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Purification and Characterization of a Laccase from the White-Rot Fungus *Trametes multicolor*

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Abstract

The wood-degrading fungus *Trametes multicolor* secretes several laccase isoforms when grown on a simple medium containing copper in the millimolar range for stimulating laccase synthesis. The main isoenzyme laccase II was purified to apparent homogeneity from the culture supernatant by using anion-exchange chromatography and gel filtration. Laccase II is a monomeric glycoprotein with a molecular mass of 63 kDa as determined by sodium dodecylsulfate polyacrylamide gel electrophoresis, contains 18% glycosylation, and has a p*I* of 3.0. It oxidizes a variety of phenolic substrates as well as ferrocyanide and iodide. The pH optimum depends on the substrate employed and shows a bell-shaped pH activity profile with an optimum of 4.0 to 5.0 for the phenolic substrates, while the nonphenolic substrates ferrocyanide and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate) show a monotonic pH profile with a rate decreasing with increasing pH.

Index entries: *Trametes multicolor*; basidiomycete; laccase; polyphenol oxidase; lignin degradation.

Introduction

Laccases (benzenediol:oxygen oxidoreductases; EC 1.10.3.2.) are multicopper-containing enzymes belonging to the blue oxidases and catalyze the one-electron oxidation of a wide variety of substrates, particularly various

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phenols, anilines, and benzenethiols, as well as metal complexes (such as ferrocene), ferrocyanide, or iodide *(1)*. While laccases are remarkably unspecific as to their reducing substrates, they strongly prefer oxygen as their oxidizing substrate, which is reduced to water in a four-electron transfer *(2–5)*. Laccase was first described from the sap of the Japanese lacquer tree *Rhus vernicifera (6)*. Probably the first report on the presence of laccase in fungi was from Laborde in 1897 *(7)*. Since then, laccases have been found in the majority of white-rot fungi described to date, and they are also produced by other types of fungi, some bacteria, and insects *(7–9)*. Fungal laccases are believed to be involved in the degradation of lignin and/or in the removal of potentially toxic phenols arising during this degradation *(3,5,10)*. In addition, fungal laccases are hypothesized to take part in the synthesis of dihydroxynaphthalene melanins or pigments *(5,11)*, in fungal morphogenesis *(12,13)*, and in phytopathogenesis and fungal virulence *(14,15)*.

Many white-rot fungi produce multiple laccase isoforms under the appropriate inductive conditions *(4,5,16)*. Most fungal laccases studied are extracellular proteins, but intracellular laccases have been detected in some fungi *(15,17)*. Typically, fungal laccases are glycosylated molecules of 60–85 kDa of which 15–20% is carbohydrate *(3,18)*, although glycosylation of fungal laccases can also be significantly higher *(19)*. Glycosylation in laccases is suggested to play a role in secretion, proteolytic susceptibility, copper retention, and activity. To date, thorough purification and characterization was performed on laccases from more than 20 different fungi *(9,18)*.

Laccases have become important, industrially relevant enzymes that can be used for a number of diverse applications, including biocatalytic purposes such as delignification of lignocellulosics and crosslinking of polysaccharides, bioremediation applications such as waste detoxification and textile dye transformation *(9)*, food technologic uses, personal and medical care applications *(18)*, and biosensor and analytical applications *(20)*. In the present study, a laccase isoenzyme secreted by the white-rot basidiomycete *Trametes multicolor*, which only recently has been identified as an excellent source of this enzyme activity *(21)*, was purified to apparent homogeneity and characterized with respect to its physicochemical and biochemical properties.

Materials and Methods

Chemicals

All chemicals were of the highest purity available. 2,2'-Azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS), *p*-anisidine, catechol, guaiacol, hydroquinone, NaI, and $K_4Fe(CN)_6$ were from Sigma (St. Louis, MO); 2,6-dimethoxyphenol (2,6-DMP) was from Fluka (Buchs, CH); bovine serum albumin (BSA) (fraction V) was from United States Biochemical (Cleveland, OH); and peptone from meat was from Merck (Darmstadt, Germany).

Organism and Culture Conditions

The wild-type strain of *T. multicolor* MB49 used was isolated from hardwood in Southern Germany and was obtained from the culture collection of the Institute of Applied Microbiology, University of Agricultural Sciences Vienna, where it is deposited under the indicated strain number. Stock cultures were maintained on potato dextrose agar plates (Difco, Detroit, MI) and transferred every 2 mo. Shake-flask cultures of the organism were grown at 25°C with continuous agitation at 110 rpm in baffled 1000-mL Erlenmeyer flasks containing 200 mL of medium consisting of 40 g/L of glycerol, 15 g/L of peptone from meat, and 1 g/L of $MgSO_4 \cdot 7H_2O$. The pH was adjusted to 5.0 with phosphoric acid prior to sterilization. Several agar plugs cut from the actively growing outer circumference of a fungal colony growing on potato dextrose plates were used as inoculum. For stimulating laccase synthesis, a sterile stock solution of $CuSO_4 \cdot 5H_2O$ (1 *M* in water) was added to the actively growing culture after 10 d of incubation so that the final concentration in the medium was 1.0 m*M (21)*. After 20–25 d of cultivation, the laccase activity reached its maximum of approx 85,000 U/L.

Purification of Enzyme

Mycelia were separated by centrifuging at 15,000*g* for 20 min at 4°C. The extracellular medium from one flask was concentrated to 20 mL using a 10-kDa ultrafiltration membrane (YM10; Millipore, Bedford, MA) and then diafiltrated against 50 m*M* formate buffer, pH 4.25 (buffer A). Any precipitate was removed by centrifugation (45,000*g* for 30 min at 4°C) and the supernatant applied to a 30-mL Q-Sepharose column (Amersham Pharmacia, Uppsala, Sweden) preequilibrated with buffer A. Laccase II was eluted at 5 mL/min by continuously decreasing the pH to 3.0 in 5 column vol by mixing buffer A with 50 m*M* formate buffer, pH 3.0. Elution was simultaneously monitored at 280 and 610 nm for protein and the type 1 copper in laccase, respectively. The laccase fractions were pooled, immediately diluted with the same volume of 100 m*M* citrate buffer (pH 5.5), to avoid denaturation of the enzyme, and concentrated as described previously to about 2 mL. The concentrated enzyme solution was applied to a Superdex 75 prep grade column (800 × 16 mm; Amersham-Pharmacia) equilibrated with 20 m*M* acetate buffer, pH 5.0, containing 200 m*M* NaCl and eluted at 2 mL/min. Active fractions were pooled, desalted, filter sterilized, and stored at 4°C.

Enzyme Activity Assay

Laccase activity was determined with ABTS as the substrate. The assay mixtures contained 0.5 m*M* ABTS, 20 m*M* sodium acetate buffer (pH 3.5), and 20-µL aliquots of appropriately diluted enzyme sample. Oxidation of ABTS was monitored by following the increase in A_{436} ($\varepsilon = 29.3\ mM^{-1} \cdot cm^{-1}$) *(22)*. One unit of laccase activity is defined as the amount of enzyme required to oxidize 1 µmol of ABTS/min at 25°C.

Determination of Protein

Protein determinations were done according to the dye-binding method of Bradford *(23)*, using BSA as the standard.

Determination of Sugar Content

The carbohydrate content of purified laccase II was estimated by the phenol sulfuric acid method using mannose as the standard *(24)*.

Electrophoretic Analyses

Denaturing sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on the Amersham Pharmacia Phast System using precast PhastGel 8–25% gels according to Laemmli *(25)*. Laccase II samples were denatured by diluting with an equal volume of Sample Buffer, Laemmli, 2× concentrate (Sigma), and incubating for 30 min at 30°C. The molecular weight was estimated by comparison with marker proteins (Molecular Standard Mixture Recombinant, 15–150 kDa; Sigma) after proteins were visualized by silver staining. Isoelectric focusing was performed on the same system using precast gels (PhastGel IEF 3–9). A low-p*I* marker protein kit (pH 2.8–6.5; Amersham Pharmacia) was used to determine p*I* values. Either proteins were stained with silver following the manufacturers instruction manual or the laccase bands were visualized by activity staining. To this end, the electrophoresis gel was incubated at room temperature with 20 m*M* acetate buffer, pH 3.5, containing 10 m*M* ABTS, with gentle shaking until green bands were clearly visible.

Determination of Molecular Weight

Molecular weight of the native protein was determined by gel filtration using a Superdex 75 HR 10/30 column (Amersham Pharmacia), equilibrated with 50 m*M* phosphate buffer, pH 7.0, containing 100 m*M* KCl. The column was calibrated with the standard proteins ribonuclease A (M_r = 13,700), carbonic anhydrase (M_r = 29,000), ovalbumin from chicken egg (M_r = 45,000), BSA (M_r = 66,000), and transferrin (81,000), each at a concentration of 2 mg/mL. The flow rate for elution was 0.5 mL/min.

Steady-State Kinetic Measurement

All measurements were made at 25°C at the pH optimum of the respective substrate using oxygen (air-saturated solutions) as the electron acceptor. Table 2 provides the extinction coefficients used. Reactions were initiated by the addition of laccase, and initial rates were obtained from the linear portion of the progress curve. All kinetic constants were calculated by nonlinear least-squares regression, fitting the observed data to the Henri-Michaelis Menten equation. To determine the kinetic constants for oxygen, the rate of oxygen consumption was measured with an oxygen electrode placed in a thermostatically controlled vessel (Rank Brothers, Cambridge, UK) at 25°C using ABTS (0.5 m*M* in 20 m*M* Na acetate buffer, pH 3.5) as the substrate.

The pH optima for various substrates were determined using air-saturated Na citrate buffer (50 m*M*, pH 2.5–6.5). The optimum temperature was determined between 15 and 70°C under standard assay condition using ABTS as the substrate.

Results and Discussion

Purification

Isolation of the laccase isoenzyme was performed from the culture supernatant of *T. multicolor* harvested after approx 20 d of cultivation when extracellular laccase activity reached its maximum. Under the growth conditions selected, *T. multicolor* secreted at least five laccase isoforms that could be distinguished because of differences in their p*I* values, which ranged from 3.0 to 6.2 (Fig. 1, lane 1). The purification protocol established for the selected laccase isoenzyme termed laccase II is based on anion-exchange chromatography and gel filtration as given in detail in Materials and Methods. Anion-exchange chromatography of the concentrated culture filtrate gave two peaks of laccase activity. The first peak contained several laccase isoenzymes with p*I* values of >3, as shown by isoelectric focusing followed by activity staining with ABTS (Fig. 1, lane 2). The second peak, which eluted at a pH of approx 3.3, contained only one laccase isoform with a p*I* of 3.0 (laccase II; Fig. 1, lane 3). This laccase isoform is the predominant laccase enzyme formed by *T. multicolor* under the growth conditions selected in this study, as is also evident from the activity staining of the crude culture filtrate (Fig. 1, lane 1). Table 1 presents a summary of a typical purification of laccase II. This two-step procedure yielded a bright blue protein that was apparently homogeneous, as judged by isoelectric focusing (Fig. 1, lane 3) and SDS-PAGE (data not shown). The purified laccase II had a specific activity of 400 U/mg under standard assay conditions using ABTS and oxygen (air) as substrates. The molecular mass of laccase II was 63 kDa as determined by SDS-PAGE, and 55 kDa according to gel filtration, indicating that the enzyme is monomeric. The p*I* was 3.0, as judged by isoelectric focusing and comparison to standard proteins. The carbohydrate content of laccase II was estimated to be 18% using the phenol–sulfuric acid method with mannose as the standard.

Kinetic Properties

In accordance with results on laccase from other fungal sources, *T. multicolor* laccase II oxidizes a range of different substrates. Table 2 lists some of these substrates and their apparent kinetic constants determined spectrophotometrically when using oxygen (air-saturated solutions) as the electron acceptor. In addition, the kinetic constants for oxygen, measured with an oxygen electrode and with ABTS as the electron donor, are given. The kinetic constants determined are quite comparable with those reported for other *Trametes* spp. *(20,26–29)*. The highest kinetic efficiency k_{cat}/K_m was found for the model substrate ABTS. Furthermore, laccase II exhibited high

Table 1
Purification of Laccase II from *T. multicolor*

Purification step	Laccase activity (U)	Protein (mg)	Specific activity (U/mg)	Yield (%)
Culture broth (concentrated)	14,000	43.4	320	100
Ion-exchange chromatography				
Peak 1	2890	ND[a]	ND[a]	20.7
Peak 2	5580	15.5	360	39.8
Gel filtration				
Peak 2	4450	11.2	400	31.8

[a] ND, not determined.

Table 2
Apparent Kinetic Constants of Laccase II from *T. multicolor* for Some Substrates

Substrate	Wavelength monitored (nm)	Extinction coefficient ($mM^{-1}\cdot cm^{-1}$)	pH measured	K_m (μM)	k_{cat} (s-1)	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)
ABTS	436	29.3 [a]	3.5	14.1	510	$36{,}100 \times 10^3$
$Fe(CN)_6^{4-}$	420	0.98[b]	2.5	40.2	856	$21{,}300 \times 10^3$
Catechol	392	1.46 [a]	4.0	845	223	264×10^3
Hydroquinone	247	21.03 [a]	4.5	362	126	346×10^3
Guaiacol	465	12.1 [a]	4.5	436	130	297×10^3
2,6-DMP	468	27.5 [a]	4.0	186	203	1090×10^3
p-Anisidine	542	1.173 [a]	5.5	1510	194	129×10^3
I^-	353	26.0 + 2.2 $[I^-]$ [e]	3.5	483,000	10.7	0.022×10^3
Oxygen	—	—	3.5	761	1080	1420×10^3

[a] Datum is from Muñoz et al. *(22)*.
[b] Datum is from Naqui and Varfolomeev *(35)*.
[c] Datum is from Xu *(1)*.

activity with either methoxy- or hydroxy-substituted phenols. The Michaelis constant K_m for oxygen was 0.76 m*M*. Considering the low solubility of oxygen in aqueous solutions under assay conditions, which roughly amounts to 0.26 m*M* *(30)*, laccase II typically is employed under nonsaturating conditions of its cosubstrate oxygen.

Laccase II also catalyzed the oxidation of inorganic ions such as ferrocyanide very efficiently with a k_{cat}/K_m of $21.3 \times 10^6\ M^{-1}\cdot s^{-1}$, which is in accordance with reports on other fungal laccases *(20, 28, 29)*. By contrast, the iodide ion is a very poor substrate with a catalytic efficiency that is approximately six orders of magnitude lower than that of ferrocyanide. A similar high K_m value, but a significantly lower turnover number k_{cat}, has been reported for the oxidation of iodide by a *Myceliophthora thermophila* laccase

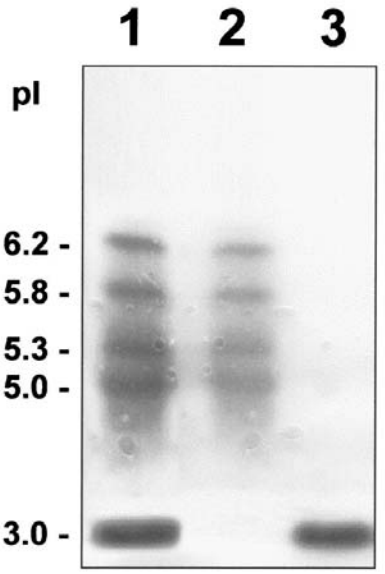

Fig. 1. Isoelectric focusing of laccase from *T. multicolor*. Bands were visualized by activity staining using ABTS as the substrate. Lane 1, culture supernatant; lane 2, laccase activity peak 1 from anion-exchange chromatography step; lane 3, purified laccase II.

(1). Despite these unfavorable kinetic constants, it has been suggested that I^- is a true substrate of fungal laccases and that catalysis is attributed to a direct interaction between I^- and the enzyme *(1)*.

pH and Temperature Dependence of Activity

The pH dependence of the activity of laccase II was determined for eight different substrates (see Fig. 2 A–H): ABTS, hydroquinone, catechol, 2,6-DMP, *p*-anisidine, guaiacol, ferrocyanide, and iodide, respectively. The pH optima varied between 2.0 and 5.0 depending on the substrate employed. The phenolic substrates examined showed bell-shaped pH activity profiles with similar pH optima of 4.0–4.5. Interestingly, the nonphenolic substrate iodide had a similar bell-shaped pH profile, while the other two nonphenolic substrates—ferrocyanide and ABTS—showed only monotonic pH profiles in which the rate decreased with increasing pH. It has been suggested recently that both OH^- inhibition at the type 2/type 3 trinuclear copper cluster of laccase and the redox potential difference between a reducing substrate and the type 1 copper (which correlates to the electron transfer rate and is favored by phenolic substrate at a higher pH) can affect the pH activity curve of a laccase *(9,31)*. Our findings are in agreement with this hypothesis. The oxidation of the nonphenolic substrates does not involve proton exchange and, hence, is mainly affected by OH^- inhibition, which increases with higher pH, resulting in the monotonic pH profiles shown in Fig. 2A,2G. Note that the

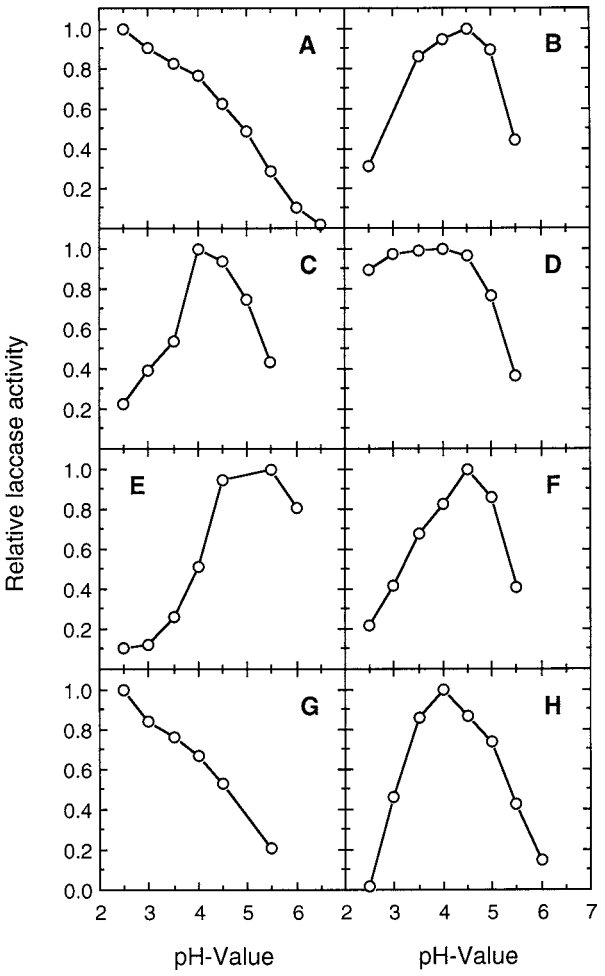

Fig. 2. Effect of pH on activity of laccase from *T. multicolor* in presence of different substrates. (**A**) ABTS (0.5 m*M*); (**B**) hydroquinone (1.0 m*M*); (**C**) catechol (1.0 m*M*); (**D**) 2,6-dimethoxyphenol (1.0 m*M*); (**E**) p-anisidine (1.0 m*M*); (**F**) guaiacol (1.0 m*M*); (**G**) ferrocyanide (1.0 m*M*); (**H**) iodide (250 m*M*).

pH activity profile of the nonphenolic substrate I^- (Fig. 2H) does not show this monotonic pH profile, but a bell-shaped curve similar to those obtained for the phenolic substrates examined (e.g., Fig. 2B). For these substrates, oxidation involves proton release and has a significant E^0 dependence on the pH. Hence, the pH profile is biphasic and possibly reflects the opposing effects of the pH-induced redox potential changes and the OH^- inhibition *(31)*.

The reaction velocity of the laccase-catalyzed oxidation of ABTS increased with increasing temperature, showing a maximum at 50°C (3 min, standard assay conditions at pH 3.5; Fig. 3). This temperature optimum is comparable with those determined for other fungal laccases *(29)*.

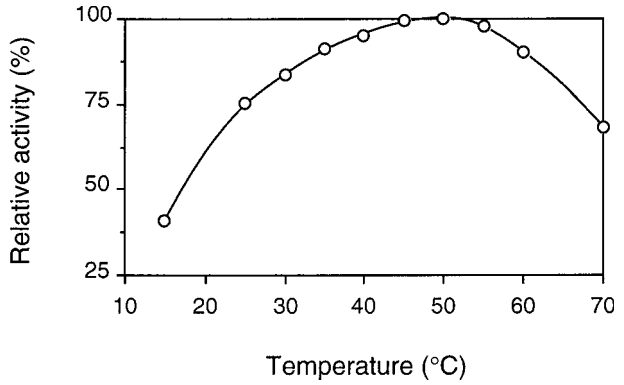

Fig. 3. Temperature dependence of laccase II activity measured under standard assay conditions. The total assay time was 3 min.

Table 3
Effect of Several Inhibitors on Laccase II from *T. multicolor*

Compound	Concentration (m*M*)	Inhibition (%)
NaN_3	0.025	96
	0.05	>98
	0.1	>98
EDTA	0.01	0
	0.1	15
H_2O_2	0.1	0
	1.0	9

Effect of Inhibitors

The effects of several putative laccase inhibitors on laccase II were studied using ABTS as the substrate at pH 3.5 (Table 3). Especially azide is a very effective inhibitor of laccase II, and complete inhibition was observed with 0.05 m*M* NaN_3. Azide is thought to bridge both the type 2 and type 3 Cu in laccase *(5,32)*. The inhibiting effect of halides, well-known inhibitors of laccases, were studied in more detail (Fig. 4). The observed halide inhibition with laccase II is in agreement with previous reports. Based on I_{50} (the concentration of an inhibitor causing 50% activity reduction), which for the sodium halides NaF, NaCl, and NaBr was 0.022, 16, and 120 m*M*, respectively, the same order of the inhibitory potency of the halides ($F^- > Cl^- > Br^-$) was observed as for other laccases *(33,34)*. This inhi-bition order has been attributed to the restricted accessibility of the copper sites for larger halides. Fluoride, which completely inhibits laccase II at a concentration of 2.5 m*M*, is thought to bind to type 2 Cu and in some instances to both type 2 and type 3 Cu *(5,33)*.

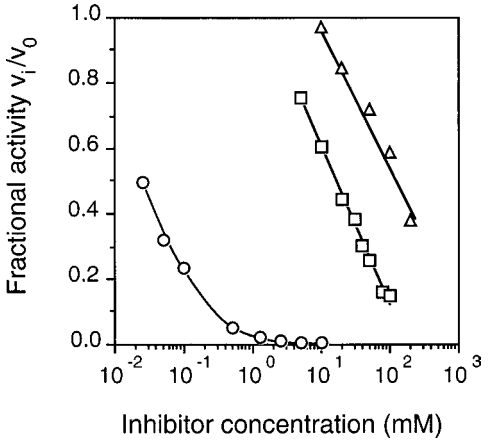

Fig. 4. Inhibition of laccase II by halides. The dose response plot shows the fractional activity v_i/v_0 as a function of inhibitor concentration (v_i, initial velocity in the presence of inhibitor; v_0, initial velocity in the absence of inhibitor). ○, Fluoride; □, chloride; △, bromide.

Conclusions

The major laccase isoform of *T. multicolor*, laccase II, was characterized and some of its physicochemical and biochemical properties were determined. Laccase II has typical biochemical characteristics, such as molecular mass, glycosylation, and kinetic properties, of laccases from basidiomycetes. Because of the high yields that are obtained in cultivations of this strain on a simple, glycerol-based medium, the *T. multicolor* enzyme could be of interest for some of the biotechnologic applications that have been suggested for laccases from other fungal sources.

Acknowledgments

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