APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1993, p. 2607–2613
0099-2240/93/082607-07$02.00/0

Vol. 59, No. 8

Purification and Characterization of a Phenoloxidase (Laccase) from the Lignin-Degrading Basidiomycete PM1 (CECT 2971)

PEDRO M. COLL, JOSE M. FERNÁNDEZ-ABALOS, JULIO R. VILLANUEVA, RAMÓN SANTAMARÍA, AND PILAR PÉREZ*

Instituto de Microbiología Bioquímica, Consejo Superior de Investigaciones Científicas, and Facultad de Biología, Universidad de Salamanca, 37008 Salamanca, Spain

Received 19 February 1993/Accepted 11 May 1993

A new lignin-degrading basidiomycete, strain PM1 (= CECT 2971), was isolated from the wastewater of a paper factory. The major ligninolytic activity detected in the basidiomycete PM1 culture supernatant was a phenoloxidase (laccase). This activity was produced constitutively in defined or complex media and appeared as two protein bands in native gel electrophoresis preparations. No enzyme induction was found after treatment with certain potential laccase inducers. Laccase I was purified to homogeneity by gel filtration chromatography, anion-exchange chromatography, and hydrophobicity chromatography. The enzyme is a monomeric glycoprotein containing 6.5% carbohydrate and having a molecular weight of 64,000. It has an isoelectric point of 3.6, it is stable in a pH range from 3 to 9, and its optimum pH is 4.5. The laccase optimal reaction temperature is 80°C, the laccase is stable for 1 h at 60°C, and its activity increases with temperature. Spectroscopic analysis revealed that the enzyme has four bound copper atoms, a type I copper, a type II copper, and a type III binuclear copper. The amino-terminal sequence of the protein is very similar to the amino-terminal sequences of laccases from *Coriolus hirsutus* and *Phlebia radiata*.

Lignin is a structurally complex aromatic biopolymer which is a major component of woody plants. It is extremely resistant to attack by most microorganisms, and chemical degradation of lignin has been the only method used for pulping in the paper-making process. In addition, the pulp and paper industry generates diluted effluents containing lignin derivatives and other phenolic compounds; these are often toxic and give the effluents their characteristic brown color. Such compounds are difficult to remove by currently available technology. Research on ligninolytic microorganisms has intensified in recent years because of their potential applications (5, 37). Microorganisms able to degrade lignin include the wood-rotting fungi (3) and, to a lesser extent, certain actinomycetes and bacteria (20). The extracellular enzymes of some white rot fungi, particularly ligninases (peroxidases) (20) and laccases (6, 18), have been shown to be highly effective in degrading lignin and lignin derivatives. Laccase (benzenediol:oxygen oxidoreductase; EC 1.10.3.2.) is a multicopper blue oxidase capable of oxidizing *ortho*- and *para*-diphenols and aromatic amines by removing an electron and a proton from a hydroxyl group to form a free radical. Laccases are widespread in nature; they have been found in many plants and fungal species (27). Several laccases have been purified and characterized biochemically (19, 44, 46), and some of the genes encoding the laccases have also been cloned and genetically characterized (14, 21, 40). The biological functions of these enzymes remain unclear; they have been implicated in different processes, such as sporulation (23), pigment production (8), rhizomorph formation (47), and lignin degradation (6, 7, 18). Even though the role of laccases in the process of lignin degradation by white rot fungi remains unclear, the latest results suggest that these enzymes are more important than was initially thought. Laccase III from *Coriolus versicolor* is able to depolymerize a preparation of soluble lignin in water (30), and it is able to oxidize veratryl alcohol, nonphenolic lignin model compounds (6), and kraft lignin (7) in the presence of certain intermediate compounds. Laccases can also catalyze the oxidative polymerization of the phenolic compounds derived from lignin, which are then easily removed (5).

In this paper we describe the isolation of a lignin-degrading basidiomycete, strain PM1 (= CECT 2971), and the purification and characterization of the major laccase released by this organism into the culture supernatant (laccase I). Basidiomycete PM1 has not been clearly classified yet because it does not sporulate. The behavior of the fungus in culture and the characteristics of the laccase closely resemble the behavior and characteristics of members of the genus *Coriolus* and their laccases. Basidiomycete PM1 laccase was found to contain the four copper ions of three different types which form the redox center of some multicopper oxidases of eukaryotic species (38). The N terminus of the PM1 laccase is very similar to the N termini of *Coriolus hirsutus* and *Phlebia radiata* laccases (21, 40).

MATERIALS AND METHODS

Organisms. Basidiomycete PM1, which has been deposited in the Spanish Type Culture Collection as strain CECT 2971 and in the British National Collection of Wood Rotting Macrofungi as strain FPRL B873, was isolated from wastewater from the paper factory of the Empresa Nacional de Celulosa, Miranda de Ebro, Burgos, Spain. *C. versicolor* was a gift from F. Laborda, and *Phanerochaete chrysosporium* BKM-F 1767 (= ATCC 24725) was a gift from T. K. Kirk. *Pleurotus eryngii* A180, *Heterobasidion annosum* A198, *Fomes fomentarius* A166, and *Schizophyllum commune* A111 were gifts from A. Martínez.

Culture conditions. Screening for lignin-degrading micro-

* Corresponding author.

organisms was done on plates containing different solid culture media supplemented with 0.5% kraft lignin (Induline AT; Sigma). Several media were used. GAE medium contained (per liter) 10 g of glucose, 1 g of asparagine, 0.5 g of yeast extract, 0.5 g of K_2HPO_4, 1 g of $MgSO_4 \cdot 7H_2O$, and 0.01 g of $FeSO_4 \cdot 7H_2O$. Basal medium I was similar to the medium used for *Phanerochaete chrysosporium* ligninase production (32); it contained (per liter) 10 g of glucose, 1.32 g of ammonium tartrate (7.2 mM), 0.2 g of KH_2PO_4, 50 mg of $MgSO_4 \cdot 7H_2O$, 10 mg of $CaCl_2$, 10 μg of thiamine, and 1 ml of a solution containing (per liter) 3 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of $MnSO_4 \cdot H_2O$, 1 g of NaCl, 100 mg of $FeSO_4 \cdot 7H_2O$, 185 mg of $CoCl_2 \cdot 6H_2O$, 80 mg of $CaCl_2$, 180 mg of $ZnSO_4 \cdot 7H_2O$, 10 mg of $CuSO_4 \cdot 5H_2O$, 10 mg of $AIK(SO_4)_2$, 10 mg of H_3BO_3, 12 mg of $Na_2MoO_4 \cdot 2H_2O$, and 1.5 g of nitrilotriacetate. The pH of basal medium I was adjusted to pH 4.5 with 20 mM dimethylsuccinate or to pH 6.5 with 50 mM MES (morpholineethanesulfonic acid). We also used basal medium II, which is the medium used for *C. versicolor* ligninase production (10). Cultures grown under nitrogen-limiting conditions contained 2.2 mM ammonium tartrate and 1% glucose. When the carbon source was limiting, the concentration of glucose was decreased to 0.2%.

Plates were incubated at 24 to 28°C for several days. Stock cultures were maintained on solid potato dextrose medium (GIBCO) and Czapec Dox medium, which contained (per liter) 30 g of sucrose, 2 g of $NaNO_3$, 1 g of K_2HPO_4, 0.5 g of $MgSO_4$, 0.5 g of KCl, 0.01 g of $FeSO_4 \cdot 7H_2O$, and 20 g of agar.

The liquid medium used for laccase production was GAE medium. To produce an inoculum, agar cubes cut from basidiomycete PM1-colonized potato dextrose agar plates were incubated in GAE medium. Then, the total contents of one flask were homogenized in distilled water and used to inoculate other flasks. Cultures were incubated at 37°C with or without shaking for 7 to 45 days. When potential laccase inducers were used, they were dissolved in 50% ethanol, sterilized by filtration, and added to the medium at different times, and the medium was gently mixed. Unless otherwise stated, the final concentration of each inducer was 1 mM. The final concentration of ethanol in the medium was always less than 0.5%, and an equivalent amount of ethanol was added to control flasks without inducer.

The other media used were YED medium (10 g of glucose per liter, 10 g of yeast extract per liter) and YEPD medium (YED medium containing 20 g of peptone per liter).

Assay for laccase activity. Lignin dephenolization in solid medium was detected as described elsewhere (45). Laccase activity was visualized on plates containing 0.02% guaiacol since laccase catalyzes the oxidative polymerization of guaiacol to form reddish brown zones in the medium.

Laccase activities in the supernatants of fungal cultures in liquid medium were determined in 50 mM sodium acetate buffer (pH 4.5), containing 1 mM guaiacol as the substrate at an incubation temperature of 37°C; 1 U of enzyme activity was defined as the amount of the enzyme which elicited an increase in A_{465} of 1 U/min. Laccase activity was also assayed in native polyacrylamide gel electrophoresis (PAGE) by soaking the gel in 50 mM sodium acetate buffer (pH 4.5) containing 2 mM guaiacol. After several minutes enzymatic bands with a brown color appeared because of the polymerization of guaiacol.

Purification of the extracellular laccase I from basidiomycete PM1. All purification steps were carried out at 4°C. The supernatant from a 40-day static culture (2 liters) of basidiomycete PM1 was filtered, concentrated 65 times, and dialyzed against 20 mM histidine-HCl buffer (pH 6.5) in Amicon ultrafiltration cells by using type YM30 membranes. The concentrated supernatant was then applied to a Sephacryl S-200 column (1.5 by 100 cm; Pharmacia) preequilibrated with 20 mM histidine-HCl buffer (pH 6.5) and eluted at a flow rate of 10 ml h^{-1}. The fractions containing laccase activity were pooled, concentrated by ultrafiltration with a Centricon 30 microconcentrator, and loaded onto an ion-exchange Mono Q HR 5/5 column (5 by 50 mm) in fast-performance liquid chromatography (FPLC) equilibrated with the same buffer. The laccase was eluted with a linear gradient of 0.0 to 0.2 M NaCl for 15 min at a flow rate of 1 ml min^{-1}, and 1-ml fractions were collected. The active fractions were dialyzed against 20 mM histidine-HCl buffer (pH 6.5) containing 1.7 M ammonium sulfate and were applied to a hydrophobicity Phenyl Superose HR 5/5 column (50 by 5 mm) in FPLC. The laccase was eluted with a linear gradient of 1.7 to 0.0 M ammonium sulfate for 30 min at a flow rate of 0.5 ml min^{-1}, and 0.5-ml fractions were collected. Enzyme purity was confirmed by sodium dodecyl sulfate (SDS)-PAGE.

PAGE of proteins. SDS-PAGE on 12% (wt/vol) polyacrylamide gels was performed by the method of Laemmli (22). Samples were treated with 1% SDS and 2% dithiothreitol and boiled at 100°C for 5 min. Proteins were visualized by silver staining (31). Nondenaturing electrophoresis was performed under the same conditions, except that SDS and dithiothreitol were omitted and samples were not boiled. In nondenaturing gels laccase activity was detected by incubating the gels with 2 mM guaiacol in 50 mM sodium acetate buffer (pH 4.5).

Isoelectric point determination. Analytical isoelectric focusing PAGE was performed with a mini-isoelectric focusing cell (model 111; Bio-Rad) by using 0.2-μg samples of purified laccase I in 5% polyacrylamide gels with a pH gradient of 2.5 to 5. Protein was visualized by silver staining or with 2 mM guaiacol in 50 mM sodium acetate buffer (pH 4.5). Markers for pH 2.5 to 6.5 were used (isoelectric focusing calibration kit obtained from Pharmacia).

Glycoprotein test. Denatured purified laccase (1 mg) was treated with 35 mU of endoglycosidase H (endo-β-*N*-acetylglucosaminidase; EC 3.2.1.96) from *Streptomyces plicatus* to eliminate possible N-linked carbohydrate and was analyzed by SDS-PAGE.

Total carbohydrate contents were determined by the phenol-sulfuric acid method (1).

Copper content. Purified enzyme was concentrated (175 μg/ml) and dialyzed against deionized water by ultrafiltration through Amicon YM30 filters, and the copper content was determined by atomic absorption spectrometry, using the graphite furnace technique and a Hitachi model Z-8000 spectrometer.

Laccase absorbance at different wavelengths was measured with a Hitachi model U-2000 spectrophotometer.

Amino-terminal amino acid sequence. The amino-terminal amino acid sequence of purified laccase was determined by stepwise Edman degradation (11) with a gas phase sequencer (Applied Biosystems model 470A); the amino acids were identified by high-pressure liquid chromatography of the phenylthiohydantoil derivatives of the amino acids obtained from the sequencer.

Enzyme characterization. Estimates of the laccase optimum pH were obtained by using 50 mM acetate buffer (pH 3.6 to 5.5) and 50 mM citrate-phosphate buffer (pH 2.6 to 7). pH stability was assayed in the pH range from 2 to 12 by using 50 mM citrate-phosphate-borate buffer. The optimum temperature and thermal stability were determined in the 20

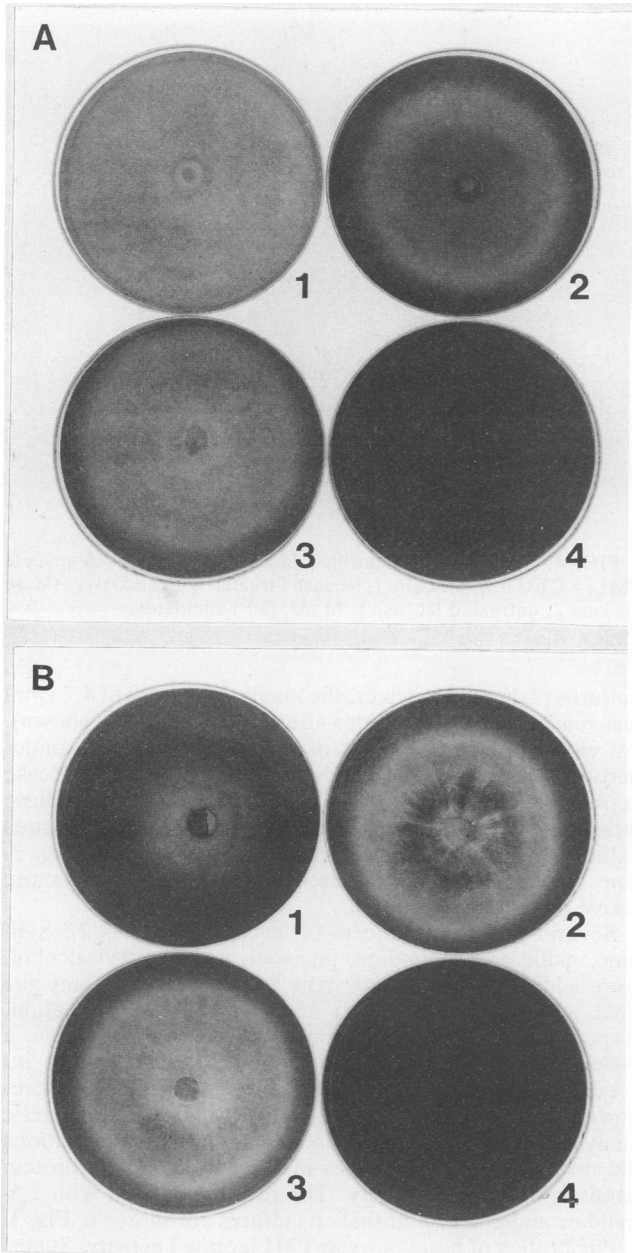

FIG. 1. Decolorization of kraft lignin by *Phanerochaete chrysosporium* (plates 1), *C. versicolor* (plates 2), and basidiomycete PM1 (= CECT 2971) (plates 3) in basal medium I (A) or GAE medium (B). Plates 4 were control plates without fungi.

to 90°C range. The K_m value was determined from Lineweaver-Burk plots by using guaiacol concentrations of 0.05 to 10 mM. The effects of several potential inhibitors on enzyme activity were determined by using concentrations of 0.005 to 10 mM. The level of enzyme activity determined in the absence of additive was considered to be 100%.

Other procedures. To estimate the M_r under native conditions, gel filtration chromatography was carried out by using a Sephacryl S-200 column (1.5 by 100 cm; Pharmacia) previously calibrated with proteins having known molecular masses as the standards.

Protein concentrations were determined as described previously (25).

RESULTS

Isolation and culture of ligninolytic microorganisms. Screening for lignin-degrading microorganisms was done in samples of the effluent wastewater from a paper factory (Empresa Nacional de Celulosa, Miranda de Ebro, Burgos, Spain). Samples diluted 1:10 or 1:50 were inoculated onto plates containing different solid media, including basal medium I and basal medium II prepared at pH 4.5 or 6.5 under conditions of nitrogen or carbon source limitation and GAE medium (pH 7.0). All media were supplemented with 0.05% kraft lignin. The plates were incubated at 28 or 37°C. Several fungi were isolated, and their ligninolytic activities were determined by using the dephenolization test of Sundman and Näse (45). The dephenolization produced by the different fungi was always compared with the dephenolization produced by *C. versicolor* and *Phanerochaete chrysosporium*.

Five potential ligninolytic fungi were isolated: PM1, PM2, PM3, PM4, and PM5. However only one of these organisms, PM1, was able to modify kraft lignin to the same extent as *C. versicolor* or *Phanerochaete chrysosporium*. The culture conditions required by PM1 were similar to those required by *C. versicolor*, except that PM1 grew better at pH 6.5. Both fungi caused kraft lignin dephenolization in basal medium I (Fig. 1A, plates 2 and 3) and in GAE medium (Fig. 1B, plates 2 and 3), while *Phanerochaete chrysosporium* caused greater dephenolization when it was grown in basal medium I (pH 4.5) (Fig. 1A, plate 1) but not when it was grown in GAE medium (pH 7.0) (Fig. 1B, plate 1).

A second screening was performed on plates containing basal medium I at pH 4.5 or 6.5 supplemented with 0.02%

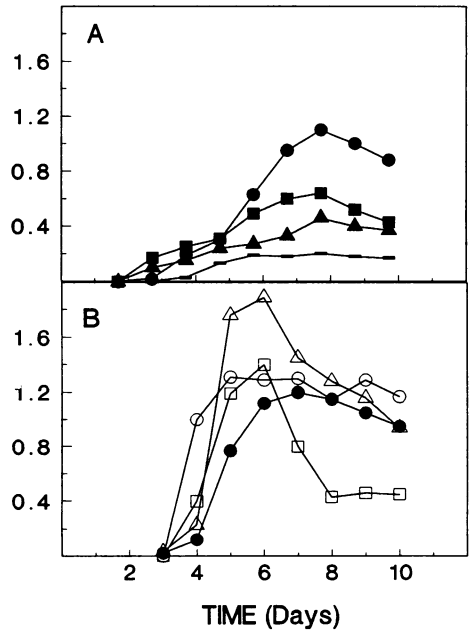

FIG. 2. Laccase activities in supernatants from basidiomycete PM1 (= CECT 2971) cultures grown in different media. (A) Symbols: ●, GAE medium; ■, YED medium; ▲, YEPD medium; ▬, basal medium I. (B) Symbols: ●, GAE medium; □, GAE medium containing 2% Tween 80; ○, GAE medium in an oxygen atmosphere; △, GAE medium containing 2% Tween 80 in an oxygen atmosphere.

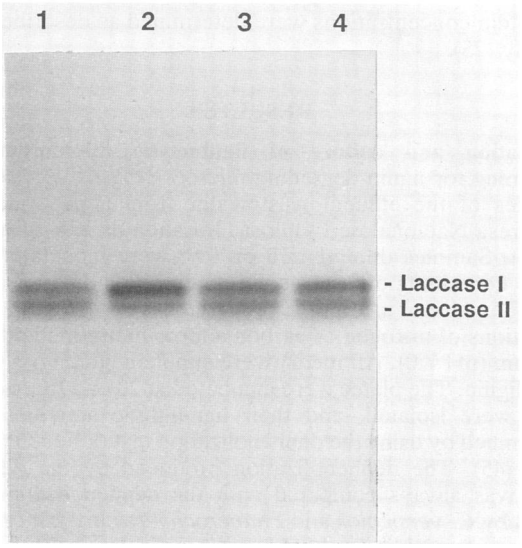

FIG. 3. Native PAGE gel developed to determine laccase activities in supernatants from basidiomycete PM1 (= CECT 2971) cultures grown statically in GAE medium (lane 2) or from shaken cultures in GAE medium alone (lane 1) or with 2,5-xylidine (lane 3) or guaiacol (lane 4).

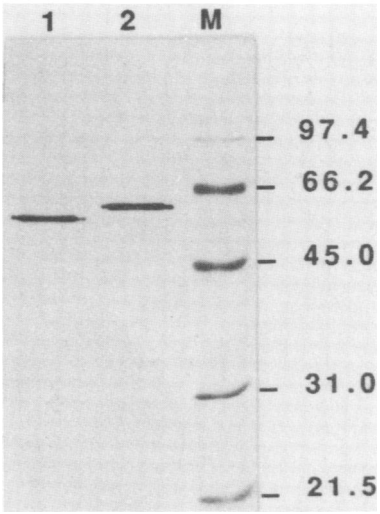

FIG. 4. SDS-PAGE of purified laccase I from basidiomycete PM1 (= CECT 2971). Lane 1, laccase I treated with endoglycosidase H; lane 2, untreated laccase I. M, M_r (10^3) standards.

Poly R-478 (Sigma), a high-molecular-weight polymeric dye used to detect extracellular oxidases. Ligninase and manganese peroxidase decolorize this dye (15). *Phanerochaete chrysosporium* caused maximum decolorization of Poly R-478 at pH 4.5, but it did not decolorize it at pH 6.5. *C. versicolor* and PM1 were able to decolorize Poly R-478 at both pHs, while the other fungi used, including *Pleurotus eryngii* A180, *H. annosum* A198, and *Schizophyllum commune* A111, did not cause any decolorization at all (data not shown).

Fungus strain PM1 was considered a basidiomycete because as determined by electron microscopy it showed doliporus in the septa, although it was impossible to classify PM1 by spore analysis. Using the method described by Stalpers (43), we selected the following characteristics: the presence of laccase, white colonies, a growth rate of 40 to 70 mm in 14 days, colorless colony reverse, crystals in the aerial mycelia, the absence of tyrosinase, and the absence of clamp connections. The most similar organism on the basis of these criteria was *Polyporus hydnoides* (Aphyllophorales). Basidiomycete PM1 has been deposited in the Spanish Type Culture Collection (Coleccion Española de Cultivos Tipo) as strain CECT 2971 and in the British National Collection of Wood Rotting Macrofungi as strain FPRL B873.

Laccase activity of basidiomycete PM1. When basidiomycete PM1 was grown in liquid culture, with or without shaking, phenoloxidase was the only ligninolytic activity detected in the supernatant. Other ligninolytic activities, such as ligninase and manganese peroxidase activities, were assayed as described previously (13, 29) in different culture media (basal medium I and basal medium II prepared at pH 4.5 or 6.5 under conditions of nitrogen or carbon source limitation and GAE medium at pH 7.0) but were not detected. Laccase was produced constitutively; total activity increased as mycelial dry weight increased. Different media were assayed for laccase production, and the highest level of activity was obtained with GAE medium (Fig. 2A). The addition of 0.2% Tween 80 or an oxygen atmosphere slightly increased laccase activity (Fig. 2B) in short-term shaken cultures (7 days). However, the maximal activity of 4.7 U/ml was reached in static cultures after 30 days (data not shown). An electrophoretic analysis of culture supernatants under native conditions revealed two possible bands with laccase activity, as determined with 2 mM guaiacol (Fig. 3); these were designated laccase I and laccase II. Shaken cultures exhibited the same level of activity in both bands (Fig. 3, lane 1), while in static cultures the upper band was more active (Fig. 3, lane 2).

Several potential inducers of laccases, such as 2,5-xylidine, gallic acid, toluidine, guaiacol, and veratryl alcohol, were added to shaken and static cultures of basidiomycete PM1 in GAE medium after 85 and 185 h of incubation, respectively, and laccase activity was measured over a 7-week period. None of the compounds significantly increased the laccase activity, in contrast to the results reported previously for other fungi (4), and electrophoretic analysis of the culture supernatants under native conditions did not disclose any changes in the pattern of the protein bands with laccase activity. The results obtained with 2,5-xylidine and guaiacol in shaken cultures are shown in Fig. 3.

Purification of basidiomycete PM1 laccase I activity. Supernatant (2 liters) from 40-day static cultures was concentrated and dialyzed, and this crude enzyme preparation was filtered on a Sephacryl S-200 column. The fractions with laccase activity were then purified in FPLC by using a Mono Q anion-exchange column (see Materials and Methods), where

TABLE 1. Purification of laccase I activity from basidiomycete PM1 (= CECT 2971)

Step	Vol (ml)	Protein concn (mg/ml)	Total activity (U)	Sp act (U/mg)	Yield (%)	Purification (fold)
Culture supernatant	2,050.00	0.27	9,020	16	100	
Ultrafiltration	12.85	11.14	12,615	88	140	5.5
Sephacryl S-200	4.42	12.98	7,940	138	88	8.6
Mono Q	1.40	8.19	4,690	409	52	25.5
Phenyl Superose	0.72	7.80	3,325	592	37	37.0

TABLE 2. Properties of purified laccase I from basidiomycete PM1 and other fungal laccases

Laccase source	M_r (10^3)	% Glycosylation	No. of copper atoms	pI	Optimum pH	pH stability	Stable temp (°C)	Reference(s)
PM1	64.0	6.5	4	3.6	4.5	3–9	60	
C. versicolor	63.0		4	3.0	4–5	7–8	50	44
C. hirsutus	63.0	15	4	3.6	4.5	6–9	50	21, 44
Phlebia radiata	64.0	5–14	2	3.9	4.5			19, 33, 40
Agaricus bisporus	100.0	15	2	3.4–4.0	5.6			46
Armillaria mellea	59.0			4.1	3.5			36

the laccase activity eluted as a single peak in the fractions corresponding to 0.13 to 0.16 M NaCl. There was always a small fraction of laccase activity (<10%) retained in the column, and it eluted at 1 M NaCl.

Further purification was accomplished with a hydrophobicity Phenyl Superose column, from which laccase activity eluted as a single peak in the fractions corresponding to 0.46 to 0.26 M ammonium sulfate. Laccase I appeared to be homogeneous when active fractions were analyzed by SDS-PAGE (Fig. 4, lane 2). A summary of the purification procedure is shown in Table 1; 37-fold purification was achieved, with a final yield of 37%. The M_r was found to be 64,000 by SDS-PAGE by using gels containing different polyacrylamide concentrations (7.5, 9.0, 10.5, and 13.5%, wt/vol) and 67,000 by calibrated gel filtration chromatography. These results suggest that the enzyme is monomeric.

Properties of basidiomycete PM1 laccase I. The main laccase I characteristics are summarized in Table 2, which also shows properties of other fungal laccases. Laccase I is a glycoprotein since it binds to concanavalin A. The total carbohydrate content was 6.5%, as determined by the phenol-sulfuric acid method by using mannose as the standard. Treatment of the denatured enzyme with endo-β-*N*-acetylglucosaminidase H decreased its size to 60 kDa (Fig. 4, lane 1), indicating that most of the carbohydrate (6.25%) is N linked. The isoelectric point was 3.6, as determined by isoelectric focusing. The K_m of the enzyme, as determined by using guaiacol as the substrate in the range from 0.05 to 10 mM, was 0.50 mM. The optimum pH was 4.5, as determined in either 50 mM acetate buffer (pH 3.6 to 5.5) or 50 mM citrate-phosphate buffer (pH 2.6 to 7). The enzyme retained 90% of its activity for 1 h at pH 3 to 9 and for 24 h between pH 7 and pH 9. The optimum temperature for laccase I activity in a 30-s reaction was 80°C.

Laccase was stable for 1 h during preincubation at 60°C (Fig. 5). Preincubation at high temperatures (37 to 80°C) seemed to activate the enzyme considerably. When the enzyme was preactivated by 15 min of incubation at 50°C, its thermal stability decreased slightly, but the optimal reaction temperature was the same (data not shown).

The copper content of the enzyme, as determined by atomic absorption spectrometry, was 3.63 molecules of copper per molecule of protein. Figure 6 shows the absorbance of laccase I at different wavelengths. The shoulder at 330 nm corresponds to a type 3 binuclear copper, and the peak at 600 nm corresponds to a type I or blue copper atom.

The effects of several potential laccase activity inhibitors (4) were determined at the optimal pH. Inactivation of PM1 laccase I by various concentrations of the potential inhibitors is shown in Table 3. The most effective inhibition was obtained with sodium azide. The effect of sodium thioglycolate was much lower, and EDTA had very little effect at the concentrations used.

Laccase I was subjected to Edman degradation to determine the N terminus of the mature protein. The sequence obtained is shown in Fig. 7 together with the N-terminal sequences of other known laccases. Strong similarities with the laccases from *C. hirsutus* and *Phlebia radiata* were found, while the N-terminal sequences of *Agaricus bisporus* and *Neurospora crassa* laccases were clearly different.

DISCUSSION

In this paper we describe the isolation of a kraft lignin-degrading fungus from the wastewater of a paper factory. This organism is a white rot basidiomycete able to oxidize phenolic compounds, such as ferulic acid or guaiacol. According to Stalpers' classification (43), the closest species was *Polyporus hydnoides*. However, DNA restriction enzyme analysis indicated that basidiomycete PM1 was different from *Polyporus hydnoides* (data not shown). The behavior of basidiomycete PM1 with respect to kraft lignin degradation, Poly R-478 decolorization, and ferulic acid polymerization was more similar to the behavior of *C. versicolor* than to the behavior of *Phanerochaete chrysosporium*, the two fungi used as model lignin degraders. The white rot fungi produce diverse enzymatic activities able to degrade lignin. *Phanerochaete chrysosporium* produces several ligninases and manganese peroxidases (29); *Phlebia radiata* produces peroxidases and a laccase (33); *C. versicolor* produces peroxidases, several laccases, and one aryl alcohol oxidase (10, 12); *Rigidosporus lignosus* (13) and *Dichomitus squalens* secrete laccase and manganese peroxidase activities (34); and *Pleurotus ostreatus* and *Pleurotus eringyi* secrete laccase (41) and aryl alcohol oxidase activities (16). When culture supernatants of basidiomycete PM1 were analyzed for the presence of ligninolytic enzymatic

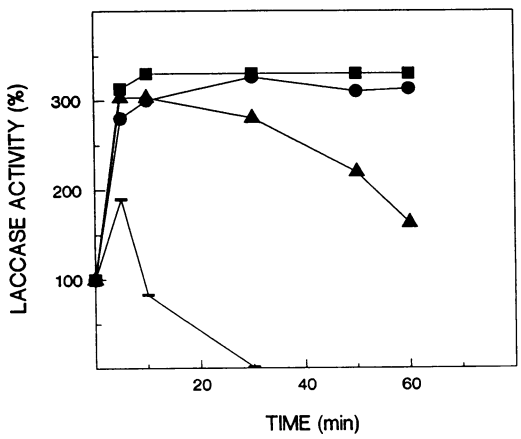

FIG. 5. Purified laccase I activity after preincubation at different temperatures. Symbols: ●, 50°C; ■, 60°C; ▲, 70°C; ▬, 80°C.

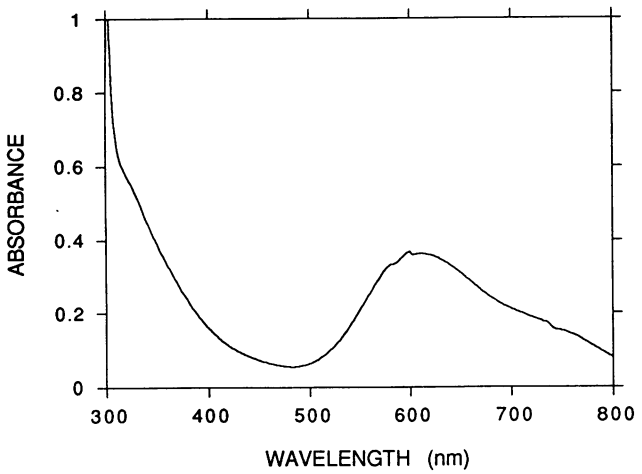

FIG. 6. Laccase I absorption spectrum at 25°C in 0.1 M phosphate buffer (pH 6.0).

activities, the only activity detected was laccase activity. However, the ability of this fungus to decolorize kraft lignin and Poly R-478 suggests that other oxidative activities are present (15). Basidiomycete PM1 laccase activity seems to be constitutive and was produced in all of the media examined, although maximal production occurred in GAE medium. The activity was not strongly increased by the presence of Tween 80 or an oxygen atmosphere in the culture, in contrast to what has been described for ligninase activities (2, 20). The activity is not inducible by compounds known to induce other basidiomycete laccases, such as 2,5-xylidine (4), guaiacol (42), toluidine (12), lignosulfonate (17), and gallic acid and ferulic acid (24). However, other constitutive laccases have been described previously (4).

The characteristics of purified PM1 laccase I are very similar to those of other white rot fungal laccases (4), and the behavior of PM1 laccase I in the presence of several inhibitors is also the same as the behavior of other laccases. The main difference between basidiomycete PM1 laccase I and other white-rot fungal laccase activities is the thermal stability of this enzyme. Other laccase activities are stable at temperatures below 50°C (9, 44, 46), while the basidiomycete PM1 laccase I is stable at 60°C; to date, no other temperature-activated laccases have been described.

The amino-terminal sequence of PM1 laccase I is very similar to amino-terminal sequences of the laccases from *C. hirsutus* and *Phlebia radiata*, while it is very different from the amino-terminal sequences of other laccases, such as those from *A. bisporus* and *N. crassa*. Both *C. hirsutus* and *Phlebia radiata* are white rot basidiomycetes; however, the laccase of *Phlebia radiata* has only two copper atoms (33), one type 1 and one type 2, and the prosthetic group pyrroloquinoline quinone (19). The laccases from *A. bisporus* (46) and *Schizophyllum commune* (9) also have two copper atoms. The spectral characteristics of PM1 laccase are similar to the spectral characteristics of the laccases containing four copper atoms (type 1, 2, and 3 configurations) and no pyrroloquinoline quinone group, such as those of *C. versicolor* (26) and *Coliolus consors* (39). Most laccases and copper oxidases with these types of copper configurations have conserved amino acids acting as copper ligands (28). Experiments to clone and sequence the PM1 laccase gene are in progress and should provide new information for establishing the evolutionary relationships among the different laccases.

TABLE 3. Effects of some laccase inhibitors on the oxidation of guaiacol by purified laccase I from basidiomycete PM1 (= CECT 2971)

Inhibitor concn (mM)	% Inhibition with the following inhibitors:			
	Sodium azide	Potassium cyanide	Thioglycolic acid	EDTA
0.005	83	0	0	
0.01	93	0	0	
0.02	97	5	1	
0.10	100	31	9	0
1.00	100	91	55	0
2.00	100	95	67	0
5.00	100	99	80	3
10.00	100	100	86	18

Microorganism	Amino terminus
Basidiomycete PM1	S I G P V A D L T I S N G A V S P
C. hirsutus	A I G P T A D L T I S N A E V S P
P. radiata	S I G P V T D F H I V N A A V S P
A. bisporus	D T - K T F N F D L V N T R L A -
N. crassa	G G G G G C N S P T N R Q C W S P

FIG. 7. Amino-terminal compositions of laccase I from basidiomycete PM1 and laccases from *C. hirsutus*, *Phlebia radiata*, *A. bisporus*, and *N. crassa*. The regions in which the amino acids are identical are enclosed in boxes. The amino acids conserved in the five proteins are indicated at the bottom by solid squares. The data for *C. hirsutus*, *Phlebia radiata*, *A. bisporus*, and *N. crassa* were obtained from references 21, 40, 35, and 14, respectively.

ACKNOWLEDGMENTS

We thank A. Martínez and A. González for their help in classifying basidiomycete PM1 (= CECT 2971). We thank N. Skinner for correcting the manuscript.

P. M. Coll and J. M. Fernández-Abalos acknowledge support from fellowships granted by the Ministerio de Educación y Ciencia, Spain. This work was supported by grant BIO88-0700 from the Comisión Interministerial de Ciencia y Tecnología, Spain, and by a grant from the Empresa Nacional de Celulosa, S.A.

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