
### Catalytic mechanisms and regulation of lignin peroxidase

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**Summary**

Lignin peroxidase (LiP) is a fungal haemoprotein similar to the lignin-synthesizing plant peroxidases, but it has a higher oxidation potential and oxidizes dimethoxylated aromatic compounds to radical cations. It catalyses the degradation of lignin models but in vitro the outcome is net lignin polymerization. LiP oxidizes veratryl alcohol to radical cations which are proposed to act by charge transfer to mediate in the oxidation of lignin. Phenolic compounds are, however, preferentially oxidized, but transiently inactivate the enzyme. Analysis of the catalytic cycle of LiP shows that in the presence of veratryl alcohol the steady-state turnover intermediate is Compound II. We propose that veratryl alcohol is oxidized by the enzyme intermediate Compound I to a radical cation which now participates in charge-transfer reactions with either veratryl alcohol or another reductant, when present. Reduction of Compound II to native state may involve a radical product of veratryl alcohol or radical product of charge transfer. Phenoxy radicals, by contrast, cannot engage in charge-transfer reactions and reaction of Compound II with H₂O₂ ensues to form the peroxidatically inactive intermediate, Compound III. Regulation of LiP activity by phenolic compounds suggests feedback control, since many of the products of lignin degradation are phenolic. Such control would lower the concentration of phenolics relative to oxygen and favour degradative ring-opening reactions.

**Introduction**

Lignin, a major constituent of plant cell walls and one of the most recalcitrant natural polymers known, can be degraded to CO₂ and H₂O under aerobic conditions by a group of specialized ‘white-rot’ fungi, and although the extracellular enzymology of degradation has been the subject of intensive investigation in recent years, the mechanisms involved remain elusive. Three enzymes have emerged as important to the process, LiP, discovered in 1983 [1, 2], a manganese-dependent peroxidase (MnP) and laccase. LiP has a higher oxidation potential compared with the other enzymes and is able to catalyse the one-electron oxidation of dimethoxylated compounds representative of the intramolecular structures of lignin, to radical cations that degrade in a manner dependent on the nature of substituent groups and reaction conditions [3, 4]. With lignin model compounds, it catalyses the C–C and ether cleavage reactions important for lignin depolymerization. However, it is also able to oxidize phenolic compounds to radicals which participate in a variety of non-enzyme-catalysed reactions, including polymerization [5, 6]. Polymerization of phenols underlies the basis of lignin synthesis and in vitro, LiP has been shown to catalyse further polymerization of lignin through its oxidative action on the phenolic side-chains of the lignin polymer [7, 8]. Polymerization is enhanced in the presence of the fungal secondary metabolite veratryl alcohol [7].

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*Abbreviations used: LiP, lignin peroxidase; HRP, horseradish peroxidase; 4-MA, 4-methoxy mandelic acid; MnP, manganese-dependent peroxidase.*

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Recently, however, it was reported that in the presence of low concentrations of \( \text{H}_2\text{O}_2 \) and veratryl alcohol, LiP would catalyse the partial depolymerization of a synthetic hardwood lignin [9]. This paper examines the catalytic mechanism of LiP and the role of veratryl alcohol, and provides a model by which lignin-depolymerizing reactions might be favoured over lignin-polymerizing reactions.

**Catalytic cycle of LiP**

LiP shares many structural features in common with horseradish peroxidase (HRP) (one iron protoporphyrin IX in the high-spin pentacoordinate ferric state with histidine coordinated as the fifth ligand [10, 11]; a high degree of sequence similarity for the active-site amino acids [12, 13] and a similar closed active-site structure [14]). The catalytic cycle is also similar; reaction with \( \text{H}_2\text{O}_2 \) yields the intermediate Compound I which is reduced back to the native state via Compound II [4, 5]. However, the Compound I of LiP develops a higher oxidation potential with \( \text{H}_2\text{O}_2 \) than HRP, the basis of which is unknown. The second-order reaction constant for Compound I formation is \( 4.2 \times 10^5 \text{ M}^{-1} \text{s}^{-1} \) [15, 16] and is controlled by an acidic ionizable group of \( pK_a = 1 \) [17] which is consistent with the unusually low pH optimum (pH 1.2) for steady-state turnover. This has a value of approx. 4 s\(^{-1}\) at pH 1.3. Rate constants for the reduction of Compounds I and II by veratryl alcohol are reported to be \( 2.62 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \) (pH 2.75) and \( 1.6 \times 10^5 \text{ M}^{-1} \text{s}^{-1} \) (pH 3.05) [18] (but see below). Compound II can also react with \( \text{H}_2\text{O}_2 \) to yield Compound III but the reaction occurs with considerably lower concentrations of \( \text{H}_2\text{O}_2 \) than is required with HRP [19–21]. The second-order rate constant determined at low \( \text{H}_2\text{O}_2 \) levels, has been calculated as \( 1 \times 10^4 \text{ M}^{-1} \text{s}^{-1} \), approximately 500 times faster than that recorded for HRP [21]. Compound III is stable [22], but in the presence of further \( \text{H}_2\text{O}_2 \) and veratryl alcohol is converted back into the native state via a so-called intermediate Compound III\(^*\) [23] with a second-order rate constant determined as \( 3.5 \times 10^5 \text{ M}^{-1} \text{s}^{-1} \) [23].

**Reaction with veratryl alcohol**

Veratryl alcohol is a fungal secondary metabolite produced at the same time as LiP [24, 25] and oxidized by LiP to yield veratraldehyde [26]. However, it also enhances catalysis by LiP [7, 27]. We have proposed that it is oxidized to radical-cation intermediates that, in their known capacity to act as diffusible charge-transfer reagents, facilitate oxidation of hydrophobic, large molecules like lignin, at the same time as promoting reaction of Compound II with an electron donor over the reaction with \( \text{H}_2\text{O}_2 \) [19, 27]. This would otherwise result in the formation of the stable intermediate, Compound III and cessation of the peroxidase cycle. The formation of veratryl alcohol radical-cation intermediates and their ability to participate in charge-transfer reaction has, however, been widely questioned. In particular, e.p.r. techniques have been unable to identify veratryl alcohol radicals as intermediaries in the LiP-catalysed oxidation of veratryl alcohol [28], and an alternative explanation for the enhancing effect of veratryl alcohol has been presented. In this veratryl alcohol converts Compound III\(^*\) back into native enzyme to restore activity [29].

However, several lines of evidence strongly suggest that the LiP-catalysed oxidation of veratryl alcohol involves radical-cation intermediates able to participate in charge-transfer reactions. Apart from veratraldehyde, ring-opened and quinone products of the oxidation of veratryl alcohol have been identified in minor amounts under aerobic conditions. These have been attributed to the reaction of oxygen with radical cation and radical intermediates respectively [30, 31], consistent with oxygen consumption measurements taken in the course of the reaction [32]. The enhancing effect of veratryl alcohol on LiP-catalysed reactions is similar to that obtained with dimethoxybenzene [19, 27], for which an e.p.r. signal corresponding to the radical cation has been identified [33]. More recently, using n.m.r. spectroscopy, evidence was obtained for the production of radical intermediates which participated in charge-transfer reactions with veratryl alcohol [34].

**Mechanism of reaction-enhancement by veratryl alcohol**

Table 1 shows that in the oxidation of the monomethoxylated compound +methoxymandelic acid (4-MA, 1200 nmole) by LiP, only 2 nmole of product (anisaldehyde) are produced until veratryl alcohol is added (1139 nmole), whereas the yield of anisaldehyde is increased to 360 nmole. Significantly, veratraldehyde from the oxidation of veratryl alcohol could not be observed, despite the fact that the concentration of veratryl alcohol was equimolar to that of 4-MA. Based on their relative redox potentials and the fact that the rate constant for the reduction of Compound I by 4-MA is \( 1.5 \times 10^7 \text{ M}^{-1} \text{s}^{-1} \) [34], we have attributed these effects to the preferential oxidation of veratryl alcohol by LiP to radical cations. The radical cations so formed oxidize 4-MA in a process of charge transfer. Products from the oxidation of 4-MA are observed because of the greater tendency for C–C...
Table I
Oxidation of 4-methoxymandelic acid in the absence and presence of veratryl alcohol

The reactions were carried out in 20 mM-phosphate buffer at pH 2.75 containing 0.5 nmoles of lignin peroxidase and either, or both, 1139 nmoles of veratryl alcohol and 1202 nmoles of 4-MA where appropriate, in a total volume of 800 ml. The reaction was initiated by adding 430 nmoles of H_2O_2 and the incubation continued for 1 h before the products were separated on a C18 reversed phase h.p.l.c. column using 40% acetonitrile in water and monitored at 278 nm.

<table>
<thead>
<tr>
<th>Substrates added</th>
<th>Veratryl alcohol</th>
<th>Veratraldehyde</th>
<th>Methoxymandelic acid</th>
<th>Anisaldehyde</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veratryl alcohol</td>
<td>594</td>
<td>318</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>4-MA</td>
<td>—</td>
<td>—</td>
<td>1116</td>
<td>2</td>
</tr>
<tr>
<td>Veratryl alcohol plus 4-MA</td>
<td>969</td>
<td>0</td>
<td>626</td>
<td>353</td>
</tr>
</tbody>
</table>

![Spectral analysis of lignin peroxidase](image)

The reaction conditions were the same as given in the legend of Table I. The spectra were recorded using a Hewlett Packard (HP 8452A) diode array spectrophotometer 1, 40 and 75 s after initiating the reaction with H_2O_2. Trace (a) 4-MA alone, (b) veratryl alcohol alone, (c) 4-MA plus veratryl alcohol.

Side-chain fragmentation in the case of 4-MA compared with C-H side-chain fragmentation in veratryl alcohol [19, 27]. The alternative explanation [29] is that veratryl alcohol converts Compound III*, formed during oxidation of 4-MA, back into its native state, without being oxidized to veratraldehyde. However, Fig. 1 shows that the steady-state turnover intermediate observed in the course of oxidation of 4-MA in the presence of veratryl alcohol is Compound II and not Compound III. When 4-MA was omitted, the steady-state turnover intermediate was Compound II, but when veratryl alcohol was omitted, the steady-state turnover intermediate with 4-MA was Compound III. Similar results were obtained when either anisyl alcohol or ferrocyanide were substituted for 4-MA. These results cannot be explained in terms of veratryl alcohol acting on Compound III* since the rate of this reaction
(3.5 × 10⁴ M⁻¹ s⁻¹ [23]) would mean that Compound III should have been observed as the steady-state intermediate and not Compound II. Compound II has also recently been reported to be the steady-state intermediate during the oxidation of Poly R in the presence of veratryl alcohol [36]. When the reduction of Compound II, formed after reduction with H₂O₂, was examined by stopped-flow techniques, the rate of reduction with veratryl alcohol was determined to be independent of concentration (1–50 mM-veratryl alcohol) and slow, in the order of 4–6 s⁻¹, at pH 3. This result is similar to that originally reported by Tien et al. [28] but dissimilar to that recently reported by Warishi et al. [18]. It is consistent with the observation of Compound II as a steady-state intermediate in the experiments described above. On the basis of these results, we propose that veratryl alcohol is oxidized by the enzyme intermediate Compound I to be a radical cation which now participates in charge-transfer reactions with either veratryl alcohol or another reductant when present. Reduction of Compound II to native state will occur provided veratryl alcohol is present. This may involve reduction by a radical product (for example, veratryl alcohol radical obtained after C–C cleavage or 4-MA radical formed after C–C cleavage of the respective radical cations) or alternatively, an interaction with veratryl alcohol or a derivative to prevent reaction with H₂O₂ to form Compound III.

**Reaction with phenols**

During the oxidation of phenolic compounds, compared with veratryl alcohol, LiP is inhibited [6]. Since inhibition can be relieved by lowering the level of H₂O₂, inhibition is considered to reflect the relative ease with which reaction of Compound II with H₂O₂ occurs in the absence of veratryl alcohol. Phenolic compounds are also preferentially oxidized by LiP compared with veratryl alcohol [6]. This means that for maximal LiP turnover and for the oxidation of non-phenolic compounds, the occurrence of phenolic compounds in the vicinity of LiP needs to be minimized.

**Model for lignin breakdown**

LiP enzymes are proposed to be fixed in space, closely associated with the fungal sheath and in the vicinity of a pool of veratryl alcohol and H₂O₂. Oxidation of veratryl alcohol yields radical cations which exchange rapidly with neighbouring molecules. Some of these dismutate to yield veratraldehyde, which may be recycled back to veratryl alcohol [38]. Others oxidize lignin. In this way, contact by LiP with phenolic compounds is minimized. However, charge transfer through the lignin polymer will produce phenolic degradation products among others, as a consequence of the LiP-initiated oxidation process. Phenolic compounds will be more easily oxidized than non-phenolic compounds, to phenoxy radicals. In the presence of oxygen they may react to form ring-opened products, which could then be metabolized intracellularly to CO₂. They may alternatively be reduced in a cycle to increase the chance of reaction ultimately with oxygen. They may otherwise couple to produce a polymer with even higher redox potential than lignin itself, and therefore less susceptibility to oxidative degradation. The accumulation of phenolic products relative to the concentration of oxygen available for reaction is likely to favour their polymerization. Such accumulation might be minimized by regulating the rate of their production. This is achieved by regulating the rate of lignin degradation through feedback control of LiP with phenolic compounds which drive the enzyme into an inactive catalytic state. Further oxidation of veratryl alcohol and hence, lignin, would be prevented until the pool of phenolic compounds was reduced. This may be achieved through the action of the phenol-oxidizing enzymes, laccase or MnP, or by further intracellular fungal metabolism [39].

Summary

A peroxidase has 1-electron oxidation as its characteristic activity, while that of cytochrome P-450 is hydroxylation. Both catalytic cycles involve similar high valency states of iron. However, a peroxidase can only accept electrons at the haem edge, while the substrate for cytochrome P-450 is bound in a precise orientation before the active state is created.

Introduction

A peroxidase is defined as an oxidoreductase acting with hydrogen peroxide as acceptor (EC 1.11). This discussion will be restricted to haem proteins such as horseradish peroxidase or the ligninase of white-rot fungi, in their characteristic action as 1-electron oxidizing agents:

\[ H_2O_2 + 2X + 211^+ \rightarrow 211O + 2X^- \]  
(Scheme 1)

Cytochrome P-450 is the collective name for haem proteins that give an Fe(II)-CO complex with a \( \gamma \)-band near 450 nm, a property that correlates with a cysteine anion (thiolate) as axial ligand to the haem [1]. The cytochromes P-450 typically act as mono-oxygenases; the covalent bond of dioxygen is broken, one O-atom being incorporated into an organic substrate, while the other is reduced to water:

\[ RH + O_2 + 2H^+ + 2e^- \rightarrow ROH + H_2O \]  
(Scheme 2)

The O-atom incorporation is often followed by an elimination or rearrangement [2]; such secondary reactions are outside the scope of this discussion.

Redox states of oxygen

The redox chemistry of oxygen underlies the catalytic cycles of these enzymes. Successive 1-electron reductions of dioxygen yield superoxide, hydrogen peroxide, the hydroxyl radical and water, with 1-electron potentials \( (E_0) \) in volts as shown below [3]:

\[
\begin{align*}
O_2 & \quad O_2^- \quad H_2O_2 \quad H_2O + HO^- \quad 2H_2O \\
& \quad -0.33 \quad +0.89 \quad +0.38 \quad +2.32
\end{align*}
\]