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

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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

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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′

shaking at 250 rpm. Laccase activity was monitored using ABTS as substrate in glycine-HCl buffer (pH 3.5) with the method as described before [18].

Purification of his-tagged protein from the supernatant of *P. pastoris* GS115 culture was done by affinity chromatography. Briefly, about 500 mL supernatant was obtained by centrifugation at 4000 g for 20 min at 4°C, and then the protein was precipitated with 80% $(NH_4)_2SO_4$ saturation at 4°C overnight. The protein was obtained by centrifugation at 12,000 g for 30 min at 4°C. The protein pellet was dialyzed with 100 mM Tris–HCl buffer (pH 7.0) with Amicon 8400 with 10-kDa cut off membranes. Finally, the dialyzed solution was concentrated to 0.5 mL with centrifugal filter unit with 10-kDa cutoff membranes. After that, it was loaded onto Ni-NTA agarose column with 5 mL bed volume equilibrated with the same buffer. Elution of laccase from the column was done with glycine-HCl buffer (pH 3.5) without using imidazole. 1 mL elution was collected in each tube, and the activity of laccase was monitored according to the method mentioned above. Quantification of protein was done according to BCA method [23].

2.5. The properties of the recombinant PPLCC2

The purity and apparent molecular weight of the protein was determined by denaturing SDS-PAGE on the 4–12% gradient gel. 200 ng proteins were loaded in SDS-PAGE to verify the homogeneity. The molecular weight was calibrated with pre-stained molecular markers RM006 (Sangon Biotech, China). Protein bands were stained with Coomassie blue R-250 (Dingguo, China). Activity staining was done with the same treatments as SDS-PAGE, except that the sample was not boiled for 5–10 min and the electrophoresis was performed at 4°C. The UV–vis spectrum of the purified laccase was recorded in 10 mM sodium phosphate buffer, pH 6.0 with Mapada UV-1100 spectrophotometer (Shanghai, China) Oxidation of ABTS, DMP and SGZ by the recombinant protein was monitored spectrophotometrically at 420 nm ($\varepsilon = 36{,}000\ M^{-1}\ cm^{-1}$); 477 nm ($\varepsilon = 14{,}800\ M^{-1}\ cm^{-1}$), and 525 nm ($\varepsilon = 65{,}000\ M^{-1}\ cm^{-1}$), respectively [18]. Optimal pH for these oxidation reactions was determined in 100 mM glycine-HCl (pH 2 to 3), and acetate buffer (pH 3.5 to 6.0). Optimal temperature for the oxidation of different substrates was determined from 30°C to 80°C, with the optimal pH. Measurement of the specific activity of the recombinant protein toward ABTS (0.4 mM) was done at 25°C, pH 3.5. Stability of the purified protein in buffers with different pH values was investigated by incubating the enzyme for 120 min at room temperature. The residual activity was determined with ABTS as substrate in 100 mM sodium acetate buffer (pH 3.5). Activity at the zero time point was determined and used as the control. Thermostability was investigated by incubating the enzyme at 25°C and 60°C in glycine-HCl buffer (pH 3.5) for different time. Residual activity was determined with ABTS as substrate in the same buffer.

Initial rate kinetics of laccase for the above substrates were determined at 25°C with the optimum pH value as just determined, using 0.001 nM enzyme and a substrate concentration range from 0.5 to 34 μM for ABTS; 0.002 nM enzyme and a substrate concentration range from 0.25 to 17.5 μM for 2,6-DMP. Kinetic parameters were obtained by nonlinear regression using a least-squares minimization (Solver function; Microsoft Excel). The confidence intervals were estimated by using an *F*-test formalism to determine the increase in the sum of the error squares that is statistically significant at the 95% level.

2.6. Bioinformatics analysis of the amino acid sequence of PPLCC2

To understand the phylogenetic relationships among white and brown rot fungi laccase, several randomly selected laccase sequences from white rot fungi and all laccase sequences from brown rot fungi were retrieved from NCBI database (http://www.ncbi.nlm.nih.gov/) or JGI official websites (http://www.jgi.doe.gov/). The analysis was done according to the previously described method [9]. Sequence alignment was done with ClustalX2 software. Possible N-glycosylation site was predicted with online website (http://www.cbs.dtu.dk/services/NetNGLYC/).

3. Results

3.1. Heterologous expression of Pplcc2 in P. pastoris GS115

After positive *Pichia* transformants were obtained, 500 mL liquid MM medium was used to express the protein with one of the transformants named as Pplcc2-1. Buffered MM medium (BMM) was first tried to maintain the pH during the fermentation, but the precipitation of Cu^{2+} by phosphate buffer interfered with the formation of active enzyme. Therefore, only MM liquid medium was used to express the protein at low temperature to minimize the degradation of the protein. The expression of laccase was under the control of methanol oxidase promoter and lasted for 11 d under the optimal conditions (1% methanol, 250 rpm and 15°C), and the activity reached its peak after incubation for 6 d (Fig. 1).

3.2. Purification of laccase with affinity chromatography

By only single affinity purification step, the heterologous protein was purified to electrophoretic homogeneity with apparent molecular weight of 67 kDa, which is very close to the theoretical molecular weight predicted by amino acid sequence (64 kDa). This enzyme was not sensitive to SDS treatment, as it could still oxidize ABTS into its green product in acid buffer (100 mM Glycine-HCl, pH 3.5) after running the gel for 1 h in SDS-PAGE buffer (Fig. 2). The minor difference of the molecular weight was most probably due to the glycosylation by *P. pastoris*, which is well known for fungal laccases and other proteins expressed in *Pichia* expression system [24], (Fig. 2a). The UV–vis spectrum of the purified PPLCC2 showed typical characteristics of multicopper oxidases including a shoulder at 325 nm (type III binuclear copper) and a peak at 614 nm (type I blue copper atom) (Fig. 2b).

3.3. Substrate preferences of PPLCC2

PPLCC2 showed different substrate preferences from PPLCC1. PPLCC2 could not oxidize SGZ, but PPLCC1 can oxidize SGZ with a second order kinetics [18]. The kinetic parameters (K_m and K_{cat}) were determined with 2,6-DMP and ABTS as substrate. The oxidation of ABTS by PPLCC2 showed a typical Michaelis–Menten kinetics, with a K_m of 22.08 ± 3.5 μM and a K_{cat} of 1213 ± 18.3 s^{-1}. The oxidation of 2,

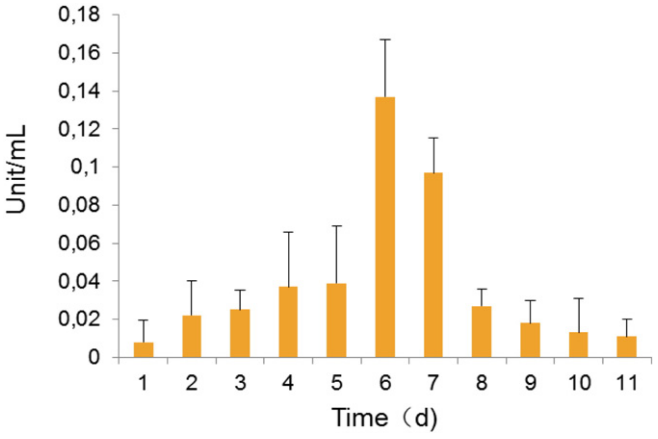

Fig. 1. Time course of PPLCC2 laccase activity in supernatant from *P. pastoris* GS115 culture. Error bars show standard deviations among the triplicate measurements.

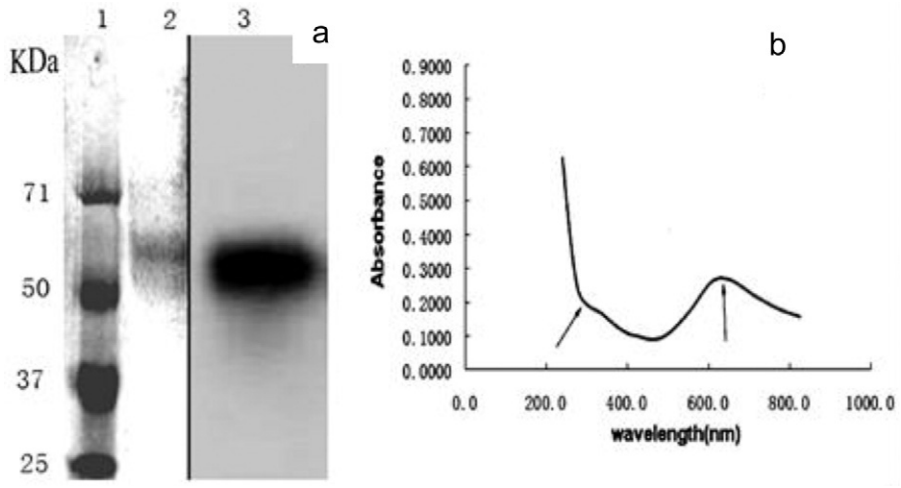

Fig. 2. SDS-PAGE and activity staining of the purified laccase protein with ABTS. (a) Lane 1, protein marker; Lane 2, purified protein; Lane 3, activity staining of the purified protein. (b) Absorption spectrum of PPLCC2. Arrows indicate the characteristic absorbance peaks corresponding to type I (614 nm) and III (325 nm) Cu(II).

6-DMP also exhibited saturation kinetics, with a K_m of 11.62 ± 2.7 μM and a K_{cat} of 293.2 ± 21.9 s^{-1} (Table 2). All of the parameters were in the normal range of laccase from white rot fungi [25].

3.4. The effects of pH on activity of PPLCC2 toward different substrates

The optimal pH for the oxidation of ABTS by PPLCC2 was determined to be 3.5 and clearly showed a bell curve tendency (Fig. 3a). This is dramatically different from PPLCC1, which showed no signs of bell cure tendency using ABTS as substrate [18]. For PPLCC1, the optimal pH for the oxidation of ABTS was determined to be less than 2.7. For the oxidation of DMP, the optimal pH was determined to be 5.0 (Fig. 3b), which is pretty close to PPLCC1 (pH 4.8).

3.5. Effects of temperature on the activity of PPLCC2

Based on the optimal pH determined for the oxidation of different substrates, the optimal temperature for the oxidation reaction was investigated. As we can see that the optimal temperature for the oxidation of ABTS and DMP were determined to be 60°C (Fig. 5b). Oxidation rate of DMP at 30°C was very slowly, and is not shown in the figure. Thermostability of PPLCC2 was investigated by incubating the enzyme at 25°C and 60°C for 7 h. As we can see that the enzyme remained stable for at least 4 h at 25°C and 60°C. The half-life of this enzyme at 60°C was longer than 6 h. Therefore, it is much more stable than most other thermostable laccases at the same temperature, which, in most cases, has a half-life of only 2 h [25]. This enzyme activity also showed a gradually increasing tendency during the treatment. The residual activity reached its peak after treatment for 4 h, which increased by about 50% compared with control (Fig. 5a). Therefore, it was proved that this enzyme is thermostable and thermoactivated one.

Table 2
The properties of two laccase (PPLCC1 and PPLCC2) from *P. placenta*.

	PPLCC1	PPLCC2
Properties		
Glycolization extent	40%	4.7%
Thermostability (half-life)	na	6 h (60°C)
Oxidation of SGZ	$K = 1.90 \times 10^5$ M^{-1} s^{-1}	(-)
Optimal pH	5.0	(-)
Oxidation of ABTS		
K_m	41 μM	22.08 μM
k_{cat}	1280 s^{-1}	1213 s^{-1}
Optimal pH	<2.6	3.5
Optimal temperature (°C)	na	60
Oxidation of DMP		
K_m	58 μM	11.62 μM
k_{cat}	1970 s^{-1}	293.2 s^{-1}
Optimal pH	4.8	5.0
Optimal temperature (°C)	na	60

(-) Negative results; na: data not available.

3.6. Bioinformatics analysis of laccase from white and brown rot basidiomycetes

13 laccase sequences from typical white rot basidiomycetes and 19 sequences from the most recently sequenced brown rot basidiomycetes were selected in the analysis. All putative signal peptides were excluded and 466 informative characters were used to construct the neighbor joining (NJ) tree (Fig. 6). Five clusters could be clearly recognized. All of them were strongly supported (bootstrap values >74%) including one white and four brown rot fungi laccase clusters. This clustering pattern does not completely follow taxonomical relationships of the species. At least, they clustered in part according to the function or the type of the wood rot. Most of the sequences from typical white rot clustered together to form the largest group. Pplcc1 and Pplcc2 clustered into two different groups with lower bootstrap values (54% and 58% respectively). Other *stricto sensu* laccases from the same species clustered together with high bootstrap values (>74%).

The alignment of PPLCC1 and PPLCC2 with other laccases with known 3-D structures indicate that the similarity between PPLCC2 to PPLCC1 is about 60%. They both have the signature sequences of *stricto sensu* laccases including 10 conserved residues of His and the Cys at Cu-sites [26] (Fig. 7). The amino acid residues in the axial position at CuT1 site are both Phe that is typical for most of the laccase from white rot fungi [26,27]. The Aspartic residue (Asp206) has been suggested to be related to the proton abstraction (phenolic substrates) or the stabilization of cation-radical (non-phenolic ABTS) in model laccases [28]. However, no difference was observed for PPLCC1 and PPLCC2 (Fig. 7). LEA/VSG segment in the vicinity of substrate binding and the T1 Cu sites has been related to the high redox properties of laccases [29]. Interestingly, no such sequences were found in all of the brown rot laccase, which might partially explain the overall low potentials of these laccases to oxidize SGZ. Glycosylation has been suggested to be related to the thermostability of laccase [26,27]. Six and four N-Glycosylation sites have been predicted in Pplcc1 and Pplcc2 respectively. This might partially explain the higher

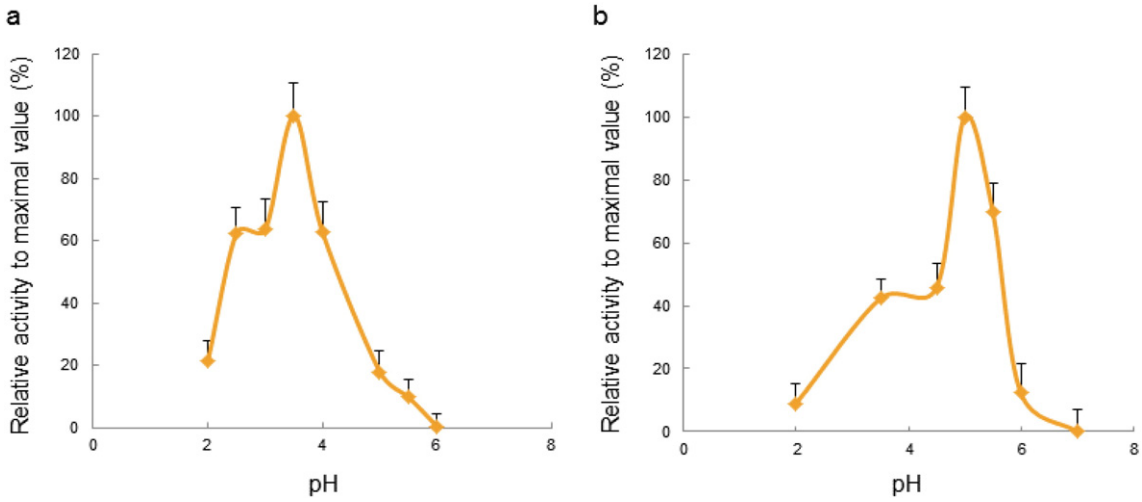

Fig. 3. The optimal pH for the oxidation of ABTS and DMP by PPLCC2. The highest activity at optimal pH was set to 100%. At each pH, the value was detected for three times. Values indicated the means for three replicates. (a) Optimal pH for the oxidation of ABTS; (b) optimal pH for the oxidation of DMP. Error bars show standard deviations among the triplicate testing.

glycosylation extent and thermostability of PPLCC1 than PPLCC2 (data not shown).

4. Discussion

The roles of laccase in the degradation of lignin has been debated for many years since the model white rot fungus *P. chrysosporium* was not found to contain typical laccase sequences [11]. However, evidences that support the involvement of lignin degradation by laccase have been accumulating [30]. Brown rot fungi were found to contain multiple laccase isoforms [17], and they were also proved to cause lignolysis by hydroxyl radicals produced by laccase/hydroquinone driven Fenton reaction at least in *P. placenta* [18]. One of the laccase isoform *Pplcc1* has been suggested to be involved in the lignolysis [18], but the property of another isoform *Pplcc2* has never been investigated. In this investigation, *Pplcc2* was expressed heterologously in *P. pastoris*, and the properties were systematically studied in order to better understand the laccase profiles in this fungus.

To express the *Pplcc2* in *P. pastoris*, two kinds of signal peptides were used to guide the secretion of protein. However, no extracellular oxidation activity was observed at all (data not shown) using its own putative signal peptide sequence. When it was changed to α-factor signal sequence from *Saccharomyces cerevisiae*, the recombinant protein was successfully secreted into the media. This is different to the situation of *Pplcc1* in the same strain, which was secreted into the medium using it own signal peptide sequence [18]. This situation is also different to what was observed for laccase from white rot fungus *Physisporinus rivulosus*. When two laccase isoforms were expressed in *P. pastoris*, the expression of rLac1 was very successful with two kinds of signal peptide. However, only negligible extracellular laccase activity could be detected for another isoform rLac2 when using the two kinds of signal peptide [31]. These results may indicate that *Pplcc2* encodes an intracellular laccase or the enzyme was attached to fungal

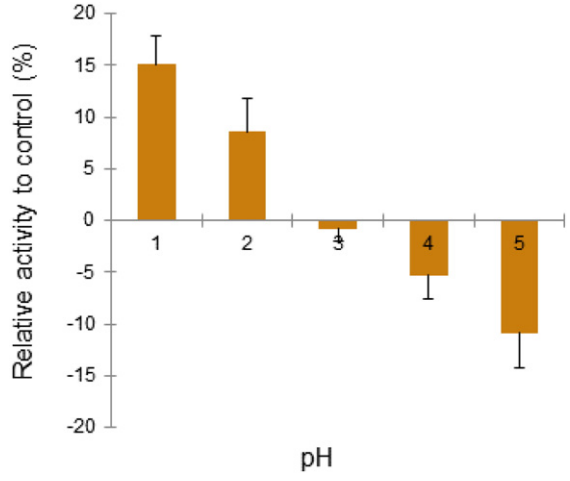

Fig. 4. Stability of enzyme in buffers with different pH values. The value indicated the relative increase or decrease of activity to the controls after treatment for 2 h. The activity at the beginning of the treatment was used as the control. Error bars show standard deviations among the triplicate testing.

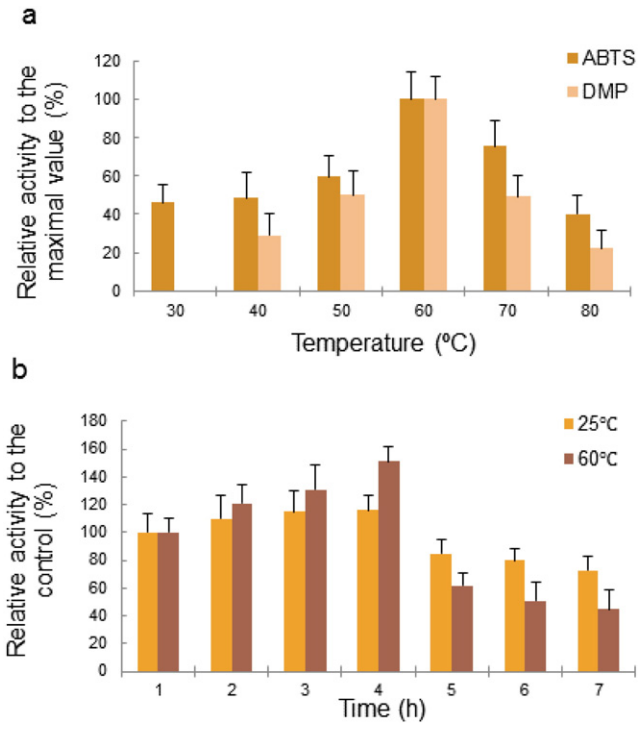

Fig. 5. The optimal temperatures for ABTS and DMP and thermostability of PPLCC2. (a) Thermostability at room temperature (25°C) and 60°C for different time and thermo-activation of the PPLCC2 by high temperature. Reactions at the beginning of the treatment at each temperature were used as control and set to 100%. (b) Optimal temperatures for ABTS and DMP. The highest activity at optimal temperature was set to 100%. Error bars show standard deviations among the triplicate testing.

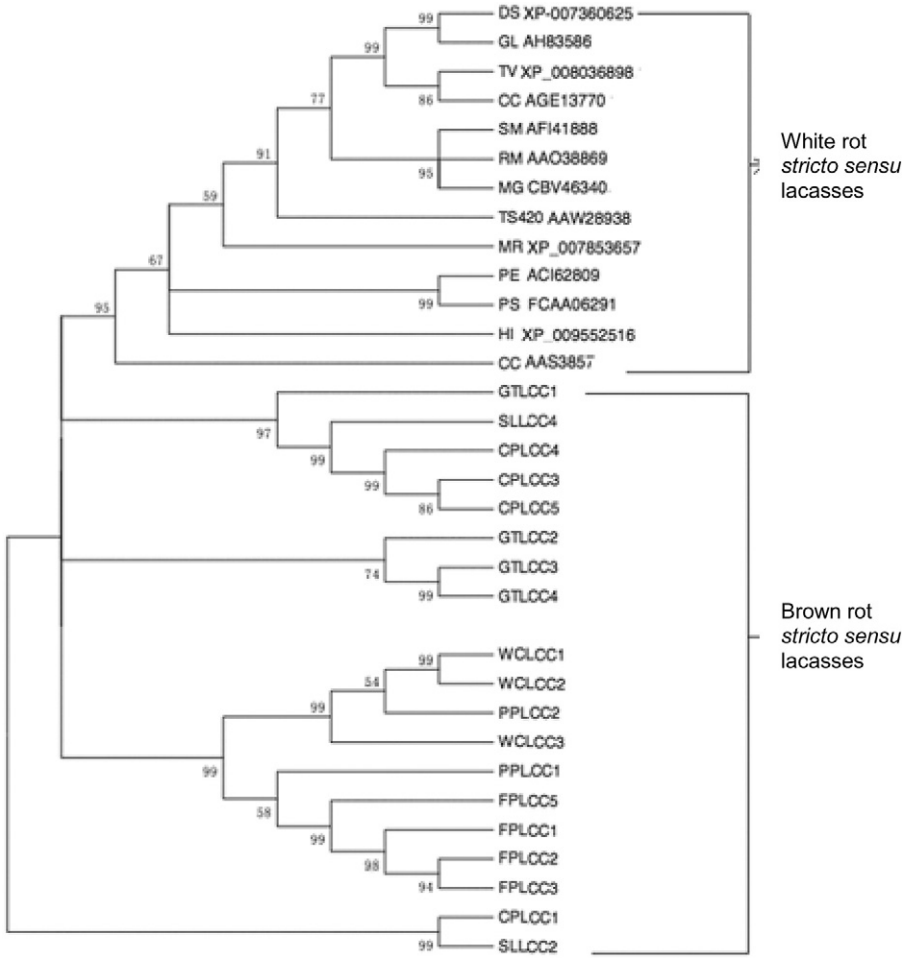

Fig. 6. Neighbor joining (NJ) tree (MEGA 5.1 software) constructed using 19 brown rot and 13 white rot laccase amino acid sequences. Bootstrap values (not less than 50%) were indicated. Groups white and brown rot laccase were indicated by vertical brackets. Species codes: CS: *Ceriporiopsis subvermispora*; CC: *Coprinopsis cinerea* or *Coriolopsis caperata*; DS: *Dichomitus squalens*; HI: *Heterobasidion irregulare*; MG: *Meripilus giganteus*; MR: *Moniliophthora roreri*; PE: *Pleurotus eryngii*; PS: *Pleurotus* sp. '*Florida*'; RM: *Rigidoporus microporus*; SM: *Steccherinum murashkinskyi*; TS: *Trametes* sp. *420*; TV: *Trametes versicolor*; PP: *Postia placenta*; FP: *F. pinicola*; GT: *G. trabeum*; SL: *Serpula lacrymans*; WC: *Wolfiporia cocos*; CP: *Coniophora puteana*.

cell wall in surrounding hyphal sheaths [32]. Intracellular or cell wall and spore-associated laccases of fungi have been suggested to be involved in the transformation of low molecular weight phenolic compounds in the cell, the possible formation of melanin and other protective compounds in cells [32]. Our transcriptional investigation of *Pplcc1* and *Pplcc2* also showed that the expression of *Pplcc2* was upregulated by several kinds of stresses, indicating the possible involvement of *Pplcc2* in the stress response [33]. This could also partially explain our previous results in which no peptides were detected outside of fungal cells for PPLCC2 in the squeezate obtained during the degradation wood wafers [18]. However, cytological studies using laccase specific antibody might shed further light on the precise location of the enzyme [32].

As SGZ is usually regarded as one of the standard substrates for laccase [25], the inability of PPLCC2 to oxidize SGZ may pose a question about whether *Pplcc2* encodes laccase. In fact, there are many laccase isoforms from white rot fungi that cannot oxidize SGZ, including LacA from *Trametes* sp. AH28-2, Lac1 from *Trametes versicolor* and so on. Only a few reports about the kinetics of SGZ for white rot fungi laccase are available now [25]. In addition, at least one study about the different substrate preferences between the native and heterologous laccase has been reported. The native laccases from *P. rivulosus* can oxidize SGZ at a slow rate, but the recombinant enzymes were unable to oxidize SGZ at all [31]. This investigation also indicates that the subtle conformational differences between native and recombinant protein might account for different catalytic properties including the substrate preferences [31]. The recently purified native laccase from brown rot fungus *F. pinicola* also showed very low affinity to SGZ [19]. Therefore, laccases from brown rot fungi might generally have low affinity to SGZ, but more data are needed to get a clear conclusion.

The presence of laccase isoforms with different optimal pH for the oxidation of ABTS in brown rot fungi indicates that these laccases might play different roles during the different stage of degradation, because brown rot fungi usually secrete large amount of oxalate in the incipient stage of degradation of aspen wafer to decrease pH of the environment to 2.0 [18]. The low optimal pH of laccase for oxidation of ABTS has also been reported in white rot fungus *Trametes villosa* [34]. However, most of the optimal pH for the oxidation of ABTS of laccase from white rot fungi falls between 3.0 and 6.0 [25]. Even though, these laccases from brown rot fungi cannot be regarded as acid laccase, because the optimal pH for the oxidation of phenolic compound such as DMP was 4.8 for PPLCC1 and 5.0 for PPLCC2, which is higher than 3.5, the typical value for acid laccase [25]. PPLCC2 was very stable in buffers with different pH values, even though the stability decreased as the pH was increased from 2.0 to 6.5. After treatment of the enzyme for 2 h, at least 90% of the activity was retained in buffer with pH 6.5. Interestingly, incubation of the enzyme

Fig. 7. Multiple sequence alignment on fungal laccases with known structure. Sequences were retrieved from PDB database. CcL: *C. cinerea* (PDB:1A65); TvL: *T. versicolor* (PDB:1KYA); PcL: Pycnoporus cinnabarinus (PDB: 2XYB). Binding sites for copper; disulfide bond; the axial ligand for T1-Cu; conserved amino acid may cause high E0 enzymes in fungal laccase. Underlined: LEA/VSG segment in the vicinity of substrate.

in acid buffer (pH 2.0) increased the activity by about 15% compared with the control (Fig. 4). Therefore, PPLCC was proved to be an acid-activated laccase.

Thermal stable laccases have great potentials in pulp, paper, textile and food industries, which have only been reported for a few laccases from white rot fungi and bacterium strains [35]. For the first time, we found that PPLCC2 also has this property, which has been related to the open conformation of the substrate-binding cavity and the conformational changes caused by increasing temperature [31]. The incubation at room temperature (26°C) could still increase the activity by about 20% after treatment for 4 h. Therefore, higher temperature induced higher activity of this enzyme, which indicates that higher temperature was favorable of the formation of more open conformation. The newly purified laccase from brown rot fungus *F. pinicola* can only retain its activity after incubation at 50°C for 1 h. Enzyme activity sharply decreased when the temperature was increased to 60°C, and no activity was detected at 80°C. The purified laccase showed nearly no activity at pH 4.5 at all [18].

Phylogenetic analysis of laccase has been performed by Hoegger et al. [9] without brown rot laccase. After that, Kües and Rühl [22] investigated the phylogenetic relationship of MCOs and laccase-like sequences from brown rot fungi *P. placenta* and *Serpula lacrymans* were included in the analysis in which laccases from white rot and brown-rot fungi intermingle with enzymes from the saprotrophic *Coprinopsis cinerea* and the ectomycorrhizal species *L. bicolor* In our investigation, more brown rot fungi laccase were used in the analysis, no other MCOs were included. Here, we showed that there was a clear differentiation between laccases from white rot fungi and brown rot fungi (Fig. 6). This result is similar to another investigation in which a clear differentiation between laccases from strong white-rot species and enzymes from *C. cinerea* and *L. bicolor* was seen, with laccases from the straw-decaying white-rot fungus *Pleurotus ostreatus* clustering in between [22]. These results suggest that laccases might evolve with the ability of fungal species to degrade the lignin.

In conclusion, this investigation together with our previous work proved the existence of two functional laccases with different properties in brown rot fungus *P. placenta*. *Pplcc1* seems to encode an extracellular laccase that might involve in the laccase/hydroquinone driven Fenton reaction. *Pplcc2* seems to encode an intracellular or cell wall associated laccase that might involve in stress response during the degradation process. The two recombinant laccase isoforms have different substrate preferences toward the traditional laccase substrate SGZ. The presence of laccase with different optimal pH indicates that they might play different roles in different stages of degradation process. It was proved that PPLCC2 is the first thermostable enzyme identified from brown rot fungi, which may have potential usage in pulp and paper, textile and food industries.

Conflict of interest

The authors declare no conflict of interest.

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