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Characterization and cloning of laccase gene from *Hericium coralloides* NBRC 7716 suitable for production of epitheaflagallin 3-*O*-gallate

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ABSTRACT

Epitheaflagallin 3-*O*-gallate (ETFGg) is a minor polyphenol found in black tea extract, which has good physiological functions. It is synthesized from epigallocatechin gallate (EGCg) with gallic acid via laccase oxidation. Various basidiomycetes and fungi were screened to find a suitable laccase for the production of ETFGg. A basidiomycete, *Hericium coralloides* NBRC 7716, produced an appropriate extracellular laccase. The purified laccase produced twice the level of ETFGg compared with commercially available laccase from *Trametes* sp. The enzyme, termed Lcc2, is a monomeric protein with an apparent molecular mass of 67.2 kDa. The N-terminal amino acid sequence of Lcc2 is quite different from laccase isolated from the fruiting bodies of *Hericium*. Lcc2 showed similar substrate specificity to known laccases and could oxidize various phenolic substrates, including pyrogallol, gallic acid, and 2,6-dimethoxyphenol. The full-length *lcc2* gene was obtained by PCR using degenerate primers, which were designed based on the N-terminal amino acid sequence of Lcc2 and conserved copper-binding sites of laccases, and 5'-, and 3'-RACE PCR with mRNA. The Lcc2 gene showed homology with *Lentinula edodes* laccase (sharing 77% amino acid identity with Lcc6). We successfully produced extracellular Lcc2 using a heterologous expression system with *Saccharomyces cerevisiae*. Moreover, it was confirmed that the recombinant laccase generates similar levels of ETFGg as the native enzyme.

1. Introduction

Laccases (1,4-diphenol: dioxygen oxidoreductase, EC 1.10.3.2) belong to a group of polyphenol oxidases found in insects [1], plants including the Japanese lacquer tree *Rhus vernicifera* [2–4], fungi, especially in white-rot fungi [4,5], and some bacteria [4]. Most fungal laccases are produced extracellularly, playing an important role in the decomposition of wood lignin [5] and the formation of humic matter [6]. They are multi-copper proteins that use molecular oxygen to oxidize various aromatic and non-aromatic compounds by a radical-catalyzed reaction mechanism [7]. They generally contain three types of copper, of which Type 1 is responsible for the blue color, oxidation of substrates, and electron extraction. The extracted electrons are transferred to Type 2 and/or Type 3 copper sites, where molecular oxygen is reduced to water. A broad range of substrates undergo laccase oxidation, including diphenols and various phenolic compounds, and substrates overlap with those of monophenol monooxygenase tyrosinase (EC 1.14.18.1) [3] and catechol oxidase (1,2-diphenol: dioxygen oxidoreductase, EC 1.10.3.1) [3]. The range of substrates accepted by laccase as a hydrogen donor is outstanding, and oxidation of syringaldazine in combination with an inability to oxidize tyrosine is a recognized indicator of laccase activity [4]. Accordingly, laccases play important roles in the textile industry [8], synthetic chemistry [7,9–11], the food industry [12], and the biodegradation of environmental pollutants [13], due to their ability to catalyze electron transfer reactions without additional cofactors.

A few reports on functional food production via laccase reactions have been published. Epitheaflagallin 3-*O*-gallate (ETFGg), a minor polyphenol in black tea extract (<0.1% (w/w)) [14], has some good physiological functions and shows, for example, antioxidant activities, anti-obesity effects via inhibition of lipase [15] and β-glycosidase, anti-inflammatory activities, and inhibitory effects on membrane Type 1 metalloprotease (MT1-MMP) [16], all of which suggest it is a promising functional food material. ETFGg is synthesized from epigallocatechin gallate (EGCg), a major polyphenol in green tea extract, with gallic acid via laccase oxidation (Scheme 1) [14,15]. However, the level of production of ETFGg from EGCg has been unsatisfactory to date, even under optimized reaction conditions.

In this study, we describe the screening of a novel laccase (Lcc2) from *Hericium coralloides* NBRC 7716, which is suitable for ETFGg production, characterization, cloning, and heterologous expression of *lcc2* in *Saccharomyces cerevisiae*.

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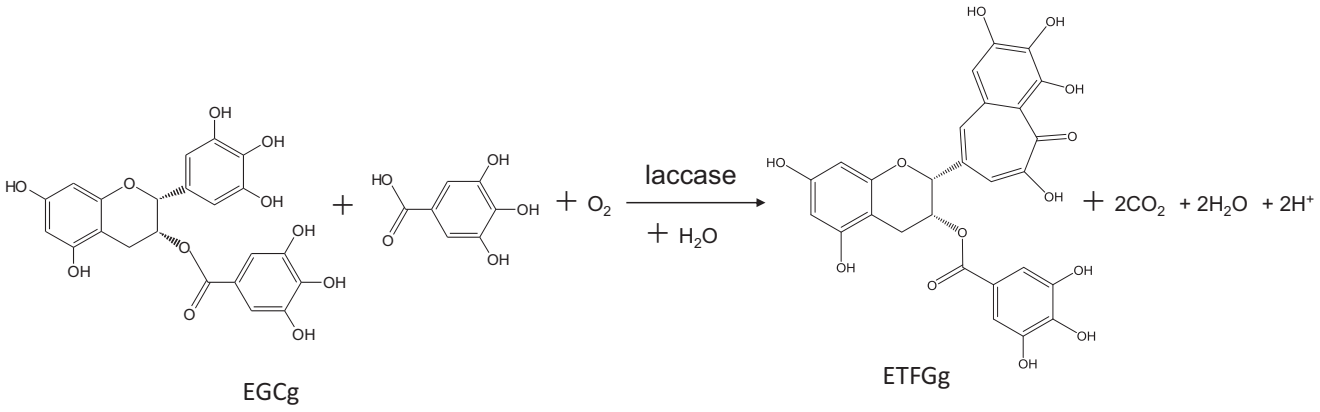

Scheme 1. Mechanism of coupling reaction of EGCg and gallic acid is described in detail in Ref. [14].

2. Materials and methods

2.1. Culture conditions and screening of fungal strains

Fungal strains (151: 36 genera, 141 strains of basidiomycetes and 5 genera, 10 strains of ascomycetes) were obtained from the Biological Resource Center (NBRC), NITE (Chiba, Japan), Toyama Prefectural Agricultural, Forestry and Fisheries Research Center (Toyama, Japan) and the American Type Culture Collection (Manassas, VA, USA), and maintained on potato dextrose agar (PDA) (Difco, Sparks, MD, USA). To induce laccase production, each strain was grown on wheat bran solid medium, consisting of 20 g of wheat bran and 20 mL of 0.1 mM $CuSO_4$ solution. Plugs of mycelium (ca. 5 mm diameter) were cut from the PDA plate cultures, and placed on the center of sterilized wheat bran solid medium in a 300 mL conical flask. Each fungus was incubated aerobically at 25 °C for 3–4 weeks. The crude extract was obtained from 40 g of loosened solid fermentation media with the addition of 50 mL of 20 mM potassium phosphate buffer (KPB) (pH 6.0) after incubation at 4 °C for 4 h, followed by filtration with paper towels and squeezing.

2.2. Purification and physicochemical characterization of laccase from H. coralloides NBRC 7716

The crude extract was obtained from 240 g solid fermentation media cultured for 2 weeks, followed by extraction with 300 mL of 20 mM KPB (pH 6.0), as reported in the Culture conditions and screening section. After centrifugation, the supernatant was fractionated with solid ammonium sulfate precipitation. The precipitate obtained with 40–80% saturated ammonium sulfate was collected, suspended in buffer containing 40% saturated ammonium sulfate, and applied to a Butyl-Toyopearl 650 M (Tosoh Corp., Tokyo, Japan) column (28 × 1200 mm) that had been equilibrated using the same buffer. The enzyme was eluted with a linear 40–0% aqueous ammonium sulfate solution gradient in buffer. Fractions with high enzyme activity were dialyzed against the buffer (pH 6.0), and applied to a DEAE-Toyopearl 650 M (Tosoh) column (28 × 500 mm). The enzyme was eluted using a linear 0–0.5 M aqueous NaCl solution gradient in the same buffer. Fractions with high enzyme activity were desalted and concentrated using a Centriprep YM-30 filter unit (Merck Millipore, Darmstadt, Germany, molecular mass cut-off 30,000 Da). The enzyme solution was applied to a gel filtration column of Cellulofine GCL2000sf (28 × 880 mm) (Seikagaku Corp., Tokyo, Japan) that had been equilibrated with the buffer containing 0.3 M NaCl (pH 6.0) and eluted at a flow rate of 0.2 mL/min. Fractions with high enzyme activity were collected, dialyzed against 20 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS) buffer (pH 7.0), and the enzyme was loaded onto a Bioassist Q column (Tosoh, 4.6 × 50 mm), which had been equilibrated with the same buffer (pH 7.0), and eluted with a linear 0–0.3 M aqueous NaCl solution gradient in the same buffer at a flow rate of 0.5 mL/min.

The purified enzyme was applied to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which was performed according to the method of Laemmli [17]. After electrophoresis, the gel was stained with Coomassie brilliant blue G-250. The N-terminal amino-acid sequence was determined by APRO Life Science Institute, Tokushima, Japan.

The molecular mass of the enzyme was determined by analytical high-performance liquid chromatography (HPLC) with a TSK-Gel G3000sw column (Tosoh, 7.5 × 300 mm) at a flow rate of 0.5 mL/min with 50 mM Tris–HCl (pH 7.0) containing 0.1 M NaCl. The molecular mass of the native enzyme was determined by comparing the retention time of laccase with that of the MW-Marker (Oriental Yeast Co., Tokyo, Japan).

Protein concentration was estimated using the Bradford method, calibrated with bovine serum albumin as a standard (Bio-Rad Protein Assay Kit; Bio-Rad, Hercules, CA, USA).

2.3. Enzyme assay

The reaction mixture consisted of 50 mM acetate buffer (pH 4.5) containing 10 mM 2,6-dimethoxyphenol (DMP) or 10 mM of all other substrates, with the exception of syringaldazine (5 mM). Generally, laccase activity was assayed spectrophotometrically at 25 °C by measuring the increase in absorbance of 2,2′,6,6′-tetramethoxydibenzo-1, 1′-diquinone (dimeric DMP) at 468 nm (ϵ = 49.6 mM^{-1} cm^{-1}) from DMP, the result of which was used to calculate the activity. One unit of enzyme was defined as the amount that converted 1 μmol of substrate in 1 min under these conditions.

Similarly, the following absorption coefficients for the oxidized compound of the substrate were used to calculate activity [nm, ϵ (mM^{-1} cm^{-1})]: 2,2′-azino-bis-(3-ethylthiazoline-6-sulphonate) (ABTS) (420, 36), syringaldazine (525, 65), *o*-guaiacol (465, 12), *o*-catechol (412, 2.2). Activities towards L-DOPA (475 nm), tyrosine (475), pyrogallol (420) and gallic acid (420) were regarded as increases in absorbance at each wavelength for the oxidized compound (POU); 1 POU indicates a 1.0 increase in absorbance in 1 min at 25 °C.

2.4. Cloning and DNA sequencing of the lcc2 gene

H. coralloides NBRC 7716 genomic DNA was prepared from vegetative mycelia cultured in 50 mL liquid PD medium for 2 weeks. Mycelia were collected from the culture, washed twice with 10 mM Tris–EDTA (TE) buffer (pH 8.0), homogenized by pestle and mortar following the addition of liquid nitrogen, and extracted with