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# Laccase Component of the *Ceriporiopsis subvermispora* Lignin-Degrading System

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**Laccase activity in the lignin-degrading fungus *Ceriporiopsis subvermispora* was associated with several proteins in the broth of cultures grown in a defined medium. Activity was not increased significantly by adding 2,5-xylidine or supplemental copper to the medium. Higher activity, associated with two major isoenzymes, developed in cultures grown on a wheat bran medium. These two isoenzymes were purified to homogeneity. L1 and L2 had isoelectric points of 3.4 and 4.8, molecular masses of 71 and 68 kDa, and approximate carbohydrate contents of 15 and 10%, respectively. Data indicated 4 copper atoms per mol. L1 and L2 had overlapping pH optima in the range of 3 to 5, depending on the substrate, and exhibited half-lives of 120 and 50 min at 60°C. They were strongly inhibited by sodium azide and thioglycolic acid but not by hydroxylamine or EDTA. The isoenzymes oxidized 1,2,4,5-tetramethoxybenzene but not other methoxybenzene congeners. A variety of usual laccase substrates, including lignin-related phenols and ABTS [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)], were also oxidized. Kinetic parameters were similar to those of the laccases of *Coriolus versicolor*. The N-terminal amino acid sequence (20 residues for L1) showed significant homology to those of laccases of other white rot basidiomycetes but not to those of the laccases of *Agaricus bisporus* or *Neurospora crassa*.**

*Ceriporiopsis subvermispora* is a white rot fungus that is unusually selective for the lignin component in its decay of wood (2, 3). The fungus apparently is relatively uncommon and has not been widely studied. Until recently, nothing was known about its ligninolytic system. In our laboratory, the fungus and its lignin-degrading enzyme system are of interest from the standpoint of biopulping, an experimental pulping process in which wood is pretreated with a ligninolytic fungus prior to pulping. With mechanical pulping, the pretreatment saves substantial energy and results in improved paper strength properties (2). *C. subvermispora* is the best of several hundred species of fungi examined for biopulping. Rüttimann et al. (27, 28) showed recently that *C. subvermispora* produces two extracellular enzyme activities which have been associated with lignin depolymerization in other fungi, viz., manganese peroxidase and laccase, whereas a third enzyme associated with lignin depolymerization in other fungi, lignin peroxidase, was not detected. Rüttimann et al. (26) demonstrated the presence of at least two laccase isoforms in cultures of a strain of *C. subvermispora*, and very recently, Lobos et al. (18) obtained evidence that four or five isoforms are produced by this strain. Our purpose here was to isolate and characterize the major laccases of *C. subvermispora*.

Laccase oxidizes phenolic units in lignin to phenoxy radicals, which can lead to the degradation of some structures (5, 6, 12, 14). In the presence of appropriate primary substrates, the effect of laccase can be greater; reportedly, laccase-primary substrate systems degrade lignin in kraft pulp (6, 8) and oxidize nonphenolic compounds that otherwise are unattacked (5). It is not yet known whether such primary substrates function in vivo in lignin biodegradation, and indeed, the actual role of laccase is not fully clarified.

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## MATERIALS AND METHODS

**Chemicals.** All chemicals were reagent grade, except for some methoxybenzenes, which were donated by P. Kersten of this laboratory.

**Organism and liquid culture conditions.** *C. subvermispora* CZ-3 was obtained from The Center for Mycology Research, Forest Products Laboratory, Madison, Wis., and maintained on potato dextrose agar. Experimental liquid cultures were grown in 2-liter Erlenmeyer flasks containing 500 ml of medium or in 125-ml Erlenmeyer flasks with 30 ml of medium. Blended mycelium grown on the medium of the experiment was used as inoculum (approximately 0.5 mg [dry weight] of mycelium per ml of culture medium). Two media were used. One was a bran-based medium which contained (per liter) wheat bran flour (ConAgra, Inc., Omaha, Nebr.) (30 g), yeast extract (Difco, Detroit, Mich.) (10 g), and a trace element solution (28) (1 ml). The medium was buffered at pH 4.5 with *trans*-aconitic acid (10 mM). The other was a minimal medium (31). The effects of additional copper ($CuSO_4$) and 2,5-xylidine in the minimal medium were examined as described in Results.

**Laccase assays.** Laccase activity was assayed in two ways. In the first, the oxidation of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma, St. Louis, Mo.) was monitored as increased $A_{420}$ (5). The reaction mixture contained 10 μl of the enzyme sample, 1 mM ABTS, and 50 mM sodium tartrate (pH 3.0) in a final volume of 1.0 ml. One unit was defined as the amount of the laccase that oxidized 1 μmol of ABTS $min^{-1}$. This assay was used to determine optimum pH and $K_m$. Substrate specificity, optimum pH, and kinetic parameters were studied by the second assay, which was based on oxygen uptake with various substrates and which used a YSI model 53 oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio) fitted with a Gilson single-port 1.5-ml reaction chamber. Both assays were performed at 25°C with 50 mM sodium tartrate buffer (pH 2 to 6). The concentration of oxygen at air saturation was assumed to be 258 nmol/ml.

The activities of laccase isoenzymes L1 and L2, which had different pH optima, were separately measured by assaying with ABTS at both pH 3 and 5. The activity at pH 5.0 was multiplied by 1.75 to give L2 activity, and L1 activity was obtained by subtracting the L2 activity from the total activity at pH 3.0 (see the "Optimum pH" section in Results).

**Enzyme isolation.** Liquid cultures (1,500 ml, bran medium) were harvested after 14 days, filtered, and concentrated 50-fold with a Minitan (Millipore, Milford, Mass.) ultrafiltration unit (10-kDa molecular cutoff; Amicon, Beverly, Mass.). The solution was frozen (−20°C), thawed, and centrifuged to remove precipitated polysaccharide. Protein was precipitated with ammonium sulfate at 90% saturation, dissolved in 100 ml of 0.01 M sodium acetate buffer (pH 6.0), dialyzed against the same buffer, and again membrane concentrated to 30 ml. The resulting solution was applied to an Accel (Millipore) anion exchange column equilibrated with 0.01 M acetate (pH 6.0), and proteins were eluted with 0.6 M acetate (pH 6.0). Fractions containing laccase activity were pooled, dia-

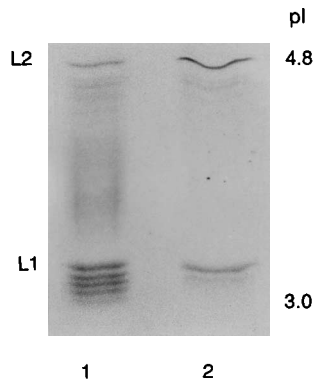


FIG. 1. Isoelectric focusing patterns of laccases with activity staining. Lane 1, liquid culture with the minimal medium; lane 2, liquid culture with the complex (bran-based) medium. Ultrafiltered, 6- to 10-fold-concentrated culture broths (4 μl) were applied directly to each lane.

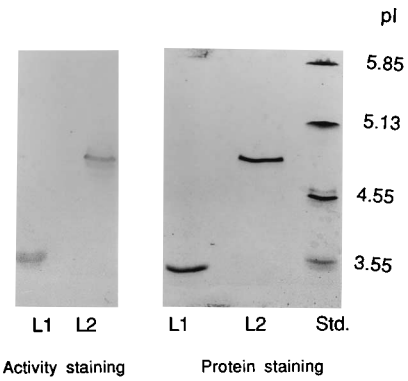


FIG. 2. Isoelectric focusing of purified laccases L1 and L2. Approximately 1 μg of protein was applied to each lane. Std., standards.

lyzed against 0.01 M sodium acetate (pH 6.0), and concentrated. This solution was subjected to anion exchange chromatography (Mono Q column; Pharmacia, Uppsala, Sweden) in a fast protein liquid chromatography (FPLC) system (Pharmacia) with a sodium acetate gradient, 0.01 to 0.6 M, pH 6.0. Fractions corresponding to two laccase peaks, designated L1 and L2, were pooled separately. The concentrated L1 solution was subjected to hydrophobic chromatography (Phenyl Superose column; Pharmacia) in the above-mentioned chromatography system and eluted with a gradient of ammonium sulfate, 1.7 to 0.0 M. The concentrated L2 solution from the FPLC was further purified by isoelectric focusing after being dialyzed and concentrated.

Isoelectric focusing was done with a Bromma 2117 Multiphor apparatus (LKB, Stockholm, Sweden) and Servalyt Precoats polyacrylamide gels (12.5 by 12.5 cm; Serva, Paramus, N.J.), pH 3 to 6. L2 was stepwise focused at 200 V, 400 V, 800 V, and 1,200 V for 1 h at each voltage. After activity staining (see below), L2 was recovered.

**Laccase characterization.** Isoelectric points were determined with 4-cm gels (Serva). Samples were dialyzed against 0.01 M sodium acetate (pH 4.0) and concentrated to about 20 U/ml (approximately 100 μg/ml). Proteins were focused for 10 min at 400 V and then for 5 min at 800 V and stained with Serva Violet (Serva). Laccase activity staining was done with 200 ppm of 4-chloro-1-naphthol in 50 mM sodium tartrate buffer (pH 4.0) (17). The protein standards were amyloglucosidase (pI 3.55), trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.13), and bovine carbonic anhydrase (pI 5.85).

Apparent molecular weights were determined by sodium dodecyl sulfate (SDS) electrophoresis with the Phast System (Pharmacia), according to the manufacturer's recommended method. The protein standards were rabbit muscle phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), chicken egg white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and chicken egg white lysozyme (14.4 kDa).

Glycosylation was examined with a glycan detection kit (Boehringer Mannheim Corporation, Indianapolis, Ind.), which was used after the purified laccases were blotted onto nitrocellulose. The sugar contents of the purified laccases were estimated from the differences in the SDS electrophoresis-determined molecular weights of the purified laccases before and after deglycosylation with hydrogen fluoride (22).

Thermal stability was studied as follows. Purified laccases L1 and L2 were separately incubated in 50 mM sodium tartrate buffer (pH 4.0) at 60°C, and the remaining activities were assayed over time (see "Laccase assay," above). The half-lives were calculated by the equation $x = x_0(1/2)^{t/g}$ or $\log x = \log x_0 - [(\log 2)/g]t$, where $x$, $x_0$, $t$, and $g$ are remaining activity, initial activity, time, and half-life, respectively.

The concentrations of the purified laccases were determined by $A_{205}$ (29). The copper content was measured as reported by Broman and Malmström (7).

N-terminal sequencing was done by the University of Wisconsin—Madison Biotechnology Center.

## RESULTS

We began by determining the development of laccase activity in two media, one chemically defined and the other based on wheat bran. The influence of $Cu^{2+}$ or the laccase inducer 2,5-xylidine on activity development in the minimal medium was assessed. Two laccase isoenzymes were then isolated from the culture broth of the bran-based medium (which gave the higher activity). The purified laccases were characterized as to pH optima, isoelectric points, presence of carbohydrate, molecular weights, and UV-visible spectra. Thermal stabilities, the effects of selected inhibitors, and kinetic parameters were then determined. The N-terminal amino acid sequence of the major isoenzyme was compared with those of other fungal laccases.

**Laccase production.** Higher levels of laccase activity were found in the complex liquid medium, even when 2,5-xylidine or $Cu^{2+}$ was added to the defined medium (9, 21). Activity development was only slightly stimulated by the xylidine (from 0.8 U/ml in unsupplemented medium to 1.1 U/ml) and not at all by $Cu^{2+}$. In the complex medium cultures, laccase activity was associated mainly with two proteins, designated L1 and L2; these were also present in the defined medium cultures, to-

TABLE 1. Recovery of laccases L1 and L2 during purification

| Purification step | Total activity (U) | Recovery (%) |
|---|---|---|
| Dialyzed culture filtrate | 5,500 | 100 |
| $(NH_4)_2SO_4$ precipitation | 3,200 | 58 |
| Accel anion exchange | 3,000 | 54 |
| Mono Q (L1) | 1,490 | |
| Mono Q (L2) | 380 | 34[a] |
| Phenyl Superose (L1) | 800 | |
| Preparative isoelectric focusing (L2) | 50 | 15[b] |

[a] This value is the recovery percentage for the Mono Q (L1) and Mono Q (L2) purification steps.

[b] This value is the recovery percentage for the Phenyl Superose (L1) and preparative isoelectric focusing (L2) purification steps.

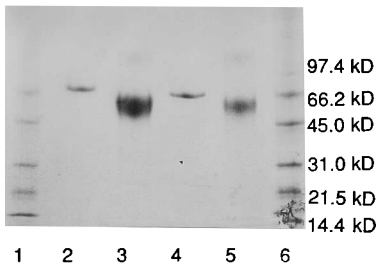


FIG. 3. SDS electrophoresis of purified laccases L1 and L2. Lanes 1 and 6, standard proteins; lane 2, purified L1; lane 3, deglycosylated L1; lane 4, purified L2; lane 5, deglycosylated L2. Approximately 2 μg of protein was applied to each lane.

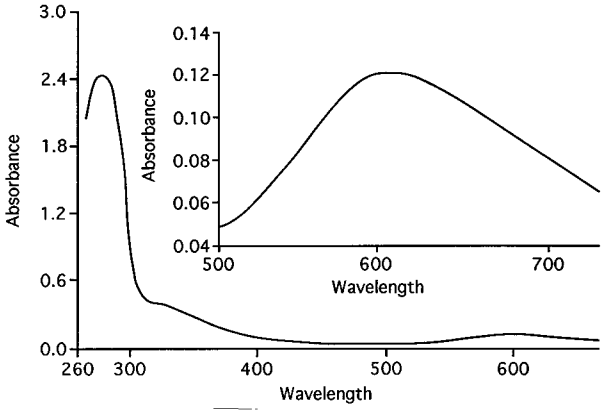


FIG. 4. Absorbance spectrum of laccase L1. The concentration of L1 was 2.3 mg/ml in 0.01 M sodium acetate buffer at pH 4.0.

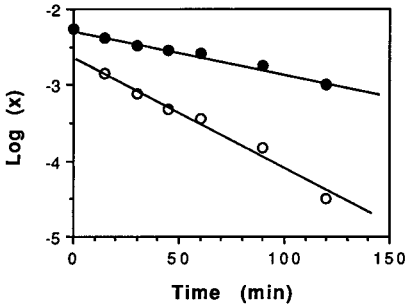


FIG. 5. Thermal stability of laccase L1 (●) and L2 (○) at 60°C. The *y* axis indicates logarithmic values of the remaining laccase activity.

gether with many others active proteins (Fig. 1). (The broad bands which appear in the middle of the gel of the minimal medium proteins [Fig. 1] were probably due to manganese peroxidase, because these bands became more prominent after $Mn^{2+}$ and $H_2O_2$ were added [not shown] [see reference 18].) The fungus produced approximately six times more L1 than L2 in the complex medium (measured by the ABTS assay), but the time courses of production were similar for both isoenzymes (not shown). Both activities appeared on day 4 and reached maxima on day 8.

**Purification of laccases.** The concentrated broth from the bran cultures was contaminated by pigments, which were largely removed by the initial freeze-thaw, the protein precipitation with ammonium sulfate, and the Accell anion exchange chromatography. Subsequent anion exchange chromatography (Mono Q) resolved two peaks of laccase activity (data not shown) which were collected separately. Both fractions (L1 and L2) were bright blue after concentration. Since fraction L2 contained some minor laccase isoenzymes which could not be separated by Phenyl Superose chromatography, L2 was cleaned up by preparative isoelectric focusing. The specific activities of purified L1 and L2 are 310 and 220 U/mg of protein, respectively. Recovery of the laccases during purification is summarized in Table 1.

**Physical characterization.** The isoelectric points of L1 and L2 are 3.4 and 4.8, respectively (Fig. 2). Both enzymes were found to be glycoproteins by the use of an enzyme immunoassay; hydrogen fluoride-deglycosylation results were in accord. Thus, SDS electrophoresis of native and hydrogen-fluoride-deglycosylated L1 and L2 revealed molecular masses of 71 kDa and 68 kDa for native L1 and L2 and 60 kDa and 61 kDa after deglycosylation (Fig. 3), indicating approximate sugar contents of 15 and 10%. The UV-visible spectrum of L1 has a shoulder at approximately 330 nm and a peak at 610 nm in addition to the near-UV peaks (Fig. 4). The ratio of $A_{280}$ to $A_{610}$ is 20, which is similar to those of the laccases of *Coriolus versicolor* (11). For the *C. versicolor* laccase, peaks at 610 nm and 330 nm are known to correspond to type 1 and type 3 Cu, respectively (11), suggesting that L1 also contains type 1 and type 3 Cu. The ratio of total copper content and type 1 copper content, which was estimated by $A_{610}$ (extinction coefficient = 4,600 $M^{-1}$ $cm^{-1}$ [19]), was 4.3. Similarly, when the copper content was calculated on the basis of the protein concentration, a value of 3.7 mol of Cu per mol of protein was indicated. These results suggest that L1 contains 4 mol of copper ions, as do the *C. versicolor* laccases (11, 19). The concentration of Cu in L2 could not be determined exactly, because of the difficulty in obtaining enough protein, but the spectrum of L2 was similar to that of L1.

**Thermal stability.** Preliminary experiments showed that L1 and L2 were maximally stable at pH 5 and 3 to 4, respectively. The thermal stabilities of L1 and L2 at 60°C were therefore investigated at pH 4.0. The logarithms of the remaining activity were plotted against time (Fig. 5); the slopes indicated half-lives of 120 and 50 min for L1 and L2.

**Inhibitor studies.** The effects of several laccase activity inhibitors were examined with ABTS as a substrate at pH 3.0. Both enzymes were 100% inhibited by sodium azide at 0.02 mM and by thioglycolic acid at 0.5 mM; hydroxylamine and EDTA were not strongly inhibitory (data not shown).

**Optimum pH.** The optimum pH of L1 and L2 for the oxidation of ABTS (Fig. 6) and 1,2,4,5-tetramethoxybenzene (TMB) (not shown) was 2 to 3. The activity of L2 with both substrates extended over a broader range; thus, at pH 5, L1

TABLE 2. Kinetics characterization laccases for the oxidation of various substrates[a]

| Substrate | Optimum pH | | $K_m$ (mM) | | $K_{cat}$ ($s^{-1}$) | | $K_{cat}/K_m$ ($mM^{-1}$ $s^{-1}$) | |
|---|---|---|---|---|---|---|---|---|
| | L1 | L2 | L1 | L2 | L1 | L2 | L1 | L2 |
| TMB | 3.0 | 3.0 | 0.9 | 6.9 | 56 | 28 | 62 | 4 |
| Dimethoxyphenol | 4.0 | 4.0 | 2.9 | 7.7 | 23 | 76 | 8 | 10 |
| Syringic acid | 3.0 | 4.0 | 0.13 | 0.10 | 31 | 78 | 240 | 780 |
| Guaiacol | 3.0 | 5.0 | 1.6 | 0.44 | 52 | 66 | 33 | 150 |
| Vanillic acid | 4.0 | 6.0 | 1.1 | 1.0 | 37 | 66 | 34 | 66 |
| Vanillin | 4.0 | 6.0 | 6.3 | 9.0 | 26 | 10 | 4 | 1 |
| Vanillyl alcohol | 3.0 | 5.0 | 1.6 | 0.61 | 47 | 37 | 29 | 60 |
| ABTS | 3.0 | 3.0 | 0.03 | 0.02 | 101 | 96 | 3,400 | 4,800 |

[a] The kinetics parameters were obtained by determining the oxygen consumption rates (see text).

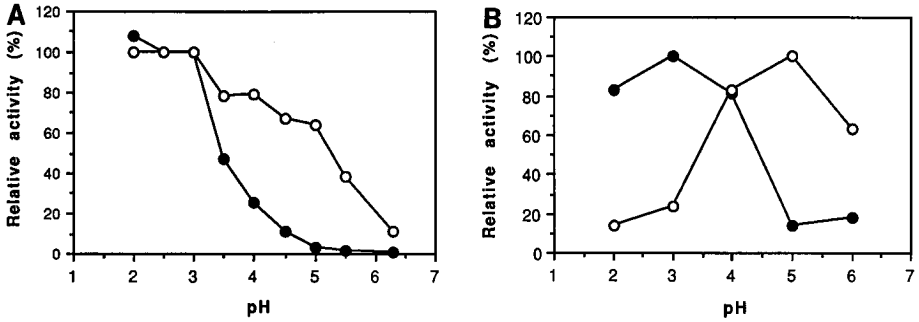


FIG. 6. The pH dependence of laccases L1 and L2 for the oxidation of ABTS (A) and guaiacol (B). The reaction mixtures contained 1 mM substrate, 50 mM sodium tartrate, and L1 (●) or L2 (○).

hardly oxidized ABTS, whereas the activity of L2 was about 60% of the pH 3.0 rate. This result made it possible to detect L1 and L2 activities separately in cultures. The optimum pH of L1 and L2 was the same for 2,6-dimethoxyphenol oxidation (pH 4.0), but with other examined phenolic compounds, the optimum of L1 was lower than that of L2 (Fig. 6 and Table 2).

**Substrate specificities.** Kinetic parameters were determined at the optimum pH for each isoenzyme (Table 2). The $K_m$ values of L1 and L2 for ABTS were lower than those for any of the other compounds, and those for syringic acid were the second lowest. The $K_m$ values of both isoenzymes for vanillin were the highest. The catalytic efficiencies (second-order rate constants, $K_{cat}/K_m$) for the substrates varied from 4 and 1 for vanillin (for L1 and L2) to 3,400 and 4,800 for ABTS. The catalytic efficiencies of the enzymes were second best with syringic acid. L1 and L2 did not oxidize tyrosine or methoxybenzene congeners with higher redox potentials than that of TMB.

## DISCUSSION

Our work complements and extends the recent report of Lobos et al. (18), who demonstrated multiple laccase isoforms in *C. subvermispora* growing in a chemically defined medium. Their laccases had pIs in the range of 3.2 to 3.5 and 4.7, which is in good agreement with the findings here. The laccases were not characterized further, although manganese peroxidases produced by the cultures were characterized in some detail. In our work, two dominant isoenzymes were produced in the complex bran medium, whereas in the minimal medium, multiple forms were detected. Both Lobos et al. (18) and we (data not shown) detected both laccase and manganese peroxidase in vivo in wood chip cultures, which in our case were biopulping runs (see reference 15 for details).

Bollag and Leonowicz (4) reported that isoenzymes of laccase of some fungi are constitutive, whereas others are induced by 2,5-xylidine. The two isoenzymes studied here apparently are in the constitutive category, since this compound (or copper) failed to significantly increase total activity.

The estimated carbohydrate contents of L1 and L2 are within the range reported for the laccases of other basidiomycetes, including *C. versicolor* (33), *Coriolus hirsutus* (16), *Phlebia radiata* (23), *Agaricus bisporus* (32), and *Armillaria mellea* (25). Yoshitake et al. (33) demonstrated that the carbohydrate moiety of the laccase of *C. versicolor* imparts resistance to proteolytic attack and elevated temperatures. Perhaps the somewhat greater thermal stability of L1 over that of L2 reflects its higher carbohydrate content.

The optimum pH of L1 for ABTS, TMB, and dimethoxyphenol was the same as that of L2, which is consistent with the findings for the laccase of *C. versicolor* (4, 13). However, the optimum pH of L1 for the other phenolic compounds was lower than those of L2 and the laccases of other fungi (4). Since the pH during the degradation of lignin in wood by *C. subvermispora* gradually decreases from 5.0 to around 3.5 (1), the different pH optima of L1 and L2 allow oxidation over a wider pH range. The maximum thermal stability of the *C. subvermispora* laccases was found at pH values (pH 3 to 5) lower than those reported for the laccases of *C. versicolor* (pH 7 to 8) and *C. hirsutus* (pH 6 to 9) (30).

The *C. subvermispora* laccases are similar to those of other ligninolytic basidiomycetes. Kersten et al. determined the kinetic parameters of two laccase isoenzymes of *C. versicolor* for the oxidation of TMB (13), finding no significant difference in their catalytic efficiencies (the second-order rate constants [$K_{cat}/K_m$] were 28 and 42 $mM^{-1}$ $s^{-1}$). The efficiencies of L1 and L2 for TMB oxidation are in this same range, although that of L1 is 15-fold greater than that of L2. The $K_m$ of L1 is lower than those of the *C. versicolor* laccases (2.2 and 1.5 mM). The *C. subvermispora* laccases are like those of *C. versicolor* in that they oxidize only the methoxybenzene congener with the lowest redox potential (TMB) (13). The laccases of ligninolytic fungi generally contain four copper ions, one type 1, one type 3, and two type 2 (11). Our laccases seem to be similar. The N-terminal sequence of L1 shows high homology with those of laccases of *C. hirsutus* (16), *C. versicolor* (20), and *P. radiata* (23) but not with those of *A. bisporus* (24) or *Neurospora crassa* (10) (Fig. 7). From these various results, we conclude that the

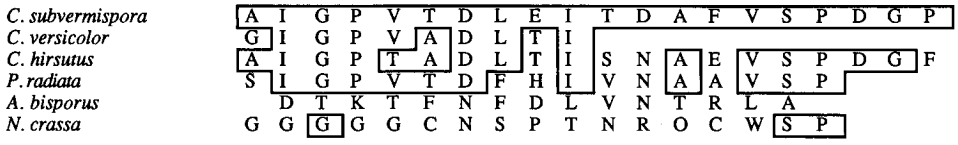


FIG. 7. Comparison of N-terminal amino acid sequence of laccase L1 from *C. subvermispora* with those of laccases from *C. versicolor*, *C. hirsutus*, *P. radiata*, *A. bisporus*, and *N. crassa*. Identical amino acid residues are boxed. The data for *C. versicolor*, *C. hirsutus*, *P. radiata*, *A. bisporus*, and *N. crassa* laccases are from references 20, 16, 23, 24, and 10, respectively.

laccases of *C. subvermispora* are similar to those of *C. versicolor* and other studied white rot fungi but not to those of more distantly related fungi.

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