Journal of Molecular Catalysis B: Enzymatic 57 (2009) 204–210

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

The effect of lignin model compound structure on the rate of oxidation catalyzed by two different fungal laccases

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ARTICLE INFO

Article history:
Received 23 May 2008
Received in revised form 20 August 2008
Accepted 3 September 2008
Available online 13 September 2008

Keywords:
Laccase
Lignin
Oxidation
Cyclic voltammetry
Docking

ABSTRACT

Laccases (EC 1.10.3.2) are multicopper oxidases able to oxidize various substrates, such as phenolic subunits of lignin. The substrate range can be widened to non-phenolic units by the use of mediators. Since discovery of the laccase-mediator system, direct reactions of lignin and laccase without mediated electron-transfer have gained much less attention. The objective of this study was to investigate lignin as a substrate for fungal laccases by using lignin model compounds. These model compounds contained guaiacylic and syringylic moieties and also compounds of guaiacylic origin at a higher oxidation level. Some of these compounds are commercially available, but most of them were synthesized. The oxidation reaction rates of the lignin model compounds were studied by monitoring consumption of the co-substrate oxygen, in reactions catalyzed by laccases from two different fungi; *Melanocarpus albomyces* and *Trametes hirsuta*, possessing different molecular and catalytic properties. These reaction rate studies were compared to physicochemical properties of the lignin model compounds: relative redox potentials determined using cyclic voltammetry and pK_a-values. Docking of syringylic and biphenylic compounds to the active sites of both laccases was performed and the resulting model complex structures were used to further interpret the reaction rate results. Reaction rates of laccases are mainly affected by the ability of a lignin model compound to be oxidized and the pK_a-value of the substrate seems to be less important. As a consequence, syringylic compounds are oxidized with the highest rates and compounds at a higher oxidation level and redox-potential, such as vanillin, are oxidized at a much lower rate. Both guaiacylic and syringylic type compounds fit well in the active sites of both laccases. Only for a biphenylic compound, steric clashes were observed, and they are likely to have an effect on the reaction rate. When the oxidation rates on the selected model compounds with the two different laccases were compared, the redox-potential difference between laccases T1 copper and the lignin model compound (ΔE) was not the only property that determined the oxidation rate. In the case of lignin model substrates, also the selectivity of a specific laccase, reflected in the k_{cat}/K_m value, plays an important role.

1. Introduction

Laccases (EC 1.10.3.2) are multicopper oxidases able to catalyze one-electron oxidation of various substrates like mono-, di-, and polyphenols, aminophenols, diamines and some inorganic compounds, with concomitant reduction of O_2 to H_2O [1,2]. Phenolic subunits of lignin, a heterogeneous polymer consisting of phenylpropane units, are the natural substrates of laccases. The laccase substrate range can be expanded to non-phenolic units by electron-transfer and hydrogen-atom-transfer mediators [3,4]. Mediators are also required when laccases are used for lignin degradation and they are probably involved in lignin biodegradation.

Laccases are common enzymes in the nature, and they are found widely in plants and fungi as well as in some bacteria and insects. Especially many fungal and some bacterial laccases, have been isolated and properly characterized structurally and biochemically [5,6]. Laccases have four copper atoms in their active site [7]. Of these four coppers, one is a type-1 (T1) copper forming a mononuclear center, a one type-2 (T2) copper and two type-3 (T3 and T3′) coppers form a trinuclear center. Classification of coppers is based

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doi:10.1016/j.molcatb.2008.09.004

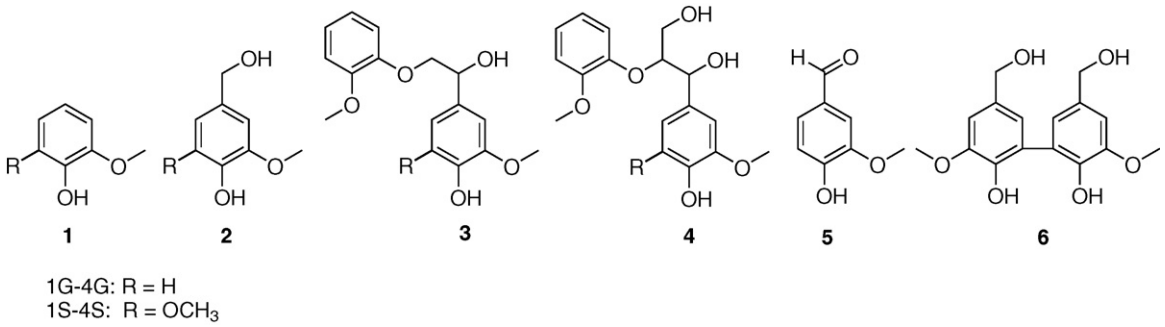

Fig. 1. Lignin model compounds used to study reaction rates with laccases. **1G**: guaiacol, **2G**: vanillyl alcohol, **3G**: guaiacylglycol β-guaiacyl ether, **4G**: guaiacylglycerol β-guaiacyl ether, **1S**: syringol, **2S**: syringyl alcohol, **3S**: syringylglycol β-guaiacyl ether, **4S**: syringylglycerol β-guaiacyl ether, **5**: vanillin, and **6**: dehydrodivanillyl alcohol.

on their spectroscopic features: the T1 copper is a paramagnetic blue copper with absorption maximum at around 600 nm, the T2 copper is a paramagnetic normal copper, and the T3–T3′ copper-copper pair is an antiferromagnetically coupled, EPR silent pair with an absorption maximum at 330 nm. The redox-potential of the T1 copper is characteristic for each laccase. The reducing substrates are oxidized near the mononuclear center: T1 copper accepts an electron from a substrate and it is further transferred to the trinuclear center along a Cys-His pathway. Transfer of four electrons results in reduction of dioxygen to two water molecules.

Biotechnological processes are becoming more interesting for the industry because of the continuing demands for less polluting processes. Such processes have been extensively searched for in the pulp and paper industry. Besides the laccase-mediator system, which has been studied intensively after its discovery and is especially a candidate solution for bio-bleaching, other pulp and paper related laccase applications have also been investigated. These studies include the use of laccase in mechanical pulp processing [8] and for modification of pulp fibers [9,10].

When developing different laccase-based applications for lignin modification, it is essential to understand the reactivity of laccases towards different types of substrates, especially towards different phenolic structures characteristic in lignin. The suitability of a chemical compound as a laccase substrate in general depends on both the properties of the substrate and the enzyme. The substrate must bind near the T1 copper site, which is largely determined by the nature and position of the substituents on the phenolic substrate, especially with bulky side chains [11,12]. Furthermore, the redox-potential (E) of the substrate must be low enough, because the rate of a laccase-catalyzed reaction has been shown to depend on the difference between the redox-potentials of the enzyme and the substrate, ΔE [laccase-substrate] [11,13–16]. The redox-potential of the substrate is determined by its chemical structure, and different substituents have different impact on the E [substrate] depending on their propensity to withdraw or donate electrons. For instance, methoxy substituents are electron-donating and increase the electron density at the phenoxy group, thus making it more readily oxidized [11,17]. Redox-potentials of laccases vary from 0.4 V to 0.8 V, and the most critical factor determining the E [laccase] is the coordination sphere of the T1 copper [14,18–20].

Concerning redox potentials of lignin model compounds, data is missing for several more complex compounds, for example the dimeric ones. This is because the radical–radical coupling, that follows the one-electron oxidation, interferes with straightforward measurements and therefore, only a couple of indirect measurement methods exist. The so-called critical oxidation potentials have been determined for guaiacol and vanillin [21,22] and the redox-potential of 2,6-dimethoxyphenol has been determined using the pulse radiolysis method [23]. Redox potentials also correlate to the Brown substituent constants (σ^+) that reflect substituent effects in aromatic electrophilic reactions [23]. Based on this correlation, the redox potentials of many substituted phenols have been calculated and compared with the kinetic data of the laccase from *Trametes villosa* (also called *Polyporus pinsitus*) [11]. Based on these studies, oxidation rate seems to be proportional to the redox-potential difference (ΔE) between T1 copper of a laccase and a substrate. Unfortunately, redox-potential calculations are not feasible for more complex lignin model compounds, for example for β-*O*-4 dimers. However, the Brown σ^+ constants also correlate with the phenols' cyclic voltammetry peak potentials [24]. The identities of the anodic peaks in the cyclic voltammogram of guaiacol have been resolved with ESR spectroscopy; the anodic peak at a lower potential refers to the oxidation of the phenol and a second peak at a higher potential to the oxidation of the aromatic ring [25].

The aim of the present study was first of all to investigate reaction rates of different lignin model compounds with a high- and low-redox laccases from the white rot fungus *Trametes hirsuta* (T1 copper redox-potential 780 mV [26]) and from the ascomycete fungus *Melanocarpus albomyces* (470 mV [27]). The used lignin model compounds included guaiacylic and syringylic compounds and compounds of guaiacylic origin with a higher oxidation level ("once-oxidized") compared to the other ones (Fig. 1). The reaction rates were determined by measuring oxygen consumption rates with oxygen electrode under identical conditions for the two different laccases on the selected model compounds. Oxygen consumption rates were then compared with the relative oxidation potentials of the lignin model compounds, determined with cyclic voltammetry, and with the phenolic pK_a-values found in the literature [28]. Furthermore, the binding of the substrates in the laccase active site was evaluated with docking studies using the 3D structure of *M. albomyces* laccase and a model *T. hirsuta* laccase structure based on the homologous *Trametes versicolor* 3D structure.

2. Experimental

2.1. Synthesis of model compounds

From the used compounds commercially available were 2-methoxyphenol (guaiacol, **1G**, Merck), 2,6-dimethoxyphenol (syringol, **1S**, Aldrich) and 4-hydroxy-3-methoxybenzaldehyde (vanillin, **5**, Merck). The other compounds were prepared using well-known methods. Corresponding aldehydes were reduced with $NaBH_4$ in ethanol to produce 4-hydroxymethyl-2-methoxyphenol (vanillyl alcohol, **2G**) and 4-hydroxymethyl-2,6-dimethoxyphenol (syringyl alcohol, **2S**). Dimeric models 1-(4-hydroxy-3-methoxyphenyl)-2-(2′-methoxyphenoxy)-1-ethanol (guaiacylglycol β-guaiacyl ether, **3G**) [29], 1-(4-hydroxy-3-methoxyphenyl)-2-(2′-methoxyphenoxy)-

1,3-propanediol (guaiacylglycerol β-guaiacyl ether, **4G**) [29], 1-(4-hydroxy-3,5-dimethoxyphenyl)-2-(2′-methoxyphenoxy)-1-ethanol (syringylglycol β-guaiacyl ether, **3S**) [30], 1-(4-hydroxy-3,5-dimethoxyphenyl)-2-(2′-methoxyphenoxy)-1,3-propanediol (syringylglycerol β-guaiacyl ether, **4S**) [31] and 5,5′-bis(hydroxymethyl)-3,3′-dimethoxy-2,2′-biphenyldiol (dehydrodivanillyl alcohol, **6**) [32] were synthesized according to previously reported methods. The synthesized products were purified by crystallization or with preparative HPLC. For preparative HPLC methanol-water was used as an eluent (isocratic flow) and following equipment: ISCO 2350 HPLC pump, Shimadzu SPD-6A UV Spectrophotometric detector, Shimadzu C-R6A Chromatopac data processor, reverse phase column (Waters SymmetryPrep C_{18}, 19 mm × 150 mm, 7 μm).

2.2. Laccase enzymes

The *M. albomyces* laccase was overproduced in *Trichoderma reesei* and purified as described earlier [27]. The *T. hirsuta* laccase was produced in its native host and purified [33].

2.3. Determination of laccase activity

The laccase activity was determined using ABTS [2,2′-azino-bis-(3-ethylbenzothiazoline)-6-sulphonate] as a substrate in 25 mM succinate buffer, pH 4.5 at 22 °C [34]. The oxidation of ABTS was monitored by absorbance measurements at 436 nm (ε = 29 300 M^{-1} cm^{-1}). The effect of ethanol on the laccase activity was measured using **1S** (ε = 19 600 M^{-1} cm^{-1} at 469 nm) as a substrate in 40 mM MES–NaOH buffer, pH 6, at 22 °C. The activity was measured in the presence of 5–50% (v/v) ethanol.

The kinetic parameters for *T. hirsuta* laccase were determined with **1G** and **1S** in 40 mM MES–NaOH buffer, pH 6, at 22 °C. The Michaelis–Menten curves for determining K_m were obtained by curve fitting analysis using Prism software 4.01 (GraphPad Software Inc., San Diego, CA).

2.4. Other reagents

Tetrabutylammonium perchlorate (TBAP, puriss.) and $LiClO_4$ (purum) were purchased from Fluka. Bu_4NOH (40 wt.% solution in water) was from Aldrich and $AgNO_3$ was provided with the reference electrode kit. The acetonitrile used for cyclic voltammetry was HPLC grade and used without further purification.

2.5. Oxygen consumption measurements

Oxygen consumption was measured with FIBOX 3 fiber-optic oxygen meter (PreSens, Regensburg, Germany) at room temperature. A model compound was dissolved in 3:7 ethanol–sodium succinate buffer (25 mM, pH 4.5) to a final concentration of 10 mM. For compound **3G**, the ratio of solvents was 1:1. Of the model compound solution, 1840 μl was pipetted into a vial (total volume 1860 μl), with a closed cap, and the solution was stirred for 10 min to let it stabilize. After that 20 μl of diluted enzyme preparation was injected through a septum in the cap, and the oxygen consumption was monitored for 10 min. An enzyme dosage of 2 nkat ml^{-1}, based on ABTS activity, was used for all measurements and the activity was optimized with **1S**, a substrate that is readily oxidized by laccases. All measurements were repeated twice and the consumption per time unit was calculated from the linear part of the oxygen consumption curve. The final value is an average of the two measurements.

2.6. Cyclic voltammetry

Measurements were performed using an Autolab PGSTAT20 potentiostat and a three electrode cell. The electrolyte used in Ag/Ag^+ reference electrode (Non-aqeous Silver/Silver Ion Reference Electrode Kit was purchased from BASi, Warwickshire, UK) was 0.01 M $AgNO_3$ and 0.1 M TBAP in acetonitrile. The working (area 1.8 cm^2) and counter electrodes were made from Pt. The measured solutions contained 2 mM of the lignin model compound and 0.1 M of $LiClO_4$ in acetonitrile. Measurements were performed with and without added base (2 mM Bu_4NOH) and the same day on which the solutions were prepared. A step potential of 0.01 V and a scan rate of 0.1 V s^{-1} were used.

2.7. Substrate docking studies

A structural model of the *T. hirsuta* laccase (Protein data bank code Q02497) was built based on the known structure of the homologous laccase from *T. versicolor* (PDB-code 1KYA [12]) sharing 91.0% sequence identity. The active site region is completely conserved in these two laccases. Sequence comparisons and structural alignments used for model building were made using the Multalin program [35] and ClustalW [36]. The *T. hirsuta* homology model was constructed using the Swiss-Pdb Viewer [37] and PyMOL (Delano Scientific) programs and was used as a starting point for the construction of models in complex with 4-hydroxy-3,5-dimethoxybenzaldehyde (syringaldehyde) created by superimposing the *T. hirsuta* laccase model and the *M. albomyces* laccase structure (PDB-code 1GW0 [38]) with the 2,5-xylidine complex structure of *T. versicolor* laccase.

3. Results and discussion

Wood lignin is composed of three different types of phenylpropane units, which differ in the amount of their methoxy substituents [39,40]. The units are: *para*-hydroxyphenyl (no methoxyls *ortho* to the phenolic hydroxyl), guaiacyl (one methoxyl *ortho* to the phenolic hydroxyl) and syringyl (two methoxyls *ortho* to the phenolic hydroxyl). Softwood lignin contains mainly guaiacyl units and hardwood lignin contains guaiacyl and syringyl units, both lignins also have low amounts of *para*-hydroxyphenyl units. The phenylpropane units are linked together, to form a seemingly randomly organized polymeric network, with several types of linkages, the most abundant being the β-*O*-4 type (45–60%). Other types of linkages are β-5, β-β, 5-5, 5-5/β-*O*-4 (dibenzodioxocin), 5-*O*-4 and β-1. Because syringyl units lack the possibility to form bonds to 5-position, the frequencies of different types of linkages differ markedly between softwood and hardwood. In the wood cell wall, condensed structures such as 5-5 and dibenzodioxocin, are localized more abundantly in the inner secondary wall than in the middle lamella [41,42].

The lignin model compounds used to study the oxidation reaction rates catalyzed by laccases from *M. albomyces* and *T. hirsuta* were chosen to represent some of the important structural patterns in wood lignin (Fig. 1). First, model compounds representing guaiacylic (**1G**–**4G**) and syringylic (**1S**–**4S**) structures were included. Secondly, the compounds varied from monomeric to dimeric and their side chain composition varied from no side chain at all (**1**) to a very bulky one, such as in β-*O*-4 dimer (**3**–**4**). The β-*O*-4 structures are often referred to reactive structures in lignin as they contain a benzylic hydroxyl group prone to react with versatile type of reactions in lignin deformation [43]. Thus, the major structural differences in the chosen lignin model compounds expected to have an impact on their oxidation catalyzed by laccases were the size and

different substituents. Model compounds with only minor structural changes were compared as well, for example *β*-*O*-4 dimers **3** lack only the hydroxymethyl group in the side chain compared to dimers **4**. Differences in substitution were expected to cause differences in the redox potentials of the lignin model compounds, which might have been reflected in the reactivity with the two different laccase catalysts. The shape of the molecules might also have had an impact on the reactivity, if this would have interfered with the proper binding of the substrate in the laccases active site.

Vanillin (**5**) and dehydrodivanillyl alcohol (**6**) were chosen for this study, because they are well-known oxidation products of vanillyl alcohol (**2G**) and therefore also at a higher oxidation level, and thus having a higher oxidation potential, compared to the other compounds. Besides this, it is interesting to study, for comparison, what would be the rate of the reaction where the primary oxidation products (i.e. the products from the first oxidation) are oxidized again. Dehydrodivanillyl alcohol (**6**) is a very interesting compound since the structure is rigid, and it is not at all clear how well it would fit to the active sites of the laccases.

From the used model compounds only **1G**, **1S** and **5** were commercially available. The others were synthesized, purified carefully and characterized. Compound **3G** was found, during the purification stage, to have a very low solubility compared to the other compounds and this was expected to have an effect on the kinetic data (below). When compared to all other compounds, **3G** indeed behaved to some extent differently than expected based on its chemical structure and this might be due to its poor solubility in watery solutions.

Oxygen consumption measurements were used to measure the oxidation rates of lignin model compounds in laccase-catalyzed reactions. A constant, saturating concentration of the substrate, high enough not to limit the reaction, and a constant dosage of the laccase were used to study differences in the reactivity of the compounds. Cyclic voltammetry was applied to estimate the relative oxidation potentials of the different substrates. The relationship of the kinetic data to the estimated oxidation potentials and the pK_a-values measured by Ragnar et al. was studied [28]. Finally, binding of these lignin-based phenolic substrates in the active sites of both laccases was studied using docking approaches in order to evaluate differences in the substrate specificity between the two enzymes.

3.1. Oxidation of lignin model compounds catalyzed by laccases

The results of the oxygen consumption measurements are presented in Table 1. When the two different laccases were compared, *T. hirsuta* laccase oxidized all model compounds with a lower oxidation level (**1**–**4**) at a more or less similar rate while *M. albomyces* laccase seemed to be more active towards the syringyl than the guaiacyl compounds. As expected, compounds **5** and **6**, which are at a high oxidation level already, were oxidized with a slower rate compared to the other compounds. The oxidation rate of **3G** was relatively slow for both *M. albomyces* and *T. hirsuta* laccases. Due to low solubility of **3G** mentioned above, the measurements were carried out in a solution containing more ethanol (50% compared to 30%). Based on control activity measurements with compound **1S**, no decrease in activity was observed in the presence of ethanol, although decreased activities have been previously observed with *Poliporus pinsitus* laccase in the presence of organic solvents [44,45].

It is possible, although unlikely, that secondary oxidation products, formed in the reaction, had an effect on the oxidation consumption results. For example, vanillin (**5**) and dehydrodivanillyl alcohol (**6**) are well-known oxidation products of vanillyl alcohol (**2G**). With *M. albomyces* laccase, their oxidation rate was very low compared to the other compounds. With *T. hirsuta*, oxidation rate of **6** was more significant and it could have had an additive effect to the oxidation rate of **2G**. Calculated from the oxygen consumption rate of **2G** and assuming that **2G** formed only **6** as a product, however, the concentration of **6** would have been only 0.5 mM after 10 min in the solution, which was used to measure oxidation rate of **2G**. From this, we conclude that the oxygen consumption values are mainly due to oxidation of the primary substrates, which were used at 10 mM concentrations.

Table 1
Oxygen consumption rates on selected lignin model compounds from laccase-catalyzed reactions.

Model compound	$c(O_2)$ ($\mu g\,l^{-1}\,s^{-1}$)	
	M. albomyces laccase	*T. hirsuta* laccase
Guaiacol (**1G**)	11.0 ± 1.2	17.1 ± 1.8
Vanillyl alcohol (**2G**)	11.8 ± 0.4	13.8 ± 0.9
Guaiacylglycol *β*-guaiacyl ether (**3G**)	4.8 ± 0.6	7.8 ± 1.0
Guaiacylglycerol *β*-guaiacyl ether (**4G**)	9.4 ± 0.8	12.8 ± 0.8
Syringol (**1S**)	22.2 ± 3.5	16.9 ± 2.0
Syringyl alcohol (**2S**)	19.8 ± 0.7	14.0 ± 0.7
Syringylglycol *β*-guaiacyl ether (**3S**)	13.8 ± 1.3	14.5 ± 1.1
Syringylglycerol *β*-guaiacyl ether (**4S**)	17.2 ± 0.1	13.4 ± 0.7
Vanillin (**5**)	0.1 ± 0.0	1.8 ± 0.0
Dehydrodivanillyl alcohol (**6**)	1.3 ± 0.1	7.3 ± 0.2

Concentration of the used model compound was 10 mM and a dosage of the laccase 2 nkat ml^{-1} (based on ABTS activity), measurements carried out at pH 4.5 and room temperature.

3.2. Characterization of lignin model compounds by cyclic voltammetry

Cyclic voltammetry was used to measure oxidizability of the lignin model compound series used in the oxygen consumption measurements. Other potential methods to characterize these properties are, for example, the critical oxidation potential method and the pulse radiolysis method. A drawback of the critical oxidation potential method is that, besides being laborious, some model compounds cannot be measured because of the narrow potential ranges. With larger molecules, also the solubility may cause problems. The pulse radiolysis method, which has been performed in water solutions, is neither a very straightforward measurement method. The aim of our measurements was to determine the relative oxidation potentials of different lignin model compounds under identical conditions. Therefore, it was not necessary to get information on the redox potentials in aqueous solutions. In addition, since most of the compounds studied are only partially soluble or insoluble in water, cyclic voltammetry in acetonitrile was used.

Interpretation of cyclic voltammograms was based on the thorough study previously made for guaiacol (**1G**) [25]. According to earlier findings, the first anodic peak in a cyclic voltammogram corresponds to the oxidation of a phenol to a phenoxy radical and the second peak to the oxidation of an aromatic ring resulting in an aryl cation. The peak indicating phenolic oxidation was only observed in the presence of base. Interestingly, we were able to observe oxidation of phenol in neutral solutions as well by decreasing the starting potential from −0.5 V to −1.0 V.

Based on the earlier study mentioned above, we assumed that in all lignin model compounds the phenolic hydroxyl is most readily oxidized. Cyclic voltammograms were determined under identical conditions for all the selected lignin model compounds in order to obtain the relative order of their oxidizability. It should be pointed out that anodic peak potentials are not strictly the same as oxidation potentials. However, anodic peak potentials provide sufficient information on the redox properties of lignin model compounds, to order the compounds based on the easiness of oxidation. As a typical example, the cyclic voltammogram of vanillyl alcohol (**2G**) in buffered and non-buffered conditions is shown in Fig. 2.

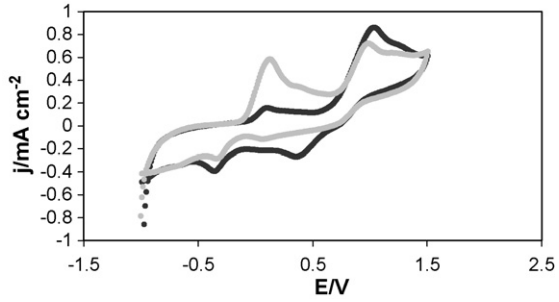

Fig. 2. Cyclic voltammogram of 2 mM vanillyl alcohol (**2G**) in acetonitrile containing 0.1 M $LiClO_4$. Reference electrode Ag/Ag^+, step potential 0.01 V and scan rate 0.1 V s^{-1}. Black line: unbuffered solution, grey line: solution buffered with 2 mM Bu_4NOH.

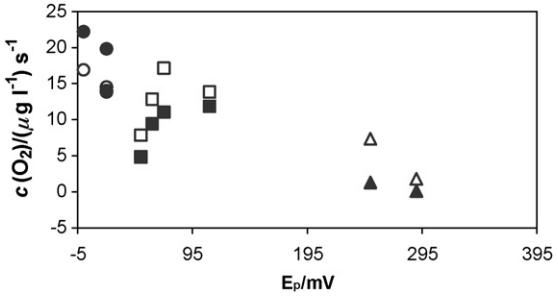

Fig. 3. Oxygen consumption rates for the studied lignin model compounds as a function of peak potential. Filled patterns indicate oxidation with *M. albomyces* laccase and empty patterns with *T. hirsuta* laccase. Circle: syringylic compound, square: guaiacylic compound, triangle: vanillin (**5**) and dehydrodivanillyl alcohol (**6**).

The observed first anodic peaks from cyclic voltammetry measurements are listed in Table 2. As expected, the results indicated that compounds **5** and **6**, which are at a higher oxidation level, were more difficult to oxidize than the other compounds. It also seemed, as expected, that syringyl compounds were more easily oxidized than guaiacyl compounds. Interestingly, the addition of a base to the measured solution decreased the oxidation potential in some cases and increased the potential in others. However, the relative order of oxidation peak potentials was the same in buffered and non-buffered solutions with all the tested compounds except for **3G**.

3.3. Comparison of oxygen consumption rates to the relative redox potentials and the pK_a-values of lignin model compounds

When comparing the oxidation rates (Table 1) it can be seen that compounds **5** and **6**, which are at a higher oxidation level and have highest oxidation potentials, were more difficult to oxidize than the other compounds by both *M. albomyces* and *T. hirsuta* laccases. The *M. albomyces* laccase, which has the lower redox-potential of the two laccases, seemed to be virtually unable to oxidize these compounds. The *T. hirsuta* laccase could oxidize them to some extent, compound **6**, which has a lower oxidation potential, being more reactive compared to compound **5**. As could be expected, the *M. albomyces* laccase oxidized the syringyl compounds faster than the corresponding guaiacyl compounds as the syringyl compounds have lower oxidation potentials than the guaiacyl compounds, based on the peak potential data (Table 2). In contrast, *T. hirsuta* laccase, with a higher redox-potential than *M. albomyces* laccase (redox-potential difference about 300 mV), oxidized the syringyl compounds with only a slightly higher rate than the guaiacyl compounds. Again, compound **3G** is an exception to the rule, and its oxidation is fairly slow by both laccases when compared to the other *β-O*-4 dimers (**3**–**4**), likely due to its poor solubility under the optimal conditions for laccase enzymes. In Fig. 3, where this comparison is illustrated, it can be seen that the model compounds form three groups according to their relative oxidation potentials. Guaiacylic and syringylic compounds form two groups and compounds **5** and **6**, which have much higher oxidation potentials compared to the other compounds, form their own group. Here, it can be clearly seen, that *T. hirsuta* laccase oxidizes at faster rate the compounds with a higher oxidation potential (guaiacylic compounds, **5** and **6**) but *M. albomyces* laccase takes over when looking at the group of syringylic compounds.

According to a comparative analysis with several fungal laccases for oxidation of a series of phenols, anilines and benzenethiols Xu [11] showed that the rate of oxidation is proportional to the redox-potential difference between the type 1 copper site and the substrate. Furthermore for phenols, the steric effect of small *ortho*-substituents, e.g. OH, OCH_3 and C_2H_5, was found to be unimportant. It is surprising that, according to our results, *T. hirsuta* laccase oxidized syringyl compounds with similar rates as the corresponding guaiacyl compounds, although the redox potentials of the syringyl compounds are substantially lower, indicating that there might be specificity differences due to the OCH_3 *o*-substituents. With the lower-redox-potential laccase of *M. albomyces*, the results are consistent with the redox-potential difference between the type 1 copper site and the substrate theory: syringyl compounds were oxidized at a clearly higher rate than the guaiacyl compounds.

In this study, reaction rates at only one concentration were measured, however, the concentration was chosen high enough (10 mM) not to limit the reaction. The K_m values of laccases are usually, with few exceptions, less than 1 mM for phenolic substrates

Table 2
Measured anodic peak potentials E_p (mV) from measured cyclic voltammograms and pK_a-values found from literature [28] for the studied lignin model compounds.

Model compound	E_p (unbuffered solution) (mV)	E_p (solution with 2 mM Bu_4NOH) (mV)	pK_a
Guaiacol (**1G**)	70	80	9.93
Vanillyl alcohol (**2G**)	110	130	9.78
Guaiacylglycol *β*-guaiacyl ether (**3G**)	50	90	–[a]
Guaiacylglycerol *β*-guaiacyl ether (**4G**)	60	70	9.88
Syringol (**1S**)	0	−10	9.98
Syringyl alcohol (**2S**)	20	10	9.87
Syringylglycol *β*-guaiacyl ether (**3S**)	20	–[b]	–[a]
Syringylglycerol *β*-guaiacyl ether (**4S**)	–[b]	40	–[a]
Vanillin (**5**)	290	330	7.40
Dehydrodivanillyl alcohol (**6**)	250	230	6.87

In cyclic voltammetry concentration of the measured model compound was 2 mM in acetonitrile containing 0.1 M $LiClO_4$, reference electrode Ag/Ag^+, step potential 0.01 V and scan rate 0.1 V s^{-1}.

[a] Value was not found.

[b] No peak observed.

[4]. Thus, it can be stated that laccases were saturated with the substrate in our kinetic measurements.

To further analyze the observed results, the kinetic parameters, K_m and k_{cat}, for the simplest syringylic and guaiacylic lignin model compounds used in the study (**1G** and **1S**) were compared. For *M. albomyces* laccase, the values were determined earlier in our laboratory [27] and for *T. hirsuta* laccase they were determined in this work (Table 3). The kinetic values were for comparison reasons, determined at pH 6, which is more optimal for *M. albomyces* laccase while *T. hirsuta* laccase would have preferred a slightly more acidic pH. On the other hand, oxygen consumption measurements were performed at pH 4.5, which again is more favorable for *T. hirsuta* laccase. For both laccases, the K_m value of **1S** was smaller compared to **1G**. However, the difference was much greater in the case of *M. albomyces* laccase. The very low K_m value of **1S** for *M. albomyces* laccase indicates that this enzyme has a high affinity for syringylic compounds. However, in order to completely evaluate the role of the substrate binding methods have to be developed to measure binding constants. With both enzymes, the k_{cat} value of **1G** was smaller than the k_{cat} of **1S**. By examining k_{cat}/K_m, the specificity constant, which is a measure for the enzyme's substrate specificity, one gets with *M. albomyces* laccase $3.6 \times 10^{-4}\,M^{-1}\,s^{-1}$ for **1G** and $1089.8 \times 10^{-4}\,M^{-1}\,s^{-1}$ for **1S** compared to *T. hirsuta* laccase, values being $24.5 \times 10^{-4}\,M^{-1}\,s^{-1}$ for **1G** and $216.2 \times 10^{-4}\,M^{-1}\,s^{-1}$ for **1S**. This indicates that both enzymes have a preference for syringyl compounds. This selectivity seems to be the most striking for *M. albomyces* laccase, more than two orders of magnitude, while for *T. hirsuta* laccase a bit less than one order of magnitude. This type of selectivity was also observed in another study comparing oxidation catalyzed by high- and low-redox laccases (*Trametes villosa* and *Myceliophthora thermophila* laccases, 790 and 460 mV) with several substituted phenols and anilines [13].

Another property that might affect the reaction rate of a phenol is its ability to deprotonate to form phenolate ion in aqueous solutions. The pK_a-values, characterizing this property, have been measured by Ragnar et al. [28] for many lignin model compounds (Table 2). Comparison of these pK_a-values with the oxygen consumption data shows, however, that there seems to be no clear correlation between these values. Actually, the pK_a-values of guaiacyl and syringyl compounds are very similar. The result is consistent with the latest results that the rate-determining step in a laccase-catalyzed oxidation is the electron-transfer from the phenolic substrate to the laccase and the resulting radical cation is rapidly deprotonated [13].

3.4. Docking

The top panel of Fig. 4 shows the known *Trametes versicolor* complex structure with 2,5-dimethylaniline (2,5-xylidine) bound in its active site [12]. This is the only complex laccase structure available so far with a small aromatic compound bound in the active site and it was used for the modeling of the lignin model compounds used in this study. In the middle panel is shown the *T. hirsuta* model structure, in which the active site is completely conserved when compared to *T. versicolor* laccase. Syringaldehyde shown in the enzymes active site was positioned based on the binding orientation of 2,5-xylidine observed in the *T. versicolor* structure, assuming a similar orientation of phenolic compounds and aniline derivatives. No steric clashes with the enzymes active site were observed. In the bottom panel the structure of *M. albomyces* laccase with syringaldehyde in the active site is shown. The orientation of syringaldehyde relative to the *M. albomyces* active site was not changed and is based on a superposition of the backbone atoms of the two different laccases. It seemed that guaiacylic and syringylic compounds used in this study fitted well in both the active site

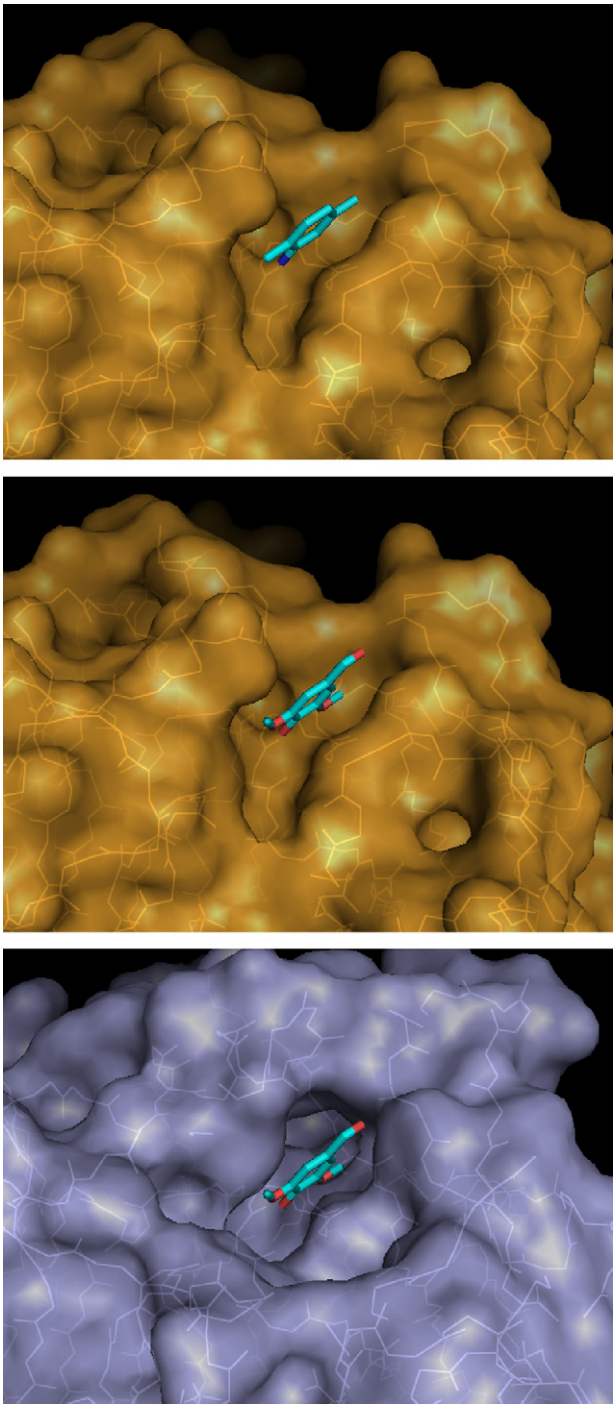

Fig. 4. Active site views of *M. albomyces* and *T. hirsuta* laccases. Top panel: molecular surface of *T. versicolor* laccase active site containing 2,5-xylidine. Middle panel: molecular surface of *T. hirsuta* laccase active site containing syringaldehyde. Bottom panel: molecular surface of *M. albomyces* laccase active site containing syringaldehyde.

of the *T. hirsuta* and *M. albomyces* laccases. Syringylic compounds, having two *o*-methoxyl groups to the phenol, might have some additional interactions with the active site, resulting in a higher affinity compared to guaiacylic compounds; this might explain the lower K_ms of syringylic type compounds compared to guaiacylic compounds (Table 3). Substituents at the *para* position to the phenolic hydroxyl moiety point outwards, therefore, it is very likely that a bulky side chain in β-*O*-4 models (**3**–**4**) does not contribute

Table 3
Kinetic parameters, K_m and k_{cat} with simple, guaiacylic and syringylic substrates for *M. albomyces* [27] and *T. hirsuta* laccases.

Compound	*M. albomyces* laccase			*T. hirsuta* laccase		
	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (10^{-4} M^{-1} s^{-1})	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (10^{-4} M^{-1} s^{-1})
Guaiacol (**1G**)	890	1900	3.6	66	970	24.5
Syringol (**1S**)	5.2	3400	1089.8	37	4800	216.2

All values have been determined at pH 6.
Specificity constant, k_{cat}/K_m, has been calculated from the determined values.

much to the binding of the substrates. Dehydrodivanillyl alcohol (**6**) is an exception to this; a simple superposition leads to steric clashes with active sites of both enzymes. Rotating the biphenylic rings to a less favorable conformation improved the fit, but one has to move the molecule relative to 2,5-xylidine to make the binding possible. For correct docking of dehydrodivanillyl alcohol (**6**) a better template structure would be required. In addition to the high oxidation potential, a different, less favorable binding mode might explain the lower activity observed for this compound. Based on these studies, the structural features explaining the low and high K_m values for the syringylic and guaiacylic compounds could not be visualized.

4. Conclusions

The redox-potential of the substrate seemed to be the most important property of the selected lignin model compounds, when different properties affecting the laccase-catalyzed oxidation reaction rates were studied. As expected, for a reaction in which the rate-determining step is the transfer of electron, the pK_a-values of the lignin model compounds did not correlate at all to the kinetic data. Binding of the substrate to the active site seemed to be a less decisive property affecting the reaction rate. However, in the case of a biphenylic model compound sterical clashes within the active site could affect the reaction rate. When the two laccases with a low and high T1 copper redox-potential were compared, differences in specificity between the two enzymes seemed to affect the substrate oxidation. This difference in specificity for lignin model compounds between enzymes is interesting, because in laccase-catalyzed oxidations, substrate specificity is often considered to be a less important factor, because of the wide substrate range including also many non-lignin type substrates, observed for this class of oxidoreductases.

Acknowledgements

Financial support from the international PhD programme in Pulp and Paper Science and Technology in Finland (PaPSaT) is gratefully acknowledged.

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