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Abstract In this paper the oxidation of milled wood lignin (MWL), catalysed by three enzymes, i.e. laccase, tyrosinase and horseradish peroxidase (HRP) was studied. The oxidation was followed by measuring the consumption of O₂ during laccase and tyrosinase treatment and of H₂O₂ during HRP treatment. Both laccase and HRP were found to oxidise lignin effectively, whereas the effect of tyrosinase was negligible. The changes in MWL molecular-weight distributions caused in the reactions were analysed by gel permeation chromatography. Both laccase and HRP treatments were found to polymerise MWL. Peroxidase treatment was found to decrease the amount of phenolic hydroxyls in MWL, whereas no such effect could be detected in the laccase-treated sample. Both laccase and HRP treatments were, however, found to increase the amount of conjugated structures in MWL. The formation of phenoxy radicals during the treatments was studied by electron paramagnetic resonance spectroscopy. Phenoxy radicals were detected in both laccase and HRP-treated samples. The amount of the formed phenoxy radicals was found to be essentially constant during the detected time (i.e. 20–120 min after the addition of enzyme).

Introduction

Oxidative enzymes are potential tools for modification of wood lignin (Grönqvist et al. 2003a). Several studies on the action of oxidative enzymes on fibre-bound lignin and on low-molecular-weight lignin model compounds have been reported (e.g. Felby et al. 1997a,b; Barsberg and Thygesen 1999; Buchert et al. 2002; Rittstieg et al. 2002; Grönqvist et al. 2003b; Lund et al. 2003). The effects of laccase on residual lignin from kraft pulp have also been investigated (Niku-Paavola et al. 2002). Despite extensive studies, the mechanisms of action of oxidative enzymes on lignin are still only partially understood.

Laccase is a multi-copper oxidase which catalyses oxidation of various aromatic compounds, especially phenols, by concomitant reduction of O₂ to H₂O (Gianfreda et al. 1999; Xu 1999). During laccase-catalysed oxidation of wood fibres, phenoxy radicals are formed in the lignin matrix (Felby et al. 1997a,b; Barsberg and Thygesen 1999; Widsten et al. 2002). Tyrosinase is also a copper-containing enzyme, being able to catalyse the hydroxylation of monophenols to *o*-diphenols and further the oxidation of *o*-diphenols to *o*-quinones (Sánchez-Ferrer et al. 1995). Both laccase and tyrosinase utilize O₂ as co-substrate. Horseradish peroxidase (HRP) catalyses the oxidative coupling of phenolic compounds (Gajhede 2001). In the HRP reaction, H₂O₂ is used as co-substrate. During the HRP catalysed oxidation of lignin, phenoxy radicals are formed (Caldwell and Steelink 1969).

Lignin, one of the three main constituents in wood, is a polyphenolic material formed by enzymatic polymerisation of coniferyl, sinapyl and coumaryl alcohols. These phenylpropanoid units are linked to each other in irregular order via ether linkages and carbon–carbon bonds (Sjöström 1993; Alen 2000).

The effects of oxidative enzymes on fibre-bound lignin have been most commonly monitored by measuring the consumption of the co-substrate, i.e. O₂ or H₂O₂, by monitoring the effects of the oxidation on the physical properties of fibres or by studying the formation of radicals (Felby et al. 1997b; Grönqvist et al. 2003b). These kinds of studies

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can give valuable information about the effects of an enzyme on the whole fibre substrate. Oxidation of model compounds, on the other hand, gives information on the substrate specificities of enzymes on different types of structures. The structure of lignin is, however, complex and thus only indicative information about the effects of the enzymes on native macromolecular lignin can be obtained by studies on model compounds.

In order to study the oxidation of native lignin, isolated lignin should be studied. There is, however, no ideal method to isolate lignin. Relatively unchanged lignin can be isolated from wood with the so-called milled wood lignin (MWL) method (Holmbom and Stenius 2000).

In this study, the role of laccase, HRP and tyrosinase in the oxidation of MWL was investigated by measuring the consumption of O₂ and H₂O₂ in the reactions and by studying the changes in molecular-weight distributions and the amount of phenolic hydroxyls in the substrate. As radicalisation of MWL is taking place during laccase and HRP-catalysed reactions, the radical formation in the samples was studied using electron paramagnetic resonance (EPR) spectroscopy in order to better understand the underlying mechanisms.

Materials and methods

Enzymes and MWL

Lignin isolated from spruce by the MWL method (Holmbom and Stenius 2000) was kindly supplied by KCL (Oy Keskuslaboratorio-Centrallaboratorium Ab, Finland). Laccase, tyrosinase and HRP were used in the treatments (Table 1). The laccase was produced and partially purified as described by Poppius-Levlin et al. (1999). The other enzymes used were commercial preparations. Laccase, peroxidase, tyrosinase, lignin peroxidase and manganese peroxidase activities were each measured at the pH used in the experiments. Laccase and Mn-peroxidase activities were determined using ABTS [2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] as substrate (Niku-Paavola et al. 1998). Peroxidase activity was analysed using guaiacol as substrate (Bergmeyer 1974; Paszczyński et al. 1985) and lignin peroxidase using veratryl alcohol as substrate (Tien and Kirk 1983). Tyrosinase activity in tyrosinase and in peroxidase preparations was measured using L-dopa (3,4-dihydroxy-L-phenylalanine) as substrate. Ty-

Table 1 Enzymes used in treatments of milled wood lignin

Enzyme	Source	Supplier	Quality	pH optimum
Laccase	<i>Trametes hirsuta</i>	VTT	Partially purified	4.5
Tyrosinase	Mushroom	Fluka	Commercial	6–7
Horseradish peroxidase	Horseradish	Fluka	Commercial	6–7

rosinase activity in the laccase preparation was measured using L-tyrosine as substrate, as laccase is known to oxidase L-dopa (Robb 1984). The activity profiles of the enzymes used are shown in Table 2.

Enzymatic treatments of MWL

MWL was dissolved in a small volume of 0.1 M NaOH, buffer (25 mM citric acid buffer at pH 4.5 and 25 mM Naphosphate buffer at pH 7.0) was added, and the pH of the solution was adjusted to 4.5 (laccase) or 7.0 (tyrosinase and HRP) with 1 M HCl. The solution was diluted with buffer to a concentration of 1 mg/ml. The substrate solution was treated with 1,000 nkat/g of enzyme at 20°C with mixing. In HRP treatments H₂O₂ was added to a final concentration of 0.5 mM in the solution. In laccase and tyrosinase treatments, no extra O₂ was added. The O₂ concentration was about 8.5 mg/l at the beginning of the reactions done in sealed 30-ml flasks filled to the brim with the MWL solution. The consumption of dissolved O₂ (laccase and tyrosinase treatments) and H₂O₂ (HRP treatments) was measured during the first 30 min of the reaction.

For the analyses of molecular weight and phenolic hydroxyl groups, the treatments were continued for an additional 23.5 h. The concentration of H₂O₂ in the HRP samples was re-adjusted to 1 mM after 2 h. No O₂ was added to the laccase- and tyrosinase-treated samples, but the samples were let to react in contact with air during the rest of the treatment. The reference treatments were performed under similar conditions but without the addition of enzyme. In HRP treatments the effect of H₂O₂ was also studied.

Formation of radicals in the MWL samples during the enzymatic treatments was studied during a 2-h reaction. The samples were prepared and treated as described above except that the laccase and tyrosinase samples were let to react in contact with air. The laccase dosage was 2,500 nkat/ml and HRP and tyrosinase dosages were 1,000 nkat/g.

Table 2 Activity profiles of the enzymes used

Measured activity	Enzyme preparation		
	Laccase ^a (nkat/ml)	Tyrosinase ^b (nkat/mg)	Horseradish peroxidase ^b (nkat/mg)
Laccase	9,571	91.5	n.d.
Peroxidase ^c	2,399	n.d.	2,060
Tyrosinase	n.d. ^d	167	n.d.
Lignin peroxidase ^c	19	n.d.	n.d.
Manganese peroxidase ^c	122	n.d.	n.d.

^aMeasurement pH 4.5

^bMeasurement pH 7.0

^cIn the presence of H₂O₂

^dn.d. No activity was detected in the preparation

^eIn the presence of Mn and H₂O₂

Analyses

The O₂ measurements were made in a closed vessel with a SensorLink PCM800 meter, using a Clark oxygen electrode. The amount of H₂O₂ left in samples after treatments with HRP was analysed by the FOX method (Wolff 1994).

The effect of the enzymatic treatments on MWL after a 24-h reaction was analysed by monitoring the changes in the molecular weights by gel permeation chromatography [(GPC) Hortling et al. 1999]. Four polystyrene sulphonate Na-salts (MW 4,800, 17,000, 41,000 and 35,000) and phenol were used as standards in calculating the molecular weights of the samples. Changes in the amount of phenolic hydroxyls were analysed according to Tamminen and Hortling (1999).

For the radical measurements aliquots of the reaction solutions were put in 3-mm diameter quartz tubes for EPR spectroscopy and immediately frozen in liquid nitrogen in order to stop the reactions. Then, X-band CW EPR spectra were recorded at -150°C on a Bruker EMX spectrometer equipped with a variable temperature BVT 2000 unit (Bruker). The *g* values were determined by standardisation with α,α' -diphenyl- β -picryl hydrazyl (DPPH). The paramagnetic species concentrations, expressed as spin/g of MWL, were calculated with a $\pm 5\%$ precision by means of a calibration curve made with a series of standard solutions of DPPH in toluene/nujol 2:1 at different dilutions. The calibration curve was obtained by plotting the areas under the EPR signals, obtained by double integration of the resonance lines, against the number of spins/cm of the EPR tube filled by the sample. For each sample, the weight of MWL dissolved in the solution filling 1 cm of the EPR tube was accurately determined and eventually the spin/g value was obtained.

Results

Co-substrate consumption as an indication of the oxidation efficiency

The oxidation of MWL catalysed by three enzymes, i.e. laccase, tyrosinase and HRP, was studied by monitoring the consumption of O₂ (for the laccase- and tyrosinase-catalysed reactions) and H₂O₂ (for the HRP-catalysed reaction) during the first 30 min of the reaction with an enzyme dosage of 1,000 nkat/g. Both in the laccase- and HRP-catalysed oxidations, a significant consumption of the co-substrate was observed with respect to the reference treatment (Figs. 1a, 2), whereas only negligible consumption of O₂ was seen in the tyrosinase-treated sample (Fig. 1b). Although the laccase preparation contained peroxidase activity (Table 2), the peroxidase present was not expected to function as H₂O₂ was not supplied during the laccase treatment.

The reactivities of the enzymes were compared by estimating the amounts of substrate oxidised after 10 min and 30 min (Fig. 3). The estimations were made by taking into account the stoichiometry of the reactions. The

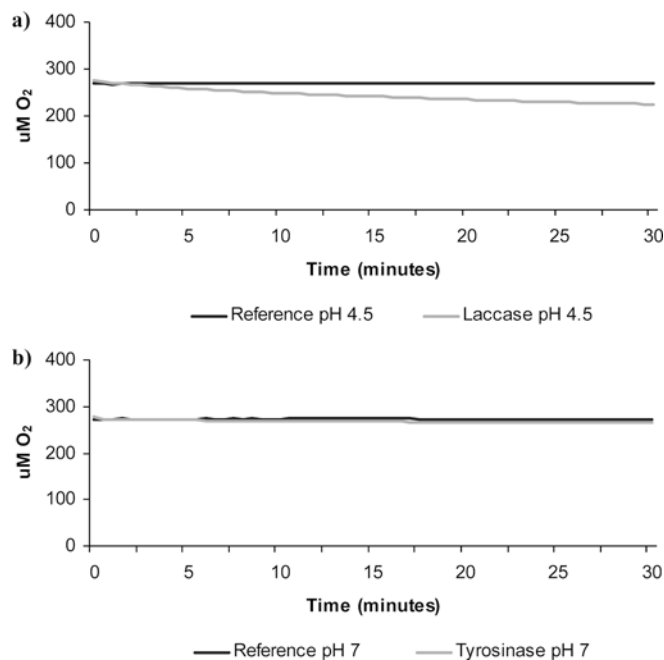


Fig. 1 The consumption of O₂ in **a** laccase-catalysed and in **b** tyrosinase-catalysed treatments of milled wood lignin (MWL) [0.1% MWL solution, pH 4.5 (laccase) or 7.0 (tyrosinase), 20°C, 1,000 nkat/g of enzyme], analysed by measurement of dissolved O₂, sampling every 30 s. Reference treatments were made as the enzymatic treatments but without the addition of enzyme

calculations were based on following assumptions: in the laccase-catalysed oxidation, 1 mol O₂ reacts with 4 mol of substrate ($\text{O}_2 + 4\text{e}^- + 4\text{H}^+ \rightarrow 2\text{H}_2\text{O}$). In the tyrosinase-catalysed reaction, 1 mol O₂ reacts with 1 mol substrate and in the HRP-catalysed reaction, consumption of 1 mol H₂O₂ indicates oxidation of 2 mol substrate ($\text{H}_2\text{O}_2 + 2\text{e}^- + 2\text{H}^+ \rightarrow 2\text{H}_2\text{O}$). The results show that after 10 min, both laccase and HRP had catalysed the oxidation of lignin, i.e. about 110 $\mu\text{mol/g}$ and 250 $\mu\text{mol/g}$. After 30 min of reaction, the amount of the oxidised substrate increased with both laccase and HRP, while the effect of tyrosinase remained negligible. The calculated amount of substrate oxidised by laccase and HRP after 30 min reaction was 205 $\mu\text{mol/g}$ and 360 $\mu\text{mol/g}$, respectively.

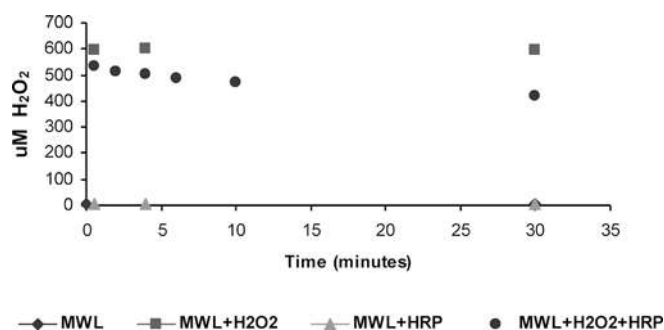


Fig. 2 The consumption of H₂O₂ in horseradish peroxidase (HRP) catalysed treatments of MWL (0.1% MWL solution, pH 7.0, 20°C, 1,000 nkat/g of HRP, 0.5 mM of H₂O₂)

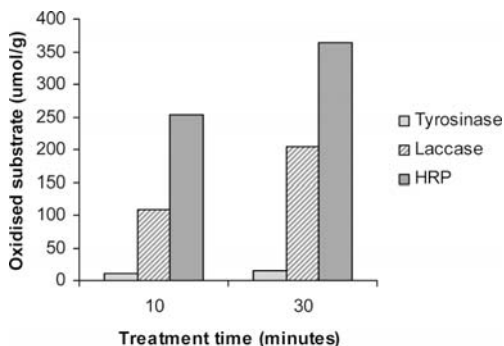


Fig. 3 MWL oxidised during 10- and 30-min treatments with laccase (pH 4.5), tyrosinase (pH 7.0) and HRP (pH 7.0). The enzyme dosage was 1,000 nkat/g

Effect of enzymatic oxidation on molecular weight and structure of MWL

The mode of action of these enzymes was also investigated by monitoring the changes in the molecular-weight distributions of the MWL substrate after 24-h treatments. Lac-

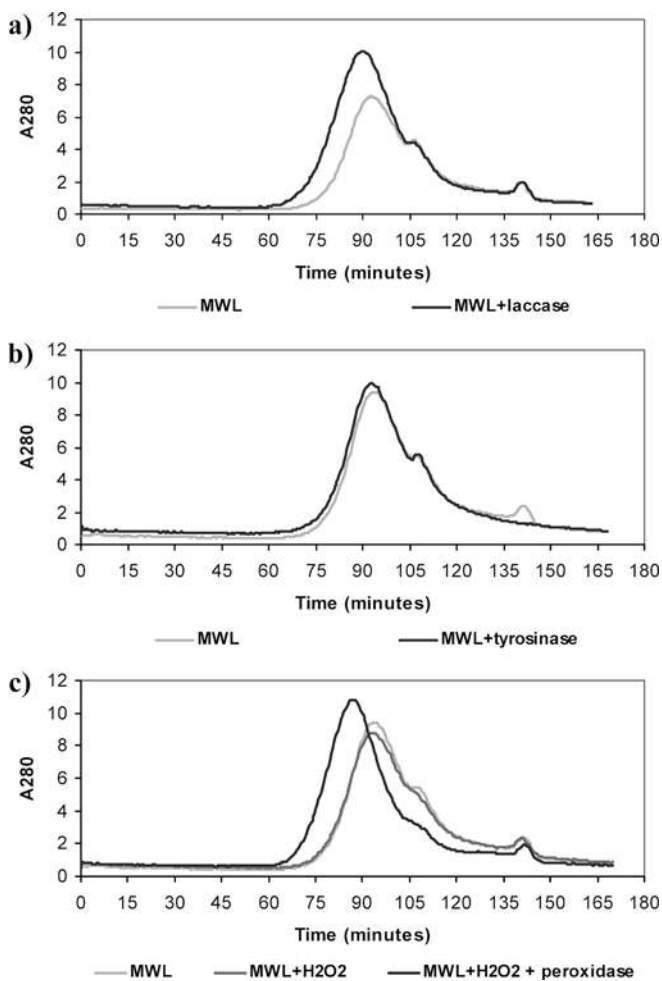


Fig. 4 Gel permeation chromatography elution curves of MWL treated with **a** laccase, **b** tyrosinase and **c** HRP [20°C, pH 4.5 (laccase) or pH 7.0 (tyrosinase and HRP), 1,000 nkat/g of enzyme]

Table 3 Effect of the enzyme treatment on the structure of lignin (1 mg/ml, 24 h, 1,000 nkat/g of enzyme at 20°C)

Treatment	Conj. lignin (mmol/g)	Tot. phenols (mmol/g)	Conj./Tot phenols (%)
Reference, pH 4.5	0.141	1.09	13
Laccase, pH 4.5	0.162	1.11	15
Reference, pH 7.0	0.174	1.28	14
Tyrosinase, pH 7.0	0.170	1.24	14
H ₂ O ₂ , pH 7.0	0.154	1.27	12
H ₂ O ₂ + horseradish peroxidase, pH 7.0	0.169	1.15	15

case and HRP treatments of MWL led to polymerisation of the MWL (Fig. 4a, c). H₂O₂ alone had practically no effect on the molecular weight of MWL. In the laccase-catalysed oxidation, the molecular weight of the fraction with the largest molecules was found to increase by about 40% (from 9,200 to 12,900) whereas in the case of HRP the increase was slightly more pronounced, i.e. about 78% (from 9,200 to 16,400). The effect of tyrosinase on MWL was negligible as expected from the reactivity measurements.

In order to further study the effects of these enzymes on MWL, the phenolic hydroxyl contents of the samples were analysed after 24-h treatments, as described by Tamminen and Hortling (1999). The amount of measured total phenols in the reference sample treated at pH 7 was about 30% higher than in the reference sample treated at pH 4.5. Hydroxyl groups are known to be sensitive to pH and thus, the effect observed is most probably due to modification of the MWL taking place at neutral pH.

The HRP-catalysed treatment reduced the phenolic hydroxyls in MWL by 9% and increased the amount of

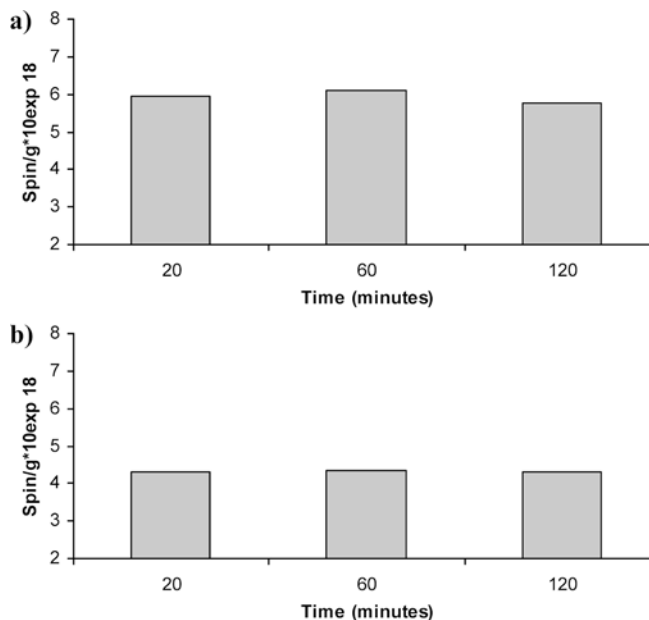


Fig. 5 The absolute amount of phenoxy radicals measured in MWL treated with **a** laccase and **b** HRP

conjugated structures by 10%. Surprisingly, no decrease in the content of phenolic hydroxyls was obtained in the laccase treatment, although the amount of conjugated structures increased by 15%. Apparently the formed phenoxy radicals were delocalised into the aromatic ring, and thus, no decrease in the phenolic hydroxyls was observed (Table 3).

EPR spectroscopy of enzymatic oxidation of MWL

To study the formation of radicals, the MWL solution was treated with laccase, HRP and tyrosinase for 2 h. Aliquots of the reaction solutions were taken at reaction times of 20, 60 and 120 min, and the amount of radicals in the samples were quantified. No detectable EPR signals were found in the reference-treated samples. As expected, no detectable signals were observed after treatment with tyrosinase (results not shown), whereas after both laccase and HRP treatments of MWL samples EPR signals typical of phenoxy radicals ($g=2.004$ and $\Delta H_{pp}=9$ G) were observed (Hon 1992). The amount of radicals in both laccase and HRP treatments was found to be essentially constant during the detected time (Fig. 5).

Discussion

The results of the present study confirm that the actions of the three lignin oxidising enzymes, i.e. laccase, tyrosinase and HRP on MWL are different.

According to the results obtained, both laccase and HRP effectively catalysed the oxidation of MWL, whereas the effect of tyrosinase was negligible. Similar results were obtained by Kaplan (1979), who reported that laccase and peroxidase can be used in oxidation of lignin, whereas tyrosinase is unreactive towards lignin. Kaplan, however, reported only about the possibility to oxidise lignin with these enzymes; he did not compare their effects. In the present study the actions of HRP and laccase on lignin were found to be clearly different. Based on the consumption of the co-substrate (O_2 and H_2O_2) and results obtained by GPC, HRP was found to modify lignin more efficiently than laccase resulting into a more intensive polymerisation of the starting material.

The different mode of action of these two enzymes was further visualised by the analysis of phenolic structures of lignin by the method of Tamminen and Hortling (1999). As both laccase and HRP react primarily with the phenolic hydroxyls groups and form the corresponding radicals, it was expected that a decrease in the quantity of phenolic hydroxyls would be obtained. Surprisingly, in the laccase-catalysed oxidation, the amount of phenolic hydroxyl groups did not decrease, although the amount of conjugated structures was found to increase. The HRP-catalysed treatment on the other hand, did reduce the phenolic hydroxyl content in MWL, with concomitant increase in the amount of conjugated structures.

In our previous studies, oxidation treatment of a lignan compound (hydroxymatairesinol) by laccase was found to decrease the phenolic hydroxyl content by 40–50% (Buchert et al. 2002). One plausible explanation for the different behaviour is the molecular weight of the sample. Lignans are low-molecular-weight substrates for laccase, whereas the molecular weight of MWL is high. In a high-molecular-weight substrate, delocalisation of formed radicals may occur and thus, the phenoxy content may remain the same as initially. According to Mai 1998 and Hüttermann et al. 2001, when a phenoxy radical in lignin is delocalised into the lignin matrix and reacts with another radical, a hydroxyl group is regenerated at its original site. According to this theory, it is possible that the amount of conjugated structures is increased, while the amount of phenols remains the same. This assumption is also supported by the report of Niku-Paavola et al. (2002), where the laccase treatment was not found to have an effect on the hydroxyl groups in residual lignin isolated from kraft pulp. Thus, it seems that the effect of laccase on low-molecular-weight substrates, such as lignans and on the more complex lignin is different.

Phenoxy radicals were detected, as expected, both in the laccase- and HRP-treated samples. The amount of radicals was found to be rather constant during the detected time. However, the amounts of radicals at the beginning of the reactions were not measured. Ferm et al. (1972) reported that the amount of radicals in sweetgum MWL increased during the first 15–25 min, whereafter it started to decrease.

As polymerisation in both the laccase- and HRP-treated samples was observed after a 24-h reaction, consumption of radicals must, however, have taken place. The polymerisation reactions can have taken place immediately after addition of laccase, i.e. during the time when the amount of radicals was not measured. Alternatively, the observed overall stability in the amount of radicals might also have been due to constant formation of new radicals while polymerisation took place. One possibility would also be that the polymerisation reactions took place after the 2-h period when the radicals were detected. Felby et al. (1997a,b) found in their radicalisation studies of beech wood fibres with laccase that of the radicals formed in a 1-h treatment, a considerable proportion was found to be stabilised in the lignin polymer. This result would indicate that the radicals detected in this study were stable and rather unreactive.

The results obtained in this study show that the mode of action of HRP and laccase seem to be different. The significance of this difference in different applications using oxidative enzymes remains to be studied.

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