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Isolation of a novel alkaline-induced laccase from *Flammulina velutipes* and its application for hair coloring

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Laccase is a member of the multi-copper oxidase family and a promising for hair coloring. In this study, we isolated a novel alkaline-induced laccase from the white-rot fungus *Flammulina velutipes* and studied the possibility to apply the enzyme for hair coloring. Laccase activity detected in the culture supernatant of *F. velutipes* was found to significantly increase when exchanging the medium to laccase inducing one whose pH was adjusted to 9.0. Three isozymes were detected by activity staining on non-denaturing SDS-PAGE. The major isozyme, Flac1, was purified from the culture supernatant after being induced at pH 9.0 by ion-exchange column chromatography. The N-terminal peptide sequence of Flac1 was determined, revealing clear homology with laccases from other white-rot fungi. Optimum pH of oxidation was found to be around pH 5.0 −6.5 regardless of several different substrates used. Oxidation activities of Flac1 to several hair dye agents as substrate showed the higher activity at pH 6.5 than that at pH 9.0. Oxidation activity was also detected at pH 9.0 which was suitable for hair coloring. When the purified Flac1 was applied for hair coloring system without using hydrogen peroxide, effective coloring was observed at the protein amount of 0.25 mg/1g of hair used. These results indicated that this alkaline-induced novel laccase isolated from the culture supernatant of *F. velutipes* might be a useful enzyme for hair color.

[**Key words:** Laccase; Enzyme induction; *Flammulina velutipes*; Optimum pH; Dye; Hair coloring]

Laccase (E.C. 1.10.3.2) is a well-known enzyme which was discovered from the lacquer tree *Rhus vernicifera* by Yoshida et al. in 1883 (1). Presently, a large number of laccases have been isolated from various fungi, plants, bacteria, and insects. Laccase is a member of the multi-copper oxidase family of enzymes containing four copper atoms in their functional units. These copper atoms are classified into three types, type 1, type 2, and type 3, according to their spectroscopic and magnetic properties. Laccase catalyzes the oxidation of substrate at the type 1 copper site, and concomitant reduction of O_2 to water occurs at a trinuclear copper cluster formed by the type 2 and type 3 copper atoms. Their wide range of substrate specificity, the use of air oxygen as the second substrate, and no production of hydrogen peroxide suggest possible industrial applications. These applications include coloring and decolorization of textile, delignification of kraft pulp, oxidative degradation of organic pollutants, and the development of biosensors and biofuel cells. Although many laccases have been isolated, there are only a few reports on the investigation of laccase with high activity under neutral or alkaline conditions (2–4). It is well known that the optimum pH of laccases changes depending on the nature of both the enzymes and the substrates. The paucity of neutral and alkaline laccases, and the dependence of optimum pH on substrate narrows its application to limited industrial fields.

One possible application of laccase is in hair coloring. Oxidation hair color, which dominates the hair color market, consists of dye precursors and an oxidizing agent. In a typical hair color product, the former is *p*-diamines and *p*-aminophenols, and the latter is H_2O_2. They form chromatic indo dyes after mixing at the time of use (5). Dye couplers, which are generally phenols, are also included to produce various colors. The coloring reactions are usually carried out at alkaline pH because the hair swells and the penetration of dyes is enhanced. However, side reactions with the hair proteins occur simultaneously because of the severe reaction condition and cause hair damage. If H_2O_2 is replaced by another oxidizing agent, e.g., laccase, the hair damage should be reduced. Laccase-based hair colors have been proposed in the literature (Zeffren, E. and Sullivan, J. F., United States Patent 1, 320, 250, 1973; and Ref. 6) but have not been realized. The coloring reaction needs to be carried out at alkaline pH to obtain sufficient color fastness. Under acid and neutral pHs, the hair is apparently colored well but easily decolorized by a shampoo treatment. Since the hair color products are usually offered to the market in series with color variations, many combinations of the dye precursors and the dye couplers are required. Therefore, it is desirable

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for a laccase to have constant pH dependency of reactions for different substrates. The optimum pH of the known alkaline laccases (2–4) shifts according to the substrates, and there are a few laccases which can oxidize diamines and phenols at the same pH condition.

It is necessary to produce a sufficient amount of enzyme for an industrial application. It is well known that the secretion of laccase is enhanced by the addition of the following to the medium: metal ions such as Cu (7–11) and Cd (7), or substrates such as vanillin and ferulic acid (12,13). Contamination with heavy metals must be avoided for cosmetic use, and laccase substrates such as vanillin result in complexes which are difficult to remove in subsequent purification steps. A production method that is simpler and low cost is required.

In this study, we found a unique laccase from the culture supernatant of the white-rot fungus *Flammulina velutipes* named Flac1. Its optimum pH is only slightly acidic or neutral (5.0–6.5) and minimally dependent on substrate [i.e., 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), *p*-phenylenediamine (PPD), *o*-aminophenol (OAP), 2,6-dimethoxyphenol (DMP) and syringaldazine (SGZ)]. The ability to oxidize variable substrates at a constant pH is a unique property of Flac1, which was considered advantageous for hair coloring. We optimized the culture condition of *F. velutipes* and succeeded in obtaining a high amount production using a laccase inducing medium in which pH was adjusted to alkaline. The purified Flac1 was obtained and characterized for the first time. A hair coloring test with Flac1 was also conducted.

MATERIALS AND METHODS

Strains and chemicals *Flammulina velutipes* NBRC 30601 was purchased from the National Institute of Technology and Evaluation (Japan). Laccase from *Rhus vernicifera* and bilirubin oxidase (BOD) from *Myrothecium verrucaria* were kindly provided by Prof. Takeshi Sakurai (Kanazawa University). Diaminotoluene sulfate and 2-chloro-1,4-phenylenediamine were purchased from Pfaltz & Bauer (USA) and Sigma-Aldrich Japan, respectively. 1,3-Dihydroxynaphthalene, 2,6-dihydroxynaphthalene, and 5-hydroxyindole were purchased from Tokyo Chemical Industry Co., Ltd. (Japan). All other chemicals of the highest grade available were purchased from Wako Pure Chemical Industries, Ltd. (Japan).

Culture conditions *F. velutipes* was inoculated onto a potato dextrose and yeast extract medium (PDY) agar plate (2.4% potato dextrose broth (Difco), 0.1% Bacto™ yeast extract (Difco), 2% agar). After cultivation for 2 weeks at 25°C, the agar plate covered with hyphae was cut into lattices of 5 mm × 5 mm, and 15 pieces were inoculated into 50 mL of malt extract, casamino acid, and yeast extract (MCY) medium (2% Bacto™ malt extract (Difco), 0.5% Bacto™ yeast extract (Difco), 0.1% casamino acid (Daigo), pH 7.0) and cultivated with shaking for 2 weeks at 25°C. The entire volume of resulting culture medium was added to 500 mL MCY medium and cultivated with shaking for another 2 weeks at 25°C.

Enzyme activities Laccase activity was spectroscopically determined by monitoring oxidation of DMP in acetate buffer (pH 5.5) at 470 nm. Optimum pH values were also determined by monitoring the oxidations of ABTS, PPD, OAP, DMP and SGZ. ABTS, PPD, and DMP were dissolved in water. OAP was dissolved in dimethyl sulfoxide (DMSO) and then Milli-Q water was added until the DMSO concentration became 10%. SGZ was dissolved in ethanol. The final concentrations of the substrates were 0.005 M, except SGZ was 0.0005 M. The oxidations were monitored at 420, 470, 420, 470 and 530 nm for ABTS, PPD, OAP, DMP and SGZ, respectively.

Enzyme induction The culture supernatant was removed by decantation and 500 mL of fresh, enzyme inducing medium (1.0% glucose, 0.1% Bacto™ yeast extract, 0.14% $(NH_4)_2SO_4$, 0.36% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.1% mineral mixed solution (1.0% $CuSO_4$, 1.0% $ZnCl_2$, 0.7% $FeCl_3 \cdot 6H_2O$, 0.5% $CoSO_4 \cdot 7H_2O$, 0.5% $MnCl_2 \cdot 4H_2O$), pH 9.2) was added to the pellet form hyphae. The cultivation was continued with shaking at 25°C for 2–3 days, and the culture supernatant was collected by decantation. This process was repeated several times.

Enzyme purification The culture supernatant was filtered with cellulose acetate membrane (0.45 μm). The pH of the filtrate was adjusted to 7.5 using sodium hydroxide aqueous solution. Five grams of DEAE-cellulose (Sigma-Aldrich) was added to 1 L of the filtrate, and stirred with shaking at 4°C for 30 min. The supernatant was removed with decantation. The adsorbed proteins were eluted with 0.1 M sodium phosphate buffer (pH 6.5) containing 1 M sodium chloride, and dialyzed against Milli-Q water at 4°C. The extract of the culture was applied onto a Q-Sepharose® column (2.6 × 10 cm, Pharmacia, FPLC system) equilibrated with 0.05 M of Tris–HCl buffer (pH 8.0), and laccase was eluted with gradient concentration of sodium chloride (0–1.0 M).

SDS-PAGE SDS-PAGE was performed according to Laemmli's method (14) with a minor modification. The sample was separated by electrophoresis without adding denaturing agent or boiling. Activity staining was performed according to the method reported by Hoopes (15).

Amino acid sequence determination The N-terminal amino acid sequence of purified Flac1 was determined using a protein sequencer (Applied Biosystems, Procise 492 HT). Proteins separated by SDS-PAGE were electroblotted on a polyvinylidene difluoride membrane, and the excised bands were directly analyzed.

Oxidation activity for diamines, aminophenols, phenols, naphthols and indoles *p*-Aminophenol (PAP), trimethylhydroquinone, naphthols and indoles were dissolved in a 10% DMSO solution, whereas other compounds were dissolved in a 1% DMSO solution. Oxidation was spectroscopically monitored in sodium phosphate buffer (pH 6.5) and Tris–HCl buffer (pH 9.0). The final concentration of the substrates and the buffer were 0.001 M and 0.1 M, respectively. A unit of enzyme activity was defined as the amount of enzyme which produced an increase in one absorbance unit per minute.

Chromogenic reactions PPD, *m*-phenylenediamine (MPD), OAP, DMP, PAP were dissolved in 10% DMSO solution. Britton and Robinson buffer (pH 6.5 and 9.0), substrates, and enzyme (2 μL) were mixed in the 96 well plate. The total volume of the reaction mixture was 100 μL, and the final concentration of the substrate, buffer, and DMSO was 0.005 M, 0.1 M, and 1%, respectively.

pH stability Britton and Robinson buffer (0.18 mL) and Flac1 solution (0.02 mL) were premixed and incubated at 30°C. After 1 h incubation, an aliquot of 0.02 mL of the premixed solution, phosphate buffer (pH 6.5), and PPD solution were mixed well and the absorbance at 470 nm was spectroscopically monitored. The final volume of the reaction mixture was 1.0 mL and the final concentration of the buffer and substrate were 0.2 M and 0.005 M, respectively.

Thermostability Flac1 solution (0.1 mL) and 0.2 M Britton and Robinson buffer which pH was adjusted to 9.0 (0.1 mL) were premixed and incubated at 30°C, 40°C, 50°C, 60°C, 70°C, and 80°C. After the elapse of a certain period of time, an aliquot of 0.02 mL of the premixed solution, phosphate buffer (pH 6.5), and PPD solution were mixed well and the absorbance at 470 nm was spectroscopically monitored. The final volume of the reaction mixture was 1.0 mL and the final concentration of the buffer and substrate were 0.2 M and 0.005 M, respectively.

Hair coloring test Polyoxyethylene (20) hydrogenated caster oil 2.0%, lactic acid 1.0%, PPD 1.0%, and hydroxyethyl cellulose 1.5% were dissolved in Milli-Q water, and the pH was adjusted to 9.0 with monoethanolamine. This base material (2 g) and Flac1 solution were mixed and applied to a bundle of human white hair (1 g, 10 cm, Beaulax, Japan) which was then placed at 30°C and 70% relative humidity for 30 min. Then the hair bundle was washed with a 1.0% solution of sodium lauryl sulfate and rinsed with water until the water became clear. After overnight drying, *L*, *a*, and *b* values of the hair bundle was determined with a colorimeter (Chromometer 3610d, Minolta, Japan). The color difference (ΔE^*ab value) from untreated hair bundle, negative control, was calculated with the following equation:

$$\Delta E * ab = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2} \quad (1)$$

RESULTS AND DISCUSSION

F. velutipes is a very popular mushroom indigenous to Japan. The gene sequences of laccase from *F. velutipes* have been reported (Genebank No. AY485826, AY450406, AB252575), and activity has been detected in culture supernatant (16,17). However, enzymatic characterization of purified laccase has not been reported since its activity is low in comparison with laccases from other white-rot fungi (18). Thus, we considered the induction of laccase. Laccase is well known to be induced by the addition of metals and substrates. Such a method has post-process complications such as an effluent problem because of environmental contamination, and substrate polymerization and difficulty of their removal. In this report we isolated and characterized a new laccase from *F. velutipes* and established optimum cultivation condition to increase the enzyme production significantly without using metals or substrates. In the MCY medium which pH was adjusted to 5.0, 6.0, 7.0, 8.0 and 9.0, laccase activity increased with increasing pH (data not shown). However, mycelium growth and pellet formation were inhibited at pH higher than 8.0. Attempts were made to induce laccase after the fungus was allowed to grow and form pellets so that a large amount of enzyme can be expressed under conditions which facilitate purification. High laccase activity was successfully obtained by exchanging the medium to a laccase inducing one (pH 9.0) after sufficient cultivation in an MCY medium (pH 7.0) until fungi grew and formed mycelial pellets (Fig. 1). In MCY medium at pH 7.0, the activity did not increase even when cultivation was extended several days. In the laccase inducing medium, the activity increased 36-fold compared to the activity observed in the MCY medium. This maximum activity could be maintained for 3–5 days