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Extracellular oxidases of *Cerrena* sp. complementarily functioning in artificial dye decolorization including laccase, manganese peroxidase, and novel versatile peroxidases

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ABSTRACT

Extracellular ligninolytic oxidoreductases produced by *Cerrena* sp. strain Ra, a soil-isolated basidiomycete with high artificial dye-decolorizing activity, were purified and characterized. One thermostable laccase, one typical manganese peroxidase, and two versatile peroxidases (VPI and VPII) were found in the culture of this fungus. The different characteristics of each enzyme enable the strain express wide-range of oxidizing activity under various conditions. VPI decolorizing activity was observed toward various kinds of dye compounds, and the activity and specificity varied depending on the oxidizing mediators added to the reaction mixture. Optimized VPI/mediator-coupling decolorizing will be widely used for industrial wastewater treatment.

1. Introduction

Over ten thousand kinds of synthetic dyes are used for textile, leather, paper, and food dyeing or other industrial applications. Total dye production in the world is estimated at about 800,000 t per year, and up to 15% of the used dyes are released into wastewater (Wesenberg et al., 2003). Although many synthetic dyes are mutagenic and carcinogenic (Chung et al., 1981; Hildenbrand et al., 1999), most of them are not removed by conventional biological treatment using microbes. In contrast, chemical treatments are effective for dye degradation, but they are very costly.

Some kinds of basidiomycetes, known as white-rot fungi, are efficient at breaking down synthetic dyes. The fungi secrete ligninolytic enzymes in order to depolymerize lignin structures of woody plants. These extracellular ligninolytic enzymes contain two types of oxidoreductases: laccase and peroxidase. Laccase and peroxidase are glycosylated proteins that catalyze the oxidation of many substrates by reducing oxygen to water or hydrogen peroxide to water, respectively (Wong, 2009). Ligninolytic peroxidases belong to the class II heme peroxidase superfamily and are classified into three types of enzymes: lignin peroxidase, manganese peroxidase, and versatile peroxidase.

Lignin peroxidase directly oxidizes a variety of phenolic and non-phenolic aromatic compounds including synthetic dyes (Ollikka et al., 1993). Veratryl alcohol (VA) is a typical non-phenolic substrate for lignin peroxidase and is oxidized to its cation radical (Khindaria et al., 1995). This cation radical can act as a diffusible redox mediator for the degradation of dyes with high redox potentials, which are not directly oxidized by lignin peroxidase (Harvey et al., 1986; Heinfling et al., 1998). Alternatively, VA can reduce the Compound II form of lignin peroxidase, as a result of dye oxidation, to its native form (Paszczynski and Crawford, 1991).

Manganese peroxidase directly oxidizes Mn^{2+} to Mn^{3+} (Glenn et al., 1986). The complex of Mn^{3+} and an organic acid acts as a diffusible oxidizer of phenolic compounds and some dyes (Heinfling et al., 1998; Kishi et al., 1994; Kuan and Tien, 1993).

Versatile peroxidase is a hybrid enzyme of lignin peroxidase and manganese peroxidase found in some species of *Pleurotus* (Kamitsuji et al., 2005; Martinez et al., 1996), *Bjerkandera* (Mester and Field, 1998; Palma et al., 2000), *Lepista* (Zorn et al., 2003), and so on. This enzyme is able to oxidize a variety of phenolic and non-phenolic substrates including Mn^{2+}, VA and different types of dyes (Tinoco et al., 2007). Moreover, it directly oxidizes some high redox-potential dyes that can be oxidized by lignin peroxidase only in the presence of VA (Heinfling et al., 1998; Kamitsuji et al., 2005; Mester and Field, 1998).

Recently, a novel peroxidase family with DyP from *Thanatephorus cucumeris* Dec 1 as a representative one was proposed. DyP has several characteristics that distinguish it from all other

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peroxidases, including a particularly wide substrate specificity especially toward hydroxyl-free anthraquinone dyes, a lack of homology to most other peroxidases, and the ability to function well under much lower pH conditions (Sugano et al., 2007).

We screened various kinds of laccase- and peroxidase-producing microorganisms decolorizing microorganisms from various natural sources with dye-decolorizing activity as an indicator, and found an alkaline laccase (Sulistyaningdyah et al., 2004), a thermostable laccase (Nakatani et al., 2010), and two bacterial peroxidases (Koga et al., 1999a, 1999b; Ogawa et al., 2004). In this study, we found that a trametoid basidiomycete, *Cerrena* sp. strain Ra, could efficiently degrade the synthetic dye Remazol Brilliant Blue R (RBBR). From *Cerrena* sp. strain Ra, one laccase, one manganese peroxidase, and two isoforms of versatile peroxidase were isolated and characterized. Each enzyme complementarily represents the oxidative activity of the strain under different conditions. In particular, one of the versatile peroxidase isoforms was analyzed in detail, specifically focusing on its synthetic dye decomposition ability.

2. Materials and methods

2.1. Isolation of dye-decolorizing microorganisms

Dye decolorizing microorganisms were isolated from soil samples by spreading soil suspended in water onto 1.5% agar plates containing 1% potato extract, 2% glucose, 0.05% RBBR (Remazol Brilliant Blue R), and 0.003% chloramphenicol (pH 6.0). These plates were incubated at 28 °C for several days, and RBBR-decolorizing colonies were collected for the following test of laccase and peroxidase activity.

2.2. Identification of strain Ra

Strain Ra underwent molecular phylogenetic analysis based on the nucleotide sequence of 18S and 28S rDNA. Sequencing of 18S rDNA was carried out using NS1 and NS8 primers as described by White et al. (1990), and sequencing of 28S rDNA was carried out using NL1 and NL4 primers as described by O'Donnell (1993).

2.3. Preparation of culture supernatants for the assay of extracellular oxidoreductase activity

Selected strains were cultivated in a liquid medium containing 1% glucose, 0.5% polypeptone (Nihon Pharmaceutical), 0.1% yeast extract, 0.1% KH_2PO_4, 0.1% K_2HPO_4, and 0.02% $MgSO_4 \cdot 7H_2O$ at pH 6.0 and 28 °C for 4–7 day with shaking (300 rpm). The culture supernatants were filtered with 1.2 μm MF-Millipore membrane filter and were used for the laccase and peroxidase activity assays described below.

2.4. Enzyme assays

During microbial screening and enzyme purification, laccase and peroxidase activities were assayed with dye decolorizing assays. For the laccase assay, the reaction mixture consisted of 0.25 mM RBBR in 100 mM malonate buffer (pH 4.5). Twenty micro-ls of enzyme were added to the reaction mixture (180 μl) in a 96-well microplate, and decreases in the absorbance at 592 nm were measured using a microplate photometer, the SpectraMax 210 (Molecular Device), at 30 °C. For the peroxidase assay, the reaction mixture consisted of 0.25 mM RBBR, 5 mM $MnSO_4$, and with or without 3 mM hydrogen peroxide in 100 mM malonate buffer (pH 4.5). Measurement of absorbance was carried out in the same way as in the laccase assay. Differences between absorbance values of reaction mixtures with and without hydrogen peroxide were used for calculation of peroxidase activity. In both cases, the concentration of the dye was calculated using the molar extinction coefficient (ε_{592})=6,170/M cm. One unit of enzymatic activity (U) is equivalent to one μmol of the dye decomposed per minute.

2.5. Purification of enzymes

Strain Ra was cultivated in 2-l flasks containing 300 ml of liquid medium. The liquid medium was composed of 5% malt extract, 0.3% yeast extract, 0.2% KH_2PO_4, 0.2% K_2HPO_4, and 0.001% $MnSO_4 \cdot 5H_2O$ (pH 6.6). Cultivation was carried out aerobically at 28 °C for 3–7 day with shaking at 120 rpm. The culture supernatant was separated from the mycelia by filtration with 1.2 μm MF-Millipore membrane filter. All of the following purification steps were carried out at 0–4 °C and the buffer used was 20 mM Tris–HCl (pH 7.4), unless otherwise stated. The clear supernatant was concentrated with an AIP-1010 ultra filtration module (MW 10,000 cut; Asahi Chemical Industry), dialyzed against the buffer, and used for the purification of extracellular oxidoreductases.

Enzyme production was determined by using the RBBR decolorizing assays described above. The dialyzed concentrated protein solution was applied to a DEAE-Sepharose FF column (50 mm × 100 mm; GE Healthcare) equilibrated with the buffer. After the column was washed with the buffer, the enzymes were eluted with a linear gradient of 0–1 M NaCl. Depending on the activity of each oxidoreductase, the elution fractions were combined into three active pools: a pool with laccase activity and two pools with peroxidase activity. The active pools were respectively dialyzed against the buffer, and loaded to a MonoQ HR 10/10 column (GE Healthcare) equilibrated with buffer and eluted with a linear gradient of 0–0.5 M NaCl. The active fractions with laccase or peroxidase activity were respectively applied to a Superose 12 column (GE Healthcare) equilibrated with buffer containing 0.2 M NaCl and separated with the same buffer. The standard proteins used were glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), adenylate kinase (32 kDa), and cytochrome c (12.4 kDa). The purified enzymes were used for characterization.

2.6. Enzyme characterization

Protein concentration was determined with a Protein Assay Kit (Bio-Rad). The molecular weight of native protein was determined by HPLC on a TSKgel G3000SW column (7.5 × 600 mm; Tosoh). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the oxidoreductases was performed on 12.5% polyacrylamide gels with SDS using the Tris-glycine buffer system. The NH_2-terminal amino acid sequences of the purified enzymes were determined by automated Edman degradation with a model 476 A sequencer (Applied Biosystems).

3. Results

3.1. Screening of microorganisms producing extracellular oxidoreductases

Many microorganisms in soil samples formed colonies on culture plates containing RBBR dye as an indicator of oxidoreductase secretion. The microorganisms decolorizing RBBR around their colonies were collected as potential producers of extracellular oxidoreductases. For each isolate, the laccase and peroxidase activity of the culture supernatant was assayed with RBBR-decolorizing assay. An isolate, labeled as Ra, showed strong RBBR-decolorizing activity and also produced oxidoreductases in its culture. Strain Ra was identified as a basidiomycete belonging to Polyporales based on