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The Enzymic Degradation of Lignin by White-Rot Fungi

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The enzymic degradation of lignin by white-rot fungi

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A possible pathway for the depolymerization of lignin is presented. The ether bond is the principal bond resulting in the polymerization of the phenylpropanoid precursors. The ether bond is very stable and unlikely to be degraded during the initial steps of depolymerization of lignin outside of the cell.

Chemical analysis of the breakdown products suggests that the main reactions leading to the depolymerization are the cleavage of the aryl–α-carbon bond or the bond between the α- and β-carbons of the alkyl side chain. Physiological experiments show that high oxygen concentrations stimulate the rate of breakdown of lignin; this could be the result of the activation of an enzyme with low affinity for oxygen such as laccase or polyphenol oxidase. It is also possible that high oxygen concentrations result in the appearance of free radicals of oxygen in the assay system.

It is proposed that laccase in the presence of hydroxyl radicals has the capacity to act as an exoligninase, removing aryl monomers that contain a free phenolic group. The mechanism of depolymerization of derivatives in which the phenolic group actually participates in an ether linkage is less clear. This may be achieved through the action of an extracellular alcohol oxidase that converts the primary alcohol to an aldehyde, resulting in the cleavage of the αC–βC bond. This enzyme may be considered an endoligninase.

Introduction

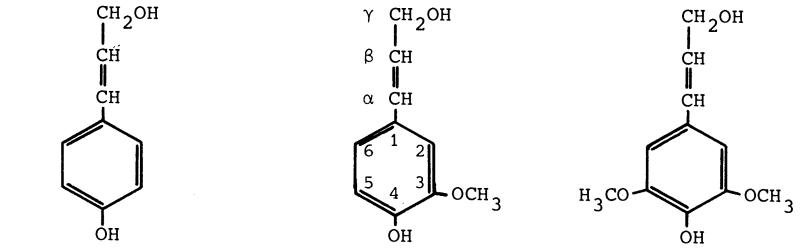
Lignin is a complex three-dimensional biopolymer, synthesized by plants mainly to provide strength and protection. In the natural state it occurs in close association with cellulose as the lignocellulose complex, which represents a very durable material highly resistant to biological degradation. The rate of breakdown of the lignocellulose complex plays an important role in controlling the flow of material through the Earth's carbon–oxygen cycle. Studies on the degradation of lignin are aimed at defining the metabolic pathway by which it is broken down and assessing the relative importance of the processs and the value of the product in the developing field of biotechnology. It is hoped that the knowledge gained will be useful in developing new processes that make fuller use of, and produce new products from, plant material that is currently treated as waste material.

Structure of lignin

It is necessary to appreciate the complex structure of lignin in order to be able to speculate on the nature of the reactions involved in its degradation. Most of our knowledge comes from sophisticated chemical analysis, which, in relation to the secondary products in plants, far outstrips the appreciation of the metabolic events leading to the synthesis or degradation of the molecules involved. The chemical analysis leading to the elucidation of the structure of lignin was pioneered by Freudenberg and coworkers (see Sarkanen & Ludwig (eds) 1971, pp. 165–240) and confirmed by using ^{1}H n.m.r. (Lundquist 1979) and ^{13}C n.m.r. (Nimz & Ludemann 1976) in addition to i.r. and u.v. spectroscopy (see Sarkanen & Ludwig (eds) 1971, pp. 165–240).

The nature of precursors of lignin

The essential features of lignin are most easily understood by considering the process by which the phenylpropanoid precursors are polymerized to form polymeric lignin. Figure 1 shows the principal building blocks of lignin. The lignin of conifers is considered to be a polymer mainly of coniferyl alcohol, whereas, in hard wood such as beech, the coniferyl alcohol



p-coumaryl alcohol coniferyl alcohol sinapyl alcohol

FIGURE 1. Monomeric precursors of lignin.

is polymerized together with equal amounts of sinapyl alcohol. *p*-Coumaryl alcohol, which contains no methoxy groups in the aromatic nucleus, is found in lignin isolated from grasses.

Oxidation of precursors

The initial reaction responsible for the polymerization of the monomers is an oxidation resulting in the removal of one electron and the production of a free radical semiquinone. This oxidation can be achieved by several enzyme systems, all of which exhibit a low level of specificity towards the nature of the phenolic substrate. Peroxidase and hydrogen peroxide would be the most favoured system (Geissman & Crout 1969) while it can also be achieved by polyphenol oxidase or laccase in the presence of oxygen (Geissman & Crout 1969). Both peroxidase and polyphenol oxidase are capable of oxidizing a monophenol and inserting a second phenolic group in a position *ortho* to the original phenol group. The role of laccase in the biosynthesis of lignin is complicated because it is doubtful whether laccase from higher plants, which would be the only form capable of playing a role in the synthesis of lignin, can oxidize monophenols (i.e. exhibit cresolase activity), whereas it is clear that laccase from basidiomycetes, which do not contain lignin and play a role in degrading lignin, readily oxidize monophenolic substrates (Mayer & Harel 1979). Clearly this interesting apparent difference between the different types of laccase requires further investigation.

Oxidative coupling of free radicals

When a diphenolic substrate is oxidized to the free radical it is possible for the free radicals to react with each other and to undergo a disproportionation reaction to yield one mole of quinone and one of diphenol. When the precursors of lignin are oxidized, the removal of a second electron is not possible and the unpaired electron becomes delocalized within the resonating structure of the molecule, which can then react as if the electron were located at either the 4-OH, C3 or C5 positions in the aryl nucleus or in the α or β carbon of the alkyl side chain. These free radicals then react with each other to form covalent bonds between either the C–O or C–C positions. The most favoured product of coupling is an ether bond between the 4-OH and βC of the alkyl side chain (β-O-4 ether) which can be seen between

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the aromatic nuclei 1 and 2 in figure 2, which is a generalized structure of lignin. This type of ether bond forms nearly 50 % of the polymerizing bonds within the lignin molecule (Adler 1977). Other ether bonds also result from oxidative coupling, notably between 4-OH and the α carbon (between derivatives 3 and 13 in figure 2) and the 4-OH and a carbon in the aromatic nucleus (between derivatives 8 and 10 in figure 2). These ether bonds constitute approximately 80 % of the intermolecular linkages between the precursors resulting in the polymerization of

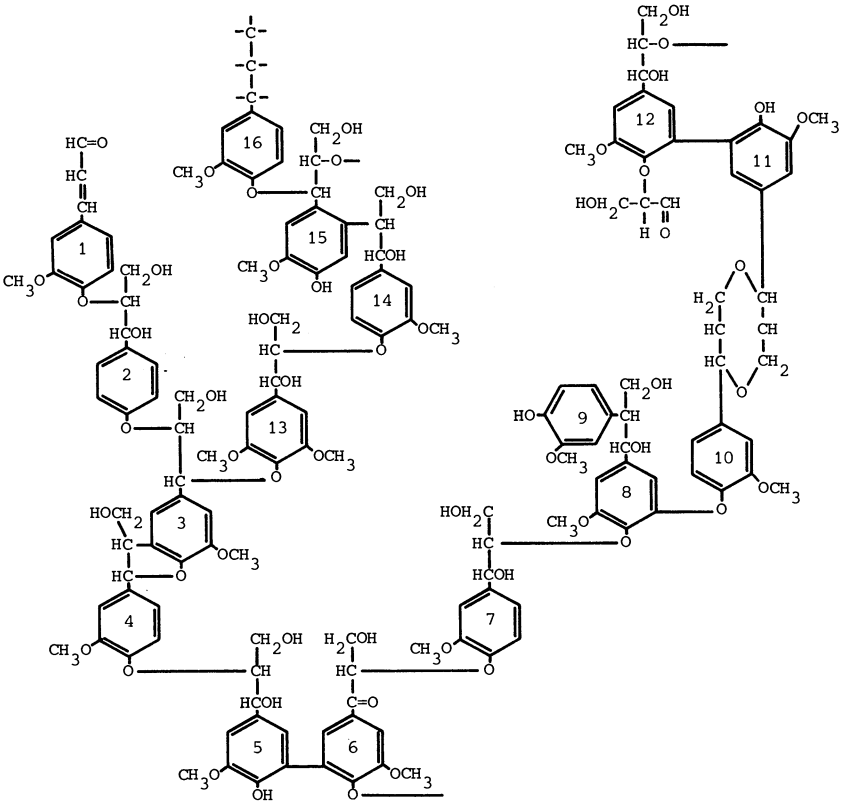

FIGURE 2. Main structural features of conifer lignin (after Adler 1977).

lignin. Two other types of bond that are readily formed during the oxidative coupling of the free radicals are direct aryl–alkyl bonds, as shown between derivatives 8 and 9 (figure 2) forming about 8 % of the polymerizing bonds, and direct coupling between the aromatic nuclei to form biphenyl bonds (between derivatives 11 and 12 in figure 2). These constitute about 10 % of the polymerizing bonds and represent the most stable bonds in the lignin molecule.

Nature of the reactive groups in lignin

The types of reactions involved in the breakdown of lignin will be determined by the nature of the reactive groups within the molecule. The most striking feature of lignin is the high content of aromatic nuclei, which are chemically stable and must be split before lignin can be completely degraded. Ring cleavage is best understood in microbial metabolism where it is achieved by enzymes known as intramolecular dioxygenases, which insert two atoms of oxygen onto carbon atoms of the ring to yield an aliphatic dicarboxylic acid (Nozaki 1979). The dioxygenase enzymes are located inside the cells and are not thought to be excreted to the extracellular medium.

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The second major type of chemical bond is the ether group involved in the polymerizing bonds and the methoxy groups. Ether bonds are very stable, are known to be resistant to acid or alkaline hydrolysis and are not prone to attack by strong oxidizing agents. From a chemical point of view they can be made less stable by inserting a strongly electronegative atom onto one of the carbon atoms participating in the ether linkage. One such element would be oxygen, the hydroxylation of the carbon atom forming a hemiketal bond, which is more readily hydrolysed than the parent ether. Such a hydroxylation could be brought about by a mixed-functional mono-oxygenase attack. These enzymes are often dependent on NADH or NADPH and are not considered to be active in the extracellular medium (Nozaki 1979).

The final chemical grouping of interest is the hydroxyl group of the primary alcohol on the γ carbon, the secondary alcohol on the α carbon and the free phenolic group in the 4 position in the aryl nucleus. The phenolic group is free on only about 20 % of the rings because of its extensive involvement in forming the polymerizing ether bonds. From a biochemical point of view all these hydroxyl groups could be oxidized to form carboxylic acid, ketone or quinone groups.

Because the polymerization of the precursors is non-enzymic and results in the creation of asymmetric centres on both the α and β carbons of the alkyl side chain (see figure 2), the resulting lignin shows no optical activity and is a racemic mixture of isomers. Such a lack of chirality could result in problems for direct enzymic attack because most enzymes exhibit a strict preference for a particular optical isomer, so it might be expected that destruction of the asymmetric centres may occur before enzymic modification.

It is apparent from knowledge of the structure of lignin that oxidative processes are likely to be favoured in the destruction of lignin.

Organisms that degrade lignin

Precise knowledge about the range of organisms that can degrade lignin remains unclear and is difficult to establish because of the experimental difficulties. However, the ability to degrade lignin rapidly does appear to be confined to saprophytic microorganisms, mainly the fungi and actinomycetes.

White-rot and soft-rot fungi

The white-rot fungi are normally considered to be able to cause extensive breakdown of all the structural components of wood, including the cellulose and lignin (Crawford 1981*b*). Most of them are basidiomycetes, although some ascomycetes have also been reported to cause white-rot type decay. *Phanerochaete chrysosporium* (= *Sporotrichum pulverulentum*) and *Coriolus versicolor* are the most extensively studied white-rot fungi, and they degrade lignin and cellulose at equal rates (Rosenberg 1980).

The soft-rot fungi, principally ascomycetes and fungi imperfecti, also cause the breakdown of the wood structure in that they produce a characteristic softening of the surface of the tissue, after attacking the moist wood. There is strong evidence to suggest that they can degrade both lignin and cellulose (Crawford 1981*b*) and in this respect they are similar to white-rot fungi. However, they differ markedly in the micromorphological relation that they have with the cell wall structure. Whereas the hyphae of the white-rot fungi penetrate into the lumen of the cell and degrade the lignocellulose complex while moving over the inner surface, the soft-rot organisms penetrate the plant cell wall and form cavities in the S_2 or middle layer of the

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secondary cell wall parallel to the cellulose microfibrils (Levy & Dickinson 1981). It is unknown whether these differences in behaviour result from differing enzymic mechanisms in the two types of organisms, but the elucidation of the enzymology would be of the greatest interest in helping to understand both the structure of the cell wall and degradation of the lignocellulose complex.

Brown-rot fungi

Brown-rot fungi are usually defined as those wood-rotting fungi that decompose wood by removing the carbohydrates and leaving lignin in a modified but undegraded state. The details of how the lignin is modified and why this increases the accessibility of the cellulose to cellulase attack are unknown, but it is of great interest and value to biotechnology. The brown-rot fungi are taxonomically very similar to the fungi that cause white-rot decay, and are mostly basidiomycetes, for example *Poria placenta* or *Paxillus involutans*.

Bacterial degradation of lignin

Numerous bacteria have been reported to decompose lignin (Crawford 1981*b*). There is clear evidence that actinomycetes (filamentous bacteria) such as *Streptomyces* and *Nocardia* can degrade lignin. However, evidence that other types of bacteria can degrade lignin is questionable. Although many bacteria such as *Pseudomonas* can metabolize monomeric ring structures very effectively, there is little evidence to suggest that they can attack polymeric lignin (Janshekar & Fiechter 1982).

Biodegradation of lignin

Most of our knowledge concerning the pathway of lignin breakdown comes from the study of degradation by the two white-rot fungi, *Coriolus versicolor* and *Phanerochaete chrysosporium*. Most of the data come from a chemical analysis of the breakdown products and modified high molecular mass residues. Little direct enzymology is currently available to help define the metabolic pathway followed. Our current understanding comes from the chemical analysis of the fate of extracted lignin, the use of radioactive lignin and artificially polymerized coniferyl alcohol and, finally, the use of low molecular mass model compounds.

Chemical modification of extracted lignin

The early research in the field concentrated on this approach. One major problem with this method is the selection of a suitable substrate. Most studies use a form of extracted lignin and many of these preparations become highly modified from the natural state when they are extracted (see Crawford 1981*a*). Klason lignin, which is the residue obtained after refluxing the lignocellulose material with dilute sulphuric acid, is highly modified and is therefore a poor substrate for lignin biodegradation studies. Braun's lignin, another form of extracted lignin, is of low molecular mass and is extracted with ethanol. It is unlikely to be extensively modified but is obtained in very low yield and may not be representative of lignin in the tissue. Finally there is milled wood lignin or 'Björkman lignin', which is obtained by ball-milling finely ground wood and extracting the lignin by using a dioxan:water mixture (Björkman 1956). It is of higher molecular mass than Braun's lignin, and so is more likely to resemble native lignin. This form of lignin is now considered to be the best available for these studies (Crawford 1981*a*).

The principal objective in these studies has been to attempt to separate the breakdown

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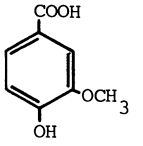
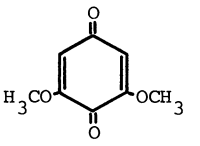
products from the residual lignin and analyse the nature of the chemical modification that has occurred. Results show that only small amounts of aromatic monomers are produced, mainly in the form of vanillic acid and 2,6-dimethoxy-*p*-benzoquinone (figure 3). The 2,6-dimethoxy-*p*-benzoquinone may arise from the cleavage of the bond between the aryl nucleus and the α-carbon in the alkyl side chain. The vanillic acid may arise from a different form of cleavage occurring between the α and β carbons in the alkyl side chain.



vanillic acid



2,6-dimethoxy-*p*-benzoquinone

FIGURE 3. Monomeric breakdown products from biodegraded lignin.

Analysis of the residual polymerized lignin shows that in about one third of the material the molecular mass had increased (Ishihara 1980). This observation shows the very close relationship between the processes of biodegradation and biosynthesis and highlights several important considerations. It is unlikely that it would be possible to obtain a high concentration of monomers as breakdown products because they are liable to be repolymerized. It may therefore be necessary to remove the products of degradation as quickly as they are formed, to establish the maximum rate of degradation. It is also possible that low molecular mass material may be modified and then repolymerized, leading to the mistaken view that such modification had taken place in the polymer.

Spectroscopic analysis of the residual high molecular mass lignin (Kirk & Chang 1975; Crawford 1981*b*) shows that extensive modification has occurred. These changes can be summarized as a loss of methoxyl groups, a decrease in aromatic protons compared with aliphatic protons, an increase in carboxylic groups and the oxidation of the alcohol group on the α carbon to a ketone. A scheme of degradation of the polymer to account for these changes is given in figure 4. Although this scheme adequately explains the chemical changes it does not take into account known enzymology, and at least two major difficulties arise. They relate to the mechanism of hydroxylation at the 2-position in the ring and the process of ring cleavage. The hydroxylation cannot be achieved by the action of laccase (Mayer & Harel 1979), although oxidation by tyrosinase may result in hydroxylation. It is more usual to consider the hydroxylation of aromatic rings to be achieved by attack by a mixed-functional mono-oxygenase, but such enzymes are usually dependent on NAD(P)H, and no evidence is available to suggest that they are extracellular. A similar situation exists with respect to the cleavage of the aromatic ring system, which is normally achieved by an intramolecular dioxygenase enzyme (Nozaki 1979), known to be present in many bacteria and some fungi but always found inside the cell. It is difficult to see how intracellular enzymes can act on highly polymerized lignin.

Studies on isolated, unlabelled lignin have also shown that laccase may be involved in the breakdown of lignin, the best evidence in support of this contention coming from the studies of Ander & Eriksson (1976) who found that a phenoloxidase-lacking mutant of *Sporotrichum pulverulentum* was unable to degrade lignin until purified laccase was added to the culture.

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Kinetic measurements with radioactively labelled lignin

There are two types of labelled lignin now in use, the first being milled wood lignin randomly labelled with ^{14}C obtained by growing the plant in the presence of ^{14}C-labelled phenylalanine. The other labelled substrate is obtained by polymerizing coniferyl alcohol with peroxidase and hydrogen peroxide. This polymer can have the label placed in the methoxy group, the

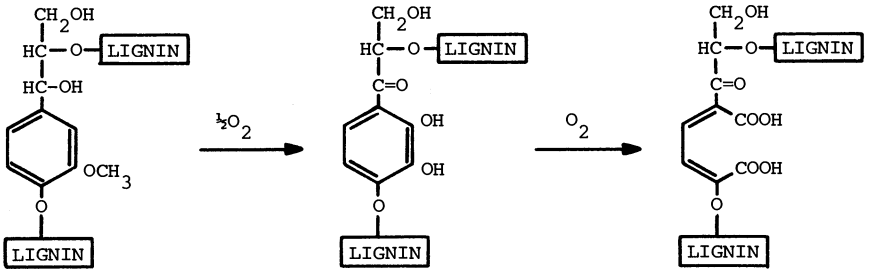

FIGURE 4. Observed structural changes in the residual high molecular mass lignin after biodegradation.

aromatic ring or the alkyl side chain. Artificial polyconiferyl alcohol is considered to be a good model for softwood lignin, and comparative studies suggest that it behaves like milled-wood lignin (Haider & Trojanowski 1980).

The development of these labelled substrates has made it possible to determine the rate at which lignin is converted to carbon dioxide and if it is assumed that the rate-limiting step is the initial depolymerization of lignin then the rate of $^{14}CO_2$ production can be considered to reflect the activity of ligninases. Several important results have come from the use of labelled substrates. The most significant is the observation that in stationary cultures the rate of lignin breakdown is increased dramatically when the oxygen concentration in the gas phase is increased to 100 % (Kirk *et al.* 1978; Bar-lev & Kirk 1981), which is five times that present in air. Oxygen concentrations in this region are often considered to be toxic because of the generation of free radicals of oxygen, but lignin-degrading microorganisms can thrive in a high-oxygen environment for long periods. It is possible that lignin can mop up free radicals before they can damage the fungal cells. The role of high oxygen does not seem to be to induce more enzyme but to make more efficient use of existing enzymes because the high rate of lignin breakdown can be obtained quickly if the oxygen level is increased from 21 to 100 % (Kirk *et al.* 1978). There seem to be two ways in which the stimulation by high oxygen levels can be explained. Firstly, the oxidase responsible may have a very low affinity for oxygen, as with polyphenol oxidase and laccase, which have K_m values for oxygen of 1 and 0.25 mM respectively (Mayer & Harel 1979). Alternatively it could be that the oxidase gives rise to different products in the presence of elevated oxygen levels; especially important in this respect would be the appearance of free radicals of oxygen.

Parallel studies (Kirk *et al.* 1978) also show that lignin breakdown is decreased if the cultures are shaken and that under these conditions high oxygen levels completely prevent lignin degradation, suggesting that failure to establish the correct spatial configuration between the fungus and the substrate prevents the lignin from protecting the living cells from oxygen-induced damage.

Other observations of note to emerge from the use of labelled lignin are that the ability of

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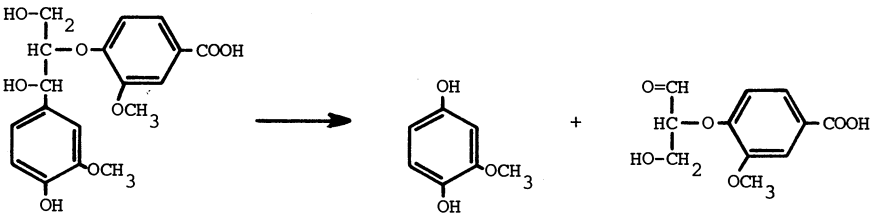
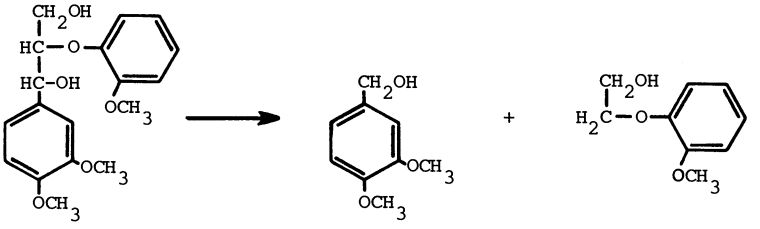
fungi to degrade lignin develops markedly only when the growth rate is limited by lack of nitrogen (Keyser *et al.* 1978) and is repressed by the addition of a nitrogen source (Weinstein *et al.* 1980). These studies also show that white-rot fungi cannot grow on extracted lignin as the sole carbon source, and their ability to degrade lignin is dependent on the presence of a second readily oxidizable substrate such as cellulose, cellobiose or glucose (Kirk *et al.* 1976).



guaiacylglycerol-β-vanillic acid ether

FIGURE 5. Cleavage by *Fusarium solani* of the aryl–alkyl bond in a model lignin dimer containing a free phenolic group (Katayama *et al.* 1980; Higuchi 1981).



3, 4-dimethoxyphenylglycerol-β-guaiacyl ether

FIGURE 6. Cleavage by *Phanerochaete chrysosporium* of the α–β carbon atoms in a model lignin dimer containing no free phenolic groups (Weinstein *et al.* 1980; Enoki *et al.* 1981).

Use of model compounds

Another experimental approach to resolving the way in which lignin is naturally degraded comes from the use of model, low molecular mass dimers or trimers of lignin precursors. The main difficulty in using this technique is to ensure that the conversion being studied is relevant to the degradation of polymeric lignin. Many microorganisms can be shown to degrade dilignols but to be quite inactive in degrading milled-wood lignin (Janshekar & Fiechter 1982).

The fate of two different dimers is of central interest. The first is guaiacylglycerol-β-vanillic acid ether, which is a dimer linked via an arylglycerol-β-aryl ether bond and containing a free phenolic group in one aryl ring (figure 5). This compound is cleaved by a rupture of the aryl-αC bond to yield the products shown in figure 5. Such a reaction could remove the 20% of the aryl rings with free phenolic groups known to exist in milled wood lignin. It is clear that such depolymerization does not alter the ether bond; it consequently does not produce a new phenolic group and cannot be self-sustaining.

3,4-Dimethoxyphenylglycerol-β-guaiacyl ether is a model dimer in which the free phenolic group is protected by a methyl group and may be considered to be a model for a part of the lignin in which all the phenolic groups are involved in ether bonds. Analysis shows that this dimer (figure 6) is depolymerized by a cleavage between the α and β carbon atoms of the alkyl side chain and, once again, the ether bond is left undegraded as would be expected from the stability of the bond.

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These two reactions provide very strong evidence that enzymic systems exist which can result in the depolymerization of polymeric lignin by the cleavage of the aryl-αC or αC–βC bond system.

Speculation on the enzymic nature of lignin depolymerization

Very little firm experimental evidence is available about the direct role of any enzyme system in the initial attack on highly polymerized lignin, but some evidence is available to implicate laccase and an extracellular alcohol oxidase. The speculations presented in this section provide a framework for the design of future experiments.

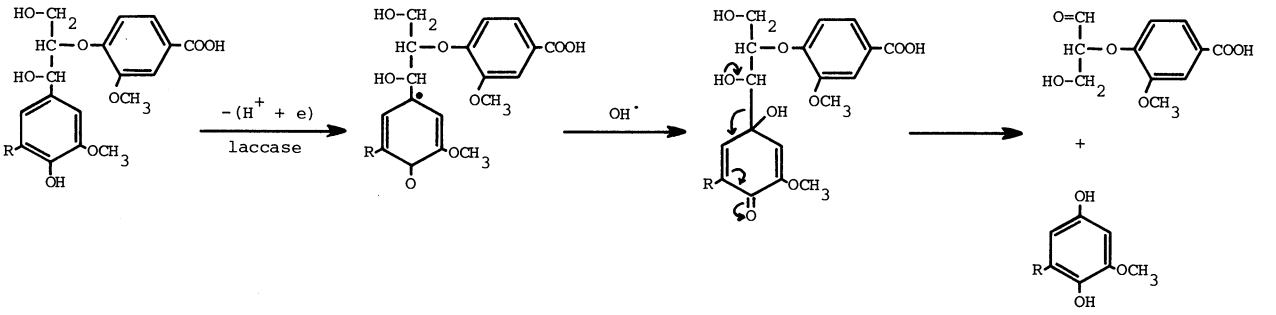

Figure 7. Oxidation of model compounds by laccase and free hydroxyl radicals.

Role of laccase

Laccase has long been implicated in both lignin synthesis and lignin degradation (Geissman & Crout 1969; Crawford 1981*b*). It has a low specificity with respect to the phenolic substrate and a low affinity for oxygen, which fit in with the known physiological behaviour of lignin breakdown (i.e. stimulation by high oxygen concentrations; see the section above on kinetic measurements).

Recent evidence has been obtained to show that oxidation of model compounds by laccase can, in the presence of hydroxyl free radicals, result in the cleavage of the aryl-αC bond as shown in figure 7. It is therefore clear that laccase in the presence of free radicals of oxygen has the capacity to remove single aromatic monomers from the polymer, provided that the aryl ring in question has a free phenolic group. Thus laccase may be able to act as an exoligninase provided that there is a source of hydroxyl free radicals, which could arise directly from the dismutation of superoxide (Fridovich 1974). The source of these free radicals is unclear but they could arise directly from laccase itself or as the result of other oxidase activity, notably cellobiose oxidase which has been suggested as a source of such radicals (Eriksson 1981).

Oxidation of a monophenol by laccase can also result in the loss of a methyl group and the production of a fully oxidized quinone as shown in figure 8. These strong oxidative reactions tend to yield highly oxidized products, which would not be substrates for intramolecular dioxygenase attack; the latter would need diphenolic groups in the ring (Nozaki 1979). Westermark & Eriksson (1974) have described the presence of cellobiose : quinone oxidoreductase in white-rot fungi that could perform the role of reducing the quinones produced by the activity of laccase to the *o*-phenols before ring cleavage occurs (see scheme in figure 9). Such interdependence between lignin degradation and cellobiose oxidation may help to explain the dependence of lignin breakdown on the presence of another oxidizable carbohydrate.

Role of extracellular alcohol oxidase

There have been several reports in the literature (Farmer *et al.* 1960; Ohta *et al.* 1979) that lignin-degrading fungi produce an extracellular alcohol oxidase that oxidizes alcohol to the aldehyde and hydrogen peroxide. This oxidation seems to prepare the alkyl chain for cleavage

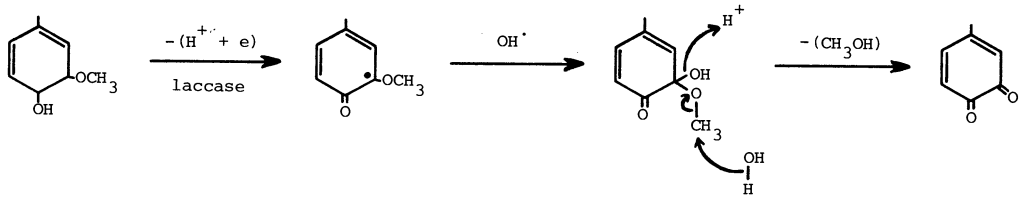

FIGURE 8. Potential pathway of demethoxylation catalysed by laccase.

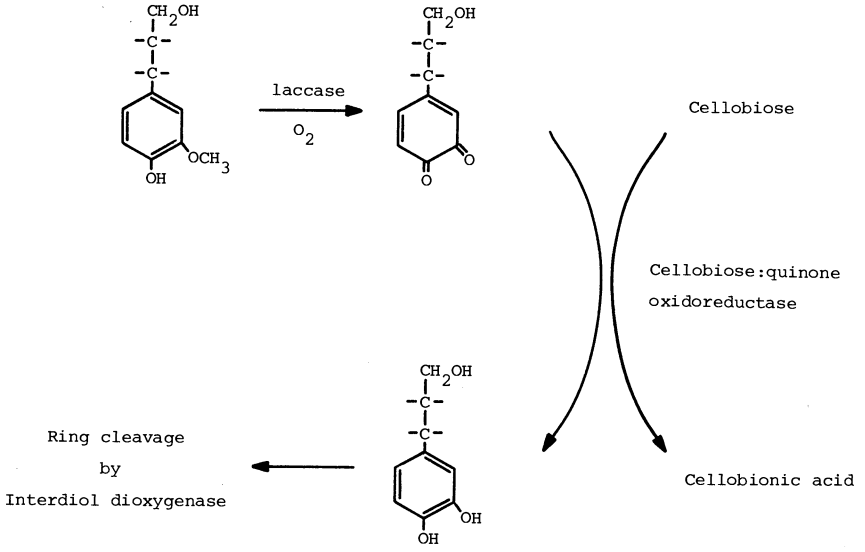

FIGURE 9. Scheme for reduction of quinones by cellobiose:quinone oxidoreductase (after Westermark & Eriksson 1974).

between the α and β carbon atoms (Crawford 1981*b*). Recent studies with model dimers have produced evidence that the oxidation of the primary alcohol group on the γ carbon atom of the alkyl chain between the two aryl groups results in the depolymerization of the dimer in a manner shown in figure 8. Thus the activity of the alcohol oxidase may be responsible for the depolymerization of aryl units in the centre of lignin in which both phenol groups are linked as ethers to neighbouring molecules by catalysing the cleavage of the α–β carbon bond. Clearly, the details of this reaction need clarification but it is possible that the alcohol oxidase could be considered to be a potential candidate for an endoligninase.

In summary it would appear that our current knowledge concerning the enzymology of lignin breakdown is far from certain but there is a possibility that we may be close to an outline understanding about the enzymes involved. It is hoped that the analysis presented in this paper will provide a stimulus for future developments in the field.

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