Properties of laccase isoenzymes produced by the basidiomycete *Ceriporiopsis subvermispora*[1]

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Laccase is one of the ligninolytic enzymes found in liquid cultures of the fungus *Ceriporiopsis subvermispora* in defined medium. As an approach to a clarification of the role of laccases during the attack on lignin by the fungus, the enzyme has been characterized further. The levels of this phenol oxidase increase 2-fold in the presence of *p*-anisidine and are severely affected when addition of either Mn(II) or Cu(II) ions to the medium is omitted. Isoelectrofocusing allowed the resolution of two laccase isoenzymes, with pIs of 3.65 and 3.59. In rich medium, laccase activity is 10-fold higher than in salt medium, and it is not affected by the external addition of *p*-anisidine or Mn(II). Four isoenzymes were detected in these cultures with pIs between 3.76 and 3.60. In a wheat bran medium, four isoenzymes with pIs in the range 3.63–3.46, plus a fifth isoenzyme of high pI (4.82), were also identified. The absorption spectrum of a pool containing the four isoenzymes from rich medium shows a maximum at 600 nm, typical of laccase possessing a type I copper atom. The molecular mass of the isoenzyme with pI 3.60 is 79 kDa, as determined by SDS/PAGE. Upon treatment with endoglycosidase F, the molecular mass of this isoform decreases to 63 kDa, indicating a high degree of glycosylation. Substrate specificity studies conducted with the four isoenzymes from rich medium and a combination of isoenzymes from salt medium showed marked differences among them. The amino-terminal sequences (24 residues) of three isoenzymes isolated from rich medium were determined. Two of them are identical, whereas the third one differs from these in three amino acid residues. The consensus sequence reveals clear homology with laccases from other microorganisms.

Lignin peroxidase (LiP[4]), manganese peroxidase (MnP) and laccase have been implicated in lignin biodegradation by white-rot fungi [1]. LiP has the unique ability to attack the non-phenolic residues of the polymer, a

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[4] Abbreviations used: LiP, lignin peroxidase; MnP, manganese peroxidase; YMPG, medium containing, among other components, yeast extract, malt extract, peptone and glucose; ABTS, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate); IEF, isoelectrofocusing.

reaction that can be considered mandatory during lignin breakdown. In turn, MnP and laccase exert their catalytic action on the phenolic residues of lignin. Laccases are blue copper-containing glycoproteins that use oxygen as electron acceptor to remove hydrogen from the phenolic hydroxyl groups. This reaction gives rise to radicals that, as demonstrated with lignin model compounds, can undergo rearrangements leading to alkyl–aryl cleavage, oxidation of benzyl alcohols, and cleavage of side chains and aromatic rings [2,3]. Recent studies have shown that in the presence of appropriate mediators, laccase can also oxidize non-phenolic substrates [4–6] as well as Mn(II) [7].

The precise role of laccase during lignin decay has not been elucidated. Some studies suggest that it contributes to substrate depolymerization [8–10], whereas others indicate that it may catalyse the coupling of degradation intermediates that are toxic to fungi [11]. Laccase participation in ligninolysis is supported by immunocytochemical methods, which show this enzyme to be localized in degraded wood cell walls [12].

Laccase is found in higher plants and fungi. Among the latter, it is widely distributed in ascomycetes, deuteromycetes and basidiomycetes [13]. Multiple isoforms of the enzyme have been found in cultures of *Trametes* (*Coriolus*) *versicolor* [14], *Panus tigrinus* [15], the basidiomycete PM1 [16] and *Rigidoporus lignosus* [17], among others, all of which have acidic p*I*s. In contrast, *Phlebia radiata* produces a single isoenzyme with a p*I* of 3.5 [18]. Some laccase genes from fungi have been cloned and sequenced [19–24].

In recent years, our group has focused its efforts on the characterization of the ligninolytic system of the basidiomycete *Ceriporiopsis subvermispora*, which is composed of MnP and laccase [25,26]. The absence of LiP in both liquid and solid cultures of this microorganism suggests that different strategies may be employed in nature for lignin breakdown. In a previous report [27], we showed that in liquid cultures of defined composition, *C. subvermispora* produces four isoenzymes of laccase with p*I*s between 3.65 and 3.45. A fifth isoform of p*I* 4.70 was irregularly detected. Several laccase isoforms were also isolated from early cultures of this fungus on wood [27]. The purpose of the present work was to characterize the laccases produced by *C. subvermispora*, as an initial approach to clarify the role of this phenol oxidase during lignin attack by this fungus.

Materials and methods

Organism

C. subvermispora strain FP-105752 was obtained from the Center for Forest Mycology Research of the Forest Products Laboratory, Madison, WI, U.S.A. The fungus was maintained on agar slants of potato dextrose agar medium (Difco).

Growth of the fungus
Experimental cultures of *C. subvermispora* FP-105752 were grown at 30 °C with agitation (180 rev./min) in Erlenmeyer flasks (125 ml) containing 30 ml of medium. They were inoculated with 13.5 mg (dry weight) of homogenized mycelium prepared as previously described [25]. The compositions of the minimal salts and YMPG media were as reported by Ruttimann et al. [25]. Unless otherwise indicated, both media contained 11 p.p.m. (195 μM) of $MnSO_4$. In the bran-based medium, the concentration of *trans*-aconitic acid, pH 4.5, trace metals and $MnSO_4$ were as in the salt medium. In addition, it contained, per litre, 30 g of wheat bran flour, 10 g of yeast extract (Difco) and 1 g of Tween 20. For purification of laccase, cultures were grown in 2 litre Erlenmeyer flasks containing 830 ml of either salt medium or YMPG broth.

Enzyme assays
Laccase activity was routinely assayed at 30 °C in 1.0 ml reaction mixtures containing 4.33 mM 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) (Sigma Chemical Co.) in 50 mM glycine, pH 3.0 [18]. One unit was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per min. For substrate specificity studies, caffeic (ε_{318} 16835 $M^{-1} \cdot cm^{-1}$), ferulic (ε_{318} 6531 $M^{-1} \cdot cm^{-1}$), sinapic ($\varepsilon_{317.5}$ 19531 $M^{-1} \cdot cm^{-1}$) and syringic ($\varepsilon_{272.5}$ 9250 $M^{-1} \cdot cm^{-1}$) acids (Aldrich) were present at a concentration of 0.1 mM. The representative molar extinction coefficients were determined in 50 mM glycine, pH 3.0. To test the effect of inhibitors, these were preincubated with the enzymes for 5 min at 30 °C, before the addition of ABTS.

Enzyme fractionation
All operations described below were performed at 4 °C. Day 15 cultures (830 ml) in either defined or YMPG media were centrifuged for 30 min at 10 000 rev./min in a type JA-20 Beckman rotor to remove the mycelium and the extracellular glucan. Solid ammonium sulphate was slowly added to the supernatant to a final concentration of 80%. The suspension was centrifuged for 20 min at 18 000 rev./min in the same rotor and the pellet obtained was resuspended in 50 mM sodium acetate, pH 4.8. After extensive dialysis against this buffer, protein was loaded onto a Q-Sepharose (Pharmacia) column (0.8 $cm^2 \times 16$ cm) previously equilibrated with the same solution. The column was first washed with three volumes of 50 mM sodium acetate, pH 4.8, and thereafter proteins were eluted with a 50 ml linear gradient of 50–500 mM of this buffer and 1 ml fractions were collected. Fractions enriched in laccase activity were pooled and dialysed against 50 mM sodium acetate, pH 4.8, before preparative isoelectrofocusing (IEF) (see below).

Isoelectrofocusing
Analytical IEF was performed over the pH range 3–6, using Servalyt Precotes 3–6 polyacrylamide gels from Serva Fine Biochemicals Inc.,

Westbury, NY, U.S.A. Gels were run and developed as reported before [27]. Standards for IEF were all from Sigma Chemical Co.: amyloglucosidase from *Aspergillus niger* (pI 3.6), glucose oxidase from *Aspergillus niger* (pI 4.2), soybean trypsin inhibitor (pI 4.6), β-lactoglobulin A from bovine milk (pI 5.1), carbonic anhydrase II from bovine erythrocytes (pIs 5.4 and 5.9) and carbonic anhydrase I from human erythrocytes (pI 6.6). After Q-Sepharose chromatography, laccases produced in YMPG medium were resolved by preparative IEF as follows: 16–20 mg of protein were applied to a 5% polyacrylamide gel (12 cm $\times$ 8 cm $\times$ 0.5 cm) that also contained 5% (w/v) glycerol, 2% (w/v) Bio-Lyte 3/5 (Bio-Rad), 300 μl of 0.1% riboflavin-5′-phosphate, 180 μl of 10% ammonium persulphate and 20 μl of TEMED. To facilitate gel formation, a fluorescent lamp was placed 5 cm above the tray containing the mixture. The anodic fluid (pH 3) contained, per litre of solution, 3.3 g of aspartic acid and 3.7 g of glutamic acid, whereas the cathodic fluid (pH 10) contained, per litre, 4 g of lysine, 4 g of arginine and 120 ml of ethylenediamine. After applying the protein sample, the gel was run at 4 °C as follows: 180 min at 200 V, 60 min at 400 V and 20 min at 600 V. Laccase isoenzymes could be easily identified in the gel owing to their intense blue color. Each band was excised with a razor blade and the activity was electroeluted overnight at 4 °C in a dialysis bag (cut-off 3500 kDa) containing 2 ml of 50 mM sodium acetate, pH 4.8.

Absorption spectrum

The enzyme pool, containing a mixture of four isoenzymes obtained after Q-Sepharose chromatography, was dialysed against 100 mM sodium acetate, pH 6.0 [21]. Then a spectrum was recorded at 30 °C, between 200 and 800 nm, in a Shimadzu UV 160 spectrophotometer.

Protein electroblotting and sequencing

After IEF, isoenzymes were loaded separately on to a 8% SDS-polyacrylamide gel and electrophoresed at 20 mA constant current. Proteins were electroblotted to poly(vinylidene difluoride) membranes [28] and sequences on an Applied Biosystems gas-phase 475 protein sequencer at the University of Wiscosin Biotechnology Center.

Endoglycosidase treatment

Digestion of laccase with this enzyme was conducted as described by Pedemonte et al. [29], with minor modifications: 50 μg of the laccase isoform with p*I* 3.60 suspended in 30 μ of water were combined with 2 μl of 10% SDS and with 25 μl of a solution containing 50 mM Tris/HCl, pH 7.5, 4% β-mercaptoethanol and 200 mM EDTA. The resulting mixture was boiled for 5 min. Then one unit (20 μl) of endoglycosidase F–*N*-glycosidase F (Sigma Chemical Co.) was added and the reaction mixture was incubated for 24 h at 30 °C.

Other procedures
SDS/PAGE was performed as reported by Laemmli [30] and the protein bands were stained using a silver stain kit (Sigma). Protein concentration was measured by the method of Bradford [31].

Results

Production of laccase in different media

In previous reports we have shown that laccase activity in cultures of the basidiomycete *C. subvermispora* in standard salt medium reaches levels in the range 0.30–0.35 U/ml [27,32]. Because one of the objectives of this work was to purify this activity for its characterization, conditions that would enhance laccase production were explored. Thus, when *p*-anisidine was added to the cultures 6 days after inoculation, a doubling in enzyme activity was observed (Table 1). Laccase levels also improved when ammonium tartrate was replaced by Casaminoacids (Difco) as nitrogen source, whereas enzyme activity was negligible when the salt medium lacked externally added Mn(II) or Cu(II) (Table 1). Enhanced levels of laccase were obtained in rich (YMPG) medium. In contrast to the results obtained in defined medium, the omission of Mn(II) or addition of *p*-anisidine to cultures in YMPG had little influence on laccase production. The highest levels of laccase were obtained in wheat bran medium (Table 1).

IEF analysis of samples withdrawn from the cultures led to the finding that the p*I*s of the isoenzymes differed with the composition of the growth medium. In day 15 cultures in defined medium, two isoforms with p*I*s of 3.65 and 3.59 were observed, whereas in YMPG four laccases with p*I*s of 3.76, 3.71, 3.65 and 3.60 were identified (Figure 1). These isoenzyme patterns did not vary throughout the growth period of the fungus. In the wheat bran-based medium, four isoforms with pIs of 3.63, 3.57, 3.52 and 3.46 and a fifth isoform with a higher pI (4.82), not present in early cultures, are clearly distinguished (Figure 1).

Table 1 Production of extracellular laccase by *C. subvermispora* in different media
Values shown correspond to the activity observed in 15 day cultures.

Medium	*Activity (units/ml)*
Salt medium	0.32
+ 1 mM *p*-anisidine	0.68
− Ammonium tartrate, + 0.3% casaminoacids	0.67
− Cu(II)	0.05
− Mn(II)	0.03
YMPG medium	2.12
+ Mn(II)	1.90
+ 1 mM *p*-anisidine	2.11
Wheat bran medium	3.85

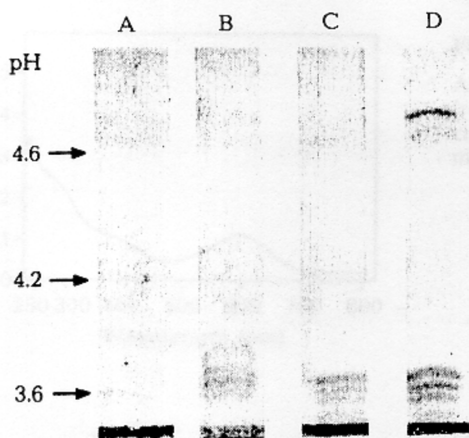

Figure 1

Isoelectrofocusing of laccases present in salt medium (A), YMPG broth (B), wheat bran medium, day 8 (C) and wheat bran medium, day 15 (D).

Table 2 Purification of laccases from cultures of *C. subvermispora* in YMPG medium

Purification step	*Volume (ml)*	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg protein)*
Culture filtrate	830	124.5	1,320	10.6
Ammonium sulphate	71	27.7	1,115	40.3
Q-Sepharose chrom.	7	17.4	1,547	88.9
IEF				
Laccase p*I* 3.76	2	0.44	48.6	109.0
Laccase p*I* 3.71	1.8	0.79	55.4	70.0
Laccase p*I* 3.65	1.5	0.42	64.3	151.0
Laccase p*I* 3.60	0.3	0.15	20.4	135.0

Purification of laccases produced in YMPG medium

The procedure described in the Materials and methods section allowed the purification of the four laccase isoenzymes detected in the YMPG medium (Table 2). Before preparative IEF, the absorption spectrum of the pool containing the four isoenzymes was determined (Figure 2). The peak observed at 600 nm, typical of copper-containing proteins, reveals a type 1 or blue copper atom, whereas the shoulder near 300 nm may indicate type 3 copper [33]. After preparative IEF, the purity of each laccase was confirmed in analytical IEF gels that were run in duplicate. One gel was developed enzymically and the other by staining with Coomassie Brilliant Blue. In each pair of gels, single bands were observed at the expected pIs (results not shown).

The laccase isoenzyme with a pI of 3.60 was randomly selected for determination of carbohydrate content. This isoform migrates in polyacrylamide gels run under denaturing conditions as a single band with a molecular mass of 79 kDa (Figure 3, lane A). After treatment with endoglycosidase F, the protein migrated in the gel to a position corresponding

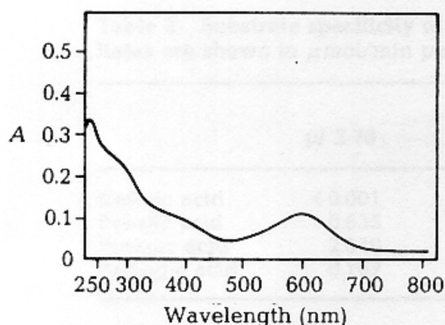

Figure 2

Absorption spectrum of the pool of laccases from YMPG medium, obtained under the conditions described in the Materials and methods section.

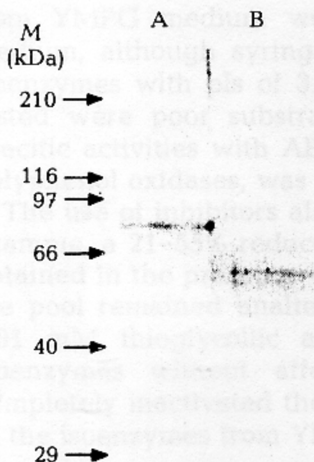

Figure 3

SDS/PAGE of one of the laccase isoenzymes (p*I* 3.60) before (A) and after (B) digestion with a mixture of endoglycosidase F and *N*-glycosidase F. Each lane contained 30 ng of laccase protein. *M*, molecular mass.

to a molecular mass of 63 kDa (Figure 3, lane B). A lower band is also visible in lane B, at a molecular mass (32 kDa) that coincides with that reported for endoglycosidase F [34].

Substrate specificity studies

Various single-ring aromatic compounds that are structurally related to lignin residues were tested as possible substrates of the four laccases from YMPG broth. For comparative purposes, a mixture of laccase isoenzymes from salt medium obtained after Q-Sepharose chromatography was also included in this analysis. The results obtained showed clear differences among the various isoenzymes, as well as between them and the pool from salt medium (Table 3). The best substrate for all the enzyme preparations was simple acid, whereas the *o*-diphenolic compound caffeic acid was poorly oxidized. In addition, the isozymes

Table 3 Substrate specificity of laccases produced by *C. subvermispora*
Rates are shown in μmol/min per mg.

	pI 3.76	*pI* 3.71	*pI* 3.65	*pI* 3.60	*Pool laccases from salt medium*
Caffeic acid	<0.001	0.165	0.303	0.196	0.010
Ferulic acid	0.635	0.191	0.432	1.094	0.035
Sinapic acid	2.640	1.524	2.440	5.350	0.164
Syringic acid	0.167	<0.001	0.241	0.555	0.015

from YMPG medium were more active than the laccases from salt medium, although syringic and caffeic acids were not oxidized by the isoenzymes with pIs of 3.71 and 3.76, respectively. The four compounds tested were poor substrates compared with ABTS (see corresponding specific activities with ABTS in Table 2). Tyrosine, a typical substrate of polyphenol oxidases, was not oxidized by either laccase preparation.

The use of inhibitors also revealed differences among the enzymes. For example, a 21–55% reduction in the activities of the four isoenzymes was obtained in the presence of 20 mM kojic acid [35], whereas the activity of the pool remained unaltered upon addition of this compound. Likewise, 0.01 mM thioglycollic acid caused a 25–32% inhibition of the four isoenzymes without affecting the pool. In contrast, 2 mM EDTA completely inactivated the pool from salt medium, decreasing the activity of the isoenzymes from YMPG only between 20 and 39%.

N-terminal sequence of three isoenzymes

The N-terminal sequences of isoenzymes with pI values of 3.71, 3.65 and 3.60, determined as described in the Materials and methods section, are shown in Figure 4. They are compared with N-terminal sequences of laccases from other fungi. *C. subvermispora* sequences share significant sequence identity with other basidiomycete laccases and many of the amino acid differences are fairly conservative, e.g. I↔L, D↔E, G↔A.

Discussion

Previously, we reported that the basidiomycete *C. subvermispora* produces MnP and laccase [25]. Because it is likely that the ligninolytic machinery of this fungus comprises the latter phenol oxidase, we decided to characterize this activity further.

We have confirmed that Mn(II) is required for laccase detection in the extracellular fluid of cultures in salt medium [25,27]. Northern blotting would help to reveal if this metal influences transcription of laccase gene(s), as it occurs with the MnP genes in *P. chrysosporium* [36]. On the other hand, the omission of Cu(II) also leads to a reduction of enzyme

	Sequence
Majority	A I G P V T D L T I T N A F V S P D G F S R Q A
C.s. (pI 3.71)	A I G P V T D I E I T D A F V S P D G P G L L K
C.s. (pI 3.65)	A I G P V T D I E I T D A F V S P D G P G L L
C.s. (pI 3.60)	A I G P V T D I E I T D A F V S P P H P P L L R
P.r.	S I G P V T D F H I V N A A V S P D G F S R Q A
PM-1	S I G P V A D L T I S N G A V S P D G F S R Q A
C.v.	G I G P V A D L T I T N A E V S P D G L S R Q A
C.h.	A I G P T A D L T I S N A E V S P D G F A R Q A
P.o.	A I G P T G D M Y I V N E D V S P D G F T R S A
A.b.	A K T R T F D F D L V N T R L A P D G F E R D T

Figure 4

Alignment of N-terminal sequences of basidiomycete laccases. Excluding *C. subvermispora* isoenzymes (pI 3.71, 3.65 and 3.60), amino acid sequences were deduced from nucleotide sequence. The majority sequence is indicated above and residues matching the consensus are shaded. Comparisons of *C. subvermispora* (*C.s.*) N-terminal sequences with published ascomycete laccases showed insufficient homology for alignment. Abbreviations used: *P.r.*, *P. radiata* (GenBank accession: X52134); *PM-1*, unidentified basidiomycete clone (GenBank accession: Z12156); *C.v.*, *C. versicolor* (no database accession, see [24]); *C. h.*, *Coriolus hirsutus* (GenBank accession: M60561); *P.o.*, *Pleurotus ostreatus* (GenBank accession: Z22591); *A.b.*, *Agaricus bisporus* (Genbank accession: L10664).

activity to negligible levels (Table 1). Up to 10-fold higher concentrations of these metals than those normally added to this medium showed no effect on enzyme activity. It is possible that the laccase apoprotein may be present in cultures lacking Cu(II), although in an inactive form, as shown by Huber and Lerch with *Neurospora crassa* [37]. Northern blotting could also give information about the mode of action of *p*-anisidine, an inducer that has proved more potent with other fungi [38]. The fact that this compound shows no effect in YMPG medium suggests that it acts at the transcription level.

The pIs of laccase isoenzymes may vary with the composition of the medium, although they are in the range reported in the literature for laccases from other fungi [8,15,39]. Isoforms with pIs of 3.65 and 3.59 were observed in salt medium. Two laccases of lower pI that are present at lower levels [27] are not visible in the gel shown in Figure 1, lane A, although occasionally they were observed throughout these studies. The four isoenzymes produced in YMPG medium have all higher pIs than those from salt medium, whereas those from wheat bran medium exhibit even higher pIs, suggesting a lower degree of glycosylation. The fifth isoform of pI 4.82 that appears in late cultures had also been identified before in defined medium [27], although the conditions favouring its production have not been reproducible.

In spite of their similar pIs, individual laccase isoenzymes produced in YMPG broth could be isolated by Q-Sepharose chromatography followed by preparative IEF. The first step allowed partial separation from MnPs and it always yielded more units than those present in the starting material, suggesting the removal of some inhibitor. Although recovery of total enzyme activity in the latter step was only about 12%, it was sufficient for our purposes. We have shown before that fast protein liquid

chromatography, a possible alternative to preparative IEF, resolves neither MnP nor laccase isoenzymes [25].

The molecular mass of most laccases from white-rot basidiomycetes is in the range 55–65 kDa [8,15,18,39]. Therefore the value of 79 kDa determined for the isozyme with pI 3.60 appears to be high. In turn, if removal of carbohydrate upon treatment with endoglycosidase F was nearly quantitative, this isoenzyme would contain about 20% carbohydrate. However, the criterion of electrophoretic mobility may not be accurate for these determinations, because it is not known with certainty how this parameter is influenced by the carbohydrate moieties of the protein.

Different substrate specificities or distinct behaviour towards enzyme inhibitors in laccase isoenzymes from the same microorganism are not unexpected [13,17]. For products of the same gene, these differences may be due to varying degrees of glycosylation. Data provided by N-terminal sequencing constitutes a first, although insufficient, approach to knowing the number of genes coding for laccase in *C. subvermispora*. At this stage it is not clear that the pI 3.71 and the 3.65 isoforms are encoded by separate genes. Peptide mapping or sequencing at the genetic level would be required for this purpose. However, their high amino-acid homology suggests that they may represent post-translational modification of the same protein. In contrast, there are numerous precedents for gene families in basidiomycetes, as in *A. bisporus* [22], *C. hirsutus* [23] and *Polyporus pinsitu* [40]. We are currently synthesizing suitable 3′ primers to amplify cDNA from *C. subvermispora*, which will then allow us to obtain a homologous probe to screen genomic libraries of the fungus.

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