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Short communication

# Phenolic compounds as likely natural mediators of laccase: A mechanistic assessment

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**Abstract**

The oxidation mechanism of non-phenolic substrates induced by laccase under catalysis by two phenolic mediators is shown to be radical.


*Keywords:* Enzymes; Laccase; Mediators; Phenols; Radicals

Laccases (EC 1.10.3.2) are oxidases mainly produced by basidiomycete fungi. By using four Cu centres, they perform the monoelectronic oxidation of reducing substrates, coupled to the $4e^-$ reduction of $O_2$ to $H_2O$ [1,2]. Because their redox potential is low and in the 0.5–0.8 V *vs*. NHE range, laccases typically use phenols as substrates, or phenolic units in lignin (phenoloxidase activity, Scheme 1), for redox compatibility [2]. This induces the well-known oxidative phenol coupling/oligomerization through aryloxyl radical intermediates [3].

Non-phenolic substrates, or the plentiful non-phenolic units in lignin, having redox potential above 1.3 V, cannot be oxidised by laccases directly. However, an indirect oxidation becomes possible in the presence of redox mediators [4]. These are oxidised monoelectronically by laccase, and in turn oxidise non-phenolics (such as benzyl alcohols) according to mechanisms unattainable to laccase, for example by H-atom transfer (HAT), thereby overcoming redox restrictions [5,6]. Several of these mediators have been described: most of them belong to the hydroxylamine (>NO–H) class, and their mechanism of oxidation has been investigated (Scheme 2) [5–7].

It has been recently found that some phenolic compounds [8,9], such as Phenol Red, catechol, hydroquinone, or a few others [10,11], when oxidised by laccase to resonance-stabilised aryloxyl radicals, become able to catalyse the oxidation of non-phenolics (Scheme 3) before being depleted in coupling or $O_2$-induced ring-cleavage reactions.

This finding assigns an expanded natural role to the phenoloxidise laccase: not only as a promoter of lignin biosynthesis, but also as a delignifying enzyme even in the absence of added 'foreign' mediators (like the hydroxylamines in Scheme 2), because phenolic compounds are common in the environment of the enzyme [9]. No specific study of the mechanism of oxidation of non-phenolic substrates induced by phenolic mediators has been reported, and the involvement of the $ArO^{\bullet}$ species as a reactive intermediate in a HAT oxidation had been plainly inferred (cf. Scheme 3) [9]. The present study aims at resolving this overlooked point.

## 1. Hammett correlation

A consolidated mechanistic tenet has been followed here, that the Hammett treatment of the substituents effect upon reactivity, as well as the determination of the kinetic isotope effect, can enable to assess the mechanism of oxidation of non-phenolic substrates, induced by laccase and catalysed by two phenolic mediators (Fig. 1) taken here as exemplary although simplified cases [8,9].

Accordingly, the effect of substituents in making faster or slower the aerobic oxidation by laccase from *Trametes villosa* (TvL), with either Phenol Red or hydroquinone as mediators (ArOH in Scheme 4), has been investigated in buffered water solution (pH 5) at 25 °C with a small series of 4-X-substituted benzyl alcohols (i.e., $Ar'CH_2OH$), having electron-donor or electron-withdrawing X-substituents. Benzylic alcohols are well-established functional models of non-phenolic (benzylic)

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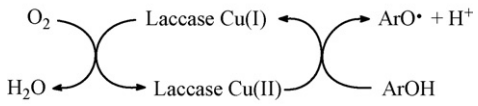
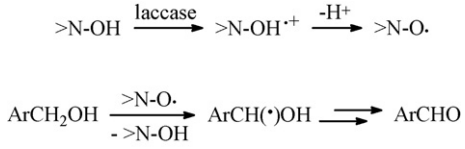
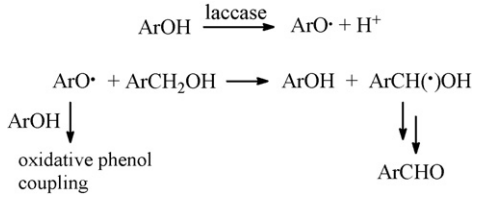



Scheme 1. The redox cycle of the phenoloxidase laccase.




Scheme 2. The role of a >NO–H mediator of laccase in the oxidation of non-phenolic substrates: the HAT route.




Scheme 3. Phenols as mediators of laccase towards non-phenolic substrates.

structural moieties in lignin [5], and mechanistic information deriving from their reactivity with laccase and mediators is considered significant for the issue of the oxidative delignification. Each one of these substituted benzyl alcohols has been pitted with the unsubstituted parent compound in separate competition experiments as already described [5], and the $k_X/k_H$ relative reactivity ratios determined by GC analysis from the amount of the two aldehydes produced (Ar'CHO and PhCHO, respectively; Scheme 4) [5], for reaction times that would ensure only a modest conversion to products (Table 1). No other products, besides Ar'CHO and PhCHO, have been observed.

A plot of the $k_X/k_H$ ratios in logarithmic form *vs.* the $\sigma^+$ parameter of the substituents in a Hammett-like treatment has allowed obtaining the $\rho$ values from the linear correlations (Fig. 2).

The small $\rho$ values obtained (i.e., −0.57 and −0.46) support the radical nature of the slow step in the oxidation

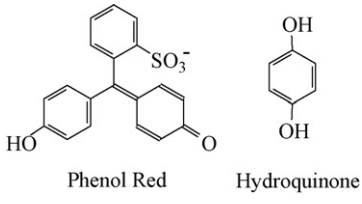
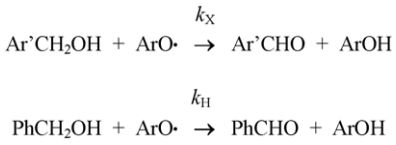
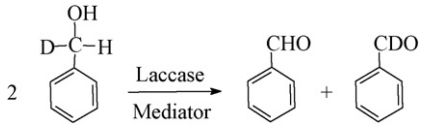



Fig. 1. Phenolic mediators investigated.




Scheme 4. Competitive experiments of aerobic oxidation of 4-X-substituted benzyl alcohols (Ar'$CH_2$OH) by TvL and phenolic mediators (ArOH).




Scheme 5. Isotope effect determination from product analysis.

reaction by laccase and phenolic mediators, in keeping with a HAT route through an intervening aryloxyl radical. This is indeed the reaction mechanism followed (Scheme 2) by aminoxyl radical intermediates deriving from >NO–H mediators. For example, with HPI (*N*-hydroxyphthalimide), HBT (1-hydroxybenzotriazole), VLA (violuric acid) and NHA (*N*-hydroxyacetamide) as mediators of TvL, we had determined $\rho$ values (*vs.* $\sigma^+$) of −0.89, −0.64, −0.41 and −0.42, respectively [5,12], that is, negative in sign and similarly small in absolute value. The negative sign of $\rho$ and the better fit invariably obtained *vs.* $\sigma^+$, are consistent with the electrophilic character of the attacking O-centred radicals [5], whereas the small but not negligible value of $\rho$ is in line with the documented and moderate H-abstraction reactivity of the aminoxyl radicals [13]. This is in sharp contrast with the high reactivity and lack of selectivity typical of another O-centred radical, such as the hydroxyl radical [14], the incursion of which in these oxidation reactions can therefore be safely excluded [15].

## 2. Isotope effect

To further support the HAT mechanism of mediation, we have determined the intramolecular primary isotope effect with properly deuteriated non-phenolic substrates in the oxidation by TvL and the two phenolic mediators (Table 2).

Determination of the relative amount of the ArCHO and ArCDO oxidation products (Scheme 5) was done by GC–MS analyses, and this enabled to reckon the $k_H/k_D$ ratios [5].

Table 1
Relative reactivity ratios obtained from competition experiments of oxidation of 4-X-substituted benzyl alcohols by TvL and two phenolic mediators (cf. Scheme 4)[a]

| Phenolic mediator | $k_{MeO}/k_H$ | $k_{Me}/k_H$ | $k_{Cl}/k_H$ | $k_{CO_2Me}/k_H$ | $k_{NO_2}/k_H$ |
|---|---|---|---|---|---|
| Phenol Red | 2.6 | 1.1 | n.d. | 0.38 | 0.30 |
| Hydroquinone | 1.7 | n.d. | 0.77 | 0.58 | 0.29 |

[a] Reaction conditions: [Ar'$CH_2$OH] = 20 mM, [PhC$H_2$OH] = 20 mM, [Med] = 10 mM, TvL = 10 U; for a reaction time of 24 h. Determined by GC.

Table 2
Laccase/mediator-induced oxidation of deuteriated non-phenolic substrates in buffered (pH 5) water solution at 25 °C, under $O_2$[a]

| Substrates | $k_H/k_D$[b] | $k_H/k_D$[c] |
|---|---|---|
| PhCHDOH | 9.0 | n.d. |
| 4-MeO$C_6H_4$CHDOH | 9.8 | 2.2 |

[a] Reaction conditions: [Subst] = 20 mM, [Med] = 10 mM, TvL = 10 U; for a reaction time of 24 h. Determined by GC/MS.
[b] With TvL and Phenol Red.
[c] With TvL and hydroquinone.

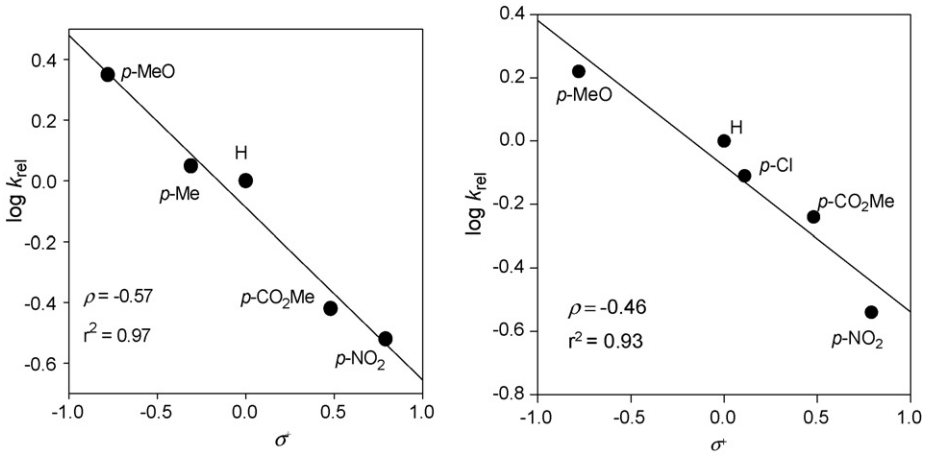



Fig. 2. Hammett-like correlations of the relative reactivity ($k_{rel}$) in the aerobic oxidation of 4-X-substituted benzyl alcohols by TvL, with Phenol Red (left) or hydroquinone (right) as mediators.

The $k_H/k_D$ ratios obtained with both the benzylic alcohols of Table 2 under mediation by Phenol Red are large in value, as expected for an HAT oxidation where H- or D-abstraction from the $\alpha$ C–H (or C–D) bond is rate determining [5]. In contrast, the $k_H/k_D$ ratio obtained under hydroquinone mediation is smaller, even though still significant for a rate-determining H-abstraction, but we do not have a sound explanation for this discrepancy at present.

## 3. Conclusion

Laccases oxidise phenolic monolignols in order to promote the first steps of lignin biosynthesis by oxidative phenolic coupling [2,3], i.e., a polymerization of phenolic substrates. As a nice case of microscopic reversibility, laccases are also involved in the reverse reaction that is the oxidative depolimerization (degradation) of lignin, by relying on mediators. Besides a number of compounds (mostly >N–OH species) that do mediate laccase but are not present in the natural environment of the enzyme [5,7], suitable phenolic compounds have been recently suggested to perform as natural mediators of laccase in the depolimerization process [8–11]. Our present results confirm that a model ArOH can be monoelectronically oxidised by laccase to ArO$^\bullet$ that, by taking advantage from appropriate stabilisation, leaves the active site and migrates intact in solution, where it undertakes a rate-determining H-abstraction oxidation of non-phenolic model substrates, as the Hammett correlation and the kinetic isotope effect do endorse. A close analogy of behaviour thus emerges between ArO$^\bullet$ and >NO$^\bullet$ species (both generated by laccase) as reactive intermediates in the HAT route of oxidation, and both types of compound are potentially fitted to the oxidative depolimerization of lignin. Natural phenolic compounds thus confer a central role to laccase in both the lignification and delignification processes, despite the seemingly lower oxidation proficiency of this enzyme with respect to other and stronger oxidising enzymes excreted by ligninolytic fungi. It is suggested that a plentiful amount of phenolic monomers drives the laccase-induced oxidation towards the polimerization process, whereas the depolimerization of lignin is enabled by residual phenolic monomers that act as ‘natural’ mediators of laccase in the oxidative degradation of non-phenolic moieties of the polymer.

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