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Influence of mediators on laccase catalyzed radical formation in lignin

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ABSTRACT

Laccases (EC 1.10.3.2) catalyze oxidation of phenolic groups in lignin to phenoxyl radicals during reduction of O_2 to H_2O. Here, we examine the influence on this radical formation of mediators which are presumed to act by shuttling electrons between the laccase and the subunits in lignin that the enzyme cannot approach directly. Treatments of three different lignins with laccase-mediator-systems (LMS) including laccases derived from *Trametes versicolor* and *Myceliophthora thermophila*, respectively, and four individual mediators, 1-hydroxybenzotriazole (HBT), N-hydroxyphthalimide (HPI), 2,2,6,6-tetramethylpiperidin-1-yloxy (TEMPO), and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were assessed by real time electron paramagnetic resonance measurements. Radical steady state concentrations and radical formation rates were quantified. LMS treatments with 500 µM N-OH type mediators (HPI or HBT) did not affect the lignin radical formation, but increased doses of those mediators (5 mM) surprisingly led to significantly *decreased* radical formation rates and lowered steady state radical concentrations. Laccase-TEMPO treatment at a 5 mM mediator dose was the only system that significantly increased steady state radical concentration and rate of radical formation in beech organosolv lignin. The data suggest that electron shuttling by mediators is not a significant general mechanism for enhancing laccase catalyzed oxidation of biorefinery lignin substrates, and the results thus provide a new view on laccase catalyzed lignin modification.

1. Introduction

Lignin, a complex, heterogeneous, and water-insoluble aromatic polymer, is one of three major components in lignocellulosic material. The structure of lignin arises from radical coupling reactions of primarily three hydroxycinnamyl alcohols: p-coumaryl, coniferyl, and sinapyl alcohols resulting in an entangled network of phenolic and non-phenolic subunits (mono-aromatic phenylpropanoid units) [1]. The water-insoluble, recalcitrant nature of lignin makes modification, separation, and general upgrading of lignin streams from biorefineries challenging [2,3]. Currently, this abundant and renewable carbon source is thus the most poorly exploited lignocellulosic biopolymer, except for being used for energy production by combustion, regardless of its potential as a raw material for sustainable manufacturing of various new products and materials [2–4].

White-rot fungi are efficient lignin degrading organisms, and enzymes expressed by them serve as biocatalysts enabling lignin modification under mild conditions [5]. Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) are capable of modifying lignin in a catalytic cycle which involves oxidation of four moles of mono-phenolic substrate (e.g. phenolic lignin subunits) by simultaneous reduction of one mole of O_2 to two moles of H_2O. Laccases are of particular interest as biocatalysts for lignin modification since they only require O_2 as opposed to other lignin modifying enzymes, which require H_2O_2. The oxidizing capacity of laccase is defined by the redox potential of the T1 copper site in the enzyme. Fungal laccases have particularly high redox potentials ranging from 0.5 to 0.8 V vs. the Normal Hydrogen Electrode (NHE) [6,7]. The laccase catalyzed oxidation of phenolic subunits, which represent 10–30% of the units in native lignin, leads to formation of phenoxyl radicals and thus "activation" of the lignin polymer [8–10]. Stabilized by the lignin matrix, these phenoxyl radicals linger in the polymer long enough to be detected and quantified by EPR spectroscopy [11,12]. The phenoxyl radicals increase the reactivity of lignin to facilitate polymerization, depolymerization, and grafting processes which are not governed by laccase catalysis [11,13,14].

The action of laccase on lignin may be expanded by use of mediators. Mediators are low weight molecular compounds which are relatively stable, although reactive, in their oxidized as well as in their reduced form (depending on the mechanism of oxidation, the oxidation of mediators may produce radical intermediates). Mediators of natural origin are already present in biomass, whilst synthetic mediators include various types of chemical compounds [15,16]. It is claimed that mediators enhance the

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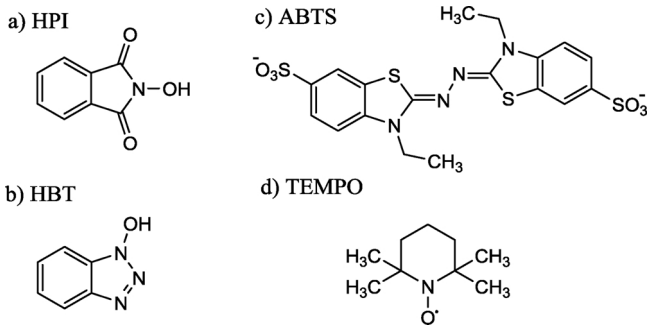

Fig. 1. Structures of synthetic mediators included in LMS treatments.

laccase catalyzed lignin modification by two mechanisms: i) The mediators act as electron transfer agents between laccase and substrate, where the oxidized form of the mediator, i.e. after being catalytically oxidized by laccase, diffuses away from the catalytic pocket and, due to its limited size, is capable of oxidizing substrates being inaccessible or too bulky for the laccase to oxidize directly [17–22]. ii) The mediator expands the oxidizing capability towards oxidation of higher-redox potential non-phenolic subunits in lignin (> 1.3 V vs. NHE) by introducing alternative reaction pathways for oxidation [18,22–25]. The different reaction pathways for oxidation of non-phenolic model substrates (e.g. benzyl alcohols) catalyzed by laccase-mediator systems via use of synthetic mediators, have been thoroughly studied [15,21,26,27]. TEMPO is a resonance-stabilized stable radical compound (Fig. 1d) which has been widely explored as a mediator: Once oxidized by laccase, the TEMPO radical forms an oxoammonium ion which reacts to oxidize non-phenolic compounds by polar addition-elimination reactions. The generated hydroxyl amine returns to the radical state either by acid induced disproportionation or by laccase oxidation. Laccase catalyzed oxidation of ABTS and N-OH type mediators, i.e. HBT and HPI (Fig. 1a–c), results in formation of reactive mediator species. The resulting $ABTS^+$ radical may react with non-phenolic substrates to create benzylic cation radical intermediates by one-electron transfer (ET) pathways, while N-oxyl radicals from oxidation of HBT and HPI create benzylic radical intermediates by hydrogen abstraction (HAT) [28,29]. These radical intermediates are less stable than phenoxyl radicals and cannot be detected by EPR without use of spin trapping techniques [11,30]. Despite the vast amount of well performed studies of laccase mediator systems (LMS) treatments on lignin and lignin model compounds, the currently available results concerning LMS treatments on lignins are often inconsistent, and a comprehensive understanding of how laccase-mediator-systems impact lignin modification is lacking [14,23]. Whether the enhancing effect by mediators on laccase catalyzed lignin modification is primarily caused by the claimed ability of mediators to function as electron shuttles or is a result of their ability to facilitate oxidation of non-phenolic units in lignin is for example rarely addressed. If mediators act as electron shuttles to oxidize bulky and inaccessible parts of lignin this would mean that a LMS treatment would enhance the rate of the radical response compared to the corresponding neat laccase induced radical formation in lignin. In this framework the objective of the present study was to investigate the influence on laccase catalyzed radical formation in lignin by synthetic mediators representing different oxidation routes. This assessment was done by measuring if any quantitative differences in stable lignin derived radicals detectable by EPR could be observed.

2. Materials and methods

2.1. Lignins

Organosolv lignin, SOL, was obtained from Sigma-Aldrich (Milwaukee, WI, USA). The beech organosolv lignin, BOL, was produced at Thünen Institute of Wood Research (Hamburg, Germany) [31]. Both SOL and BOL lignins had a high lignin content comprising 94 wt% and 87.4 wt% of Klason lignin, respectively.

The lignin from wheat straw, WSL, was obtained from hydrothermally pretreated wheat straw that had been exhaustively enzymatically treated with a Cellic® CTec2 enzyme cocktail from Novozymes (Bagsværd, Denmark): The wheat straw was first hydrothermally pretreated in a pilot plant facility for 10 min at 190 °C according to [32]. The resulting insoluble fiber fraction was then repeatedly treated (3 rounds of treatment) with Cellic® CTec2 for 48 h at 4.5% of wheat straw dry matter, pH 5.1, 50 °C with 0.02 wt% sodium azide added as preservative. The Cellic® CTec2 was added in a ratio of 0.5% by weight (liquid) to the pretreated wheat straw fiber (dry matter). In between each enzyme treatment the suspension was centrifuged for 20 min at 5350*g* and the resulting pellet was washed twice with deionized water. The final wheat straw lignin pellet was washed, dried at 70 °C, ground and sieved. This treatment increased the content of Klason lignin in the wheat straw lignin (WSL) sample from 25.9% to 43.7 wt%. The remaining content consisted of 37.8 wt% cellulose, 9.2 wt% hemicellulose, and 6.1 wt% ash.

2.2. Laccases

The fungal laccases were derived from the basidiomycete *Trametes versicolor* (Tv) and the ascomycete *Myceliophthora thermophila* (Mt). The Tv laccase was purchased from Fluka (St. Gallen, Switzerland), and the Mt laccase kindly donated from Novozymes (Bagsværd, Denmark).

2.3. Laccase activity assay

Determination of laccase doses were based on spectrophotometric monitoring of the oxidation of syringaldazine at 530 nm ($\varepsilon = 6.5 \cdot 10^4\ M^{-1}\ cm^{-1}$) at 25 °C. The assay reaction was carried out in 0.25 mM syringaldazine, 10 vol% ethanol, 25 mM sodium acetate (pH 5.0) using a suitable amount of enzyme. Enzyme activity was expressed in units: One Unit (U) was defined as the amount of enzyme required to catalyze conversion of 1 µmol of substrate (syringaldazine) per minute at the assay reaction conditions.

2.4. Mediators

Four mediators, 1-hydroxybenzotriazole (HBT), N-hydroxyphthalimide (HPI), 2,2,6,6-tetramethylpiperidin-1-yloxy (TEMPO) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Fig. 1), all obtained from Sigma-Aldrich (Steinheim, Germany), were tested in combination with each of the laccases on each of the 3 lignin samples. Each mediator was solubilized in Milli-Q water to 10 mM and adjusted to pH 5 with 0.5 mM NaOH. The mediator solutions were then added individually in equimolar amounts to a resulting concentration of 500 µM in the relevant reaction mixtures. The experiments employing 5 mM of final mediator concentration were prepared similarly, but on the BOL lignin substrate only.

2.5. Laccase-mediator study of radical formation in lignin suspensions

Lignin suspensions were prepared by adding Milli-Q water resulting in a 10 wt% lignin suspension, including pH adjustment to pH 5 with 0.5 M NaOH. The slurry was heated to 60 °C, left overnight at 5 °C and re-adjusted to pH 5. The laccase treatments were conducted in aliquots of 800 µl slurries carried out in darkness at 25 °C. To ensure oxygen saturation, the samples were vigorously shaken at 900 rpm and sealed with gas permeable, water proof membranes (ABgene, Surrey, UK). Laccases were dosed in 2.0 U/g of solid (DM) (Units according to the syringaldazine assay) and solutions of mediator was added to a concentration of 500 µM (or additionally for assessment on the BOL lignin substrate to 5 mM). For each sample, an amount of 50 µl of suspension was repeatedly drawn from the slurry for immediate EPR measurements