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Research Article

A Laccase with HIV-1 Reverse Transcriptase Inhibitory Activity from the Broth of Mycelial Culture of the Mushroom *Lentinus tigrinus*

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A 59 kDa laccase with inhibitory activity against HIV-1 reverse transcriptase (IC_{50} = 2.4 μM) was isolated from the broth of mycelial culture of the mushroom *Lentinus tigrinus*. The isolation procedure involved ion exchange chromatography on DEAE-cellulose and CM-cellulose, and gel filtration by fast protein liquid chromatography on Superdex 75. The laccase was adsorbed on both types of ion exchangers. About 95-fold purification was achieved with a 25.9% yield of the enzyme. The procedure resulted in a specific enzyme activity of 76.6 U/mg. Its N-terminal amino acid sequence was GIPDLHDLTV, which showed little similarity to other mushroom laccase and other *Lentinus tigrinus* strain laccase. Its characteristics were different from previously reported laccase of other *Lentinus tigrinus* strain. Maximal laccase activity was observed at a pH of 4 and at a temperature of 60°C, respectively. This study yielded the information about the potentially exploitable activities of *Lentinus tigrinus* laccase.

1. Introduction

Laccases (benzenediol oxygen oxidoreductase; EC 1.10.3.2) are glycosylated multicopper oxidases. A laccase was first discovered from the Japanese tree *Rhus vernicifera* more than one century ago [1]. Laccases are widely distributed among plants [2], insects [3], bacteria [4, 5], and basidiomycetous and ascomycetous fungi [6–8]. They have been purified from cultured mycelia of various mushrooms [9–13]. In recent years, studies of laccases have been carried out owing to biocatalysts for industrial effluents, bioremediation, colour and phenolic removal, and hair dyeing [14–16].

Proteins with HIV-1 reverse transcriptase inhibitory activities have been purified and characterized from mushrooms including laccase [17–19], antifungal proteins [20], ribonucleases [21], ubiquitin-like peptides [22], and lectins [23].

Lentinus tigrinus is the white rot fungus, which are the most efficient lignin degraders in nature and are capable of producing laccases and ligninolytic peroxidases [13, 24]. Hence, most attention has been paid to its lignin-degrading strain, such as the hybrid Mn peroxidase of *L. tigrinus* 8/18 [25], an efficient PAH degrading strain of *L. tigrinus* [26], as well as the inducible enzyme including cellulose, xylanases (Elisashvili, Khardziani, Tsiklauri, and Kachlishvili, 1999), Mn peroxidase [25], and laccase [27, 28].

In view of the observation that laccases from other strains (Strain 8/18, Strain CBS 577.79, and Strain BKM F3616D) exhibit different molecular mass and characteristics [29–32], and no attention on its medicinal effects, this prompted us to isolate a laccase with inhibitory activity against HIV-1 reverse transcriptase from the mycelium of the *L. tigrinus* HPXG59 and compare its characteristics with laccases isolated from other *L. tigrinus* strains reported earlier.

2. Materials and Methods

2.1. Purification and Analysis of Laccase. The mycelium of the mushroom *Lentinus tigrinus* HPXG59 was incubated at 25°C in a shaker for 7 days. The incubation broth was collected

Table 1: Purification of laccase from mycelial extract of *Lentinus tigrinus*.

Chromatographic fractions	Total activity (U)	Total protein content (mg)	Specific laccase activity (U/mg)	Yield (%)	Purification fold
Extract	1480.8	1828.1	0.81	100.0	1.0
D3	939.8	89.5	10.5	63.5	13.0
CM2	578.3	12.6	45.9	39.1	56.7
SU1	383.0	5.0	76.6	25.9	94.6

and centrifuged (12000 g, 20 minutes, 4°C). To 500 mL of the broth, 5.05 mL of 1 M Tris-HCl buffer (pH 7.2) was added before ion exchange chromatography of the broth on a 2.5 × 25 cm column of DEAE-cellulose (Sigma) in 10 mM Tris-HCl buffer (pH 7.2). After the flow through fraction (D1) had been eluted, the column was eluted stepwise with 0.1 M NaCl and 1 M NaCl in the starting buffer to yield fractions D2 and D3. Fraction D3 was subsequently subjected to ion exchange chromatography on a 2.5 × 20 cm column of CM-cellulose (Sigma) in 10 mM NH_4OAc (pH 4.5) when the flow through fraction (CM1) had all been eluted, the column was eluted with a linear concentration (0-1 M) gradient of NaCl in 10 mM NH_4OAc buffer (pH 4.5). The first adsorbed fraction (CM2) was then applied to a Superdex 75 HR 10/30 FPLC column (Amersham Biosciences). The column was eluted with 0.2 M NH_4HCO_3 buffer (pH 8.5). The first peak (fraction SU1) constituted purified laccase.

2.2. Assay of Laccase Activity. Laccase activity was assayed by measuring the oxidation of 2, 7′-azino-bis [3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt (ABTS). A modification of the method of Shin and Lee [40] was used. An aliquot of enzyme solution was incubated in 1.3 mL of 67 mM sodium acetate buffer (pH 4.5) containing 1.54 mM ABTS at 30°C. One unit of enzyme activity was defined as the amount of enzyme required to produce an absorbance increase at 40 nm of one per min per mL of reaction mixture under the aforementioned conditions.

2.3. Molecular Mass Determination. Molecular mass was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and FPLC gel filtration. SDS-PAGE was carried out in accordance with the procedure of Laemmli and Favre [41], using a 12% resolving gel and a 5% stacking gel. At the end of electrophoresis, the gel was stained with Coomassie brilliant blue. FPLC gel filtration in 0.2 M NH_4HCO_3 buffer (pH 8.5) at a flow rate of 24 mL/h and with a fraction size of 0.8 mL was carried out using a Superdex 75 column which had been calibrated with molecular mass standards (GE Healthcare).

2.4. Analysis of N-Terminal Amino Acid Sequence. Amino acid sequence analysis was carried out using an HP G1000A Edman degradation unit and an HP1000 HPLC system [20].

2.5. Assay of pH and Temperature Optima. In the assay for optimal pH value, a series of solution of ABTS in buffers with different pH values was used. The assay buffers were prepared in 0.1 M NaOAc buffer (pH 3.0, pH 4.0, and pH 5.0), 0.1 M MES buffer (pH 5.0, pH 6.0, and pH 7.0) and 0.1 M HEPES buffer (pH 7.0, pH 8.0, and pH 9.0). The assay temperature was 37°C. To determine the optimal temperature, the reaction mixture was incubated at 20°C to 90°C.

2.6. Assay for HIV-1 Reverse Transcriptase Inhibitory Activity. The assay for HIV reverse transcriptase inhibitory activity was carried out according to instructions supplied with the assay kit from Boehringer Mannheim (Germany). The assay takes advantage of the ability of reverse transcriptase to synthesize DNA, starting from the template/primer hybrid poly (A) oligo (dT) 15. The digoxigenin- and biotin-labeled nucleotides in an optimized ratio are incorporated into one of the same DNA molecule, which is freshly synthesized by the reverse transcriptase (RT). The detection and quantification of synthesized DNA as a parameter for RT activity follows a sandwich ELISA protocol. Biotin-labeled DNA binds to the surface of microtiter plate modules that have been precoated with streptavidin. In the next step, an antibody to digoxigenin, conjugated to peroxidase, binds to the digoxigenin-labeled DNA. In the final step, the peroxidase substrate is added. The peroxidase enzyme catalyzes the cleavage of the substrate, producing a colored reaction product. The absorbance of the samples at 405 nm can be determined by using a microtiter plate (ELISA) reader and is directly correlated to the level of RT activity. A fixed amount (4–6 ng) of recombinant HIV-1 reverse transcriptase was used. The inhibitory activity of the isolated laccase was calculated as percent inhibition as compared to a control without the laccase [18].

3. Results and Discussion

3.1. Purification of Laccase. The mycelial pellet extract of *Lentinus tigrinus* was fractionated by ion exchange chromatography on DEAE-cellulose. Laccase activity was undetectable in fraction D1, low fraction D2, and concentrated in fraction D3 (Table 1). D3 was resolved on CM-cellulose into a large unadsorbed fraction CM1, a small adsorbed fraction CM2, and a large adsorbed fraction CM3 (Figure 1). Laccase activity was indiscernible in CM1 and mainly located in CM2. Fraction CM3 had very little activity (Table 1). Gel filtration of CM2 yielded two peaks of roughly equal size (collected as fraction SU1 and SU2) together with a small peak SU3 adjacent to SU2 (Figure 2). Laccase activity was enriched in SU1. There was residual activity in SU2 and no activity in SU3 (Table 1). Peak SU1 represented the purified laccase. About 95-fold purification was achieved with a

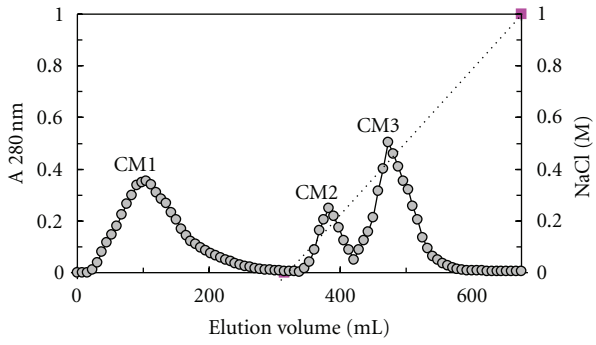

Figure 1: Ion exchange chromatography on CM-cellulose column (2.5 × 20 cm). Sample: fraction of *Lentinus tigrinus* incubation broth that was adsorbed on DEAE-cellulose. Fraction size: 8 mL. Starting buffer: 10 mM NH_4OAc buffer (pH 4.5). Dotted line across right-hand side of chromatogram indicates linear NaCl concentration (0-1 M) gradient used to CM2 and CM3.

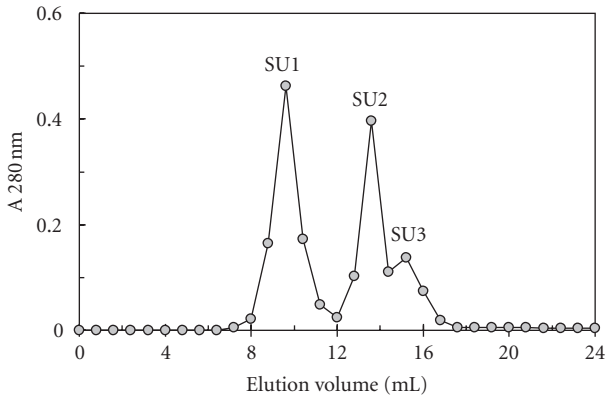

Figure 2: FPLC gel filtration on Superdex 75 hR 10/30 column. Sample: fraction CM2. Buffer: 0.2 M NH_4HCO_3 buffer (pH 8.5). Fraction size: 0.8 mL. Flow rate: 0.4 mL/min.

25.9% yield of the enzyme. The purified laccase exhibited an activity of 76.6 U/mg (Table 1).

The chromatographic behavior of *Lentinus tigrinus* laccase on cationic and anionic exchangers was similar to that of *Tricholoma mongolicum* laccase [35]. They were both adsorbed on DEAE and CM ion exchangers. But it was differed from laccase from *Lentinus edodes* [36–38] and other mushrooms [18, 19, 42] (Table 3). The purity was determined by SDS-PAGE. This observation suggests that this enzyme is a monomeric protein (Figure 3).

3.2. Determination of Molecular Mass. SU1 appeared as a single band with a molecular mass of 59 kDa in SDS-PAGE (Figure 3) and as a single peak with the same molecular mass upon re-chromatography on Superdex 75 (data not shown). Its molecular mass was close to those of most laccases.

The laccase from *L. tigrinus* demonstrated a molecular mass of 59 kDa, which is less than those (63 kDa and 69.1 kDa) of *L. tigrinus* reported earlier [29, 32, 33], while it still among the range reported for most other mushroom laccases [17, 35, 37–39, 43, 44]. A wide range of molecular masses from 30 to 70 kDa is demonstrated by laccase from mushroom (Table 3).

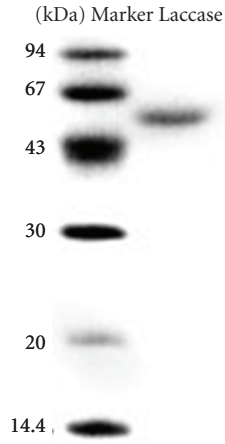

Figure 3: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis results. Left lane: molecular mass markers from Ge Healthcare. From top downward: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14.4 kDa). Right lane: purified laccase (fraction SU1).

Table 2: N-terminal sequence comparison of *Lentinus tigrinus* laccase and other mushroom laccases.

Mushroom laccase	N-terminal sequence
Lentinus tigrinus (this study)	GIPDLHDLTV
Panus tigrinus 8/18	AVGPVADLTVTNANISPDGF
Lentinus edodes L54 laccase	YGQTVSENLFIVN
Lentinus edodes SR-1 laccase	AIGPVTDLHIVN
Lentinus edodes laccase	AGTSHFADL
Trametes versicolor laccase I	AIGPVASLV
Trametes versicolor laccase II	GIGPVADLT
Trametes versicolor laccase III	GIGPVADLT
Tricholoma mongolicum laccase	GIGPVADLYVGNRIL
Tricholoma giganteum laccase	DDPQQAVIDD
Coriolus hisutus I	AIGPTADLTISNA
Coriolus hisutus II	GIGTKANLVI
Pycnoporus cinnabarinus laccase	AIGPVADLTLTNA
Agaricus bisporus laccase	DTKTFNFDLVNTRLA
Pleurotus eryngii laccase	AVGPVLGPDA
Pleurotus ostreatus laccase	AIGPTGNMYIVNE
Ganoderma lucidum	GQNGDAVP

Identical corresponding amino acid residues are underlined.
N-terminal sequence of laccases other than *Tricholoma matsutake* laccase are taken from reference.

3.3. N-Terminal Amino Acid Sequence. The N-terminal amino acid sequence was GIPDLHDLTV. The N-terminal sequence of *Lentinus tigrinus* laccase showed little similarity to other mushroom laccases (Table 2). The N-terminal sequence of *L. tigrinus* laccase bears resemblance to published mushroom *Trametes versicolor* laccase [45]. However, there

Table 3: Comparison of biochemical characteristics and activities of laccase from *Lentinus tigrinus* and other mushroom laccases.

	Lentinus tigrinus HPXG59	*Panus tigrinus* CBS 577.79	*Panus tigrinus* 8/18	*Panus tigrinus* BKM F3616D	*Tricholoma mongolicum*	*Lentinus edodes*	*Lentinus edodes* (lacI)	*Lentinus edodes* (lacII)	*Pleurotus eryngii*	*Hericium erinaceus*	*Tricholoma giganteum*
Molecular mass (kDa)	59	69.1	63	—	66	67	72	58	34	63	43
Chromatographic behavior on											
DEAE-ion exchange	AD	—	AD	AD	AD	UN	AD	—	AD	AD	UN
CM-ion exchange	AD	—	—	—	AD	UN	—	AD	UN	UN	AD
Q-ion exchange	—	AD	AD	—	UN	—	AD	—	AD	AD	—
Affi-Gel blue gel	—	—	—	—	—	UN	—	—	—	—	AD
Optimum pH	4.0	3.75	4.8	7.0	2-3	4.0	4	3	—	5	4
Optimum temperature (°C)	60	55	60	65	30	70	40	40	70	50	70
HIV-1 reverse transcriptase inhibitory activity (IC_{50})	2.4 μM	—	—	—	0.65 μM	7.5 μM	—	—	2.2 μM	9.5 μM	2.2 μM

AD: adsorbed. UN: Unadsorbed. —: not determined or not attempted.

References: *Panus tigrinus* CBS 577.79 [29], *Panus tigrinus* BKM F3616D [30], *Panus tigrinus* 8/18 [31–34], *Tricholoma mongolicum* [35], *Lentinus edodes* [36], *Lentinus edodes* (lacI) [37], *Lentinus edodes* (lacII) [38], *Pleurotus eryngii* [39], *Hericium erinaceus* [19], *Tricholoma giganteum* [18].

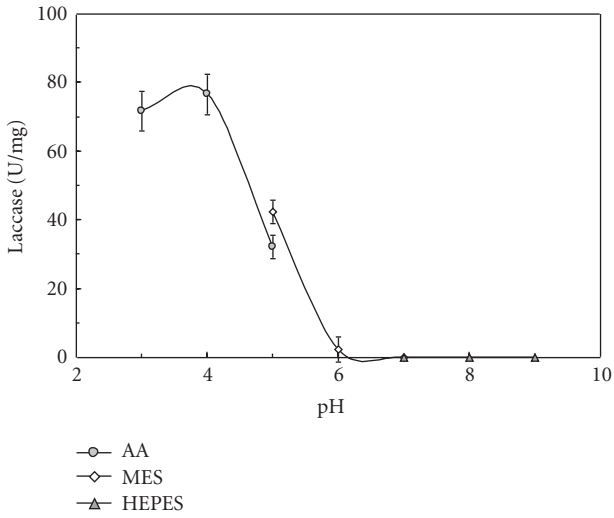

Figure 4: Effect of pH on activity of *Lentinus tigrinus* laccase.

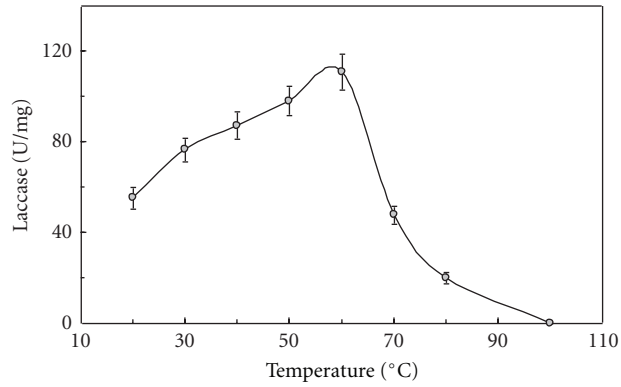

Figure 5: Effect of temperature on activity of *Lentinus tigrinus* laccase.

was only a slight similarity to the N-terminal sequences of *L. tigrinus* reported earlier [32]. It is noteworthy that laccases from different *L. tigrinus* strains may have distinct N-terminal sequence.

3.4. Optimal pH and Temperature. The activity increased slightly as the ambient pH was elevated to 4. There was a precipitous decline in activity when the pH was 5. Activity was barely detectable at pH 6 and indiscernible at and above pH 7 (Figure 4). The laccase activity rose steadily as the ambient temperature was elevated from 20°C to 60°C. The activity dropped abruptly when the temperature rose to 70°C. At 100°C no activity remained (Figure 5).

L. tigrinus laccase requires a temperature of 60°C to express maximal activity. In this aspect it resembles laccases from *Clitocybe maxima* and *Tricholoma matsutake* which have the optimum temperature at 60°C. A pH of 4 is optimal for the activity of *L. tigrinus* laccase requires an acetic pH for activity, like laccases from a number of mushrooms including *Agaricus blazei*.

3.5. HIV-1 Reverse Transcriptase Inhibitory Activity. The laccase inhibited HIV-1 reverse transcriptase by 27.7% and 86.3% when the laccase concentration was 1.5 μM and 15 μM, respectively. The IC_{50} value was 2.4 μM. A comparison of IC_{50} value towards HIV-1 RT from mushroom laccase is shown in Table 3.

Previously, some mushroom proteins such as laccase [17–19, 35, 36, 42, 43], lectins [46], ubiquitin-like proteins [47], ribosome-inactivating proteins [20, 48], and antifungal proteins [20]inhibited the activity of HIV-1 reverse transcriptase. The *L. tigrinus* laccase also possesses inhibitory activity with an IC_{50} value around 2.4 μM. The mechanism of the HIV-1 reverse transcriptase inhibitory activity of the *L. tigrinus* laccase may be protein-protein interaction [49]. Though some mushroom laccases have shown antiproliferative activity [17, 35, 43], the *L. tigrinus* laccase was devoid of antiproliferative and antifungal activities.

3.6. Comparison with Other Lentinus tigrinus Laccases. A 64 kDa laccase was purified from both liquid- [32] and solid-state cultures [31] of *Panus tigrinus* 8/18, using ammonium sulfate, anion exchange on DEAE-Toyopearl 650S, gel filtration on Sephadex G-100, and anion exchange chromatography on a Mono Q column using an FPLC chromatographic system [31]. And the structure of the laccase was solved at 1.5 Å [50].

Another 69.1 kDa laccase was isolated from mycelia of *P. tigrinus* CBS 577.79, by employing ultrafiltration (cut-off 10 kDa), chromatography on Q-Sepharose sn Superdex 75 [29]. Cadimaliev has reported a laccase which is isolated by chromatography on TEAE-cellulose and DEAE-Toyopearl 650 M from *P. tigrinus* BKM F3616D [30]. However, its molecular mass and N-sequence has not been described. The laccases isolated by Leontievsky and Golovleva differ from the laccase isolated in the present study and the laccase purified by Daniele Quaratino in biological activities, such as in molecular mass, optimum temperature, and optimum pH (Table 3). It is hard to compare the N-sequence, since there is no report about the laccase isolated by Leontievsky, Golovleva, and Cadimaliev.

4. Conclusions

In summary, the *L. tigrinus* laccase was found to be different from those of previous laccase in N-terminal sequence, molecular mass, optimal pH, and optimal temperature. The laccase inhibited HIV-1 reverse transcriptase with IC_{50} value of 2.4 μM but was devoid of any antifungal or anti-prolif-er-a-tive activity. Previously isolated *L. tigrinus* laccases have not been so tested [29, 30, 32]. This study yielded information about the potentially exploitable activities of *L. tigrinus* laccase.

Acknowledgments

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