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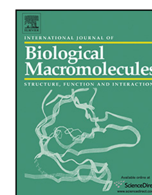

Enhancement of catalysis and functional expression of a bacterial laccase by single amino acid replacement

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ABSTRACT

Structure–function relationships underlying laccases properties are very limited that makes these enzymes interesting for protein engineering approaches. Therefore in the current study, a thermostable laccase that was isolated from *Bacillus* sp. HR03 with the ability of bilirubin oxidation besides its laccase and tyrosinase activity is used. The extensive application of this enzyme is limited by its low expression level in *Escherichia coli*. Based on sequence alignments and structural studies, three single amino acid substitutions, D500G, D500E, D500S and a glycine insertion, are introduced using site-directed mutagenesis to evaluate the role of Asp^{500} located in the C-terminal segment close to the T1 copper center. Substitution of aspartic acid with less sterically hindered, conserved residue such as glycine increase k_{cat} (2.3 fold) and total activity (7.3 fold) which is accompanied by a significant increase in the expression level up to 3 fold. Biochemical characterization and structural studies using far-UV CD and fluorescence spectroscopy reveal the importance of C-terminal copper-binding loop in the laccase functional expression and catalytic efficiency. Kinetic characterization of the purified mutants toward 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), syringaldazine (SGZ) and bilirubin, shows that substrate specificity is left unchanged.

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1. Introduction

Engineering of metalloproteins by means of site-directed mutagenesis presents interesting new opportunities for the study of structure-function relationships in proteins. Understanding these relations has great importance in the development of new nanobiotechnological devices [1]. Information on redox proteins has emerged from the study of a class of proteins known as laccases-type [2,3].

Laccases (benzenediol-oxygen oxidoreductase; EC 1.10.3.2) belong to the family of blue multicopper oxidases, a large group of enzymes characterized by the presence of at least four copper ions distributed in cupredoxins domains. The catalytic centers consist of three structurally and functionally distinct copper clusters that are defined according to their spectroscopic properties: type-1 (T1) or blue copper, type-2 (T2), and two type-3 (T3) copper ions [4]. These enzymes follow a bi-bi ping-pong mechanism, the substrate is oxidized by the T1 copper atom, the electron is conducted through the core of the protein to the T2/T3 site, and four electrons are used to reduce dioxygen to water. Although laccases exhibit broad specificity, their typical substrates are electron-rich phenols or aryl amines. The oxidation efficiency of the enzyme depends on the redox potential difference between the substrate and T1 Cu (II), known as the electrochemical driving force of the electron transfer [5]. Laccases are widespread in nature and have been found in many plants and fungi as well as in some bacteria [6]. These enzymes have been implicated in many diverse physiological functions such as morphogenesis, pathogenesis, lignin synthesis, and lignin degradation [7]. Their occurrence, characterization, functions and applications have been extensively reviewed [8–10].

We have recently published a report on the cloning, expression and characterization of a thermostable laccase from a local *Bacillus* sp. HR03 (accession no. FJ663050) [2], which exhibited bilirubin oxidation ability along with laccase and tyrosinase activity. The isolated enzyme was expressed as a precipitate in the form of inclusion bodies. Previous attempts to recover the soluble laccases were reported unsuccessful [11]. Therefore, in the present study, we decided to use structure-guided mutagenesis to enhance the functional expression and catalytic efficiency of this industrially

Abbreviations: ABTS, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); SGZ, syringaldazine; T1 copper, type-1 copper; CD, circular dichroism; IPTG, isopropyl-β-D-1-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride.

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important enzyme in the most widely used host (*Escherichia coli*). Three single amino acid replacements (D500G, D500E, D500S) and a glycine insertion have been made at D500 located in C-terminal T1 copper binding loop. Biochemical characterization and structural studies using far-UV CD, fluorescence spectroscopy and bioinformatics were also carried out.

2. Materials and methods

2.1. Materials

All chemical reagents were of analytical grade or higher. The growth media and reagents were purchased from Liofilchem (Roseto degli Abruzzi, Italy). The plasmid extraction kit was obtained from R-real Biotech Corporation (Banqiao, Taiwan). The oligonucleotide sequences were synthesized by Bioneer (Deajeon, South Korea). The LONG high fidelity PCR system (DNA polymerase), Dpn1, isopropyl-β-D-1-thiogalactopyranoside (IPTG), and PCR reagents were purchased from Fermentas Life Sciences (Vilnius, Lithuania). The Q-Sepharose FF was obtained from Amersham Biosciences (Piscataway, USA). ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)), SGZ (SGZ; 4-hydroxy-3, 5-dimethoxybenzaldehyde azine) and bilirubin were purchased from Sigma (St. Louis, MO). All the other chemicals were obtained from Merck (Darmstadt, Germany).

2.2. Bacterial strains, plasmid, and growth conditions

Laccase gene was cloned into pET21a (Novagen, USA), an expression plasmid. *E. coli* DH5α and BL-21 (DE3; Stratagene) were used as hosts for the cloning and protein expression, respectively. All strains were grown in Luria–Bertani (LB) medium at 37 °C.

2.3. Bioinformatics analysis

The modeling was carried out by submitting the deduced protein sequence of laccase to the Swiss Model server (http://swissmodel.expasy.org//swissmodel.html). Multiple sequence alignment was performed with the Clustal W version 1.82. Energy minimization obtained by HyperChem version7.0 molecular modeling programs. ASA View (http://gibk26.bio.kyutech.ac.jp/jouhou/shandar/netasa/asaview/) server was used to determine the molecular surface accessibility of the protein. Hydrogen bonds and salt bridges of the wild type and its variants were investigated using PIC (http://pic.mbu.iisc.ernet.in/) and WHAT IF (http://swifi.cmbi.ru.nl) servers. The molecular representation of the enzyme structures was determined by PyMOL viewer. Kinetic constants were calculated using Prism software version 5.04 (available at www.graphpad.com).

2.4. Site-directed mutagenesis

Three single amino acid substitutions and an insertion were created using QuikChange method described by Fisher and Pei [12]. Plasmid pET21a (+) that contained the wild-type laccase gene was used as a template and the following four sets of synthetic primers were designed:

D500G, F 5′-GAGCATGAAGACTAT**GGT**ATGATGAGACCGATG-3′
D500G, R 5′-CATCGGTCTCATCATA**CCA**TAGTCTTCATGCTC-3′
D500S, F 5′-GAGCATGAAGACTAT**TCT**ATGATGAGACCGATG-3′
D500S, R 5′-CATCGGTCTCATCAT**AGA**ATAGTCTTCATGCTC-3′
D500E, F 5′-GAGCATGAAGACTAT**GAG**ATGATGAGACCGATG-3′
D500E, R 5′-CATCGGTCTCATCAT**CTC**ATAGTCTTCATGCTC-3′
Gly insertion, F 5′-GAGCATGAAGACTAT**GGT**GACATGATGAGACCG-3′
Gly insertion, R 5′-CGGTCTCATCATGTC**ACC**ATAGTCTTCATGCTC-3′

The PCR products were treated with DpnI at 37 °C for 16 h. The DpnI-digested DNA was transformed into *E. coli* DH5a via the calcium chloride method [13]. All mutants were screened and confirmed directly by DNA sequencing. Plasmids with desired mutations were then transformed in to *E. coli* BL21 (DE3).

2.5. Expression and purification

E. coli BL21 (DE3) cells carrying the wild type laccase and its variants were grown in 10 mL Luria–Bertani (LB) medium supplemented with ampicillin (100 μg mL^{-1}) at 37 °C with shaking (180 rpm). Subsequently, 2 liter shaking flask containing 500 mL Terrific Broth (TB) supplemented with ampicillin were inoculated with 5 mL of the prepared culture and incubated at 37 °C, shaking at180 rpm. Expression was induced at an optical density (OD_{600}) of 1.6 by adding 0.1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and 2.0 mM CuSO4. The cells were incubated overnight at 18 °C in a shaking incubator (140 rpm). Cells were harvested by centrifugation (15 min, 6000 × *g*, 4 °C) and the pellets were resuspended in 50 mM potassium phosphate buffer, pH 7.6 containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5 mM CuSO4. The cells were disrupted by sonicating on ice and the cell debris was removed by centrifugation (18 min, 14,000 × g, 4 °C). The supernatant was heated at 70 °C for 15 min and the denatured proteins were removed by centrifugation (10 min, 10,000 × *g*, 4 °C). The supernatant was loaded on a Q-Sepharose FF (Amersham Biosciences) column, which had been equilibrated with 20 mM potassium phosphate buffer, pH 7.6. Elution was performed with a linear NaCl gradient (0–1 M) in the potassium phosphate buffer pH 7.6. Fractions containing laccase activity were collected (activity was measured using ABTS as a substrate), concentrated, and desalted by Amicon ultrafiltration (membrane cut-off 10 kDa, Millipore, USA). Protein concentration was determined using a Bradford assay [14], and bovine serum albumin was used as standard. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 12% polyacrylamide running gel [15].

2.6. Enzyme activity

Laccase activity was measured spectrophotometrically at room temperature. Oxidation of 2 mM ABTS and 0.05 mM SGZ were followed in 100 mM citrate/phosphate and potassium phosphate buffer pH 4.0 and 7.0, respectively. The increase in absorbance was measured at 420 nm (ε = 36,000 M^{-1} cm^{-1}) for ABTS, and 525 nm (ε = 65,000 M^{-1} cm^{-1}) for SGZ [16,17]. Oxidation of 400 μM bilirubin (dissolved in 2 N NaOH) in 200 mM Tris buffer (pH 8.4) was monitored through the decrease in absorbance at 440 nm (ε = 56,300 M^{-1} cm^{-1}) [18,19]. One unit is defined as the amount of enzyme that oxidizes 1 μmol of substrate per min. Kinetic parameters (K_m, V_{max}, k_{cat}) were determined using the Michaelis–Menten equation. The assay was performed at least in triplicate.

2.7. Circular dichroism (CD) studies

The far-UV CD spectrum of purified laccase was determined in 100 mM potassium phosphate buffer, pH 7.0 using Jasco J-715 spectropolarimeter (Jasco Corporation, Japan). The enzyme was extensively dialyzed against same buffer at a protein concentration of 0.2 mg/mL. Results are presented as molar ellipticity $[\theta]$ (deg cm^2 $dmol^{-1}$), based on a mean amino acid residue weight (MWR) assuming average weights of 113. The molar ellipticity $[\theta]$ was calculated from the formula $[\theta]_\lambda = (\theta \times 100\,\mathrm{MWR})/(cl)$, where c

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