Biochimie 148 (2018) 46–54

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

# An extracellular yellow laccase from white rot fungus *Trametes* sp. F1635 and its mediator systems for dye decolorization

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ARTICLE INFO

*Article history:*
Received 9 December 2017
Accepted 23 February 2018

*Keywords:*
*Trametes*
Laccase
Purification
Laccase-mediator system
Dye decolorization

ABSTRACT

A novel extracellular laccase was purified from fermentation broth of the white rot fungus *Trametes* sp. F1635 by a three-step protocol including two consecutive ion-exchange chromatography steps on DEAE-Sepharose and SP-Sepharose, and a final gel-filtration on Superdex 75. The purified laccase (TsL) was a monomeric protein with the molecular mass of 64.8 kDa. It demonstrated high oxidation activity of $4.00 \times 10^4$ U/mg towards ABTS. Its N-terminal amino acid sequence was AIGPVADLTIINNAV which was unique and sharing high similarity of other fungal laccases. TsL was a yellow laccase based on absorption spectrum analysis. It demonstrated an acidic pH optimum of 2.6 and temperature optimum of 50 °C towards ABTS. The $K_m$ and $V_{max}$ values towards ABTS were estimated to 18.58 μM and 1.35 μmol/min, respectively. TsL manifested effective decolorization activity towards eriochrome black T (EBT), remazol brilliant blue R (RBBR), malachite green (MG), and eriochrome black T (EBT) (over 60%). Violuric acid (VA) and acetosyringone (AS) were the optimal mediators for the laccase in dye decolorization. Results suggest that TsL demonstrates great potential for dye decolorization and water treatment.



## 1. Introduction

The white rot fungi (WRF) can efficiently decay the lignin in wood [1]. They cause the rotted wood to feel moist, soft, or stringy and appear white or yellow. In resent years, accumulating literature suggest that there are great potential for WRF application since they can degrade not only lignin but also a wide variety of environmentally pollutants, such as synthetic dyes, industrial wastewater and aromatic pesticides [2,3]. There are many different ligninolytic enzymes that are involved in the decay of wood by white rot fungi, including laccases (Lac, E.C. 1.10.3.2), lignin peroxidases (LiP, E.C. 1.11.1.14), manganese-dependent peroxidases (MnP, E.C. 1.11.1.13) and versatile peroxidases (VP, E.C. 1.11.1.16), etc [4]. It has been reported that among various enzymes, laccases represent great potential for biotechnological and environmental applications [3,5].

Laccases (benzenediol: oxygen oxidoreductase) are multicopper oxidase enzymes belonging to the superfamily of multicopper oxidases. They are widely distributed in bacteria, plants and especially fungi [3]. In general, laccases are extracellular glycoproteins and present mostly as monomers, dimers or tetramers with a typical molecular weight range of 60–80 kDa for the monomer and a carbohydrate content of 15–20% [6–8]. Typical laccases (blue laccases) have four copper atoms which are distributed in three different copper centers (Type 1, 2 and 3): Type 1 (T1) or blue copper center, Type 2 (T2) or normal copper center, and Type 3 (T3) or coupled binuclear copper center. Spectral characteristic studies reveal that the T1 site of laccases imparts a light blue color to the enzyme solutions confirms optic absorption at around 600 nm, T2 site is invisible in electron absorption spectra, and T3 site can be identified by the presence of a shoulder close to 330 nm [7,9]. The copper atom in T1 site is responsible for the color of the enzyme (blue), while copper atoms in the type 2 and 3 centers are involved in the catalytic reactions with various substrates [5,10]. Non-typical

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https://doi.org/10.1016/j.biochi.2018.02.015
0300-9084/

laccases lacking of T1 site are described as yellow laccases.

Laccases exhibit various functions including cross-linking of monomers, degradation of polymers and ring cleavage of aromatic compounds. They are green catalysts and can oxidize a variety of compounds like carbohydrates, aromatic and non-aromatic compounds by a one-electron transfer mechanism using molecular oxygen as the electron acceptor [11]. On the other hand, recalcitrant substrates with high redox potentials are hard to oxidize by laccases alone. Therefore, some redox mediators, such as 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), are added with laccases to enhance the oxidation activities. These mediators act as electrons shuttles involving in the oxidation of complex substrates (such as lignin polymers) that can not enter the active site of laccases [11]. Laccases and laccase-mediator systems (LMS) possess great potentials in decolorization and detoxification of textile industry wastewater and environmental remediation applications [12,13].

WRF species from genus *Trametes* have been well studied since they are good laccase producers such as *T. hirsute*, *T. trogii*, and *T. versicolor* [3,14–16]. In the present study, we aimed to purify a novel extracellular laccase with different enzymatic properties from fermentation broth of our newly isolated *Trametes* sp. F1635. Enzymatic properties and LMS for dye decolorizing application were also investigated.

## 2. Materials and methods

### 2.1. Fungal strain

Fruiting bodies of a wild *Trametes* mushroom were collected from Mount Wutai, Shanxi province, China. Pure mycelial culture, numbered as strain F1635, was obtained in Petri dishes containing potato dextrose agar (PDA) medium at 28 °C. The fungus was cultured at 28 °C, stored at 4 °C, and monthly transferred to fresh PDA slants, in the Key Laboratory of Urban Agriculture (North China) of Ministry of Agriculture, Beijing University of Agriculture. Fresh culture was raised for the experimentation.

### 2.2. Identification of strain F1635

Taxonomical identification was carried out based on morphological and molecular characteristics. Molecular identification was based on Internal Transcribed Spacer (ITS) region (ITS1-5.8S-ITS2) analysis using ITS1 (5′ TCCGTAGGTGAACCTGCGG 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) primers [17]. *Trametes* sp. F1635 was cultured in Petri dishes containing PDA medium at 28 °C for 5 d, followed by total genomic DNA extraction of mycelia using a genomic DNA extraction kit (TIANGEN, China). The ITS region was amplified by PCR in a volume of 25 μL under standard conditions [18]. Subsequently, the PCR production was sequenced by Sangon Biotech (Shanghai) Co., Ltd. (China), and compared with ITS sequences obtained from GenBank using the Blast tool in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic tree was made by the MEGA 6.0 software using the neighbor-joining (NJ) method with the bootstrap value 1000.

### 2.3. Laccase production and purification

Laccase production was carried out using potato dextrose medium with the fed-batch fermentation method in a fermentation tank (5 L) at 28 °C for 100 h. The fermentation broth was initially filtered to remove mycelial debris using cotton gauze filter. After centrifugation at 4 °C and 9000 rpm for 15 min, the supernatant was dialyzed in distilled water overnight. Subsequently, the crude laccase solution was applied to anion exchange chromatography on a column of DEAE-Sepharose (2.5 cm × 30 cm) previously eluted with the starting buffer (10 mM Tris-HCl buffer, pH 8.5). After sampling, the solution was eluted successively with 0, 100, and 300 mM NaCl in the same buffer. All obtained fractions were monitored for laccase activity, and laccase rich fraction (D2) were pooled and dialyzed for further purification on cation exchange chromatography of SP-Sepharose (10 mM HAc-NaAc, pH 3.8). After removal of an unadsorbed fraction (SP1), two adsorbed fractions (SP2 and SP3) were eluted with 50 and 150 mM NaCl in the starting buffer, respectively. Laccase active fraction SP3 was finally applied to gel filtration by fast protein liquid chromatography (FPLC, GE Healthcare, USA) on a Superdex 75 gel filtration column (0.15 M $NH_4HCO_3$ buffer, pH 8.5) using an AKTA Purifier (GE Healthcare, USA). The second fraction (SU2) eluted constituted the purified laccase from *Trametes* sp. F1635 (abbreviated as TsL).

### 2.4. Assay for laccase activity

Laccase activity was determined spectrophotometrically using 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as the substrate [19]. Enzyme solution (5 μL) was mixed with 0.6 mM ABTS solution (195 μL, in 50 mM sodium acetate buffer, pH 4.5) at 37 °C (water bath) for 5 min, followed by an addition of 10% TCA (190 μL) to end the reaction. One enzyme unit (U) was defined as the amount of enzyme required to produce an increase of one absorbance unit at 420 nm per minute per milliliter of the reaction mixture under the assay conditions. All treatments were performed in triplicate [20].

### 2.5. Determination of molecular mass

FPLC-gel filtration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were performed for molecular mass (*Mr*) determination. During the purification protocol, molecular mass standards (GE Healthcare, USA) were applied to the FPLC chromatography. The standard curve of Log *Mr* vs elution volume was obtained. *Mr* of the active enzyme can be calculated based on the curve. SDS-PAGE was performed using the standard procedure with a 12% resolving gel and a 5% stacking gel [20]. After the electrophoresis, the gel was stained with Coomassie brilliant blue (CBB) R-250. Another standard curve of Log *Mr* vs relative mobilities of molecular mass standards (Genview, USA) was obtained. *Mr* of the present purified laccase was evaluated based on the two curves.

### 2.6. N-terminal and inner amino acid sequencing

After SDS-PAGE, the purified enzyme on the gel was transferred to a polyvinylidenedifluoride (PVDF, Bio-Rad, USA) membrane by electro-blotting and stained with CBB R-250. The stained band was then excised and analyzed by the automated Edman degradation method using an HP G1000A Edman degradation unit (Hewlett Packard Company, USA) and an HP1000 HPLC system (Hewlett Packard Company, USA) [21].

After electrophoresis and visualization, the protein band of purified laccase TsL on SDS-PAGE was recovered and digested overnight using trypsin. Subsequently, digestion products were eluted and analyzed by nano liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS, Agilent, USA) for inner amino acid sequencing. The data were acquired using Xcalibur software (Thermo Electron, USA). Sequence homologues were searched using the BLAST/NCBI database [19].