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The white-rot fungus *Pleurotus ostreatus* secretes laccase isozymes with different substrate specificities

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Abstract: Four laccase isozymes (LCC1, LCC2, LCC3 and LCC4) synthesized by *Pleurotus ostreatus* strain V-184 were purified and characterized. LCC1 and LCC2 have molecular masses of about 60 and 65 kDa and exhibited the same pI value (3.0). Their N termini were sequenced, revealing the same amino acid sequence and homology with laccases from other microorganisms. Laccases LCC3 and LCC4 were characterized by SDS-PAGE, estimating their molecular masses around 80 and 82 kDa, respectively. By native isoelectrofocusing, their pI values were 4.7 and 4.5, respectively. When staining with ABTS and guaiacol in native polyacrilamide gels, different specificities were observed for LCC1/LCC2 and LCC3/LCC4 isozymes.

Key words: ABTS, guaiacol, isozyme, N-terminal sequence, *Pleurotus*, phenoloxidase

INTRODUCTION

White-rot basidiomycetes are microorganisms able to degrade lignin efficiently. However, the degree of lignin degradation with respect to other wood components largely depends on the environmental conditions and the fungal species involved. Studies aimed at understanding the mechanisms of lignin degradation by fungi have revealed the complexity of the enzymatic systems because there is more than one path to lignin degradation and the enzymatic machinery of the various microorganisms is different (Hatakka 1994).

Two classes of oxidative enzymes, namely laccases (phenoloxidases) (Thurston 1994, Leonowicz et al 2001) and peroxidases (lignin and manganese peroxidase) (Farrell et al 1989, Datta et al 1991, Reddy 1993, Reddy and D'Souza 1994, Cullen 1997) have received the greatest attention. The role of laccases recently has been reevaluated because new information on their biodegradative mechanisms has been obtained in several fungal species (Bourbonnais and Paice 1990, 1992, Archibald and Roy 1992, Leonowicz et al 2001). Moreover, some genera of basidiomycetes, such as *Pleurotus* spp., were found to lack lignin peroxidases (Fukushima and Kirk 1995, Galliano et al 1988, 1991), indicating that different enzymes are probably involved in lignin biodegradation and that, among these enzymes, laccases could play a key role. Studies on the enzymes secreted by the basidiomycete fungus *Pleurotus ostreatus* have shown that the concerted action of laccase and aryl-alcohol oxidase, produces significant reduction in the molecular mass of soluble lignosulphonates (Marzullo et al 1995).

The preferential degradation of lignin by *P. ostreatus* strain V-184 has been demonstrated in our laboratory, and the fungus has been shown to degrade wood and lignocellulosics efficiently (Delgado et al 1992, Ginterová et al 1992, Klibansky et al 1993). In the present work, we describe the identification and characterization of four laccase activities secreted by strain V-184 and report on differences in the reactivity toward specific substrates for laccase determinations among this family of isolated laccase isozymes.

MATERIALS AND METHODS

Microorganisms and culture media.—P. ostreatus strain V-184 was kindly supplied by Dr. A. Ginterová from the Feedstuff Institute in Bratislava, Slovakia; it was propagated on 2% malt-extract agar in our fungal collection.

Two liquid culture media were used: a complete medium described by Mansur et al (1997) and a synthetic medium, previously used to grow *Phanerochaete chrysosporium* (Kirk et al 1986). The pH of the synthetic medium was adjusted to 4.5 or 6.5 with 20 mM sodium-2,2-dimethylsuccinate and

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50 mM MES buffers, respectively. Cultures were incubated at 28 C in 500 mL Erlenmeyer flasks containing 150 mL of culture medium and agitated at 100 rpm for 14–16 days.

Protein, biomass and enzyme activity determinations.—The protein concentration was determined using the BioRad protein assay kit (BioRad, Hercules, California), following the manufacturer's instructions, with bovine serum albumin as standard. Biomass was estimated gravimetrically. Spectrophotometric assays of laccase activity were carried out with 1 mM guaiacol or 10 mM 2,2′-azino-bis-(3-ethylbenzthiazolinesulphonate) (ABTS) as substrates, in 100 mM sodium acetate buffer (pH 5.0). When guaiacol was used, the absorbance was monitored at 465 nm (ε = 12 000 M^{-1} cm^{-1}); when ABTS was the substrate, the absorbance was monitored at 436 nm (ε = 29 300 M^{-1} cm^{-1}). Aryl-alcohol oxidase activity (AAO) also was assayed spectrophotometrically, as the oxidation of veratryl (3,4-dimethoxybenzyl) alcohol to veratraldehyde, monitored at 465 nm (ε = 9300 M^{-1} cm^{-1}). The reaction mixtures contained 10 mM veratryl alcohol in 100 mM sodium phosphate, pH 6.0. One U of enzyme activity is defined as the amount of enzyme releasing 1 μmol.·min^{-1} oxidized product at 25 C in both enzymatic determinations. The lignin and manganese peroxidase activities were measured by the methods described in Mansur et al (1997). Colorimetric determinations were performed on a Shimadzu recording spectrophotometer (Model UV 160, Shimadzu, Kyoto, Japan). Each assay was done in triplicate.

Glucose concentration determination.—The glucose concentrations were determined by the glucose oxidase method (Lloyd and Whelan 1969).

Electrophoresis and isoelectric focusing.—SDS-PAGE was performed by the method of Laemmli (1970), using 12% polyacrylamide gels. Myosin (202 kDa), β-galactosidase (116.3 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumine (66.0 kDa) and ovalbumin (45 kDa) (BioRad, Milan, Italy) were used as standards. Gel staining was performed with either a silver staining kit (BioRad, Milan, Italy), or using first guaiacol and then ABTS at 1 mM and 10 mM respectively, in 100 mM sodium acetate buffer (pH 5.0) (Niku-Paavola et al 1990).

Analytical isoelectric focusing polyacrylamide gel electrophoresis (IEF) (Görg et al 1980) was performed with a mini-isoelectric focusing cell (Model 111, BioRad, Milan, Italy) by loading 0.2 μg of protein in 5% polyacrylamide gels containing 20% ampholytes (range 2.5–9.0) (BioRad, Milan, Italy). The anode and cathode solutions were 0.04 M aspartic acid and 0.1 M NaOH, respectively. The laccase isozymes were visualized by staining with 2 mM guaiacol in 0.1 M sodium acetate buffer (pH 5.0).

Purification of the extracellular activity of P. ostreatus *strain V-184.*—Supernatant (2 liters) from a 14-day-old culture was clarified by filtration through 45 μm filters (Millipore Corporation, Bedford, Massachusetts) and concentrated 10-fold by ultrafiltration in a BIO 2000 hollow fiber cartridge (BioFlow, Glasgow, United Kingdom) with a 10 kDa cut-off membrane. Proteins in the samples were precipitated with 85% ammonium sulfate, dissolved in 5 mL of 10 mM acetate buffer (pH 5.0), and dialyzed for 12 h against the same buffer (5 L). Crude enzyme samples (2 mL) were loaded onto a column of DEAE-Biogel (BioRad, Milan, Italy), equilibrated with 10 mM sodium acetate, pH 4.5. Proteins were eluted with a linear gradient of 0–1 M NaCl in the same buffer. The eluate was monitored for absorbance at 280 nm and laccase activity. The fractions with enzymatic activity were pooled, dialyzed against 10 mM sodium acetate, pH 4.5, and stored at −70 C.

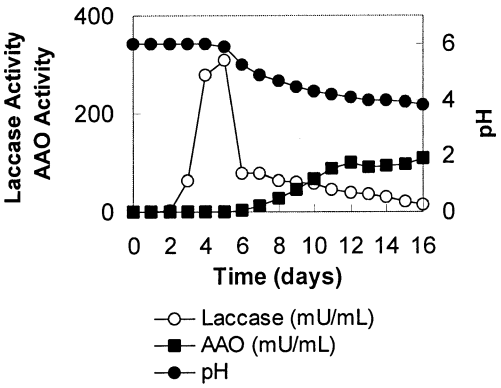

FIG. 1. Laccase and aryl-alcohol oxidase (AAO) activities produced by *Pleurotus ostreatus* strain V-184 in complete medium. Laccase activity was measured using ABTS as substrate. Note that enzymatic activities determinations were calculated in mU/mL.

Amino acid composition and sequence analysis.—Amino acid composition was determined with a Biotronik Photometer autoanalyzer (Model BT 7025, Cambridge, United Kingdom), after hydrolysis of 10 μg protein in 6 M HCl. The N-terminal sequences of laccases LCC1 and LCC2 were determined by automated Edman degradation in an Applied Biosystems 477A (Foster City, California) pulsed-liquid protein sequencer with 120A online phenylthiohydantoin analysis.

Statistics.—All experiments were performed two or more times, and the measurements were highly reproducible. The standard deviation in all the analytical assays was always less than 10%.

RESULTS

Ligninolytic activities.—Phenoloxidase and aryl-alcohol oxidase were the ligninolytic activities detected in the supernatants when *P. ostreatus* strain V-184 was grown in aerated cultures. Other ligninolytic activities, such as lignin and manganese peroxidases, were not detected under these experimental conditions (data not shown).

Aryl-alcohol oxidase activity was detected only when the strain V-184 was grown in complete medium under aeration. Maximal activity (100 mU/mL) was reached after 12 d of growth (FIG. 1). Laccase activity peaked on Day 5, reaching 310 mU/mL when the fungus was grown in complete medium. This lev-

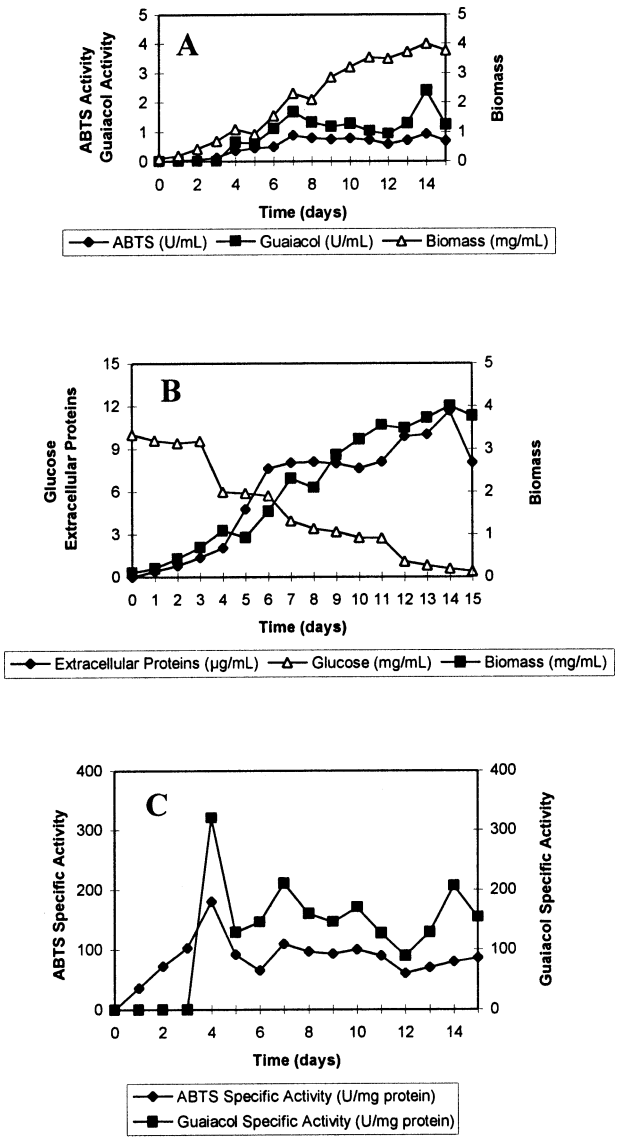

FIG. 2. Different parameters monitored during a 15 d growth of *P. ostreatus* strain V-184 in synthetic medium (pH 6.5).

A. Biomass estimated gravimetrically, and Laccase activity (U/mL) measured using ABTS and Guaiacol as substrates.

B. Extracellular Proteins, Glucose and Biomass measured during the experiment.

C. ABTS and Guaiacol Specific Activities (U/mg of total protein).

el of enzyme activity fits with a pH value around 6 (FIG. 1). After 5 d of growth, a decrease in pH was observed (from 6 at the beginning to 4 at the end of the growth), which was paralleled by a 10-fold decrease in the laccase activity level (from 310 mU/mL to 30 mU/mL; FIG. 1). This could indicate that laccase activity was related to the pH value of the culture medium. To confirm this, we performed experiments

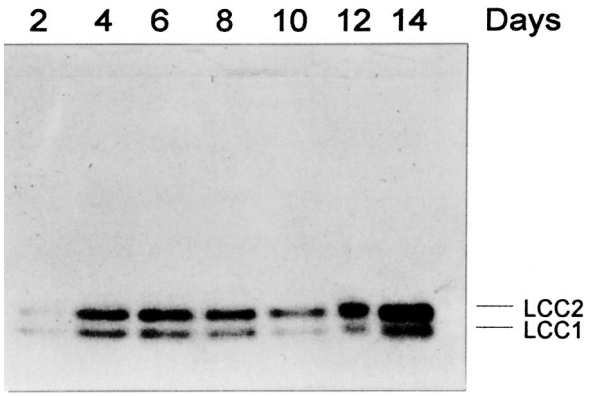

FIG. 3. Native 12% polyacrilamide gel electrophoresis, from samples of different days of growth using synthetic medium. The same amount of total extracellular protein was loaded in each lane. Staining was performed with 1 mM Guaiacol in 100 mM sodium acetate buffer (pH 5.0).

in synthetic medium at two different pH, 4.5 and 6.5. Laccase activity at pH 4.5 was low (data not shown). However, at pH 6.5, laccase activity was higher than those observed at pH 4.5, reaching 2.4 U/mL and 0.8 U/mL at the end of the experiments measured with guaiacol and ABTS as substrates, respectively (FIG. 2A).

Synthesis of laccases appeared to be constitutive (Scheel et al 2000) because total activity increased proportionally with the biomass production, measured as mycelial dry weight (FIG. 2A). The exponential growth extended from Day 2 to Day 14, which was accompanied with increased laccase production (FIG. 2A).

The extracellular protein concentration increased in the same way as the laccase activity during growth, mainly due to laccase production (FIG. 2B). This was confirmed when specific activities were calculated, reaching up to 180 and 300 U/mg of protein, with ABTS and guaiacol, respectively (FIG. 2C). Glucose, the only carbon source available to the culture, was consumed during the exponential growth, from a starting concentration of 10 mg/mL to 0.4–0.6 mg/mL at the end of the incubation period (FIG. 2B).

Proteins in supernatants from different days were analyzed by PAGE in native gels, which were stained either with guaiacol or ABTS. Curiously, only when the staining of the zymogram was performed with guaiacol, could we detect two well-differentiated bands, corresponding to two laccase isoforms. They were called laccase 1 (LCC1), the band with higher electrophoretic mobility, and laccase 2 (LCC2). The same pattern of enzyme bands was detected during the entire period of growth using synthetic medium, although the intensity of the bands increased at the end of the experiment (FIG. 3).

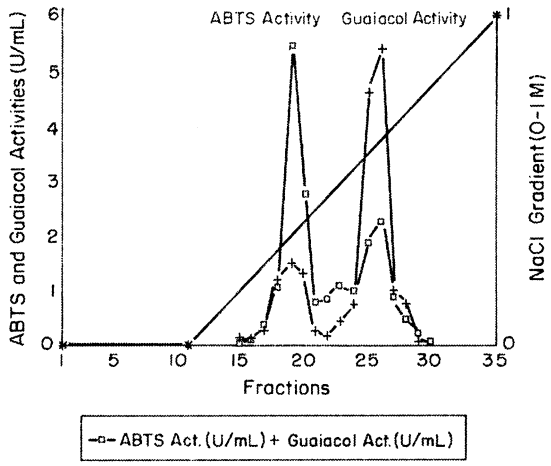

FIG. 4. Laccase activity (U/mL) measured with ABTS and Guaiacol from the two peaks detected when a linear gradient 0–1 M of NaCl was applied to DEAE-Biogel column. Fractions (2 mL) were collected.

Purification and characterization of the laccases.— When the supernatant from a 14-day-old culture was concentrated by ultrafiltration, followed by ammonium sulphate precipitation, two bands corresponding to new laccase isoforms were visible on a native electrophoresis gel. These two new bands were detected only when ABTS was used for the gel staining and were named laccase 3 (LCC3), and laccase 4 (LCC4) (FIG. 5A). When the total proteins were loaded on a DEAE column, two peaks showing laccase activity were eluted at 0.3 M and 0.6 M NaCl linear gradient in a DEAE column (FIG. 4). The fractions corresponding to each peak, were analyzed spectrophotometrically either with ABTS or guaiacol. It is of interest to note that the activity that eluted at 0.3 M NaCl showed much higher reactivity for ABTS than for guaiacol, whereas the second activity (eluted at 0.6 M NaCl) showed higher reactivity for guaiacol than for ABTS (FIG. 4). These results were in agreement with the enzymatic affinities observed when samples corresponding to each peak were analyzed in PAGE native gels (FIG. 5A).

Due to the strong binding of the laccase isoforms to the DEAE bed matrix, only one chromatographic step was necessary to purify each pair of enzymes to near homogeneity. The laccases were purified according to the procedure summarized in TABLE I. An 11.8-fold purification was achieved, with a final yield of 20.4%. Analytical IEF native gels were performed to further characterize the laccases present in the two peaks eluted from the DEAE column (FIG. 5B). When proteins were stained with guaiacol, two bands corresponded to LCC3 and LCC4, with a pI value of 4.7 and 4.3, respectively (FIG. 5B, lane 2). In the case of LCC1 and LCC2 (FIG. 5B, lane 3), there was only one very intense band with a pI of 3.0.

To estimate the monomeric molecular weight of the four laccase isoforms, each band was eluted and subjected to SDS-PAGE, the molecular weight of LCC1 and LCC2 were estimated at 60 and 65 kDa respectively (FIG. 5C), and 80 and 82 kDa for LCC3 and LCC4 (data not shown). Consequently, we conclude that, in our culture conditions using the synthetic medium at pH 6.5, we could identify up to four laccase isozymes with different mobility patterns in native gel electrophoresis, due to their different pI and molecular weights.

Finally, the purification procedure let us determine the N-terminal sequence of both LCC1 and LCC2. Each isoenzyme showed the same amino acid sequence, which when compared to other fungal laccases exhibited a number of highly conserved amino acids (TABLE II).

DISCUSSION

Laccase and other ligninolytic activities previously have been reported to be related to the stationary phase of growth in different fungi and that ligninolytic activities often are triggered due to nutrient lim-

TABLE I. Purification of laccases from *Pleurotus ostreatus* strain V-184

Purification step	Total extracellular protein (mg)	Total laccase activity* (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture supernatant	34.3	5355	156.1	100	1
Ultrafiltration (10-kDa filter)	14.0	6730	480.7	125	3
$(NH_4)_2SO_4$ precipitation	4.0	3035	758.8	57	5
DEAE-Biogel eluate					
1st peak	0.2	840	4200	15	
2nd peak	0.4	290	725	5.4	
Total	0.6	1130	1883	20.4	11.8

* Laccase activity was measured using ABTS as substrate.

TABLE II. Comparison of N-terminal amino acid sequences of the two laccase isoenzymes LCC1 and LCC2 from *P. ostreatus* with those of other fungal laccases

Microorganism	N-terminal amino acid sequence 1 5 10 15
Pleurotus ostreatus LCC1 and LCC2	A T Y X L L N V L I X L N - -
Pleurotus ostreatus laccase I	A I G P T G D M Y I V N E D V
Pleurotus ostreatus laccase II	A I G P A G N M Y I V N E D V
Pleurotus eryngii laccase I	A X K K L - D F H I I N N - -
Pleurotus eryngii laccase II	A T K K L - D F H I I N N - -
Agaricus bisporus laccase I	K T R - T F D F D L V N T - -
Agaricus bisporus laccase II	D T K - T F N F D L V N T - -
Schyzophyllum commune	A L G P V G N L P I V N K E I
Basidiomycete PM1 laccase	S I G P V A D L T I S N G A V
Phlebia radiata laccase	S I G P V T D F H I V N A A V
Coriolopsis gallica laccase	S I G P V A - L T I S N - V -
Trametes villosa laccase I	G I G P V A D L T I T N A A V
Trametes villosa laccase II	A I G P V A S L V V A N A P V
Trametes villosa lcc3	S I G P V T E L D I V N K V I
Trametes villosa lcc4	A I G P V T D L T I S N G D V
Trametes villosa lcc5	A I G P V T D L T I S N A D V
Trametes versicolor laccase I	A I G P V A S L V V A N A P V
Trametes versicolor laccase II	G I G P V A D L T I T N A A V
Trametes versicolor laccIIIc	G I G P V A D L T I T N A E V
*Trametes sp. Lcc1*I	A V G - E A D L T I T N A V V
Trametes sp. Laccase II	S I G P V A D M T I S N A E V
Trametes sp. Laccase III	A V G P V T D L T I S N A N V
Trametes trogii	A I G P V A D L V I S N G A V
Ceriporiopsis subvermispora laccase	A I G P V T D L E I T D A F V
Pycnoporus cinnabarinus	A I G P V A D L T L T N A A V
Pycnoporus cinnabarinus lcc3-2	A I G P K A D L V I S D A V V

The data for *P. ostreatus*, *P. eryngii*, *A. bisporus*, *S. commune*, basidiomycete PM1, *P. radiata*, *C. gallica*, *T. villosa*, *T. versicolor*, *Trametes* sp., *T. trogii*, *C. subvermispora*, and *P. cinnabarinus* were obtained from Giardina et al. 1995, 1996; Muñoz et al. 1997; Perry et al. 1993; Hatamoto et al. 1999; Coll et al. 1993; Saloheimo et al. 1991; Calvo et al. 1998; Yaver et al. 1996; Yaver and Golightly 1996; Bourbonnais et al. 1995; Iimura et al. 1992; Mansur et al. 1997; Garzillo et al. 1998; Fukushima and Kirk, 1995; Eggert et al. 1996; Temp et al. 1999, respectively. The X corresponds to indeterminations. Dashes indicate gaps introduced to maximize alignment.

itation (Kirk and Farrell 1987, Higuchi 1990, Cullen 1997). Our results indicate that another mechanism might govern the production of laccase activity during the exponential growth phase, especially when this activity appears to be closely correlated with biomass production. The time course of laccase production in synthetic medium at pH 6.5, showed significant differences with respect to other laccases from *P. ostreatus* (Palmieri et al 1997). The maximum laccase activity was reached later during the fungal growth (FIG. 2A, Day 14) with respect to that of *Pleurotus ostreatus* laccases (Palmieri et al 1997). This behavior suggests a different physiological role for these enzymes in the two different *Pleurotus* species. It is known that a low pH is preferable for ligninolytic enzymes production (Kirk and Farrell 1987, Higuchi 1990, Cullen 1997). However, our results showed that a low pH is detrimental for the production of active laccase, perhaps due to their susceptibility to acidic proteases.

In addition to laccases, *P. ostreatus* strain V-184 also produces AAO activity (FIG. 1) in agreement with the results obtained from *P. eryngii* (Guillén et al 1992). In *Pleurotus* species, AAO appears to be constitutive because it is produced in different growth phases and culture conditions (Guillén et al 1992). The relationship between AAO and laccase activities in strain V-184 is still unknown. It is possible that other enzymes, not detected in this assay, could act synergically with AAO and laccase in the degradation of lignin.

Differences in the reactivity of laccases from either the same or different species toward various substrates have been observed (de Vries et al 1986, Sterjiades et al 1993) despite the highly conserved catalytic copper sites found in all known laccase sequences (Thurston 1994). This has been demonstrated

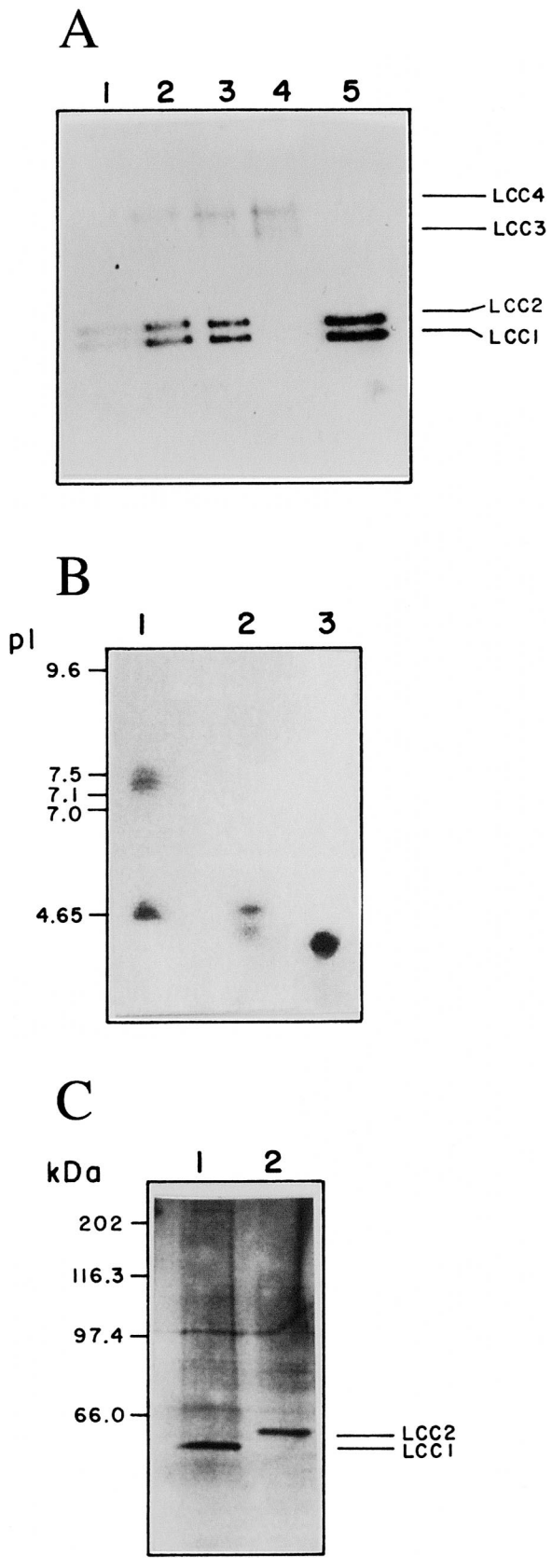

FIG. 5. Biochemical characterization of different laccases produced by a 15-day-old culture of *P. ostreatus* strain V-184 in synthetic medium at pH 6.5.

here for laccases LCC1-LCC2 and LCC3-LCC4, which clearly show different substrate affinities (FIGS. 4, 5B). Determination of more laccase amino acid sequences will help to develop a better understanding of the structure-function relationships that govern substrate specificities and functions of laccases in different biological systems. The recent reports of two new classes of laccases, named "white" (Palmieri et al 1997) and "yellow" (Leontievsky et al 1997a, b), let us say that much more complex mechanisms of action may exist within the same laccase family of enzymes, assuming that yellow laccase is formed as a result of blue laccase modification by products of lignin degradation.

Laccases from *P. ostreatus* strain V-184 were separated into four isoforms, which show different elution properties and mobilities in SDS-PAGE, native PAGE and native IEF. The four laccases exhibit similar characteristics to those of known laccases from other fungi. In fact, molecular mass, pI and N terminus sequence lie well within the range determined for other laccases (Thurston 1994). Because the N terminus sequence from LCC1 and LCC2 are identical and the molecular mass of LCC1 (60 kDa) is smaller than that of LCC2 (65 kDa), they may differ only in their glycosidation pattern. The identical sequences of the N termini let us also suggest that both can be encoded by the same gene. However, the N termini from LCC1 and LCC2 differ greatly from those of other fungal laccases (TABLE II). It is worth

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A. Native 12% polyacrilamide gel electrophoresis of samples from different purification steps.

1. Culture supernatant. 2. Concentrated ultrafiltrate. 3. $(NH_4)_2SO_4$ precipitate. 4. First peak eluted at 0.3 M NaCl from the DEAE-Biogel (laccases LCC3 and LCC4). 5. Second peak eluted at 0.6 M NaCl from the DEAE-Biogel, (laccases LCC1 and LCC2). The same amount of total extracellular protein was loaded in each lane. The gel was stained first with Guaiacol and then with ABTS (10 mM and 1 mM in 100 mM sodium acetate buffer pH 5.0, respectively).

B. Native IEF from samples of the two peaks with laccase activity eluted from the DEAE-Biogel column. 1. IEF standards. 2. Sample from the first eluted peak. 3. Sample from the second eluted peak. The gel was stained only with guaiacol (10 mM in 100 mM sodium acetate buffer pH 5.0).

C. Silver stained 12% polyacrilamide gel of laccases LCC1 and LCC2. LCC1 and LCC2 eluted from polyacrilamide native gel, were loaded in lanes 1 and 2, respectively. Myosin (202 kDa), β-galactosidase (116.3 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumine (66.0 kDa) and ovalbumin (45 kDa) were used as standards.

noting that the laccase isozymes among strains from the same species (*P. ostreatus*) and *P. eryngii*, have only three amino acids in common. Nevertheless, it is interesting to point out the highly conserved hydrophobic amino acids in position 10 and the conserved Asn in position 12 (TABLE II).

The characteristics of LCC1 and LCC2 of *P. ostreatus* strain V-184 are typical among fungal laccases. Most of them are monomeric proteins with molecular masses between 50 and 80 kDa (Bollag and Leonowicz 1984, Thurston 1994, Yaropolov et al 1994, Mayer and Staples 2002). Some exceptions include the laccases from *Agaricus bisporus* (Wood 1980) and *Trametes villosa* (Yaver and Golightly 1996), which present two subunits, and laccase I from *Podospora anserina* (Durrens 1981), which is composed of four subunits.

The results presented here for *P. ostreatus* strain V-184 laccases are consistent with the hypothesis that these phenoloxidases also have a wide range of substrate specificity in vivo. Hydroxylation of lignin substructures may represent a strategy to assist laccases in the biodegradation of lignin in vivo. Moreover, the possibility of converting recalcitrant molecules in efficiently degraded substrates could assist in optimizing the potential biotechnological applications of this class of enzymes.

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