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Research paper

Functional expression enhancement of *Bacillus pumilus* CotA-laccase mutant WLF through site-directed mutagenesis

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ABSTRACT

Bacterial laccases are potential enzymes for biotechnological applications, such as detoxification of industrial effluents, decolorization of textile, and dimerization of phenolic acids, due to their remarkable advantages, including broad substrate spectrum, high thermostability, wide pH scope, and tolerance to alkaline environments. L386W/G417L/G57F (abbreviated as WLF), a good mutant of CotA-laccase from *Bacillus pumilus* W3, has been constructed and reported by our laboratory with highly improved catalytic efficiency. However, the low-functional expression level of mutant WLF in *Escherichia coli* was a shortcoming. Three mutants, namely, K317N/WLF, D501G/WLF, and K317N/D501G/WLF, were constructed through site-directed mutagenesis to improve the functional expression of WLF in this study. The soluble and active expression of D501G/WLF and K317N/D501G/WLF in *E. coli* enhanced 4.48-fold and 3.63-fold level, respectively. The K317N/WLF failed to increase the soluble expression level, but slightly improved the stability of CotA-laccase. Results showed that not only the position 501 is significant for functional expression of *B. pumilus* W3 CotA, but also these mutants still remained its high thermostability, resistance of alkaline with salt, and conspicuous decolorizing efficiency. This work is the first to improve the soluble expression of *B. pumilus* CotA-laccase in E. coli by site-directed mutagenesis. The D501G/WLF and K317N/D501G/WLF will be suitable candidates for biotechnological applications.

1. Introduction

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) belong to the family of blue multicopper enzymes that oxidize a range of substrates, such as phenols, anilines, arylamines, ascorbic acid, and some inorganic compounds [1,2]. The broad substrate range enables laccases to become candidates for many industrial and biotechnological applications [3], such as decolorization of synthetic dyes and bioremediation. The molecular structure of laccases contains three types of copper ions according to their magnetic and spectroscopic properties, namely, type1 (T1), type2 (T2), and double type3 (T3) copper ions [4]. The center of T1 copper is the site of substrate oxidation and in charge of electron transfer. The T2 site is a mononuclear center formed by type 2 copper, and the T3 site is composed of two strongly coupled type 3 copper ions. The trinuclear T2/T3 cluster is made up of one T2 copper ion and two T3 copper ions, which bind and reduce molecular oxygen to water [5,6].

Laccases are widespread in fungi, higher plants [3], insects [7,8], and bacteria [9,10], but only fungal laccases are currently used in industrial processes [3]. Fungal laccases are usually unstable at high temperature and alkaline condition, thereby limiting their practical applications to waste – water treatment [11]. The pH value of most waste – waters from textile industries is neutral to alkaline (range from 7 to 11). Bacterial laccases have high potential in commercial application because of their high stability under drastic conditions [12]. The first isolation of prokaryotic laccase was from *Azospirillum* sp. in 1993. Then, laccases were gradually discovered from *Pseudomonas* sp., *Escherichia coli*, *Streptomyces* sp., *Bacillus subtilis*, *B. licheniformis*, and *B. pumilus* [11,13]. CotA-laccases are the most popular among these bacterial laccases. CotA is a highly thermostable laccase; moreover, the CotA-laccase from *B. subtilis* is mostly studied in structure and functional characterization [14,15]. Water-soluble enzyme can be produced through heterologous expression with high thermostability and alkaline activity. *E. coli* is the most common host for protein expression because of its easy genetic manipulation, rapid growth rate, and short post-translational modification, such as glycosylation [12]. However, these laccases are also mainly expressed as the form of inclusion bodies in *E. coli*. Previous attempts to recover soluble laccases were unsuccessful [16]. In recent years, site-directed mutagenesis technology was used to improve functional expression and achieved success in *B. licheniformis* [5] and *Bacillus* sp. HR03 [17]. Enhancing the functional expression of laccases will contribute to its industrial applications.

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A report on L386W/G417L/G57F (abbreviated as WLF), which is an excellently constructed mutant from wild-type (WT) CotA-laccase of *B. pumilus* W3, has been recently published to improve the catalytic efficiency. WLF is appropriate for biotechnological applications because of its high activity and thermostability in decolorizing industrial dyes [6]. However, the low functional expression of WLF in *E. coli* showed a limitation; thus, the site-directed mutagenesis method was performed to enhance its soluble expression in this study.

2. Materials and methods

2.1. Materials

2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid; ABTS), ampicillin, acid red 1, and acid blue 129 were all purchased from Sigma − Aldrich Co. (St. Louis, MO, USA). Methyl blue, methyl green, and malachite green were purchased from Sinopharm (Shanghai, China). Acetosyringone was purchased from Macklin (Shanghai, China). The bacterial DNA extraction kit, fast multi-site mutagenesis system, and isopropyl-β-D-thiogalactoside (IPTG) were purchased from TransGen (Peking, China). All other media and chemicals were standard reagent grade.

2.2. Bacterial strains, plasmids, and culture conditions

B. pumilus W3, the laccase-producing bacterial strain, was isolated by our laboratory [18] and deposited at the China Center for Type Culture Collection (CCTCC No. M2015018). Mutant WLF was constructed by our laboratory [6]. pColdII (Takara, China), a cold shock expression plasmid vector, was used to clone the CotA-laccase genes. Meanwhile, *E. coli* DH5α cells (Takara, China) were used for subcloning procedures. *E. coli* BL21(DE3) cells (Takara, China) were used as hosts for protein expression. All strains were grown in Luria-Bertani (LB) medium at 37 °C.

2.3. Bioinformatics analysis

All protein sequences were searched from the NCBI server (https://www.ncbi.nlm.nih.gov). Multiple sequence alignment was presented by Clustalx version 1.81 and DNAMAN. The homology modeling was performed by submitting the deduced protein sequence of laccase to the Swiss Model server (http://swissmodel.expasy.org/interactive). Among the known 3D CotA models, the *B. pumilus* W3 CotA-laccase shares maximum protein sequence identity (69.17%) with the *B. subtilis* CotA-laccase (PDB code:1GSK). Swiss-pdb viewer 4.0.1 server, a 3D protein structure-viewing software, was used to calculate the hydrogen bonds and the force field energy of the protein.

2.4. Site-directed mutagenesis

The construction of two single amino acid substitutions (K317N/WLF and D501G/WLF) and one double amino acid substitution (K317N/D501G/WLF) were attributed to the QuickChange method described by Fisher and Pei [19]. Plasmid pColdII, which contains the WLF mutant gene, was served as template, and two sets of synthetic primers were designed as follows:

K317N, F 5′-AACGATTGTTTTAAACAATAAGGCAGGC-3′
K317N, R 5′-GTTTAAAACAATCGTTTGGTTTTCGTAA-3′
D501G, F 5′-AACACGAGGATTATGGCATGATGCGGCC-3′
D501G, R 5′- CCATAATCCTCGTGTTCTAATATGTGAC-3′

The D501G/WLF was used as template for the double-mutant (K317N/D501G/WLF). These genes were amplified using the PCR program recommended by Fast Mutagenesis System of Transgen Company (www.transgen.com.cn). The program was adjusted as: 4 min at 94 °C, 25 cycles of 20 s at 94 °C, 20 s at 55 °C and 3 min at 72 °C, and final extension at 72 °C for 10 min to clone purpose genes. To select positive transformants easily, PCR products were digested at 37 °C for 1 h by DMT enzyme, which can digest methylated template. Then, the digested products were transformed into *E. coli* DH5α. All mutants were screened and confirmed further by DNA sequencing. Plasmids with a mutant gene were then transformed into *E. coli* BL21(DE3) for protein expression.

2.5. Expression and purification of laccases

E. coli BL21(DE3) cells carrying the wild-type laccase and other variants genes were grown in 3 ml Luria-Bertani (LB) medium supplemented with ampicillin (100 μg ml^{-1}) at 37 °C and 200 rpm for 10 h. Then, overnight pre-culture (1 ml) was inoculated into fresh 50 ml LB culture medium containing ampicillin (100 μg ml^{-1}) and incubated at 37 °C in a shaking incubator (200 rpm). When the optical density at 600 nm (OD_{600}) reached 0.5, the shake flasks were incubated at 15 °C for 30 min in a stationary state. Later, the IPTG (0.4 mM) and $CuSO_4$ (0.25 mM) were added to shake the flasks. After further incubation at 15 °C with shaking (200 rpm) for 24 h, the cells were harvested by centrifugation (8000 rpm, 10 min, 4 °C) and the pellets were re-suspended in 20 mM phosphate buffer (pH 7.0) containing 0.5 mM $CuSO_4$. Then, the cells were disrupted by sonification on ice for 15 min twice and the cell debris were removed by centrifugation (8000 rpm, 20 min 4 °C). Subsequently, the supernatant was incubated at 70 °C for 15 min and the denatured proteins were removed by centrifugation (10000 rpm, 10 min, 4 °C) [5,17]. Each recombinant pColdII plasmid including CotA mutant gene encodes a fusion-protein containing His_6-tag of NH_2-terminal to simplify the purification process. These fusion proteins were purified by immobilized metal-affinity chromatography. A HiTrap™ HP 1 ml column and AKTA purifier (AKTA avant 25), which was purchased from GE Healthcare (Uppsala, Sweden), were used to load the supernatant and purify the target protein. The column was equilibrated earlier with 20 mM phosphate buffer (pH 7.0), and then the supernatant was loaded and washed by a linear imidazole gradient (0–500 mM) with phosphate buffer (pH 7.0) under normal conditions. Fractions with laccase activity were measured using ABTS as substrate. The same fractions having laccase activity were pooled, and then desalted and concentrated by HiTrap™ Desalting column (5 ml) and ultrafiltration centrifuge tube. Protein concentration was assayed by an Easy Protein Quantitative Kit (Bradford). Bovine serum albumin was used as standard [20]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), which uses 12% polyacrylamide running gel, was performed to analyze recombinant laccase expression [21]. Non-denaturing PAGE (omitting the SDS, β-mercaptoethanol, and heat treatment) was used to confirm the single-target protein band. The bacterial proteins from *E. coli* BL21(DE3) strain with empty pColdII plasmid were used as controls.

2.6. Laccase activity assay

Laccase activity was measured spectrophotometrically at 50 °C using ABTS as substrate. The reaction mixture (3 ml total volume) contained 0.5 mM ABTS, 100 mM sodium citrate/phosphate buffer (pH 3.6), and 0.1 ml diluted purified laccase. The oxidation of ABTS resulted in an absorbance increase at 420 nm (ε_{420} = 36,000 M^{-1} cm^{-1}) [5]. One unit is defined as the amount of enzyme that oxidizes 1 μmol of substrate per min. Kinetic parameters K_m and k_{cat} were determined at 50 °C and pH 3.6 using the ABTS assay with concentration of 10 − 1000 μM ABTS. The data were fitted to the Michaelis–Menten equation by non-linear regression (Origin8.5 software). The assay was performed in triplicate.

2.7. Characterization of purified laccases

2.7.1. Optimum pH and pH stability

The effect of pH on the purified laccases was determined through a