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Oxidation Capacity of Laccases and Peroxidases as Reflected in Experiments With Methoxy-Substituted Benzyl Alcohols

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Abstract

A set of methoxy-substituted benzyl alcohol (MBA) congeners were examined regarding susceptibility to oxidation by *Trametes versicolor* laccase, *T. versicolor* lignin peroxidase and horseradish peroxidase: 2,4,5-trimethoxybenzyl alcohol (TMBA), 3,4,5-TMBA, 2,3,4-TMBA, 2,5-dimethoxybenzyl alcohol (DMBA), 3,4-DMBA, and 2,3-DMBA. The corresponding methoxy-substituted benzaldehydes were strongly predominant as products on enzymic oxidation. This together with different reaction rates and redox potentials makes the MBAs suitable as substrates in the characterization of ligninolytic enzymes. For fungal laccase, the reaction rate order was: 2,4,5-TMBA >> 2,5-DMBA > 3,4-DMBA > 3,4,5-TMBA ~ 2,3,4-TMBA ~ 2,3-DMBA. Horseradish peroxidase displayed a similar reactivity order. Oxidation of some of the MBAs with laccase and horseradish peroxidase was only observed when the reactions were carried out at low pH and with relatively high-substrate concentration. 3,4-DMBA (veratryl alcohol) was the best substrate for lignin peroxidase and the reaction rate order was: 3,4-DMBA > 2,4,5-TMBA ~ 3,4,5-TMBA > 2,5-DMBA > 2,3,4-TMBA ~ 2,3-DMBA. The oxidation experiments with different MBAs elucidate the potential of the enzymes as oxidants in various applications.

Index Entries: Lignin peroxidase; horseradish peroxidase; laccase; methoxy-substituted benzyl alcohols.

Introduction

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are glycosylated blue copper-containing phenol oxidases that are widely distributed in higher plants and fungi *(1,2)*. Laccase catalyzes the one-electron

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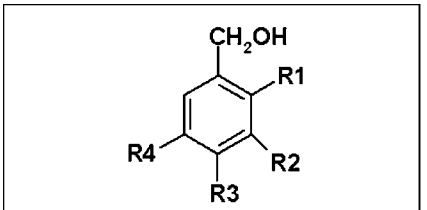
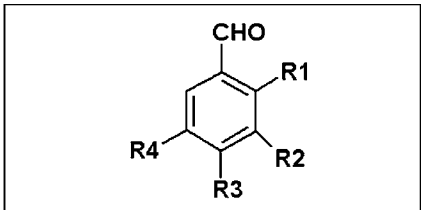
oxidation of a variety of phenolic compounds, as well as diamines and hexacyanoferrate, concomitantly with the four-electron reduction of molecular oxygen to water. The catalytic ability of laccases has been related to the difference in redox potential between the substrate and the enzyme (3,4). The action of laccase is usually limited to the types of substrates mentioned earlier and the enzyme is often described as not being active with nonphenolic lignin model compounds. However, in the presence of low-molecular mass mediators, laccase can be employed for the oxidation of a variety of nonphenolic aromatic compounds (5). Thus, laccases have very broad substrate specificity regarding the reducing substrate and have an interesting potential as industrial enzymes. They are attracting considerable interest for a variety of biotechnological applications, such as organic synthesis/transformation (6), textile processing, delignification of pulp, manufacture of new materials, construction of biosensors and biofuel cells, and detoxification (7–9).

Laccases produced by the white-rot fungus *Trametes* (*Coriolus*, *Polyporus*) *versicolor* have a relatively high-redox potential (0.7–0.8 V) (1). The catalytic site of the enzyme comprises four copper ions, one type 1 (T1), one type 2 (T2), and two type 3 (T3) copper ions. The T1 copper oxidizes compounds via one-electron abstraction and transfers electrons to the T2 and T3 copper ions. The T2 and T3 copper ions form a trinuclear cluster responsible for oxygen binding and reduction (1,10). The catalytic mechanism of laccase is still not fully clear. Lignin peroxidase (LP; EC 1.11.1.14) and horseradish peroxidase (HRP; EC 1.11.1.7) are heme-containing enzymes displaying a similar catalytic cycle. A single two-electron oxidation of the peroxidase by hydrogen peroxide is followed by two single-electron oxidations of the reducing substrate by the peroxidase (11). A comparison based on the oxidation of methoxy-substituted benzenes suggested that LP is a stronger oxidant than HRP, which in turn is a stronger oxidant than *T. versicolor* laccase (3). Laccase oxidized only 1,2,4,5-tetramethoxybenzene, which has a relatively low half-wave potential (12).

Methoxyl-substituted aromatic substrates are of interest considering that lignin is formed mainly from two methoxylated precursors, coniferyl alcohol and sinapyl alcohol (13). In addition, 3,4-dimethoxybenzyl alcohol (DMBA; veratryl alcohol) is produced as a secondary metabolite by lignin-degrading fungi (14). The investigation of the oxidation of methoxy-substituted benzyl alcohols (MBAs) is therefore relevant for the elucidation of lignin biodegradation in nature. The oxidation of veratryl alcohol has been examined using laccase-mediator systems (5,15,16), HRP (17,18), and LP (19–21). Valli et al. (22) and Koduri and Tien (23) have studied the oxidation of 4-methoxybenzyl alcohol and 3,4,5-trimethoxybenzyl alcohol (TMBA) by LP. The oxidation of 3,4,5-TMBA with syringaldehyde as mediator in reactions catalyzed by laccase (16) and the oxidation of 2,4-DMBA with 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1-hydroxybenzotriazole (HBT) as mediators (24) have also been reported.



	R1	R2	R3	R4
2,3-DMBA	OCH_3	OCH_3	H	H
2,5-DMBA	OCH_3	H	H	OCH_3
3,4-DMBA	H	OCH_3	OCH_3	H
2,3,4-TMBA	OCH_3	OCH_3	OCH_3	H
2,4,5-TMBA	OCH_3	H	OCH_3	OCH_3
3,4,5-TMBA	H	OCH_3	OCH_3	OCH_3



	R1	R2	R3	R4
2,3-DMBD	OCH_3	OCH_3	H	H
2,5-DMBD	OCH_3	H	H	OCH_3
3,4-DMBD	H	OCH_3	OCH_3	H
2,3,4-TMBD	OCH_3	OCH_3	OCH_3	H
2,4,5-TMBD	OCH_3	H	OCH_3	OCH_3
3,4,5-TMBD	H	OCH_3	OCH_3	OCH_3

Fig. 1. Methoxysubstituted benzyl alcohols used as substrates (MBAs) and their predominating conversion products on enzymic oxidation (MBDs).

We have investigated a set of MBAs (Fig. 1) as substrates with respect to oxidation by LP, HRP, and laccase. In contrast to substrates such as 1,2,4,5-tetramethoxybenzene *(3)*, the MBAs are advantageous in the respect that the methoxy-substituted benzaldehydes (MBDs) are produced as predominant products. This makes the MBAs suitable for kinetic studies. As expected based on theoretical considerations, it was found that one of the MBAs studied, 2,4,5-TMBA, was particularly well suited as substrate. The results from the oxidation experiments with MBAs provide a basis for judgment of the capacity of the enzymes as oxidants in various applications.

Methods

Chemicals

MBAs (Fig. 1), MBDs (Fig. 1), catechol (1,2-dihydroxybenzene), hydroquinone (1,4-dihydroxybenzene), ABTS, and HBT were obtained from Sigma-Aldrich (Steinheim, Germany). Procedures for the preparation of 1-(4-hydroxy-3,5-dimethoxyphenyl)-1-propanol *(25)* (mp 94.9–96.4°C; mp 96–97°C is reported in Pew and Connors *[25]*) and 1-(4-hydroxy-3,5-dimethoxyphenyl)-1-propanone *(26)* (mp 109.8–110.3°C; mp 109–110°C is reported in Pew and Connors *[26]*) have been described in the literature.

The preparation of bis(2,4,5-trimethoxyphenyl)methane has been described by von Usslar and Preuss *(27)* and Birch et al. *(28)*. We obtained *bis*(2,4,5-trimethoxyphenyl)methane by treatment of 2,4,5-TMBA with 0.06 *M* hydrochloric acid at room temperature. Precipitated *bis*(2,4,5-trimethoxyphenyl)methane was filtered off. Mp 99.5–100.5°C (mp 102–102.5°C is reported in *[27]*). ^{1}H NMR (400 MHz, $CDCl_3$, TMS, 20°C): δ 3.75 (6H, s, OCH_3), 3.81 (6H, s, OCH_3), 3.84 (2H, s, CH_2), 3.88 (6H, s, OCH_3), 6.58 (2H, s, H-Ar), and 6.66 (2H, s, H-Ar).

Table 1
TLC and UV-Visible Data for the MBAs and Their Oxidation Products

Compound	R_f	Color[a]	λ_{max} (nm)	ε
2,3-DMBA	0.40	Purple	274	1600
2,5-DMBA	0.44	Green	289	3000
3,4-DMBA	0.22	Pink	276	2800
2,3,4-TMBA	0.30	Red	270	900
2,4,5-TMBA	0.20	Blue	289	4400
3,4,5-TMBA	0.20	Brown	270	810
2,3-DMBD	0.77	Orange	260	9400
			322	2400
2,5-DMBD	0.78	Orange	258	8500
			355	4300
3,4-DMBD	0.58	Yellow	277	11000
			308	9200
2,3,4-TMBD	0.66	Yellow	285	14000
2,4,5-TMBD	0.42	Yellow	277	11000
			344	8500
3,4,5-TMBD	0.61	Yellow	286	11000

[a]Observed after spraying with formalin/H_2SO_4 solution.

Thin-Layer Chromatography Analyses

Thin layer chromatography (TLC) was performed using silica gel plates (Kieselgel 60F_{254}, Merck, Darmstadt, Germany). The eluent was toluene-ethyl acetate (2:1) (R_f values: MBAs and MBDs, *see* Table 1; 1-(4-hydroxy-3,5-dimethoxyphenyl)-1-propanol, 0.19; 1-(4-hydroxy-3,5- dimethoxyphenyl)-1-propanone, 0.42; *bis*(2,4,5-trimethoxyphenyl)-methane, 0.47). Spots were observed using ultraviolet (UV) light and by spraying with formalin/H_2SO_4 (1:9) and subsequent heating.

UV-Visible Spectroscopy

UV-visible spectra were recorded with a UV-1601PC (Shimadzu, Tokyo, Japan), unless otherwise stated. The compounds were first dissolved in 95% ethanol (to 60 m*M*) and then diluted to a suitable concentration with water. The λ_{max} and ε_{max} values are: MBAs and MBDs, *see* Table 1; catechol, λ_{max} 275 nm, ε_{max} 2400; hydroquinone, λ_{max} 289 nm, ε_{max} 2600. UV-visible spectra for 1-(4-hydroxy-3,5-dimethoxyphenyl)-1-propanol (λ_{max} 272 nm, ε_{max} 1800) and 1-(4-hydroxy-3,5-dimethoxyphenyl)-1-propanone (λ_{max} 299 nm, ε_{max} 10,000) were recorded using a Cary 4 UV-visible spectrophotometer (Varian, Palo Alto, CA).

Enzyme Preparations

T. versicolor laccase was obtained from Jülich Fine Chemicals GmbH (Jülich, Germany). The fungal laccase was purified to homogeneity using

anion-exchange chromatography and 0.1 *M* phosphate buffer (pH 6.0), as reported previously *(29)*, except that DEAE Sepharose Fast Flow resin (Amersham Biosciences, Uppsala, Sweden) was used instead of DEAE A50 Sephadex. The purity of the preparation was judged from sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequent staining with Coomassie brilliant blue. The laccase activity was determined by using ABTS and the reactions were monitored at 414 nm ($\varepsilon = 3.6 \times 10^4$) *(30)*. The reaction medium was 50 m*M* acetate buffer, pH 5.2, and the reaction temperature was 23°C. One unit equals the formation of 1 µmol ABTS radical cation per min. The protein concentration was determined with the dye assay (Bio-Rad, Hercules, CA) and bovine-serum albumin was used as the standard.

Lacquer tree (*Rhus vernicifera*) laccase and HRP VI (Reinheitzahl [RZ, A_{403}/A_{275}] 3) were obtained from Sigma Chemical Co. (St. Louis, MO). The HRP activity was determined in the same way as the laccase activity except that H_2O_2 was added to a concentration of 0.4 m*M*.

LP was produced by *T. versicolor* PRL572 and purified to homogeneity as previously described *(31)*. The activity was determined using a 2 m*M* veratryl alcohol solution and the veratraldehyde (3,4-dimethoxybenzaldehyde [DMBD]) formation was monitored based on the absorbance at 310 nm as described previously *(32)*. One unit equals the formation of 1 µmol veratraldehyde/min.

Screening for Acid Instability of MBAs

Solutions (10 m*M*) of the MBAs (Fig. 1) in 0.2 *M* HCl were kept at 23°C overnight. TLC analysis did not show any product formation, except for 2,4,5-TMBA. Treatment of 2,4,5-TMBA with 0.2 *M* HCl resulted in the formation of *bis*(2,4,5-trimethoxyphenyl)methane (*cf.* reports by von Usslar and Preuss *[27]* and Birch et al. *[28]*). Therefore, the effect of acidic conditions on 2,4,5-TMBA was investigated further. 2,4,5-TMBA (40 m*M* solution) was treated with citrate-phosphate buffers at pH 2.2, 3.0, 4.0, and 5.0. Samples for TLC analysis were taken after 15 min, 30 min, 1 h, 3 h, and 24 h. Treatment at pH 2.2 resulted in dimer formation after 15 min, whereas dimer formation at pH 3.0 was detected only after 1 h or longer. At pH 4.0 and 5.0, dimer formation was detected in samples taken after 3 and 24 h, respectively. Thus, dimer formation occurs even under weakly acidic conditions. However, both TLC and spectrophotometric analyses indicated that the rate of dimer formation at pH 3.0–5.0 was so slow that the results of the enzymic oxidations were not affected.

Examination of Oxidation Reactions of MBAs Using UV-Visible Spectroscopy and TLC

The enzymic reactions were monitored at 23°C by UV-visible spectrophotometry using wavelength scans (230–450 nm). The reactions were performed with a substrate concentration of 0.5 m*M* at pH 3.0, 5.2, and 6.0.

The concentrations of LP and HRP were 4 and 120 µg/mL, respectively, and the reactions were initiated by adding enzyme to the reaction mixture, which was 0.4 m*M* with respect to H_2O_2. For fungal laccase, the reaction medium was air-saturated and the enzyme concentration was 330 µg/mL. Control samples (no enzyme added) were examined with and without H_2O_2. The cuvets were sealed to avoid evaporation of water. Spectra were recorded every 2nd h.

In a second series of experiments, TLC was employed to verify the results obtained by UV-visible spectrophotometry. The enzymic reactions were carried out with 20 m*M* substrate concentration at pH 4.0 (50 m*M* acetate buffer) and 23°C overnight. For LP and HRP, enzyme and H_2O_2 were added in portions to decrease the effect of enzyme inactivation and to obtain enough product for TLC analysis. For fungal laccase, 3,4-DMBD was detected by TLC as a product from 3,4-DMBA in an experiment with a 3,4-DMBA concentration of 40 m*M* and an enzyme concentration of 1 mg/mL at pH 3.0 and at 23°C for 4 d. After the reaction, the mixture was extracted with 0.5 volume of ethyl acetate and a sample of the extract was applied to a TLC plate. Controls that did not contain any enzyme were examined as well.

The oxidation of 2,4,5-TMBA with tree laccase (*R. vernicifera)* was also examined. The reaction medium was 50 m*M* acetate buffer (pH 4.0). The substrate concentration was 2 m*M* and laccase concentrations up to 0.025 units/mL (as determined with ABTS at pH 5.2) were tested. The samples were monitored for 48 h, but no reaction was observed (UV spectroscopy).

Comparison of the Oxidation Rates of MBAs

The oxidation rates of the different MBAs were monitored based on the formation of MBDs using UV-visible spectrometry. The increase in absorbance was recorded at 322 nm for 2,3-DMBA (ε = 2400), 355 nm for 2,5-DMBA (ε = 4300), and 344 nm for 2,4,5-TMBA (ε = 8500). 3,4-DMBA (ε = 9200), 2,3,4-TMBA (ε = 5500), and 3,4,5-TMBA (ε = 6300) were monitored at 310 nm. The initial absorbance change was used to calculate the rate of product formation (µmol/min). Oxidation by LP was studied using reaction mixtures containing 1 m*M* substrate, 50 m*M* citrate-phosphate buffer (pH 3.0), 5 µg/mL LP, and 0.4 m*M* H_2O_2. HRP was investigated in the same manner as LP, except that the concentration of substrate was 10 m*M* and that the enzyme concentration (HRP) was 160 µg/mL. Laccase reaction mixtures contained 28 m*M* substrate, 50 m*M* citrate-phosphate buffer (pH 3.0) and 1 mg/mL fungal laccase. The highest reaction rate observed for each enzyme was arbitrarily set to 100.

Determination of Kinetic Constants With 2,4,5-TMBA and 3,4-DMBA as Substrates

The oxidation rates of 2,4,5-TMBA and 3,4-DMBA catalyzed by LP, HRP, and fungal laccase were determined at 23°C by UV-visible spectrophotometry

using 50 m*M* acetate buffer (pH 4.0) as the reaction medium. The H_2O_2 concentration in the peroxidase-catalyzed reactions was 0.4 m*M*. The initial apparent reaction rates (V_0, μ*M*/min) were calculated based on the initial increase of the absorbance at 344 nm (ε = 8500) for 2,4,5-TMBA and at 308 nm (ε = 9200) for 3,4-DMBA. The curves were of Michaelis-Menten type and the steady-state apparent Michaelis constant, K_m, was determined by fitting V_0 and the substrate concentration to the Michaelis-Menten equation with the Prizm program of GraphPad Software (San Diego, CA). All the experiments were repeated in duplicate and the standard deviations of the determination of the apparent k_{cat} and K_m values are given.

With LP, the 2,4,5-TMBA concentration was 0.01–1.2 m*M* and the enzyme concentration was 1.6 μg/mL. With HRP, the 2,4,5-TMBA concentration was 1–26 m*M* and the enzyme concentration was 0.1 μg/mL. For both peroxidases, the reactions were monitored for 5 min. With fungal laccase, the 2,4,5-TMBA concentration was 1–40 m*M* and the enzyme concentration 0.1 μg/mL. All the experiments with laccase were carried out in air-saturated solutions and the reactions were monitored for 60 min.

With LP, the 3,4-DMBA concentration was 0.01–1.4 m*M* and the enzyme concentration was 1.6 μg/mL. The reactions were monitored for 2 min. With HRP, the 3,4-DMBA concentration was 1-32 m*M* and the enzyme concentration was 100 μg/mL. The reactions were monitored for 15 min. With fungal laccase, the 3,4-DMBA concentration was 4–60 m*M*, the enzyme concentration was 100 μg/mL, and the reactions were monitored for 60 min.

pH Profiles

The initial reaction rates at 23°C were monitored using UV-visible spectrophotometry. The reaction media were 50 m*M* citrate-phosphate buffers and the pH range was 2.2–7.0. For LP and HRP, the concentration of H_2O_2 was 0.4 m*M*. The pH profiles with catechol (based on the decrease in absorbance at 275 nm), hydroquinone (based on the decrease in absorbance at 289 nm), 1-(4-hydroxy-3,5-dimethoxyphenyl)-1-propanol (based on the increase in absorbance at 295 nm [formation of 1-{4-hydroxy-3,5-dimethoxyphenyl}-1-propanone]), and ABTS (based on the increase in absorbance at 414 nm [formation of ABTS radical cation]) as substrates were determined for LP, HRP, and fungal laccase. The pH profile with ABTS as the substrate was also determined for tree laccase. In all cases mentioned earlier, the substrate concentration was 0.5 m*M*. The pH profiles with 2,4,5-TMBA (based on the increase in absorbance at 344 nm [formation of 2,4,5-trimethoxybenzaldehyde {TMBD}]) as the substrate were determined for LP, HRP, and fungal laccase. The pH profile with 3,4,5-TMBA (based on the increase in absorbance at 300 nm [formation of 3,4,5-TMBD]) as substrate was determined for LP. In reactions with 2,4,5-TMBA and 3,4,5-TMBA, the substrate concentration was 1 m*M*. The pH profiles for 2,5-DMBA (based on the increase in absorbance at 355 nm [formation

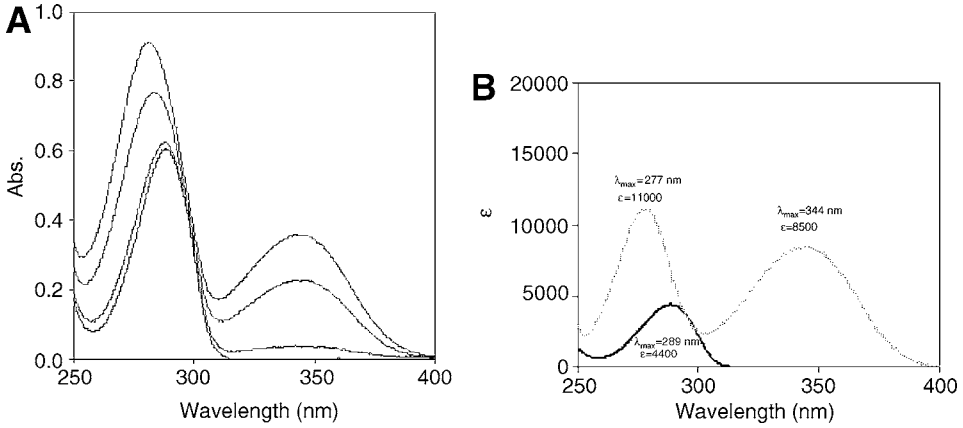


Fig. 2. (A) UV-visible spectral changes for a reaction with 2,4,5-trimethoxybenzyl alcohol (TMBA) catalyzed by *T. versicolor* laccase. After initiation of the reaction by addition of laccase (the initial spectrum [λ_{max} 289 nm] is shown as a black line [bottom]), the absorbance gradually increased (the gray lines represent spectra recorded after 1.5 h [second from bottom], 15 h [second from top] and 22 h [top]). The reaction was performed in 50 m*M* acetate buffer, pH 5.2, with 0.14 m*M* 2,4,5-TMBA and 56 µg/mL laccase in the reaction mixture. The yield of 2,4,5-TMBD was 3% after 1.5 h, 19% after 15 h, and 30% after 22 h. **(B)** UV-visible spectra for 2,4,5-TMBA (black line) and 2,4,5-TMBD (gray line).

of 2,5-DMBD]) and 3,4-DMBA (based on the increase in absorbance at 310 nm [formation of 3,4-DMBD]) as substrates (in a concentration of 10 m*M*) were determined for HRP. The concentration of LP was 0.4–2 µg/mL. For HRP, the enzyme concentration was 0.02–2 µg/mL, except when 2,5-DMBA and 3,4-DMBA were substrates. In the latter two cases, the enzyme concentration was 200 µg/mL. The laccase concentration was 0.07–50 µg/mL.

Results

To investigate if the MBA congeners (Fig. 1) served as substrates for LP, HRP, and laccase, the UV-visible spectral changes of the reaction mixtures obtained in experiments with the different MBAs were examined. UV-visible spectroscopy provides conclusive results as reactants and products differ distinctly from each other as concerns UV-visible properties (data are given in Table 1 and an example is provided in Fig. 2). Product identification was in general complemented by TLC examinations (data regarding R_f values and spot colors are given in Table 1). No formation of oxidation products was observed unless enzymes were added to the reaction mixture. Reactions were found to occur for all combinations of the six MBAs and LP, HRP and fungal laccase, but the oxidation of some of the MBAs by HRP and laccase was extremely slow. A low pH and a relatively high concentration of the MBAs had in general a beneficial impact on the reactions. In reaction mixtures containing HRP and 2,3-DMBA, 2,3,4-TMBA

Table 2
Relative Rate of Oxidation of Methoxy-Substituted Benzyl Alcohols by LP, HRP, and *T. versicolor* Laccase Determined by UV Spectroscopic Measurements[a]

	Relative rate of oxidation		
Substrate	LP/H_2O_2	HRP/H_2O_2	Laccase/O_2
2,3-DMBA	13[b]	≤0.02	≤0.01
2,5-DMBA	24[b]	0.05[b]	0.05[b]
3,4-DMBA	100[b]	0.03[b]	0.02[b]
2,3,4-TMBA	13[b]	≤0.02	≤0.01
2,4,5-TMBA	40[b]	100[b]	100[b]
3,4,5-TMBA	36[b]	≤0.02	≤0.01

[a]The rate of product formation with the best substrate is arbitrarily set to 100 for each enzyme. For reaction conditions, *see* Methods.
[b]The identity of the product was verified by TLC analysis.

or 3,4,5-TMBA, spectral changes were not observed within 24 h at pH 5.2 or 6.0, but reactions (very slow) were observed at pH 3.0. Similarly, in reaction mixtures containing laccase and 2,3-DMBA, 3,4-DMBA, 2,3,4-TMBA, or 3,4,5-TMBA, spectral changes were not observed within 24 h at pH 5.2 or 6.0, but reactions (very slow) were observed at pH 3.0. The spectral changes observed with fungal laccase and the best substrate, 2,4,5-TMBA, are illustrated in Fig. 2. Oxidation caused by LP and HRP led to similar spectral changes. Figure 2 shows that the enzymic oxidation caused a shift of λ_{max} from 289 to 277 nm and a new peak appeared at 344 nm. UV-visible and TLC examinations showed that the sole predominating oxidation product was 2,4,5-TMBD. With respect to product formation, the other MBA congeners exhibited analogous results with LP, HRP, and *T. versicolor* laccase; the products generated were the corresponding MBDs (UV-visible and TLC examinations).

When the laccase-catalyzed oxidation of 2,4,5-TMBA was allowed to proceed more extensively (the reaction mixture contained 1 m*M* substrate, 220 µg/mL fungal laccase and 50 m*M* acetate buffer, pH 4.0, and was incubated at 23°C for 24 h), the yield of 2,4,5-TMBD was about 80% (UV spectrometry). TLC analysis still showed only one product: 2,4,5-TMBD. When the reaction was allowed to continue for further 24 h, there was only a slight increase in the yield of 2,4,5-TMBD.

LP could oxidize all of the MBAs at a substantial rate (Table 2). With LP, the reaction rate order was: 3,4-DMBA > 2,4,5-TMBA ~ 3,4,5-TMBA > 2,5-DMBA > 2,3,4-TMBA ~ 2,3-DMBA. The reaction rates at pH 3 were 23 nmol/min with 2,5-DMBA and 94 nmol/min with 3,4-DMBA, when the substrate concentration was 1 m*M* and the concentration of LP was 5 µg/mL.

With HRP, the reaction rate order was 2,4,5-TMBA >> 2,5-DMBA > 3,4-DMBA > 2,3-DMBA ~ 3,4,5-TMBA ~ 2,3,4-TMBA (Table 2). The rates of

Table 3
Comparison of Apparent Kinetic Parameters for LP, HRP, and *T. versicolor* Laccase With 2,4,5-TMBA and 3,4-DMBA as Substrates at pH 4.0

	2,4,5-TMBA			3,4-DMBA		
Enzyme	k_{cat} (/min)	K_m (m*M*)	k_{cat}/K_m (/m*M*/min)	k_{cat} (/min)	K_m (m*M*)	k_{cat}/K_m (/m*M*/min)
LP	135 ± 2	0.047 ± 0.003	2900	260 ± 3	0.115 ± 0.005	2300
HRP	2700 ± 200	8 ± 1	340	5 ± 0.1	26 ± 1	0.2
laccase	600 ± 200	73 ± 9	8	<1	>100	–

HRP-catalyzed oxidations at pH 3.0 were 7 nmol/min with 2,5-DMBA and 4 nmol/min with 3,4-DMBA, when the substrate concentration was 10 m*M* and the enzyme concentration was 160 µg/mL. An oxidized intermediate of HRP interfered with the determination of 2,3-DMBD. This could possibly lead to an overestimate of the oxidation rate of 2,3-DMBA by HRP.

With fungal laccase, the reaction rate order was: 2,4,5-TMBA >> 2,5-DMBA > 3,4-DMBA > 3,4,5-TMBA ~ 2,3,4-TMBA ~ 2,3-DMBA (Table 2). The reaction rates for fungal laccase at pH 3.0 were 0.7 nmol/min with 2,5-DMBA and 0.2 nmol/min with 3,4-DMBA, when the substrate concentration was 28 m*M* and the enzyme concentration was 1 mg/mL. The laccase-catalyzed oxidation of 2,4,5-TMBA proceeded at a 15-fold higher rate when the substrate concentration was raised from 1 to 16 m*M*, indicating a nearly linear relationship with the concentration change. With 3,4-DMBA as substrate for fungal laccase, the oxidation rate at pH 3.0 was 54 times higher when the substrate concentration was raised from 0.4 to 28 m*M*. Spectral changes in experiments with 2,4,5-TMBA as substrate were not observed with *R. vernicifera* laccase.

ABTS and HBT were studied as mediators for *T. versicolor* laccase in the oxidation of MBAs. The concentrations of ABTS and HBT were 1 and 0.8 m*M*, respectively. The pH range studied was 4.0–5.2. All the six MBAs (Fig. 1) were oxidized by the laccase-ABTS system and TLC analysis showed that the products were the corresponding MBDs. The effect of HBT on the oxidation of 2,4,5-TMBA to 2,4,5-TMBD was also studied. The oxidation rate was 87-fold faster when HBT was present in a reaction mixture in which the concentration of 2,4,5-TMBA was 1 m*M*.

Based on the oxidation experiments with the MBAs (Table 2), 2,4,5-TMBA and 3,4-DMBA were chosen as substrates in further kinetic studies and the results from the determination of apparent kinetic constants (k_{cat}, K_m and k_{cat}/K_m) are collected in Table 3. It is notable that for both 2,4,5-TMBA and 3,4-DMBA, LP had a comparatively low K_m. With all the three enzymes, the K_m value for 2,4,5-TMBA was lower than for 3,4-DMBA.

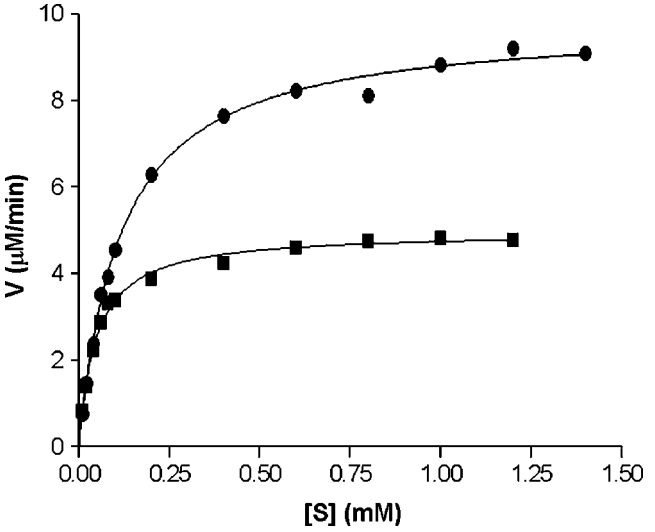


Fig. 3. LP-catalyzed oxidation of 2,4,5-TMBA (■) and 3,4-DMBA (●) at pH 4.

HRP and laccase had several 100-folds higher k_{cat} for 2,4,5-TMBA than for 3,4-DMBA. In contrast, LP had higher k_{cat} for 3,4-DMBA than for 2,4,5-TMBA. The clear difference in this regard is shown by the Michaelis-Menten plots for LP with 2,4,5-TMBA and 3,4-DMBA as substrates (Fig. 3).

The effect of pH on the initial oxidation rates of nonphenolic (Fig. 4A–C) and phenolic substrates (Fig. 4D–F) catalyzed by LP, HRP, and laccase was studied. With LP, an optimum at pH 3.0 was always observed for the initial reaction rates, regardless whether the substrate was nonphenolic (Fig. 4A) or phenolic (Fig. 4D).

With HRP, 3,4-DMBA, 2,5-DMBA, and 2,4,5-TMBA showed monotonic pH profiles; the rates decreased with increasing pH (Fig. 4B) (data for 2,5-DMBA are not shown as they are practically identical to those obtained with 3,4-DMBA). ABTS exhibited a bell-shaped pH profile in the pH range 2.2–7.0 and displayed an optimum at pH 4.0 (Fig. 4B). All phenolic substrates (Fig. 4E) showed pH profiles with optima in the range 6.0–7.0.

With *T. versicolor* laccase, the two nonphenolic substrates 2,4,5-TMBA and ABTS showed monotonic pH profiles; the rates decreased as the pH increased (Fig. 4C). With *R. vernicifera* laccase, ABTS showed an optimum at pH 3.0 (Fig. 4C). The three phenols exhibited bell-shaped pH profiles with an optimum at pH 4.0 (Fig. 4F).

Discussion

Enzymic oxidation of MBAs invariably resulted in formation of MBDs as the prevalent products. Even though LP, HRP, and *T. versicolor* laccase could catalyze the oxidation of all the different MBA congeners

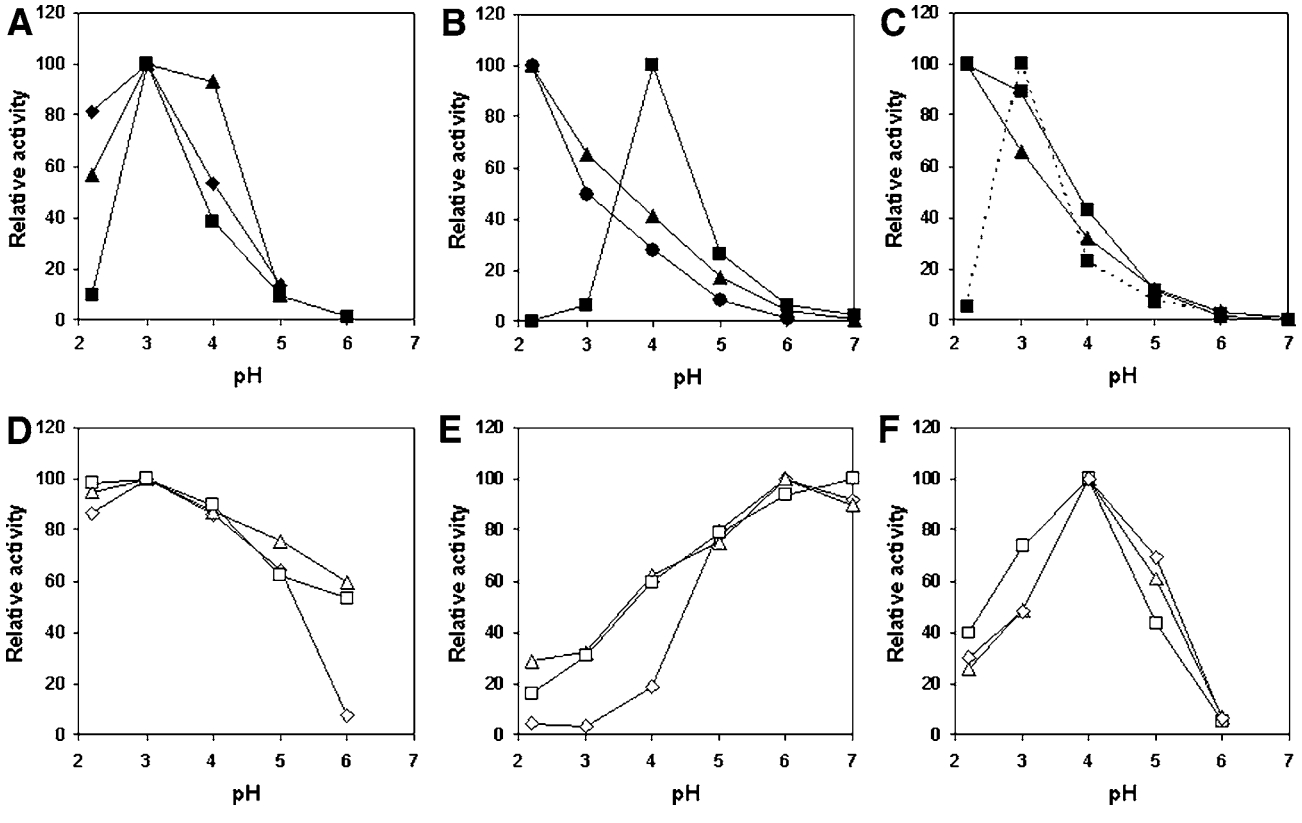


Fig. 4. (A–C) show the pH profiles for the relative initial rates of the oxidation of nonphenolic substrates catalyzed by LP/H_2O_2 **(A)**, HRP/H_2O_2 **(B)**, *T. versicolor* laccase/O_2 **(C**, solid line), and *R. vernicifera* laccase (**C**, dashed line). The substrates are 2,4,5-TMBA (▲), ABTS (■), 3,4,5-TMBA (◆), and 3,4-DMBA (●) **(D–F)** show the pH profiles for the relative initial rates of oxidation of phenolic substrates catalyzed by LP/H_2O_2 **(D)**, HRP/H_2O_2 **(E)**, and *T. versicolor* laccase/O_2 **(F)**. The substrates are catechol (Δ), hydroquinone (❑), and 1-(4-hydroxy-3,5-dimethoxyphenyl)-1-propanol (◇).

studied (Fig. 1), the rate of oxidation was very different (Table 2). For HRP and laccase, fastidious reaction conditions, such as low pH (3.0) and a relatively high concentration of substrate (10–28 m*M*), were required to obtain even very slow reactions with some of the MBAs.

The finding that a high-substrate concentration was required to obtain a detectable reaction rate for laccase and HRP may be explained in terms of high K_m values for these two enzymes compared with LP (Table 3). It may be possible to modify HRP and laccase by means of site-directed mutagenesis to obtain enzymes with lower K_m. Significant changes of the K_m and k_{cat} of laccases modified by site-directed mutations have been reported *(33)*.

HRP concurred with *T. versicolor* laccase in the respect that 2,4,5-TMBA was by far the best substrate, whereas LP catalyzed the oxidation of 3,4-DMBA with higher rate. The catalytic efficiency of LP, discussed in terms of k_{cat}/K_m values (Table 3), was of the same order of magnitude with both 2,4,5-TMBA and 3,4-DMBA as substrates. In contrast, the k_{cat} and the catalytic efficiency for HRP and fungal laccase were several order of magnitudes higher with 2,4,5-TMBA than with 3,4-DMBA as substrate. Thus, there seems to be a fundamental difference regarding oxidation of the MBAs between HRP and fungal laccase on one hand, and LP on the other hand.

In contrast to laccase from *T. versicolor*, equivalent or even twofold higher doses of *R. vernicifera* laccase did not catalyze product formation from 2,4,5-TMBA. This is in all probability related to the difference in redox potential between *R. vernicifera* (around 0.4 V) and *T. versicolor* laccase (around 0.8 V) *(1)*. Because MBAs are neither phenolic compounds nor diamines, they are not conventionally regarded as substrates for laccase. Laccases are normally used in combination with mediators in oxidization experiments with nonphenolic compounds *(5)*.

Kersten et al. *(3)* observed enzymic oxidation of several compounds in a set of methoxy-substituted benzene congeners with *Phanerochaete chrysosporium* LP. HRP and *T. versicolor* laccase were found to catalyze the oxidation of few (HRP) or only one (laccase) of the methoxy-substituted benzenes studied. The enzymic oxidation of the methoxy-substituted benzenes was performed at low pH (3.0) and with relatively low substrate concentration (0.2 m*M*). With 1,2,4,5-tetramethoxybenzene, which was found to serve as substrate for all three enzymes, the K_m for LP was found to be considerably lower than for HRP and laccase *(3)*, which is analogous to our results with 2,4,5-TMBA and 3,4-DMBA. That 1,2,4,5-tetramethoxybenzene served as substrate for all three enzymes was attributed to its comparatively low half-wave potential (0.81 V *[12]*) compared with, for example, methoxybenzene (1.76 V *[12]*), which was resistant to oxidation by all three enzymes *(3)*. The catalytic action of a set of fungal laccases *(4)* has also been discussed in terms of the redox potential displayed by the enzymes in relation to the redox potential determined for the reducing substrate.

Benzyl alcohol has been reported to possess a redox potential of 2.4 V, whereas the redox potential of MBAs was found to be lower, for example, 1.7 V for 4-methoxybenzyl alcohol *(34)*. The redox potential of three of the DMBAs used in this study has been determined *(35)*: 2,5-DMBA, 1.33 V (vs normal hydrogen electrode); 3,4-DMBA, 1.36 V; 2,3-DMBA, 1.39 V. The present study showed that the oxidation rate order of these DMBAs with *T. versicolor* laccase and HRP is the following one: 2,5-DMBA > 3,4-DMBA > 2,3-DMBA. However, for LP, 3,4-DMBA was the best substrate (Table 2). Increasing rates of the oxidation of DMBAs by HRP and laccase follow decreasing redox potential, whereas the oxidation experiments indicate a special relationship between LP and 3,4-DMBA (veratryl alcohol). This compound is a secondary metabolite of *P. chrysosporium (14)*. There are different hypotheses regarding the role of veratryl alcohol in lignin biodegradation *(36)*. Veratryl alcohol may protect LP from inactivation by hydrogen peroxide by prevention of the formation of the inactive form Compound III. Veratryl alcohol may serve as the reducing substrate for Compound II to complete the catalytic cycle back to the native form of the enzyme. Furthermore, veratryl alcohol may act as a one-electron redox mediator in lignin biodegradation. Interestingly, site-directed mutagenesis of LP has suggested the existence of an electron-transfer pathway between Trp171 and the heme group, and that Trp171 is important for the oxidation of veratryl alcohol but not all reducing substrates *(37)*. Site-directed mutagenesis of manganese peroxidase supported the role of Trp171 in the oxidation of veratryl alcohol by LP *(38)*. Multiple alignments of peroxidase sequences early on indicated that LPs specifically contained this conserved Trp, located near the proximal His in the F helix *(39)*. Thus, LP is known to possess a conserved amino-acid residue that may be responsible for the interaction with veratryl alcohol.

The oxidation of the MBAs was favored by low pH (2.0–3.0) for all the three enzymes. The oxidation of veratryl alcohol by LP has previously been shown to be favored by low pH *(21,37,40)*. However, LP will eventually become instable if the pH is too low. Regarding laccase, Bourbonnais and Paice *(5)* reported that veratryl alcohol was not oxidized at pH 5.0. In the present study, a pH of 3.0 rather than 5.2 or 6.0 was found to be required in order to observe any laccase-catalyzed oxidation of veratryl alcohol (3,4-DMBA). HRP showed an optimum at higher pH with ABTS than with 2,4,5-TMBA, 3,4-DMBA, or 2,5-DMBA as substrate (Fig. 4B), which suggests that the optimum for ABTS oxidation observed at pH 4.0 is not a consequence of enzyme instability at low pH (2.0–3.0).

The pH optima for LP-catalyzed oxidation of phenolic substrates did not differ from those of nonphenolic substrates, whereas the optima for HRP-catalyzed and *T. versicolor* laccase-catalyzed oxidation of phenolic substrates were observed at higher pH than those for nonphenolic substrates (Fig. 4). The pH profiles obtained with fungal laccase and phenolic and nonphenolic substrates were studied by Xu *(41)*, who proposed that

the pH activity profiles of fungal laccase was the result of two opposing effects, namely the redox potential difference among the reducing substrate and the T1 copper of laccase, which would be favored by higher pH for a phenolic substrate, and the binding of a hydroxide ion to the T2/T3 copper center of laccase, which would result in inhibition and decreased activity at higher pH. The changes in E^0 with pH of fungal laccases were found to be rather modest *(41)*. The contribution of hydroxide inhibition would consequently result in a monotonic pH activity profile for a nonphenolic substrate, as observed for *T. versicolor* laccase-catalyzed oxidation of ABTS and 2,4,5-TMBA (Fig. 4C).

The fact that the enzymes studied display different pH profiles for the same substrate, as observed for the oxidation of ABTS or phenolic substrates (Fig. 4), suggests that not only substrate-specific but also enzyme-specific factors need to be taken into account to explain the pH profiles. Indeed, site-specific mutagenesis of LP resulted in a novel form that displayed a shift in the pH/activity profile, at least regarding one substrate *(37)*. More research is required to elucidate the detailed mechanisms behind the pH profiles.

1-(4-Hydroxy-3,5-dimethoxyphenyl)-1-propanol was found to serve as a substrate for LP, HRP, and laccase (Fig. 4). In a series of experiments with HRP at different pH (4.5, 5.0, and 7.0), 1-(4-hydroxy-3,5-dimethoxyphenyl)-1-propanone was obtained in about 80% yield. HRP-catalyzed dehydrogenation of 1-(4-hydroxy-3,5-dimethoxyphenyl)-1-propanol has previously been reported to produce 1-(4-hydroxy-3,5-dimethoxyphenyl)-1-propanone in high yield *(26)*. Dehydrogenation of the structurally closely related compound 1-(4-hydroxy-3-methoxyphenyl)-1-propanol resulted in the formation of a dioxepin as a major product *(25)*.

It appears from the examples discussed earlier that the MBA congeners are suitable in studies of oxidation with different enzymes or combinations of enzymes and mediators. One reason for this is that the oxidation of the MBAs produces the corresponding MBDs as the sole predominating products. Second, the MBAs exhibit characteristic UV properties that make it possible to follow the progress of the oxidation reactions by UV measurements. Third, the set of MBAs selected represents different requirements as concerns oxidation potential and this makes it possible to do comparative studies of the capability of the different enzymes as oxidation catalysts. For example, 2,4,5-TMBA could be used to discriminate among laccases from different sources, such as *T. versicolor* and *R. vernicifera*. We think that the MBAs are useful as substrates not only in studies of enzyme properties but also in connection with the design of processes based on enzymic catalysis aiming at detoxification of waste effluents *(7)*, pulp bleaching *(42)*, and production of chemicals *(43)*. The comparisons of the different enzymes show that LP/H_2O_2 is an efficient catalyst as judged from the oxidation experiments with MBAs. However, in certain applications selectivity is of importance and in such cases HRP/H_2O_2 and laccase/O_2 may serve as suitable oxidants.

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