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Laccase/mediated oxidation of a lignin model for improved delignification procedures

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Abstract

Laccase oxidises the phenolic but not the non-phenolic subunits of lignin. Redox mediators drive laccase towards the oxidation of non-phenolic subunits, particularly the benzyl alcohol groups. In search for non-polluting delignification strategies of wood pulp for paper making, oxidations by laccase/mediator systems have been performed on Adlerol, a non-phenolic α-hydroxyl-β-arylether model of lignin. The mechanism and extent of these mediated oxidations of Adlerol to Adlerone (α-carbonyl) have been investigated and compared with chemical oxidations. The more valuable mediator of laccase results to be violuric acid, which performs a radical oxidation of Adlerol. Earlier studies had revealed that Adlerone is efficiently degraded under, for example, kraft cooking conditions, whereas Adlerol resists degradation. Thus, a pre-oxidation of the α-hydroxyl-β-arylether subunits in wood pulp by the laccase/VLA system appears promising for weakening the network of lignin, thereby activating it towards subsequent oxydelignification treatments.

Keywords: Laccase; Mediators; Lignin degradation; Oxidation; H-atom abstraction; Radicals; Electron transfer; Radical cations

1. Introduction

The industrial preparation of paper requires separation and degradation of lignin in wood pulp, but environmental concerns urge to replace conventional and polluting chlorine-based delignification/bleaching procedures [1]. Oxygen delignification processes have been industrially introduced [2], but pre-treatments of wood pulp with ligninolytic enzymes might provide milder and cleaner strategies of delignification that are also respectful of the integrity of cellulose [1].

Lignin is a three-dimensional biopolymer which, in association with cellulose, reinforces and protects the plants cell wall [3]. White-rot fungi perform a selective oxidative delignification in woody tissues by secreting several enzymes [3,4], such as lignin peroxidase (LiP) [5], manganese peroxidase (MnP) [6], and laccase (EC 1.10.3.2) [7]. Laccase is a family of 'blue-copper' oxidases containing four copper ions. With respect to the more powerful oxidants

Abbreviations: HBT, 1-hydroxybenzotriazole; HPI, *N*-hydroxyphthalimide; VLA, violuric acid; NHA, *N*-hydroxyacetanilide; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) salt; TEMPO, 2,2′,6,6′-tetramethyl-piperidine-*N*-oxyl; $Co^{III}W$, potassium 12-tungstocobaltate^{III}; CAN, cerium^{IV} ammonium nitrate

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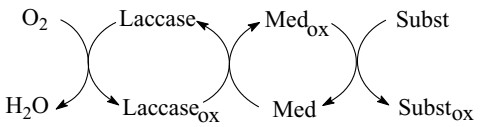


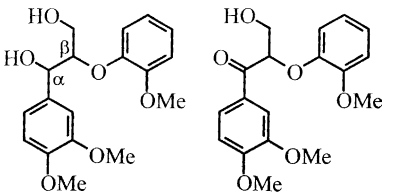
Scheme 1. The role of a mediator of laccase activity.

LiP and MnP, laccase has a lower redox potential (ca. 0.7–0.8 V/NHE) and therefore its targets are the easier to oxidise, but less abundant (15–20%) [3] phenolic subunits of lignin [4,7]. Strangely enough, and despite its apparently limited scope as a phenoloxidase, laccase is widely produced by ligninolytic fungi, and plays an important role in the natural oxygen-dependent degradation of lignin [4,7].

This apparent paradox appears to have an explanation. In fact, the substrate range of laccase may be extended to non-phenolic structures by the use of easily oxidisable mediators [8].

In a laccase/mediator system (Scheme 1) the enzyme oxidises the mediator [8–10], and the resulting Med_{ox} species can oxidise even non-phenolic substrates by resorts to mechanisms, e.g. radical ones [10,11], which differ from the single electron transfer route followed by the enzyme [7]. A number of artificial mediators of laccase has been described [9,11,12], and the oxidation mechanism followed by the Med_{ox} species of each mediator has been investigated [10,11,13]. Mediators having the N–OH functionality give the best performances [9–15]. In nature, appropriate mediators might promote laccase towards the oxidation of the non-phenolic moieties of lignin [4,14–16], such as the abundant (40%) networking β-O-4 linkages [17], thereby explaining why some fungi do not excrete LiP or MnP [18,19], and yet carry out an extensive delignification by relying only on laccase [4]. Laccase is more readily available and easier to manipulate than LiP and MnP, and laccase/mediator systems are beginning to find practical applications [20–22].

Important information deriving from earlier studies of one of us [23] is that the oxidative degradation of a β-O-4 arylether model of lignin is accelerated ca. 10^6 times during sulfate cooking whenever an α-carbonyl group is also present in the model structure. Because the oxidation of benzyl alcohols (or ethers) to α-carbonyl derivatives occurs extensively with laccase/mediator systems under mild conditions [10,11,13,24], we have attempted a pre-modification of the pulp structure by introducing α-carbonyl groups through a suitable laccase/mediated oxidation, in order to weaken the lignin network and foster its subsequent cleavage, thereby demonstrating the feasibility of an enzymatic bleaching of the pulp (viz. Lignozym®-process [12]). Here, we describe the oxidation of the non-phenolic α-hydroxyl-β-arylether Adlerol (i.e. 1-(3,4-dimethoxyphenyl)-3-hydroxy-2-(2-methoxy-phenoxy)propan-1-ol), a known model of lignin [21], into its α-carbonyl-derivative Adlerone (i.e. 1-(3,4-dimethoxyphenyl)-3-hydroxy-2-(2-methoxyphenoxy)propan-1-one) by laccase and mediators endowed with different structure. We then compare these results with those of some non-enzymatic oxidations.



Adlerol **Adlerone**

2. Experimental procedure

2.1. Materials

Adlerol and Adlerone were already available in the laboratory [13]. Reagents and mediators were commercial products (Carlo Erba or Aldrich), or prepared as previously reported [25].

2.2. Enzyme preparation

Laccase from a strain of *Trametes villosa* (viz. *Poliporus pinsitus*) (Novo Nordisk Biotech) was purified by ion-exchange chromatography on Q-Sepharose by elution with citrate buffer [11]; laccase having an absorption ratio A_{280}/A_{610} of 20–30 was considered sufficiently pure [26]. An activity of 9000 U/ml was determined spectrophotometrically by the standard reaction with ABTS [27].

2.3. Laccase-mediated oxidations

The oxidation reactions were performed at room temperature in a mixed solvent [25], prepared with a buffered (pH 5; 0.1 M in sodium citrate) water solution (1.5 ml) and 1.5 ml of dioxane (hereafter: 50%-dioxane), that was purged with O_2 for 30 min prior to the addition of the reagents [11]. In general, 60 μmol of Adlerol were incubated with 10 U of laccase, along with 20 or 60 μmol of mediator in 3 ml of 50%-dioxane, for 24 h at room temperature, an atmosphere of oxygen being kept in the reaction vessel by means of a hemi-inflated latex balloon. At the end of the reaction the internal standard was added, the reaction flask heated shortly in a steam bath and, following a work-up with ethyl acetate and concentration to a small volume, the molar amounts of Adlerone and residual Adlerol determined by HPLC analysis, suitable response factors being determined and used. The oxidation yields (Table 1) were calculated with respect to the initial molar amount of Adlerol.

2.4. Chemical oxidations

Adlerol (30 μmol), dissolved in 0.2 ml MeCN, was oxidised with $K_5Co^{III}W_{12}O_{40}$ (viz. $Co^{III}W$) (60 μmol) in 2 ml citrate buffer (i.e. 10%-MeCN) at room temperature for 4 days; conventional work-up with $CHCl_3$ or ethyl acetate preceded HPLC analysis. In the oxidations with 'preformed' $ABTS^{2+}$, 20 μmol of ABTS were dissolved in 1.5 ml of 2 M H_2SO_4; 40 μmol of $(NH_4)_2Ce^{IV}(NO_3)_6$ (viz. CAN) dissolved in 1.5 ml of 2 M H_2SO_4 were added, and the red colour of $ABTS^{2+}$ developed immediately; 60 μmol of Adlerol dissolved in 0.10 ml MeCN and 1 ml of 2 M H_2SO_4 were quickly added, and the resulting solution stirred at room temperature until the red colour of $ABTS^{2+}$ had turned blue (i.e. $ABTS^{\bullet+}$) (ca. 5 min). Dilution with MeOH:water 1:1 and HPLC analysis followed. Oxidation of Adlerol

Table 1
Oxidations of Adlerol with laccase/mediator systems or chemical oxidants, at room temperature[a]

Oxidant	Solvent	Conditions	Reaction time	Adlerol recovered (%)	Adlerone yield (%)[b]
Laccase	50%-dioxane	Without mediator	24 h	99	0
Laccase/TEMPO	50%-dioxane	3:1 Adlerol:mediator[c]	24 h	85	10
Laccase/HBT	50%-dioxane	3:1 Adlerol:mediator[c]	24 h	80	18
Laccase/HBT	50%-dioxane	1:1 Adlerol:mediator[c]	24 h	53	45
Laccase/HPI	50%-dioxane	3:1 Adlerol:mediator[c]	24 h	81	15
Laccase/NHA	50%-dioxane	3:1 Adlerol:mediator[c]	24 h	75	22
Laccase/VLA	50%-dioxane	3:1 Adlerol:mediator[c]	24 h	45	55
Laccase/VLA	50%-dioxane	1:1 Adlerol:mediator[c,d]	24 h	25	73
Laccase/VLA	50%-dioxane	1:1 Adlerol:mediator[e]	24 h	19	81
Laccase/VLA	50%-dioxane	1:1 Adlerol:mediator[c]	6 h	77	22
Laccase/ABTS	50%-dioxane	1:1 Adlerol:mediator[c,e]	24 h	97	1
$ABTS^{2+}$[f]	2 M H_2SO_4[g]	1:3 oxidant:Adlerol	5 min	87	8
$Co^{III}W$	10%-MeCN	2:1 oxidant:Adlerol	4 days	95	1[h]
Ishii method[i]	Ethyl acetate	1:1 Adlerol:HPI	24 h	100	0
Ishii method[i]	Ethyl acetate	10:1 Adlerol:HPI	24 h	99	1

[a] See Section 2 for conditions.
[b] Molar yield vs. initial moles of Adlerol.
[c] Molar ratio, with 10 U of laccase.
[d] Initial incubation with 7 U of laccase was followed by additional 3 U after 16 h of reaction.
[e] Initial incubation with 15 U of laccase was followed by additional 5 U after 16 h of reaction.
[f] Pre-formation of $ABTS^{2+}$ is by reaction of ABTS with CAN, in a 1:2 molar ratio (see Section 2).
[g] Containing 2% MeCN (v/v).
[h] Veratryl aldehyde (3%) was also formed.
[i] With $Co(OAc)_2/O_2$, see Section 2.

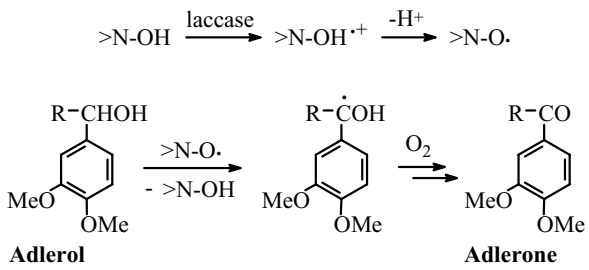
(15 μmol) with HPI (1.5 μmol), $Co(OAc)_2$ (1.2 μmol) and *m*-chlorobenzoic acid (1.5 μmol) (i.e. the 'Ishii method') [28] in 3 ml ethyl acetate was run at room temperature for 24 h. Washings with water preceded HPLC analysis.

2.5. HPLC analysis

A Hewlett-Packard 1050 HPLC system (pump, detector, and solvent delivery system), equipped with a Supelcosil LC-18-DB 25 cm × 4.6 mm column and a HP 3395B integrator, was employed. The analyses were carried out at 0.5–0.7 ml/min flow rate with a 1:1 water:MeOH mixed solvent as the eluent. Quantitation of Adlerone and of Adlerol were achieved by using benzophenone as the internal standard; the compounds were identified by their retention time and UV-spectrum. Prior to the HPLC analysis, the concentrated sample from the reaction, containing the standard, was diluted 30 times with the mobile phase and filtered through a 0.2 μm Teflon syringe filter (Superchrom Varisep).

3. Results and discussion

For solubility reasons the oxidation of Adlerol with laccase/mediator systems was run in 50%-dioxane [25], where the substrate is fully dissolved and the enzymatic activity is largely preserved [29]. A control experiment confirmed that Adlerol, being a non-phenolic substrate, is not oxidised by laccase in the absence of a mediator (Table 1). Preliminary trials were run with a 3:1 Adlerol:mediator molar ratio, but a 1:1 ratio was then adopted with the more promising mediators. The best results are those with mediator VLA, and this confirms previous findings [21]. Higher yields of Adlerone are however obtained when a second aliquot of laccase is added 16 h after the first aliquot (total reaction time, 24 h), and/or the amount of laccase is doubled. No other products besides Adlerone were detected with any of the laccase/>N–OH-type mediator systems (HBT, HPI, NHA, VLA) tested, whose reactivity features have been already described [10,11,25]. The present experimental outcome is consistent with a radical oxidation route that proceeds by H-abstraction from the benzylic C–H bond geminal to the α O–H bond of Adlerol (Scheme 2).

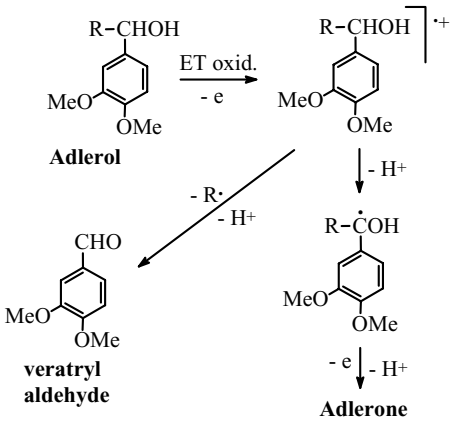


Scheme 2. A radical route of oxidation.

The leading intermediate of this radical route is the >N–O• species, originating from laccase interaction with the mediator (i.e. Med_{ox} in Scheme 1). Full support for this radical mechanism has been provided with other benzylic alcohols [10,11,30,31], and certainly the easy oxidation of VLA to the corresponding >N–O• species [11], as well as its considerable persistency [32], play a relevant role in the success of this radical oxidation. The performance of the laccase/TEMPO system appears lower than that of laccase/VLA (Table 1). TEMPO, i.e. a stable >N–O• radical, is oxidised by laccase to the oxoammonium ion [13,24], and follows an ionic oxidation route. Even lower is the efficiency of the laccase/ABTS system, a non-N–OH mediator [10,11], which follows an electron-transfer (ET) route of oxidation.

Independent comparison is made with two chemical oxidants that follow a single electron transfer oxidation route, namely, the Co^{III}-complex ($Co^{III}W$) and the $ABTS^{2+}$ ion, 'preformed' by oxidation of ABTS with a Ce^{IV} salt [30]. Their oxidation of Adlerol is poor. With $Co^{III}W$ it was possible to detect both the Cα–Cβ cleavage product, i.e. veratryl aldehyde, and the Cα–H cleavage product, i.e. Adlerone. This evidence is in keeping with the ET route of oxidation (Scheme 3) already documented [10,33].

Finally, the Ishii method [28], a well established Co^{III}-catalysed radical procedure that enables to obtain carbonyl derivatives from benzylic alcohols in high yields [34], fails in the present case despite the common involvement of a >N–OH-type co-oxidant, such as HPI. Solubility problems of Adlerol might reduce the efficiency of this particular procedure.



Scheme 3. An ET route of oxidation.

We have demonstrated that the laccase/VLA system is very valuable for the α-oxidation of lignin model Adlerol. This radical procedure is superior to the chemical oxidants tested, which follow an ET route of oxidation; it is also superior to the laccase/TEMPO system, which follows an ionic oxidation route [13]. Combined with the previous information, indicating that the α-ketone Adlerone is fully degraded during cooking, whereas Adlerol is unreactive [23], a pre-oxidation of wood pulp with laccase and VLA appears a promising strategy for weakening the networking structure of lignin. It would enable a subsequent oxygen-delignification treatment to proceed more efficiently [2], thus providing a mild and chlorine-free bleaching of the pulp. Trials with industrial pulps are underway and will be reported in due course. Preliminary results, however, show that the pre-treatment of an unbleached pulp sample with laccase/VLA reduces the kappa number from 21 to 16, thereby making the subsequent oxygen-delignification stage easier [35].

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