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Purification and Properties of Laccase of the White-rot Basidiomycete *Coriolus hirsutus*

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Laccase produced by *Coriolus hirsutus* was purified to electrophoretic homogeneity by acetone precipitation, Sephacryl S-200 HR chromatography, DEAE Sepharose CL-6B chromatography, and Mono Q HR 5/5 chromatography. The purification of laccase was 46.6-fold with an overall yield of 23.7%. Laccase from this fungus was a monomeric glycoprotein with 16% carbohydrate content, and has an isoelectric point of 4.2, and molecular mass of 78 kDa, respectively. The N-terminal amino acid sequence of the enzyme showed significant homology to those of laccases from *Coriolus versicolor*, *Pycnoporus cinnabarius*, and an unidentified basidiomycete, PM1. The highest rate of 2,2,-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) oxidation by laccase was reached at 45°C, and the pH optima of the enzyme varied depending on the substrate in the range of 2.0 to 4.5. The enzyme was stable at 60°C for 5 h and lost 80% activity at 80°C in 30 min. The enzyme oxidized a variety of usual laccase substrates including lignin-related phenol, and had the highest affinity toward ABTS. Under standard assay conditions, the apparent K_m value of the enzyme toward ABTS was 8.1 μM. The enzyme was completely inhibited by L-cysteine and sodium azide, but not by potassium cyanide, SDS, and thiourea.

Key words: Extracellular laccases, *C. hirsutus*

Laccases (EC 1.10.3.2) are copper-containing enzymes catalyzing the oxidation of a broad number of phenolic compounds and aromatic amines by using molecular oxygen as the electron acceptor (25). These enzymes were identified for the first time in the Japanese lacquer tree, *Rhus vernicifera*, and they were widely distributed among plants and fungi (12, 19). Fungal laccases, in addition to being related to different physiological processes (28), were involved in lignin degradation together with lignin peroxidase (LiP) and manganese dependent peroxidase (MnP) (13, 16). Laccases oxidized phenolic units in lignin to phenoxy radicals, which can lead to the degradation of some structures (2, 3, 15). In the presence of appropriate primary substrates, the effect of laccases could be greater. In the presence of ABTS, for instance, laccase from *Trametes* (*Coriolus*) *versicolor* oxidized nonphenolic lignin model compounds, which are not substrates for laccase alone. This strategy recently led to the development of laccase-based treatment for pulp bleaching which represents the first promising biotechnological application of a process based on a single ligninolytic enzyme.

Based on the patterns of ligninolytic enzymes, white-rot fungi could be divided into two groups, those producing only laccase and MnP and those producing LiP in addition to laccase and MnP. These two groups have been related with the ability of white-rot fungi to preferentially degrade lignin from woody plant cell walls and to mineralize efficiently a synthetic lignin preparation (12). White-rot basidiomycete *C. hirsutus* decolorized several recalcitrant dyes such as bromophenol blue, Congo red, and Poly R-478, effectively and secreted several potent extracellular laccases and peroxidases (27). In this work, we describe the purification and characterization of one of these laccases.

Materials and Methods

Microorganism

C. hirsutus P04 was obtained from the Fungal Culture Collection of Taejon University (FCCTU). The fungus was maintained on a potato dextrose agar plate at 4°C, from which they were transferred to new plates and incubated at 28°C for 6 days before use in experiments. For laccase production, incubations were carried out at 28°C by inoculating 100 ml of potato dextrose broth shaken in 250 ml flasks with *C. hirsutus* mycelia by agitation.

Chemicals

Sephacryl S-200 HR, DEAE Sepharose CL-6B,

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molecular mass (M_r) markers for gel filtration chromatography, and isoelectric point markers were supplied by Sigma. Standard M_r proteins for SDS-PAGE were obtained from Boehringer-Mannheim. All other chemicals used were of reagent grade.

Enzyme assay

Laccase activity was assayed by measuring the oxidation of 100 μM ABTS buffered with 20 mM sodium acetate buffer, pH 4.0. Formation of the cation radical was monitored at 420 nm ($\varepsilon_{max} = 3.6 \times 10^4$ $cm^{-1}M^{-1}$). An aliquot of enzyme solution was incubated in 3 ml of 20 mM sodium acetate buffer (pH 4.0) containing 100 μM ABTS at 25°C. One unit of enzyme activity was defined as the amount of enzyme required for the oxidation of 1 μmol of ABTS per minute in the reaction mixtures.

Purification of enzyme

Unless otherwise stated, all procedures were performed at 4°C. The culture filtrate was used for the enzyme purification after filtration through Whatman No. 1 filter paper. The aqueous solution was centrifuged to remove particles. Cold acetone (-10°C) was added to give 65% saturation and the precipitated proteins were collected by centrifugation at 28,000 × *g* for 30 min. After removing the acetone by nitrogen purging, the precipitate was redissolved in a minimal volume of 20 mM sodium acetate buffer, pH 4.5 (buffer A). The redissolved protein was loaded onto a Sephacryl S-200 HR column (2.5 × 120 cm) equilibrated with buffer A. The enzyme was eluted with the same buffer at a flow rate of 30 ml/h, collecting 10 ml fractions. These were assayed for enzyme activity and those containing the highest activity were collected. The previously retained fractions were concentrated in an Amicon stirred cell by using a PM-10 membrane and applied to a DEAE Sepharose CL-6B column (2.8 × 15 cm) which was equilibrated with buffer A. The column was subsequently washed with 100 ml of equilibration buffer, the enzyme fractions were then eluted with a linear concentration gradient of 0 - 0.3 M NaCl in the same buffer at a flow rate of 30 ml/h. The fractions containing laccase activity were collected, concentrated, desalted, and applied to an FPLC system with a Mono Q HR 5/5 column.

Homogeneity of the enzymes was confirmed by SDS-PAGE and protein concentration was determined by a modified method of Lowry *et al.* (18) with bovine serum albumin as the standard protein. The carbohydrate content was determined according to the method proposed by Dubois *et al.* (8), using glucose as a standard carbohydrate.

Molecular-mass determination

The native M_r of purified protein was estimated by gel filtration chromatography on a Superose 12 HR 10/30 column (volume 24 ml) mounted on a Pharmacia FPLC system. Standard proteins of known M_r were used to calibrate the column. SDS-PAGE was performed on a polyacrylamide gel with concentrations ranging from 5 to 20%, according to the method proposed by Laemmli (17). Coomassie brilliant blue G-250 was used for staining according to the method of Neuhoff *et al.* (21).

Analytical isoelectric focusing

The isoelectric point of the enzyme was determined on an isoelectric focusing gel (IEF-PAGE) with a gradient of pH 3 to 10 (125 by 65 mm, 0.4 mm thick; Bio-Rad, USA) as described previously (14). The isoelectric point of laccase was determined by comparison with a protein standard mixture.

N-terminal amino acid sequencing

Purified laccase was electroblotted directly from an SDS-PAGE gel to a polyvinylidene difluoride membrane (Sequi-Blot PVDF; Bio-Rad, USA), and sequence determination was made at the Korea Basic Science Institute using an Applied Biosystems Procise 491 automatic sequencer (Applied Biosystems Inc., USA).

Temperature and pH optimum

The above assay with ABTS as substrate was also used to determine the optimum temperature of *C. hirsutus* laccase. To estimate the optimum pH of the enzyme, activity was measured with ABTS and 2,6-dimethoxyphenol in 0.1 M citrate-phosphate buffer. All determinations were performed in duplicate, with an average sample mean deviation of the reported values less than 3%.

Kinetic calculations

Rates of substrate oxidation were determined by means of spectrophotometry using molar extinction coefficients of various substrates. The molar extinction coefficients determined in 20 mM sodium acetate buffer (pH 4.0) were 35,645 $cm^{-1}M^{-1}$ at 470 nm for 2,6-dimethoxyphenol and 6,400 $cm^{-1}M^{-1}$ at 436 nm for guaiacol. The extinction coefficients of sinapinic acid, ferulic acid, and vanillic acid were used as described previously (14). The K_m values were determined by measuring initial velocity. All kinetic studies were performed at least three times and the kinetic data were fitted to a hyperbola using the Michaelis-Menten equation. The best values were determined by linear least-square regression analysis.

Results and Discussion

Purification of enzymes

The enzyme was purified to homogeneity from the

Table. 1. Purification of extracellular laccase from *C. hirsutus*

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purificatio factor (fold)
Culture filtrate	555.2	295.6	1.88	100	1.0
Acetone precipitation	520.5	183.1	2.84	93.8	1.5
Sephacryl S-200 HR chromatography	476.7	51.2	9.31	85.9	5.0
DEAE Sepharose chromatography	146.6	2.2	66.64	26.3	35.4
Mono Q HR 5/5 chromatography	131.5	1.5	87.67	23.7	46.6

culture filtrate of *C. hirsutus* as summarized in Table 1. Three chromatographic steps were required to purify. During the first step, using Sephacryl S-200 HR blue pigment from Azure B was separated from laccase. At the end of the process, laccase was purified 46.6-fold with a yield of 23.7%.

Physical characterization

The M_r of the native enzyme was estimated to be 85 kDa by gel filtration chromatography on Superose 12 HR. SDS–electrophoresis of the enzyme revealed an M_r of 78 kDa (Fig. 1A). These data indicate that the enzyme is a monomeric protein similar to those described for other fungal laccases. Most of them are monomeric proteins with molecular masses between 50 and 80 kDa (1, 28, 31). Some exceptions include laccase I of *Podospora anserina* (9), which is composed of four subunits and laccases of *Agaricus bisporus* (29) and *Trametes villosa* (32), composed of two subunits. The isoelectric point of laccase was 4.2 (Fig. 1B). The isoelectric point of the enzyme lies well within the range determined for other fungal laccases (10, 11, 20, 24, 32). The enzyme contained about 16 % of total carbohydrate. The carbohydrate content of the enzyme was within the range reported for laccases of other basidiomycetes, including *P. cinnabarinus* (10), *C. subvermispora* (11), *Phlebia radiata* (22), *A. bisporus* (29), and *C. versicolor* (33). The function of the carbohydrate chains was not clear. The carbohydrate may not be necessary for enzyme activity and extracellular secretion of laccase. It had been reported that N-linked carbohydrate chains of laccase III of *C. versicolor* played important roles in enzyme protection from proteolysis (33). The N-terminal amino acid sequences of the enzyme (15 residues) was shown in Fig. 2. Seventy three percent identity was found in comparison to laccases from *P. cinnabarinus* (10) and the basidiomycete PM1 (5) and laccase III from *C. versicolor* (4), 67% identity to laccase II from *C. versicolor* (4), 60% identity to *C. subvermispora* laccase I (11) and *C. versicolor* laccase I (4), and 53% identity to laccase of *P. radiata* (26). All of these fungi are wood-rotting basidiomycetes belonging to the class of white-rot fungi. Laccases of isolated non-wood-rotting fungi such as *A. bisporus* (24) were significantly different.

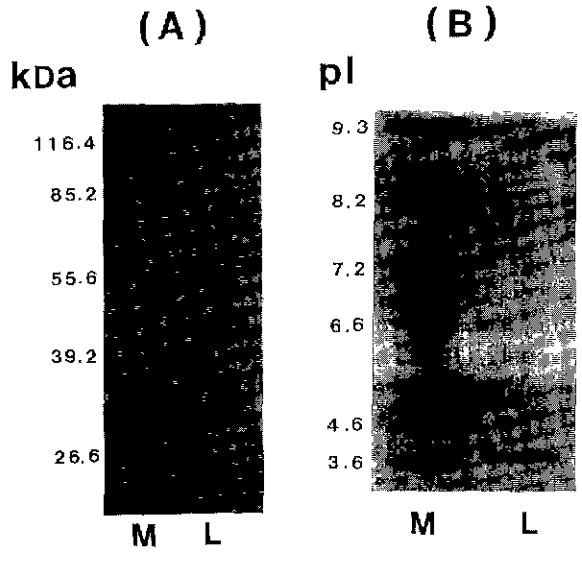

Fig. 1. Electrophoresis of laccase from *C. hirsutus*. Protein was stained with Coomassie brilliant blue G-250. (A) SDS-PAGE of laccases. Lanes: M, standard M_r marker proteins; L, purified laccase. (B) Isoelectric focusing of laccase. Lanes: M, standard pI marker proteins; L, purified laccase.

Effects of pH and temperature

The effect of pH on the enzyme activity was examined at pH values ranging from 2.0 to 7.0 with ABTS and 2,6-dimethoxyphenol as the substrate. Laccase had different pH optima depending upon the substrate. The optimum pH of the enzyme for oxidation of ABTS and 2,6-dimethoxyphenol was 2.0 and 4.5, respectively (Fig. 3). These results were similar to the findings for laccases of *C. subvermispora* (11). The optimum temperature for ABTS oxidation was 45°C (Fig. 4). The effect of temperature on the stability of the enzyme was measured. As shown in Fig. 5, the enzyme lost 80% of its activity at 80°C for 30 min. However, the enzyme showed remarkable stability at 60°C for 5 h.

Substrate specificities

The ability of the enzyme to catalyze oxidation of

Coriolus hirsutus laccase	A I G P T A D L T I S N A E V
Agaricus bisporus laccase I (23)	K T R - T F D F D L V N T R L
Agaricus bisporus laccase II (23)	D T K - T F N F D L V N T R L
Basidiomycete PM1 laccase (5)	S I G P V A D L T I S N G A V
Ceriporiopsis subvermispora laccase 1 (11)	A I G P V T D L E I T D A F V
Pleurotus eryngii laccase I (20)	A X K K L - D F H I I N N
Pleurotus eryngii laccase II (20)	A T K K L - D F H I I N N
Phlebia radiata laccase (26)	S I G P V T D F H I V N A A V
Pycnoporus cinnabarinus laccase (10)	A I G P V A D L T L T N A A V
Trametes versicolor laccase I (4)	A I G P V A S L V V A N A P V
Trametes versicolor laccase II (4)	G I G P V A D L T I T D A A V
Trametes versicolor laccase III (4)	G I G P V A D L T I T D A E V

Fig. 2. Comparison of N-terminal amino acid sequences of laccase from *C. hirsutus* with those of other fungal laccases. Identical residues were boldfaced and underlined. Numbers in parentheses indicate references and dashes indicate gaps introduced to maximize alignment.

aromatic compounds was investigated. The enzyme was able to oxidize several phenolic compounds in standard assay conditions (Table 2). ABTS, sinapinic acid and ferulic acid were the preferred substrates of the enzyme. The relationship between enzyme activity and substrate concentration was the Michaelis-Menten type. The apparent K_m values of the enzyme for ABTS determined from a Lineweaver-Burk plot were estimated to be 8.1 μM under the standard enzyme assay conditions, which was lower than those for any other compounds tested. The catalytic efficiencies (V_{max}/K_m) for the substrates varied from 0.2 for guaiacol to 1,028.2 for ABTS. The catalytic efficiency of the enzyme was second best with sinapinic acid. However, the enzyme did not oxidize catechol, which is a well-known substrate of laccase. Also, this enzyme did not react to a lignin-related model compound, vanillic acid, which was readily attacked by lac-

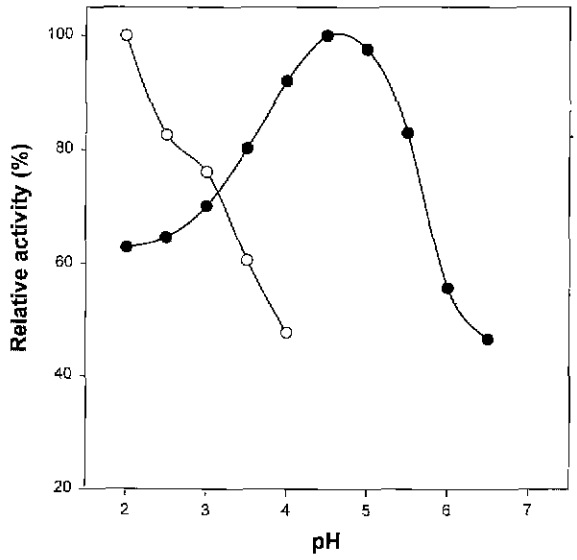

Fig. 3. The pH-dependence of laccase activity. (○) ABTS as a substrate; (●) 2,6-dimethoxyphenol as a substrate. The enzyme activity measured at various pH values under the standard assay conditions with 0.1 M citrate-phosphate buffer.

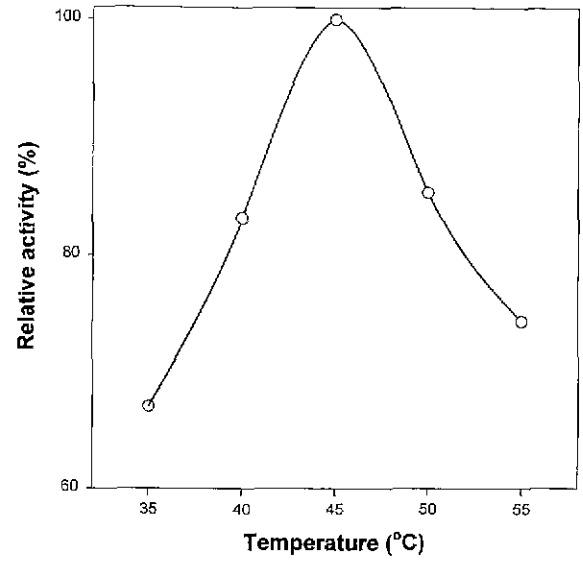

Fig. 4. Effect of temperature on *C. hirsutus* laccase activity. Enzyme activity was assayed in 20 mM sodium acetate buffer (pH 4.0) at various temperatures.

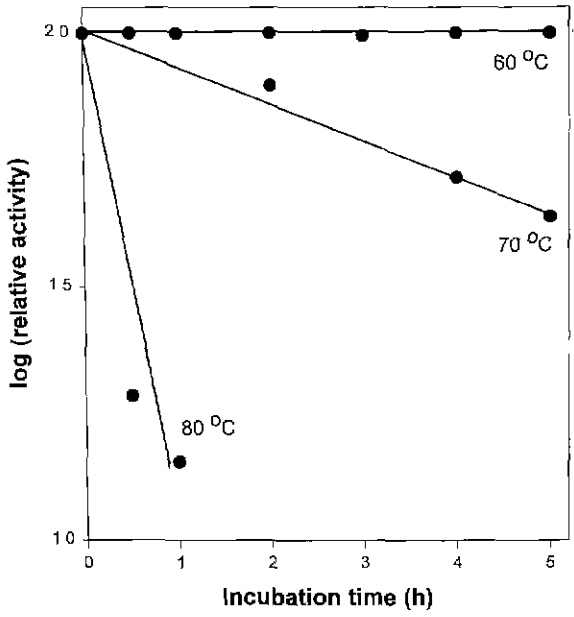

Fig. 5. Thermal stability of the laccase. The stability of the enzyme was measured by incubating the enzyme at defined temperatures prior to the assay. After the substrate was added, the remaining activity of laccase was determined.

cases of *C. subvermispora* (11). It had been reported that significant differences in redox potential existed among fungal laccases and that the V_{max} values of reactions catalyzed by these enzymes were positively correlated to the differences in the redox potential between the laccases and the substrates (30).

Effects of inhibitors

The sensitivities of laccase of *C. hirsutus* towards several putative laccase inhibitors were very similar to that seen with the laccase from *P. cinnabarinus* (10). The enzyme was completely inhibited by 1.0 mM L-cysteine, a classical inhibitor of phenol oxidase-type activities and 1.0 mM sodium azide, the most effective inhibitor of oxidative enzyme reactions. The metal binding agent potassium cyanide and a specific laccase inhibitor, thiourea, inhibited laccase activity incompletely. The inhibition of laccase activity by cysteine, sodium azide, and potassium cyanide indicated that the reaction mode of laccase is an oxidative process. The enzyme was completely inhibited by 5.0 mM EDTA and SDS (Table 3). These results not only suggest the presence of a catalytic metal center such as copper in the enzyme protein, but also the possibility that this catalytic metal center plays an important role in the enzyme reaction (28).

Table. 2. Kinetic constants of laccase from *C. hirsutus*

Substrate	Kinetic constant		
	K_m(μm)	V_{max}(U/mg)	V_{max}/K_m
ABTs	8.1	8328.5	1028.2
2,6-Dimethoxyphenol	15.8	36.3	2.3
Ferulic acid	2369.8	7418.4	3.1
Guaiacol	175.6	38.0	0.2
Sinapinic acid	16.2	314.7	19.4
Catechol	ND	-	-
Vanillic acid	ND	-	-

ND, not determined

Table. 3. Effect of various chemicals on the activity of *C. hirsutus* laccase. Enzyme activity was measured at the standard assay-condition with ABTS as the substrate

Chemical	Concentration (mM)	Inhibition (%)
L-Cysteine	0.1	15.3
	1.0	100
	5.0	100
EDTA	0.1	43.6
	1.0	98.5
	5.0	100
Sodium azide	0.1	98.6
	1.0	100
	5.0	100
Potassium cyanide	0.1	30.8
	1.0	85.6
	5.0	98.9
SDS	0.1	16.9
	1.0	98.2
	5.0	100
Thiourea	0.1	44.3
	1.0	81.4
	5.0	99.3

Acknowledgments

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