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Laccase of *Coriolus zonatus*

Isolation, Purification, and Some Physicochemical Properties

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Abstract

Laccase is one of the lignolytic enzymes found in liquid cultures of the fungus *Coriolus zonatus* in defined medium. The enzyme was isolated from culture liquid and characterized. Laccase from *C. zonatus* is a single-chain protein with a molecular mass of 60 kDa. Carbohydrate moiety of enzyme consisted of mannose, galactose and *N*-acetyl-glucosamine in a ratio of 6:2:0.6, respectively, and comprised 10% of the entire molecule. Isoelectric point was detected at pH 4.6. Laccase was found to have a pH optimum of 4.9 and temperature optimum of 55°C. Substrate specificity studies were conducted with catechol, K-ferrocyanide, hydroquinone, and sinapinic acid as substrates. The highest efficiency of catalysis was observed with sinapic acid as the substrate. The kinetic constants k_{cat} and K_m of this reaction were 624 s^{-1} and 7 μ*M*, respectively.

Index Entries: Laccase; enzyme purification; *Coriolus zonatus*; substrate specificity; thermostability; pH stability.

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Introduction

Lignin biodegradation is attracting the attention of many researchers because of its significance for efficient bioconversion of lignocellulosic residues into useful chemicals and its ability to enhance the digestibility of these residues as feed for ruminants *(1)*. In nature, lignin is degraded by various microorganisms, particularly, white rot fungi. These fungi produce extracellular lignin-degrading enzymes such as ligninase, Mn-peroxidase, and laccase *(2)*.

Laccases (*p*-diphenol: oxygen oxidoreductase; EC 1.10.3.2) are blue copper-containing glycoproteins that use oxygen as the electron acceptor to remove hydrogen from phenolic hydroxyl groups. This reaction leads to the formation of free radicals that, as demonstrated with model lignin compounds, can undergo rearrangements leading to alkyl-aryl cleavage, oxidation of benzyl alcohols, and cleavage of side chains and aromatic rings *(3)*. It has also been shown that laccase can oxidize nonphenolic substrates in the presence of appropriate redox-mediators *(4,5)*.

The precise role of laccase in lignin degradation is now under investigation. Some researchers suggest that laccase contributes to substrate depolymerization *(6)*, whereas others indicate that it may catalyze the coupling of degradation intermediates that are toxic to fungi *(7)*. Laccase's key role in lignin decay is supported by immunochemical methods, which show this enzyme to be localized in degraded-wood cell walls (8).

Our previous studies have shown that *Coriolus zonatus* causes extensive lignin degradation. Consequently, we have focused our efforts on the investigation of enzymes that excrete during decay by this fungus, especially on extracellular laccase. The aim of this work is to isolate homogeneous laccase preparations and to characterize their physicochemical and biochemical properties in comparison with other fungal and plant laccases.

Materials and Methods

All chemicals used as buffers and substrates were commercial products of at least reagent grade, unless otherwise indicated.

Fungal Culture

C. zonatus (Nees.: Fr.) Quel. 093 producing highly active extracellular laccase was found by screening cultures of basidial fungi from the collection of the Komarov Botanical Institute, Russian Academy of Sciences.

C. zonatus was grown in 250-mL Erlenmeyer flasks containing 150 mL of medium. The medium used had the following composition (g/L): 3 peptone, 10 glucose, 0.6 KH_2PO_4, 0.001 $ZnSO_4$, 0.4 K_2HPO_4, 0.0005 $FeSO_4$, 0.05 $MnSO_4$, 0.5 $MgSO_4$. For submerged cultivation, this medium was supplemented with 0.15 g/L of $CuSO_4$. In all experiments the pH of the medium was adjusted to 6.0. The media were sterilized by heating to 120°C

for 45 min. After cooling, they were inoculated with a mycelial implant taken from a preculture grown on 2% agar and brewer's wort diluted 1:4 (v/v) with water. After incubating at 26–28°C for 8–10 d in 250-mL Erlenmeyer flasks containing 150 mL of medium and ceramic beads, mycelium grown in the surface culture was disrupted into small fragments by ceramic beads suitable for inoculation (10–15 mL/flask).

This stage of surface cultivation was followed by submerged cultivation at 26–28°C on a rotary shaker for 10–12 d. The culture liquid was then filtered through a porosity 1-sintered-glass filter, and the filtrates containing the enzyme were collected.

Purification Method

All procedures described subsequently were performed at 4°C. Culture filtrate was saturated with ammonium sulfate to a final concentration of 90%, and after 120 min with gentle stirring, the filtrate was centrifugated for 30 min at 8000*g*. The pellet obtained was resuspended in distilled water and was applied onto a Sephadex G-25 (Pharmacia, Uppsala, Sweden) column (2×100 cm) previously equilibrated with 5 m*M* K-phosphate buffer, pH 6.0. Fractions enriched in laccase activity were pooled and loaded onto a DEAE-Toyopearl 650 M (Toyo Soda, Tokyo, Japan) column (2 × 20 cm) previously equilibrated with the same buffer. Proteins were eluted with a 150-mL linear gradient of 5–200 m*M* of the same buffer, and 3-mL fractions were collected. For further purification, fractions with laccase activities were dialyzed against 5 m*M* K-phosphate buffer, pH 6.0, and then applied onto a DEAE-Toyopearl 650 M column (2 × 20 cm) previously equilibrated with the same buffer. Proteins were eluted with a 250-mL linear gradient of 5–200 m*M* of the same buffer and 1-mL fractions were collected.

High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) was performed on a column (0.1 × 30 cm) of Superose 12 (Pharmacia) previously equilibrated with 50 m*M* K-phosphate buffer, pH 6.5. This step was used either for enzyme purification to a homogeneous state or for molecular mass determination *(9)*. In the latter case, the columns were calibrated with standard proteins: papain, 23,400; chymotrypsin, 25,000; formate dehydrogenase, 43,000; bovine serum albumin (BSA), 66,000; aldolase, 158,000. V_0, volume of the mobile phase (column's free volume), was 6 mL, determined according to the elution profile of blue dextran. V_t, the total volume of the packed bed (the elution volume of tryptophan molecules), was 17.5 mL.

Enzyme Purification Control

Enzyme purification control was carried out with HPLC or polyacrylamide gel electrophoresis (12.6% acrylamide in 0.2*M* Tris-glycine buffer at pH 8.3) under denaturing or nondenaturing *(10)* conditions. Proteins were stained with a solution of 0.1% Coomassie blue in 5% acetic acid. The

molecular mass of enzyme under denaturing conditions was estimated with reference to standard proteins: aprotinin, 6500; a-lactalbumin, 14,200; trypsin inhibitor, 20,000; trypsinogen, 24,000; carbonic anhydrase, 29,000; glyceraldehyde-3-phosphate dehydrogenase, 36,000; ovalbumin, 45,000; BSA, 66,000 (Sigmamarker for low molecular weight range, Sigma, St. Louis, MO).

Isoelectric Point (pI) Determination

Analytical isoelectrofocusing was performed over the pH range 4.0–10.0, using the ampholines from Pharmacia according to previously used methods (10).

Amino Acid and Carbohydrate Composition

Amino acid composition was determined after the hydrolysis of laccase in 6*M* HCl at 110°C for 24 h. A SYKAM S 432 amino acid analyzer (Gleilich, Germany) using postcolumn derivatization with orthophthaldialdehyde was utilized.

Carbohydrate content determination involved derivatization of the monosaccharides, released on acid hydrolysis, to *N*-(4-methylcoumarin-7-yl) glycamines, and their subsequent analysis by reverse-phase HPLC with fluorimetric quantitation using a spectrofluorimeter Hitachi MPF-3 (Hitachi, Tokyo, Japan) *(11)*.

Adsorption and Electron Paramagnetic Resonance Spectra

Homogeneous laccase was dialyzed against 5 m*M* K-phosphate buffer, pH 6.0, and the spectrum was recorded at 25°C between 900 and 200 nm, using a Hitachi-557 spectrophotometer (Hitachi, Tokyo, Japan).

Electron Paramagnetic Resonance (EPR) spectra were recorded using an RE 1306 radiospectrometer (Zavod Eksperimental'nogo Priborostroenia, Tchernogolovka, Russia) at 9.4 GHz at 77 K. All samples were in 50 m*M* K-phosphate buffer, pH 6.0.

Determination of Copper

Copper analyses were carried out with 2,2'-biquinoline (Merck, Darmstadt, Germany) as the colorimetric reagent using a standard procedure *(12)*.

Laccase Assay

Laccase activity was usually determined spectrophotometrically in a reaction medium containing 0.1% catechol (Sigma) in 100 m*M* acetate buffer, pH 4.9, at room temperature *(13)*. The volume of reaction mixture was 2 mL. The activity was expressed as $A_{410} \times$ dilution/(mg of protein · min). The protein concentration was determined by the Lowry method *(14)*.

Substrate Specificity

When substrate specificity was investigated, the laccase activity was determined by oxygen consumption using a Clark electrode in a hermeti-

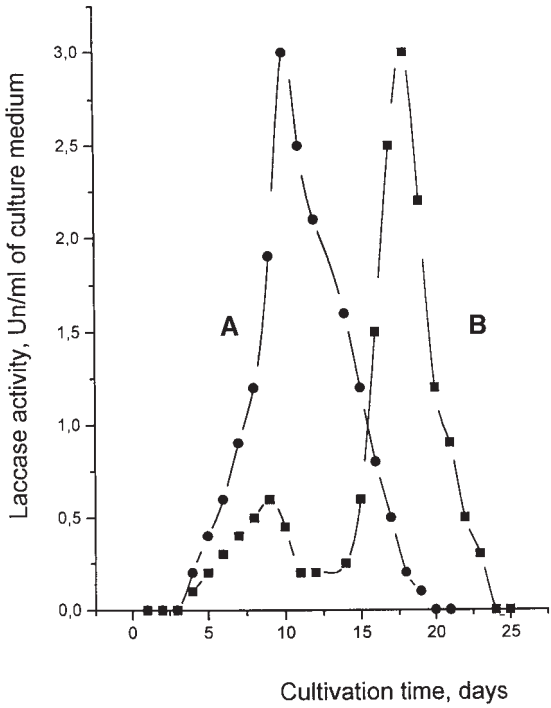

Fig. 1. Time course of laccase activity in the medium of submerged (1) and surface (2) cultures of *C. zonatus*.

cally seated cell (final volume 0.7 mL) at 25°C with constant stirring. The substrates—catechol (Sigma), hydroquinone and K-ferrocyanide (Merck), 3,5-dimethoxy-4-hydroxycinnamic (sinapic) acid (Aldrich, Steinheim, Germany)—were dissolved in 50 m*M* phosphate buffer, pH 4.9. The reaction was started by the addition of laccase. The final concentration of laccase in the cell was $2 \times 10^{-8} M$. The concentration of oxygen was accepted to be 260 µ*M* (according to the evaluation of the Henry coefficient: 773 A/mol per kg of water). The velocities of reactions were expressed in µ*M* O_2/(mg of protein · min). The kinetic parameters (K_m and k_{cat}) of the enzymatic reaction catalyzed by laccase were calculated according to Michaelis-Menten kinetics.

Results and Discussion

During the growth of *C. zonatus,* it was shown that this strain produced both extracellular and intracellular oxidases such as peroxidase, laccase, and phenoloxidase. The maximum content of extracellular enzymes corresponded to the end of the stationary phase or the beginning of decay (16–18 d for surface culture). In the course of cultivation, we typically observed two peaks of laccase synthesis in surface culture and one peak in submerged cultures (Fig. 1). The highest level of laccase activity was observed

Table 1
Scheme of Purification of *C. zonatus* Laccase

Steps of purification	Specific activity (U/mg of protein)	Total activity (U)	Purification factor
Initial preparate	0.4	18,000	1.0
Precipitation with $(NH_4)_2SO_4$	1.0	14,000	2.5
Gel filtration on Sephadex G-25	23.0	12,000	57.5
Ion-exchange chromatography on DEAE-Toyopearl 650 M	97.0	11,000	242.5
Rechromatography on DEAE-Toyopearl 650 M	178.0	8240	445.0

between d 7 and 10 under conditions of submerged cultivation. The activity of extracellular laccase in cultures in elaborated medium reached levels in the range of 2.5–3.0 U/mL. In the present work, we investigated only extracellular laccases, isolated from culture filtrate after submerged cultivation.

Table 1 summarizes the four-step purification procedure. The crude filtrate was a relatively intense brown. After the first purification step (precipitation with ammonium sulfate), a considerable portion of pigments was removed but the preparates still were light brown. This color was probably the result of the presence of oxidized phenolic compounds as well as pigments. After the second step (gel filtration on Sephadex G-25), the degree of purification was increased (purification factor: 57) but without significant changes in the sample's color. The DEAE-Toyopearl 650 M chromatography permitted the separation between pigments and two laccase isoenzymes. After the fourth step of purification, we obtained blue preparations of laccase 1, purified 445-fold. It should be mentioned that molecular mass of laccase 1 was 60 + 2 kDa and laccase 2 was 75 + 2 kDa estimated by HPLC (Fig. 2A). But the amount of laccase 2 as well as its activity were insignificant, and for further investigations we used only laccase 1. The value of molecular mass determined by HPLC was confirmed by results of electrophoresis under denaturing conditions: 58 + 3 kDa (Fig. 2B). The molecular mass of laccase *C. zonatus* was of the close values as for some other fungal laccases: *Coriolus versicolor*, 62 kDa *(15)*; *Coriolus hirsutus*, 55 kDa *(15)*; *Ceriporiopsis subvermispora*, 79 kDa *(16)*. The result of electrophoresis under denaturing conditions indicated that laccase 1 was a single polypeptide chain protein (Fig. 2B).

Table 2 presents the amino acid composition of laccase. The main difference between this laccase and other fungal laccases was in the content of glycine. Such high glycine content could not be explained by the composition of growing media. With sucrose in place of glucose as the source of carbon, the enzyme produced has the same amino acid composition (data not shown). In spite of the high glycine content, the value of p*I* was 4.6. Note that the p*I* of laccases isolated from relative strains belonging to genus *Coriolus (15)* are in the same range, which is 3.5–4.9.

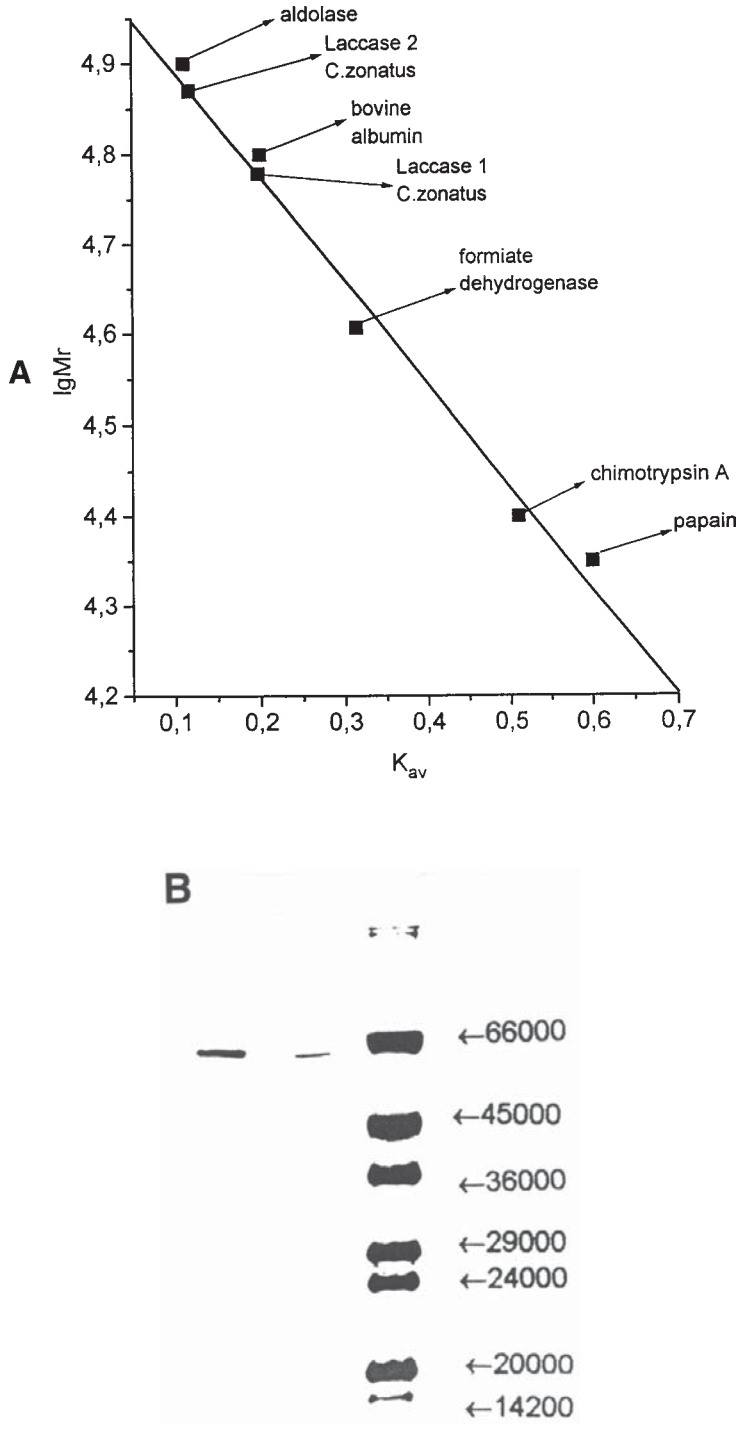

Fig. 2. **(A)** Molecular mass determination of laccase *C. zonatus* by HPLC. M_r, molecular mass (kDa) and K_{av}, true partition coefficient. **(B)** sodium dodecyl sulfate-polyacrylamide gel electrophoresis of laccase *C. zonatus*. The lanes contained 5 and 30 µg (1 and 2) of laccase protein. *M*, molecular mass (kDa).

Table 2
Amino Acid Composition of *C. zonatus* Laccase

Amino acid residue	Number of amino acid residues/ molecule of laccase
Asx	56
Thr	35
Ser	39
Glx	48
Gly	102
Ala	52
Val	33
Met	5
Ile	20
Leu	31
Tyr	4
Phe	15
Lys	14
His	16
Arg	13

Like all known fungal laccases, the laccase investigated was a glycoprotein containing 10% carbohydrate per molecule of protein. Carbohydrate moiety consisted of mannose, *N*-acetyl-glucosamine, and galactose in a ratio of 6:2:0.6, respectively.

The laccase molecule contained 3.5–3.9 copper atoms as determined by the 2,2'-biquinoline method. Probably the one copper atom was binding to the protein very tightly and could not be removed completely under spectrophotometrical determination by the 2,2'-biquinoline method. Consequently, the values of copper obtained were slightly lower than was expected. The laccase spectrum featured adsorption peaks at 280 and 610 nm and a shoulder at 330 nm. Thus, laccase has copper type 1 atoms, which impose the intense blue of the enzyme (peak at 610 nm). It also contains two copper type 3 atoms, which form a binary complex with adsorption (shoulder) at 330 nm (Fig. 3). All EPR spectra were performed at <20 mW, but scanning of microwave power in the range of 5–50 mW did not produce dramatic changes in the spectra of proteins observed. Hence, all measurements were conducted using up to 20 mW of microwave power and a modulation amplitude of 1.0–2.7 G. Laccase EPR spectra were typical of other fungal laccases and laccase of *Rhus vernicifera (17)* (Fig. 4). Analysis of EPR spectra showed the existence of two copper atoms of types 1 and 2. The parameters of the first type of copper were determined as $g_{\perp} - 2.061$, $g_{||} - 2.200$, and $A_{||} - 8.103 \times 10^{-3}\ cm^{-1}$. These values were in close agreement with those for other laccases *(17)*. As it was previously shown for plant laccases, the surrounding of copper type 1 atoms could be determined more precisely when copper atom type 2 was removed from the protein globule and the nitrogen ligands were rearranged to square configuration.

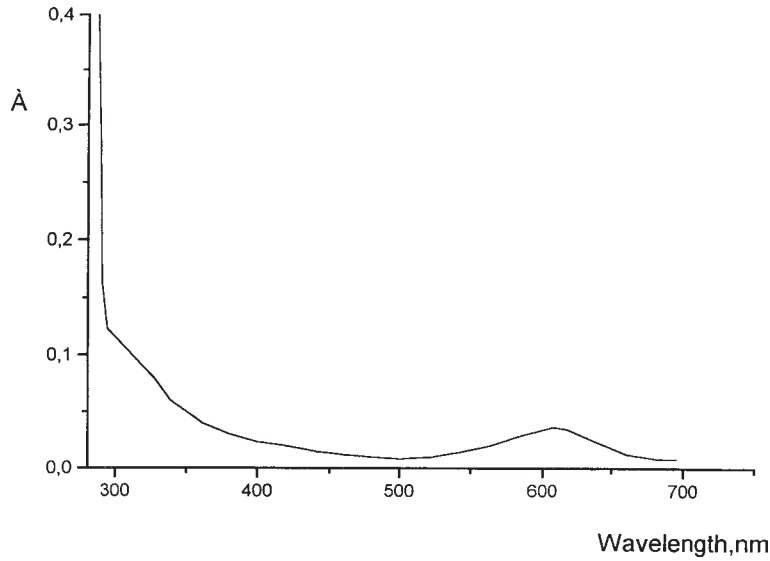

Fig. 3. Adsorption spectrum of laccase, obtained under the conditions described in the Materials and Methods section.

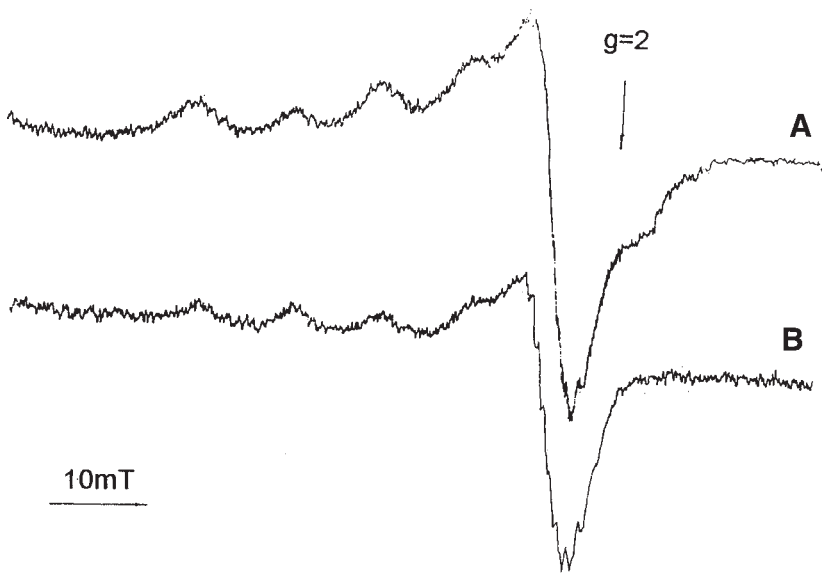

Fig. 4. EPR spectra of native **(A)** and copper type 2–depleted laccase **(B)** at 77 K. Modulation amplitude: 0.27 mT; microwave power: 5 mW. All samples were in 50 m*M* K-phosphate buffer, pH 6.0.

The superhyperfine splitting from four equivalent nitrogen atoms could then be measured more precisely. These observations were confirmed in our case too (Fig. 4B), and the value of A_N was calculated to be 1.04 mT.

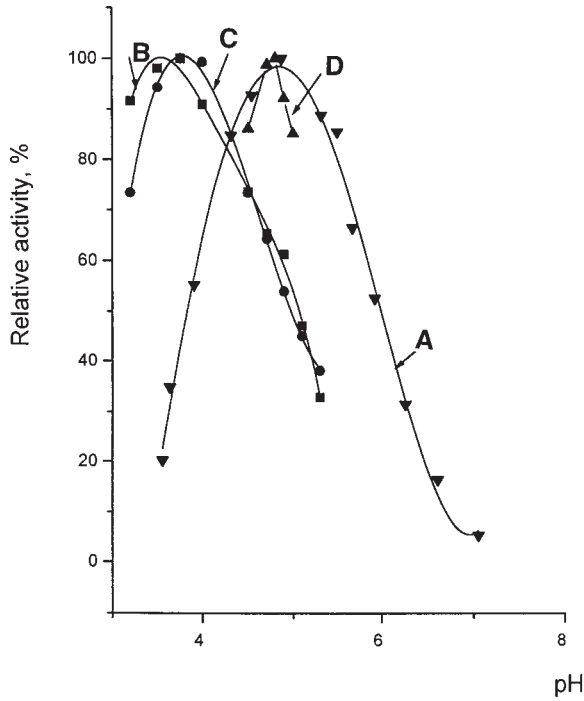

Fig. 5. Effect of pH on laccase activity measurement. Laccase activity was measured spectrophotometrically, as indicated in the Materials and Methods section. Buffers used were 100 m*M* universal, pH 3.5–7.0 **(A)**; 100 m*M* citrate-phosphate, pH 3.2–5.3 **(B)**; 100 m*M* citrate, pH 3.2–5.3 **(C)**; 100 m*M* acetate, pH 4.4–5.1 **(D)**. All buffers contained 0.5*M* KNO_3.

Investigations of laccase pH dependency were carried out over the pH range 3.5–7.0, and catechol was used as a substrate. Reaction mixtures contained 0.5*M* KNO_3 in order to eliminate the influence of ionic strength on the enzyme activity. It was shown that enzyme activity did not depend on the concentration of KNO_3 in the range of 0.5–1.0*M*. Note that pH optimum was shifted toward the acetic range: 3.7–3.9 for citrate-phosphate and 3.8–4.0 for citrate buffers in comparison with universal (4.9–5.1) and acetate buffers (4.8–5.0) (Fig. 5).

Laccase from *C. zonatus* possessed the highest value of activity at 55°C (Fig. 6).

Stability of laccase was investigated in 100 m*M* K-phosphate buffer, pH 4.9, at two temperatures: 25 and 40°C (Fig. 7). The enzyme lost 15–20% of its activity at 25°C and 35–40% at 40°C during 60 h of incubation. Incubation of the enzyme in different buffers—K-phosphate, pH 4.9; K-citrate-phosphate, pH 3.75; K-acetate, pH 4.8; and K-citrate, pH 3.75—showed that the remaining enzyme activity was 65, 15, 10, and 5%, respectively (Fig. 8). Thus, laccase from *C. zonatus* was the most stable in phosphate buffer.

Table 3 shows that hydroquinone and catechol *(15)*, specific substrates of *p*-diphenol oxidases, but not tyrosine, a typical substrate of polyphenol

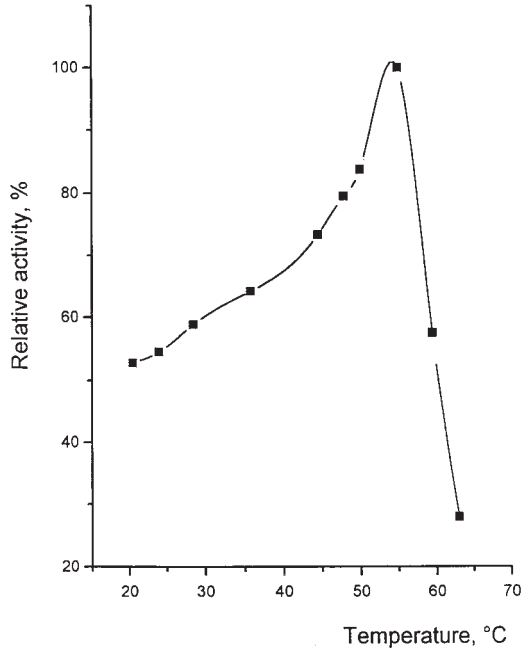

Fig. 6. Effect of temperature on laccase activity measurement. Laccase activity was measured spectrophotometrically, as indicated in the Materials and Methods section. Buffer used was 100 m*M* acetate, pH 4.9.

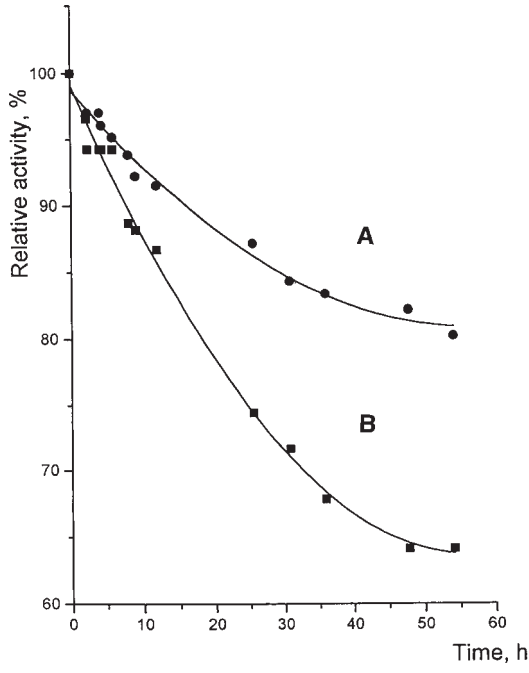

Fig. 7. Thermostability of laccase *C. zonatus* at different temperatures: **(A)** 25°C; **(B)** 40°C. Laccase activity was measured spectrophotometrically, as indicated in the Materials and Methods section, at room temperature. Buffers used were 100 m*M* phosphate, pH 4.9 (for incubation) and 100 m*M* acetate, pH 4.9 (for laccase assay).

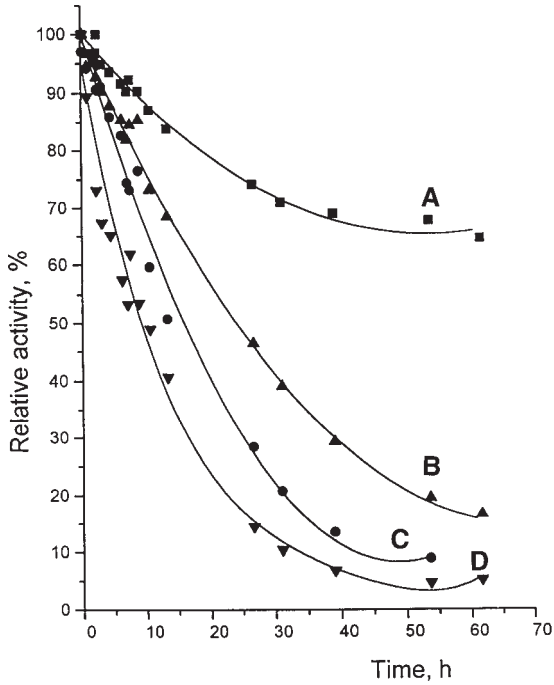

Fig. 8. pH stability of laccase *C. zonatus* at 40°C. Laccase activity was measured spectrophotometrically, as indicated in the Materials and Methods section, at room temperature. Buffers used were 100 m*M* phosphate, pH 4.9 **(A)**; 100 m*M* citrate-phosphate, pH 3.75 **(B)**; 100 m*M* acetate, pH 4.8 **(C)**; 100 m*M* citrate, pH 3.75 **(D)**.

Table 3
Determination of K_m and k_{cat} for Laccase from *C. zonatus*

Substrate	K_m (μ*M*)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1}/M^{-1})
Tyrosine	0	0	0
$K_4[Fe(CN)_6]$	202	517	25.6×10^5
Catechol	197	423	21.5×10^5
Hydroquinone	86	485	56.8×10^5
Sinapinic acid	7	624	891.4×10^5

oxidases, are oxidized by laccase from *C. zonatus*. These results confirmed that the enzyme investigated is laccase. Like other fungal laccases, this enzyme also catalyzed the oxidation of inorganic ions such as ferrocyanide with high catalytic constants (K_m, 202 μ*M*; k_{cat}, 517 s^{-1}). This enzyme showed high levels of catalysis efficiency in comparison with other fungal and plant laccases *(15)*. Note that the highest catalytic constant and the lowest Michaelis constant, 7 μ*M* and 624 s^{-1}, respectively, were found for the model lignin compound: sinapinic acid (Table 3). These results show that laccase *C. zonatus* is involved in lignin biodegradation processes with high efficiency.

Thus, laccase from *C. zonatus* was characterized and some of its physicochemical and biochemical properties were determined. This enzyme is pH- and thermostable in a wide range of pHs (3.5–6.5) and temperatures (20–50°C) and has high catalytic constants and wide substrate specificity.

Acknowledgment

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