Journal of Molecular Catalysis B: Enzymatic 99 (2014) 20–25

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

An extracellular laccase with antiproliferative activity from the sanghuang mushroom *Inonotus baumii*

Jian Sun[a,b], Qing-Jun Chen[a], Meng-Juan Zhu[b], He-Xiang Wang[b,**], Guo-Qing Zhang[a,*]

[a] College of Biological Sciences and Engineering, Key Laboratory of Urban Agriculture (North) of Ministry of Agriculture, Beijing University of Agriculture, Beijing 102206, China
[b] State Key Laboratory of Agro-Biotechnology and MOA, Key Laboratory of Soil Microbiology, College of Biological Sciences, China Agricultural University, Beijing 100193, China

ARTICLE INFO

Article history:
Received 22 April 2013
Received in revised form 4 October 2013
Accepted 5 October 2013
Available online 17 October 2013

Keywords:
Inonotus baumii
Laccase
Enzyme purification
Characterization
Antiproliferative activity

ABSTRACT

We described the purification and characterization of a novel extracellular laccase from the traditional Chinese medicinal mushroom *Inonotus baumii* with antiproliferative activity. The laccase (IBL) was purified from fermentation broth of *I. baumii* by employing initial filtration and centrifugation steps, followed by three ion-exchange chromatography steps comprising DEAE-cellulose, CM-cellulose, and Q-Sepharose, and a final gel-filtration step by fast protein liquid chromatography (FPLC) on Superdex 75. The purified enzyme was a monomeric protein with a molecular mass of 66 kDa calculated by FPLC and SDS-PAGE. It possessed an N-terminal amino acid sequence of AIGPVDEV (SPIN: C0HJB2), a temperature optimum of 20 °C, pH optima of 2.4 and 3.2 toward ABTS and guaiacol respectively, and *Km* values of 1.31 mM and 2.27 mM toward ABTS and guaiacol respectively at pH 2.4 and 30 °C. The ranking of its oxidative activity toward various aromatic substrates was ATBS > guaiacol > 4-methylcatechol > 4-hydroxyindole > catechol > hydroquinone > 2,6-dimethoxy-phenol (19.6%) > pyrogallol > ferulic acid > N, N-dimethyl-1, 4-phenylenediamine. Cu^{2+} can enhance the enzyme activity of 10.8–14.6 fold in the ion concentration range of 1.25–10 mM. IBL manifested antiproliferative activities toward HepG2 and L1210 cells with IC_{50} values of 2.4 μM and 3.2 μM, respectively, but is devoid of inhibitory activity toward HIV-1 reverse transcriptase.

1. Introduction

Laccase (*ρ*-diphenol: dioxygen oxidoreductase, EC 1.10.3.2) is a multicopper oxidase that is widespread among plants, bacteria, and especially fungi [1,2]. It catalyzes the oxidation of a broad range of organic and inorganic substrates, including aromatic amines, ascorbate, diamines, diphenols, and polyphenols [3]. As one of the oldest enzymes first described at 1883, laccase can be divided into two major groups with clear differences, including those in higher plants and those in fungi [1]. Laccases from higher plants play very important roles in lignin biosynthesis, whereas fungal laccases are involved in wood degradation, pigmentation, and pathogenesis [2]. Recently, the occurrence and properties of the laccases have been comprehensively reviewed due to their wide applications in pulp and paper, textile, pharmaceutical industries [4].

Inonotus baumii (used to be identified as *Phellinus baumii*), commonly called 'Sanghuang' in China and 'meshimakobu' in Japan, is a famous traditional Chinese medicinal mushroom used in China, Japan, Korea, and other Asian countries for centuries [5]. Technically, it belongs to Order Hymenochaetales, Family Hymenochaetaceae. As a famous herbal medicine, *I. baumii* has been described to be effective on a diversity of ailments, including anti-cancer, anti-diabetes, hepatoprotection, improving blood circulation, alleviating gastroenteric disorder, etc. [6,7]. In the present study, we report for the first time the purification and characterization of an extracellular laccase from fermentation broth of *I. baumii*. Enzyme characteristics and in vitro antiproliferative and anti-virus studies are also investigated.

2. Materials and methods

2.1. Strain and culture condition

Strain MW0801 was isolated from fresh fruiting bodies of *Inonotus* sp. occurring on the tree truck of *Syringa reticulata* in the campus of China Agricultural University (Beijing, China), and collected in Agricultural Culture Collection of China (ACCC52850). The fungus was cultured at 26 °C, stored at 4 °C, and monthly transferred to fresh PDA slants which contained (g/L): potato, 200; glucose, 20; and agar, 20. For purification of the laccase, strain MW0801 was

* Corresponding author. Tel.: +86 10 8079 7308; fax: +86 10 8079 7308.
** Corresponding author. Tel.: +86 10 6273 2578; fax: +86 10 6273 2578.
E-mail addresses: hxwang@cau.edu.cn (H.-X. Wang), zhanggq1001@gmail.com (G.-Q. Zhang).

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http://dx.doi.org/10.1016/j.molcatb.2013.10.004

inoculated into the liquid PD media which contained (g/L): potato, 200 and glucose, 20. The media were cultured using an orbital shaking incubator at 200 rpm and 26 °C for 10 days. Then, the fermentation broth was collected for further laccase purification.

2.2. Identification by rDNA sequence analysis

Total genomic DNA of Strain MW0801 mycelia was extracted using the CTAB method [8]. The ITS region (ITS-1, 5.8S, and ITS-2) was amplified by PCR, using universal primers ITS1 (5′ TCCGTAGGTGAACCTGCGG 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′). PCR reactions were performed in a volume of 50 μL under standard conditions [9]. The PCR production was sequenced by Beijing Genomics Institute (Beijing, China), and compared with ITS sequences in GenBank using the Blast tool in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3. Assay for laccase activity

The activity of laccase was spectrophotometrically determined using ABTS (2, 2′-azinobis [3-ethylbenzothiazolone-6-sulfonic acid] diammonium salt) as the substrate [10]. In brief, enzyme solution (5 μL) was mixed with 1 mM ABTS solution (145 μL, in 50 mM sodium acetate buffer, pH 5.2) at 30 °C for 5 min, followed by ending the reaction by an addition of 10% TCA (250 μL). The change in the absorbance due to the oxidation was monitored at 405 nm for enzyme activity. One unit (U) of enzyme activity was defined as the amount of enzyme required to produce one absorbance increase at 405 nm per minute per milliliter of the reaction mixture under the assay conditions. Protein concentration was determined according to Bradford using a protein assay kit (Bio-Rad Lab, Richmind, California, USA) with bovine serum albumin (BSA) as the standard [11]. All determinations were performed in triplicate.

2.4. Purification of extracellular laccase

Fermentation broth containing laccase was initially filtered through absorbent gauze, followed by centrifugation at 8000 rpm and 4 °C for 15 min. Subsequently, the supernatant was dialyzed with distilled water and further purified by three successive steps of ion exchange chromatography, firstly on DEAE-cellulose (10 mM Tris–HCl buffer, pH 8.0), secondly on CM-cellulose (10 mM sodium acetate buffer, pH 5.2), and finally on Q-Sepharose (10 mM Tris–HCl buffer, pH 7.6). The laccase fraction was ultimately purified by FPLC on a Superdex 75 HR 10/30 gel filtration column (0.2 M NH_4HCO_3 buffer, pH 8.5).

2.5. Molecular mass determination by SDS-PAGE and FPLC gel filtration

The molecular mass (*Mr*) of the purified laccase was determined using both sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and FPLC-gel filtration. SDS-PAGE was carried out following the protocol of Laemmli and Favre [12] with a 12% resolving gel and a 5% stacking gel. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250. The *Mr* was calculated based on SDS-PAGE and FPLC-gel filtration [10].

2.6. Determination of N-terminal amino acid sequence of purified laccase

After SDS-PAGE and membrane transfer procedures, N-terminal amino acid sequence analysis was performed using an HP G1000A Edman degradation unit and an HP1000 HPLC system [13].

2.7. Determination of pH and temperature optima of purified laccase

In this assay, pH optimum of IBL was determined using ABTS and guaiacol as substrates, respectively. A series ABTS and guaiacol solution in different pH value was used instead of the ABTS solution at pH 5.2 in the standard enzyme assay. The assay buffers were prepared in citric acid-Na_2HPO_4 buffers (pH 2.2–8.0). In the assay for temperature optimum determination, the reaction mixture was incubated at different temperature including 4, 20, 40, 50, and 60 °C instead of 30 °C in the standard enzyme assay. All determinations were performed in triplicate.

2.8. Determination of pH stability and thermostability of purified laccase

In the pH stability assay, enzyme solutions were previously incubated in 50 mM citric acid-Na_2HPO_4 buffers at different pH values (2.4, 3.2, 5.2, and 7.2, respectively) for different durations (10, 20, 30, 40, 50, and 60 min, respectively) at 4 °C. Subsequently, the residual laccase activity was triplicately assayed using the standard assay. In the thermostability assay, enzyme solutions were previously incubated at various temperatures (50, 60, 70, and 80 °C, respectively) for various durations (10, 20, 30, 40, 50, and 60 min, respectively). The residual laccase activity was measured in triplicate using the standard assay after the reaction mixture had been cooled down to room temperature.

2.9. Assay for enzyme kinetic and substrate specificity of purified laccase

The Michaelis-Menten constants of the purified laccase were determined using ABTS at pH 2.4 and guaiacol at pH 3.2 in various concentiration (0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mM) and at 30 °C. The *Km* values were obtained from a Lineweaver–Burk plot [14]. To determine the substrate specificity of the purified laccase, seven aromatic substrates (at 5.0 mM concentration) instead of ABTS were used in the standard enzyme assay at optimal pH (pH 2.4, 50 mM citric acid-Na_2HPO_4 buffers) and 30 °C. The assayed substrates included 2,6-dimethoxy-phenol, 4-hydroxyindole, 4-methylcatechol, catechol, ferulic acid, guaiacol, hydroquinone, N,N-dimethyl-1,4-phenylenediamine, pyrogallol, and tyrosine. The substrate oxidation rate was followed by measuring the change in absorbance using the molar extinction coefficient (ε) obtained from the literature [15]. All determinations were performed in triplicate. Laccase activity toward ABTS was regarded as 100%.

2.10. Assay for metal ions and EDTA on laccase activity

To estimate metal ions and EDTA on enzyme activity, equal volumes of the purified laccase solution were pre-incubated with metal ions or EDTA solutions (at a final concentrations of 1.25, 2.5, 5.0, and 10 mM, respectively) at 4 °C for 1 h before the standard laccase assay was performed. The chemical reagents of metal ions were including KCl, $CaCl_2$, $CdCl_2$, $CuCl_2$, $FeCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$, and $AlCl_3$. Control samples were assayed without the metal ions. All determinations were performed in triplicate.

2.11. Assay of anti-proliferative activity and HIV-1 reverse transcriptase inhibitory activity of purified laccase

Anti-proliferative activity of the purified laccase toward tumor cell lines hepatoma HepG2 and mouse lymphocytic leukemia L1210 was tested by using the MTT assay as described by Fang et al. using heat-inactivated IBL as negative control [16]. Inhibitory activity toward human immunodeficiency virus type 1 (HIV-1) reverse