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Characterization of an extracellular laccase, PbLac1, purified from *Polyporus brumalis*

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ABSTRACT

Polyporus brumalis (strain ibrc05015) secreted high amounts of laccases (Lacs) in liquid medium. With 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) as a substrate, Lac activity was 7.72 U ml^{-1} and this strain secreted a maximum 0.23 mg ml^{-1} of total protein. The enzyme, PbLac1 was purified to homogeneity using hydrophobic and anion-exchange chromatography. The purified PbLac1 had a molecular mass of 63.4 kDa as determined by polyacrylamide-gel electrophoresis. PbLac1 oxidized a wide range of substrates such as 3,4-dihydroxy L-phenylalanine (L-DOPA) and catechol, but not tysorine. The activity of PbLac1 was increased by addition of 10.0 mM Cu^{2+}. PbLac1 could decolorize several industrial dyes, such as Remazol Brilliant Blue R known as model dyes of environmental xenobiotics. In addition, PbLac1 decolorized a wide range of substrates, such as the carcinogen, Poly R-478, in the presence of violuric acid as mediator. The E° value of PbLac1 was 0.80 V ± 0.01 *versus* normal hydrogen electrode, which is a very high redox potential compared to those of other basidiomycetous Lacs. These results suggest the potential utility of PbLac1 for industrial applications.

Introduction

Laccase (Lac; *p*-benzenediol:dioxygen oxidoreductase, EC 1.10.3.2) belongs to a group of polyphenol oxidases that contain copper atoms in their catalytic centers. Lac catalyzes the oxidation of single-electron from phenolic substrates or aromatic amines. Many organisms possess Lac-encoding genes with a wide range of biological roles. Plant Lacs are involved in the formation of the lignin polymers by radical-based mechanisms (Sterjiades *et al.* 1992; Liu *et al.* 1994; Ranocha *et al.* 2002; Hoopes & Dean 2004). Fungal Lacs are involved in various biological processes, such as lignin degradation (Leonowicz *et al.* 2001), fruiting-body formation (De Vries *et al.* 1986), and human and plant pathogenesis (Burke & Cairney 2002; Nosanchuk & Casadevall 2003; Langfelder *et al.* 2003). In addition, Lacs have potential industrial applications for processes such as pulp bleaching (Bourbonnais *et al.* 1995; Call & Mücke 1997), dye decolorization (Chivukula & Renganathan 1995), polymer synthesis, and biosensing (Gomes *et al.* 2004). Lacs also have the ability to degrade environmentally persistent xenobiotics such as chlorophenols (Ullah *et al.* 2000; Ahn *et al.* 2002), pesticides (Amitai *et al.* 1998; Maruyama *et al.* 2006), and endocrine-disrupting chemicals (Fukuda *et al.* 2001; Tsutsumi *et al.* 2001;

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Abbreviations: *ABTS, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; BPB, Bromophenol Blue; L-DOPA, 3,4-dihydroxy L-phenylalanine; EDTA, ethylenediaminetetraacetic acid; ITS, internal transcribed spacer; Lac, Laccase; NBB, Naphthol Blue Black; NHE, normal hydrogen electrode; RBBR, Remazol Brilliant Blue R; 2,6-DMP, 2,6-dimethoxyphenol.*

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Junghanns et al. 2005; Tamagawa et al. 2007). More recently, new applications have been suggested for Lacs, for example, as cathode catalysts for biofuel cells (Kamitaka et al. 2007b). These applications require a highly efficient Lac production system.

Lacs have been purified from many basidiomycetous species (Baldrian 2006), and there are some reports on the screening of fungal strains for high Lac production (Morisaki et al. 2001; Kiiskinen et al. 2004). Among white-rot fungi, *Trametes versicolor* and *Pycnoporus cinnabarinus* of the *Polyporaceae* family have been studied in particular detail (Bourbonnais et al. 1995; Eggert et al. 1996; Han et al. 2005). Lacs from *T. versicolor* and *P. cinnabarinus* have high redox potentials (Reinhammar 1984; Xu et al. 1996; Sigoillot et al. 2004). Therefore, Lacs from *Polyporaceae* constitute attractive candidates for industrial applications including pulp bleaching, pulping and its waste-water treatment (Manzanares et al. 1995; Geng & Li 2002).

Because of their potential in industrial applications, several attempts have been made to produce large amounts of their Lacs including heterologous expression of recombinant Lacs. There have been several reports of heterologous expression of active recombinant Lacs including the use of hosts, such as methylotrophic yeast *Pichia pastoris* (Jönsson et al. 1997; Guo et al. 2006), *Yarrowia lipolytica* (Madzak et al. 2005), and *Aspergillus oryzae* (Hoshida et al. 2005). Nevertheless, in case of *P. cinnabarinus*, the natural production of Lac reached the best production level compared to heterologous expression (Sigoillot et al. 2004).

In this study, we screened freshly isolated strains of white-rot fungi, primarily belonging to the family *Polyporaceae* and the genus *Polyporus*, in order to find strains capable of producing large amounts of Lacs with high redox potentials comparable to that of *T. versicolor*. From this screening, a strain of *Polyporus brumalis* that produced high amount of Lac was identified, and a Lac was purified and characterized subsequently from its culture. These results demonstrated its potential for industrial applications such as bioremediation and biofuel cells.

Materials and methods

Chemicals

Unless otherwise stated, all chemicals were purchased from Wako Pure Chemicals (Osaka, Japan), and were certified reagent grade.

Strains used in this study

We collected several strains from northern Japan (Aomori, Akita and Iwate Prefectures). These strains are shown in Table S1. They were deposited to National Institute of Technology and Evaluation (NITE) Biological Resource Center (NBRC). Strains obtained from the National Institute of Agrobiological Science (NIAS) and the NITE of Japan are shown in Table S2.

Separation of mycelia

The internal tissues from fruiting bodies were inoculated on 1.5 % agar plates with 0.25 × MYPG medium containing 0.25 % Bacto malt extract (Difco, MI, USA), 0.1 % Bacto yeast extract (Difco, MI, USA), 0.1 % tryptone peptone (Difco, MI, USA), and 0.5 % glucose. For isolation of the strains, mycelia were grown at 25 °C and repeatedly transferred to new 0.25 × MYPG agar medium at interval of 5–10 d.

Culture conditions

For the initial screening of strains producing high Lac activity, mycelia were inoculated on 0.25 × MYPG agar plates. After 4–10 d of growth, top agar containing 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; Sigma, MO, USA) as a substrate for Lac was overlaid on the MYPG plates and strains with high Lac activity were visually selected by coloration of ABTS. For the second screening, five blocks (7 mm diameter) were cut from 0.25 × MYPG agar-plate cultures of each selected strain and inoculated into 100 ml of 0.5 × MYPG [0.5 % Bacto malt extract (Difco, MI, USA), 0.2 % Bacto yeast extract (Difco, MI, USA), 0.2 % tryptone peptone (Difco, MI, USA), and 1.0 % glucose] liquid media. The cultures were incubated at 25 °C with shaking (7 cm stroke, 75 rpm) and the Lac activity was measured 7-d intervals for 42–70 d.

Enzyme assays

Lac activity was measured using ABTS as substrate. The reaction mixture, containing 1.0 mM ABTS in McIlvaine buffer (pH 4.0) in a total volume of 100 μl, was incubated for 10 min. The reaction was stopped by the addition of an equal volume of 5 % (v v^{-1}) trichloroacetic acid. Oxidation of ABTS was measured by monitoring the increase in absorbance at 420 nm. Formation of cation radicals was detected by measuring the absorbance increase at 420 nm ($\varepsilon 420 = 36\,000\ M^{-1}\ cm^{-1}$). One unit of Lac activity was defined as the amount of enzyme that catalyzed the oxidation of 1.0 μmol of ABTS in 100 μl of reaction mixture at 30 °C in 1 min.

Protein assays

Protein concentration was measured by the Bradford method using the Protein Assay Reagent (5 × concentrate; Cytoskeleton Co., CO, USA). Bovine serum albumin (BSA; Sigma, MO, USA) was used as standard. Protein concentration was monitored by photometrically light absorbance at 595 nm.

Purification of PbLac1

To obtain Lac for purification, mycelia from three Erlenmeyer flasks were transferred to a 2-l Erlenmeyer flask containing 500 ml of 1 × MYPG [1.0 % Bacto malt extract (Difco, MI, USA), 0.4 % Bacto yeast extract (Difco, MI, USA), 0.4 % tryptone peptone (Difco, MI, USA), and 2.0 % glucose] medium and incubated at 25 °C with rotary shaking (120 rpm) for 16 d. All chromatography steps were carried out at 4 °C. Column chromatography was performed with a FPLC system (GE Healthcare, Amersham Bioscience, UK). The culture filtrate was concentrated to 200 ml using an ultrafiltration cell Model 8400 (Millipore, MA, USA) and an ultrafiltration membrane (10.0 kDa cut-off; Millipore). Ammonium sulfate was added to 30 % (w v^{-1}) saturation. The supernatant was applied to a Hiprep phenyl column (GE Healthcare) that was equilibrated