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Purification, molecular characterization and reactivity with aromatic compounds of a laccase from basidiomycete Trametes sp. strain AH28-2

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Abstract A recently isolated basidiomycete, Trametes sp. strain AH28-2, can be induced to produce a high level of laccases when grown on a cellobiose-asparagine liquid medium. After induction by kraft lignin, two major isozymes were detected in the fermentation supernatant of the fungus. The principal component laccase A, which accounts for about 85% of the total activity, can be purified to electrophoretic homogeneity by three chromatographic steps: DEAE-Sepharose FF, Superdex-200 and Mono-Q. The solution containing purified laccase is blue in color, and the ratio of absorbance at 280 nm to that at 600 nm is 22. The molecular mass of laccase A is estimated to be 62 kDa by SDS-PAGE, 57 kDa by FPLC, and measured as 58,522 Da by MALDI mass spectrum. Laccase A is a monomeric glycoprotein with a carbohydrate content of 11–12% and an isoelectric point of 4.2. The optimum pH and temperature for oxidizing guaiacol are 4.5 and 50°C, respectively. The half-life of the enzyme at 75°C is 27 min. The enzyme shows a good stability from pH 4.2 to pH 8.0. The $K_m$ values of the enzyme toward substrates 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), guaiacol and 2,6-dimethoxyphenol are 25, 420 and 25.5 $\mu$M, respectively, and the corresponding $V_{max}$ values are 670, 66.8, and 79 $\mu$M min$^{-1}$ mg$^{-1}$, respectively. Laccase A activity is strongly inhibited by 0.1 mM NaN₃ or 0.1 mM cyanide. Two units of laccase A alone is able to completely oxidize 100 $\mu$mol 2,6-chlorophenol in 6 h. In the presence of 1 mM ABTS and 1-hydroxybenzotriazole, 15.0 U laccase A is able to oxidize 45% and 70% of 50 $\mu$mol fluorene in 12 and 18 h, respectively. The laccase A gene was cloned by a PCR method, and preliminary analysis of its sequence indicates 87.0% similarity to the corresponding segment in the phenoloxidase gene from Coriolus hirsutus.

Introduction

Lignin is a structurally complicated biopolymer composed of phenylpropanoids. It is quite stable and resistant to degradation under normal conditions. It is well established that the white-rot fungi are most effective in lignin degradation in nature, synthesizing a ligninolytic system including lignin peroxidase (Lip), manganese peroxidase (Mnp) and laccase, which are responsible for the degradation of lignin. The well-studied white-rot fungus Phanerochaete chrysosporium produces Lip, Mnp and laccase during its metabolic processes. Lip is regarded as the critical enzyme for lignin degradation and has shown to oxidize non-phenolic lignin substructure accounting for more than 90% of the total. However, the white-rot fungi Dichomitus squalens and Ceriporiopsis subvermispora can degrade lignin efficiently without expressing detectable Lip (Péréc and Gold 1991; Rüttimann-Johnson et al. 1997). Most white-rot fungi capable of degrading lignin can synthesize Mnp and laccase. Actually, the combination of Mnp and laccase is much more common than that of Lip and Mnp. However, the white-rot basidiomycete Coriolopsis rigida secretes no detectable Lip or Mnp but enough extracellular laccase (Saparrat et al. 2002); the laccase alone has been demonstrated to be capable of degrading lignin, including the non-phenolic moieties. Additional studies also indicate that laccase plays a key role in the lignin degradation process (Otterbein et al. 2000).

Laccase (benzenediol:oxygen oxidoreductase; EC 1.10.3.2) is a polyphenol oxidase that contains four coppers, and is able to oxidize its substrates, ortho-, para-diphenol and aromatic compounds containing hydroxyl and amine groups, while simultaneously catalyzing the reduction of one dioxygen molecule to two molecules of...
water (Thurston 1994). In the presence of appropriate redox mediators, such as 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) and 1-hydroxybenzotriazole (HBT), laccase also catalyzes the oxidation of non-phenolic lignin model compounds (Bourbonnais and Paice 1990) and degrades polycyclic aromatic hydrocarbons (Pickard et al. 1999) and various dye pollutants. Because of the significance of potential applications in biopulping, kraft lignin bleaching or degradation of aromatic pollutants and wastewater treatment, laccase draws considerable attention from researchers. Many laccases have been purified and characterized (Schneider et al. 1999; Cambria et al. 2000; Min et al. 2001; Jung et al. 2002). Some laccase genes have been cloned (Giardina et al. 1995, 1999; Yaver et al. 1996; Otterbein et al. 2000; Kim et al. 2001), and the corresponding recombinant proteins have also been expressed in some cases. However, the mechanism of fungal degradation of lignin by laccase remains to be defined. Therefore, purification and characterization of laccase from novel wood-rotting fungi will help shed light on this mechanism.

The white-rot fungus *Trametes* sp. strain AH28-2, recently isolated in China from rotting wood, is able to selectively degrade lignin components of natural rice straws, and shows strong ability to decrease chemical oxygen demand in the wastewater of pulping with straw (Wu et al. 2002). Preliminary studies indicate that this fungus can be induced to produce large amounts of laccase on a defined medium without secreting detectable Lip and tyrosinase (Xiao et al. 2001). In order to investigate the ligninolytic system of *Trametes* sp. strain AH28-2 and the role of its laccase in lignin degradation, laccase A was purified and characterized, and its reactivity with aromatic compounds was studied.

**Materials and methods**

**Fungal strain**

*Trametes* sp. strain AH28-2 was obtained from the culture collection of the School of Life Sciences, Anhui University, China. Pure strain was grown at 28°C for 5 days on CPDA slants [CPDA medium contains: 20.0 g glucose, 1.0 g KH₂PO₄, 1.5g MgSO₄·7H₂O, 50 μg vitamin B₁, 15.0 g agar powder and 1,000 ml potato extract liquid (20%)] and then maintained at 4°C and inoculated once every 3 months.

**Growth conditions and induction of laccase production**

For studies of laccase production, 5–6 cylinders (diameter, 10 mm) of this strain grown on CPDA plates were used to inoculate 250 ml Erlenmeyer flasks containing 100 ml liquid cellulose-asparagine medium. The liquid culture medium contained (per liter): 10 g cellulose, 10 ml glycerol, 1.5 g l-asparagine, 1.0 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 0.01 g CaCl₂, 0.001 g FeSO₄·7H₂O, 0.1 g Na-HPO₄·5H₂O, 0.002 g CuSO₄·5H₂O, 0.0275 g adenine, and 50 μg vitamin B₁. The culture was incubated at 28°C on a rotary shaker at 120 rpm. A 72-h-old liquid culture was homogenized using a sterilized blender. The volume of inoculum was 10 ml per 200 ml culture medium, which was then cultivated under the same conditions. After 3 days, kraft lignin (Sigma, St. Louis, Mo.) was added to a final concentration of 0.1% (w/v) to stimulate laccase production. Maximum laccase activity was obtained after another 2–3 days.

**Enzyme assay**

Laccase activity was usually assayed in two ways (Xiao et al. 2001). First, the oxidation of ABTS was monitored as increased A₄₇₀ at 25°C. The reaction mixture (in a total volume of 3 ml) contained 0.1 ml enzyme solution at different dilutions and 0.5 mM ABTS in sodium tartarate buffer (pH 4.0). The assay was also performed using guaiacol as substrate in a mixture containing 1 ml laccase dilution, 1 mM substrate and 50 mM succinic acid-NaOH buffer (pH 4.5). One unit was defined as the amount of laccase that oxidized 1 μmol substrate per minute. Assays were carried out in triplicate for each dilution and on two different dilutions. Standard deviation did not exceed 8% of the average values.

**Enzyme purification**

Unless otherwise stated, all procedures were performed at 4°C. The culture fluid for the enzyme purification was first filtered through six layers of sterile gauze. The aqueous solution (1,080 ml) was centrifuged at 6,000 g for 30 min and then concentrated to 50 ml in a Minitan Ultrafiltration System with a low-binding regenerated cellulose membrane (Millipore, Bedford, Mass.). The concentrate was centrifuged at 12,000 g for 20 min and the supernatant was then dialyzed against buffer A (10 mM citrate-NaHPO₄, pH 6.0) overnight followed by centrifugation as before. A three-step chromatography procedure followed: (1) the supernatant was applied to a DEAE-Sepharose FF column (10×200 mm, Amersham Pharmacia, Uppsala, Sweden) pre-equilibrated with buffer A. The column was then washed with approximately 100 ml buffer A to remove melanin and polysaccharide, and eluted with a linear gradient of (NH₄)₂SO₄ (0–1 M in buffer A; flow rate 0.8 ml min⁻¹); (2) fractions containing laccase activity from step 1 were pooled and concentrated to a volume of 3–5 ml in an Amicon ultrafiltration stirred pressure cell equipped with a 47 mm 30,000 molecular-weight cut-off filter. The concentrate was subjected to gel filtration using a Hiloald 26/60 Superdex 200 preparative grade column (Amersham Pharmacia) pre-equilibrated with buffer B [10 mM citrate-NaHPO₄, 0.15 M (NH₄)₂SO₄, pH 6.0]. Fractions were eluted from the column with buffer B; (3) fractions containing laccase activity from step 2 were pooled, desalted, dialyzed against buffer A and applied to FPLC with a Mono-Q HR 10/10 preparative grade column (Amersham Pharmacia) pre-equilibrated in buffer A. Fractions eluted with a linear gradient of (NH₄)₂SO₄ (0–0.5 M in buffer A; flow rate 1.0 ml min⁻¹) were collected. The active fractions were pooled and dialyzed against buffer A. Finally, enzyme purity was assessed by SDS-PAGE.

**Enzyme characterization**

SDS-PAGE analysis of proteins was performed on 10% or 12% polyacrylamide gels (Laemmli 1970). Proteins were stained with Coomassie Brilliant Blue R250 (Fluka, Buchs, Switzerland). Native-PAGE was performed by incubating the gel at 25°C in buffer A containing 10 μM syringaldazine (Sigma) [pre-dissolved in dimethylsulfoxide (DMSO)]. The molecular mass of denatured laccase was determined by FPLC with gel filtration on a Superdex 200 HR10/30 preparative grade column (Amersham Pharmacia), and also by MALDI mass spectrum using a model BIFLEX III MALDI-time-of-flight mass spectrometer (Bruker Biospin, Billerica, Mass.) with α-cyano-4-hydroxycinnamic acid as matrix. The isoelectric point was estimated by isoelectric focusing using a Bio-Rad Fast Gel System (Bio-Rad, Hercules, Calif.) with Bio-Rad wide-range ampholytes (pH 3–9) and Pharmacia low-range ampholytes (pH 2.5–5.0) as standards. The carbohydrate