

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Lignin-degrading enzymes

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The substantial potential applications of lignin-degrading microbes and enzymes have spurred research on lignin biodegradation in recent years. As described here, that research has led to the discovery in the basidiomycete *Phanerochaete chrysosporium* of the first lignin-degrading enzymes and elucidation of their mode of action. A family of powerful extracellular peroxidase isoenzymes has been the focus of most investigations. The key catalytic reaction of these glycoproteins, in the presence of hydrogen peroxide, is one-electron oxidation of aromatic nuclei, generating unstable aryl cation radicals. These decompose via a number of reactions, which have been elucidated with dimeric model compounds for lignin. The involvement of carbon-centred and peroxyl free-radical intermediates has been established. The peroxyl intermediates result from the addition of molecular oxygen to the C-centred radicals. Strong evidence for a classical peroxidase-type catalytic cycle of the ligninases has been obtained. The major research need is to identify the full complement of enzymes needed to degrade lignin to small fragments; this degradation is not accomplished by the isolated ligninases or by the crude extracellular mixture of enzymes secreted by cultures as they degrade lignin.

Introduction

Potential applications of the microbes and enzymes that degrade lignin are substantial. They include pulping wood, bleaching pulps, modifying fibre surfaces of mechanical pulps, converting by-products lignins to useful chemicals, upgrading feeds, freeing the carbohydrates in lignocellulosics for bioconversions, and treating various lignin-derived wastes. Recognition of these potentials during the last decade, coupled with the general enthusiasm for things 'biotechnical', has promoted funding for lignin biodegradation research, and accelerated the rate of research progress. In the past four years, basic research has resulted in the discovery of the first lignin-degrading enzymes and the elucidation of their mode of action. My purpose in this brief overview is to review these advances, and to discuss research needs.

The nature of lignin and its location within and between the cell walls of woody plants are now generally known. It is sufficient to remind the reader that lignin makes up 20–30% of most woods, and 5–15% of most agricultural crop residues. It is a complex, polydisperse polymer composed of phenylpropane units linked through five to six major types of C—C and C—O—C bonds. The aromatic rings in this natural plastic are substituted with methoxyl groups, and the interunit linkages lack stereoregularity. For reviews on lignin, the reader is referred to Adler (1977), Janshekar & Fiechter (1983), Saka & Goring (1985), and Sarkanen & Ludwig (1971). The polymer is a formidable substrate for microbes; indeed, its degradation apparently does not provide sufficient energy to support growth of the degrading organism. Even so, lignin is decomposed readily by certain microbes in the presence of carbohydrate

energy sources. Recent investigations in Switzerland have demonstrated surprisingly high rates of lignin breakdown by the fungus *Phanerochaete chrysosporium* (Ulmer *et al.* 1984); the rates are comparable with those seen with less formidable biopolymers such as cellulose.

Phanerochaete chrysosporium is a higher basidiomycete, one of a large group of white-rot wood-decay fungi and litter-degrading fungi. These organisms appear to be major degraders of lignin. *Phanerochaete chrysosporium* has properties that make it attractive for research. It is unusual among white-rot fungi in having a high temperature optimum for growth (40° C), in growing and degrading lignin rapidly, in producing copious asexual spores, in readily producing the sexual fruiting structure in the laboratory, and in having no laccase activity. Consequently, it has been studied widely, and today far more is known about its degradation of lignin than about that of any other microbe.

Research on lignin degradation by this fungus began in the early 1970s. That work has resulted in a description of the following: (*a*) chemical changes in lignin and identification of low molecular mass products formed from lignin during its biodegradation (Chen & Chang 1985); (*b*) the culture parameters important for complete mineralization of lignin, and the attendant physiology (Kirk & Shimada 1985); and (*c*) the pathways of initial degradation of lignin model dimeric compounds (Higuchi 1985). Here I discuss subsequent research that has resulted in discovery and characterization of the first lignin-degrading enzymes and their mode of action. It is useful by way of introduction, however, to review the model-compound work that made the discovery of ligninases possible.

Degradation of lignin model compounds by ligninolytic cultures of *P. chrysosporium*

Considerable effort in several laboratories in the early 1980s went into describing the degradative pathways of dimeric model compounds by cultures of *P. chrysosporium* grown under conditions optimized for complete mineralization of lignin (Higuchi 1985). The objective was to define specific reactions that could be used as probes (assays) for lignin-degrading enzymes. Many different models were investigated, and their pathways of degradation were deduced from product identification. The initial degradative reaction of one type of model provided the desired probe that led to discovery of the lignin-degrading enzymes. That type of model represents the β-1, or 1,2-diarylpropane-1,3-diol substructure of lignin. The useful reaction, C_α–C_β cleavage (= C_1—C_2 cleavage) in the propyl side chain, was already known to be important in the fungal degradation of lignin from studies of the degraded lignin polymer (Chen & Chang 1985). Even in studies with the intact cultures, that cleavage reaction revealed some unusual biochemical features (Nakatsubo *et al.* 1982; Nakatsubo & Kirk 1983; figure 1): (*a*) the reaction proceeded with a lack of stereoselectivity: the synthetic models each were mixtures of four stereoisomers, and all were degraded at the same rate; (*b*) formation of a C_α–C_β double bond or a C_α-carbonyl group was ruled out by retention of deuterium at C_α and C_β in the products; (*c*) the reaction appeared to be oxygenase catalysed, in that oxygen was incorporated from O_2 at C_β, but hydroxylation before cleavage was ruled out by the deuterium retention. Some of these features were puzzling, but we had in any event the basis for a simple enzyme assay.

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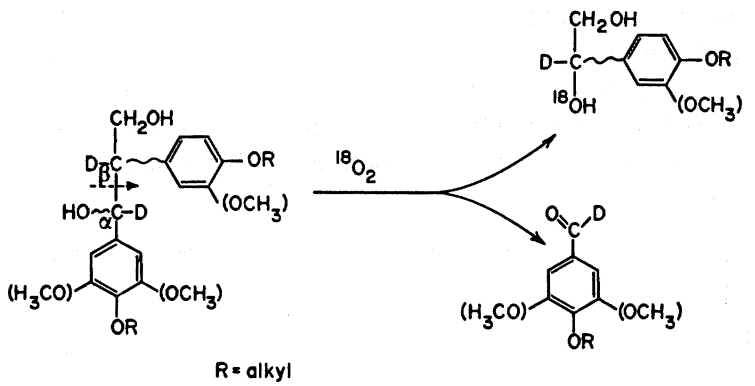

FIGURE 1. Lignin dimeric model compounds of the 1,2-diarylpropane-1,3-diol type are degraded by *Phanerochaete chrysosporium* via an initial cleavage between C_α and C_β in the propyl side-chains. Cleavage yields an aromatic aldehyde product from the C_α moiety, and a phenylglycol product from the C_β moiety. Studies with several different models (summarized in this scheme) provided insight into the mechanism. The facts that the new hydroxyl group in the phenylglycol product is derived from molecular oxygen, that the hydrogens (deuteriums) on C_α and C_β are retained during cleavage, and that the cleavage exhibits no stereoselectivity are illustrated.

DISCOVERY AND CHARACTERIZATION OF LIGNIN-DEGRADING ENZYMES

By using formation of the aromatic aldehyde product from a β-1-type of model as assay, we found the responsible enzyme in late 1982 (Tien & Kirk 1983). Glenn *et al.* (1983) reported the same or a closely related enzyme. Both groups found that the activity occurred in the extracellular culture fluid of *P. chrysosporium*, and required H_2O_2 for activity. We looked for the enzyme in the extracellular culture fluid because the chemical studies had indicated that C_α–C_β cleavage occurs *in the lignin polymer*, and therefore outside the cells (Kirk & Chang 1975). The suggestion that H_2O_2 might be required followed from separate studies showing that extracellular H_2O_2 is produced by ligninolytic cultures of *P. chrysosporium* and that it is required for lignin degradation (Faison & Kirk 1983; Kutsuki & Gold 1982). Like the reaction in intact cultures, the reaction catalysed by the enzyme resulted in incorporation of oxygen from O_2 into the phenylglycol product of cleavage (Tien & Kirk 1984). It was particularly important that the new enzyme, isolated initially by native gel electrophoresis, partly depolymerized methylated lignin, releasing aromatic aldehydes indicative of C_α–C_β cleavage (Tien & Kirk 1983).

We then purified the enzyme by ion-exchange chromatography (Tien & Kirk 1984); this process was straightforward because it is the dominant extracellular protein in glucose-grown cultures. We have referred to the enzyme simply as ligninase. Gold *et al.* (1984) also purified an enzyme that is the same or a very similar protein, and refer to it as 'diarylpropane oxygenase'.

The purified ligninase was found to contain a single protohaem IX prosthetic group, and to have a molecular weight of approximately 42000 and an isoelectric point of 3.5 (Tien & Kirk 1984). It is a glycoprotein, with about 15% carbohydrate (consisting of the neutral sugars mannose, xylose, glucose and galactose (M. Tien, T. K. Kirk & R. Farrell, unpublished results), and presumably glucosamine. The enzyme is apparently a classical peroxidase, albeit an especially powerful one.

In recent work, Renganathan *et al.* (1985), Leisola *et al.*(1985) and we (Kirk *et al.* 1986*a*)

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have shown that there are in fact several haemoproteins with ligninase activity. Kirk *et al.* (1986*a*) purified six ligninase proteins from the extracellular fluid of *P. chrysosporium*, by using anion-exchange HPLC (figure 2). Our unpublished work shows that these multiple ligninases all have similar catalytic activity and similar molecular masses, are all glycoproteins, and all cross-react with a polyclonal antibody to the dominant isoenzyme. They differ somewhat in

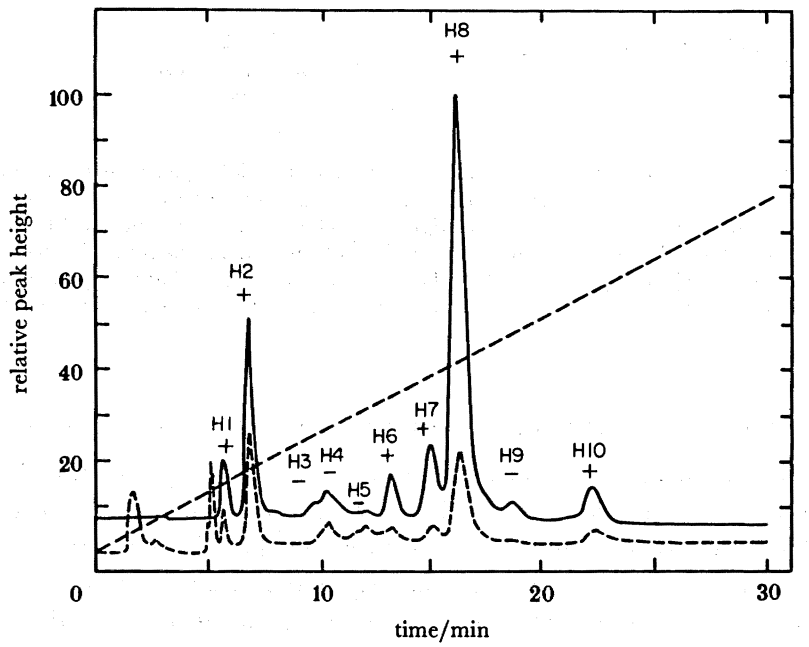

FIGURE 2. High-performance liquid chromatography profiles of extracellular fluid from a five-day flask culture of *P. chrysosporium* strain BKMF-1767. Column effluent was monitored at 409 nm (—), at which haems absorb strongly, and at 280 nm (-----) for total protein. Haemoprotein peak designations (H1-H10) are given (Kirk *et al.* 1986). Ligninase peaks are indicated by '+'. The sloping line shows the acetate gradient used in the chromatography (Kirk *et al.* 1986).

specific activities and in other properties, as discussed by R. Farrell (this symposium). Renganathan *et al.* (1985) isolated three ligninase proteins and found them to be similar in molecular mass, to be glycoproteins, to have similar catalytic activities, and to differ in specific activities. Leisola *et al.* (1985) separated four ligninase proteins by isoelectric focusing, but did not characterize them further. The dominant isoenzyme in our work, H8 (figure 2), is the one that we originally purified (Tien & Kirk 1983). As shown in figure 2, four haemoproteins (absorbing at 409 nm) without ligninase activity are also resolved by this chromatographic procedure (H3, H4, H5, H9). We have found in unpublished work that these proteins have the peroxide-dependent Mn^{2+}-oxidizing activity described by Kuwahara *et al.* (1984) and by Huynh & Crawford (1985). They are discussed below in the section on research needs. Finally, a few non-haem proteins of unknown catalytic activity are also resolved (figure 2); the haemoproteins, however, are clearly the dominant extracellular proteins.

Mode of action of ligninase on its substrates

The purified ligninases catalyse a variety of seemingly disparate oxidations in addition to the originally described C_α–C_β cleavage (Gold *et al.* 1984; Tien & Kirk 1984; figure 3). All the reactions require H_2O_2, and some involve hydroxylation with molecular oxygen (C and E, figure 3). Seeking an underlying mechanism for ligninase, therefore, did not seem at first to be straightforward. However, the variety of reactions catalysed suggested that radical

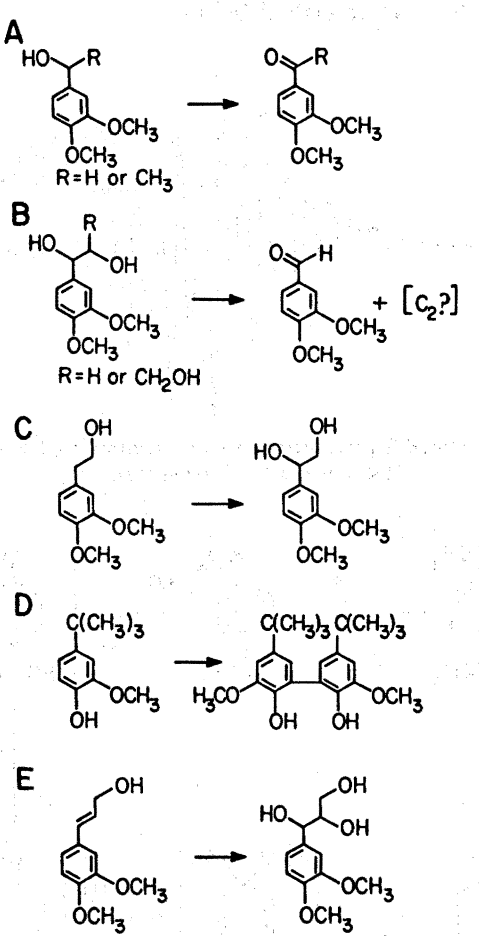

Figure 3. Purified ligninase H8 catalyses several different reactions in lignin-related compounds, in addition to the C_α–C_β cleavage shown in figure 1. Reactions C and E involve O_2, and all require H_2O_2.

chemistry might be involved (Kersten *et al.* 1985). This proved to be the case, and the investigation of the radical reactions involved has provided insight into the underlying mechanism of ligninase. The discovery that radicals are involved was made with surprisingly simple ligninase substrates.

Thus, we found that certain methoxybenzenes are readily oxidized by ligninase (Kersten *et al.* 1985). Several of the methoxybenzene congeners are oxidized by one electron to aryl cation radicals, which are relatively stable at the low pH optimum for ligninase (pH 2.5–3). The diagnostic electron-spin resonance (ESR) spectrum for the cation radical from ligninase oxidation

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of 1,2,4,5-tetramethoxybenzene is shown in figure 4. Cation radicals from the methoxybenzenes are not stable, and decompose. The 1,4-congener, for example, decomposes by reacting with water, yielding methanol and *p*-benzoquinone. We suggested a mechanism for that reaction, and have since verified (P. J. Kersten & T. K. Kirk, unpublished results) the involvement of water in the reaction as originally postulated (figure 5). This mechanism indicates that alkoxyl groups other than methoxyls will be eliminated as the corresponding alkanol, which has proved to be the case. Thus *p*-diethoxybenzene yields ethanol (Kersten *et al.* 1985), and recent work shows that the glyceryloxy substituent in lignin can be eliminated (Kirk *et al.* 1986*b*), as described below.

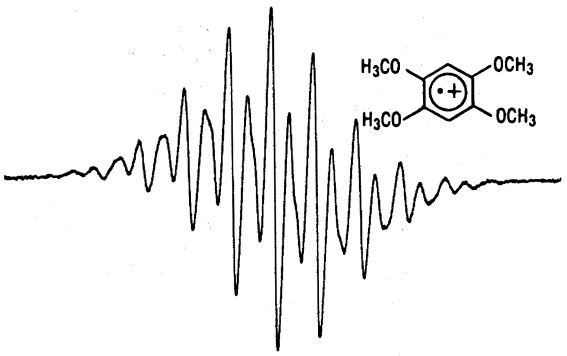

FIGURE 4. The ESR spectrum obtained from reaction mixtures containing 1,2,4,5-tetramethoxybenzene, ligninase H8, and H_2O_2 (Kersten *et al.* 1985).

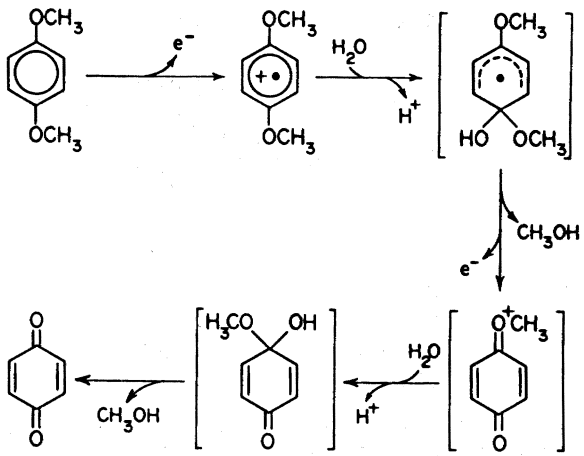

FIGURE 5. Scheme showing two sequential one-electron oxidations of 1,4-dimethoxybenzene and addition of water with loss of methanol, following oxidation by ligninase–H_2O_2 (Kersten *et al.* 1985). The intermediate cation radical and products *p*-benzoquinone and methanol have been identified.

Kinetic evidence indicates that the cation radicals are the direct products of ligninase oxidation, rather than products of a reverse disproportionation reaction following an initial two-electron oxidation (Kersten *et al.* 1985). A true steady-state radical concentration is observed, and that concentration varies linearly with ligninase concentration.

Interpretation of the results with the methoxybenzenes was made relatively easy by reference to literature descriptions of related cation radicals and their decomposition products in chemical

systems. A key reference was the work of Snook & Hamilton (1974), who studied the one-electron oxidation of phenylalkanols by Fenton's reagent or by peroxydisulphate. Their work in particular led us to propose that most, if not all, ligninase-catalysed reactions involve cation-radical intermediates (Kersten *et al.* 1985). A similar suggestion was made by Schoemaker *et al.* (1985). Our subsequent work has supported this interpretation of the ligninase mechanism.

Evaluating the mechanism of lignin model degradation by ligninase, however, was not straightforward. We knew that H_2O_2 was consumed during all reactions, and that, in some, O_2 was consumed as well. The stoichiometries were complicated by the fact that the models underwent sequential branching reactions with ligninase. To circumvent these problems, we prepared a new dimer, 1-(3,4-dimethoxyphenyl)-2-phenylethanediol, that is degraded readily by ligninase to give only two products, veratraldehyde and benzaldehyde (Hammel *et al.* 1985). Because ligninase does not degrade unsubstituted benzyl moieties, we knew that enzyme attack could only be on the dimethoxy-substituted aromatic ring. Thus we had a relatively simple system for clarifying the mechanism of the important C_α–C_β cleavage.

We found that cleavage proceeded aerobically or anaerobically (Hammel *et al.* 1985). Anaerobically, a stoichiometric amount of H_2O_2 is required, whereas in air, a substoichiometric amount of H_2O_2 is required, but a compensating amount of O_2 is consumed. Work with spin-trapping agents and ESR spectrometry showed the presence of carbon-centred α-hydroxybenzyl radicals during the anaerobic cleavage reaction, and of peroxyl radicals and superoxide anion radicals during cleavage in air. A radical spin-trapping agent decreased the amount of benzaldehyde but not veratraldehyde formed during ligninase cleavage, clearly by trapping the intermediate α-hydroxybenzyl radical, which would ordinarily decompose to benzaldehyde (Hammel *et al.* 1985). This indicated that free radicals are involved in the cleavage and are not incidental.

On the basis of these various experiments, a scheme was formulated for the reactions following ligninase oxidation of the special dimer (Hammel *et al.* 1985; figure 6). Initial formation of a cation radical from the methoxylated aromatic ring is followed by spontaneous C_α–C_β cleavage, with formation of an aromatic aldehyde, and an α-hydroxybenzyl radical. The major pathway (the right branch in figure 6) involves formation of veratraldehyde and elimination of the α-hydroxybenzyl radical itself. However, the ESR results indicate that some of the α-hydroxy-3,4-dimethoxybenzyl radical is also formed (the left branch in figure 6). In the absence of oxygen (and of spin-trapping agent), the α-hydroxybenzyl radicals are further oxidized, perhaps by ligninase, to the aromatic aldehyde. When present, O_2 adds to the radical as shown to give an α-hydroxylbenzylperoxyl radical, which evidently decomposes with elimination of superoxide anion radical. The latter dismutates, yielding H_2O_2; this accounts for the consumption of less than stoichiometric quantities of added H_2O_2 in aerobic reactions.

The involvement of radical intermediates in C_α–C_β cleavage has now been confirmed with additional special model compounds (Hammel *et al.* 1986). These models (figure 7) differ from the preceding one in having no hydroxyl group at C_β, and instead having 0, 1, or 2 methyl groups at C_β. The rationale for the methyl substitution was to stabilize the C_β-centred radicals expected from cleavage. Degradation of these models under both anaerobic and aerobic oxidation by ligninase–H_2O_2 was studied. The C_β-centred radicals formed were identified by ESR spectrometry. The key results from studying the products were the following (figure 7): (*a*) methyl groups at C_β indeed stabilized the C_β-centred radicals: the model with no methyl

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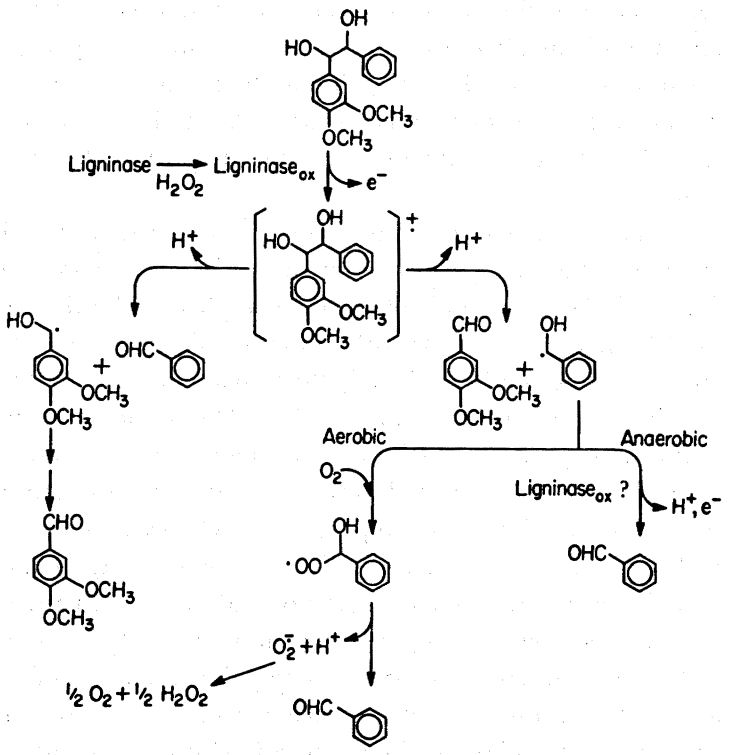

FIGURE 6. Proposed scheme for aerobic and anaerobic cleavage of 1-(3,4-dimethoxyphenyl)-2-phenylethanediol by ligninase H8. Reactions of the α-hydroxy-3,4-dimethoxybenzyl radical, indicated at the left with two arrows, are proposed to be analogous to those shown at the right for the α-hydroxybenzyl radical.

group at C_β was in part oxidized at C_α and in part C_α–C_β cleaved; (*b*) under anaerobic conditions, coupling of the C_β-centred radicals to give diphenylalkanes was quantitatively important; and (*c*) the β,β-dimethyl-substituted radical added oxygen to form (after a one-electron reduction, perhaps by the enzyme) cumene hydroperoxide, which was identified as a major product. These results establish the intermediacy of carbon-centred and peroxyl radicals at C_β in C_α–C_β cleavage by ligninase.

Having established the involvement of radicals in C_α–C_β cleavage, and having gained insight into the roles of H_2O_2 and O_2, we turned our attention to model compounds containing the major interunit linkage found in lignin: the β-*O*-4, or arylglycerol-β-aryl ether linkage (Kirk *et al.* 1986*b*). We sought to predict and interpret the results of ligninase oxidation of such models based on the above results. The β-*O*-4 ether models received considerable attention in elegant work by Umezawa & Higuchi (1985*a–d*), who studied their degradation in intact ligninolytic cultures of *P. chrysosporium*. The effect of purified ligninase H8 on such models had also been examined to some extent in our laboratory (Tien & Kirk 1984). The β-*O*-4 substructures present an interesting problem for ligninase-catalysed degradation, because oxidation of the aromatic nucleus in the arylglycerol moiety is expected to lead to C_α–C_β cleavage or to C_α-oxidation, as we found with purified ligninase (Tien & Kirk 1984), whereas oxidation of the other nucleus could lead to 4-*O*-dealkylation, as Umezawa & Higuchi (1985*a–d*) have studied. We have attempted to assess the relative importance of these two consequences of ligninase catalysis with a series of specially designed model compounds. The consequences of ligninase oxidation are illustrated in figure 8. We observed C_α–C_β cleavage, C_α-oxidation,

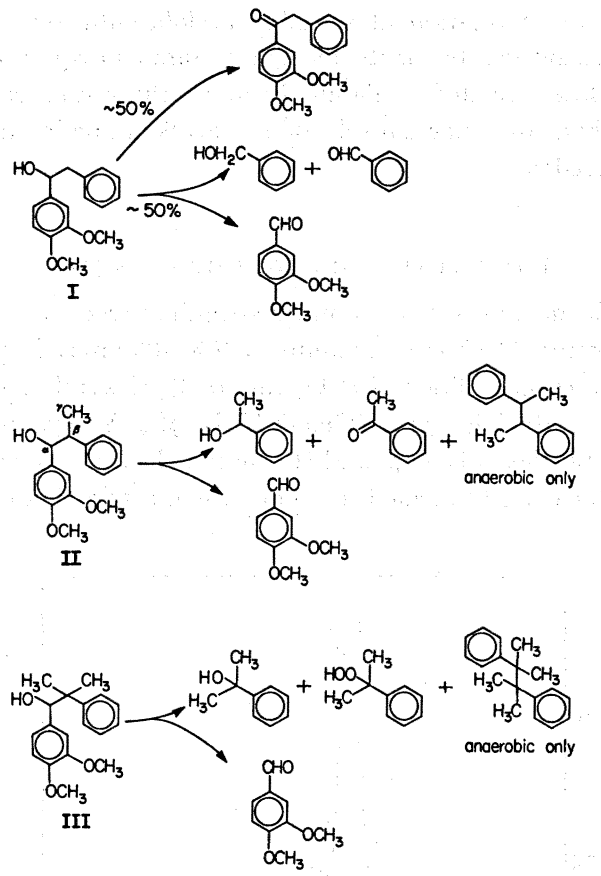

FIGURE 7. Reaction products formed on ligninase H8-catalysed oxidation, in the presence of H_2O_2, of three diarylpropane-type dimer model compounds under aerobic and anaerobic conditions. The models differ only in the number of methyl groups at C_β (Hammel *et al.* 1986).

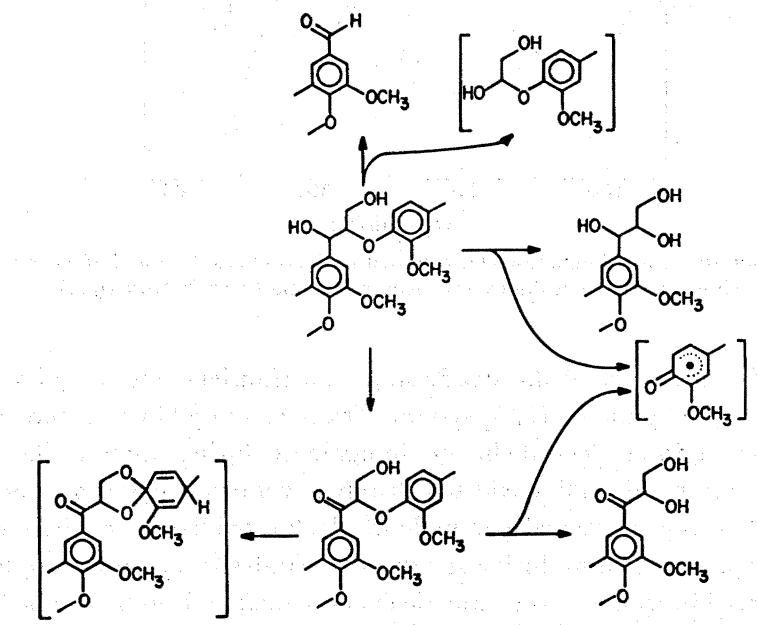

FIGURE 8. Oxidation of the arylglycerol-β-aryl ether substructure by ligninase H8–H_2O_2 leads to several different products via more than one pathway. The major pathway is C_α–C_β cleavage, shown at the top. Compounds in brackets are unstable; that at lower right is hypothetical. The scheme is based on results described in Kirk *et al.* (1986).

aryl-*O*-dealkylations, and formation of unstable cyclohexadienones. Our results indicate, however, that C_α–C_β cleavage is by far the most important consequence of ligninase oxidation in the models most closely related to lignin. Even so, the results of Umezawa & Higuchi (1985*a–d*) point clearly to an important role for 4-*O*-dealkylation in intact cultures, indicating that further study is needed.

The catalytic cycle of ligninase

Initial spectral studies demonstrated that purified ligninase resembles horseradish peroxidase and related haemoproteins. The Soret maximum at 408–409 nm is shifted to 418–420 nm, and the absorptivity decreases on addition of H_2O_2 (figure 9); this shift and decrease are reversed by adding substrates or reducing agents (Tien & Kirk 1984; Renganathan *et al.* 1985). These spectral changes resemble those of compound II (the one-electron oxidized form) of horseradish peroxidase. Further study has confirmed that ligninase is a peroxidase.

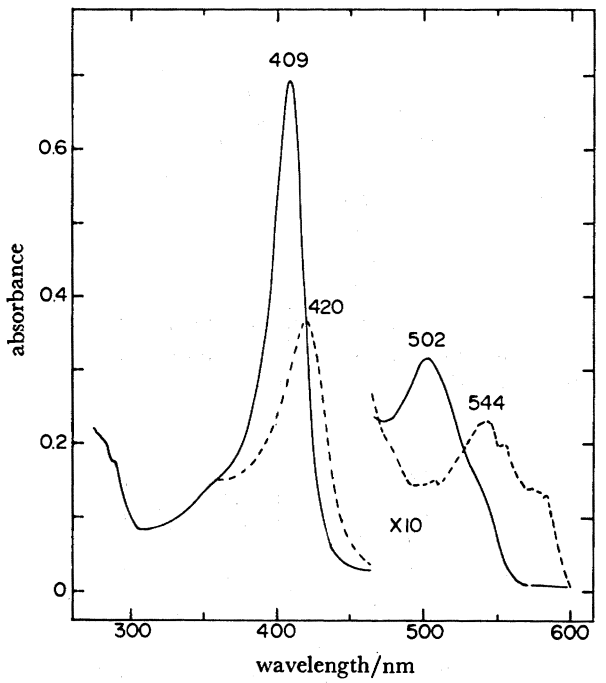

Figure 9. Absorption spectrum of ligninase H8, recorded in the presence (—) and absence (- - - - -) of H_2O_2. Numbers above peaks are wavelength maxima (Tien & Kirk 1984).

Tien *et al.* (1986) investigated the steady-state and transient-state kinetics of oxidation of veratryl alcohol by the ligninase–H_2O_2 system. Transient-state kinetics studies were possible because of the distinct Soret spectral changes in ligninase during catalysis. Results established the following: (*a*) veratryl alcohol is oxidized cleanly to veratraldehyde when present in excess, one mole of H_2O_2 being consumed per mole of alcohol oxidized; (*b*) the enzyme operates through a ping-pong mechanism during steady-state catalysis; (*c*) peroxidase-like compounds I and II are observed in transient-state kinetic experiments; and (*d*) free-radical intermediates are not detected (even though they are with other substrates, as described above). Various kinetic parameters were also determined.

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The results are interpreted to indicate that ligninase reacts first very rapidly with H_2O_2 to form compound I (two-electron oxidized form of peroxidases). Compound I then reacts with veratryl alcohol to form veratraldehyde and resting enzyme. The data indicate that compound II (the one-electron oxidized form of peroxidases) is not involved in this oxidation. Nevertheless, in the absence of veratryl alcohol, compound II is formed in a relatively slow reaction that probably involves oxidation of peroxide by compound I. Whether the oxidation of veratryl alcohol proceeds via one two-electron step, or via two sequential, rapid one-electron steps could not be distinguished. Even so, the previous work with other substrates suggests that the latter possibility obtains. It was also predicted that with substrates such as those discussed in the foregoing, which clearly are oxidized by one electron, compound II will be found to play a role. The conclusion is that ligninase acts in a manner entirely analogous to other peroxidases. Thus the catalytic cycle depicted in figure 10 is probable in ligninase oxidation of lignin. In this cycle, ligninase catalyses either two one-electron oxidations, or one two-electron oxidation; presumably the former dominates.

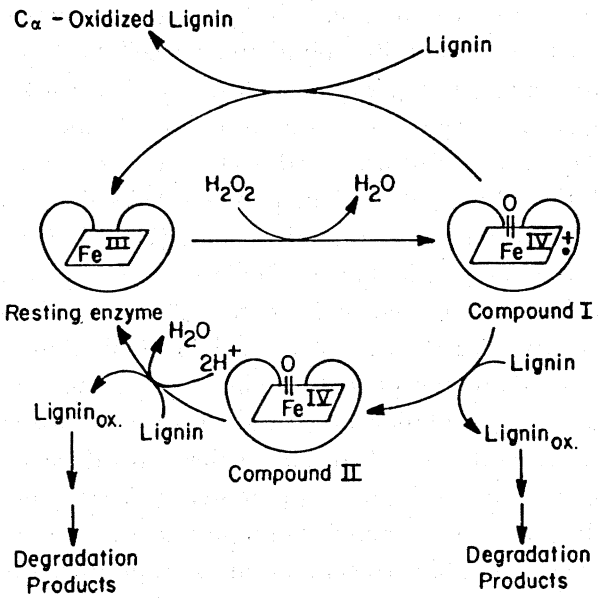

FIGURE 10. Probable catalytic cycle of the peroxidase ligninase. The resting enzyme (left) is first oxidized by two electrons by H_2O_2, forming compound I. Compound I then oxidizes lignin in two one-electron steps, with intermediate formation of compound II (lower), or in a single two-electron oxidation (oxidation of C_α groups) without participation of compound II (upper). The former is probably more important. The scheme is based on several different investigations (see text).

RESEARCH NEEDS

The major research need at present is to identify the full complement of enzymes needed to degrade lignin to assimilable fragments. Ligninase H8 does not accomplish this completely. In fact, it is clear that an important consequence of ligninase action is to produce new phenolic hydroxyl groups from the β-*O*-4 substructures, and that the phenol-oxidizing activity of the enzyme will result in *polymerization* of such structures. It seems clear, therefore, that there must be some activity present to prevent such polymerization. The cellobiose-quinone oxidoreductase ('CBQase') reported by Westermark & Eriksson (1974, 1975) reduces quinones, and perhaps

phenoxy radicals as well, to the corresponding phenols, reversing phenol-oxidizing enzyme activity. Thus when present, CBQase might serve this function. However, CBQase is not always produced during lignin mineralization (Ander & Eriksson 1975; T. Kellerher, T. W. Jeffries & T. K. Kirk, unpublished results) and therefore is not required for the process; in the absence of CBQase, other enzymes with similar activity might be produced.

The enzyme(s) responsible for producing H_2O_2 need to be identified and characterized. Greene & Gould (1984) suggested that fatty acyl-coenzyme A oxidase activity is involved in H_2O_2 production. On the other hand, Ramasamy *et al.* (1985) presented evidence that glucose oxidase is the responsible enzyme. Neither group, however, has demonstrated that *extracellular* H_2O_2 is produced by these enzymes, and it is the extracellular H_2O_2 that must be accounted for. An extracellular Mn-peroxidase purified by Paszczynski *et al.* (1986) oxidizes NADPH, glutathione and other substrates, reducing O_2 to H_2O_2. P. J. Kersten & T. K. Kirk (unpublished results) recently discovered another extracellular enzyme that produces H_2O_2 from O_2 during oxidation of various low molecular weight saccharides. The physiological source of extracellular H_2O_2, however, is as yet unknown.

There is also evidence that enzymes other than the ligninase isoenzymes are involved in attack on the polymer. Huynh & Crawford (1985) found an extracellular H_2O_2-dependent aromatic methyl ether demethylase activity in ligninolytic cultures of *P. chrysosporium*. The substrate on which the demethylating activity was detected was 2-methoxy-3-phenylbenzoic acid; the effect of this activity on more lignin-like substrates needs to be investigated. Other enzymes that are secreted by the ligninolytic cultures also might play a role in lignin degradation; a H_2O_2-dependent esterase active on aromatic acid methyl esters (Huynh & Crawford 1985), and a manganese-dependent peroxidase are included. The latter enzyme has recently been purified and studied by Glenn & Gold (1985) and by Paszczynski *et al.* (1986). It is a protoporphyrin IX haemoprotein that oxidizes Mn^{2+} to Mn^{3+} or a higher oxidation state; the oxidized Mn can, in turn, oxidize various dyes, and phenols to phenoxy radicals. As mentioned above, the enzyme also reduces O_2 during oxidation of certain substrates (Paszczynski *et al.* 1986). As mentioned previously, we recently identified four of these Mn-dependent peroxidases in the culture fluid of *P. chrysosporium* (figure 2); they apparently are isoenzymes. The function, if any, of this Mn-dependent phenol-oxidizing activity in lignin degradation is as yet unclear.

Interestingly, the crude mixture of extracellular enzymes produced by ligninolytic cultures of *P. chrysosporium* does not catalyse the fragmentation of lignin to a greater extent than the purified ligninase H8 (T. K. Kirk, unpublished results). Several possibilities for this can be suggested: (*a*) that some enzyme component is inactivated during preparation; (*b*) that ligninase or other enzyme is inactivated during catalysis; (*c*) that key enzyme components reside on the cell surfaces and are absent in the cell-free preparations; and (*d*) that cofactors produced continuously by the cells are absent in the isolated preparation. We consider possibilities (*c*) and (*d*) to be most likely.

Many other research needs are apparent in the *P. chrysosporium* system. Much more must be learnt about the ligninases themselves; regulation and maximization of their production; properties of the various isoenzymes; the catalytic cycle of one-electron oxidations; the effects of ligninase oxidation on lignin substructures not yet studied; the structure of the active site, in particular the haem environment; the importance of glycosylation; and the amino acid sequences and three-dimensional structures of the enzymes. In addition, the ligninolytic systems of other fungi need to be characterized and compared with that of *P. chrysosporium*.

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Despite these gaps in current knowledge, progress in recent years has been rapid, and we now know a great deal about the lignin-degrading enzymes. Enough is known, in fact, to begin exploring their biotechnical exploitation in some of the many potential applications, as described by R. Farrell in this symposium.

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Discussion

J. M. Lynch (*Glasshouse Crops Research Institute, Littlehampton, West Sussex, U.K.*). Mixed-culture fermentations are potentially useful if a second species could take up products and intermediates that would derepress enzymes. In the cellulase systems, we have been unable to demonstrate this by the addition of a cellobiose-utilizing yeast to *Trichoderma* (D. M. Gaunt, A. P. J. Trinci & J. M. Lynch, unpublished results) but certainly the potential is there. However, mixed culture fermentations will be technically difficult to exploit on an industrial scale, and may not be economic.

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