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Induction, Isolation, and Characterization of Two Laccases from the White Rot Basidiomycete *Coriolopsis rigida*

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Previous work has shown that the white rot fungus *Coriolopsis rigida* degraded wheat straw lignin and both the aliphatic and aromatic fractions of crude oil from contaminated soils. To better understand these processes, we studied the enzymatic composition of the ligninolytic system of this fungus. Since laccase was the sole ligninolytic enzyme found, we paid attention to the oxidative capabilities of this enzyme that would allow its participation in the mentioned degradative processes. We purified two laccase isoenzymes to electrophoretic homogeneity from copper-induced cultures. Both enzymes are monomeric proteins, with the same molecular mass (66 kDa), isoelectric point (3.9), N-linked carbohydrate content (9%), pH optima of 3.0 on 2,6-dimethoxyphenol (DMP) and 2.5 on 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), absorption spectrum, and N-terminal amino acid sequence. They oxidized 4-anisidine and numerous phenolic compounds, including methoxyphenols, hydroquinones, and lignin-derived aldehydes and acids. Phenol red, an unusual substrate of laccase due to its high redox potential, was also oxidized. The highest enzyme affinity and efficiency were obtained with ABTS and, among phenolic compounds, with 2,6-dimethoxyhydroquinone ($DBQH_2$). The presence of ABTS in the laccase reaction expanded the substrate range of *C. rigida* laccases to nonphenolic compounds and that of $MBQH_2$ extended the reactions catalyzed by these enzymes to the production of H_2O_2, the oxidation of Mn^{2+}, the reduction of Fe^{3+}, and the generation of hydroxyl radicals. These results confirm the participation of laccase in the production of oxygen free radicals, suggesting novel uses of this enzyme in degradative processes.

Lignin is an aromatic heteropolymer of phenyl-propanoid units which confers structural rigidity to woody plant tissues and protects them from microbial attack (25). To depolymerize and mineralize lignin, white rot fungi have developed a nonspecific oxidative system including several extracellular oxidoreductases, low-molecular-weight metabolites, and activated oxygen species (47). The ability of white rot fungi to degrade a wide number of organopollutants is in part due to the action of this nonspecific system (42). Extracellular enzymes involved in the degradation of lignin and xenobiotics by white rot fungi include several kinds of laccases (34, 49), peroxidases (8, 33), and oxidases producing H_2O_2 (20, 32, 50).

The enzymatic composition of the ligninolytic system depends on the fungal species, with laccase being the common component (24, 43). For this reason, a wide number of studies have focused on demonstrating the participation of laccase in significant ligninolytic events which were first attributed to other enzymes of the ligninolytic system. These events include the oxidation of nonphenolic lignin units, which comprise ca. 80% of the polymer, the generation of the H_2O_2 required for both peroxidase activities and hydroxyl radical ($^{\cdot}OH$) formation, and the production of Mn^{3+} from the Mn^{2+} present in lignocellulose. Mn^{3+} and $^{\cdot}OH$ are low-molecular-weight ligninolytic agents which are believed to play a key role in the initial attack of lignocellulose when the ligninolytic enzymes cannot penetrate through the plant cell walls (17, 30). Laccase catalyzes directly the oxidation of phenolic lignin units and a wide number of phenolic compounds and aromatic amines, with molecular oxygen as the electron acceptor, which is reduced to water (49). In the presence of certain white rot fungi metabolites or artificial substrates acting as mediators, the substrate range of laccase was extended to nonphenolic lignin units (4, 15). These laccase-mediator systems have been shown to degrade not only lignin but also several aromatic and aliphatic xenobiotics (29, 39). The participation of laccase in the production of reduced oxygen species, i.e., superoxide anion radical ($O_2^{\cdot -}$), H_2O_2, and $^{\cdot}OH$, has been demonstrated through the oxidation of lignin-derived hydroquinones (19, 22). The oxidation of Mn^{2+} by laccase has been described to occur both directly (26) and through the oxidation of hydroquinones as a consequence of $O_2^{\cdot -}$ generation (21, 38).

Coriolopsis rigida is a white rot fungus that has been studied with regard to its capacity to degrade lignin from wheat straw (9) and the aliphatic and aromatic fractions of crude oil from artificially contaminated soils (13). Except for the production of an ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)]-oxidizing activity by *C. rigida* in solid medium (46), which suggests the secretion of ligninolytic enzymes, the ligninolytic system of this fungus remains unknown. In this study we describe the purification and characterization of two laccase isoenzymes from *C. rigida* grown under liquid culture conditions. As is the case with the efficient lignin degrader *Pycnoporus cinnabarinus* (16), *C. rigida* produced laccase as the sole

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ligninolytic enzyme under the culture conditions used in this study. This characteristic makes *C. rigida* an interesting model for studying alternative mechanisms by which laccase degrades lignin and xenobiotics. We concentrate our attention here on the oxidative capabilities of laccase, including the production of strong oxidants which would be implicated in these degradative processes.

MATERIALS AND METHODS

Chemicals. The compounds used as laccase substrates, 2-deoxyribose, 1,10-phenanthroline, 2-thiobarbituric acid (TBA), superoxide dismutase (SOD), catalase, and horseradish peroxidase, were purchased from Sigma-Aldrich. 2,6-Dimethoxy-1,4-benzohydroquinone ($DBQH_2$) was prepared by reducing the corresponding quinone with sodium borohydride (1). Stock solutions of hydroquinones were prepared in nitrogen-saturated deionized water acidified with 2 mM HCl and kept frozen at −80°C until use. H_2O_2 (Perhydrol, 30%) and endo-β-*N*-acetylglucosaminidase were obtained from Merck and Boehringer, respectively. All other chemicals used were of analytical grade.

Fungal strain and culture conditions. *C. rigida* CLPS 232 (Spegazzini Institute Culture Collection) was isolated from decaying wood collected in a subtropical Argentine rain forest (28). This strain has been deposited in the Spanish Type Culture Collection (CECT 20449). Stock cultures were kept at 4°C on 2% (wt/vol) malt extract-agar slants supplemented with yeast extract (0.4%) and *Populus* wood chips. The production of extracellular ligninolytic enzymes was carried out in a C-limited–yeast extract medium (20) supplemented with 5 g of peptone $liter^{-1}$. The addition of 150 μM $CuSO_4$ on the third day of incubation was assayed as an inducer of laccase activity (14). Homogenized pellets from 7-day-old shaken cultures were used to inoculate 1,000-ml Erlenmeyer flasks containing 200 ml of medium (3.5 mg ml^{-1}). The flasks were incubated at 28°C in a rotary shaker at 160 rpm. Samples were taken periodically from four replicate flasks, and the mycelium was separated from the culture liquid by centrifugation at 20,000 × *g* and 4°C during 10 min.

Analysis of protein, reducing sugars, and enzymatic assays. Extracellular protein was determined by Bradford method by using Bio-Rad protein assay (product number 500-0006) and bovine serum albumin as the standard. Reducing sugars were assayed by Somogyi and Nelson method, with glucose as the standard (48). Unless otherwise stated, laccase activity was measured by using 5 mM 2,6-dimethoxyphenol (DMP) in 100 mM sodium tartrate buffer (pH 3.0; ε_{469} = 27,500 M^{-1} cm^{-1}, referred to as the DMP concentration). Peroxidase activity was assayed as laccase activity in the presence of 0.1 mM H_2O_2. Aryl-alcohol oxidase activity was estimated by 3,4-dimethoxybenzaldehyde (veratraldehyde) formation (ε_{310} = 9,300 M^{-1} cm^{-1}) from 5 mM veratryl alcohol in 100 mM phosphate buffer (pH 6). Lignin peroxidase activity was determined by the H_2O_2-dependent veratraldehyde formation from 2 mM veratryl alcohol in 100 mM sodium tartrate buffer (pH 3), and the reactions were started by the addition of 0.4 mM H_2O_2. Manganese peroxidase activity was estimated by measuring the formation of Mn^{+3}-tartrate complex (ε_{238} = 6,500 M^{-1} cm^{-1}) during the oxidation of 0.1 mM Mn^{2+}($MnSO_4$) in 100 mM sodium tartrate buffer (pH 5) in the presence of 0.1 mM H_2O_2. All of the oxidation rates were determined at room temperature (22 to 25°C). International enzymatic units (in micromoles per minute) were used.

Laccase purification. Preliminary characterization of laccase from crude enzyme preparation obtained by culture liquid ultrafiltration (see below) was carried out to obtain enough information to design the purification process. The pH stability of laccase activity was studied in 100 mM borate-citrate-phosphate buffer (pH 2 to 12) at room temperature. The isoelectric point of the laccases was determined by zymograms on 5% polyacrylamide gels with a thickness of 1 mm by using a pH range from 3 to 10 (Bio-Rad Ampholine). The anode and cathode solutions were 1 M phosphoric acid and 1 M sodium hydroxide, respectively. The pH gradient formed was measured on the gel by means of a contact electrode. Protein bands with laccase activity were detected by using 5 mM DMP in 100 mM sodium tartrate buffer (pH 3) after the gels were washed for 20 min with the same buffer.

C. rigida laccase was purified from 15-day-old cultures containing $CuSO_4$. The culture liquid, separated from the mycelia by centrifugation at 20,000 × *g*, was 7.5-fold concentrated and dialyzed against 10 mM sodium acetate (pH 4.5) by ultrafiltration (Filtron; 5-kDa cutoff membrane). This crude enzyme preparation was applied to a Bio-Rad Q-Cartridge equilibrated with the same buffer at a flow rate of 1 ml min^{-1}. Retained proteins were eluted for 50 min with a NaCl gradient from 0 to 350 mM. Fractions of 2 ml were collected in tubes containing 0.15 ml of 500 mM phosphate buffer (pH 7). Fractions with the laccase activity were pooled and concentrated (Filtron Microsep; 3-kDa cutoff), and samples of 0.2 ml were applied to a Superdex 75 (Pharmacia HR 10/30) column equilibrated with 200 mM phosphate buffer (pH 7) at a flow rate of 0.4 ml min^{-1}. The laccase peak was pooled, concentrated (Filtron Microsep, 3-kDa cutoff), and dialyzed against 10 mM sodium acetate (pH 4.5), and 1-ml samples were applied to a Mono-Q anion-exchange column (Pharmacia HR 5/5) equilibrated with the same buffer containing 20 mM NaCl. Both laccase isoenzymes were eluted with a linear NaCl gradient from 20 to 100 mM for 55 min at a flow rate of 0.8 ml min^{-1}. Laccase peaks were collected, concentrated, and stored at −80°C.

Properties of purified laccases. The molecular mass of the laccase isoenzymes was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration. SDS-PAGE was performed with 7.5% polyacrylamide gels by using low-molecular-mass standards (Bio-Rad). Gel filtration was carried out on Superdex 75 as described above. The column was calibrated with aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), and RNase A (13.7 kDa). The N-carbohydrate content of purified laccases was derived from the difference in molecular mass (estimated by SDS-PAGE) found before and after treatment of laccase isoenzymes with endo-β-*N*-acetylglucosaminidase. Isoelectric focusing was performed as described above but with a 2.5 to 5.5 pH range, which was achieved by mixing 85 and 15% Bio-Rad Ampholines from pH 2.5 to 5 and from pH 3.5 to 10, respectively. Protein bands after SDS-PAGE and isoelectric focusing were stained with Coomassie blue R-250. N-terminal sequences of laccase isoenzymes were determined by automated Edman degradation of 5 μg of protein in an Applied Biosystem protein sequencer (Procise 494; Perkin-Elmer). The UV-visible spectra of native isoenzymes were recorded in 5 mM sodium phosphate (pH 7.0).

Substrate specificity. We tested 23 compounds as substrates of the *C. rigida* laccases. This was qualitatively explored by changes in the optical absorbance spectra of the reaction mixtures which contained 400 μM potential substrate, 50 mU of purified isoenzymes ml^{-1}, and 100 mM sodium tartrate buffer (pH 5). The kinetic constants of laccase isoenzymes were calculated for DMP, ABTS, 2-methoxy-1,4-benzohydroquinone ($MBQH_2$), and $DBQH_2$. The production of the ABTS cation radical, MBQ, and DBQ were estimated at 436 nm (ε = 29,300 M^{-1} cm^{-1}), 360 nm (ε = 1,252 M^{-1} cm^{-1}), and 397 nm (ε = 562 M^{-1} cm^{-1}), respectively. These reactions were performed in 0.1 M tartrate buffer (pH 3).

Transformation of nonphenolic compounds by *C. rigida* laccases. The reactions were carried out in 100 mM tartrate buffer (pH 5) and contained 150 μM concentrations of either 3,4-dimethoxyphenylacetic (homoveratric) acid (HVA) or veratryl alcohol (as nonphenolic lignin model compounds), 150 μM ABTS (as laccase mediator), and 25 mU of laccase ml^{-1} (estimated with 5 mM ABTS in the same buffer). Samples were taken periodically, the pH was lowered to 2 with 7.4 M H_3PO_4, and the samples were frozen (−20°C) until analysis. Quantitative determination of HVA, veratryl alcohol, and veratraldehyde was performed by high-pressure liquid chromatography with standard calibration curves for each compound. Reaction samples (20 μl) were injected into a Spherisorb S5ODS2 C_{18} column (Hichrom) heated at 40°C, with 10 mM phosphoric acid-methanol (70/30) as a mobile phase and a 1-ml min^{-1} flow rate. The UV detector was operated at 280 nm. The reaction blanks contained heat-denatured laccase.

Oxygen activation and Fe^{3+} reduction. Oxygen activation during the oxidation of 100 μM $MBQH_2$ by 25 mU of purified laccase isoenzymes ml^{-1} (estimated with 500 μM $MBQH_2$ in 20 mM phosphate buffer [pH 5]) was evaluated after full hydroquinone oxidation (which was followed at 360 nm) as both H_2O_2 and $^{\cdot}OH$ production. H_2O_2 was estimated by the oxidation of phenol red by horseradish peroxidase (20). Before the addition of phenol red, samples were heated at 90°C for 20 min to inactivate laccase (a treatment that does not change the H_2O_2 concentration). Enzyme inactivation was required because phenol red was oxidized by both laccase isoenzymes (see below). $^{\cdot}OH$ production was estimated as the formation of TBA-reactive substances (TBARS). In this case, $MBQH_2$ was oxidized by laccase in the presence of 200 to 220 μM Fe^{3+}-EDTA (freshly prepared) and 2.8 mM deoxyribose. TBARS were determined at the end of laccase reaction as described by Gutteridge (23). The composition of the laccase reaction for Fe^{3+} reduction studies was the same as that used for $^{\cdot}OH$ production, except that deoxyribose was replaced by 1.5 mM 1,10-phenanthroline. Formation of Fe^{2+}-phenanthroline chelate was determined spectrophotometrically (ε_{510} = 12,110 M^{-1} cm^{-1}) (2). The reaction blanks for H_2O_2, TBARS, and Fe^{2+}-phenanthroline determinations contained heat-denatured laccase.

RESULTS

Production of ligninolytic enzymes. We monitored extracellular laccase activity, protein, and reducing sugars in *C. rigida* cultures for 30 days (Fig. 1). The presence of Cu^{2+} in the

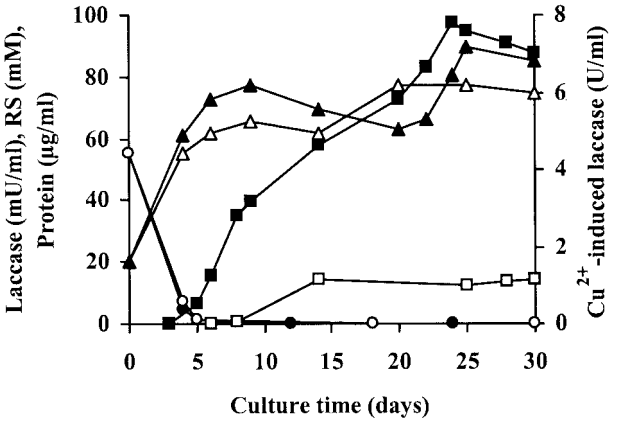


FIG. 1. Time course of laccase activity (□), protein (△), and reducing sugars (RS) (○) in the extracellular fluid of *C. rigida* cultures grown in the absence and presence of copper (open and closed symbols, respectively). The results shown are from one experiment typical of four.

culture medium did not affect the rate of glucose consumption. The pH of the culture medium (5.8) decreased slightly during vegetative growth in the presence or absence of Cu^{2+}. After glucose depletion the pH increased and remained close to neutral until the end of the experiments (data not shown). Laccase activity appeared earlier and was much higher in the presence of Cu^{2+} (ca. 500-fold). H_2O_2 did not alter the oxidation of DMP, indicating the lack of peroxidase activity. Manganese peroxidase, lignin peroxidase, and aryl-alcohol oxidase were not detected either. Zymograms of laccase after isoelectric focusing of crude enzyme preparations obtained from cultures carried out in the absence or presence of Cu^{2+} resulted in a single band with a pI of 3.9. The activity band from Cu^{2+}-induced cultures corresponded to the major protein band, seen after staining the gel with Coomassie blue R-250 (data not shown). The optimal pH of laccase activity in crude enzyme preparation from Cu^{2+}-induced cultures was 3. Laccase was stable from pH 6 to 10 at room temperature for 24 h, retaining 50 and 40% activity at pH 5 and 4, respectively.

Purification of laccase. We purified two proteins with laccase activity from Cu^{2+}-induced cultures (Table 1). During the first chromatographic step (Q-Cartridge) the laccase activity was separated from the most plentiful impurities, which include a brown pigment absorbing strongly at 280 nm (Fig. 2A). During Superdex 75 chromatography, laccase activity was detected as a symmetrical peak (Fig. 2B) which still contained several contaminating proteins as revealed by electrophoresis

TABLE 1. Purification of laccase isoenzymes from *C. rigida*

Purification step	Protein (mg liter^{-1})	Activity (U liter^{-1})	Sp act (U mg^{-1})	Yield (%)	Purification factor (fold)
Culture liquid	118	5,200	44	100	1.0
Ultrafiltration	105	5,100	49	98	1.1
Q-Cartridge	61	4,800	79	92	1.8
Superdex 75	39	3,900	100	75	2.3
Mono-Q (LacI)	7.5	810	108	16	2.5
Mono-Q (LacII)	11.6	1,300	112	25	2.5
Mono-Q (Total)	19.1	2,110	110	41	2.5

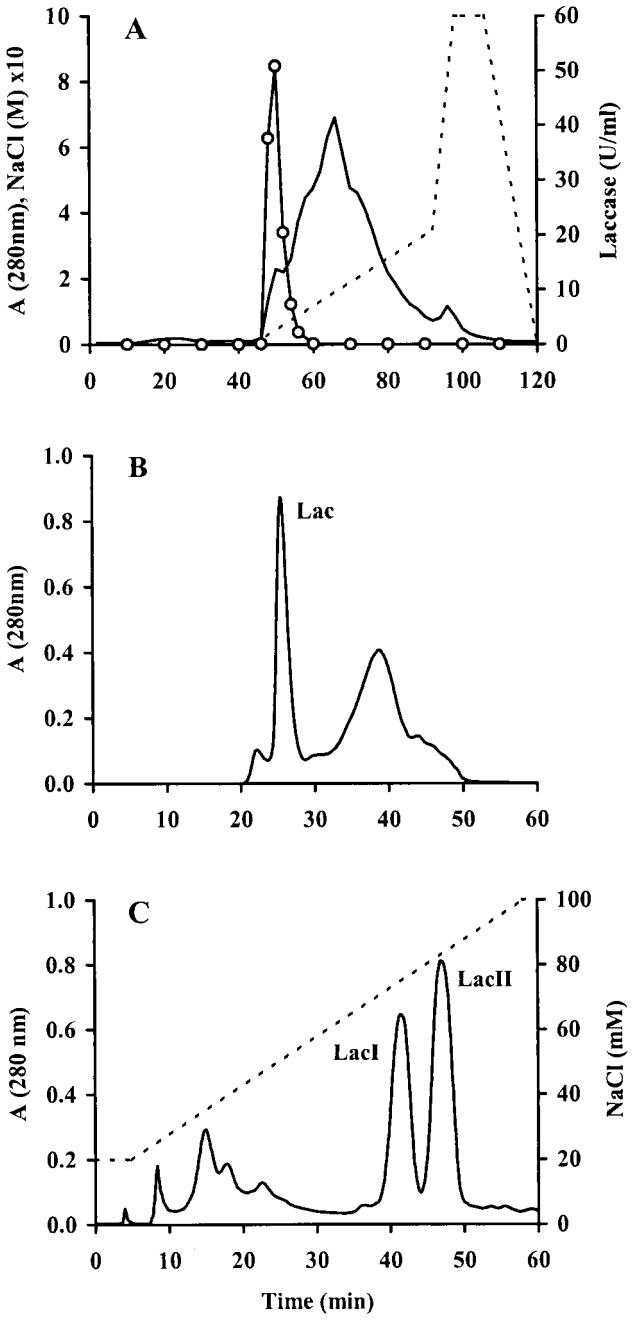
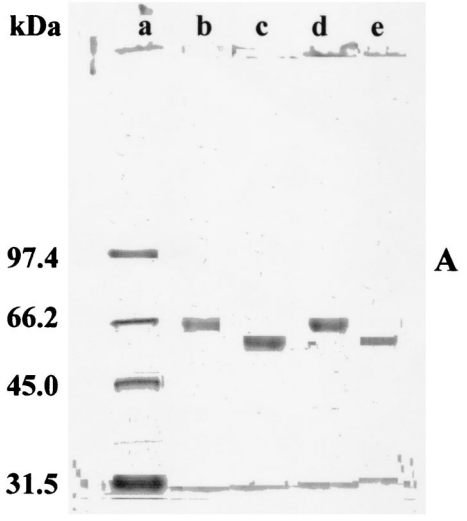


FIG. 2. Purification of copper-induced laccases from *C. rigida* by chromatography on a Q-Cartridge (A), Superdex 75 (B), and Mono-Q (C). Absorbance at 280 nm (solid line), the NaCl gradient (dashed line), and the laccase activity (○) are as indicated.

(data not shown). Chromatography through the Mono-Q column resolved two laccase activity peaks: LacI and LacII (Fig. 2C). At the end of the process, LacI and LacII had been purified 2.5-fold with yields of 16 and 25%, respectively (Table 1).

Properties of laccase. The molecular masses of LacI and LacII were 55 kDa as estimated by gel filtration chromatography and 66 kDa as determined by SDS-PAGE (Fig. 3A), indi-



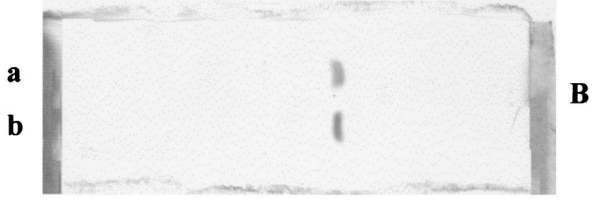


FIG. 3. Estimation of the molecular mass and pI of laccase isoenzymes from *C. rigida*. (A), SDS-PAGE of LacI (lane b), deglycosylated LacI (lane c), LacII (lane d), deglycosylated Lac II (lane e), and low-molecular-mass Bio-Rad standards (lane a). (B), Isoelectric focusing of LacI (lane a) and LacII (lane b).

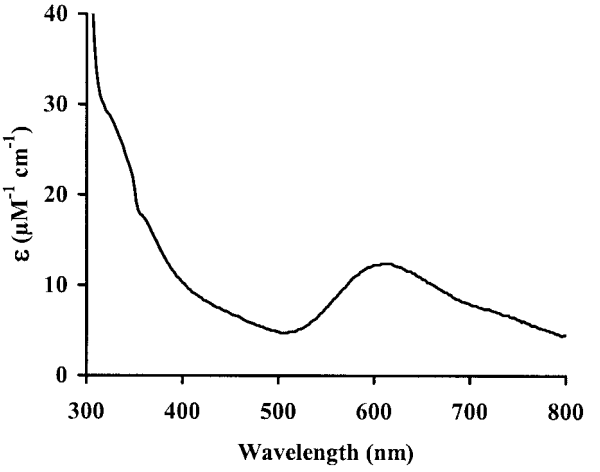


FIG. 4. Absorption spectrum of LacI and LacII from *C. rigida*. The protein concentration was 7.58 nM (considering a molecular mass of 66 kDa).

cating that these are both monomeric proteins. Based on the molecular mass lost after deglycosylation, we concluded that LacI and LacII are glycoproteins containing 9% N-linked carbohydrate. Analytical isoelectric focusing showed a pI of 3.9 for both enzymes (Fig. 3B). The N-terminal amino acid sequences of LacI and LacII were identical: AIGPKADMTIT DGAVSPDGFERQAI. The UV-visible spectra of LacI and LacII had typical characteristics of copper-containing enzymes (49), including a shoulder at 320 nm (type III binuclear copper) and a peak at 614 nm (type I blue copper atom) (Fig. 4). The optimum pH values of both enzymes, estimated in 100 mM tartrate buffer, were 2.5 and 3 for the oxidations of ABTS and DMP, respectively.

Substrate specificity. The substrate specificity of *C. rigida* laccases was qualitatively studied by changes in the absorption spectra of reaction mixtures (Table 2). Both laccase enzymes had a wide substrate specificity, oxidizing several methoxyphenols, catechol, nonsubstituted and methoxy-substituted *p*-hydroquinones, phenolic aldehydes and acids (some of them being lignin depolymerization products), an aromatic amine, ABTS, and phenol red. No activity was observed on tyrosine, phenol, cloro- and nitro-substituted phenols, and the nonphenolic compounds HVA and veratryl alcohol. The highest enzyme affinity and efficiency of the oxidation reaction were obtained with ABTS and, among phenolic compounds, with $DBQH_2$ (Table 3).

Transformation of nonphenolic compounds with the laccase-ABTS system. HVA was transformed into veratryl alcohol and veratraldehyde by both laccases in reactions containing ABTS (Fig. 5), with similar results obtained with both enzymes. No HVA was present in the reaction mixture after 24 h, and veratryl alcohol was the major product (Fig. 5A). The concentration of veratryl alcohol then slowly decreased with a corresponding increase in veratraldehyde. The laccase-ABTS system also could oxidize veratryl alcohol (Fig. 5B).

Production of activated oxygen species with the laccase-

TABLE 2. Substrate specificity of *C. rigida* laccases

Compound	Wavelength[a] (nm)
Tyrosine	NC
Phenol	NC
2-Methoxyphenol (guaiacol)	464
3-Methoxyphenol	NC
4-Methoxyphenol	253
DMP	469
4-Nitrophenol	NC
4-Chlorophenol	NC
Pentachlorophenol	NC
1,2-Benzenediol (catechol)	396
1,4-Benzohydroquinone	248
$MBQH_2$	360
$DBQH_2$	392
4-Hydroxy-3-methoxybenzaldehyde (vanillin)	230
4-Hydroxy-3,5-dimethoxybenzaldehyde (syringaldehyde)	370
4-Hydroxy-3-methoxybenzoic (vanillic) acid	248
4-Hydroxy-3,5-dimethoxybenzoic (syringic) acid	296
3,4-Dihydroxybenzoic (protocatechuic) acid	308
HVA	NC
3,4-Dimethoxybenzyl (veratryl) alcohol	NC
4-Methoxyaniline (anisidine)	500
ABTS	436
Phenol red	610[b]

[a] New absorption maxima observed after appropriate incubation periods. NC, no changes in the reaction spectrum.

[b] Spectrum recorded after the addition of NaOH (0.2 M final concentration).

TABLE 3. Kinetic constants of laccase isoenzymes from *C. rigida*[a]

Substrate	K_m (μM)		k_{cat} (s^{-1})		k_{cat}/K_m (s^{-1} M^{-1})	
	LacI	LacII	LacI	LacII	LacI	LacII
DMP	328	348	106	94	3.2×10^5	2.7×10^5
ABTS	12	11	77	78	64.1×10^5	71.3×10^5
$MBQH_2$	216	229	127	105	5.9×10^5	4.6×10^5
$DBQH_2$	89	107	187	143	21.0×10^5	13.4×10^5

[a] Laccase isoenzymes with a molecular mass of 66 kDa were used for the calculation of the k_{cat}.

$MBQH_2$ system. The oxidation of lignin-derived hydroquinones by laccase from *Pleurotus eryngii* leads to the accumulation in the reaction mixture of H_2O_2 (22) and, in the presence of Fe^{3+}-EDTA, to the reduction of Fe^{3+} and the production of $^{\cdot}OH$ (19). These findings were tested with both laccases from *C. rigida* during the oxidation of $MBQH_2$. To verify that semiquinone radicals produced by laccase from $MBQH_2$ (4 $MBQH_2 + O_2 \rightarrow 4\ MBQ^{\cdot -} + 2\ H_2O$) reduced O_2 to $O_2^{\cdot -}$($MBQ^{\cdot -} + O_2 \leftrightarrow MBQ + O_2^{\cdot -}$), we measured the effect of SOD and Mn^{2+} on quinone production rate. We found that the catalysis of $O_2^{\cdot -}$ dismutation by SOD ($O_2^{\cdot -} + 2\ H^+ \rightarrow O_2 + H_2O_2$) and the reduction of $O_2^{\cdot -}$ by Mn^{2+} ($O_2^{\cdot -} + Mn^{2+} + 2\ H^+ \leftrightarrow H_2O_2 + Mn^{3+}$) enhanced quinone production rate 1.4 and 2.9 times, respectively (Fig. 6A). The concentration of H_2O_2 after full oxidation of $MBQH_2$ in the absence of any factor promoting semiquinone autoxidation was 8.3 μM (Fig. 6B, control experiment). In the presence of SOD and Mn^{2+},

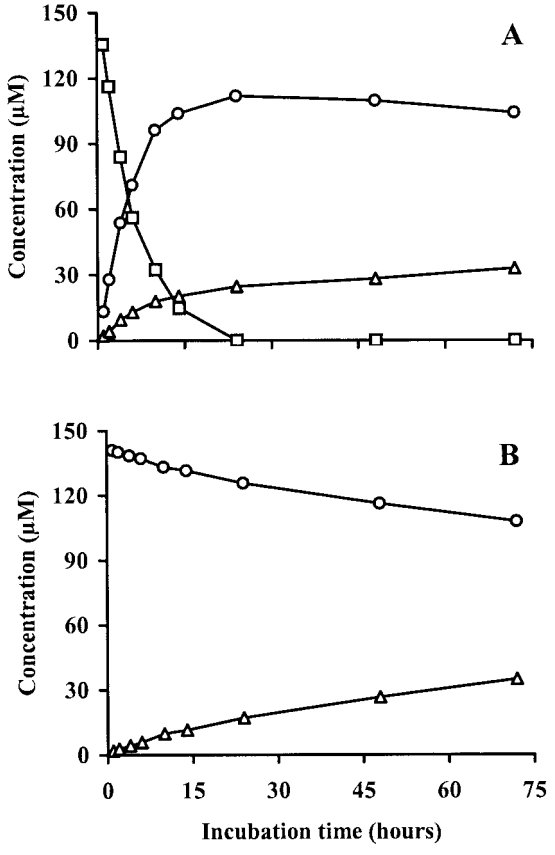


FIG. 5. Transformation of HVA (A) (□) and veratryl alcohol (B) (○) into veratraldehyde (△) by the laccase-ABTS system. The results shown correspond to LacI experiments (no significant differences were observed with LacII) and are the means of three replicates (standard deviations were <10% of the value of the point with which they were associated).

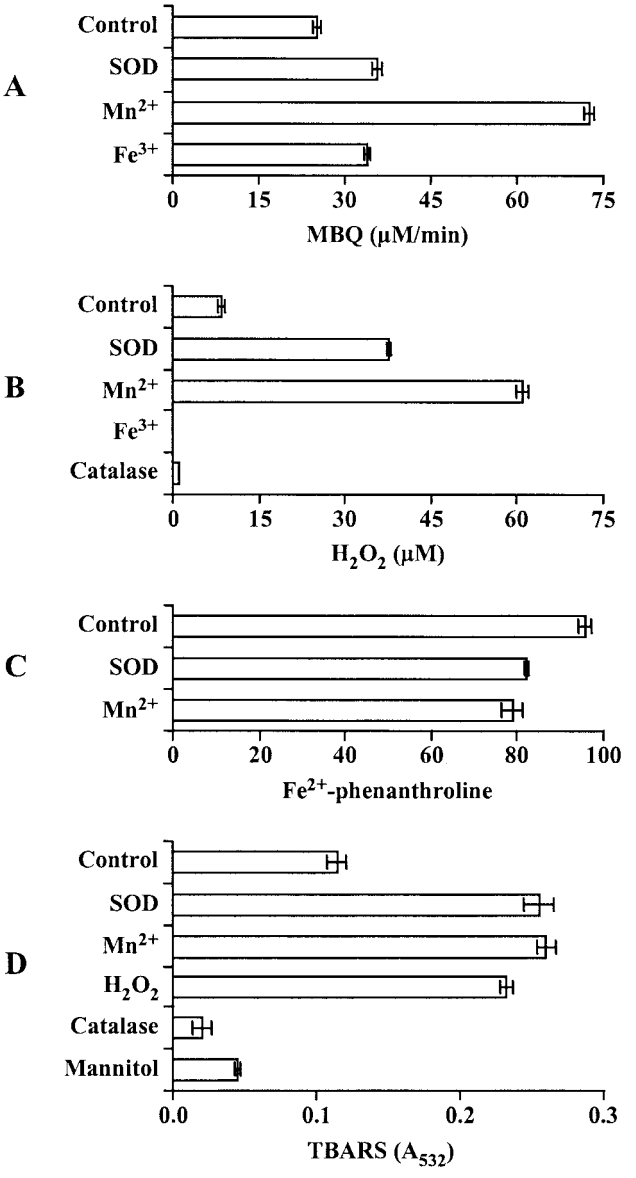


FIG. 6. Effect of different metal ions and activated oxygen species scavengers on the production rate of quinone (A), H_2O_2 production (B), Fe^{3+} reduction (C), and TBARS generation (D) during the oxidation of $MBQH_2$ by LacI and LacII. Except for quinone production rate determination experiments (A), which were performed with 500 μM $MBQH_2$, all of the reactions contained 100 μM $MBQH_2$ and 25 mU of laccase ml^{-1} (measured with 500 μM $MBQH_2$). In addition, in panel C the reaction mixtures contained Fe^{3+}-EDTA and 1,10-phenanthroline, and in panel D the reaction mixtures contained Fe^{3+}-EDTA and 2-deoxyribose. Where indicated, 100 U of SOD ml^{-1}, 100 μM Mn^{2+}, 100 to 110 μM Fe^{3+}-EDTA, 100 U of catalase ml^{-1}, 20 μM H_2O_2, and 5 mM mannitol also were present in the reactions. The results shown are those obtained with LacI, the differences found with LacII not being significant. The error bars represent ±95% confidence limits.

H_2O_2 levels increased 4.5 and 7.3 times, respectively. H_2O_2 production was confirmed with catalase, which decreased H_2O_2 concentration to negligible values.

To produce $^{\cdot}OH$ via Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$), Fe^{3+} reduction was first investigated. Between $O_2^{\cdot -}$ and semiquinone radicals, the latter are the main agents reducing Fe^{3+} ($MBQ^{\cdot -} + Fe^{3+} \rightarrow MBQ + Fe^{2+}$) during the oxidation of $MBQH_2$ by *P. eryngii* laccase (19). Fe^{3+}-EDTA increased the quinone production rate (Fig. 6A), suggesting that the reaction of Fe^{3+}-EDTA with semiquinone radicals was taking place. The production of Fe^{2+}-phenanthroline complex confirmed the existence of this reaction (Fig. 6C). Promotion of semiquinone autoxidation by SOD and Mn^{2+} decreased Fe^{2+}-phenanthroline levels 14 and 18%, respectively. Finally, production of TBARS from deoxyribose, evidencing $^{\cdot}OH$ generation, was demonstrated (Fig. 6D). The presence of SOD and Mn^{2+} increasing H_2O_2 levels (Fig. 6B) exerted a positive effect on TBARS production (2.3-fold increase). Similar results were obtained with exogenous H_2O_2 (twofold increase). In contrast, catalase and the $^{\cdot}OH$ scavenger mannitol exerted the opposite effect (5.4- and 2.5-fold decreases, respectively).

DISCUSSION

We characterized the enzymatic composition of the ligninolytic system of the white rot fungus *C. rigida*. This fungus secreted only laccase, even though peptone, which has been shown to induce peroxidases in *Bjerkandera* and *Pleurotus* species (31, 36), was a component of the culture medium. The production of laccase by white rot fungi is widespread and is the sole ligninolytic enzyme produced by some, e.g., the basidiomycete PM1 isolated from the wastewater of a paper mill (10), *P. cinnabarinus* (16), *Pycnoporus sanguineus* (45), and *Coriolopsis gallica* (7). White rot fungi may produce a number of laccase isoenzymes, some constitutively and others after induction (3, 37). Copper induces laccase at the level of gene transcription in *Trametes versicolor* (11), *Phanerochaete chrysosporium* (14), and *Pleurotus ostreatus* (40), and we found a similar effect in *C. rigida* (Fig. 1). This activity was due to two enzymes with the same pI, molecular mass, N-linked carbohydrate content, N-terminal sequence, absorption spectrum, and optimum pH. Only slight differences were found in their kinetic constants. Since the *C. rigida* CLPS 232 strain that we used is dikaryotic (28), additional work is required to determine whether these two isoenzymes are allelic variants of the same gene, derive from different posttranslational modifications of the same protein, or result from two separate genetic loci.

Compared with other fungal laccases, the characteristics of purified *C. rigida* laccases are typical. Most laccases are monomeric glycoproteins showing a molecular mass of between 50 and 80 kDa (49, 52). The pI of *C. rigida* laccases (pH 3.9) are in the acidic range reported for laccases from other white rot fungi, including *P. eryngii* and *P. ostreatus* (pI 2.9 to 4.7) (38, 41) and *Pycnoporus cinnabarinus* (pI 3.7) (16). The N-terminal sequence of *C. rigida* laccases shows identities of >70% with those from *Trametes trogii* laccase (80%) (GenBank accession number AJ294820), basidiomycete PM1 (76%) (10), *T. versicolor* laccase II (72%) (6), *Trametes villosa* laccase 1 (72%) (53), and *Pycnoporus cinnabarinus* (71%) (16), whereas it is quite different from those of other white rot fungi such as *Pleurotus eryngii* lacase II (31%) (38) and *Agaricus bisporus* Lcc1 and Lcc2 (28 and 24%, respectively) (44). As expected for laccase-like enzymes, both *C. rigida* isoenzymes have no activity toward tyrosine and have a wide substrate specificity oxidizing several hydroxy- and methoxy-substituted phenols and aromatic amines. One unusual characteristic of these laccases was their ability to oxidize phenol red, a compound that is not oxidized usually by laccases because of its comparatively high redox potential. According to Eggert et al. (16), the absence of peroxidases in white rot fungi could select for laccases with higher redox potential.

The participation of *C. rigida* laccases in the oxidation of nonphenolic compounds, the production of partially reduced oxygen species, and the oxidation of Mn^{2+} all required the presence of adequate laccase substrates. To study the oxidation of the nonphenolic lignin model compounds HVA and veratryl alcohol, we used ABTS as a laccase mediator (4). Our results (Fig. 5) are similar to those reported for *P. eryngii* laccase and show that the laccase-ABTS pair catalyzed first the Cα-Cβ cleavage of HVA and then the conversion of veratryl alcohol into veratraldehyde (35). The laccase-ABTS pair is an efficient system for the demethylation and delignification of Kraft pulp (5), the transformation of polycyclic aromatic hydrocarbons (12), and the decolorization of synthetic dyes (51).

We showed the reduction of O_2 by *C. rigida* laccases giving rise to H_2O_2 and $^{\cdot}OH$ production by using $MBQH_2$ as substrate (Fig. 6). These results confirm the involvement of laccase in oxygen activation, which has been described only in studies of the *P. eryngii* laccase (19, 22). In this setting, the semiquinone radicals produced by laccase act as reducing agents of both O_2 and Fe^{3+}. Once $O_2^{\cdot -}$ and Fe^{2+} are formed, stepwise reduction of the former by either $O_2^{\cdot -}$ (dismutation reaction) or Mn^{2+} and then by Fe^{2+} generates H_2O_2 and subsequently $^{\cdot}OH$. We infer the oxidation of Mn^{2+} by laccase-$MBQH_2$ system from the increases in quinone production rate and H_2O_2 levels in the presence of Mn^{2+} (Fig. 6A and B, respectively). The reduction of Fe^{3+} and the oxidation of Mn^{2+} by low-molecular-weight agents, such as semiquinone and $O_2^{\cdot -}$ radicals, is important in the early stages of lignocellulose degradation, since the enzymes directly catalyzing these reactions (cellobiose dehydrogenase and peroxidases, respectively) cannot access these metals in lignocellulose due to steric hindrance (18). The production of $^{\cdot}OH$ by laccase suggests novel uses for this enzyme and also for white rot fungi for the degradation of organopollutants. Hydroxyl radicals are an important component of many oxidation processes that mineralize various toxic organic compounds (27).

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