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A homodimeric laccase with unique characteristics from the yellow mushroom *Cantharellus cibarius*

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Abstract

The aim of the present study was to isolate a laccase from fruiting bodies of the yellow mushroom *Cantharellus cibarius*. The fruiting body extract was subjected to a purification protocol that involved ion exchange chromatography on DEAE-cellulose, affinity chromatography on Affi-gel blue gel and Con A–Sepharose, and gel filtration by fast protein liquid chromatography on Superdex 75. The laccase was unadsorbed on DEAE-cellulose and Affi-gel blue gel and adsorbed on Con A–Sepharose. The laccase was composed of two identical subunits each with a molecular mass of 46 kDa. The laccase exhibited a temperature-dependent rise in activity over the temperature range 20–50 °C. When the temperature was raised above 60 °C there was a fall in enzyme activity. The enzyme manifested maximal activity at pH 4. At and above pH 6 there was a dramatic reduction in activity. The unique features of this fruiting body laccase compared with previously reported mycelial laccases include homodimeric nature, a distinctive N-terminal sequence, a higher optimal pH, and adsorption on only ConA–Sepharose among the various chromatographic media tested.

Keywords: Mushroom; Fruiting bodies; Isolation; Laccase

Mushrooms constitute a rich source of proteins many of which have important biological activities. Antifungal proteins [1], ribonucleases [2], ubiquitin-like proteins [3], ribosome inactivating proteins [1,4], lectins [5–9], and laccases [10–28] are a few examples of these mushroom proteins. The activities of some of these proteins are evident from their names, while ubiquitin-like proteins and lectins display antiproliferative, immunomodulating, and HIV-1 reverse transcriptase-inhibiting activities [3,6,8,29]. Besides proteins, mushrooms also elaborate polysaccharide–peptide complexes with immunoenhancing and antitumor activities [30,31].

Laccases are a class of ligninolytic enzymes that find applications in biotechnology [32–36]. The mushroom laccases reported in the literature [10–28] originate from cultured mycelia. Similar work has not been conducted on fruiting bodies.

A ubiquitin-like peptide with ribonucleolytic activity toward various polyhomoribonucleotides has been purified from the fruiting bodies of the yellow mushroom *Cantharellus cibarius* [37]. The present report of a laccase from the yellow mushroom furnishes an addition to the existing literature on this wild mushroom.

Materials and methods

Fresh fruiting bodies (2 kg) of the yellow mushroom *Cantharellus cibarius* were extracted with distilled water (3 ml/kg) using a Waring blender. Tris–HCl buffer (1 M, pH 7.4) was added to the supernatant obtained by centrifuging (13,000*g*, 20 min) the homogenate until the concentration of Tris attained 10 mM. The supernatant was then loaded on a DEAE-cellulose (Sigma) column (5 × 20 cm) in 10 mM Tris–HCl buffer (pH 7.4). The unadsorbed fraction (D1) with laccase activity was saved. The adsorbed fraction (D2) was eluted with 0.8 M NaCl added to the 10 mM Tris–HCl buffer. Fraction D1 was then subjected to affinity chromatography on an Affi-gel blue gel (Bio-Rad) column (2.5 × 20 cm) in 10 mM Tris–HCl buffer (pH 7.4). The unadsorbed fraction (B1) with laccase activity was collected. Adsorbed proteins were eluted with 1.2 M NaCl added to the 10 mM Tris–HCl

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buffer and collected as fraction B2. Fraction B1 was applied on a Con A–Sepharose (Amersham Biosciences) column (2.5 × 20 cm) in 50 mM Tris–HCl (pH 7.4) buffer containing 0.5 M NaCl, 10 mM $CaCl_2$, and 10 mM $MgCl_2$. Unadsorbed proteins (fraction Con A1) were eluted with the buffer while adsorbed proteins (fraction Con A2) were eluted with 0.4 M methyl-α-D-glucopyranoside added to the 50 mM Tris–HCl buffer. Fraction Con A2 was fractionated on a Superdex 75 HR 10/30 FPLC column (Amersham Biosciences) using an AKTA Purifier system (Amersham Biosciences). The first peak eluted constituted purified laccase.

Assay of laccase activity. Laccase activity was assayed by measuring the oxidation of 2,7′-azinobis[3-ethylbenzothiazolone-6-sulfonic acid] diammonium salt (ABTS). A modification of the method of Shin and Lee [13] 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 405 nM of one per min per ml of reaction mixture under the aforementioned condition.

Molecular mass determination by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and by FPLC-gel filtration.

Molecular mass determination by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and by FPLC-gel filtration. SDS–PAGE was carried out inaccordance with the procedure of Laemmli and Favre [38], 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 was carried out using a Superdex 75 column which had been calibrated with molecular mass standards (Amersham Biosciences).

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.

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 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 enzymes catalyze the cleavage of the substrate, producing a colored reaction product. The absorbance of the samples at 405 nm can be determined using 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 milk protein was calculated as percent inhibition as compared to a control without the protein [29].

Results

Laccase activity was located in D1 and subsequently in B1 derived from D1 (Table 1). B1 was separated on Con A–Sepharose into a large unadsorbed fraction Con A1 with negligible laccase activity and an adsorbed fraction Con A2 with laccase activity (Fig. 1, Table 1). Con A2 was resolved on Superdex 75 into two peaks, SU1 and SU2 (Fig. 2). The first and smaller peak, SU1, was enriched in laccase activity (Table 1). It exhibited a molecular mass of 92 kDa by gel filtration (Fig. 2) and 46 kDa by SDS–PAGE (Fig. 3). The laccase exhibited very little N-terminal sequence similarity to other mushroom laccases (Table 2). The activity of the purified laccase increased steadily from 20 to 50 °C,

Table 1
Yields and laccase activities of aqueous extract and various chromatographic fractions (from 1.5 kg fresh fruiting bodies)

Chromatographic fraction	Yield (mg)	Laccase activity (μ/mg)
Extract	5230	0.65
D1	1680	1.28
D2	1970	<0.1
B1	822	1.79
B2	386	<0.1
ConA1	457	0.59
ConA2	97.4	7.54
S1	22.8	19.86
S2	42.4	1.35

Laccase activity was determined at 30 °C.

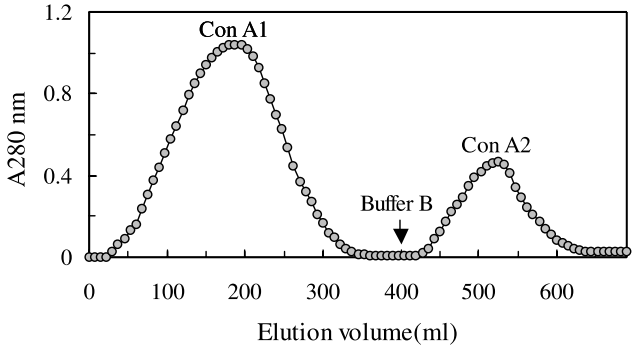

Fig. 1. Ion exchange chromatography on Con A–Sepharose. Sample: fraction of fruiting body extract unadsorbed on DEAE-cellulose and subsequently unadsorbed on Affi-gel blue gel. Column dimensions: 2.5 × 20 cm. Buffer: 50 mM Tris–HCl buffer containing 0.5 M NaCl, 10 mM $CaCl_2$, and 10 mM $MgCl_2$ (pH 7.4). Fraction size: 7 ml. Buffer B used to elute the adsorbed proteins contained 0.4 M α-methyl-D-glucopyranoside.

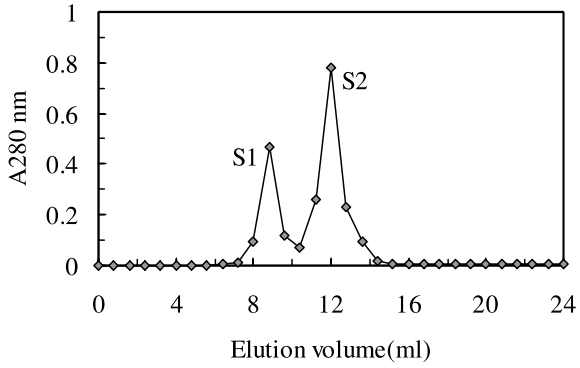

Fig. 2. Gel-filtration by fast protein liquid chromatography on a Superdex 75 HR 10/30 column. Sample: peak Con A2 from Con A–Sepharose column. Buffer: 0.2 M NH_4HCO_3 (pH 8.5). Flow rate: 0.4 ml/min. Fraction size: 0.8 ml.

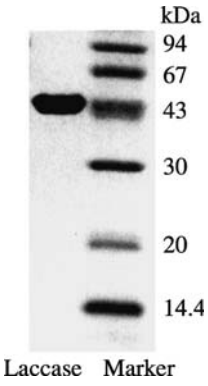

Fig. 3. SDS–PAGE results. Right lane: molecular mass markers (Amersham Biosciences), from top downward: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14.4 kDa). Left lane: yellow mushroom laccase.

Table 2
N-terminal sequence comparison of laccases from *Cantharellus cibarius* and other mushrooms

Cantharellus cibarius laccase	GCCNCGHA
Coriolus versicolor laccase	GIGTK·· ·A
Coriolus versicolor laccase	AIGPT·· ·A
Agaricus bisporus laccase I	KTRTFDFD
Agaricus bisporus laccase II	DTKTFNFD
Basidiomycete PM1 laccase	SIGPV·· ·A
Cariporiopsis subvermispora laccase I	AIGPVTDL
Pleurotus eryngii laccase I	AXKKLDFH
Pleurotus eryngii laccase II	ATKKLDFH
Phlebia radiata laccase	SIGPVTDF
Pycnoponus cinnabarinus laccase	AIGPV·· ·A
Trametes versicolor laccase I	AIGPV·· ·A
Trametes versicolor laccase II	GIGPV·· ·A
Trametes versicolor laccase III	GIGPV·· ·A

Identical amino acid residues are underlined.

(·), group introduced to maximize sequence similarity. N-terminal sequences of laccases other than *Cantharellus cibarius* laccase are taken from reference [13].

remained high at 60 °C, and then underwent a temperature-dependent decline as the temperature was further elevated to 70 °C and then 80 °C (Fig. 4). The enzyme

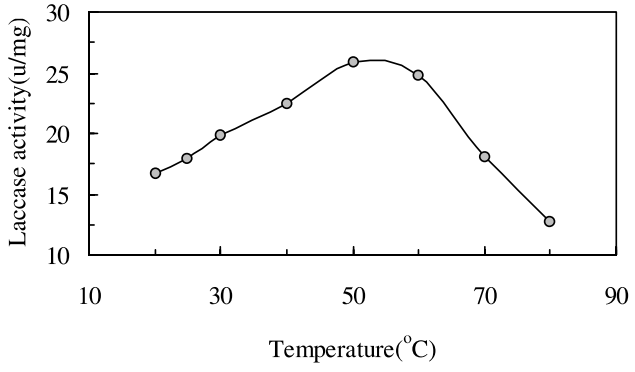

Fig. 4. Effect of temperature on laccase activity of yellow mushroom laccase.

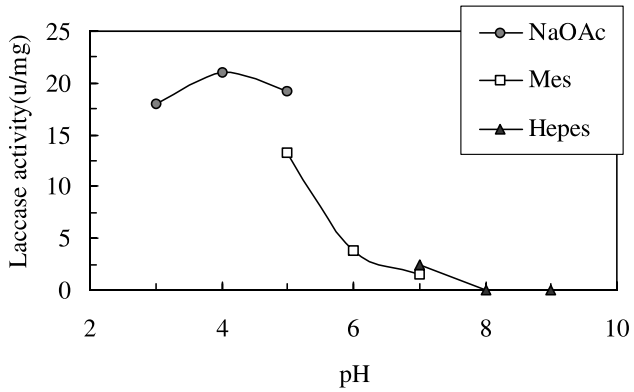

Fig. 5. Effect of pH on laccase activity of yellow mushroom laccase.

activity was maintained at a high level over the pH range 3–5, with the maximal activity at pH 4. The activity was considerably attenuated at pH 6 and pH 7. At and above pH 8 activity was indiscernible (Fig. 5). The laccase lacked any inhibitory activity on HIV-1 reverse transcriptase when tested at 10 μM.

Discussion

The yellow mushroom laccase isolated in this study is composed of two identical subunits. This is a unique characteristic because previously reported laccases are monomeric [10–28]. The molecular mass of yellow mushroom laccase is thus larger compared with laccases described in the literature.

The optimum pH for yellow mushroom laccase is 4, higher than the value of 2–3 for laccases from a number of mushrooms [10,13]. The dramatic reduction in laccase activity with an increase in pH is similar to previous findings on its counterparts in other mushrooms [13]. Yellow mushroom laccase resembles *Coriolus hirsutus* laccase [13] in thermolability. However, considerable activity of the former laccase is retained at 60 °C while in the latter laccase about 40% of the activity disappears at 60 °C.

In contrast to laccases from *Coriolus hirsutus* [13] and *Rigidoporus lignosus* [15] which are adsorbed on cationic and anionic exchangers, yellow mushroom laccase is unadsorbed on DEAE-cellulose. Unlike some mushroom proteins such as ribosome inactivating proteins [1,4], antifungal proteins [1], and ribonucleases [2] which are adsorbed on Affi-gel blue gel, yellow mushroom laccase is unadsorbed on these affinity chromatographic media. Affinity chromatography on ConA–Sepharose is useful for the purification of yellow mushroom laccase by adsorbing the laccase, leaving inactive materials in the adsorbed fraction.

Unlike ubiquitin-like proteins [1], ribosome inactivating proteins [2], and lectins [29] from mushrooms,

yellow mushroom laccase is devoid of HIV-1 reverse transcriptase-inhibiting activity.

Yellow mushroom laccase possesses an N-terminal sequence with little resemblance to those of reported mushroom laccases. Thus, it is a distinctive laccase in many aspects. The mushroom laccases described in the literature were isolated from cultured mycelia. On the other hand, yellow mushroom laccase was purified from fruiting bodies.

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