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Characterization of a Novel Laccase Produced by the Wood-Rotting Fungus *Phellinus ribis*

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The white-rot fungus *Phellinus ribis* produced a single form of laccase, which was purified to apparent electrophoretic homogeneity from cultures induced with 2,5-xylidine. This protein was a dimer, consisting of two subunits of 76 kDa as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Carbohydrate analysis revealed that the enzyme contained about 28% carbohydrate content. The laccase appeared to be different from other known laccases by the UV–visible absorption spectrum analysis. One enzyme molecule contained one copper, one manganese, and two zinc atoms. The laccase showed optimal activity at pH 4.0–6.0, 5.0, and 6.0 with 2,6-dimethoxyphenol, ABTS [2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)], and syringaldazine, respectively. The enzyme preferably oxidized dimethoxyphenol and aromatic amine compounds. The stability of the laccase was low at acidic pH, whereas it showed high stability at neutral pH and mild temperature. The N-terminal amino acid sequence revealed a very low homology with other microbial laccases. With some substrates, the addition of manganese and H_2O_2 resulted in a remarkable increase in the oxidation rate. Without an appropriate phenolic substrate, the enzyme could not oxidize Mn(II) in the presence of H_2O_2 or pyrophosphate.

Key Words: **laccase; phenol oxidase; white-rot fungi.**

The degradation of lignin results from the nonspecific oxidation reactions of enzymes, mainly present in white-rot basidiomycetes (1). Ligninolytic enzymes include manganese peroxidases, lignin peroxidases, and laccases. Laccases and manganese peroxidases catalyze the formation of radical intermediates from the high-molecular-weight lignin, but they oxidize only phenolic components of lignin. Lignin peroxidases are very effective on the oxidation of both phenolic and nonphenolic substances (2).

Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are polyphenol oxidases that catalyze the oxidation of phenolic compounds and aromatic amines using molecular oxygen as the electron acceptor (3). They are typically multicopper blue oxidases containing 2–4 copper atoms per molecule and are widely distributed in many plants and fungi. For many years, it has been regarded that laccases play a role in lignin degradation by the way that they oxidize phenolic components of them. Recently, it has been suggested that laccases can also oxidize nonphenolic lignin components in the presence of an appropriate radical mediator. Some artificial substrates (4, 5) and fungal metabolites (6) have been studied for radical mediators. It is also well known that radical mediators, such as manganese ion Mn(III) (7–9), the cationic radical of veratryl alcohol (10), and activated oxygen species (hydroxyl radical and superoxide anions) (8, 11, 12), participate in the reactions of lignin peroxidases and manganese peroxidases. Therefore, ligninases play a role in the production of radical mediators, directly or indirectly, for lignin degradation.

Fungal laccases usually contain four copper ions, which are distributed at three distinct sites. Type 1 site (blue copper) exhibits an intense absorption at about 600 nm, owing to the charge transfer between Cu (II) atom and the sulfur ligand of a cysteine residue. The type 2 copper shows a very weak absorption and functions as a one-electron acceptor. The type 3 site consists of two copper centers and it is responsible for a

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shoulder near 330 nm. The type 3 coppers function as a two-electron acceptor. All of these copper ions are involved in the catalytic activities of laccases. Structures and functions of many fungal laccases were reviewed by Thurston (3). Laccase genes and cDNA sequences from a number of basidiomycetes (13–22) and several ascomycetes (1, 23) have been cloned and characterized.

In this study, a laccase from *Phellinus ribis* was purified and characterized after induction with 2,5-xylidine. It was examined with its spectral properties, metal contents, catalytic properties, and N-terminal amino acid sequence. Furthermore, the enzyme activity was dependent on manganese ion Mn(II) and H_2O_2.

MATERIALS AND METHODS

Cell culture. *Phellinus ribis* CBS 579.50 was cultivated on malt agar plates (malt extract 20 g, peptone 1 g, glucose 20 g per liter) at 30°C for 2 weeks and conserved at 4°C. *Phellinus ribis* was inoculated in potato dextrose media and incubated at 30°C on a reciprocal shaker. After 120 h growth, 2,5-xylidine was added into the media to a final concentration of 0.5 mM, and growth was allowed for another 60 h.

Laccase purification. The culture liquid was collected through stacks of filter paper (Whatman Ltd). Proteins were precipitated with 80% saturation of ammonium sulfate. The precipitate was dissolved in a small quantity of 50 mM tartarate buffer (pH 5.0) and dialyzed overnight against the same buffer at 4°C. This crude extract was concentrated using an Amicon PM-100 ultramembrane filter. After the addition of ammonium sulfate at the final concentration of 1 M, proteins were loaded on a phenyl–Sepharose (Amersham Pharmacia Biotech) column (2.5 × 30 cm) and eluted with a linear gradient from 1 to 0.5 M ammonium sulfate. Fractions containing laccase activity were pooled, concentrated by the Amicon PM-10 membrane, and reconstituted in 50 mM Mes[2] buffer (pH 6.5). The concentrate was applied on a Resource-Q (Amersham Pharmacia Biotech) column (1 ml) and eluted with a linear gradient from 0 to 0.5 M NaCl. Active fractions were pooled and concentrated by ultrafiltration using an Amicon PM-10 membrane. The enzyme purity was confirmed by sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE).

Enzyme assays. Laccase activity was assayed at room temperature using 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,6-dimethoxyphenol (DMP), and syringaldazine. The assays were performed spectrophotometrically by measuring the increase in A_{420} for the ABTS oxidation (ϵ_{420} = 36,000 M^{-1} cm^{-1}), A_{470} for the DMP oxidation (ϵ_{470} = 35,645 M^{-1} cm^{-1}), and A_{530} for the syringaldazine oxidation (ϵ_{530} = 65,000 M^{-1} cm^{-1}) in 50 mM Mes buffer, pH 6.0 (24).

Laccase activity was measured spectrophotometrically at a given specific wavelength in 50 mM Mes buffer (pH 6.0) with a 0.1 mM substrate concentration of *o*-dianisidine (420 nm), syringic acid (272 nm), guaiacol (460 nm), 4-methoxyphenol (455 nm), 3-methoxyphenol (450 nm), ferulic acid (318 nm), vanillic acid (261 nm), sinapinic acid (376 nm), and catechol (276 nm) (25).

The optimal pH value for the laccase activity was determined in tartarate, Mes, and Hepes buffer to adjust an adequate pH range from 2.5 to 8.0. The activity was measured with DMP unless otherwise stated.

Lignin peroxidase activity was assayed by the oxidation of 5 mM veratryl alcohol to veratraldehyde (ϵ_{310} = 9300 M^{-1} cm^{-1}) in 50 mM Mes buffer, pH 6.0. Manganese peroxidase activity was assayed with 0.01% phenol red (ϵ_{610} = 4460 M^{-1} cm^{-1}) or measured by the formation of Mn^{3+}–tartarate complex in 50 mM tartarate buffer, pH 4.5.

Tyrosinase activity was measured at 274 nm at 30°C using L-tyrosine as substrate. The assay was performed with a final concentration of 0.3 mM L-tyrosine in 0.1 M Mes buffer (pH 6.0).

Protocatechuate dioxygenase activity was assayed by the decrease in absorbance at 290 nm using 0.6 mM protocatechuic acid as substrate. After 2 h of incubation at 30°C, the dioxygenation reaction was measured (26).

Protein determination. Protein concentration was determined by a bicinchoninic acid protein Assay (Sigma) with bovine serum albumin as standard.

UV–visible absorbance spectra. Absorption spectra were determined between 200 and 800 nm at room temperature in 50 mM Mes buffer (pH 6.0) with a Beckman spectrophotometer.

Determination of molecular weight. The molecular mass of denatured laccase was determined by polyacrylamide gel slab electrophoresis in 0.1% SDS. For the determination of molecular mass, the gel electrophoresis was carried out using phosphorylase b (M_r 97,400), bovine serum albumin (M_r 66,200), aldolase (M_r 39,200), triose phosphate isomerase (M_r 26,600), soybean trypsin inhibitor (M_r 21,500), and lysozyme (M_r 14,400) as molecular mass markers. Protein bands were visualized by Coomassie brilliant blue staining.

The apparent molecular mass of native enzyme was determined by the size exclusion chromatography using a prepacked Sepharose 6 HR10/30 column (Amersham Pharmacia Biotech). The enzyme was eluted from the column with 50 mM Mes (pH 6.0) and 50 mM NaCl at a flow rate of 0.4 ml/min. As molecular mass markers, myosin (M_r 200,000), β-galactosidase (M_r 116,250), phosphorylase b (M_r 97,400), serum albumin (M_r 66,200), and ovalbumin (M_r 45,000) (BioRad) were used.

Endoglycosidase treatment. The purified laccase was digested as described by Salas *et al.* (24); 10 μg protein was mixed in 25 μl solution containing 50 mM Tris/HCl (pH 7.5), 4% β-mercaptoethanol, and 200 mM EDTA. After the mixture was boiled for 5 min, 1 unit (5 μl) endoglycosidase F (Roche Molecular Biochemicals) was added, and then the reaction mixture was incubated for 24 h at 30°C.

Lectin assay. Terminal sugar moiety of the purified laccase was determined colorimetrically using a DIG glycan differentiation kit and standard control glycoproteins supplied from Roche Molecular Biochemicals. Proteins were directly spotted on an Immobilon membrane and detected immunologically using digoxigenin-conjugated lectins from the manufacturer's instruction. The following lectins were used: *Galanthus nivalia* agglutinin, specific for terminal mannose, *Sambucus nigra* agglutinin, specific for sialic acid-α-2,6-galactose, *Maackia amurenais* agglutinin, specific for sialic acid-α-2,3-galactose; peanut agglutinin, specific for galactose-β-1,3-*N*-acetylglucosamine, and *Datura stramonium* agglutinin, specific for galactose-β-1,4-*N*-acetylglucosamine. The assay was duplicated with two independent preparations of purified protein samples.

Protein sequence analysis. The N-terminal amino acid sequence of the purified laccase was determined by a Procise Protein sequencing system (Applied Biosystems) at the Korea Basic Science Institute (Seoul, Republic of Korea). After the separation by SDS–polyacrylamide gel electrophoresis, proteins were electroblotted on a polyvinylidene difluoride membrane. The laccase band was excised and analyzed directly.

Measurement of transition metal content. Metal content was qualitatively and quantitatively measured with an inductively coupled plasma mass spectrometer at the Korea Basic Science Institute (Seoul, Republic of Korea).

[2] Abbreviations used: Mes, 4-morpholineethanesulfonic acid; ABTS, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid; DMP, 2,6-dimethoxyphenol.

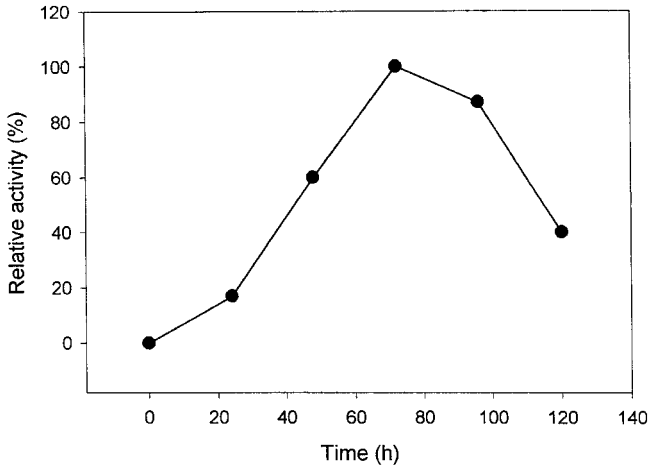

FIG. 1. Induction of the extracellular laccase activity of *P. ribis* in the secondary growth phase after the addition of 2,5-xylidine. The enzyme activity in aliquots of the culture supernatant was measured over time with DMP at pH 5.5.

RESULTS

Production of the Laccase

When *P. ribis* was cultivated in glucose-supplemented media under nitrogen-deficient conditions, the culture supernatant displayed a single laccase band that was detected by active staining with DMP on a native polyacrylamide gel. Neither lignin peroxidase activity nor manganese peroxidase activity was detected from the culture supernatant. However, the strain grew very slowly under the given conditions. Therefore, 2,5-xylidine was added to complete media to induce the optimal production of laccase in the secondary growth phase. It has been reported that the addition of 2,5-xylidine induces several times more the laccase activities of *Trametes* species (27, 28). 2,5-Xylidine was also able to act as a laccase inducer of *P. ribis*. Aliquots of the culture supernatant were taken over time after the addition of 2,5-xylidine, and the inducible laccase activity was measured (Fig. 1). A maximum activity was observed at about 72 h and only a single form of laccase was detected at various induction stages. It was proved that *P. ribis* possessed a single laccase, induced with or without the addition of 2,5-xylidine.

Purification of Laccase

The culture broth, after 72 h induction with 2,5-xylidine, was fractionated by the precipitation with 30–80% ammonium sulfate. The precipitate was dissolved in 50 mM tartarate buffer (pH 5.0) and filtered through a YM-100 membrane with a cutoff value of 100 kDa. The concentrate was loaded on a phenyl–Sepharose column. The laccase was eluted with a linear gradient of ammonium sulfate, and active fractions were collected. The enzyme was further purified by Resource Q chromatography. A single peak of enzyme activity was observed, as eluted with a linear gradient of NaCl. The result of laccase purification was summarized in Table I. About 24% of the total enzyme activity was recovered with 500-fold purification.

Physical Properties

Both native and SDS–PAGE analysis of the purified laccase resulted in a single band of protein. The subunit molecular mass of the *P. ribis* laccase was estimated to be 76,000 by SDS–polyacrylamide gel electrophoresis. The apparent molecular mass of the purified native enzyme was estimated to be 140,000 by size exclusion chromatography, suggesting that the native laccase is a homodimer. A treatment of the purified protein with deglycosylase F resulted in a molecular mass of 55,000, which indicated that the laccase was glycosylated with about 28% carbohydrate content.

The UV–visible spectrum of the purified enzyme did not show any peak near 600 nm for the type 1 blue copper site that is characteristic of most laccases (Fig. 2). Apparently the absence of a type 1 copper site caused the colorless solution of the purified enzyme. Recently, it was reported that the laccase of *Pleurotus ostreatus* also lacks the type 1 copper site (24). The UV-visible spectrum of the *P. ribis* laccase was different from that of the *P. ostreatus* laccase. The latter shows a broad absorption peak at about 400 nm, whereas the former does not display this peak.

Chemical Properties

The terminal sugar moieties of the purified enzyme were examined by the lectin-binding assay. A *G. nivalis* agglutinin was specifically bound to the purified

TABLE I

Purification of an Extracellular Laccase from *Phellinus ribis*

	Total activity[a] (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)
Culture broth	59,460	620	96	100
$(NH_4)_2SO_4$ precipitate	42,180	92	458	71
Phenyl–Sepharose[b]	9,430	0.84	22,450	16
Resource Q	7,722	0.32	48,262	13

[a] The laccase activity was determined with DMP at pH 5.5. A unit of the enzyme activity is defined as the amount of enzyme needed to oxidize 1 μmol of DMP per min. Activity values represent means of triplicate measurements (sample mean deviations $\leq$5%).

[b] Prior to application on a Phenyl–Sepharose column, the sample was concentrated by ultrafiltration using an Amicon YM-100 membrane.

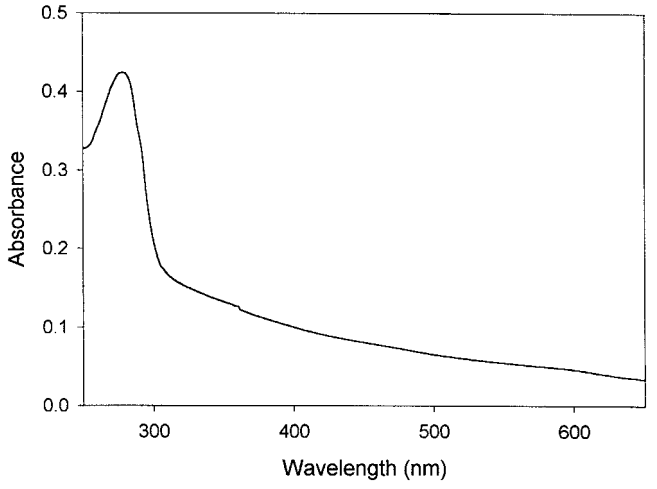

FIG. 2. UV–visible absorption spectrum of the purified laccase from *P. ribis*. The assay contained 300 μg laccase in 1 ml of 50 mM Mes buffer (pH 6.0).

TABLE III

Kinetic Constants of the *Phellinus ribis* Laccase

Substrate	K_m[a] (μM)	k_{cat}[a] (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})
2,6-Dimethoxyphenol	38 ($\pm$0.3)	1.3 ($\pm$0.2) $\times 10^5$	3.4 $\times 10^3$
ABTS	207 ($\pm$7)	8.0 ($\pm$0.5) $\times 10^4$	3.9 $\times 10^2$
Syringaldazine	11 ($\pm$0.2)	7.2 ($\pm$0.4) $\times 10^5$	6.5 $\times 10^4$

[a] The enzyme activity assay was performed in 50 mM Mes buffer (pH 6.0). The molecular weight, which was used to calculate the k_{cat} values, was 76,000. All values were calculated by the linear regression (correlation coefficient $\geq$0.98) of double reciprocal plots, $1/v$ vs 1/[S], from every set of triplicate measurements. Each standard deviation is shown in parentheses.

enzyme, which indicated that the terminal sugar moieties consisted of mannose-α−(1,2)-, α−(1,3)-, or α-(1,6)-mannose. Other lectins showed negative results.

The inductively coupled plasma mass spectrometry showed that the enzyme contained zinc, copper, and manganese ions. Iron, nickel, or molybdenum was not detected. The quantitative analysis of zinc and copper resulted in 2.0 $\pm$ 0.3 zinc atoms and 0.9 $\pm$ 0.2 copper atoms per protein molecule. One protein molecule also contained 0.4 manganese atoms. The analysis of metal ion contents suggested that the enzyme consisted of zinc and copper in a ratio of 2:1 and that a certain amount of manganese ion was probably released from the enzyme at the purification steps. Manganese ion may act as a diffusible oxidant in the radical reaction of the enzyme.

The N-terminal amino acid sequence of the purified protein was determined up to 15 amino acids as AIVSTPLLIPNANCL. The N-terminal amino acid sequences of the purified protein and several fungal laccases were aligned using the GenBank database of the BLASTP program (Table II). The *P. ribis* laccase showed a low homology, suggesting that this enzyme is distinct from other laccases.

Catalytic Properties

The reaction kinetics of the enzyme were determined using ABTS, syringaldazine, and DMP (Table III). Syringaldazine showed the lowest K_m value of 11 μM. During the substrate oxidation, the enzyme did not produce H_2O_2. The production of H_2O_2 was assayed in the presence of horseradish peroxidase. The enzyme oxidized neither tyrosine nor protocatechuic acid. Therefore, the enzyme activity appeared to originate from laccase.

In order to determine the substrate specificity of laccase, the oxidation potential was examined with various substrates (Table IV). The enzyme easily oxidized *o*-dimethoxyphenols, such as DMP, syringaldazine, syringic acid, and sinapinic acid, and it displayed a low activity with mono-methoxyphenols, such as 4-methoxyphenol, 3-methoxyphenol, guaiacol, ferulic acid, and vanillic acid. The enzyme was also able to oxidize *o*-dianisidine and catechol.

The inhibitory effects of metal chelators and sodium azide were examined at the oxidation of DMP (Table V). Metal chelators such as thioglycolic acid and EDTA inhibited the enzyme activity irreversibly. Sodium

TABLE II

N-Terminal Amino Acid Sequences of the *Phellinus ribis* Laccase and Some White Rot Fungal Laccases

Microorganisms	N-Terminal amino acid sequences	References
Phellinus ribis	A **I** V S T P L **L** I P N A N C L	
Coriolus hirsutus	A **I G P** T **A D L T I S N A** E **V**	41
Basidiomycete PM1	S **I G P V A D L T I S N** G A **V**	29
Pleurotus ostreatus POXA1	A **I G P** T G **D** M Y **I** V **N** E D **V**	24
Trametes versicolor	G **I G P V A D L T I S** D **A** E **V**	42
Ceriporiopsis subvermispora	A **I G P V** T **D L** E **I** T D **A** F **V**	43
Phlebia radiata	S **I G P V** T **D** F H **I** V **N A** A **V**	45

TABLE IV

Activities of Laccase from *P. ribis* Assessed against Various Substrates[a]

Substrate	Concentration (mM)	Laccase (U/ml)
2,6-Dimethoxyphenol	0.1	49.5 ± 2.1
Syringaldazine	1.0	62.6 ± 1.8
ABTS	1.0	48.2 ± 1.2
Syringic acid	1.0	23.2 ± 1.8
Sinapinic acid	1.0	12.6 ± 0.9
4-Methoxyphenol	1.0	3.6 ± 0.18
3-Methoxyphenol	1.0	2.7 ± 0.21
Guaiacol	1.0	4.8 ± 0.15
Ferulic acid	5.0	4.2 ± 0.12
Vanillic acid	5.0	3.6 ± 0.24
o-Dianisidine	1.0	22.5 ± 1.2
Cathecol	1.0	16.4 ± 0.09

[a] The enzyme activity assay was performed in 50 mM Mes buffer (pH 6.0). Values are presented as the means of ± SD of three parallel incubations.

azide also inhibited the enzyme activity. Sodium azide (10 mM) or 1 mM thioglycolic acid totally inhibited the enzyme activity, whereas EDTA did not show significant inhibition up to 5 mM.

Effect of pH and Temperature

The optimal activity for the oxidation of ABTS and syringaldazine was obtained at pH 5.0 and 6.0, respectively. The optimal activity for 2,6-dimethoxyphenol was observed at a broad pH range between 4.0 and 6.0 (Fig. 3). When the purified enzyme was incubated for 12 h at pH 6.0, 37°C, the activity was not altered. However, as the residual activity at pH 3.0 indicated a $t_{1/2}$ of 2.7 h, the enzyme activity decreased rapidly at lower pH.

For thermal effects, we examined the enzyme activity at 30°C, pH 6.0 after preincubation at different temperatures (Fig. 4). The enzyme activity was stable for 90 min during preincubation at 50°C. Figure 4 also showed that preincubation at high temperature seems to activate the enzyme considerably. A similar result was already reported in the basidiomycete PM1 laccase (29). When we determined the enzyme activity at different temperatures without preincubation of the enzyme, the optimal temperature was 65°C and the enzyme retained 50% of its activity after 48 min (data not shown).

Enzyme Activity in the Presence of Hydrogen Peroxide and Manganese Ion

Different substrates were used to examine the effects of manganese ion Mn(II) and H_2O_2 on the *P. ribis* laccase activity (Table VI). The enzyme activity for the oxidation of DMP increased 120% in the presence of H_2O_2. The addition of H_2O_2 and manganese Mn(II) ion resulted in 165% increase of the enzyme activity. However, manganese Mn(II) ion did not alter the enzyme activity in the absence of H_2O_2. Similar effects of H_2O_2 and manganese ion were observed with ABTS and syringaldazine.

However, the *P. ribis* laccase did not oxidize Mn(II) to Mn(III) without an appropriate phenolic substrate. The addition of H_2O_2 or pyrophosphate into the reaction mixture did not cause the oxidation of Mn(II) in the absence of an appropriate phenolic substrate.

DISCUSSION

Some fungal species excrete several laccase isozymes that are induced either under ligninolytic or nonligninolytic conditions. Under the given culture conditions, only a single laccase was induced from *P. ribis*. Neither lignin peroxidase nor manganese peroxidase was induced in the secondary growth phase of *P. ribis*.

The laccase was purified and characterized from *P. ribis*. The protein molecular mass and the inhibitory effects of a copper-chelating agent (i.e., thioglycolic acid) and sodium azide are consistent with the characteristics of other white-rot fungal laccases (24, 28, 30, 31). However, the protein contains more carbohydrate contents (28%) than other white-rot fungal laccases (2–12%). The optimal enzyme activity was observed between pH 4.0 and pH 6.0 according to phenolic substrates such as ABTS (pH 5), 2,6-dimethoxyphenol (pH 4–6), and syringaldazine (pH 6). This is a higher pH optimum than those of other laccases. The enzyme activity increased during incubation at 55°C and was relatively stable at a higher temperature in neutral pH. A similar thermal effect has been reported in the basidiomycete PM1 laccase (29).

TABLE V

Effects of Putative Laccase Inhibitors on the Laccase Activity of *Phellinus ribis*

	Relative activity (%)[a]		
Concentration (mM)	Sodium azide	Thioglycolic acid	EDTA
0	100	100	100
0.02	90	82	100
0.05	79	79	100
0.1	58	58	100
0.5	25	25	100
1	15	15	100
5	0	0	100
10	0	0	91

[a] The result is the mean value of every set of triplicate measurements, of which the variation is less than 5%. The assay began with the addition of 2 mM DMP. The reaction mixture containing inhibitors incubated at 30°C for 5 min and the assay was then started by adding enzyme solution.

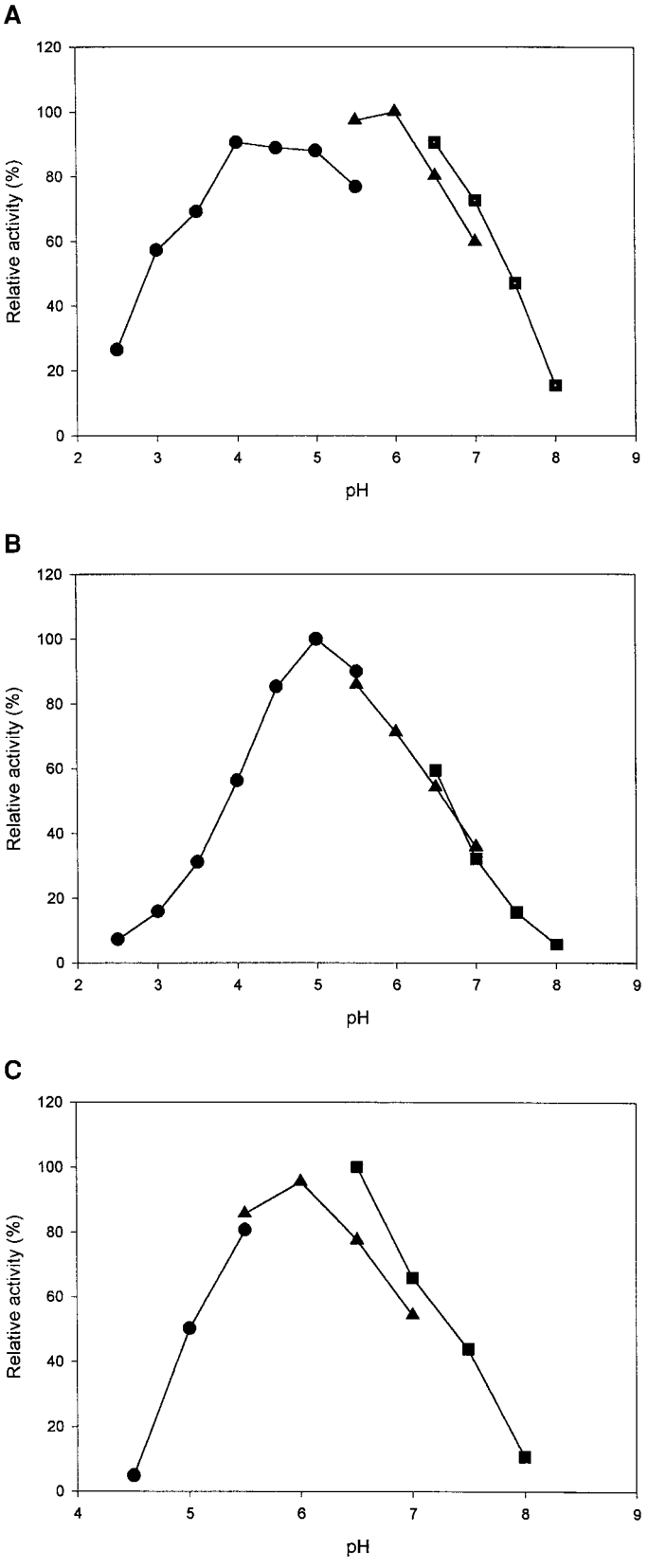

FIG. 3. pH optimum curve of purified *P. ribis* laccase. (A) 2,6-Dimethoxyphenol, (B) ABTS, (C) syringaldazine. To obtain adequate pH range, 50 mM tartarate (circle), Mes (triangle), and Hepes buffer (square) were used. Enzyme activity was measured by adding 1 mM of each substrate.

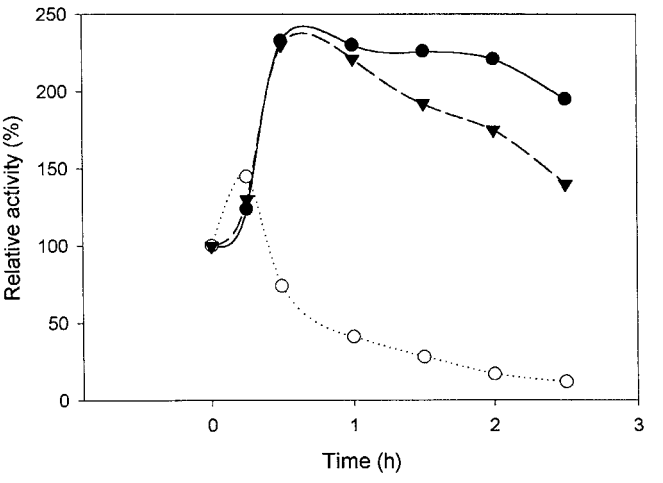

FIG. 4. Thermal effects on purified *P. ribis* laccase after preincubation at 50 (closed circles), 55 (closed triangles), and 65°C (open circles). One hundred percent activity refers to 100 U/ml by the enzyme assay using 2 mM DMP in 50 mM Mes (pH 6.0) at 30°C. The variation was less than 5%.

The *P. ribis* laccase showed a lower affinity for ABTS and oxidized it slower than 2,6-dimethoxyphenol and syringaldazine. The highest activity was observed with syringaldazine. These oxidation behaviors indicate that the laccase oxidizes methoxy-substituted phenolic compounds, such as 2,6-dimethoxyphenol and syringaldazine, more easily than unsubstituted ones, for example, ABTS. Introduction of OH, OCH_3, or CH_3 groups into the aromatic system renders the compound more easily oxidized by laccase (32, 33). In view of electron-donating effects, O-substituents of phenolic compounds are influential on laccase activity (34). Methoxyl groups donate an electron easily to introduce a one-electron oxidation. The redox potentials of phenolic compounds are also affected by the additive electron donating effects of multiple methoxyl substituents (35). Accordingly, the enzyme oxidized dimethoxyl phenols such as DMP, syringaldazine, sinapinic acid and syringic acid more easily than monomethoxyl compounds, such as guaiacol, 3-methoxyphenol, 4-methoxyphenol, ferulic acid, and vanillic acid.

It seems that the enzyme catalytic site possesses a low ionization potential due to the lack of a type 1 copper site. The purified enzyme lacks an absorption peak near 600 nm and exhibits a minor shoulder near 330 nm, perhaps responsible for the type-3 binuclear copper site. However, metal content analysis revealed

TABLE VI

Effects of Manganese ion Mn(II) and Hydrogen Peroxide on *P. ribis* Laccase Activity[a]

Substrates (1 mM)	Increased activity (%)[b]		
	+0.5 mM H_2O_2	+1 mM Mn(II)	+0.5 mM H_2O_2 +1 mM Mn(II)
DMP	20 ± 1.2[c]	0 ± 0.4	65 ± 2.5
ABTS	11 ± 0.7	0 ± 0.3	24 ± 1.5
Syringaldazine	25 ± 0.9	0 ± 0.5	75 ± 3.8

[a] The enzyme activity was measured with the addition of H_2O_2 and/or manganese ion Mn(II) in 50 mM Mes buffer (pH 6.0).

[b] The effects of supplements were determined in the relative percentage activity. Increase activity was calculated by differences in enzyme activity between the untreated and the treated with supplements.

[c] Values are presented as the means of ±SD of three parallel incubations.

only one copper atom per protein molecule, whereas two copper ions Cu(II) are necessary for the type-3 binuclear copper center. The protein contains additionally two zinc atoms that may act in place of a copper ion at the catalytic site.

The N-terminal amino acid sequence showed a very low homology with those of other white-rot fungal laccases. The PCR using primers (forward, CAC(T) TGG CAC(T) GGN TTC(T) TTC(T) TTC(T) CA; and reverse, A(G)TG G(A)CT G(A)TG G(A)TA CCA A(G)AA NTG) was not successful, suggesting that the laccase gene of *P. ribis* does not contain the consensus of two copper binding regions of white fungal laccases (36). In another case, total mRNA obtained after the 2,5-xylidine induction was used as a template for RT-PCR, but the RT-PCR using primers (forward, GGC ACT TT(T/C) TGG TA(T/C) CAC AG(T/C) CAC CTC; and reverse, GTG GCC GTG CAA GTG GAA GGG GTG AGG), which were designed for highly conserved histidinyl rich regions responsible for other copper binding sites (16), was not successful either. The PCR experiments suggest that the *P. ribis* laccase is distinct from other known laccases.

The *P. ribis* laccase does not belong to the blue copper proteins, according to its spectral properties. However, it is evident that this enzyme possesses the properties of a phenol oxidase. It is unable to oxidize tyrosine and protocatechuate and does not form hydrogen peroxide as a reaction product. The enzyme is active for the oxidation of phenolic compounds as do most laccases. The enzyme activity is specifically inhibited by thioglycolic acid or sodium azide. It is remarkable that the enzyme activity increases with the addition of manganese Mn(II) ion and hydrogen peroxide. In most cases, hydrogen peroxide inactivates laccases, but external hydrogen peroxide increases the activity of the *P. ribis* laccase to a certain extent without additional manganese Mn(II) ion. The analysis of metal contents revealed that the protein contained manganese atom, but the enzyme activity was not affected with the addition of manganese Mn(II) ion in the absence of hydrogen peroxide or pyrophosphate. Manganese Mn(III) ion is generally regarded as a diffusible oxidant, and manganese peroxidases catalyze the oxidation of Mn(II) to Mn(III) in the presence of hydrogen peroxide. Compared with that, the *P. ribis* laccase requires an appropriate phenolic substrate for the manganese oxidation in the presence of hydrogen peroxide. The *P. ribis* laccase is distinguished from laccases of *Trametes versicolor* which are able to oxidize Mn(II) in the absence of phenolic substrates (37), and those of *Pleurotus eryngii,* which produce hydrogen peroxide and Mn(III) at the oxidation of hydroquinone (38). The *P. ribis* laccase appears not to oxidize manganese Mn(II) ion itself, but to produce phenolic radicals to participate in a cascade reaction for the manganese oxidation. The fact that the *P. ribis* laccase was able to oxidize phenol red with the addition of hydrogen peroxide presumably suggested that phenolic radical and/or manganese ion, which is released from the enzyme, participates in the phenol red oxidation through a cascade reaction.

In conclusion, *P. ribis* produces neither manganese peroxidase nor lignin peroxidase. This strain excretes only a single laccase with a low oxidation potential. The laccase presumably oxidizes phenolic or nonphenolic lignin compounds with a high ionization potential through a cascade reaction in the presence of adequate radical mediators (4, 39, 40). Considering that the laccase oxidizes manganese ion and phenolic substrates in the presence of hydrogen peroxide, the enzyme may play a role in the degradation of lignin under nonligninolytic conditions. Further studies on the structural and catalytic properties of the enzyme would be necessary for elucidation of a novel *P. ribis* laccase.

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