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Laccase Isoenzymes of *Pleurotus eryngii*: Characterization, Catalytic Properties, and Participation in Activation of Molecular Oxygen and Mn^{2+} Oxidation

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Two laccase isoenzymes produced by *Pleurotus eryngii* were purified to electrophoretic homogeneity (42- and 43-fold) with an overall yield of 56.3%. Laccases I and II from this fungus are monomeric glycoproteins with 7 and 1% carbohydrate content, molecular masses (by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) of 65 and 61 kDa, and pIs of 4.1 and 4.2, respectively. The highest rate of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) oxidation for laccase I was reached at 65°C and pH 4, and that for laccase II was reached at 55°C and pH 3.5. Both isoenzymes are stable at high pH, retaining 60 to 70% activity after 24 h from pH 8 to 12. Their amino acid compositions and N-terminal sequences were determined, the latter strongly differing from those of laccases of other basidiomycetes. Antibodies against laccase I reacted with laccase II, as well as with laccases from *Pleurotus ostreatus*, *Pleurotus pulmonarius*, and *Pleurotus floridanus*. Different hydroxy- and methoxy-substituted phenols and aromatic amines were oxidized by the two laccase isoenzymes from *P. eryngii*, and the influence of the nature, number, and disposition of aromatic-ring substituents on kinetic constants is discussed. Although both isoenzymes presented similar substrate affinities, the maximum rates of reactions catalyzed by laccase I were higher than those of laccase II. In reactions with hydroquinones, semiquinones produced by laccase isoenzymes were in part converted into quinones via autoxidation. The superoxide anion radical produced in the latter reaction dismutated, producing hydrogen peroxide. In the presence of manganous ion, the superoxide anion was reduced to hydrogen peroxide with the concomitant production of manganic ion. These results confirmed that laccase in the presence of hydroquinones can participate in the production of both reduced oxygen species and manganic ions.

Laccases (benzenediol:oxygen oxidoreductases [EC 1.10.3.2]) are copper-containing enzymes catalyzing the oxidation of a broad number of phenolic compounds and aromatic amines by using molecular oxygen as the electron acceptor, which is reduced to water (44). These enzymes were identified for the first time in *Rhus vernicifera*, the Japanese lacquer tree, and they are widely distributed among plants and fungi (26, 36). Fungal laccases, in addition to being related to different physiological processes (50), are involved in lignin degradation together with lignin peroxidase (LiP) and manganese peroxidase (MnP) (27). Although the production of laccase is a common characteristic of white rot fungi, the most efficient lignin-degrading organisms, ligninolytic peroxidases have been the subject of much more study. This is due to the fact that the most extensively studied white rot fungus, *Phanerochaete chrysosporium*, does not produce detectable levels of laccase under the culture conditions generally used (51). However, the simultaneous production of laccase, LiP, and MnP by *P. chrysosporium* has been recently reported with cellulose as the carbon source (49).

Based on the patterns of ligninolytic enzymes, white rot fungi could be divided in two groups, those producing only laccase and MnP and those producing in addition LiP. These two groups, respectively, have been related with the ability of white rot fungi to degrade preferentially lignin from woody plant cell walls and to mineralize efficiently a synthetic lignin preparation (26). *Pleurotus eryngii* is a representative species from the laccase-MnP group (40), which includes most species studied to date. This fungus produces both in liquid cultures and under solid-state fermentation conditions aryl-alcohol oxidase (AAO), in addition to Mn^{2+}-oxidizing peroxidase and laccase (9, 21, 35). AAO provides the H_2O_2 necessary for peroxidase activity, through a system based on aromatic aldehyde redox cycling (20, 22, 24). The Mn^{2+}-oxidizing peroxidase from *P. eryngii* does not require Mn^{2+} to close its catalytic cycle, as described for *P. chrysosporium* MnP (53), but it oxidizes Mn^{2+} very efficiently. In addition, this enzyme exhibits Mn-independent activity on phenolic and nonphenolic aromatic compounds (35); the latter was believed to be a sole characteristic of LiP based on studies on ligninolytic peroxidases from *P. chrysosporium* (30). Bourbonnais and Paice (6) described the oxidation of nonphenolic lignin model compounds by laccase from *Trametes versicolor* in the presence of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) or other artificial laccase substrates (often known as mediators). This mechanism was confirmed in *P. eryngii*, showing that the enzyme-ABTS couple is necessary for the degradation of nonphenolic compounds (34). In this sense the identification of 3-hydroxyanthranilate acting as a natural laccase mediator in cultures of *Pycnoporus cinnabarinus* has been recently reported (12). This laccase mediator system represents an alternative mechanism for oxidation of nonphenolic lignin units (which comprise up to 85% of the polymer).

AAO and Mn^{2+}-oxidizing peroxidase from *P. eryngii* have been already purified and characterized (21, 35). In order to complete our knowledge of the ligninolytic system of this fungus, in the present paper we report the isolation of two laccase

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isoenzymes which were previously detected (37). Our aim was to compare both isoenzymes in terms of physicochemical and catalytic properties. Due to the lack of data supporting the involvement of laccase in some ligninolytic events other than the direct oxidation of lignin units, laccase isoenzymes were also compared in their ability to produce reduced oxygen species during oxidation of hydroquinones. This new aspect was taken into account after our recent finding on the existence of quinone redox cycling in *P. eryngii* and the involvement in this process of the laccase activity present in fungal cultures (23). In general, redox cycling implies the production of active oxygen species through the continuous reduction and oxidation of compounds of different natures (29). Quinone redox cycling in *P. eryngii* consists of cell-bound divalent reduction of quinones, followed by extracellular laccase-mediated oxidation of hydroquinones into semiquinones, which autoxidized to a certain extent, producing superoxide anion radical ($O_2 \cdot^-$).

MATERIALS AND METHODS

Fungal strains and culture conditions. For laccase production, *P. eryngii* IJFM A169 (= CBS 613.91 and ATCC 90787) was cultivated in a 5-liter fermentor (Microferm M-F-105; New Brunswick Scientific Co., Inc.) with 3 liters of medium at 26 to 28°C, 200 rpm, and aeration at 0.5 liter min^{-1}. The culture medium contained, per liter, 10 g of glucose, 2 g of ammonium tartrate, 1 g of KH_2PO_4, 1 g of yeast extract, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of KCl, and 1 ml of mineral solution containing, per liter, 100 mg of $B_4O_7Na_2 \cdot 10H_2O$, 70 mg of $ZnSO_4 \cdot 7H_2O$, 50 mg of $FeSO_4 \cdot 7H_2O$, 10 mg of $CuSO_4 \cdot 5H_2O$, 10 mg of $MnSO_4 \cdot 4H_2O$, and 10 mg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$. Inoculum was prepared by cultivating the fungus in 1-liter flasks with 200 ml of the above medium (26 to 28°C and 200 rpm) and then homogenizing 5-day-old mycelium. The dry weight of inoculum was 1 g per liter of medium. For immunoblot analysis, in addition to *P. eryngii*, *Pleurotus ostreatus* (CBS 411.71), *Pleurotus pulmonarius* (CBS 507.85), *Pleurotus floridanus* (MUCL 28518), *T. versicolor* (IJFM A136), *Bjerkandera adusta* (CBS 595.78), and *Ganoderma australe* (IJFM A130) were cultivated in 1-liter flasks, under the same conditions used to prepare inocula.

Enzyme assays. Unless stated otherwise, laccase (EC 1.10.3.2) activity was determined by measuring the increase in A_{436} with 5 mM ABTS as the substrate in 100 mM sodium acetate buffer, pH 4.5 (ε_{436} = 29,300 M^{-1} cm^{-1}). For kinetic studies the reaction was carried out at the optimal pH of each laccase isoenzyme (4 and 3.5 for laccases I and II, respectively). AAO (EC 1.1.3.7) activity was assayed by the oxidation of 5 mM veratryl(3,4-dimethoxybenzyl) alcohol to veratraldehyde (ε_{310} = 9,300 M^{-1} cm^{-1}) in 100 mM sodium phosphate buffer, pH 6. MnP (EC 1.11.1.13) activity was assayed with 0.01% phenol red as the substrate (ε_{610} = 4,460 M^{-1} cm^{-1}) under conditions described by Paszczynski et al. (39). All enzyme assays were carried out at room temperature. One unit of enzyme activity was defined as the amount of enzyme oxidizing 1 μmol of substrate min^{-1}.

Laccase purification. Seven-day-old culture liquid was 20-fold concentrated by ultrafiltration (Fillron 5-kDa-cutoff membrane). The extracellular polysaccharide produced by the fungus was precipitated with 30% ethanol (final concentration) and separated by centrifugation (13,000 rpm; Sorvall SS-34 rotor), and the ethanol was removed under reduced pressure. After this step, the culture liquid was concentrated again 20-fold by the same procedure. Samples of 1 ml were applied to a Sephadex G-100 column (Pharmacia K 16/100) equilibrated with 100 mM sodium acetate buffer, pH 5.5, and eluted at a flow rate of 0.8 ml min^{-1}. Fractions containing laccase activity were pooled, concentrated, and dialyzed by ultrafiltration against 10 mM sodium acetate buffer, pH 5.5 (Filtron Macrosep 3-kDa-cutoff membrane). The enzyme was then applied to a Mono-Q anion-exchange column (Pharmacia HR 5/5) and eluted with 10 mM sodium acetate buffer at pH 5.5 (buffer A) and 1 M NaCl in the same buffer (buffer B) at a flow rate of 0.8 ml min^{-1} with the following elution gradient (expressed as percentages of buffer B): 0%, 4 min; 0 to 25%, 30 min; 25 to 100%, 4 min; and 100 to 0%, 2 min. Fractions containing laccase activity were found in two major peaks, called laccases I and II, which were pooled and processed separately. Laccases I and II were concentrated and dialyzed against buffer A (Filtron Microsep, 3-kDa cutoff) and loaded onto a Mono-Q column (flow rate of 0.8 ml min^{-1}). The elution gradient used for laccase I was the following (expressed as percentages of buffer B): 0%, 4 min; 0 to 8%, 2 min; 8%, 15 min; 8 to 25%, 5 min; 25 to 100%, 4 min; and 100 to 0%, 5 min. In the case of laccase II the following gradient (expressed as percentages of buffer B) was used: 0%, 4 min; 0 to 14%, 4 min; 14%, 15 min; 14 to 25%, 5 min; 25 to 100, 4 min; and 100 to 0%, 5 min. Laccases I and II were concentrated and applied to a Superose 12 column (Pharmacia HR 10/30) equilibrated with 10 mM sodium acetate buffer, pH 5.5, containing 150 mM NaCl and eluted at a flow rate of 0.5 ml min^{-1}. The peaks containing laccase isoenzymes were collected, concentrated, and stored at −20°C.

Analytical procedures. Reducing sugars were assayed by the method of Somogyi (48), using glucose as a standard. Protein concentration was determined by the method of Bradford (8), using bovine serum albumin as a standard.

The total H_2O_2 produced during oxidation of hydroquinones by laccase was estimated by two different procedures. The first method used addition of 2.5 U of horseradish peroxidase (HRP; Sigma) per ml of laccase reaction mixture and used as an electron donor a phenol which was not a substrate of laccase (phenol red, 0.01%). After 10 min, NaOH was added (0.2 M final concentration), and the absorbance was read at 610 nm. Samples lacking HRP were used as blanks. A standard curve of H_2O_2 was prepared with dilutions of 30% Perhydrol (Merck) processed in the same way. The H_2O_2 concentration in the commercial solution was calculated from its absorbance at 230 nm (ε = 81 M^{-1} cm^{-1}). In the second method, H_2O_2 production after complete hydroquinone oxidation was estimated by monitoring oxygen consumption with a Clark-type electrode. Once oxygen consumption ceased, 100 U of catalase (Sigma) was added and the production of O_2 was measured (heat-denatured catalase was used in blanks). The amount of H_2O_2 present in samples was estimated considering the stoichiometry of catalase reaction ($2H_2O_2$:$1O_2$). Oxygen electrode was calibrated by the addition of known amounts of commercial H_2O_2 to laccase reaction mixtures containing 100 U of catalase per ml and lacking hydroquinone.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% polyacrylamide gels was performed by the method of Laemmli (32) using trypsin (21.5 kDa), carbonic anhydrase (31.0 kDa), ovalbumin (45 kDa), bovine serum albumin (66.2 kDa), and phosphorylase *b* (97.4 kDa) as molecular mass standards (Bio-Rad). Isoelectric focusing (IEF) was performed on 5% polyacrylamide gels with a thickness of 1 mm and a pH gradient from 2.5 to 5.5 (prepared with Pharmacia Ampholine by mixing 85% from pH 2.5 to 5 and 15% from pH 3.5 to 10). The anode and cathode solutions were 1 M phosphoric acid and 1 M sodium hydroxide, respectively. Protein bands after SDS-PAGE were stained with a commercial kit (Silver Stain Plus; Bio-Rad) and after IEF were stained with Coomassie brilliant blue R-250. Besides SDS-PAGE, the molecular masses of native laccases were determined by gel filtration (Superose 12 column). Laccase isoenzymes were eluted as described above. The column was calibrated with carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), and β-galactosidase (116 kDa) as standards (Sigma).

Carbohydrate content of laccase isoenzymes was determined by two different procedures: (i) the anthrone reagent method, using glucose as a standard (52), for total carbohydrate content, and (ii) SDS-PAGE before and after deglycosylation with endo-β-*N*-acetylglucosaminidase H (Boehringer Mannheim), for N-linked carbohydrate content.

The N-terminal sequences of laccase isoenzymes were determined by automated Edman degradation of 20 μg of protein in an Applied Biosystems 477A pulsed-liquid protein sequencer with a model 120A on-line phenylthiohydantoin analyzer. The amino acid composition was determined with a Biochrom 20 analyzer (Pharmacia) after hydrolysis of 10 μg of enzymes with 6 M HCl at 110°C for 24 h.

Substrate specificity of laccases. The phenols and amines used in specificity studies were purchased from Aldrich Chemical Co. Initial rates of product formation in reaction mixtures containing purified laccase isoenzymes and the different substrates were measured spectrophotometrically, at the wavelengths shown in Table 2. These wavelengths and the molar extinction coefficient (ε) of each reaction were determined previously as follows: different concentrations of substrates (50 to 400 μM) were treated with 50 mU of a partially purified laccase preparation (containing a mixture of laccases I and II from Sephadex G-100), in 100 mM sodium acetate buffer, pH 4.5; the UV-visible spectrum corresponding to the beginning of the reaction was subtracted from that obtained after complete oxidation of substrate, and the wavelength used to calculate ε was that showing the highest differences between spectra. In the case of guaiacol the ε used was that described elsewhere (39).

Anti-laccase I antibody. Laccase I purified as described above was used as an immunogen. Rabbits were injected subcutaneously with 150 μg of laccase protein in phosphate-buffered saline (PBS) buffer (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl, and 140 mM NaCl) mixed with an equal volume of complete Freund's adjuvant (Difco). Two more doses, containing the same enzyme concentration but Freund's incomplete adjuvant, were injected intramuscularly at 2- to 3-week intervals.

Immunoblot analysis. After SDS-PAGE in 12% polyacrylamide gels of 8-day-old concentrated culture liquid from the different *Pleurotus* species, the proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell) in the Bio-Rad system for 1 h at 80 V, using as a buffer 25 mM Tris-HCl, 192 mM glycine, and 20% methanol. The membranes were blocked by incubation with 5% skim milk in PBS buffer (solution A) for 1 h at room temperature. Then, the membranes were incubated overnight with anti-laccase I antiserum at 1:500, 1:250, and 1:100 dilutions in solution A and washed twice with PBS for 10 min. After that, they were incubated again in solution A containing anti-rabbit immunoglobulin G–peroxidase (Bio-Rad) conjugate at a 1:1,000 dilution in solution A for 2 h, washed three times with PBS for 10 min, and developed in the same buffer with 0.5 mM diamine benzidine, 0.8 mM chloronaftol, and 0.1 mM H_2O_2.

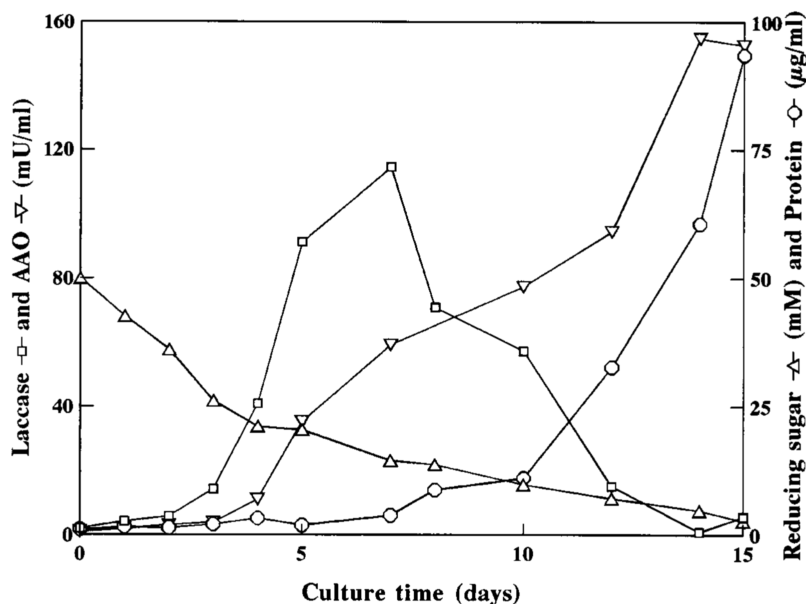


FIG. 1. Time course of laccase and AAO activities, protein, and reducing sugar in cultures of *P. eryngii.*

RESULTS

Laccase production and purification. In a previous study it was found that *P. eryngii* produced two major proteins showing laccase activity (laccases I and II), one of them (laccase II) being induced by wheat straw alkali lignin and vanillic and veratric acids (37). In order to scale up laccase production from 250-ml flasks, *P. eryngii* was cultivated in a 5-liter fermentor with 3 liters of the same culture medium. As shown in Fig. 1, laccase was produced during growth and maximal activity was found after 7 days. Cultivation of *P. eryngii* in the fermentor increased laccase levels 2.3-fold compared with levels in 250-ml flask cultures. The AAO profile and activity levels were similar to those previously reported (21), and peroxidase activities were not detected under these conditions.

Laccases I and II were purified to homogeneity according to the procedure summarized in Table 1. Four and three chromatographic steps were required to purify laccases I and II, respectively. During the first step, with Sephadex G-100, an orange pigment produced by the fungus (37) was separated from laccase. As shown in Fig. 2, laccase isoenzymes (including a minor activity which could be a modification product of laccase I or II) were separated during the second chromatographic step (Mono-Q column). The purification process for both laccases was continued in the same column but under different elution conditions (Fig. 3). A final step, using Superose 12, was necessary to complete the purification of laccase I. At the end of the process, laccase I had been purified 40-fold, with a yield of 30%, and laccase II had been purified 45-fold, with a yield of 26% (Table 1).

TABLE 1. Purification of laccase isoenzymes from *P. eryngii*[a]

Step	Activity (U)	Protein (mg)	Yield (%)	Sp act (U/mg)	Purification factor (fold)
Culture liquid	250.0	19.60	100.0	12.8	1.0
Ultrafiltration	237.5	5.02	95.0	47.3	3.7
Sephadex G-100	228.4	1.55	91.3	147.4	11.5
Mono-Q (1st)					
Laccase I	128.3	0.26	51.3	492.3	38.5
Laccase II	82.4	0.25	32.8	329.6	25.7
Mono Q (2nd)					
Laccase I	85.5	0.17	34.2	502.9	39.3
Laccase II	65.7	0.12	26.3	547.5	42.8
Superose 12 (laccase I)	75.0	0.14	30.0	535.7	41.9

[a] Laccases were purified from a 3-liter culture. Activity was determined with 5 mM ABTS as the substrate.

Properties of laccases I and II. Based on gel filtration chromatography on Superose 12, the molecular masses of native laccases I and II were estimated to be 56 and 70 kDa, respectively. Values obtained by SDS-PAGE were 65 and 61 kDa, respectively, indicating that both isoenzymes are monomeric proteins (Fig. 4A). Analytical IEF showed homogeneous protein bands with pIs of 4.2 and 4.1 (laccases I and II, respectively) (Fig. 4B). Anthrone analysis revealed that laccases I and II are proteins with a carbohydrate moiety (7 and 1%, respectively). Based on the molecular masses of both isoenzymes after N-deglycosylation, the N-linked carbohydrate contents of laccases I and II were estimated to be 5 and 1%, respectively. After freezing of purified isoenzymes, a second protein band sporadically appeared in SDS-PAGE, showing the same molecular mass as deglycosylated isoenzymes. This phenomenon was not observed when the laccases were previously treated with endo-β-N-acetylglucosaminidase H, suggesting the loss of carbohydrate moiety during the freezing-melting process.

The UV-visible spectra of native isoenzymes showed typical characteristics of copper-containing enzymes (50). Spectra included a shoulder at 330 nm, corresponding to a type 3 binuclear copper, and a peak at 600 nm, corresponding to a type 1 blue copper atom (data not shown). The 280-nm/600-nm absorbance ratio for both *P. eryngii* isoenzymes was around 15, a

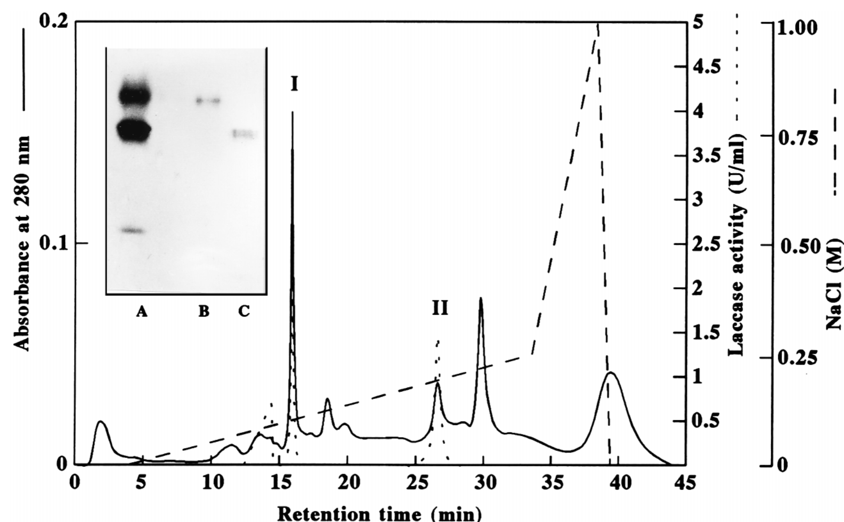


FIG. 2. Elution profile of laccase isoenzymes and protein of *P. eryngii* during Mono-Q chromatography, after a first purification step in Sephadex G-100. The inset shows zymograms of laccase isoenzymes before (lane A) and after Mono-Q chromatography: laccase I (lane B) and laccase II (lane C).

value similar to those described for other fungal laccases (15, 25).

The N-terminal sequences of laccases I and II (12 residues) are shown in Fig. 5 (the second amino acid of laccase I could not be identified). The amino acid compositions of laccases I and II presented some differences, mainly in Thr, Ser, and Ile contents. The numbers of amino acid residues of laccases I and II, estimated on the basis of molecular masses of 65 and 61 kDa, respectively, were as follows, respectively: 60 and 76 Asx, 77 and 40 Thr, 88 and 38 Ser, 49 and 50 Glx, 45 and 43 Gly, 64 and 49 Ala, 42 and 33 Val, 8 and 10 Met, 25 and 39 Ile, 30 and 47 Leu, 10 and 13 Tyr, 27 and 31 Phe, 12 and 18 His, 15 and 14 Lys, 33 and 28 Pro, and 15 and 22 Arg residues.

The reactions of both isoenzymes with the antibodies obtained against laccase I were similar (with 10 to 300 ng of protein). Additionally, immunoblot analysis of culture filtrates from other *Pleurotus* species (*P. pulmonarius*, *P. ostreatus*, and *P. floridanus*), *T. versicolor*, *B. adusta*, and *G. australe* using laccase I antibodies also shows cross-reactions, suggesting homology between their laccases (data not shown).

The effect of pH on the initial rate of ABTS (5 mM) oxidation by laccase isoenzymes was studied with 100 mM acetate buffer (pH range, 3 to 5). The highest rate was obtained at pH 4.0 (laccase I) and 3.5 (laccase II). When kept for 24 h at either 25 or 4°C and different pH values (100 mM borate-citrate-phosphate buffer; pH range, 2 to 13), laccase I was stable between pHs 5 and 7, with about 60% of activity remaining at pH 8 to 12. However, laccase II lost activity at all the pH values assayed, reaching the highest oxidation rates (70% of initial activity) at pH 8 to 12. The effect of temperature on laccase activity was determined over the range 25 to 85°C, at the optimum pH of each isoenzyme, with 100 mM sodium acetate buffer and 5 mM ABTS. Maximal values for laccases I and II were obtained at 65 and 55°C, respectively. Thermal stability was determined at fixed intervals during a 30-min incubation period in 100 mM sodium phosphate buffer, pH 7. Laccase I was stable for 10 min up to 45°C, had a half-life of 30 min at 50°C, and was nearly completely inactivated (3% remaining activity) when kept for the whole period at higher temperatures (60 to 65°C). Laccase II was stable for 10 min up to 50°C, at 55°C had a half-life of 25 min, and after 30 min at a higher temperature (65°C) presented a higher residual activity (10%) than laccase I.

Substrate specificity of laccases. Based on the results of a previous substrate specificity study carried out with a semipurified laccase preparation (37), kinetic constants of each isoenzyme were calculated with those compounds which were relatively well oxidized. The kinetic constants of laccases I and II corresponding to some substituted phenols and aromatic amines are listed in Table 2. The affinities ($1/K_m$) of both isoenzymes for the compounds tested were similar except for 2,6-dimethoxyphenol, *p*-hydroquinone (QH_2), and methyl-*p*-hydroquinone ($MeQH_2$), which presented higher affinity in the case of laccase II, and catechol in the case of laccase I. However, the maximum velocity (V_{max}) of reactions catalyzed by laccase II was always lower. As a consequence, V_{max}/K_m values showed that laccase I was more efficient oxidizing all the compounds assayed.

Comparing the kinetic constants obtained with *p*-methoxyphenol and *p*-anisidine, it was found that the affinity of both isoenzymes was higher for phenols than for aromatic amines and, although the oxidation rate of the *p*-methoxyphenol was lower in both cases, laccase I was more efficient oxidizing the phenol and laccase II was more efficient oxidizing the amine. K_m and V_{max} values similar to those found with *p*-methoxyphenol were obtained with *p*-aminophenol.

The influence of the nature and number of aromatic-ring substituent was considered in the case of substituted phenols, and the results were similar for both isoenzymes. With respect to the nature of the substituents (hydroxyl or methoxyl), the oxidation rates of catechol and QH_2 were higher than those of their corresponding methoxylated counterparts, i.e., guaiacol (*o*-methoxyphenol) and *p*-methoxyphenol. In the case of *o*-substituted phenols, i.e., catechol and guaiacol, both isoenzymes presented higher affinity for hydroquinones, whereas with *p*-substituted phenols (QH_2 and *p*-methoxyphenol) the affinity was higher for methoxyphenols. With respect to the number of substituents, the comparison between guaiacol and 2,6-dimethoxyphenol, as well as between QH_2 and $MeQH_2$, revealed that the addition of a second methoxyl group to guaiacol increased both affinity and V_{max}, and the same effect was observed when a methyl group was added to QH_2. On the

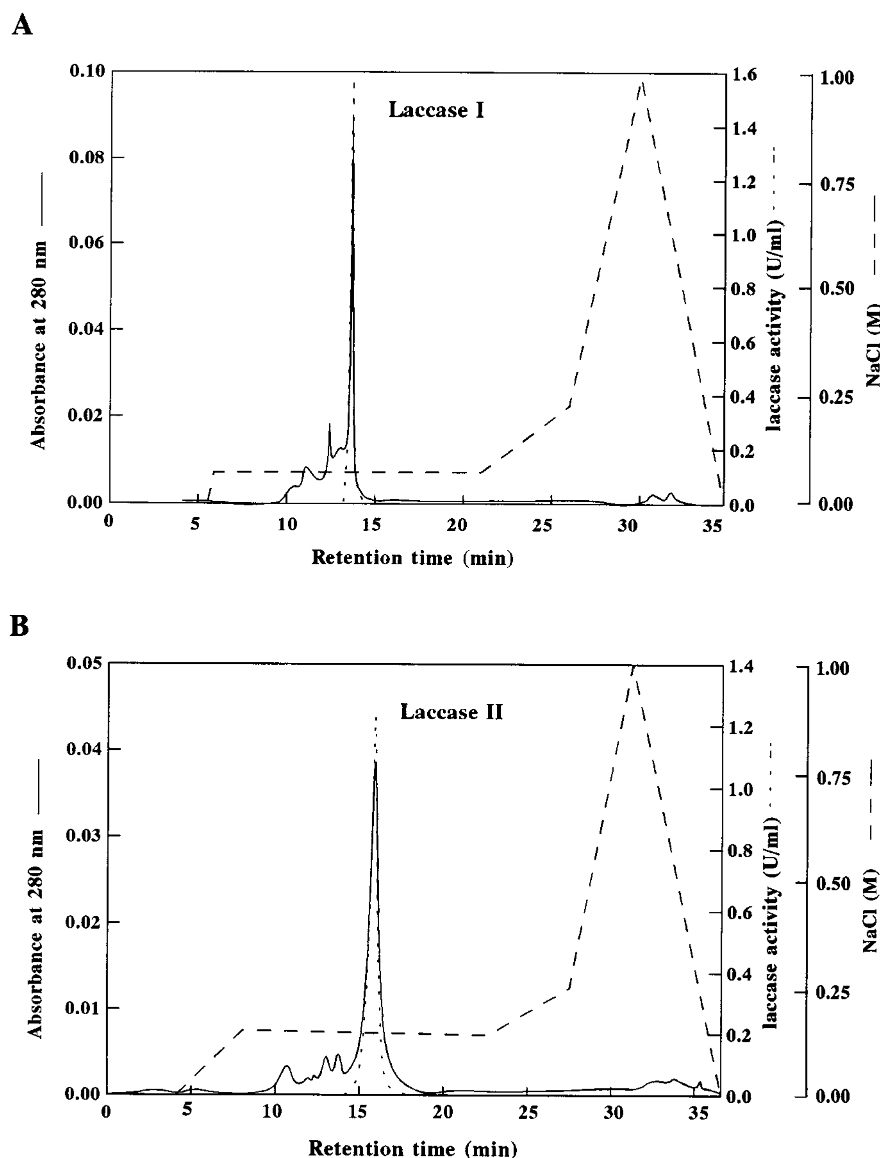


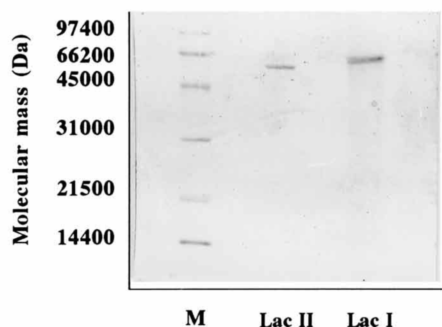
FIG. 3. Elution profiles of *P. eryngii* protein and laccases I (A) and II (B) after the second step in the Mono-Q column.

other hand, the position of the substituents on the aromatic ring (*ortho* or *para*) affected the K_m and V_{max} of each isoenzyme in a different way, as deduced from the results obtained with hydroquinones (catechol and QH_2) and methoxyphenols (guaiacol and *p*-methoxyphenol).

In our previous study on laccase from *P. eryngii* it was found that *m*-methoxyphenol was not oxidized by an enzyme preparation containing a semipurified laccase. To find if this compound is at least recognized by the enzyme, it was tested as inhibitor of laccase I with 2,6-dimethoxyphenol as a substrate. Competitive inhibition was found, with a K_i value of 2.28 mM.

Production of superoxide anion by laccases I and II. Laccase catalyzes the monovalent oxidation of different substituted phenols to the corresponding phenoxy radicals. In the case of hydroquinones (reaction 1), these radicals (semiquinones [$Q^{\cdot-}$]) are converted into quinones (Q) by different reactions, including monovalent oxidation by molecular oxygen with the concomitant production of $O_2^{\cdot-}$ (autoxidation; reaction 2). Thus, the involvement of laccase isoenzymes in the activation of oxygen during oxidation of QH_2 and $MeQH_2$ was studied. It was done by estimating the effect of superoxide dismutase (SOD) and Mn^{2+} on the quinone production rate and H_2O_2 generation from $O_2^{\cdot-}$. It is well established that SOD shifts the equilibrium of the semiquinone autoxidation reaction toward the right (reaction 2) by reacting with $O_2^{\cdot-}$ (reaction 3) (38, 54). By analogy, it was expected that reduction of $O_2^{\cdot-}$ by Mn^{2+} (reaction 4) (2) would have the same effect on the semiquinone autoxidation reaction. That SOD and Mn^{2+} do not exert any effect on the laccase activity itself (monovalent oxidation of substrates), was demonstrated using a monophenolic compound (2,6-dimethoxyphenol). SOD and Mn^{2+} increased quinone formation rates (Table 3), suggesting that semiquinones produced by laccase isoenzymes were in part converted into quinones via autoxidation. Regardless of the efficiency of SOD or Mn^{2+} in removing $O_2^{\cdot-}$, the higher quinone formation rate in the presence of Mn^{2+} can be under-

A



B

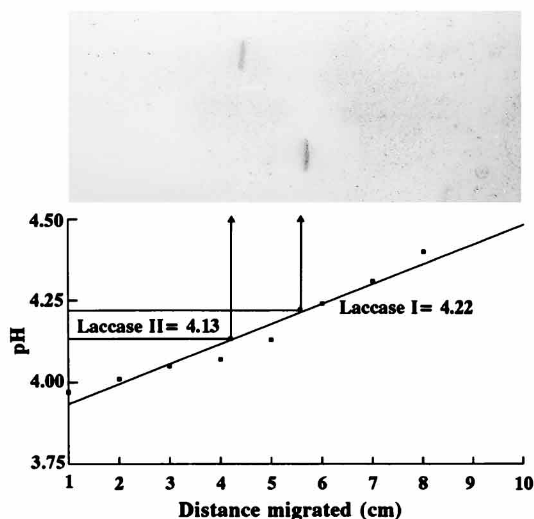
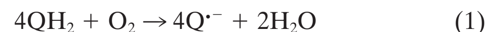
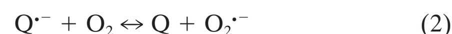


FIG. 4. Estimation of the molecular mass (by SDS-PAGE) (A) and isoelectric point (B) of laccase (Lac) isoenzymes from *P. eryngii*. Lane M, molecular mass standards.

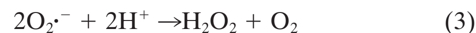
stood taking into account that, in addition to laccase, the Mn^{3+} produced during reaction 4 is able to oxidize hydroquinones.



$$4QH_2 + O_2 \rightarrow 4Q^{\cdot -} + 2H_2O \quad (1)$$



$$Q^{\cdot -} + O_2 \leftrightarrow Q + O_2^{\cdot -} \quad (2)$$



$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (3)$$

$$Mn^{2+} + O_2^{\cdot -} + 2H^+ \rightarrow Mn^{3+} + H_2O_2 \quad (4)$$

The above mechanism was confirmed by testing H_2O_2 production during hydroquinone oxidation by laccases I and II, by the HRP-phenol red procedure. Since hydroquinones are substrates of HRP, H_2O_2 levels were estimated after full oxidation of hydroquinones by laccases (spectrophotometrically confirmed) to avoid interferences or underestimations. Low levels of H_2O_2 were produced in the control experiment (Table 4). However, when SOD and Mn^{2+} were present, H_2O_2 levels considerably increased, evidencing $O_2^{\cdot -}$ formation. No H_2O_2 was found when HRP was omitted.

Besides the study of the involvement of both laccase isoenzymes in the activation of oxygen, data concerning O_2 consumption during the oxidation of $MeQH_2$ by laccase I ratified the mechanism by which SOD and Mn^{2+} increased the quinone production rate during oxidation of hydroquinones. In the absence of any agent promoting the autoxidation reaction (control experiment) (Fig. 6), oxygen was consumed at a rate of 7.33 μM/min, with the same activity level of laccase I as in the experiment whose results are shown in Table 3. The presence of SOD and Mn^{2+} increased oxygen consumption 1.45 and 4.15 times, respectively (Fig. 6), which is quite well correlated with the increase in quinone production rate shown in Table 3 (1.49 and 4.49 times, respectively). In parallel experiments, it was also found that the consumption of oxygen by laccase, acting on monophenolic compounds, was not affected by the presence of SOD or Mn^{2+}. Therefore, the more plausible explanation for the effect of these agents on the laccase-mediated transformation of hydroquinones into quinones is the promotion of the semiquinone autoxidation reaction. In the experiments involving oxygen consumption, the total amount of H_2O_2 produced during oxidation of 100 μM $MeQH_2$ by laccase I under different conditions (the absence and presence of either SOD or Mn^{2+}) was estimated also by the use of catalase as described in Materials and Methods. The

A	X	K	K	L	-	D	F	H	I	I	N	N	*Pleurotus eryngii* laccase I
A	T	K	K	L	-	D	F	H	I	I	N	N	*Pleurotus eryngii* laccase II
K	T	R	-	T	F	D	F	D	L	V	N	T	*Agaricus bisporus* laccase I (41)
D	T	K	-	T	F	N	F	D	L	V	N	T	*Agaricus bisporus* laccase II (41)
A	I	G	P	T	A	D	L	T	I	S	N	A	*Coriolus hirsutus* laccase (31)
A	I	G	P	T	G	D	M	Y	I	V	N	E	*Pleurotus ostreatus* laccase I (18)
A	I	G	P	A	G	N	M	Y	I	V	N	E	*Pleurotus ostreatus* laccase II (17)
S	I	G	P	V	A	D	L	T	I	S	N	G	Basidiomycete PM1 laccase (10)
A	I	G	P	V	T	D	L	E	I	T	D	A	*Ceriporiopsis subvermispora* laccase (16)
S	I	G	P	V	T	D	F	H	I	V	N	A	*Phlebia radiata* laccase (45)
A	I	G	P	V	A	S	L	V	V	A	N	A	*Trametes versicolor* laccase I (7)
G	I	G	P	V	A	D	L	T	I	T	N	A	*Trametes versicolor* laccase II (7)
G	I	G	P	V	A	D	L	T	I	T	N	A	*Trametes villosa* laccase I (58)
A	I	G	P	V	A	S	L	V	V	A	N	A	*Trametes villosa* laccase II (58)
A	I	G	P	V	A	D	L	T	L	T	N	A	*Pycnoporus cinnabarinus* laccase (13)

FIG. 5. Comparison of N-terminal sequences of the two laccase isoenzymes from *P. eryngii* with those of other fungal laccases. Identical residues are boxed. Numbers in parentheses indicate references. Dashes indicate gaps introduced to maximize alignment.

TABLE 2. Kinetic constants of laccase isoenzymes from *P. eryngii*[a]

Compound	λ (nm)	ε (M^{-1} cm^{-1})	Laccase I			Laccase II		
			K_m (mM)	V_{max} (U/mg)	V_{max}/K_m	K_m (mM)	V_{max} (U/mg)	V_{max}/K_m
p-Aminophenol	246	15,627	0.8	5.0	6.2	1.0	0.8	0.8
p-Anisidine	542	1,173	3.1	20.6	6.6	3.3	17.9	5.4
p-Methoxyphenol	253	4,990	0.8	10.8	13.5	0.9	0.8	0.9
Guaiacol	465	12,100	7.6	5.9	0.8	8.0	1.3	0.2
Catechol	392	1,456	2.2	32.1	14.6	4.1	15.4	3.8
QH_2	247	21,028	4.6	21.2	4.6	2.5	2.6	1.0
$MeQH_2$	250	21,112	2.1	122.2	58.2	1.6	8.4	5.2
2,6-Dimethoxyphenol	468	27,500	1.4	54.2	38.7	0.4	3.3	8.2

[a] Reactions of laccases I and II with the different compounds were carried out in 100 mM sodium acetate buffer at pHs 4 and 3.5, respectively. The λ and ε values used for spectrophotometric estimation of reaction products are shown.

results shown in Fig. 6 corroborated those obtained with the HRP-phenol red method (Table 4).

DISCUSSION

Among enzymes related to lignin degradation, *P. eryngii* produces a single isoform of AAO (21), up to five isoforms of Mn^{2+}-oxidizing peroxidases (33), and, as shown here, at least two isoforms of laccase. The N-terminal sequences of the above peroxidases are different, indicating that probably they are true isoenzymes encoded by different genes. Although the selective induction of laccase isoenzymes has been described previously in other white rot fungi (4, 58), in *P. eryngii* only the levels of laccase II increased when lignin-related compounds were present in the culture medium (37), probably because both laccases are also encoded by different genes.

The results obtained here show that laccases I and II are similar in terms of molecular mass, pI, optimum pH and temperature for ABTS oxidation, thermal stability, N-terminal sequence, and antigenic determinants. However, significant differences exist with regard to pH stability, carbohydrate contents, kinetic constants, and amino acid composition (the last suggesting two different genic products). Laccase II is less stable at neutral and acidic pH values, has a lower carbohydrate content, and is less efficient than laccase I in oxidizing all the substrates studied. The characteristics of laccases I and II of *P. eryngii* are not atypical among fungal laccases. Most of them are monomeric proteins with molecular masses between 50 and 80 kDa (4, 50, 57). Some exceptions include the laccases from *Agaricus bisporus* (55) and *Trametes villosa* (58), which present two subunits, and laccase I from *Podospora anserina* (11), which is composed of four subunits. The isoelectric point and carbohydrate content of laccase isoenzymes of *P. eryngii* lie well within the ranges determined for other fungal laccases (13, 16, 43, 58). Besides, the laccases of the different *Pleurotus* species studied here have common antigenic determinants. However, some characteristics of *P. eryngii* laccases are not similar to those of other white rot fungi. First, Bollag and Leonowicz (4) reported that the xylidine-induced isoenzymes from *Fomes annosus*, *Pholiota mutabilis*, *T. versicolor*, and *P. ostreatus* were more active oxidizing 2,6-dimethoxyphenol, chlorophenol, and several phenolic acids than the constitutive isoenzymes of the same fungi. Although laccases of *P. eryngii* have been purified from noninduced cultures, laccase II, which is the only one induced by lignin-related compounds and, to a much lesser extent, by xylidine (37), is less active than laccase I on lignin-related compounds (Table 2). Second, the N-terminal amino acid sequences of *P. eryngii* laccases differ greatly from those of other fungal laccases (Fig. 5). It is worth noting that the laccase isoenzymes of *P. ostreatus*, a species related to *P. eryngii*, have in common only 3 of the amino acids compared.

We have recently reported (37) that a partially purified *P. eryngii* laccase preparation, containing both isoenzymes, oxidized phenolic compounds having a redox potential of ≤0.6 V. Moreover, the competitive inhibition of laccase I by *m*-methoxyphenol shown here reveals that the lack of activity on this compound is due to its comparatively high redox potential, around 0.62 V. It has been recently shown that significant differences in redox potential exist among fungal laccases (0.5 to 0.8 V) and that the V_{max} values of reactions catalyzed by these enzymes are positively correlated to the differences in redox potential between the laccases and the substrates (56). Although an estimation of laccase potential based on its ability to oxidize different phenols should be made with caution, the results from oxidation of different substrates, together with the higher values of V_{max} for laccase I than for laccase II, indicate that the redox potential of laccase I should be around 0.6 V, higher than that of laccase II. The results from the study of substrate specificity also show the influence of phenolic compounds substituents on both K_m and V_{max} of laccase isoenzymes. Only in the case of laccase I, most factors increasing V_{max} also increase enzyme affinity. For instance, when hydroxyl or methoxyl radicals are changed from the *ortho* to the *para* position (catechol versus QH_2 and *o*-methoxyphenol versus *p*-methoxyphenol), *o*-methoxyl is replaced by *o*-hydroxyl radical (guaiacol versus catechol) and methyl or methoxyl groups

TABLE 3. Quinone production rates during oxidation of QH_2 and $MeQH_2$ (100 μM) by laccases (Lac) I and II, in the absence (control) and presence of SOD (90 U ml^{-1}) and Mn^{2+} (100 μM)

Hydroquinone	Mean quinone production rate ± 95% confidence limit (μM min^{-1})					
	Control		SOD		Mn^{2+}	
	Lac I	Lac II	Lac I	Lac II	Lac I	Lac II
QH_2	1.1 ± 0.0	1.1 ± 0.0	1.6 ± 0.0	1.6 ± 0.0	4.8 ± 0.2	4.9 ± 0.1
$MeQH_2$	14.5 ± 0.2	10.3 ± 0.1	21.7 ± 0.1	13.2 ± 0.3	65.3 ± 0.1	42.0 ± 0.2

TABLE 4. Hydrogen peroxide generation (HRP-phenol red procedure) by laccases (Lac) I and II during the complete oxidation of QH_2 and $MeQH_2$ (100 μM) in the absence (control) and presence of SOD (90 U ml^{-1}) and Mn^{2+} (100 μM)

Hydroquinone	Mean hydrogen peroxide concn ± 95% confidence limit (μM min^{-1})					
	Control		SOD		Mn^{2+}	
	Lac I	LacII	Lac I	Lac II	Lac I	Lac II
QH_2	0.3 ± 0.0	0.5 ± 0.0	12.0 ± 0.1	8.2 ± 0.5	34.0 ± 0.8	28.1 ± 1.1
$MeQH_2$	2.4 ± 0.1	4.4 ± 0.1	26.4 ± 0.6	26.2 ± 0.4	49.8 ± 1.1	48.6 ± 1.5

are added to the aromatic ring (QH_2 versus $MeQH_2$ and guaiacol versus 2,6-dimethoxyphenol, respectively). This positive correlation between affinity and V_{max} was also found in AAO from *P. eryngii* (21).

After noting that aromatic aldehyde redox cycling is an efficient process for extracellular H_2O_2 production in *P. eryngii* (20, 22), we began to investigate the mechanisms by which this fungus could generate the $O_2^{\cdot -}$ needed for production of OH· through the Haber-Weiss reaction. Together with chelated Mn^{3+}, OH· is considered to be one of the oxidizing agents involved in the initial attack of lignocellulosic materials when ligninolytic enzymes cannot penetrate plant cell walls (3, 14, 28). In addition, this radical is able to react with simple aromatic compounds, producing reactions similar to those undergone by aromatic radicals generated by ligninolytic enzymes (19). Other possible roles of $O_2^{\cdot -}$ in relation to lignin degradation are mentioned below. The first evidence in *P. eryngii* about a mechanism of $O_2^{\cdot -}$ generation (similar to that of H_2O_2 production), in which quinones and laccase are, respectively, the electron carriers and the extracellular enzyme enabling partial reduction of oxygen (instead of aromatic aldehydes and AAO), has been recently described (23). By using four different quinones, it was found that the whole process is nonspecific and that the extent of oxygen activation depends on the type of quinone (23). After laccase I and II purification, the study of substrate specificity demonstrated that both isoenzymes can participate in $O_2^{\cdot -}$ production via hydroquinone oxidation and therefore in quinone redox cycling. Although in the course of these experiments Mn^{2+} was used as a tool for evidencing semiquinone autoxidation reactions, the results obtained also indicate that Mn^{3+} is produced by laccase in the presence of hydroquinones.

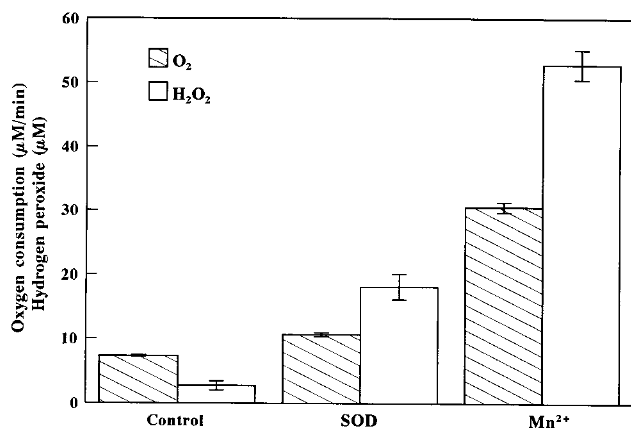


FIG. 6. Oxygen consumption rate and total H_2O_2 levels (measured by the use of catalase as described in Materials and Methods) during oxidation of $MeQH_2$ (100 μM) by laccase I (methylquinone production rate, 14.5 μM min^{-1}) in the absence (control) and presence of SOD (90 U ml^{-1}) and Mn^{2+} (100 μM). Means and 95% confidence limits are shown.

The hydroquinone-mediated mechanism for laccase generation of $O_2^{\cdot -}$ represents an alternative to those described for ligninolytic peroxidases (5, 42, 47). In the case of lignin degradation, it has been suggested that the $O_2^{\cdot -}$ formed during autoxidation of aromatic radicals can be subsequently involved in aromatic ring breakdown (46). As described here, another reaction of $O_2^{\cdot -}$ could be Mn^{2+} oxidation (2), as an alternative to peroxidase-mediated oxidation. Mn^{2+} oxidation by *T. versicolor* laccase in the presence of phenolic substrates had been reported by Archibald and Roy (1). Whether some active oxygen species are involved in this reaction, as described here for *P. eryngii* laccase in the presence of hydroquinones, has still to be investigated. In the light of the results presented, the role of laccase in lignin degradation deserves additional research since it could be responsible for oxidation of both phenolic and nonphenolic units and generation of active oxygen species.

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