Oxidative Mechanisms Involved in Lignin Degradation by White-Rot Fungi

Rimko ten Have*,† and Pauline J. M. Teunissen‡

Division of Industrial Microbiology, Department of Agrotechnology and Food Sciences, Wageningen University and Research Centre, P.O. Box 8129, 6700 EV Wageningen, The Netherlands, and Genencor International B.V., Archimedesweg 30, 2333 CN Leiden, The Netherlands

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I. Introduction

Lignin is a heterogeneous polymer that occurs in woody and vascular tissues. It is formed by a random polymerization reaction of *p*-coumaryl alcohol, 3-(4-hydroxyphenyl)propen-1-ol, radicals, and their methoxy-substituted derivatives.[1]

White-rot fungi are among the best degraders of lignin. They owe their name to the specific bleaching process that occurs during the fungal degradation of wood. This review focuses on the oxidative manners that these fungi use to degrade lignin, as described in various articles from ca. 1981 onward.

Several review articles on this subject have been written of which the following references are just a selection.[2−12] To avoid excessive overlap with other review articles, this review is especially comprehensive on parts in which experimental data seems to conflict with the current theory. This review aims at providing new insights in oxidative lignin degradation for those who are familiar with this field of research. In addition, this review intends to introduce other interested readers to several basic aspects of white-rot fungi and their oxidative mechanisms to degrade lignin.

II. History of Lignin Degradation

A. Lignin Degradation by White-Rot Fungi

Lignin forms a matrix surrounding the cellulose in woody cell walls, which protects the hemicellulose and cellulose, together this is called holocellulose, from microbial depolymerization. White-rot fungi are the only known organisms that can completely break down the lignin to carbon dioxide and water.[12] However, lignin cannot be degraded as a sole source of carbon and energy. Degradation of lignin by white-rot fungi enables them to gain access to the holocellulose, which is their actual carbon and energy source.[13] Presumably, this is the real purpose for lignin degradation.

* To whom correspondence should be addressed. Phone: +31 317-484976. Fax: +31 317-484978. E-mail: Rimko.tenHave@imb.FTNS.wau.nl.
† Wageningen University and Research Centre.
‡ Genencor International B.V.

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Rimko ten Have was born in 1971 in The Netherlands. He began studying chemistry in 1988 at Larenstein International Agricultural College and obtained his B.Sc. degree in 1992. Subsequently he studied molecular sciences at the Wageningen Agricultural University to obtain his M.Sc. degree. In 1995 he joined the group of Professor J. A. M. de Bont and conducted research on lignin peroxidase-mediated biotransformations useful in the biocatalytic production of vanillin under the supervision of Dr. J. A. Field and received his Ph.D. degree in 2000. From there on he has occupied a postdoctoral position and studied new ways to improve the mushroom yield as obtained during cultivation of *Agaricus bisporus*. His hobbies are chess, cultivating passion flowers, and growing the white-rot fungus *Pleurotus ostreatus* (Oyster mushroom, see cover).

Pauline J. M. Teunissen was born on August, 22, 1970. She obtained her M.Sc. degree in Food Technology in 1994 at the Wageningen Agricultural University, Wageningen, The Netherlands. From 1994 to 1999 she studied the role of natural chlorinated hydroquinone metabolites produced by ligninolytic fungi at the division of Industrial Microbiology, Department of Agrotechnology and Nutritional Sciences, Wageningen University. This research resulted in a Ph.D thesis entitled "The Role of Natural Chlorinated Hydroquinone Metabolites in Ligninolytic Fungi". From 1999 onward the author has been working as a scientist at Genencor International B.V., Leiden, The Netherlands.

B. Evidence for an Oxidative Manner of Degradation

In 1975 a chemical study on degraded lignins was published, showing that the degraded lignin contained oxidized side chains and aliphatic residues resulting from oxidative cleavage of aromatic rings. These findings lead to the speculation that white-rot fungi use extracellular oxygenases for lignin degradation.[14]

Subsequent studies with the white-rot fungus *Phanerochaete chrysosporium* indicated that the organism combusts ^{14}C-labeled lignin in part to $^{14}CO_2$. Bar-Lev[15] discovered in 1981 that an atmosphere containing O_2 and N_2 in an 80:20 ratio enhanced lignin degradation 5-fold if compared to normal air. This increase was shown not to be attributable to an enhanced fungal respiration rate.[15]

A pure oxygen atmosphere markedly enhanced the production of H_2O_2 in these fungal cultures.[16] The production of H_2O_2 showed a temporal relationship with lignin degradation,[17] suggesting a role for H_2O_2 in lignin degradation. This was further evidenced by the observation that the rate of lignin mineralization in the presence of catalase was only 8.6% of the value obtained with boiled catalase.[16] This showed that the oxidant H_2O_2 was indeed very important in lignin degradation.

Wood, the natural growth substrate of white-rot fungi, contains Fe^{2+} which together with H_2O_2 generates the extremely reactive hydroxyl radical ($HO^•$),[18] which may react with virtually every organic molecule available. Therefore, studies on a possible involvement of the $HO^•$ in lignin degradation were undertaken.

It appeared that $HO^•$ scavengers such as mannitol[17] and benzoate[16,17] inhibited lignin mineralization. The presence of the scavenger did not negatively affect the respiration rate of glucose by the fungus,[16,17] suggesting that fungal growth was not affected by the $HO^•$ scavenger. These findings indicated that $HO^•$ contributed to lignin degradation. This conclusion, however, has been questioned by Kirk et al.[19]

C. Discovery of Lignin Peroxidase and Reevaluation of the Role of $HO^•$

In 1983 Tien et al. discovered that white-rot fungi excrete lignin peroxidase (LiP). The enzyme uses H_2O_2 for activity and is capable of oxidizing and cleaving lignin and lignin model compounds. This was supposed to be the key reaction of lignin degradation rather than the previously supposed active oxygen species that are derived from H_2O_2.[20]

This finding lead Kirk et al.[19] to reevaluate the role of $HO^•$ in lignin degradation. They argued that the $HO^•$ scavengers used in previous studies[16,17] may not be specific and may cause an unanticipated effect. They found that $HO^•$ was indeed able to oxidize lignin model compounds, but the oxidation products differed evidently from those formed in whole fungal cultures. Additionally, these fungal products were clearly attributable to the activity of LiP. This finding demonstrated that $HO^•$ is not involved in a predominant cleavage reaction in lignin degradation.[19] A disadvantage of this study is that the lignin model compound studied easily fits the catalytic site of LiP, unlike lignin, favoring reactions between LiP and the lignin model compound. Therefore, this study does not rule out that $HO^•$ is involved in the initial attack of lignin.

Recent studies demonstrated that a $HO^•$-producing system might indeed be involved in lignin degradation.[21−25] The enzyme cellobiose dehydrogenase can oxidize a variety of di- and oligosacharides[21,24] and may transfer the released electrons to a large series of one-electron and two-electron acceptors[21] of which

Fe^{3+} and O_2, respectively, are examples. The reduction products generate $HO^{\bullet}$,[18,23] which reacts with nonphenolic lignin model compounds,[21−23,26] resulting in demethoxylation and the concomitant introduction of phenolic sites to the once nonphenolic model compound. This reaction is important because the insertion of additional phenolic groups in lignin makes it easier to oxidize the polymer.

The discovery of LiP initiated the search for other oxidative enzymes and led to the discovery of manganese peroxidase (MnP), an enzyme that oxidizes Mn^{2+} to the oxidant Mn^{3+}.[12] Besides these two peroxidases, there is also a phenol oxidase, laccase, that was already described long before the discovery of LiP. This enzyme alone did not appear to be potent enough to oxidize the nonphenolic predominant part of lignin. Therefore, the discovery of a cofactor that expanded the substrate spectrum to nonphenolic aromatics was considered a breakthrough.[27]

To further demonstrate that these three oxidative enzymes are involved in lignin degradation, mutants were made that lacked the ability to produce either LiP,[28] MnP,[29] or laccase.[30] Addition of the purified enzyme, which was lacking due to the mutation, could partially restore the lignin-degrading ability of the fungal culture.[29,30] This finding is important because the aspecific manner in which these mutants were obtained may have caused several essential disfunctional genes.

D. Environmental Conditions Inducing Lignin Degradation

Throughout the years extensive research has been conducted on the physiological onset of lignin degradation. The lignin-degrading system of white-rot fungi is composed of extracellular enzymes, such as ligninolytic peroxidases and H_2O_2-generating oxidases, and low molecular weight cofactors.[12] Nutritional and culture parameters have been investigated which influence the onset of the production of the typical ligninolytic enzymes lignin peroxidase (LiP) and manganese peroxidase (MnP).

Studies with *P. chrysosporium* showed that lignin degradation only occurs during secondary metabolism, which is triggered by the depletion of nutrient nitrogen, carbon, or sulfur.[12,31,32] It was suggested that N-limited growth conditions were natural for fungi, since wood contains only low levels of nitrogen.[33] However, recent studies have shown that not all white-rot fungal species and strains are N-regulated. The addition of organic NH_4^+ and/or L-amino acids to the growth medium did not repress ligninolytic activity in *Bjerkandera adusta* ssp. and *Coriolus versicolor*.[34,35] In fact, high-N media stimulated biomass yields and peroxidase production in these N-unregulated fungal species.[34−38]

Lignin degradation is greatly influenced by the presence of nitrogen; in addition, increasing the oxygen tension in cultures has a strong multiple activating effect on ligninolysis.[12,15] Not only is an increase in the titer of the lignin-degrading system and H_2O_2-producing system observed, but also observed is an increase in the activity of the existing ligninolytic system.[15] Faison and Kirk[39] detected higher activities of LiP in cultures of *P. chrysosporium* grown under oxygen compared to cultures under air atmosphere. More recently, Kotterman et al.[40] showed that the most important factor influencing the biodegradation of anthracene, which is most likely oxidized by ligninolytic enzymes, is oxygen. A good explanation for the stimulatory effect of elevated oxygen levels is the enhanced endogenous production of H_2O_2 in fungal cultures.[16,41] H_2O_2 is essential for the activity of peroxidases and was shown to be the rate-limiting step for peroxidase catalyzed polyaromatic hydrocarbon oxidation.[41,42]

One would expect that culture agitation would stimulate lignin degradation, since the oxygen pressure is increased. However, lignin oxidation and LiP production is strongly inhibited in agitated cultures compared to static cultures.[39,43,44] Most likely, restricted oxygen availability is the bottleneck if fungi grow as submerged pellets rather than as mycelial mats.[44]

Many other environmental parameters have been identified including the effect of pH, the choice of buffer, and the concentration of certain minerals (especially calcium and manganese).[12] The importance of manganese and other low molecular weight compounds on lignin degradation will be discussed in the next section.

E. Low Molecular Weight Compounds Involved in Lignin Degradation

Mediators have been considered essential for the oxidation of lignin by the ligninolytic enzyme system since native lignin in wood is inaccessible for large enzymes such as LiP and MnP.[45,46] Various low molecular weight compounds were identified which could play vital roles in the ligninolytic enzyme system of white-rot fungi.[47−51]

Theoretical and electron microscopic studies demonstrated that enzymes as large as peroxidases and laccases cannot have direct contact with lignin, due to the pore size of the cell wall. Therefore, a role of low molecular weight cofactors has been proposed. The importance of cofactors in the oxidation of lignin model compounds was observed early on during in vitro experiments. LiP and MnP were only able to oxidize various lignin model compounds and synthetic lignin if an appropriate cofactor was present.[52−54] It is difficult to demonstrate degradation of (synthetic) lignin by MnP or LiP because the reaction conditions favor both depolymerization and repolymerization. Important experimental conditions that need optimization are the following: the H_2O_2 and the lignin concentration, O_2, and the presence of a suitable mediator.[52]

1. Manganese

Manganese is a natural wood compound which is present in a relatively high concentration (approximately 10−100 mg/kg of dry wood).[55] The first line of evidence stressing the importance of Mn^{2+} is that MnO_2 precipitates accumulate during fungal decay.[56] Moreover, lignin degradation by several white-rot fungi is strongly enhanced by Mn^{2+} since it stimulates the MnP production and functions as a substrate for MnP. The Mn^{3+}, generated by MnP, acts

as a mediator for the oxidation of various phenolic compounds.[52,54,59−61]

Mn^{2+} also affects LiP production. LiP titers are generally lower in the presence of Mn^{2+}.[58] A possible reason for the decreased LiP activity is the lowered oxygen stress due to the scavenging of peroxyl radicals by Mn^{2+}.[62,63] It is well established that LiP activity is highly stimulated under a pure oxygen atmosphere.[39,62] Additionally it was shown that Mn^{2+} induces manganese−superoxide dismutase (Mn−SOD), which further contributes to minimizing the oxidative stress.[62]

2. Veratryl Alcohol

Veratryl alcohol (3,4-dimethoxybenzyl alcohol, VA) is synthesized de novo from glucose.[48,49] The production of VA starts in the early phase of secondary metabolism in parallel with the onset of LiP production. In *P. chrysosporium*, VA production is triggered by N-limitation, in contrast to *Bjerkandera* sp. strain BOS55 in which nitrogen has no regulatory effect on the VA biosynthesis.[12,38,64] Mester et al.[38] showed the regulatory effect of Mn^{2+} on the endogenous VA level in both *Bjerkandera* sp. strain BOS55 and *P. chrysosporium*. Manganese clearly inhibits the endogenous production of VA in both fungi.

The de novo biosynthesis of VA proceeds via the shikimate pathway yielding aromatic amino acids such as L-phenylalanine as an important intermediate.[65] ^{14}C-Isotope trapping experiments showed that in addition to phenylalanine, cinnamate, benzoate, and benzaldehyde are biosynthetic precursors of VA in *P. chrysosporium* and *Bjerkandera* sp. strain BOS55.[50,66] L-Phenylalanine is probably deaminated by phenylalanine ammonia lyase resulting in the production of cinnamate. A Claisen cleavage of the cinnamate results in the formation of benzoate and/or benzaldehyde. Benzoate or benzaldehyde is hydroxylated and methylated forming veratrate or veratraldehyde. Both can be reduced back to VA.[66]

Also, monomeric lignin degradation products such as 3- and 4-hydroxybenzoate, protocatechuate, vanillate, isovanillate, and veratrate were found to serve as precursors in VA biosynthesis in *P. chrysosporium, Bjerkandera* sp. strain BOS55 and *Pycnoporus cinnabarinus*.[50,67,68] Even 3,4-dimethoxycinnamyl alcohol, which was not considered to be a biosynthetic precursor, can be converted to VA.[43,69] This indicates that several alternative pathways are present for VA production in addition to the de novo biosynthesis from glucose. When lignin degradation products are used for VA production, the de novo synthesis appears to be repressed as shown for *Phlebia radiata*.[70]

VA is most likely the physiological substrate of LiP. As mentioned earlier, both the production of VA and the production of LiP is triggered by the onset of secondary metabolism.[12] The addition of VA to the cultures of various white-rot fungi has repeatedly been found to increase LiP titers,[39,71,72] which is explained by protecting LiP from H_2O_2-dependent reactions by offering a good reducing substrate.[71,73] By regulating the production of VA, Mn^{2+} has an indirect effect on LiP.[38]

An important role of VA is that it acts as a mediator for electron-transfer reactions in the LiP-catalyzed oxidation[74] of compounds that are hard to oxidize,[75] do not readily bind to LiP,[76] or are too large to fit in the catalytic site.[77] In the proposed mechanism LiP catalyzes the one-electron oxidation of VA to the VA cation radical, $VA^{+\bullet}$. The latter oxidizes the second substrate by one electron, concomitantly reducing $VA^{+\bullet}$ back to VA.

3. Oxalate

Oxalate is produced by white-rot fungi as a major aliphatic organic acid.[78,79] The two enzymes oxaloacetase and glyoxylate oxidase that catalyze the hydrolysis of oxaloacetate and the oxidation of glyoxylate, respectively, are responsible for the biosynthesis of oxalate.[80]

Despite the de novo oxalate production, white-rot fungi do not accumulate the acid to such a great extent but rather decompose it to carbon dioxide and formate.[81] The enzyme responsible for decomposition is oxalate decarboxylase. An important feature is that LiP as well as MnP are capable of decomposing oxalate in the presence of VA or Mn^{2+}, respectively.[76,82,83]

The cleavage of oxalate results in the formation of carbon dioxide and the formate anion radical ($CO_2^{\bullet-}$), which is further oxidized by O_2 to give CO_2 and superoxide ($O_2^{\bullet-}$ or $HOO^{\bullet}$) under aerobic conditions. The active oxygen species formed in the reaction of the formate anion radical and O_2 are suggested to directly participate in the oxidation of lignin.[84,85] In addition, superoxide can dismutate, resulting in the formation of H_2O_2 and O_2.[86] This reaction accounts for the observed oxidation of phenol red and kojic acid by MnP in the presence of Mn^{2+} and oxalate without exogenous addition of H_2O_2.[87,88] This suggests that oxalate may be regarded as a passive sink for H_2O_2 production.

The reduction of $VA^{+\bullet}$ or Mn^{3+} by oxalate suggests that as long as oxalate coexists with lignin-degrading enzyme systems, it would inhibit lignin degradation. Such an effect was indeed demonstrated by showing that oxalate strongly reduced the lignin mineralization rate in ligninolytic fungal cultures.[76,82,83,89,90] On the basis of this information it seems remarkable that the lignin content in biobleaching experiments decreases only significantly upon addition of small amounts of oxalate.[91]

4. 2-Chloro-1,4-dimethoxybenzene

White-rot fungi produce a wide range of organohalogen metabolites.[92] The most common organohalogens produced are chlorinated anisyl metabolites (CAM) and chlorinated hydroquinone metabolites (CHM).[92,93] CAM were shown to have an important physiological function in lignin degradation, serving as substrates for aryl alcohol oxidase involved in extracellular H_2O_2 production.[2] Furthermore, Mester et al.[50] elucidated a part of the biosynthetic pathway of CAM in *Bjerkandera* sp. strain BOS55, showing stimulation of CAM production by phenylalanine, benzoate, 4-hydroxybenzoate, and cinnamate.

The biosynthetic pathway of CHM involves the formation of at least 9 different CHM metabolites that have been found in 11 genera of Basidiomycetes.[92,93] Among these CHM metabolites, chlorinated 1,4-dimethoxybenzenes such as 2-chloro-1,4-dimethoxybenzene, 2,6-dichloro-1,4-dimethoxybenzene, tetrachloro-1,4-dimethoxybenzene, and tetrachloro-4-methoxyphenol are identified.[93−95]

2-Chloro-1,4-dimethoxybenzene (2-Cl-1,4-DMB) is a substrate for LiP, indicating a possible active function in the wood decomposition process.[96] Like VA it can function as a redox mediator.[77,96,97] EPR results showed that a relatively long-lived 2-Cl-1,4-$DMB^{+\bullet}$ is formed in the LiP-catalyzed oxidation of 2-Cl-1,4-DMB.[97] This would indicate the ability of the 2-Cl-1,4-$DMB^{+\bullet}$ to diffuse away from the enzyme active site and act as a diffusible redox mediator. Results indicated that 2-Cl-1,4-DMB can substitute VA in the LiP-catalyzed oxidation of azo dyes, the polymeric dye Poly R-478 (used as a model for lignin because like lignin it does not fit in the catalytic site), 4-methoxymandelic acid, *p*-anisyl alcohol, and oxalate.[77,96,97] Except for the azo dyes, none of these compounds were oxidized in the absence of 2-Cl-1,4-DMB or VA.[75,77,98] Therefore, these compounds are called terminal substrates.[77]

The formation of oxidation products derived from 2-Cl-1,4-DMB was inhibited until the second substrate was completely oxidized. This inhibition of product formation indicates that the terminal substrates and azo dyes reduce the 2-Cl-1,4-$DMB^{+\bullet}$ back to regenerate 2-Cl-1,4-DMB with concomitant oxidation of the substrates.

It is not yet certain whether 2-Cl-1,4-DMB is actually involved in lignin degradation because its biosynthesis does not coincide with the production of LiP, as shown for VA.[49] Moreover, it seems not to be oxidized by LiP at physiological pH values.[99]

III. Gene Regulation and Characteristics of LiP and MnP

A. Characteristics of Peroxidases

The two types of peroxidases were first discovered in *P. chrysosporium*.[20,100,101] LiPs and MnPs are known to be families of isozymes occurring as extracellular glycosylated proteins.[102−104 105] The ratio between the isozymes changes with the culture age and culture conditions.[72,103,104,106,107] They have molecular weights ranging from 35 to 47 kD and isoelectric points ranging from 2.8 to 5.3.[102−105,108,109] The absorption spectrum of the native enzyme has a very distinct absorbance maximum at 406−409 nm due to the presence of a single heme group (protoporphyrin IX).[105,109−111]

LiP and MnP have a typical catalytic cycle, as also observed for other peroxidases.[60,61,111,112] One molecule of H_2O_2 oxidizes the native enzyme by withdrawing two electrons, forming compound I. The latter may be reduced back in two single-electron oxidation steps to the native form via an intermediate compound II. In the case of LiP, reduction of compound II is the rate-limiting step in the catalytic cycle. For this reason, this compound is considered to be less potent than LiP compound I. Because the reduction of compound II is relatively slow, it is long available for a reaction with H_2O_2 leading to inactive enzyme, known as compound III which characterized as a complex between LiP and superoxide.[60,113]

Other fungal enzymes may supply the required hydrogen peroxide for peroxidase activity. As part of their ligninolytic system, white-rot fungi produce H_2O_2-generating oxidases,[2,12] like glucose oxidases,[114,115] glyoxal oxidase,[116] and aryl alcohol oxidase.[117] White-rot fungi that lack H_2O_2-generating oxidases rely on the oxidation of physiological organic acids such as oxalate and glyoxylate which indirectly results in H_2O_2.[79]

Also, the reduction of quinones to their corresponding hydroquinones and the subsequent autoxidation or enzymatically catalyzed oxidation may yield H_2O_2 due to the involvement and reduction of O_2.[118,119]

MnP is different from the other peroxidases due to the structure of its binding site. MnP oxidizes Mn^{2+} to Mn^{3+}, which cannot be replaced by other metals at physiological concentrations.[112] At the time of the discovery of MnP, it was demonstrated that certain aliphatic organic acids such as lactate and oxalate stimulated the Mn^{2+} oxidation rate.[110,112,120] These organic acids, e.g., oxalate and to a lesser extent malonate and glyoxylate were shown to be produced as de novo metabolites by white-rot fungi.[51,79,80,121] These acids are able to chelate Mn^{3+} resulting in a relatively stable complex. The complexed Mn^{3+} can then oxidize phenolic lignin model compounds and various phenols through phenoxy radical formation.[52,59]

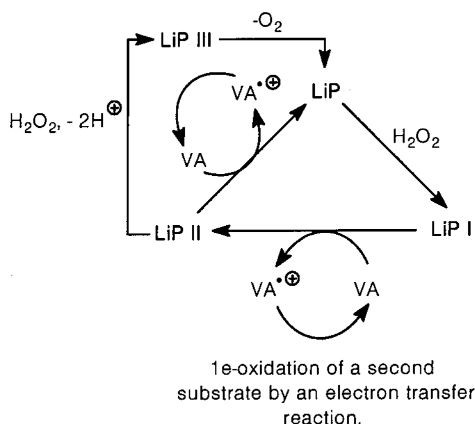
LiP is capable of oxidizing various phenolic and nonphenolic substrates with a calculated ionization potential, a measure for the ease to abstract an electron from the highest occupied molecular orbital, of up to 9.0 eV.[122]

LiP has been shown to oxidize fully methylated lignin and lignin model compounds as well as various polyaromatic hydrocarbons.[42,52,123] Among the oxidation reactions catalyzed by LiP are the cleavage of the $C_\alpha - C_\beta$ and aryl C_α bond, aromatic ring opening, and demethylation.[42] One secondary metabolite, veratryl alcohol (VA), has been the focus of many studies. VA is an excellent substrate for LiP and enhances the oxidation of otherwise poor or terminal LiP substrates.[52,124]

Three major roles of VA have been suggested so far. As described before, VA may act as a mediator in electron-transfer reactions.[75,125,126] Second, VA is a good substrate for compound II; therefore, VA is essential for completing the catalytic cycle of LiP during the oxidation of terminal substrates.[127] Third, VA prevents the H_2O_2-dependent inactivation of LiP[128,129] by reducing compound II back to native LiP. In addition, if the inactive LiP compound III is formed, the intermediate $VA^{+\bullet}$ is capable of reducing LiP compound III back to its native form (see Chart 1).[130,131]

Only a few compounds such as 3,4-dimethoxytoluene, 1,4-dimethoxybenzene, 3,4,5-trimethoxybenzyl alcohol, and 2-Cl-1,4-DMB have been found to replace the function of VA as a cofactor of LiP.[77,127,132] Since,

Chart 1. Catalytic Cycle of LiP[a]



[a] The VA radical cation is shown to function as a redox mediator and a revertant of inactive LiP compound III to native LiP.

VA is the most frequently occurring secondary metabolite produced by white-rot fungi, its physiological relevance has never been questioned.

The introduction of transient state kinetic studies has given more information about the enzyme mechanisms. Although the mediation theory is not ruled out by these experiments, the underlying oxidation mechanism by these peroxidases is more complex than anticipated. First of all, we have to distinguish between substrates which are only oxidized by compound I or by both compounds I and II. Mn^{2+} is the best reducing substrate for compounds I and II of all MnP isozymes tested.[8,87,105,109] Although compound I can directly oxidize a few phenolic compounds such as 2,6-dimethoxyphenol, guaiacol, and a phenolic tetrameric lignin model compound,[133,134] Mn^{2+} is absolutely required to reduce compound II back to the ferric form.[60,61] Mn^{2+} overcomes H_2O_2-dependent inactivation by completing the catalytic cycle of MnP.

For LiP, veratryl alcohol was demonstrated to be a good substrate for compounds I and II.[127,135] On the other hand, anisyl alcohol and manganese are the substrate for only compound I.[127,136]

Recently, peroxidases were isolated that can oxidize Mn^{2+} as well as aromatic compounds. Both *Bjerkandera* sp. BOS55 and *Pleurotus eryngii* excrete these unusual enzymes.[137,138] The hybrid enzymes oxidize Mn^{2+} much faster than organic compounds, regarding the measured k_{cat} values. The latter are determined by the rate-limiting step of the oxidation, which is the reduction of compound II to the native enzyme. The fact that a small amount of Mn^{2+} enhanced the oxidation of VA in the case of the *Bjerkandera* enzyme has been explained by a similar mechanism to that of the oxidation of *p*-anisyl alcohol in the presence of VA.[127] The small amount of a reducing substrate for compound II facilitates the turnover of the enzyme.[137] It would be interesting to gain knowledge on the structure of such a hybrid enzyme because that would provide valuable information on structural features of the substrate-binding site(s).

B. Structure

The crystal structures of MnP and LiP from *Phanerochaete chrysosporium* have been solved.[139,140] The biggest difference between the two enzymes, as far as their structure is concerned, was found in the substrate-binding site. For MnP, the acidic amino acids aspartic acid and two glutamic acids were proposed as manganese-binding residues.[141] Glu 35, glu 39, and Asp 179 in MnP isozyme H4 were involved in the binding of Mn^{2+} and electron transfer as demonstrated by site-directed mutagenesis.[133] The same binding site occurs in the hybrid peroxidase from *Pleurotus eryngii*.[138] Interestingly, mutation of serine168 to tryptophane in MnP isozyme H4 enabled the engineered enzyme to even oxidize VA.[142]

The substrate-binding site of LiP has not been determined with certainty yet. According to the crystal structure, the so-called heme access channel was proposed to be the substrate-binding site.[139] By mutating glutamic acid in this access channel, a slight decrease in the oxidation of VA was observed.[143] More significant changes in the oxidation of VA occurred when site-directed mutagenisis was performed in another site of LiP. Upon mutating tryptophane 171, LiP did not oxidize veratryl alcohol, suggesting that the substrate-binding site is more surface exposed and the electron abstracted from the reducing substrate should take a long-range electron transport route within the enzyme in order to reach the oxidized heme.[144]

The corresponding tryptophane is also present in the hybrid peroxidase from *Pleurotus eryngii*.[138] However, the existence of more than one binding site cannot be excluded because mutation of tryptophane 171 did not affect the oxidation of ABTS.[144]

C. Regulation of LiP and MnP Genes

After the discovery of the ligninolytic peroxidases, the isolation of the responsible genes from various white-rot fungi has been attempted.[8,145−148] Several regulatory elements have been described in the promoter regions of LiP and MnP. Promoter regions of LiP or MnP genes studied so far contain cAMP response elements (CRE) to notice starvation.[8,148] In addition, differential regulated expression of certain isozymes of LiP and/or MnP has been observed as a response to carbon or nitrogen limitation.[149]

Aside from starvation, the presence of Mn^{2+} is essential for MnP gene expression in *P. chrysosporium* and *Dichomitus squalens*.[57,150] The observation is in agreement with the general finding that elevated manganese levels are beneficial for the production of MnP in many white-rot fungi.[58] Putative metal response elements (MRE) were found in *P. chrysosporium* which are similar to a gene that encodes for a mouse metalloprotein.[8,151] However, the transcription of different MnP isozymes showed variable dependencies on the presence of Mn^{2+}.[152] The addition of Mn^{2+} highly stimulated the gene expression of two MnP isozymes (MnP1 and MnP2), but to a lesser extent that of the third one (MnP3). Moreover, in some white-rot fungi from the genus *Pleurotus*, proteins with MnP activity were found in cultures lacking Mn^{2+} and addition of Mn^{2+} was found to inhibit MnP production.[105]

LiP gene transcription in *P. chrysosporium* was not suppressed by Mn^{2+},[109] and the expression is not

stimulated by the presence of its cofactor, VA; rather a slight repression of LiP gene transcription has been detected in *P. chrysosporium*.[128] It seems that the presence of VA[129] or L-tryptophane[71] in fungal cultures just results in a higher amount of active LiP, compensating for the repressive effect on gene transcription.

Also putative xenobiotic response elements and heat shock elements have been found in the promoter region of LiP and MnP genes, respectively.[8,148] the xenobiotic response elements are similar to those found in mammals; however, no xenobiotic chemicals have yet been tested to induce LiP gene expression so far. The addition of various chemicals such as H_2O_2, ethanol, sodium arsenite, 2,4-dichlorophenol, as well as heat treatment stimulated the MnP production and MnP gene expression probably through the heat shock elements.[153,154] However, when manganese was omitted, only a very small increase in the MnP mRNA was detected due to the respective chemicals or the heat treatment and no MnP protein could be found in the culture fluid.

IV. Gene Regulation and Characteristics of Laccase

A. Characteristics of Laccases

Laccase belongs to the copper oxidase family. The enzyme is generally larger than the peroxidases, having a molecular weight around and beyond 60 kD.[155] As with other extracellular enzymes, laccases are glycosylated. Laccases can catalyze four single-electron oxidation steps coinciding with the reduction of O_2 to H_2O.

Laccases contain four coppers per enzyme, representing three different types, and therefore, each type has a distinct role in the oxidation of laccase substrates.[156,157] The type 1 copper is suggested to be involved in the reaction with the substrates. The type 1 copper has an absorption maximum at the wavelength of 610 nm which gives the enzyme the typical blue color. The type 2 copper and the two type 3 coppers cluster in a triangular form which are involved in the binding and in the reduction of O_2 as well as the storage of electrons originating from the reducing substrates. The type 2 copper does not show visible absorbance, while the type 3 coppers have an absorption maximum at 330 nm.

Laccase oxidizes phenolic lignin model compounds directly.[158,159] The presence of a suitable mediator expands the substrate spectrum to nonphenolics as well. Among these mediators are ABTS,[27,160] 1-hydrobenzotriazole (1-HBT),[161−163] and violuric acid,[164,165] but also natural mediators have been investigated such as 4-hydroxybenzoic acid, 4-hydroxybenzyl alcohol,[166] and 3-hydroxyanthranilate.[167]

There are fungi that produce laccase as the sole ligninolytic enzyme; therefore, natural laccase mediators should be produced in order to degrade nonphenolic lignin. Collins et al.[168] were able to demonstrate that the laccase-catalyzed oxidation of benzo[*a*]pyrene was strongly enhanced upon addition of a low molecular weight fraction obtained from a *Trametes versicolor* culture. An interesting possibility is that laccase uses liberated phenolic lignin fragments as mediators in the oxidation of the remaining lignin. However, the clarification of the exact role of laccase in lignin degradation requires further study.

B. Structure of Laccase

Several amino acid sequences of laccases are known.[169] The similarity among them is about 20−60%. The copper-binding sites appear to be conserved: they are found in every laccase. These sites are composed of a cysteine and several histidines.

There are significant differences in the redox potential of laccases.[157] It was proposed that a region related to the type 1 copper is responsible for determining the redox potential of the enzymes. Only one amino acid appears to be involved in determining the redox potential, and this amino acid is related to the copper-binding site nearest to the C-terminus. Eggert et al.[170] distinguished three classes of laccase having different redox potentials. Class 1 contains a methionine, class 2 contains a leucine, while class 3 laccases is characterized by a phenylalanine. When this phenylalanine was mutated, the redox potential was lowered.[171]

C. Regulation of Laccase Genes

The gene regulation of laccase production is different from that of peroxidases. First of all, the production of laccases is not suppressed by a high nutrient content. In many species, the use of nutrient-rich media resulted in higher laccase titers if compared to nutrient limited media. Second, two different laccases can be distinguished on the basis of gene expression. There are so-called constitutive laccases, while the other group of laccases is inducible.[172,173] Induction of laccase has been observed at the level of transcription and translation upon addition of copper, xylidine, veratric acid, etc.[172,174]

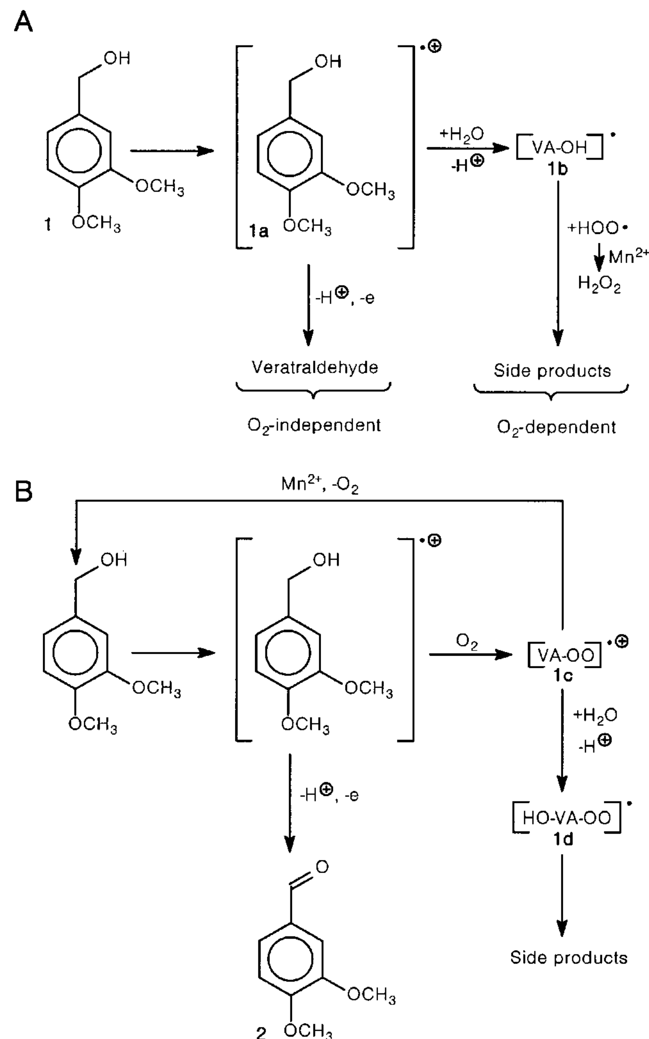
V. Oxidation Reactions Catalyzed by LiP

A. Veratryl Alcohol

LiP oxidizes VA (**1**) predominantly to 3,4-dimethoxybenzaldehyde (veratraldehyde, VAD, **2**). The reaction mechanism involves formation of $VA^{+\bullet}$,[175] which may loose a proton to form $VA^{\bullet}$. The latter may react with oxygen to VAD[176] or may alternatively be oxidized further by LiP to a cation, which readily decomposes to VAD.[177,178]

Under argon, LiP catalyzes both oxidation steps and VAD is the sole product.[132,177] In the presence of O_2, several side oxidation products, in a total molar yield of 30%, are formed, and the reaction mechanism involved here has been studied in detail.[177,179] It has been proposed that the formation of these side products was dependent on $HOO^{\bullet}$ by reacting with intermediate radicals.[177] This role of $HOO^{\bullet}$ was questioned by Shimada et al.,[179] who stated that the spontaneous decay of superoxide[86] would be to fast to act like a reactant. Recently, the mechanism has been revised because it contained two inconsistencies.[180]

Scheme 1. (A) Schematic Representation of the Old Mechanism Involved in VA Side Product Formation. (B) Current Revised Mechanism



The first step in the previous mechanism is the addition of H_2O to $VA^{+\bullet}$ (see Scheme 1A)[177] suggesting that side product formation is H_2O-dependent rather than O_2-dependent as observed. If it is assumed that the initial attack has indeed been performed by H_2O, then radical intermediates would still be available for reaction pathways that do not lead to VAD. In such a case the absence of O_2 would also yield side products. However, experiments performed under anaerobic conditions demonstrated that VAD was the sole product. The same inconsistency occurs in the reaction mechanism accounting for product formation from the veratryl alcohol methyl ether.[178]

If it is supposed that the initial attack of H_2O to $VA^{+\bullet}$ is correct, then the second step in which Mn^{2+} is hypothesized to lower the formation of side products by scavenging $HOO^\bullet$ is inconsistent. Once H_2O has reacted with the $VA^{+\bullet}$, an irreversible route toward side products has been taken. It is not possible to form either VA or VAD from a VA radical to which water was added ($HO-VA^\bullet$). The consequence of this rationale is that it is not possible to explain the observed decrease in side products by Mn^{2+} without assuming that there are other products formed from $HO-VA^\bullet$ than those that were found without Mn^{2+}. This contradicts with experimental observations because no other products are formed in the presence of Mn^{2+}.[181] For these reasons an alternative mechanism that accounts for VA side product formation has been proposed of which a schematic representation is depicted in Scheme 1B.[180] As indicated in Scheme 1B, the mechanism toward side products starts with the attack of O_2 to $VA^{+\bullet}$ yielding $VAOO^{+\bullet}$. The latter may be reduced back to VA by Mn^{2+} or may react with H_2O toward the side products. A consequence of this mechanism is that Mn^{2+} is expected to decrease the VA oxidation rate. This decrease may be difficult to measure because it is probably small.

B. Lignin Model Compounds

1. Model for 7% of the Linkages in Lignin

Several compounds that are models for the linkages in lignin have been used to demonstrate the involvement of extracellular excreted peroxidases and laccase in lignin degradation.

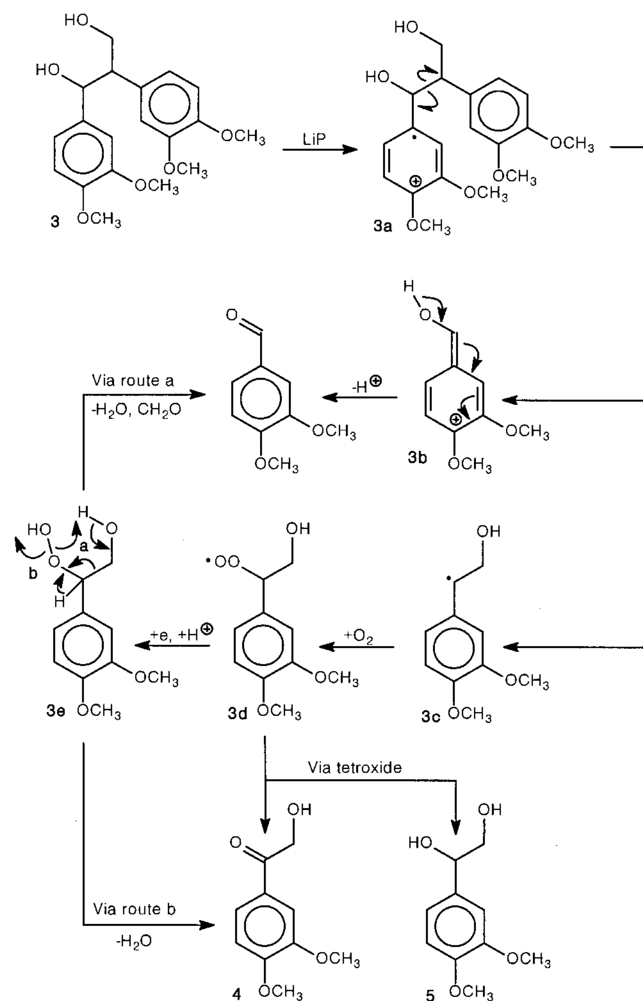
The involvement of LiP was demonstrated for the first time in 1983 by showing that lignin models were enzymatically oxidized and cleaved to smaller fragments.[20]

Lignin model compound **3** (see Scheme 2) accounts for 7% of the linkages in lignin and is readily oxidized by LiP. This oxidation step yields a radical cation which subsequently decomposes chemically to the observed products. A subsequent study, in which a similar (the degree of substitution with methoxy groups differs, but this is anticipated not to affect the reaction mechanism) lignin model compound was used, the incorporation of ^{18}O from labeled oxygen was demonstrated in C_α of 1-(3′,4′-dimethoxyphenyl)-ethanediol, the corresponding ketol, and benzaldehyde.[111] Other studies with comparable lignin model compounds indicate that under anaerobic conditions ^{18}O from labeled water is incorporated in the diol.[100,182]

Tien and Kirk[111] suggested that VAD is formed directly from **3** or via diol **5**. Formation of ketol **4** was rationalized by further oxidation of **5**. If the follow up oxidation of **5** is indeed important in the mechanism, then also ring cleavage products are expected in analogy to those found upon oxidizing VA by LiP.[178] The occurrence of such products has not been described, but this information is valuable to validate this route in the proposed mechanism. Another way to test the mechanism is that the molar yield of ketol **4** should increase during oxidation of **3** by LiP and the molar yield of diol **5** should concomitantly decrease.

On the basis of the current knowledge of cleavage mechanisms,[183−185] it is also possible to propose an alternative cleavage mechanism. As shown in Scheme 2, the radical cation **3a** cleaves homolytically yielding fragments **3b** and **3c**. A reaction between **3c** and O_2 yields peroxyl radical **3d**, which can decompose via two different routes: by coupling with another peroxyl radical forming an instable tetroxide intermediate which may decompose to **4** and **5**[186] or by the uptake of an electron and a proton yielding hydro-

Scheme 2. Proposed Cleavage Pathway of a Model Compound That Represents 7% of the Linkages in Lignin upon Oxidation by LiP in the Presence of O_2



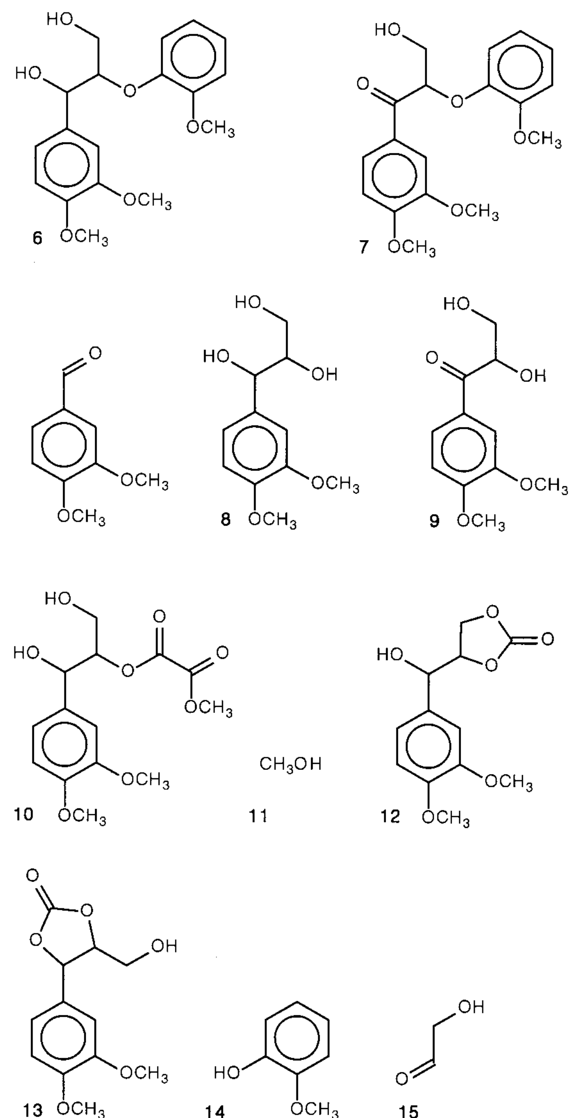
peroxide **3e**. The occurrence of hydroperoxides as intermediates is likely since they have been shown as reaction products of LiP-catalyzed reactions.[183,187] Hydroperoxide **3e** may decompose chemically to **4** or VAD. From this mechanism it is expected that formation of **4** is strongly dependent on the presence of O_2, and this may therefore be used as a criterion to validate the mechanism.

2. Model for 50–60% of the Linkages in Lignin

The oxidation of lignin model compound **6** by LiP to various products (**7**−**15**) has been studied by various groups (see Chart 2).[20,100,111,188−193] The main product is the corresponding benzaldehyde which is formed in a molar yield of 80%,[100] demonstrating that LiP can cleave the predominance of the lignin linkages.

The cleavage mechanism of model compound **6** (β-O-4 model) has been studied in detail by Tien and Kirk,[111] who performed experiments under an $^{18}O_2$ atmosphere. Incorporation of an ^{18}O in a C_β fragment would be rationalized by homolytic cleavage of the radical cation intermediate similar to the mechanism depicted in Scheme 2. Although they could clearly detect VAD, it appeared impossible to identify any

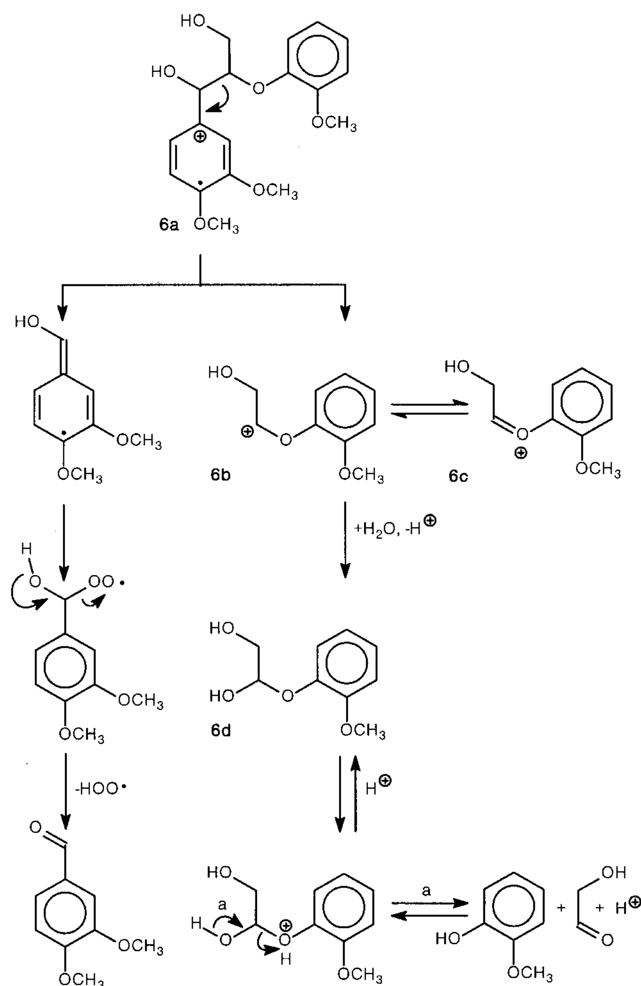
Chart 2. Products Identified upon Oxidation of 6[a]



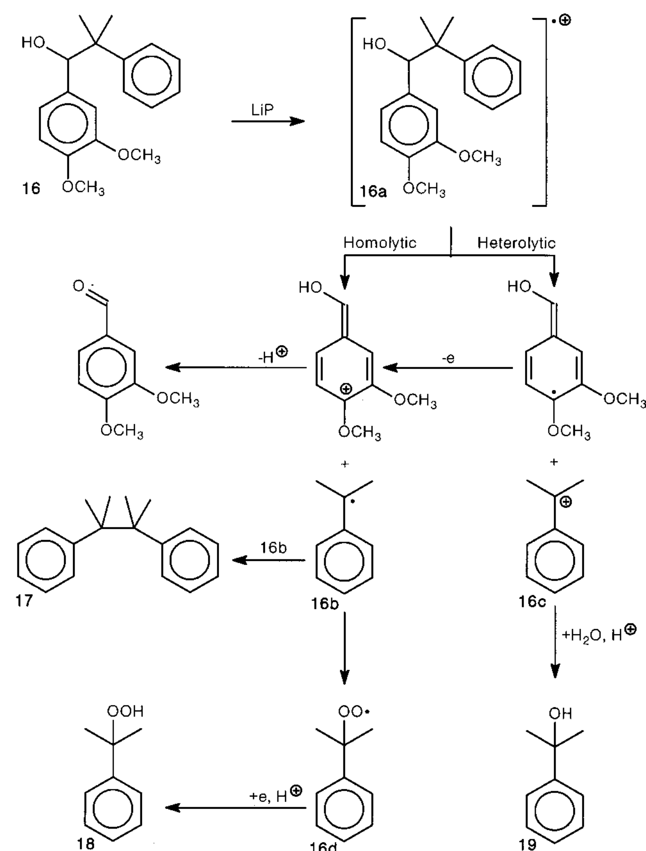
[a] The model represents 50−60% of the linkages in lignin. In most papers slightly different model compounds have been described which differ in the degree of substitution (with methoxy groups) or in the nature of the substituents, but this is anticipated not to affect the formation of the depicted products.

fragments from the C_β moiety. For that reason the model compound was modified. Replacing the hydroxy group bound to C_γ by a phenyl group lead to the identification of additional cleavage products, but ^{18}O incorporation in C_β could not be evidenced conclusively. On the basis of the results obtained with another model compound which is structurally similar to **3** (β-1 model), Tien and Kirk[111] presumed that ^{18}O incorporation would also in this case occur in C_β of the β-O-4 model compound **6**, indicating that this view is still open for debate. If this view is correct, then the radical cation **6a** undergoes homolytic cleavage leading to the formation of a radical with the structure R−HC$^\bullet$−O−R′. However, if it is incorrect, then **6a** undergoes heterolytic cleavage as depicted in Scheme 3 leading to the formation of a cation which is characterized by the structure R−HC$^+$−O−R′ (**6b**). Formation of the latter is thermodynamically much more favorable than the formation of the homolytic cleavage product with structure

Scheme 3. Proposed Heterolytic Cleavage of Radical Cation 6a to Smaller Fragments



Scheme 4. Oxidation of Model 16 by LiP[a]



[a] Intermediate radical fragments couple in the absence of O_2 to a dimer. In the presence of O_2, the radical fragment is a precursor for cumene hydroperoxide.

R–HC•–O–R′.[194] This stresses that the conclusion drawn by Tien and Kirk,[111] corresponding radical cations of β-O-4 and β-1 models cleave alike, needs more evidence because experimental evidence is lacking and moreover, there is a reason to question the current view.

This consideration is extra relevant because also other β-O-4 model compounds that were used in subsequent studies[53,188,193] are supposed to undergo homolytic cleavage based upon the work by Tien and Kirk.[111] Logically, a change in this view has consequences for the reaction mechanisms that have been published so far on this subject, and therefore, additional experiments are needed.

Regarding the experimental difficulties that Tien and Kirk[111] experienced upon studying the oxidation of **6**, further work on this compound is not recommended. Likely these difficulties may be circumvented by making structural adjustments to **6**. The replacement of the C_γ hydroxy group by a phenyl group as described before[111] is not ideal because this stabilizes both an unpaired electron or a cation on C_β as present in a possible cleavage fragment formed after homolytic or heterolytic cleavage, respectively.

3. Hydroperoxides as Oxidation Products and Roles of O_2

Upon oxidation of lignin model compound **16**, one of the products formed under air was identified as cumene hydroperoxide (**18**).[183] The formation of this product is accounted for in Scheme 4. The mechanism toward **18** involves homolytic cleavage of **16a**, yielding radical fragment **16b** which may react further with O_2 to peroxyl radical **16d**. The latter takes up an electron and a proton, forming the identified hydroperoxide.

Two possible electron donors were considered to be involved in the reduction of **16d**. These were either fresh substrate or LiP compound II.[183] The chemical reduction step by LiP is probably not important because fresh substrate is much more abundant. This suggests that substrate-derived peroxyl radicals are, like LiP, involved in the oxidation process. At that moment, however, there was no evidence available for the presumed O_2-dependent consumption of additional substrate[183] such as reported for 1-(3′,4′-dimethoxyphenyl)propene (DMPP).[184]

A future study may therefore concentrate on the rate in which **16** is consumed in the absence of O_2 or in the presence of the peroxyl radical scavenger Mn^{2+}.[186] It is expected that these two conditions decrease the consumption rate of **16**.

Besides a possible role in the consumption of additional substrate, O_2 is also important in preventing the intermediate cumene radical **16b** to couple. This is evidenced by the observation that in the absence of O_2 the dimer, 2,3-diphenylbutane (**17**), was observed as a major reaction product.[183] Such a dimerization under anaerobic conditions was also

observed using 1-(3′,4′-dimethoxyphenyl)acetone (DMPA) as a LiP substrate,[184] suggesting that O_2 may also serve a role in overcoming repolymerization of nonphenolic radical fragments to new lignin. Preventing repolymerization is also performed enzymatically as evidenced by the reduction of intermediate phenoxy radicals and radical cations by fungal cellobiose dehydrogenase.[21]

Finally, O_2 is essential for the $C_\alpha-C_\beta$ cleavage reaction of alkyl side chains as they do occur in DMPP, DMPA, and 1-(4′-acetoxy-3′-methoxyphenyl)-propene (isoeugenyl acetate, IEA).[184,185]

C. Indirect Oxidation of Aromatic Compounds by Mediation

Recent research indicates that organized arrays of lignin substructures, as present in wood, provide pathways for the transfer of electrons.[195] This finding suggests that a radical cation generated at the outside of lignin can travel deeper in the polymer by a series of intramolecular electron-transfer reactions. This insight is of interest especially since the micropores in wood are probably too small for penetration by large enzymes such as LiP,[46] and therefore, these findings suggest that LiP may also be effective while it is present at the outside of lignin.

An electron-transfer reaction, this phenomenon is called mediation, has also been shown to occur between aromatic compounds. Mediation only takes place if the conditions are thermodynamically favorable. Generally, the ionization potential (IP) of the mediator, the energy needed to abstract an electron from the highest occupied molecular orbital, should exceed that of the substrate. There is an exception. If the reaction between the mediator and the substrate is in balance, the reaction may be shifted to the right by a fast irreversible follow up reaction such as decarboxylation or C−H bond cleavage.[74,196] The chance for such a reaction to occur is enhanced by a longer lifetime of the mediator radical cation.[75,197]

During mediation the mediator is efficiently recycled upon oxidation of the substrate[185,196,198] and the conversion of the substrate is markedly enhanced if compared to conditions in which LiP alone catalyzes the oxidation of the substrate.[74,185]

1. p-Anisyl Alcohol

The compound *p*-anisyl alcohol (AA) is a terminal substrate for LiP. It appeared that addition of small amounts of VA significantly enhanced the consumption of AA.[73,74,96,127] At first this finding was explained by proposing that VA functioned as a redox mediator.[74] In a subsequent study the stimulating effect of VA on the AA oxidation was explained by VA completing the catalytic cycle of LiP allowing subsequent turnover and overcoming H_2O_2-dependent inactivation of LiP.[127] At this moment it is generally accepted that VA acts as a cosubstrate rather than a mediator during oxidation of AA, but the role of VA may be more complex as described below.

A good method to discriminate between VA functioning as a mediator or as a cosubstrate is by calculating the ratio between consumed AA and VA. In the case of mediation, the ratio has to be greater than 1 because the mediator is effectively recycled. In the alternative mechanism in which VA functions as a cosubstrate, this ratio ranges between 0 and 1 due to competition for oxidation by compound I. If there is no competition, the expected ratio is at best 1.

We took the liberty of calculating this ratio using published data, and it appears that at very low initial VA concentrations the ratio between consumed AA and VA ranges from 2.0 to 5.6,[73,74,96,127] which is clearly above the best value of 1.0 as expected according to the theory published by Koduri et al.[127] These calculated values appear to be in agreement with the mediation theory as proposed by Harvey et al.[74] At higher initial VA concentrations this ratio is in the expected range between 0 and 1,[96] which is in agreement with the current theory.

On the basis of the available literature data, it seems that the AA oxidation mechanism depends on the applied initial VA concentration. The finding that the efficiency of mediation depends on the VA concentration has been observed before upon studying the oxidation of isoeugenyl acetate (IEA). Increasing VA concentrations (up to 3 mM) resulted in a decrease in the ratio between consumed IEA and VA from 34 to 6.[185] This may be explained by the rationale that a possible reverse reaction between $IEA^{+\bullet}$ and VA (and subsequent follow up to VA derived products) is dependent on the VA concentration and may be minimized by lowering the initial VA concentration.

During future research an experiment is recommended in which the ratio between consumed AA and VA is studied at a series of different VA concentrations in order to clarify the described discrepancy.

More peculiar findings have been obtained using 1,4-dimethoxybenzenze (1,4-DMB) instead of VA as a mediator. This compound probably has an even lower redox potential than VA, and still ratios between consumed AA and 1,4-DMB are at best 2.0[96] or even 6.0.[74] Thus, it is well above the expected range between 0 and 1 if it solely were to act as a cosubstrate. A satisfactory explanation has been provided by Harvey et al.,[74] who stated that the $AA^{+\bullet}$ reacts rapidly and irreversibly via C−H bond cleavage, which is in contrast to the 1,4-DMB radical cation which is quite stable.[199] Likely, the long lifetime enables the 1,4-DMB radical cation to play a role as redox mediator.[74,75]

The radical cation of another redox mediator, 2-Cl-1,4-DMB,[96,97] was found to be reduced by AA,[97] resulting in recycling of the mediator and formation of AAld.[96]

2. Oxalate

LiP does not oxidize oxalate directly, but in the presence of VA $^{14}CO_2$ from ^{14}C-oxalate is evidently formed and at the same time the formation of VAD from VA is noncompetitively inhibited. This indicated that oxalate did not compete with VA for the same binding site as present in LiP. The operating mechanism is rationalized by an electron-transfer reaction between the enzymatically formed $VA^{+\bullet}$ and oxalate, resulting in decarboxylation of oxalate and reduction

of $VA^{+\bullet}$ back to VA.[76] This mechanism has been confirmed by an EPR experiment showing that the $VA^{+\bullet}$ signals are significantly smaller in the presence of oxalate.[200]

3. 4-Methoxymandelic Acid

Another carboxyl group containing model substrate for studying mediation is 4-methoxymandelic acid (MMA). Oxidation of the latter was significantly enhanced in the presence of only catalytic amounts of VA or 1,4-DMB.[74] Tien et al.[75] showed that increasing VA concentrations did not inhibit the oxidation of MMA and that the enzyme-bound $VA^{+\bullet}$ signal, detected by EPR, decreased upon addition of MMA. Also, the finding that VAD was not found as a VA oxidation product during MMA consumption is consistent with VA-mediated electron transfer.[198]

The observation that homogeneous $VA^{+\bullet}$ does not react with MMA did not fit within the redox mediation theory, and therefore, Candeias et al.[197] proposed that the $VA^{+\bullet}$ can exist as an enzyme-bound species, which was confirmed by Khindaria et al.[200] The enzyme-bound radical cation presumably has in this state either a longer lifetime or a higher reduction potential than in bulk solution.[197] The lifetime of free $VA^{+\bullet}$ has been measured and was around 59 ms,[176,197] and that of the enzyme-bound species appeared, indeed, higher as indicated by a lifetime of 0.54 s,[200] which is sufficient to explain the observed redox mediation.[75]

The shorter the lifetime of the MMA radical cation, the better is the chance for mediation to occur.[197] The lifetime of $MMA^{+\bullet}$ is probably too short because it rapidly and irreversibly cleaves of CO_2, finally yielding AAld as the sole product,[74,196] shifting the balance toward oxidation of MMA.

4. Phenolics

The fact that during the oxidation of phenolics LiP activity is lost has triggered a lot of scientific effort to reveal the underlying mechanism.[126,201−203]

Koduri et al.[126] investigated the oxidation of 2-methoxyphenol (guaiacol) ($K_m = 160\ \mu M$) and showed that the presence of guaiacol resulted in reversion of compound III back to native LiP, but this process was not as effective as in the presence of VA. Guaiacol appeared to be a better reducing substrate than VA for both compounds I and II. The oxidation of guaiacol was also studied in the presence of VA. In those cases lag phases in the formation of the VA oxidation product, VAD, were found that were proportional to the applied guaiacol concentration. The oxidation rate after the lag phase was the same as that obtained without guaiacol. Together this indicated that guaiacol could be oxidized directly by LiP or indirectly via an electron transfer between $VA^{+\bullet}$ and guaiacol.

Chung et al.[201,202] investigated the oxidation of phenol ($K_m = 810\ \mu M$), which showed a different oxidation pattern in the presence of VA ($K_m = 200\ \mu M$) if compared to the results obtained with guaiacol.[126] During phenol oxidation there were also lag phases in VAD formation, but now the remaining VAD formation rate after this time period was reduced significantly as explained by LiP compound III accumulation. Phenol, which is like guaiacol, a very good reductant for both LiP compounds I and II, but nonetheless LiP is inactivated during the reaction.

The mechanism involved in inactivation of LiP during the oxidation of phenol was mainly due to the accumulation of compound III, which was attributed to the inability of phenol or phenoxy radicals to revert compound III back to ferric LiP.[202]

Radical cations such as those from methoxybenzenes are involved in the reactivation of inactive LiP compound III, a complex between LiP and superoxide.[131,204] The radical cations may abstract an electron from the bound superoxide, resulting in native LiP and oxygen. These radical cations may alternatively generate superoxide from H_2O_2.[205] Superoxide itself is a very efficient superoxide scavenger as indicated by the very fast dismutation reaction between two superoxide species yielding H_2O_2 and O_2.[86] This allows the remarkable possibility that superoxide, which may be formed as a result of LiP activity, can also participate in reverting LiP compound III back to native LiP. This theory has been evidenced by the finding that the enzyme superoxide dismutase enhances the lifetime of LiP compound III.[113]

VI. Oxidation Reactions Catalyzed by MnP

The oxidation of phenolic compounds, which contain only one phenolic ring, by enzymatically generated Mn^{3+} has been studied in great detail.[59,206] Also, phenolic counterparts of lignin model compounds **3** (β-1 model)[207] and **6** (β-O-4 model)[53] have been described. The formation of the observed products from the β-1 model has been satisfactory accounted for by a mechanism in which Mn^{3+} oxidizes both the substrate and intermediate substrate-derived radicals.[207]

Upon oxidation of the β-O-4 model, the corresponding phenoxy radical was formed, which was proposed to undergo homolytic cleavage.[53] However, also a subsequent oxidation step to a cation followed by heterolytic cleavage is consistent with the published experimental findings. Heterolytic cleavage is a more likely event because the carbocation next to the oxygen is more stable than the corresponding carbon-centered radical,[194] but this theoretical statement needs experimental support.

VII. Laccase

A. Oxidation of Veratryl Alcohol

As soon as it was clear that laccase together with ABTS could oxidize VA, mechanistic studies were undertaken. It appeared that laccase first rapidly oxidizes ABTS to its radical cation.[160,208,209] The subsequent formation of VAD takes a much longer period of time than could be logically expected from the fast oxidation rate of ABTS.[27,160,209,210] The formation of VAD was therefore not the result of a electron-transfer reaction between VA and the ABTS radical cation.[27,160]

The sole product of the oxidation reaction was VAD.[208,210] As described before, VA is also a good

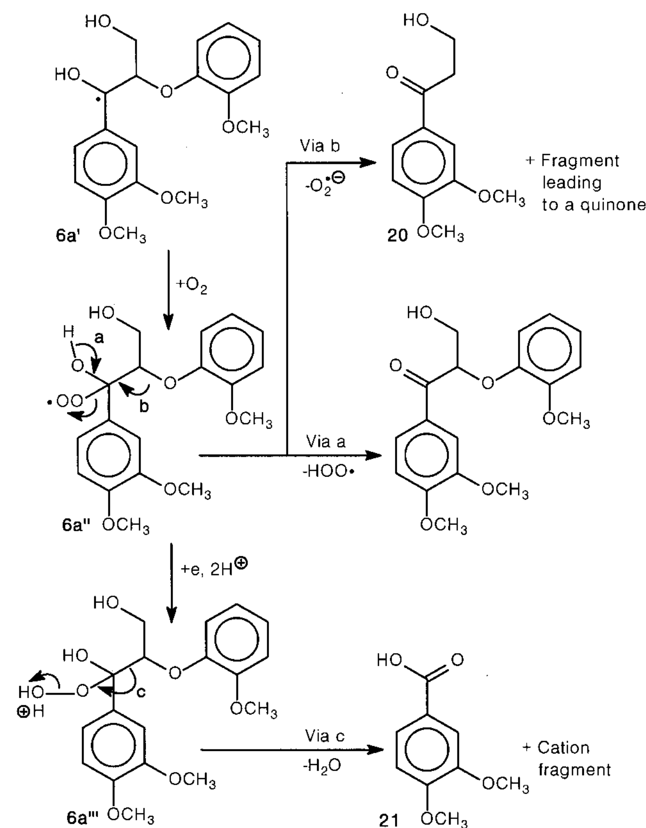
substrate for LiP, which oxidizes it to VAD but also to several side products as explained by a mechanism that involves radical cation intermediates. Because VAD was the only product from the laccase ABTS reaction, it was proposed that the reaction did not occur via radical cation intermediates but via a hydrogen abstraction mechanism as postulated by Muheim et al.[160] The VA radical formed reacts indeed extremely fast with oxygen to VAD.[176] However, Muheim et al.[160] also showed that oxidation of VA by LiP in the presence of ABTS also yields VAD as the sole product. They also showed that laccase/ABTS did not oxidize 1,4-dimethoxybenzene whereas LiP alone oxidizes it to a radical cation. Again, combination of LiP, ABTS, and 1,4-dimethoxybenzene did not result in oxidation of 1,4-dimethoxybenzene.[160] It is stated that these findings alone are not sufficient to propose a hydrogen abstraction mechanism which is based on two arguments that are described in the following paragraphs.

It is conceivable that the above-described results were previously interpreted as a difference between a compound with and one without C_α hydrogen atoms, leading to the proposal of a different oxidation mechanism in the presence of ABTS. However, there is another difference between the two compounds which is the stability of the corresponding radical cations. If it is assumed that 1,4-DMB$^{+\bullet}$ was formed during this reaction, it is likely reduced back to 1,4-DMB by ABTS because 1,4-DMB$^{+\bullet}$ is, unlike VA$^{+\bullet}$,[176,197,200] stable enough[199] and the ionization potential of ABTS is low enough to allow such a reaction.

After publication of the hydrogen abstraction theory by Muheim et al.[160] in 1992, it was already known that it was possible to minimize side product formation from VA by changing the experimental conditions such as applying anaerobic conditions, addition of Mn^{2+}, or by increasing the pH.[177] These findings are explained by a shift in importance in the radical cation mechanism.[180] Therefore, only the finding that ABTS also suppressed VA side product formation is not enough evidence to propose a hydrogen abstraction mechanism. Nevertheless, this mechanism of hydrogen abstraction was generally accepted until 1998.

In 1998 Bourbonnais et al.[209] showed in an electrochemical study that ABTS can be oxidized to its dication which can oxidize VA to VAD. The reduction rate of ABTS^{2+} by VA was found to be very slow (k = 170 $M^{-1}\cdot s^{-1}$) which provided a good explanation for the observation that the oxidation of VA to VAD takes several hours even if the enzyme oxidizes ABTS to its radical cation within a few minutes.[209] In a subsequent study, the substrate spectrum of ABTS^{2+} was expanded to benzyl alcohol, *p*-anisyl alcohol, and 1-phenyl-1-ethanol. Though, an electron-transfer reaction from the aromatic alcohol appears to be thermodynamically unfavorable, the reaction was proposed to be driven forward as a result of the deprotonation of the aromatic alcohol radical cation intermediate. This was evidenced by the finding that even the ABTS radical cation, which was unreactive

Scheme 5. Proposed O_2-Dependent Manner of Product Formation from Radical 6a′, Formed upon Oxidation by Laccase/1-HBT



at pH values below 7.0, could clearly oxidize VA to VAD at higher pH values.[211]

B. Nonphenolic Lignin Model Compounds

Also, nonphenolic lignin model compounds are oxidized by laccase in the presence of a suitable mediator.[27,160−163,165,212,213] Similar model compounds have also been used in studies with LiP. Unlike LiP, the laccase mediator system oxidizes **6** (see Chart 2) predominantly at C_α forming **7** in a molar yield ranging between 70% and 82%.[161,165] C_α−C_β cleavage was only a minor event as indicated by a 4−5% molar yield of 3,4-dimethoxybenzoic acid (**21**). VAD was not among the cleavage products.[161] The formation of products may be explained by mechanism involving both hydrogen abstraction[160] and radical cation chemistry.[212]

By assuming the involvement of radical cations there are four minor products that can be accounted for.[212,214] Among these products are **8**, **9**, and **12**. The formation of product **12** has been explained elsewhere[189,190,215] and is proposed to be formed via an unstable intermediate that contains both a hydroperoxide and a dioxetane group.[189] The involvement of the latter has been shown before in peroxidase-catalyzed reactions.[216]

In the case of experiments with 1-HBT as a mediator, 1-HBT is oxidized to a radical[162,217] which may react with substrates by abstracting a hydrogen atom. This makes radical **6a′** (see Scheme 5) the most predominant intermediate in the reaction mechanism. This radical probably reacts rapidly with O_2

forming peroxyl radical **6a″**. It is proposed that the abundance of peroxyl radicals, in the laccase-catalyzed oxidation, is essential in the formation of acid **21**. This proposal is based on the fact that **21** is not formed in LiP-catalyzed reactions[20,100,111,188−192] because the decay of radical cation **6a** via $C_\alpha-C_\beta$ cleavage appears to be more important than deprotonation. Therefore, radical **6a′** and the corresponding peroxyl radical formed thereof are much less abundant in LiP-catalyzed reactions.

The subsequent route toward **21** involves the uptake of an electron by **6a″**, followed by an acid-catalyzed chemical cleavage of the hydroperoxide intermediate **6a‴**. It is also possible to explain the formation of other abundant products by starting with peroxyl radical **6a″**.

As shown in Scheme 5, the elimination of HOO• via route a yields the main product **7**. Alternatively, cleavage route b may be taken, which yields product **20** and a fragment involved in the formation of a quinone which has indeed been identified,[212] thus evidencing this route.

VIII. Self-Propagating Chemical Reactions Initiated by Oxidative Enzymes

A. Lipid Peroxidation

Some white-rot fungi do not excrete LiP but are nonetheless able to degrade lignin and nonphenolic lignin model compounds.[218,219] Some species that do excrete LiP appear able to degrade recalcitrant polyaromatic hydrocarbons (PAHs) that have an IP which is clearly above the threshold of LiP.[220,221] Together, this suggests that white-rot fungi also possess another way to oxidize lignin. The underlying mechanism has been studied in detail.[218−223]

It appeared that a mixture of unsaturated fatty acids, Mn^{2+}, and MnP cause lipid peroxidation.[220] During this process carbon-centered lipid radicals are formed which react with oxygen-forming peroxyl radicals.[219,222] These can react with other lipid molecules to propagate and accelerate the oxidation of the fatty acid,[219] but alternatively they oxidize other organic compounds.[222] Since these lipid peroxyl radicals are quite stable, they can diffuse over relatively long distances, suggesting that they may be involved in the primary attack of lignin[219,222] because they can, unlike the peroxidases, enter the microporosity of the lignin cellulose matrix.[155]

During lipid peroxidation of a nonphenolic lignin model, a β-1 model,[218] similar products were found as those observed during oxidation by laccase/1-HBT.[161] There was one exception, however, which is further oxidation of 4-ethoxy-3-methoxybenzaldehyde to the corresponding acid during lipid peroxidation,[218] whereas further oxidation was not observed with laccase/1-HBT.[161] This indicates that the oxidative power of lipid peroxidation is superior to that of the laccase/1-HBT system.

B. 1-(3′,4′-Dimethoxyphenyl)propene

Recently, it has been proposed that the consumption of DMPP proceeds both via substrate-derived peroxyl radicals and by LiP.[184] This has been evidenced by a decreased initial DMPP consumption rate under anaerobic conditions. The consumption of DMPP was also inhibited by the peroxyl radical scavenger Mn^{2+}.[184]

It is not likely that the observed inhibition can be alternatively explained by a difference in the H_2O_2 concentration, which would be less in the absence of O_2 and in the presence of Mn^{2+} because the K_m of LiP for H_2O_2 is 100 μM[99] (at the applied pH of 3.0) which is well below the applied initial H_2O_2 concentration.[184]

Another piece of evidence pointing to such a mechanism in which O_2 accelerates the consumption of additional substrate is the presence of a hydroperoxide as a product which was found upon oxidation of lignin model compound **16** by LiP.[183] Hydroperoxides are formed from peroxyl radicals by the uptake of an electron and a proton. This oxidation step by the substrate-derived peroxyl radical led us to propose that the presence of hydroperoxide intermediates is indicative for O_2-dependent chemical reactions that accelerate the consumption of substrate, but this needs further experimental support.

IX. Concluding Remarks

In this review the most important findings are described on the different oxidative manners that white-rot fungi use as have been published from 1981 onward. The discovery of the peroxidases and suitable mediators for laccase has triggered a lot of follow up research on gene regulation, biochemical characterization, and the oxidation of a large variety of model compounds via different oxidation mechanisms.

It would be very interesting if it were possible to somehow quantify the relative contribution of each mechanism in the degradation of lignin by white-rot fungi. This, however, may be very difficult because the mechanisms probably operate synergistically.

At several places in the review we describe conflicting literature data, inconsistent theories, or alternative interpretations of published findings. In some cases more experimental work is recommended which is summarized in the following paragraphs.

The effect of Mn^{2+} and anaerobic conditions on the LiP-catalyzed oxidation rate of VA and model compound **16** may help to clarify the possibility that O_2-dependent chemical reactions are involved in additional consumption of these substrates.

The mechanism of AA oxidation by LiP in the presence of VA seems to depend on the initial VA concentration. This aspect has not been covered by the available literature, and therefore, more experimental work on this subject is encouraged.

Finally, we describe that the cleavage mechanism of β-O-4 lignin model compounds has not been determined irrefutably. In theory there are two possible ways in which the $C_\alpha-C_\beta$ bond of a β-O-4 model-derived radical cation can cleave, either homolytic or heterolytic. The current view, based on the presumption that β-1 and β-O-4 models cleave alike, is that the radical cation undergoes homolytic cleavage of the $C_\alpha-C_\beta$ bond. The alternative heterolytic

cleavage is thermodynamically more favorable, pointing out that there is a reason to question the current view. As indicated in the review, the β-O-4 model such as **6** would need modification to ensure the identification of crucial cleavage products. It is proposed that a cyclic ether (obtained by attaching the C_γ hydroxy group in **6** to either one of the two rings) may be suitable for this kind of research. The β-O-4 model compounds are used as a model for 50–60% of the linkages in lignin which clearly merits future research on the way in which LiP cleaves C_α–C_β bonds in these models.

We hope that this paper encourages scientists to provide new data regarding the subjects as described in this paragraph and elsewhere in the review.

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