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Characterization and decolorization ability of a laccase from *Panus rudis*

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Abstract

A laccase from *Panus rudis* was produced constitutively in defined shaken liquid culture without induction. The purified enzyme of 58 kDa contained 8% carbohydrate and had an isoelectric point of 3.5. The optimal pH of the enzyme is 3.5 and the optimal temperature is 60 °C with ABTS as the substrate. The K_m of ABTS is 0.10 mM. The first 20 residues at the amino terminus were determined and the cDNA sequence encoding the enzyme was isolated by RT-PCR. The highest identity of the predicted amino acid sequence is 67% with the sequence of laccase from *Lentinula edodes*. The enzyme had excellent ability to decolorize anthraquinone dye (Acid Green 27) without any redox mediators, as well as azo and indigo dyes (Acid Violet 7 and Indigo Carmine) mediated by ABTS. Compared with other laccases in dyes decolorization, a very small amount of PrL could lead to effective dyes decolorization.

Keywords: Laccase; *Panus rudis*; Characterization; Decolorization; cDNA

1. Introduction

Laccases (benzenediol:oxygen oxidoreductases, EC1.10.3.2) are polyphenol oxidases that catalyze the oxidation of a wide range of substrates. In the oxidation reaction, oxygen is reduced to water by four electrons. Laccases belong to the blue multicopper oxidases family including the ascorbate oxidase and mammalian plasma protein ceruloplasmin. They are considered to be the simplest representative of the family since they are monomeric and display a single mononuclear and a trinuclear center with total four copper atoms [1]. Laccases are widely spread in nature. Although the first laccase was found in a lacquer tree, most laccases were discovered and studied from fungal sources [2]. Laccases were also reported to be widespread in bacteria [3–5]. Fungal laccases have various biological functions in degradation of lignin, morphogenesis and pathogenesis [1,2]. Their catalytic properties and broad substrate specificity make fungal laccases have great potential in varied industrial and environmental applications including pulp delignification [6,7], textile dye bleaching [8,9] and xenobiotics degradation [10–12].

Synthetic dyes are widely used in industries and about 10% of them released by industrial effluents, which cause serious environmental pollution [13]. It has been well known that white rot fungi could be used to decolorize textile dye effluents and the involved laccases play an important role in the process [14–19]. The enzymatic treatment is simpler and more efficient than the usage of fungi cultures. So, laccases become an attractive option for biobleaching [20–25]. Some fungi laccase cDNA sequences have been cloned and heterologously expressed in yeast or aspergillus [26–31]. It should promote the biotechnology application of laccases in dye effluents treatment.

In previous studies, we found a new fungal source of extracellular laccase, *Panus rudis*, which produces this enzyme in a great yield without inducers. The enzyme was purified and partially characterized [32]. In this report, the enzymatic characters and the cDNA sequence of the protein were determined. We found that a very small amount of the protein could decolorize synthetic dyes. The remarkable decolorization ability of the enzyme suggests that it may have great potential in textile wastewater treatment.

Abbreviations: ABTS, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid); LeL, laccase from *Lentinula edodes*; PrL, laccase from *Panus rudis*; TvL, laccase from *Trametes versicolor*

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2. Materials and methods

2.1. Organisms and culture conditions

P. rudis 5.34 was obtained from China General Microbiological Culture Collection Center. The strain was maintained on a CPDA agar plate at 4 °C. Agar cubes cut from agar plate were inoculated in 150 mL shake flasks with 50 mL liquid medium and incubated with 150 rpm at 28 °C for 4 days. After aseptic homogenization of the culture, the mycelial suspension was inocubated to the same medium at a 15% (v/v) concentration and cultivated at 39 °C until the maximum laccase activity was reached. The liquid medium contained (L^{-1}) 0.01 M HAc–NaAc buffer (pH 4.5), glucose 10 g, $CaCl_2$ 0.1 g, KH_2PO_4 2 g, $MgSO_4$ 0.25 g, ammonium tartrate 0.5 g, trace element solution 10 mL (L^{-1}: aminoacetic acid 0.5 g, $MgSO_4$ 3.0 g, NaCl 1.0 g, $FeSO_4{\cdot}7H_2O$ 0.1 g, $CoCl_2{\cdot}6H_2O$ 0.18 g, $ZnSO_4{\cdot}7H_2O$ 0.1 g, $CuSO_4$ 0.1 g, $AIK(SO_4)_2{\cdot}12H_2O$ 0.01 g, $MnSO_4{\cdot}H_2O$ 0.01 g, H_3BO_3 0.01 g, $Na_2MoO_4{\cdot}5H_2O$ 0.01 g). Cloning procedures and DNA amplification were performed using *Escherichia coli* DH5α, which was grown at 37 °C on LB medium containing 50 μg/mL ampicillin.

2.2. Chemicals and reagents

Restriction enzymes, DNA polymerase, dNTP mixture, M-MLV reverse transcriptase, RNase inhibitor and pMD 18-T vector were purchased from TaKaRa Biotechnology Co., Ltd. RNA isolation reagent TRIZOL was purchased from Shanghai Sangon Co., Ltd. Primers were synthesized in Shanghai Sangon Co., Ltd. Plasmid extraction and agarose gel purification kit were purchased from Shanghai Huashun Co., Ltd. Three synthetic dyes Acid Green 27, Acid Violet 7 and Indigo Carmine were purchased from Aldrich Chemical Co., Inc.

2.3. Protein assays

Protein concentration was determined by the Bio-Rad Protein Assay Reagent with bovine serum albumin as a standard. SDS-PAGE was performed on polyacrylamide gel at a concentration of 12%. Protein was stained with Coomassie brilliant blue R-250. The isoelectric point of the enzyme was determined on isoeletric focusing gel (IEF-PAGE) with a pH gradient of 3–10. Carbohydrate contents of PrL were determined by endoglycosidase treatment. The purified protein (5 μL, 1 mg/mL) was mixed in 25 μL solution containing 30 mM citrate buffer (pH 5.5), 2 μL 10% SDS and 1 μL β-mercaptoethanol. After the mixture was boiled for 5 min, 25 mU endoglycosidase H (Roche Molecular Biochemicals) was added, and then the reaction mixture was incubated for 24 h at 37 °C. The carbohydrate content was estimated from the shift rates on SDS-PAGE.

2.4. Enzyme activity assays

Laccase activity was assayed at room temperature using 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate. The assay mixture contained 2 mM ABTS and 0.1 M sodium acetate buffer (pH 4.5). Oxidation of ABTS was followed by absorbance increase at 420 nm ($\varepsilon = 36{,}000\ M^{-1}\ cm^{-1}$). One unit (U) of laccase activity was defined as the amount of enzyme that catalyzed the formation of 1.0 μmol of product per minute. All assays were performed in duplicate, with an average sample mean deviation of less than 10%.

2.5. Enzyme characterization

Estimates of the laccase optimum pH were measured with ABTS in 50 mM sodium citrate buffer (pH 3.0–6.5). The optimum temperature was determined in the 20–75 °C ranges. The K_m value was determined using Lineweaver–Burk plots with ABTS concentration of 0.5–2.0 mM.

2.6. N-terminal amino acid sequencing

Purified laccase was electroblotted directly from an SDS-PAGE gel to a polyvinylidene difluoride membrane (PVDF, Bio-Rad), and N-terminal amino acid sequence was determined at the Analysis Center of Beijing University.

2.7. RNA isolation

Cells was grown for 3 days in liquid culture. The mycelium was separated from the culture fluid by filtration through filter paper and immediately frozen in liquid nitrogen. The frozen mycelium was ground to a powder in a mortar containing liquid nitrogen and transferred into a 1.5 mL Eppendorf tube. TRIZOL reagent is used for RNA isolation and the procedure was carried out according to the instructions. All of the solutions and appliances were treated with diethyl pyrocarbonate and sterilized by autoclave.

2.8. RT-PCR

The sequence of the forward primer (5′-GCNAT(T/C/A)GGNCCNGTN ACNGA(T/C)(T/C)TNCA(T/C)AT(T/C/A)G-3′) was based on the N-terminal amino acid sequence AIGPVTDLHIV previously obtained. The reverse primer (5′-NA(A/G)NGC(A/G)TC(G/A)TANGTNGG(A/G)(A/C)ANA-3′) corresponds to the amino acid sequences LCPTYDAL adjacent C-terminal, which is conserved among some other fungal laccases sharing highly homologous N-terminal amino acid sequences. Reverse transcription reaction was performed in 25 μL incubation mixtures containing 5 μL total RNA, 4 μL oligo(dT) (20 μM), 2000 U of M-MLV reverse transcriptase, 1.5 μL 10 mM dNTP, 1 μL RNase inhibitor (40 U/μL) and 5× buffer. The RNA was first mixed with the oligo (dT). Then, the solution was heated at 70 °C for 10 min and quickly cooled on ice. After the addition of the other components, reaction was conducted at 42 °C for 90 min. The reaction solution (8 μL) was used for PCR amplification in 50 μL mixture containing 10× PCR buffer, 5 U of Taq DNA polymerase, 0.4 mM dNTP and 1.6 mM each of forward and reverse primers. The PCR program consisted of an initial cycle of denaturation (95 °C, 3 min), annealing (50 °C, 1 min) and prolonged extension (72 °C, 20 min), followed by 34 cycles of denaturation (94 °C, 0.5 min), annealing (50 °C, 1 min) and extension (72 °C, 2.5 min). A final 10 min extension step at 72 °C was also included. The RT-PCR product was gel purified and subcloned into the pMD 18-T vector. Plasmids extracted from transformants were verified by restriction enzyme analysis and checked by sequencing (Shanghai GeneTech Co., Ltd.). Sequence alignment was carried out with the CLUSTAL W program. The 3D model for PrL was built on the basis of the reported crystal structure of *Trametes versicolor* laccase (code 1GYC), using the SWISS-MODEL (First Approach model).

2.9. Dyes decolorization

Dye solutions were prepared by dissolving 250 mg of the respective dye in 1 L distilled water. The reaction mixture for decolorization experiments contained 1 mL of dye solution, proper enzyme preparation in total 6 mL of 100 mM sodium citrate buffer (pH 4.0). ABTS (4.2–25.0 μM) was added as the mediator when it was needed. The reaction mixture was incubated at 45 °C for indicated time periods shaking at 100 rpm. Decolorization activity was determined by monitoring the decrease in absorbance on a spectrophotometer at the absorbance maximum of each dye (the absorbance maximum of Acid Green 27, Acid Violet 7 and Indigo Carmine are 410, 520 and 610 nm, respectively).

2.10. Nucleotide sequence accession number

The nucleotide sequences reported in this paper have been deposited with GenBank under accession no. AY439331.

3. Results

3.1. Characterization of PrL

When *P. rudis* was grown in defined shaken liquid culture, laccase was produced constitutively. The enzyme PrL was purified to homogeneity (one band on SDS-PAGE gel at 58 kDa) from the

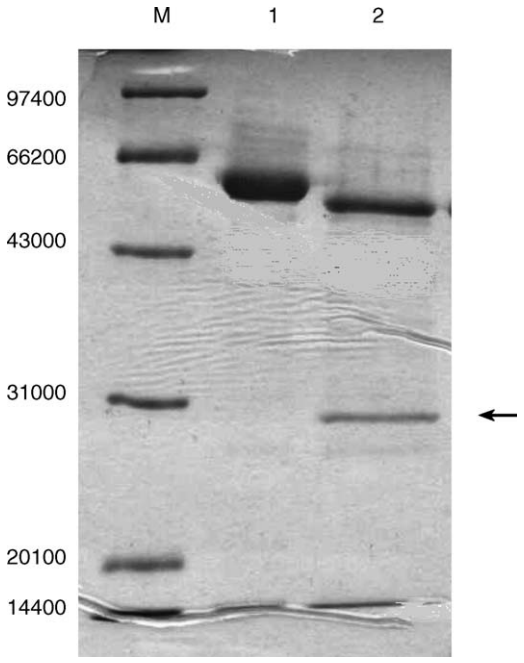

Fig. 1. SDS-PAGE of PrL deglycosylation (M, molecular weight markers; 1, purified PrL; 2, purified PrL digested with endoglycosidase H. The arrow shows the protein band of endoglycosidase).

culture and the specified activity of purified PrL was 460 U/mg (with ABTS). After deglycosylation, the molecular mass of the protein decreased to 53 kDa (Fig. 1), suggesting approximately 8% carbohydrate contents. On an isoelectric focusing gel, the enzyme had an isoelectric point of 3.5. The optimum pH for PrL was 3.5 and the optimum temperature was 60 °C with ABTS as substrate. The K_m value of the enzyme for ABTS was determined to be 0.10 mM.

3.2. cDNA sequence

The first 20 residues at the amino terminus of mature PrL are AIGPVTDLHIVNDNIAPDGF by sequencing. The cloned cDNA sequence contains 1479 bp and codes for 493 amino acids. The deduced N-terminal amino acid sequence corresponds exactly to the previously obtained sequence. There may be an estimated 5–10 residues of C-terminal deletion because the reverse primer was designed with the conserved amino acid sequences adjacent to C-terminal. The calculated molecular mass is 53.23 kDa corresponding with the deglycosylated protein. The calculated isoelectric point is 5.04, which differs from the experimental value (p*I* 3.5). The deduced protein has five potential *N*-glycosylation sites (Asn-X-Thr/Ser), at positions 217, 387, 435, 471 and 477 residues, respectively. The highest identity of PrL is 67% with the sequence of *Lentinula edodes* laccase (BAB83131). The second is 66% identity with the sequence of *T. versicolor* laccase (AAL00887, AAC49828 and AAA86659) and *Trametes villosa* laccase (AAC41687).

3.3. Amino acid comparison and homology model of PrL

A multiple sequence alignment of laccases revealed that PrL had all of the conserved copper ligands (10 His and 1 Cys) at the active sites together with some sequences close to those copper-binding motifs in laccases. On the basis of a comparison with the amino acid sequence of the laccase from *T. versicolor* (TvL), which had 66% identity with PrL sequence and the crystal structure containing a full complement of coppers [33], two disulfide bridges (Cys85-Cys487 and Cys117-Cys205) are also conserved in PrL. His396, Cys452 and His457 of PrL are predicted to be involved in the trigonal coordination of the type 1 copper. The axial position of T1 copper site is Phe, which is postulated to

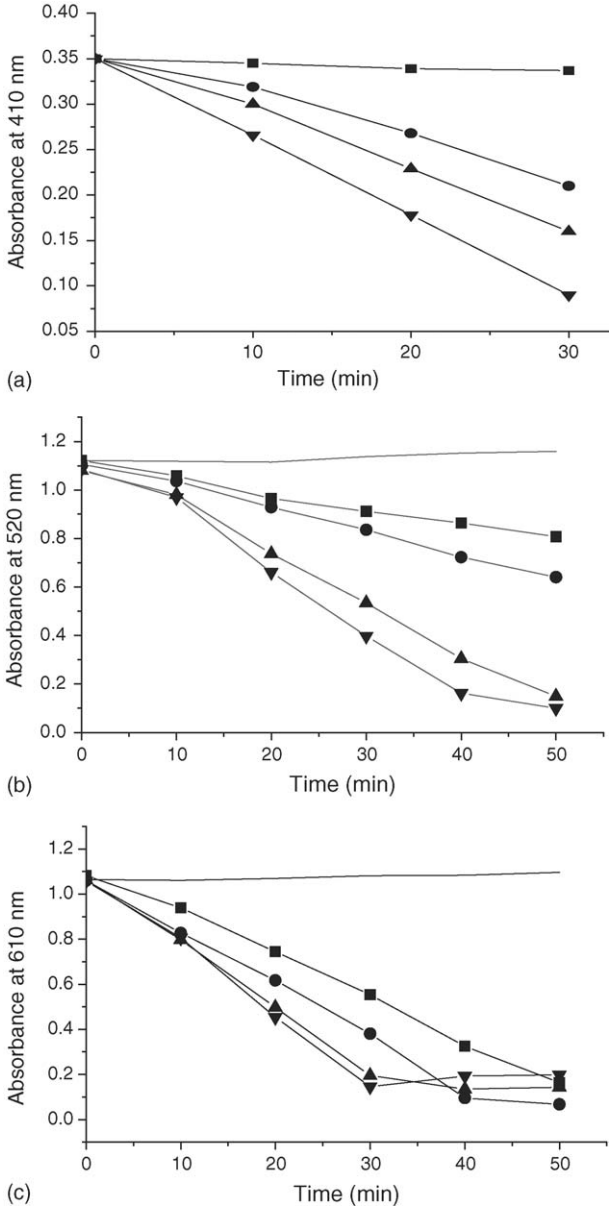

Fig. 2. Decolorization of three synthetic dyes by PrL. (a) Decolorization of Acid Green 27 by laccase at different protein concentration: (■) control with heat inactive enzyme 0.016 μg/mL; (●) 0.004 μg/mL; (▲) 0.008 μg/mL; (▼) 0.016 μg/mL. (b) Decolorization of Acid Violet 7 by PrL with different ABTS concentration: (—) control, without ABTS; (■) 4.2 μM ABTS; (●) 8.4 μM ABTS; (▲) 16.7 μM ABTS; (▼) 25.0 μM ABTS). (c) Decolorization of Indigo Carmine by PrL with different ABTS concentration: (—) control, without ABTS, (■) 4.2 μM ABTS, (●) 8.4 μM ABTS, (▲) 16.7 μM ABTS, (▼) 25.0 μM ABTS).

be responsible for the highest redox potential in fungal laccase [34,35]. The trinuclear T2/T3Cu site is normally coordinated with eight histidines in a highly conserved pattern of four His-X-His. His64 and His399 are predicted to be coordinated with T2 Cu and the other six histidines (His66, 109, 111, 401, 451 and 453) with T3 Cu pair in PrL. In the structure of high E° TvL, a hydrogen bond between Glu460 and Ser113 caused a longer Cu1-N (His458) bond at T1 site, which made the copper more electron deficient and increased the redox potential [33]. Ser113 and Asp459 at the corresponding positions of PrL made it possible to form a hydrogen bond. So, PrL was expected to have a high redox potential like TvL. The model of PrL displayed a similar pattern of fold and copper coordination with TvL and accorded with those predicted results mentioned above. But *N*-glycosylation seemed unlikely at two positions (Asn387 and 477) among the five potential sites because of steric reason based on the model.

3.4. Dye decolorization by PrL

Three synthetic dyes with typical chromophores (anthraquinone, azo and indigo) could be decolorized by PrL. Anthraquinone dye Acid Green 27 could be directly decolorized (40% with 0.005 μg/mL protein in 0.5 h; 74%, with 0.016 μg/mL protein in 0.5 h) alone and the decolorization rate was proportional to the enzyme concentration (Fig. 2a). When the enzyme concentration was about 1.7 μg/mL in reaction mixture, the decolorization of azo and indigo dyes, Acid Violet 7 and Indigo Carmine could be observed (26 and 12% after 1 h, respectively). However, in the presence of redox mediator ABTS (16.7 μM), only 0.005 μg/mL PrL could obviously decolorize the two dyes (47 and 81%, respectively, in 0.5 h). And the increase of decolorization rate was proportional to the ABTS concentration (Fig. 2b and c). The optimum temperatures of three dyes decolorization were about 70 °C and the optimum pH were pH 3.5–4.5.

4. Discussion

Laccases generally are 520–550 amino acids in length, which contain a N-terminal secrete signal sequence of 16–23 amino acids and the mature protein has three domains. Learning from the sequence-homology analysis of laccases, the percent identities from different origins is significantly different, range from 13 [36] to 99% [37]. The differences of the laccase primary sequences may be related to their organism resources and biological functions. Several fungi laccases had already been reported in the literature about the cDNA cloning and heterologous expression, such as laccase from *Trametes* sp. expressed in the yeast *S. cerevisiae* [26], laccase from *Pycnoporus cinnabarinus* expressed in *Aspergillus niger* [27], laccase from *T. versicolor* expressed in the yeast *Yarrowia lipolytica* [28], laccase from *Fome lignosus* expressed in *Pichia pastoris* [29], etc. The heterologous expression of PrL cDNA in *P. pastoris* also has been done [38].

Some fungal laccases as well as laccase mediator systems are efficient in dyes decolorization. Different dyes were decolorized by different laccase at different rates. The decolorization rate depends on the structure and the redox-potential of the enzyme as well as the structure of the dye [8]. The fact that the PrL can directly decolorize anthraquinone dye and decolorize azo and indigo dyes mediated by ABTS is the same with the laccase from *Trametes vercicolor* [9]. The reason may be that the redox potential of the two dyes are higher than that of type 1 Cu of PrL or the two dyes could not access the type 1 Cu active site because of their steric structure. The small molecule ABTS can transfer electrons between the enzyme and the two dyes as redox mediators and oxidizes the two non-substrate dyes after oxidation by laccase to cation radical. The initial decolorization rates of the three dyes by crude laccase from *T. vercicolor* were 150, 80 and 85 mg/L h, respectively. And the laccase activity in culture medium is 162 U/L [9]. The initial decolorization rates of the same three dyes by purified PrL were 62, 39 and

Table 1
Decolorization of some industrial dyes by fungi laccases

Dyes	Dyes concentration (mg/L)	Laccase	Laccase concentration (μg/mL)	Reaction condition	Decolorization rate (%)	Reference
Acid Green 27	41.7	PrL	0.016	45 °C, 30 min	74	
	20	Laccase from *M.t.*	91	30 °C, 16 h	66	[25]
	20	Laccase from *P.p.*	43	30 °C, 16 h	71	[25]
	20	Laccase from *T.v.*	6.8	30 °C, 16 h	74	[25]
Indigo Camine (Acid Blue 74)	41.7	PrL	1.7	45 °C, 60 min	12	
	41.7	PrL	0.005	45 °C, 30 min, 16.7 μM ABTS	81	
	20	Laccase from *M.t.*	91	30 °C, 16 h	30.9	[25]
	20	Laccase from *P.p.*	43	30 °C, 16 h	83	[25]
	20	Laccase from *T.v.*	6.8	30 °C, 16 h	91.7	[25]
Acid Violet 7	41.7	PrL	1.7	45 °C, 60 min	26	
	41.7	PrL	0.005	45 °C, 30 min, 16.7 μM ABTS	47	
Diamond Black	20	Laccase from *S.r.*	13.5	40 °C, 55 min	100	[20]
Chicago Sky Blue	25	Laccase from *P.c.*	0.82	2 h	100	[22]
RBBR	200	Laccase from *L.e.*	7.2	90 min	60	[24]

M.t., *Myceliophthora thermophile*; *P.p.*, Polyporus pini situs; *T.v.*, *Trametes versicolor* [25]; *S.r.*, *Sclerotium rolfsii* [20]; *P.c.*, *Pycnoporus cinnabarinus* [22]; *L.e.*, *Lentinula edodes* [24].

68 mg/L h, respectively, but the enzyme concentration used in this study of decolorization is very low (0.005–0.016 μg/mL, 2–7 U/L), about 1/50 of *T. vercicolor* laccase concentration. A very small amount of PrL could lead to effective synthetic dyes decolorization. The result (Table 1) compared with other laccase in decolorization clearly shows the outstanding decolorization ability of the enzyme, which indicated that PrL was a promising industrial enzyme for dyes wastewater treatment.

Besides the excellent ability of decolorization, PrL might have stable Cu coordination and have little sensitivity to Cu^{2+} and other metal ions [32]. It is value in wastewater treatment that PrL was not inhibited by different metal ions because industrial effluents are generally complex mixture of various mental salts. In several fungi known as the best laccase producers, laccase production is usually inducible [39,40]. Most inducers of laccase are toxic and expensive and are difficult to remove during the laccase concentration and purification procedure. *P. rudis* secretes a high level of PrL in a defined shaken liquid medium without using laccase inducers, such as 2,5-xylidine, ferulic acid, veratrylalcohol, vanillin, vanillic acid and lignin [32]. So, the production of PrL by *P. rudis* is simple and cheap. In addition, *P. rudis* is edible mushroom so that it is safe to human in environment and industry production. Therefore, PrL and its producer *P. rudis* are favorable and promising for environmental or biotechnological applications.

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