Purification and Characterization of Laccase from Wood-Degrading Fungus *Trichophyton rubrum* LKY-7

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A new wood-degrading fungus *Trichophyton rubrum* LKY-7 secretes a high level of laccase in a glucose-peptone liquid medium. The production of laccase by the fungus was barely induced by 2,5-xylidine. The laccase has been purified to homogeneity through three chromatography steps in an overall yield of 40%. The molecular mass of the purified laccase was about 65 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The purified laccase had the distinct blue color and had basic spectroscopic features of a typical blue laccase: two absorption maxima at 278 and 610 nm and a shoulder at 338 nm. The N-terminus of the laccase has been sequenced, revealing high homology to laccases from wood-degrading white-rot fungi such as *Ceriporiopsis subvermispora*. The enzyme had a "low" redox potential (0.5 V vs normal hydrogen electrode), yet it was one of the most active laccases in oxidizing a series of representative substrates/mediators. Compared with other fungal laccases, the laccase has a very low Km value with ABTS [2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid] as a substrate and a very high Km value with violuric acid as a substrate. The laccase has the isoelectric point of 4.0. The laccase had very acidic optimal pH values (pH 3-4) while it was more stable at neutral pH than at acidic pH. The laccase oxidized hydroquinone faster than catechol and pyrogallol. The oxidation of tyrosine by the laccase was not detectable under the reaction conditions. The laccase was strongly inhibited by sodium azide and sodium fluoride.

INTRODUCTION

Laccase, a copper-containing phenoloxidase that could oxidize a wide range of phenolic compounds and aromatic amines, has been found in most white-rot fungi (15). It is plausible that such a widely distributed enzyme plays an important role in fungal

degradation of lignin. Actually, laccase has been demonstrated to be essential in the degradation of lignin by a white-rot fungus *Pycnoporus cinnabarinus* (7, 8). However, the highest redox potential of laccases reported so far is about 0.8 V that is too low to oxidize non-phenolic lignin substructures, although laccase is able to oxidize some compounds such as 1-hydroxybenzotriazole (HBT) and N-hydroxyacetanilide (NHA) whose redox potentials are above 0.8 V (16). Moreover, laccase is too large to penetrate the plant cell walls for lignin degradation. A fungal metabolite 3-hydroxyanthranilic acid (3-HAA) was demonstrated to serve as a mediator to enable laccase to depolymerize synthetic lignin, yet it was later demonstrated that 3-HAA does not play an important role in the degradation of lignin by *P. cinnabarinus* (3, 11). Therefore, at present, it is unclear how a white-rot fungus uses laccase for lignin degradation. Characterization of laccase from new wood-degrading fungi could help reveal the mechanisms of fungal degradation of lignin.

Compared with LiP and MnP, laccase is easy to produce. Laccase uses atmospheric oxygen as the electron accepter, whereas LiP and MnP require hydrogen peroxide for their activities. These properties have made laccase a very attractive enzyme for industrial applications such as enzymatic pulp bleaching. Cumulative efforts in purification and characterization of new laccases could lead us to a better understanding of the laccase catalysis, thus facilitating the commercial applications of laccase in various industrial processes.

We have screened a wood-degrading fungus *Trichophyton rubrum* LKY-7 from a hardwood chip pile. Under the culture conditions, this fungus produces large amounts of laccase without expressing detectable LiP. In this paper, we describe the purification and characterization of laccase produced by *T. rubrum* LKY-7 as a first step toward understanding the role of laccase in lignin degradation by this fungus.

MATERIALS AND METHODS

Organism. A new fungal strain was isolated from a decayed hardwood chip pile in the vicinity of Chonnam, Korea. The fungus was found to have the closest similarity to *Trichophyton rubrum* as determined by a GC-FAME (gas chromatography-fatty acid methyl ester) technique (Microbe Inotech Labs, Inc., St. Louis, MO) and designated as *Trichophyton rubrum*, strain LKY-7.

Culture conditions. Two culture media were studied using mycelium block and spores of *T. rubrum*, LKY-7. One was glucose-peptone medium that contained the following (per liter): glucose, 30g; peptone, 10g; KH_2PO_4, 1.5g; $MgSO_4 \cdot 7H_2O$, 0.5g; $CuSO_4 \cdot 7H2O$, 20mg; thiamine-HCl, 2mg. Another culture medium was the one described by Kirk et al.

(8). The pH value of the media was adjusted to 5.0 with 1N HCl or 1N NaOH. The cultures were incubated at 29℃ for 8 days either on a rotary shaker (150 rpm) or in an incubator without shaking.

Enzyme assays. Laccase activity was determined by measuring the oxidation of ABTS [2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) spectrophotometrically. For kinetic studies, laccase activity was measured by O_2 electrode for 1-hydroxybenzotriazol (HBT), violuric acid (VA), N-hydroxy acetanilide (NHA), and methyl syringate (MS). [TrL] was estimated assuming an e = Abs280/(g/l) of 1. Redox potential (E °) was measured with $K_3Fe(CN)_6/K_4Fe(CN)_6$ in 10 mM MES, pH 5.5 at 20℃ as previously reported by Xu et al. (17), except that the laccase turnover switch-off by anaerobicity was replaced by 50 mM NaF.

Laccase purification. Six-day-old cultures were filtered to remove the fungal mycelia and then concentrated by ultrafiltration in an Amicon stirred cell with a PM membrane (10 kDa molecular weight cut off). The concentrate was frozen (-20℃), thawed, and filtered with a 0.4 μm membrane filter to remove the insoluble material. This filtrate was dialyzed against 20 mM sodium acetate (NaOAc) buffer (pH 5.0). The laccase purification from crude extracllular cultures was performed by three steps chromatography, Q-sepharose column (Pharmacia), Phenyl-sepharose hydrophobic column (Pharmacia), and gel-filteration with Superdex-75 column (Pharmacia).

Enzyme electrophoresis and isoelectric focusing. To determine the purity and apparent molecular weight of *T. rubrum* LKY-7 laccase (TrL), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the Laemmli method (10) with 10% polyacrylamide gel in vertical slab gel apparatus (Parmacia). Visualization was performed by silver staining. The isoelectric point of TrL was determined on a 5.0% polyacrylamide slab gel with a pH gradient of 2.5-7.5 in a Multiphor apparatus (Pharmacia). Protein bands were visualized by Coomasie brilliant blue R-250.

Copper content and N-terminal amino acid sequence. According to a literature procedure, copper content was determined through titration of TrL with 2,2'-biquinoline (14). N-terminal amino acid sequence was determined by KMBr.

Thermal stability and pH dependence.

Substrate specificity and inhibition studies.

RESULTS

Laccase production. In the basal medium, the fungus grew very slowly and expressed a very low level of laccase under both shaking and static culture conditions (data not shown). When the fungal mycelia were used for the cultivation in the glucose-peptone medium under the shaking condition, laccase activity was detectable after 2 days, peaked (ca. 7 U/ml) on day 6, and then declined (Fig. 1). The fungus secreted higher amounts of laccase under a shaking condition than under a static condition when either the fungal mycelia or the spores were used. The cultivation of the fungal mycelia also gave higher laccase activities than that of the fungal spores.

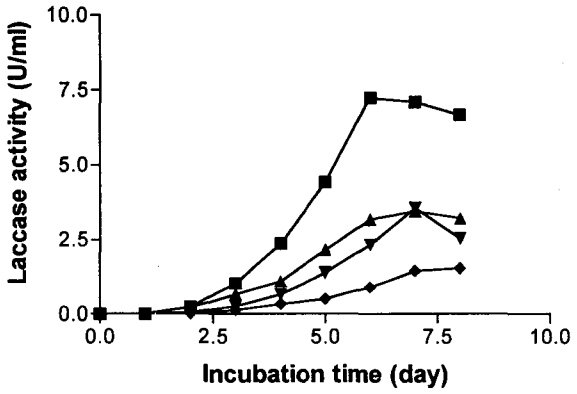

Fig. 1. Laccase production by *T. rubrum* LKY-7 grown in a glucose-peptone medium at the following culture conditions. Starting with the fungal mycelia at a shaking condition (■), starting with the fungal mycelia at a static condition (▲), starting with the fungal spores at a shaking condition (▼), starting with the fungal spores at a static condition (◆). Values are the average of three independent experiments and the maximal mean deviation is 7% of the values.

Purification of laccase. The laccase was purified to homogeneity (single band measured by SDS-PAGE) according to the procedure summarized in Table 1. Three chromatographic steps were required to purify TrL to the homogeneity. Q-sepharose chromatography removed almost all brown-colored substances in the culture concentrate and yielded a single fraction with high a laccase activity. Subsequent phenyl sepharose chromatography resolved two fractions of laccase, one with a high laccase activity and another with a very low laccase activity. The fraction with the high laccase activity, being blue in color, was further purified to the homogeneity with a gel filtration chromatography. At the end of the purification, the pure TrL was obtained in an overall 40% yield.

TABLE 1. Purification of extracellular laccase from *T. rubrum* LKY-7

Purification step	Volume (ml)	Activity (unit)	Protein (mg/ml)	SP act (U/mg)	Yield (%)	Purification factor(fold)
Culture filtrate	5,400	13,500	0.25	10	100	
Concentration(10-kDa filter)	52	10,900	2.70	78	81	8
Q-sepharose	25	8,600	2.50	173	64	17
Phenyl sepharose	20	6,400	1.50	210	48	21
Superdex-75	10	5,400	1.80	300	40	30

Properties of TrL. The purified TrL exhibited a single band on SDS-PAGE (Fig. 2). Compared with molecular weight standards, the molecular mass of TrL was estimated to be about 65 kDa that is consistent with most of fungal laccases with molecular masses between 50 and 80 kDa (2, 5, 7, 9, 13, 14). The isoelectric point (pI) of TrL was determined to be 4.0 by analytic isoelectric focusing.

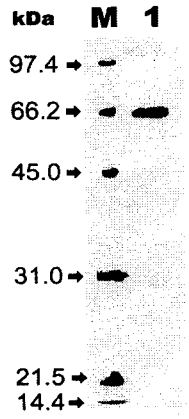

Fig. 2. SDS-PAGE profile of TrL. Protein was visualized by silver stain. Lane M : protein marker and lane 1: the purified TrL.

The purified TrL had a UV-Visible spectrum with two absorption maxima at 278 and 610 nm and a shoulder at 338 nm, with absorption of 0.22, 0.009, and 0.005, respectively (in a 1-cm quartz cuvette) (the spectrum not shown). There were 2.5±0.7 copper atoms per protein subunit in TrL, as determined by titration with biquinoline (6, 17). At pH 5.5, TrL had an E °of 0.54±0.02V vs NHE (Normal Hydrogen Electrode).

The N-terminal sequence of TrL was AIGPVADLHITDDTIAP (sequenced by KMBr)

that showed the closest similarity (70%) to *C. subvermispora* laccase (7). About 65% similarity was found in comparison to the laccase II and III from *T. versicolor* (1) and *D. squalens* (13). About 53% similariy was found to laccases from *T. villosa* (18), basidiomycete PM1 (2), and *C. hirsutus* (9), and about 47% similarity to laccase I from *T. versicolor* (1) and the laccase from *P. radiata* (14).

Temperature stability and pH optimum. The laccase was very stable below 50℃, whereas it was inactivated very rapidly over 70℃ and completely inactivated within 10 min at 80℃. TrL had an optimal pH value of 3.0 with ABTS as the substrate and an optimal pH value of 3.5 with guaiacol as the substrate. At 20℃ and in B&R buffer, TrL was more stable at pH 5-7 than at pH 3-4 and was more thermally stable at pH 6 than at pH 3 (data not shown).

Substrate specificity and inhibition pattern. The substrate specificity of the purified TrL was examined with various compounds (data not shown). Like other fungal laccases (5, 13), TrL was able to oxidize ABTS and various phenolic compounds. Interestingly, TrL oxidized hydroquinone (4-hydroxyphenol) much faster than 2-substituted phenols. Oxidations of catechol and pyrogallol were very slow and no oxidation of tyrosine was detectable.

In an effort to further investigate the catalytic properties of TrL, the kinetic parameters (K_m and k_{cat}) were determined with five substrates at pH 3 or their optimal pH values. As shown in table 2, the order of the K_m values for the substrates is ABTS < MS < NHA < HBT < VA. Compared the K_m values of other fungal laccases (12, 16), the K_m value of TrL for ABTS is very low, whereas that for VA is relatively high. The K_m values of TrL for MS, HBT and NHA are in the same range of other fungal laccases. The order of the k_{cat} values for the substrates is NHA > ABTS > VA > MS > HBT.

TABLE 2. Kinetic properties of TrL with selected substrates[a]

Substrates	K_m(mM)	k_{cat}(min^{-1})	k_{cat}/Km($M^{-1}Min^{-1}$)	pH_{opt}
ABTS	0.045±0.008	620±30	1.410^{f}	3.0
MS	0.380±0.07	330±20	8.610^{b}	3.0
HBT	24±1	160±10	6.910^{d}	-
VA	58±3.1	600±18	1.010^{g}	-
NHA	2.3±0.2	1100±20	4.710^{b}	4.0

[a] The K_m/k_{cat} values are the reseults of nonlinear regression fitting of Michaelis-Menten equation on 6 to 10 experimental reading that covered the whole rate-[substrate] profile (from the initial, linear phase to the satrurated phase).

The effects of several laccase inhibitors were determined with guaiacol (5 mM) as a substrate in 50 mM sodium citrate buffer (pH 3.5). 0.1 mM of sodium azide resulted in the complete inhibition of TrL, whereas other inhibitors required at least ten times concentration of sodium azide for the complete inhibition. In addition to sodium azide, sodium fluoride appeared to be a potent inhibitor as well. 1.0 mM of sodium fluoride completely inhibited TrL activity (data not shown).

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