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Research Article

Molecular characterization of a novel thermostable laccase PPLCC2 from the brown rot fungus *Postia placenta* MAD-698-R

Hongde An [a], Tingting Xiao [a], Huan Fan [b], Dongsheng Wei [a,*]

[a] Department of Microbiology, College of Life Science, Nankai University, Tianjin, PR China
[b] Tianjin Institute of Animal Husbandry and Veterinary Research, Tianjin, PR China

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ABSTRACT

Background: Laccase has been considered important for the degradation of lignocellulose by wood rot fungi. The properties and functions of laccase in white rot fungi have been investigated extensively, but those from brown rot fungi remain largely unknown. In this paper, a laccase isoform *Pplcc2* from the brown rot fungus *Postia placenta* MAD-698-R was expressed heterologously in *Pichia pastoris GS115*, purified and the properties of the enzyme were determined.
Results: The molecular weight of the protein was determined to be 67 kDa using SDS-PAGE. It cannot oxidize syringaldazine (SGZ), but it can oxidize 2,2′-azino-di-(3-ethylbenzothialozin-6-Sulfonic acid) (ABTS) and 2,6-dimethoxyphenol (DMP). Specific activity for ABTS was 1960 ± 19 Unit/mg. The catalytic constant (k_{cat}) was 1213 ± 18.3 s^{-1} for ABTS and 293.2 ± 21.9 s^{-1} for DMP. K_m was 22.08 μM for ABTS and 11.62 μM for DMP. The optimal pH for the oxidation of ABTS and DMP was 3.5 and 5.0 respectively. The optimal temperature for the oxidation of ABTS and DMP was 60°C.
Conclusions: This is the first identified thermo activated and thermostable laccase in brown rot fungi. This investigation will contribute to understanding the roles played by laccases in brown rot fungi.

1. Introduction

White rot fungi are considered to be one of the major terrestrial carbon recyclers on earth, especially in forest ecosystem [1,2]. High oxidation potential class II peroxidases (PODs), including lignin, manganese and versatile peroxidases, have been generally accepted as the enzymes responsible for the mineralization of lignin in white rot fungi in order to gain access to cellulose [3,4]. Accordingly, three kinds of PODs are distributed very commonly in white rot fungi [5]. Another terrestrial carbon recycler, brown rot fungi, can also utilize hemicellulose and cellulose, but they are not considered to be able to mineralize lignin [6]. Accordingly, activities and sequences of PODs have never been detected in brown rot fungi [5]. The mechanism with which brown rot fungi use to access hemicellulose and cellulose is thought to be the cleavage of lignin by hydroxyl radicals produced by non-enzymatic extracellular Fenton reaction [7,8].

Laccases (EC1.10.3.2, *p*-diphenol:oxygen oxidoreductase) belong to a superfamily of multicopper oxidases (MCOs) [9], and they have been proved to be able to directly oxidize phenolic rings to phenoxy radicals alone or cleave non-phenolic units similar to those in lignin with the aid of low molecular weight mediators *in vitro* [10]. Therefore, laccase is used to be considered as the fourth contributor for the degradation of lignin [1] in wood rot fungi. However, this opinion has been debated for many years since the model white rot fungus, *Phanerochaete chrysosporium*, was found to lack typical laccase sequence [5,11]. The absence of laccase in *P. chrysosporium* indicates that it may not be essential for the mineralization of lignin in wood rot fungi, but the roles played by laccase in the cleavage of lignin components in wood rot fungi may be underestimated. Especially, the roles played by laccase in degradation of lignin in brown rot fungi need further investigations. The detection of laccase activity by the old Bavendamm reaction always gave negative results for brown rot fungi [12], but most white rot fungi gave positive results [13]. Therefore, brown rot fungi were not considered to have functional laccase in the old opinions. Due to the absence of class II peroxidase sequences and laccase activities, brown rot fungi have not been considered to be able to degrade lignin for a long time [13,14].

However, the situations about laccase activities and sequences in brown rot fungi have been changed in the past few years. Laccase activities have been detected from several brown rot fungi with typical substrates such as ABTS, DMP and SGZ in the culture fluid in the past few years [15]. The recent whole genome sequencing projects not only confirmed the absence of PODs, but also revealed the presence of *stricto sensu* laccase sequences in nearly all selected brown

* Corresponding author.
E-mail address: weidongsheng@nankai.edu.cn (D. Wei).
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rot fungi [5,16,17]. However, the properties and functions of laccase in these brown rot fungi are poorly understood. When this manuscript was being prepared, only one laccase (PPLCC1) from *Postia placenta* has been characterized and proved to encode laccase capable of oxidizing typical laccase substrates (*Pplcc1*, Protein ID 111314) [18]. After that, the most recent work by Park et al. further extended the knowledge about the properties of laccase from another brown rot fungus *Fomitopsis pinicola* [19]. All these results indicate that laccases exist in the brown rot fungi, but the roles of laccases in brown rot fungi especially in the process of degradation of lignocelluloses have only been investigated in our previous investigation [18].

At the same time, the old opinion about the inability of brown rot fungi to degrade lignin is also challenged by NMR analysis of brown-rot aspen and spruce wafers. Many of the inter-β-monomer linkages of lignin actually disappear during brown rot by *Gloeophyllum trabeum* [20] and the content of major arylglycerol-β-aryl ether inter-unit linkage in the lignin is reduced by more than half during the brown rot by *P. placenta* [21]. Therefore, at least the two model brown rot fungi have been proved to be able to actually cause significant lignolysis without the involvement of PODs. As laccases from white rot fungi have been proved to be capable of oxidizing lignin components alone or with the help of mediators, it is reasonable to infer that once laccase-like sequences from brown rot fungi are proved to encode enzymes with similar catalytic properties to those coming from white rot fungi, laccase might also play certain roles in the degradation of lignin. Undoubtedly, laccases from brown rot fungi might also play important roles in other processes such as the pigmentation and detoxification [22].

Unfortunately, the properties of laccase from brown rot fungi have only been investigated in very few species [18,19]. More information about the catalytic properties is needed to get a relatively integral picture about the laccase from brown rot fungi. Toward this, the second laccase-like sequence *Pplcc2* (PID62097) in *P. placenta* was expressed in *Pichia pastoris* GS115 and characterized. Another motivation for us to characterize this enzyme is that the expressed sequence tag (EST) of this gene was detected in the genome data [16], but the peptides were not detected during the degradation of aspen wafers for this laccase-like sequence [18]. Therefore, it is necessary to determine whether it encodes true laccase and what properties it might have. These results will provide new knowledge to laccase from different sources and help us to better understand the roles played by laccase in lignin cleavage or other processes in fungi.

2. Materials and methods

2.1. Enzymes and chemicals

All chemicals were of analytical grade. Recombinant restriction enzymes were obtained from New England Biolabs (Ipswich, MA, USA). All nucleic acid manipulation kits were purchased from Tiangen Biotech (Beijing, China). Pichia expression kit was obtained from Invitrogen (Carlsbad, CA, USA). All substrates used in the determination of laccase activity were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA).

2.2. Brown rot fungus and culture conditions

P. placenta strain MAD-698-R was kindly provided by Hammel KE (USDA Forest Product Lab, Madison, WI, USA). It was maintained on malt and yeast extract plates (MY) (15 g malt extract, 2 g yeast extract, and 20 g agar per liter) at 28°C. For degradation of wood substrates, two aspen wafers (dimension: thickness × width × length = 2 mm × 10 mm × 20 mm) were put on top of the confluent *Postia* cultures on MY plates. The plate and wafers were separated by two layers of nylon nets to avoid the contamination of wafers by the media. After the mycelia fully colonized the wafers, they were harvested and used for total RNA extraction.

2.3. RNA extraction, cDNA synthesis and gene cloning

RNA extraction was performed according to the instructions of RNA prep pure Plant kit (Tiangen, China) with slight modifications. In order to extract fungal RNA from aspen wafers, 100 mg wafers were dipped into a 2-mL screwed tube with 1.5 mL HL lysis buffer (provided by the kit) and about 200 mg acid washed glass beads (0.5 mm, Biospec Product, USA). Wafers were disrupted using Mini-bead beater (Biospec Product, USA) for four cycles of beating. Each cycle includes beating at maximum speed for 30 s and cooling down on ice for 3 min. All disrupted materials were pooled and filtered through spin filter provided by the kit. The following steps were done according to the instructions of the kit. Genomic DNA was removed by treatment of total RNA with RNase-free DNaseI (Takara, China) at 37°C for 30 min. The integrity of RNA was analyzed by electrophoresis, and quantification was performed with NanoDrop 2000 UV–vis Spectrophotometer (Thermo Scientific, USA).

First-strand cDNA was synthesized by using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Takara, China) according to the manufacturer's instructions. The resultant cDNA mixture was diluted 1:10 with RNase-free distilled deionized water, and 1 μL was used for reverse transcription PCR. To clone the full length open reading frame of *Pplcc2*, one primer pair (LacF/LacR) was designed according to the genome sequence of *P. placenta*. The forward primer (LacF) was designed to exclude the putative original signal leader sequence (20 amino acids), and the reverse primer (LacR) was designed to include 6 histidines at N-terminal to facilitate the purification steps (Table 1). PCR reaction was performed with the following parameters: 94°C for 10 min, 30 cycles of 94°C for 30 s, 58°C for 30 s, 72°C 2 min, and a final extension at 72°C for 10 min.

2.4. Pichia transformation, heterologous expression and purification

PCR product was digested with *EcoR*I and *Not*I and inserted into pPIC9K vector. The secretion of protein was guided by the leader sequence of yeast α factor. The resultant reaction mixture was transformed into *Escherichia coli* DH5α competent cells. Positive clones were screened by colony-PCR with primer pair 5AOX/3AOX (Table 1), and the correctness of the sequence was confirmed by DNA sequencing. Transformation of *P. pastoris* was carried out by electroporation with Gene Pulser Xcell electroporation system (Bio-Rad, USA) according to the manufacturer's instructions. *Pichia* transformants appeared after growing on minimal dextrose (MD) plates (1.34% yeast nitrogen base, 4×10^{-5}% biotin, 2% glucose) for 4 d. Colonies were transferred onto inductive minimal methanol (MM) plates (1.34% yeast nitrogen base, 4×10^{-5}% biotin, 0.5% methanol, 0.2 mM $CuSO_4$ and 0.2 mM ABTS, natural pH) for the detection of laccase activity. Sterile methanol (200 μL) was added daily to induce the expression of laccase until dark green color appeared around the transformants. Large-scale expression in liquid medium was done according to the instructions of the manual, except that 0.2 mM $CuSO_4$ was included in MM liquid medium. The culture (500 mL in a 2-L flask) was incubated at 15°C in the presence of 1% methanol with

Table 1
Primers used in this study, the underlined and italic nuclear acids represent the enzyme restriction sites. The boldface represents the nuclear acids encode the six histidines.

Name	Sequence
LacF	5′-CG*GAATTC***CATCACCATCACCATCAC**CACTTAGGTCCCATAACGGAG-3′
LacR	5′-AA*TGCGGCCG*CCTAGTAATCGGACACAGGGAGC-3′
5AOX	5′-GACTGGTTCCAATTGACAAGC-3′
3AOX	5′-GCAAATGGCATTCTGACATCC-3′