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Overexpression and characterization of a thermostable, pH-stable and organic solvent-tolerant *Ganoderma fornicatum* laccase in *Pichia pastoris*

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ABSTRACT

The newly defined laccase gene, *lac1*, from the medicinal white-rot fungus *Ganoderma fornicatum* 0814 was cloned and expressed in *Pichia pastoris*. The corresponding open reading frame has 1563 nucleotides and encodes a protein of 521 amino acids, including a 21-residue secretion signal peptide. The absorption spectrum of the purified laccase, rLac1, indicated that it is a yellow laccase rather than the common blue laccase. The optimum pH of rLac1 was 2.5, 3.0 and 3.0 for ABTS, DMP and guaiacol, respectively. The kinetic parameter K_m of rLac1 was 103.9, 276.7 and 1263.4 μM for ABTS, DMP and guaiacol, respectively. The optimal temperature for rLac1 was 55 °C for all of the substrates. rLac1 is stable up to 60 °C and in the pH range 2.5–10.0. Sodium azide and thioglycolic acid strongly inhibited rLac1 activity, but it was not affected by EDTA. rLac1 retained more than 80% activity in 50% (v/v) ethanol, methanol, DMF and DMSO after a 3-h incubation at 25 °C. Less than 20% of activity was found in acetone and acetonitrile. The results show that rLac1 is thermostable, pH-stable and tolerant to organic solvents. These properties suggest that rLac1 has potential uses in industrial applications.

1. Introduction

Lignocellulosic biomass, consisting of cellulose, hemicellulose and lignin, represents the most-abundant carbohydrate polymers in nature. Among these components, lignin is the least susceptible to biological degradation because it is a cross-linked macromolecule and consists of various substructures that appear to repeat indiscriminately. White-rot fungi generally exhibit higher ligninolytic activity than other microorganisms due to the presence of laccases, a group of copper-containing oxidizing enzymes [1,2]. The laccase uses molecular oxygen to oxidize a variety of substrates from aromatic to inorganic compounds and produce water as the sole by-product [3]. The potential industrial applications of laccases include (1) dye-bleaching in the textile and dye industries, (2) biobleaching of pulp, (3) detoxification of pollutions and bioremediation, (4) construction of biosensors and biofuel cells and (5) fruit juice processing [4,5].

Industrial processes usually require enzymes to maintain active under extreme pH conditions or high concentrations of organic solvents in which enzymes often lose their activity due to improper folding [6]. Unfortunately, the current laccases from fungi, such as *Podospora anserine* [7], *Agaricus bisporus* [8], *Trametes versicolor* [9] and *Ganoderma lucidum* [10], are not suitable for commercial purposes. In addition, low yields and undesirable cellulolytic enzymes that coexist with laccase limit full-scale laccase applications. Recently, an enhancement of stability features of *Pleurotus ostreatus* laccase was obtained using a semi-rational mutagenesis [11]. The ideal enzymes for industrial applications should be thermostable, pH-stable and organic solvent-tolerant.

Ganoderma are pathogens to perennial crops [12] and are known for their degrading capability of a variety of hardwoods [13]. Several papers have reported on the purification and biochemical characterization of laccases from *G. lucidum* [10,14,15]. Although the laccase from *G. lucidum* showed stability over the pH range 4.0–10.0, it was only applied at temperatures lower than 40 °C, which is not applicable to general industrial process [15–17]. In this paper, the full-length gene of a laccase from *Ganoderma fornicatum* was cloned and expressed in the methylotrophic yeast, *Pichia pastoris*. The biochemical and kinetics properties of this recombinant laccase were investigated.

2. Materials and methods

2.1. Strains and media

G. fornicatum 0814, deposited in the American Type Culture Collection (ATCC 76536), was acquired from the culture collection of the Laboratory of

Abbreviations: ABTS, 2,2-azinobis-3-ethylbenzthiaoline-6-sulfonic acid; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; DMF, dimethylformamide; DMP, 2,6-dimethoxyphenol; DMSO, dimethylsulfoxide; DTT, dithiothreitol; TGA, thioglycolic acid.

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Applied Microbiology, Department of Biochemical Science and Technology, National Taiwan University and maintained on potato dextrose agar (Difco, Detroit, MI). *Escherichia coli* DH5α (GIBCO-BRL Life Technologies, Grand Island, NY) was used for plasmid manipulation and was grown in LB medium (Difco). *P. pastoris* KM71H (*Arg*$^+$, *Mut*^{s}) and pPICZαA plasmid were purchased from Invitrogen (Carlsbad, CA). The minimal medium (MM), containing 0.1 mM $CuSO_4$ and 0.2 mM ABTS, for transformants screening and the buffered glycerol-complex medium (BMGY) for the *P. pastoris* culture were prepared according to the manufacturer's instruction. The modified buffered methanol-complex medium (mBMMY) for induction was prepared according to the description by Chen et al. [18].

2.2. Genomic DNA isolation and laccase gene cloning

Genomic DNA isolation from *Ganoderma* has been described previously [19]. The primer set, GPF1 (5′-AAC ATG GGA CAT GCC ATG GC CAG-3′) and GPR1 (5′-TCA TTG GTC GTT GGG CGA GAG CGC GT-3′), was designed according to 5′ untranslated region (UTR), and the 3′ end of the known laccase gene sequence of *G. lucidum* (AY485825) and was used to amplify the laccase genes.

2.3. Cloning of laccase cDNA and plasmid construction

For isolating the total RNA, *G. fornicatum* 0814 was cultured for three days in yeast extract peptone dextrose (YPD medium; Difco) with 0.2 mM $CuSO_4$ and 0.2% sawdust extract at 25 °C on a rotary shaker (120 rpm). The total RNA was isolated by using TRIzol (Invitrogen). Laccase cDNAs were amplified by the ThermoScript™ RT-PCR System (Invitrogen) and Easy-A DNA polymerase (Stratagene, Kirkland, WA). The PCR products were cloned into yT&A vectors (Yeastern Biotech, Taipei, Taiwan) and transformed into *E. coli* DH5α for sequencing. The cloned laccase gene was blasted with known laccase genes from GenBank by Bioedit XP (Ibis Therapeutics, Carlsbad, CA). The laccase gene was cloned into the *EcoR* I/*Xba* I site of the pPICZαA vector, and the resulting expression plasmid was designated as pPICZαlac1.

2.4. Transformation and expression

The transformation of *P. pastoris* KM71H was performed based on the method of Wu and Letchworth [20]. The transformants were selected under pressure of 100 μg ml^{-1} zeocin (Invitrogen) on YPD agar medium supplemented with 1 M sorbitol. The candidates were transferred to minimal medium plate containing 0.2 mM ABTS and the laccase-secreting strains were screening by the formation of green pigment. The colonies with dark green halos were inoculated in 15 ml BMGY medium at 30 °C and shaken at 250 rpm for 24 h. The cultures were collected and resuspended in 3 ml mBMMY medium containing 0.2 mM $CuSO_4$ at 30 °C in a shaking incubator (250 rpm). One-percent (v/v) methanol was added every 24 h to induce laccase production.

2.5. Laccase activity assay

The laccase activity was assayed according to the method of Soden et al. [21], with minor modification. The reaction mixture contained 0.1 ml enzyme and 0.1 ml ABTS (1 mM) and was dissolved in 100 mM citrate buffer (pH 3). After incubation at 30 °C for 5 min, the change in absorbance at 420 nm was recorded with a spectrophotometer. Guaiacol and DMP (Sigma) were also used as laccase substrates for enzyme activity [22]. One unit of laccase activity was defined as the amount of enzyme that oxidized 1 μmol of substrate/minute.

2.6. Purification

After centrifuging at 3000 × *g* for 15 min to separate the cells, the extracellular laccase was concentrated and dialyzed with 20 mM Tris buffer (pH 7.4) in a vivaflow 200 filtration module (Sartorius, Hannover, Germany) at 4 °C. The protein concentrate was fractionated by anionic exchange chromatography on a Q-Sepharose XL column (2.5 cm × 40 cm, GE Healthcare, Pittsburgh, PA). The column was equilibrated with 20 mM Tris buffer (pH 7.4) and eluted using the same buffer with NaCl (0.05–1 M) at a flow rate of 2 ml/min. The fractions were monitored by absorbance at 280 nm and by laccase activity. The active fractions were pooled and concentrated by ultrafiltration. The buffer was changed to 20 mM Tris buffer (pH 7.4). This solution was applied to a gel filtration column (Sephacryl S-200 HR column, 1.6 cm × 90 cm, GE Healthcare) with the same buffer containing 0.1 M NaCl at a flow rate of 1 ml/min. The protein concentration was determined using the Bio-Rad protein assay (Hercules, CA) with bovine serum albumin as the standard.

2.7. Deglycosylation

The purified recombinant laccase was treated with 0.5 IU of endoglycosylase H (Endo H, New England Biolabs, Beverly, MA) for 24 h at 37 °C according to the manufacturer's instructions. For the zymogram analysis, the denaturing process prior to the Endo H treatment was eliminated.

2.8. Gel electrophoresis, zymography and spectrum

SDS–PAGE was performed using 10% (w/v) polyacrylamide gels with a Mini-Protein III system (Bio-Rad). The proteins were visualized in the gel by Coomassie blue and Pierce glycoprotein staining kit (Thermo, Rockford, IL). The zymography was performed according to the method of Larrondo et al. [23]. The absorption spectrum of 0.5 mg ml^{-1} laccase was measured from 200 to 750 nm at 25 °C in 20 mM Tris buffer, pH 7.4.

2.9. Laccase characterization

The optimum temperature and pH were determined by performing enzymatic assays at different temperatures (30–75 °C) and pH levels (2.5–8.0). The pH level was adjusted using the following buffers: McIlvaine's citrate-phosphate buffer (pH 2.5–7.0), phosphate buffer (pH 7.0–8.0) and ammediol buffer (pH 8.0–10.0). The pH stability was examined after the enzyme was incubated at a pH for 24 h at 25 °C. The thermostability of laccase was investigated at pH 5.0 and various temperatures for 9 h.

The effects of various concentrations of EDTA, TGA, sodium azide, SDS, β-mercaptoethanol and DTT on the activity of laccase were evaluated with the routine ABTS assay. The effect of inhibitors on the laccase activity was investigated by determining the residual activity of the mixture.

The organic solvent-tolerance test was performed at 25 °C for 3 h in the presence of 10 and 50% (v/v) of several organic solvents, including acetonitrile, acetone, ethanol, methanol, DMF and DMSO. The activity was assayed as described above with ABTS.

The kinetic parameters of the purified laccase were calculated from the initial oxidation rates of 15.6–500 μM ABTS, 15.6–1000 μM DMP and 250–2000 μM guaiacol. The reactions were carried out in McIlvaine's buffer (pH 2.5 for ABTS; pH 3.0 for DMP and guaiacol) at 30 °C. All of the kinetic studies were performed at least three times, and the kinetic data were fitted to hyperbola using the Michaelis-Menten equation.

2.10. Nucleotide sequence accession numbers

The complete genomic DNA and mRNA sequences of the laccase gene from *G. fornicatum* 0814 were submitted to the GenBank database under the accession numbers DQ914875 and DQ914876, respectively.

3. Results and discussion

3.1. Cloning of G. fornicatum 0814 laccase gene and its deduced amino acid sequence analysis

GPF1 and GPR1 were designed according to 5′UTR and the 3′ end of the known laccase-gene sequence of *G. lucidum*. The resultant PCR products using *G. fornicatum* 0814 genomic DNA as template were about 2 kb in size. The RT-PCR products of the laccase gene, *lac1*, were about 1.5 kb in size. The alignment of the cDNA and *lac1* of *G. fornicatum* 0814 shows *lac1* consists of 2091 bp in the coding region, including nine introns. The intron loci of *G. fornicatum* 0814 laccase-gene sequence were similar to those of *G. lucidum*. The intron lengths (57, 51, 56, 62, 61, 55, 58, 54 and 63 bases) are typical of fungal introns (49–85 bases) [24].

The characteristics of the deduced amino-acid sequence of the *lac1* gene are shown in Fig. 1. The cDNA open reading frame encodes a putative pre-protein of 521 amino acids. The predicted 21 amino-acid signal sequence was identified on the basis of the occurrence of the consensus peptidase recognition site consensus, Ala-X-Ala [25]. The N-terminal amino acid sequence of most fungal laccases (A/G-I-G-P-V/T) appears to be conserved [26,27]. The amino acid sequence of Lac1 was similar to those of laccases from other white rot fungi, such as *G. lucidum* (89%), *Flammulina velutipes* (79%), *Lentinus tigrinus* (76%), *Pycnoporus cinnabarinus lac1* (75%) and *T. versicolor lcc1* (74%). All of the amino-acid sequences contain five conserved Cys and ten His residues, which are vital features of known laccases [28]. Based on the structure model using *L. tigrinus* laccase (PDB id: 2QT6) as the template, Cys-106 and Cys-510 in Lac1 form a disulfide bridge. Cys-138 and Cys-227 also form a disulfide bridge, and the conserved residue Cys-475 is involved in the ligation of type I copper. The residue that is located ten amino acids downstream of the conserved cysteine in the laccase

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MARLQSLLPYFSLLLAASAYAAIGPVANLTISDAEIAPDGFTRAAVVVNG 50
VSPGPLIKGNKGDRFQINVVNQLTNHTMSKTTSIHWHGLFQEGTNWADGP 100
AFVTQCPIASGNSFLYDFRVPDQAGTFWYHSHLSTQYCDGLRGPLVVYDP 150
HDPLAHMYDVDDDSTVITLTEWYHTAAQLGRRFPVGDANSTLINGLGRST 200
ATPTADLAVVNVTQGKRYRFRLVSMACDPSFNFSIDGHDLTVIEADGVET 250
QPVTVSTITIFAAQRYSFVLTANQTIDNYWIRANPAFGDVGFAGGLNSAI 300
LRYDGAAPIEPVTSQQSTQILLNETDLHPYVPKKTPGKPKKGGVDLALNM 350
VFGFNTTNFFINNATFVPPTVPVLLQILSGAQSAQDLLPAGSVYTLPKNA 400
SIEITFPANANAAGSPHPFHLHGHTFAVVRSAGSTAYNYDNPVWRDTVST 450
GLASANDNVTIRFQTDNPGPWFLHCHIDFHLNAGFAVVLAEDVPDVAYAN 500
PVPQEWKNLCPTYDALSPNDQ* 522
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Fig. 1. The deduced amino-acid sequences of the laccase from *G. fornicatum*. The dotted line indicates the signal peptide. The possible N-glycosylation sites are highlighted in gray. The straight lines and the associated numbers indicate the conserved residues and the type of copper binding, respectively. The residue indicated by the arrow most affects the redox potential of the cupric ion.

has a major effect on the redox potential of the cupric ion [29]. Xu et al. proposed that laccases harboring phenylalanine residues at this position featured Type I copper centers which demonstrated high redox potential [30]. In this study, the ten amino acids downstream of the conserved Cys residue existing within the laccases encoded by the *lac1* are phenylalanines, suggesting that the laccases from *G. fornicatum* 0814 may demonstrate high redox potential and good ligninolytic activity. The laccase sequence from *G. fornicatum* 0814 features eleven N-glycosylation sites (N-X-S/T), whereas no O-glycosylation was found. The NetNGlyC 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) predicts nine potential N-glycosylation sites for Lac1 (N28, N75, N189, N211, N273, N323, N355, N399 and N458 residues).

3.2. Overexpression of laccases in P. pastoris

The transformants were screened by the formation of green halos on minimal methanol plates supplemented with ABTS at 30 °C. Transformants with dark green halos were chosen and expressed in shaken flasks. The clone expressing the highest level of laccase activity, designated as P4-4, was used for the remainder of the study. The activity of P4-4 subsequent to a 7-day induction reached $3.46 \pm 0.09\,U\,ml^{-1}$, which is 29 times higher than the activity of *G. fornicatum* 0814 native laccase ($0.12 \pm 0.01\,U\,ml^{-1}$). Low yields of recombinant laccases have been reported in yeasts and filamentous fungi *Trichoderma reseei* and *Aspergillus* sp. [9,21,27,31,32], due to the difficulty of heterologous expression in an active form [9,31].

3.3. Protein purification and characterization

The purification of the recombinant laccase from the culture medium is summarized in Table 1. The purified enzyme appeared as a single band on the SDS–PAGE gel (Fig. 2A). The theoretical molecular mass of the unglycosylated laccase, as calculated from the amino acid sequence, is 54.1 kDa. However, the molecular mass is 63.7 kDa, determined by SDS–PAGE. The difference in the molecular mass is due to its glycosylation as evidenced by glycostaining (Fig. 2B). After treatment with Endo H, the protein had an appar-

Table 1
Purification of rLac1.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery[a] (%)	Purification factor[b]
Culture filtrate	5763	367.20	15.69	100	1
Ultrafiltrate	5029	267.17	18.82	87.3	1.2
Q-Sepharaose XL	1352	31.23	43.29	23.5	2.8
Sephacryl S-200	941	15.87	59.27	16.3	3.8

[a] All recovery values are expressed in terms of activity units in the crude taken as 100%.
[b] The data obtained is a mean of three purifications.

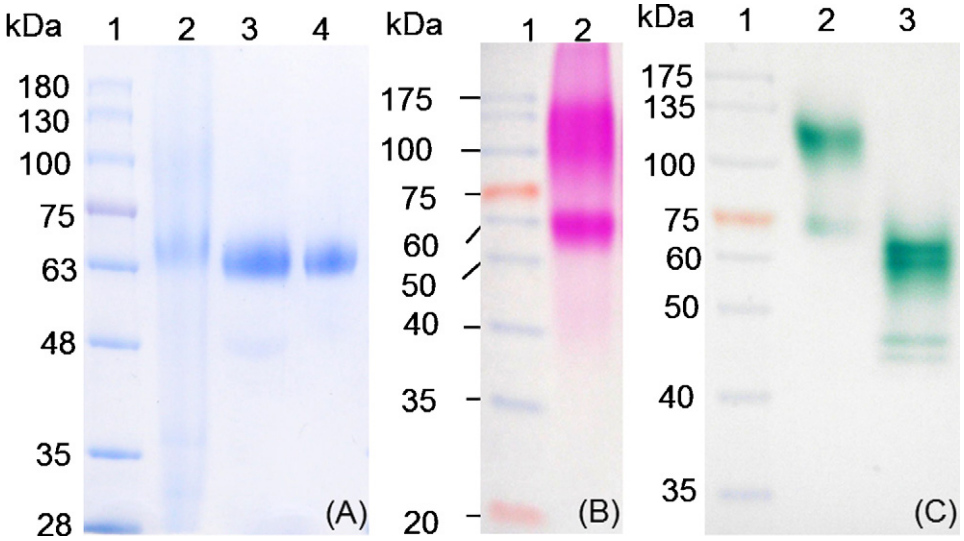

Fig. 2. SDS–PAGE results of samples collected from the purification steps, stained with Commassie Blue (A), glycoprotein stain (B) and zymogram stain with ABTS as a substrate (C) of rLac1, purified or deglycosylated with Endo H. (A) Lane 1, molecular weight markers; lane 2, 6 μg of ultrafiltrate; lane 3, 3.8 μg of the concentrated active fractions from the Q-Sepharaose column; lane 4, 1.3 μg of purified rLac1. (B) Lane 1, molecular weight markers; lane 2, purified rLac1 (1 μg). (C) Lane 1, molecular weight markers; lane 2, purified rLac1 (0.1 μg); lane 3, purified rLac1, deglycosylated with Endo H (0.1 μg).

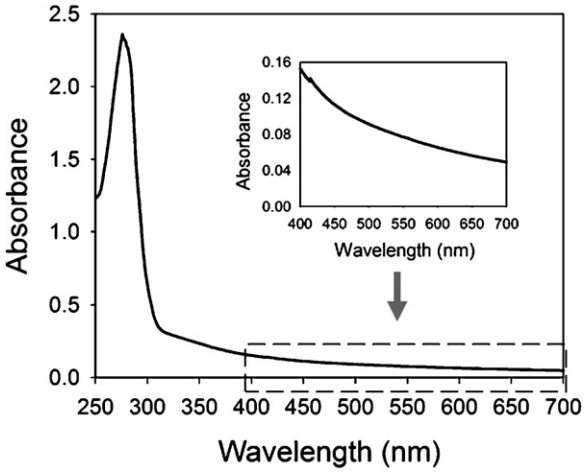

Fig. 3. UV/visible spectrum of purified rLac1.

ent molecular mass of 55 kDa, suggesting the presence of around 15.8% carbohydrate. The carbohydrate content of fungal laccases range from 10% to 25% [33]. The zymography of recombinant laccases is shown in Fig. 2C. The theoretical isoelectric focusing (pI) is 5.3. Glycosylation was found to play an important role to the enzyme stability, which protected enzyme from proteolytic degradation [34]. However, the glycosylation of fungal laccases is a major factor affecting the yield in overexpression [9,33].

The concentrated enzyme solution is yellowish-brown. The absorption spectrum of the purified laccase in the range 200–700 nm lacked the typical absorbance peak at 610 nm of type-I copper blue laccases (Fig. 3). The A_{280}/A_{610} ratio of 36 was higher than those typically found for blue laccases (15–20) [35]. These results suggest the recombinant laccase is a yellow laccase rather than a blue one. Yellow laccases are able to catalyze the oxidation of non-phenolic aromatic compounds in the absence of exogenous mediators [35,36].

3.4. Characteristics of the recombinant laccase

The optimal pH and temperature for the rLac1 using ABTS, DMP and guaiacol as substrates is shown in Fig. 4. The optimum pH of rLac1 was 2.5, 3.0 and 3.0 for ABTS, DMP and guaiacol, respectively. The difference in the optimal pH for ABTS and phenolic substances, such as DMP and guaiacol, is typical for laccases and reflects the different oxidation mechanisms that depend on the substrate [37]. Independent of the substrates, the optimal temperature of rLac1 was 55 °C, which is much higher than that for *G. lucidum* (20 °C) [15].

The thermostability and pH stability of rLac1 is illustrated in Fig. 5. rLac1 retained more than 80% of its activity in solutions with pH ranging from 2.5 to 10.0 after a 24 h incubation at 25 °C. The thermostability was investigated at pH 5.0 for 9 h at temperatures between 40 °C and 70 °C. rLac1 retained more than 70% of its activity after a 3 h incubation at temperatures up to 60 °C, but the activity decreased to less than 20% at 70 °C. rLac1 maintained at least 80% residual activity after a 9-h incubation at 50 °C. Most of the currently known laccases are stable at pH between 6.0 and 7.0, but experience a tremendous loss in their enzyme activity if subjected to a more-acidic or more-alkaline environment [33,38]. Only a laccase isozyme from *G. lucidum*, GaLc3, has been reported to be stable at 20 °C for 1 h in the pH range from 4.0 to 10.0 [15]. The rLac1 revealed greater than 95% relative activity, in variant pH ranging from 3 to 10 for 24 h at 25 °C.

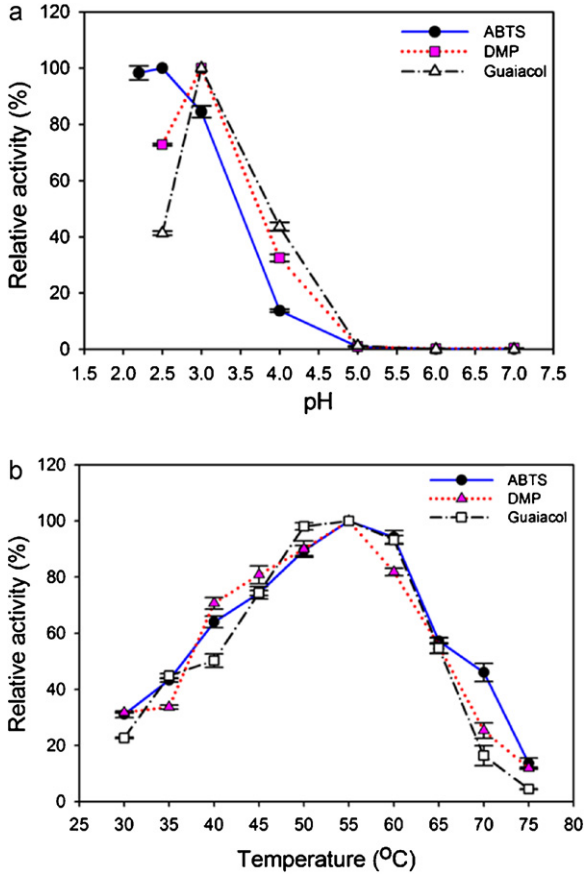

Fig. 4. The optima pH (A) and temperature (B) of the recombinant laccases rLac1. (A) The effect of pH on the laccase activity was measured in McIlvaine's buffer (pH 2.5–7.0) at 30 °C. The activity of 100% was that which was measured at the optimal pH. (B) The effect of temperature on the laccase activity was measured at the optimal pH from 30 to 75 °C. The activity of 100% was that which was measured at the optimal temperature. Each point represents the mean ± SD ($n = 3$).

We also evaluated the effects on the activity of recombinant laccase of water-miscible organic solvents (acetonitrile, acetone, ethanol, methanol, DMF and DMSO), which are used in a variety of industrial applications, on the activity of recombinant laccase. The effect of various organic solvents on laccase activity was investigated using ABTS as the substrate due to its stable radical cation in water/organic solvent mixtures in water-organic solvent mixtures [39]. The recombinant laccase showed a good stability in the presence of 10% (v/v) organic solvents. The rLac1 retained more than 80% activity in 50% (v/v) ethanol, methanol, DMF and DMSO after a 3 h incubation at 25 °C, whereas its activity was <20% in acetone and acetonitrile (Fig. 6).

Table 2 lists the effects of various enzyme inhibitors on the laccase activity. rLac1 activity was most inhibited by 0.05 mM sodium azide and DTT, β-mercaptoethanol and SDS at concentrations of 2.5 mM. No significant activity loss was observed when 5 mM of EDTA was applied, whereas the copper chelator TGA at 2.5 mM inhibited the activity significantly, suggesting that the copper ion plays an important role in regulating the activity of rLac1.

3.5. Kinetics of the recombinant laccase

The kinetic parameters of rLac1, obtained using ABTS, DMP and guaiacol as substrates are summarized in Table 3. The K_m value was 103.9, 276.7 and 1263.4 μM for ABTS, DMP and guaiacol, respectively. The kinetic parameters obtained for ABTS, DMP and guaiacol fall within the ranges reported for fungal laccases [33]. The

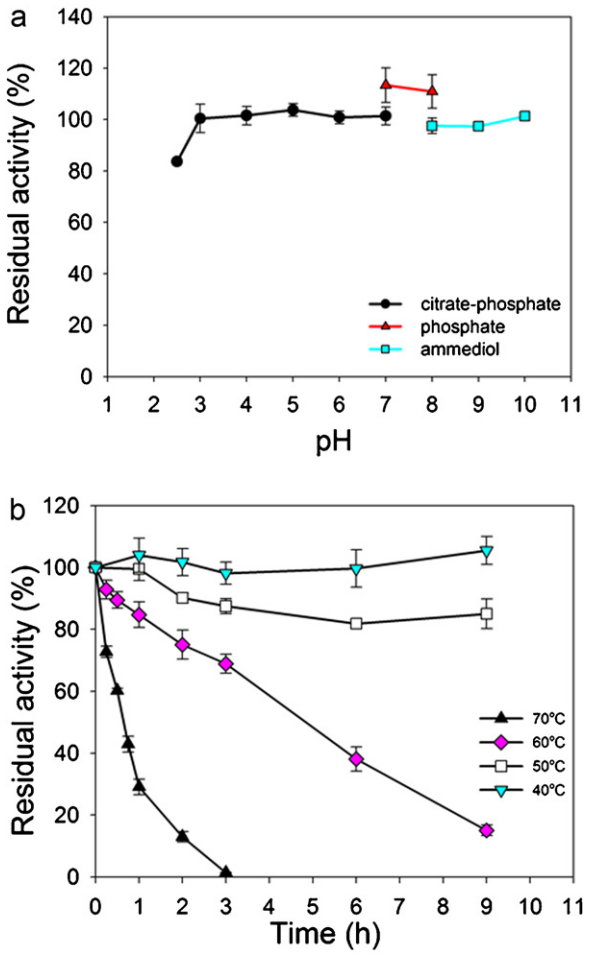

Fig. 5. The effect of pH stability (A) and thermal stability (B). (A) The laccase activity at 0.1 M citrate buffer (pH 3.0) and 30 °C was set as 100% = 6.1 ± 0.2 U ml^{-1}. (B) The laccase activity in 0.1 M citrate buffer (pH 3.0) and 30 °C was set as 100% = 10.0 ± 0.3 U ml^{-1}.

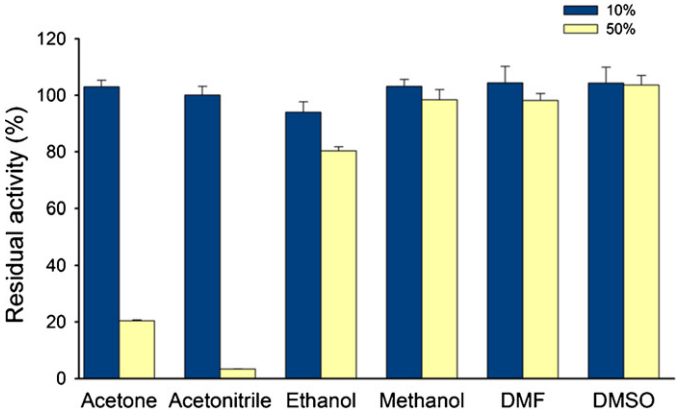

Fig. 6. The organic solvent-tolerance of the recombinant laccases rLac1. The laccase activity in 0.1 M citrate buffer (pH 3.0) and 30 °C was set as 100% = 10.0 ± 0.2 U ml^{-1}.

substrate binding preference varies significantly among fungal laccases. The highest k_{cat} was found for ABTS. The K_m was significantly lower than that for DMP and guaiacol. The purified rLac1 oxidized ABTS more efficiently than DMP and guaiacol. The introduction of a second methoxyl group as found in DMP, resulted in an almost 35-fold increase in k_{cat}/K_m as compared to the monomethoxylated substrate guaiacol. This increase can be explained by the strong electron-donating effect of the two methoxyl substituents and the favorable redox potential of the substrate DMP [40]. The enzyme may have specific interactions with these substrates, which may be correlated to their structures.

Table 2
The effect of inhibitors on the activity of rLac1.

Inhibitor	Concentration (mM)	Residual activity (%)[a]
None	–	100
Sodium azide	0.0005	68.4 ± 2.2
	0.005	20.0 ± 2.4
	0.05	3.4 ± 0.3
DTT	0.05	84.9 ± 1.0
	0.5	30.4 ± 1.3
	2.5	0.7 ± 0.1
β-Mercaptoethanol	0.05	95.4 ± 1.7
	0.5	60.4 ± 2.1
	2.5	1.6 ± 0.2
SDS	0.05	99.6 ± 1.0
	0.5	37.3 ± 1.9
	2.5	1.2 ± 0.2
EDTA	0.5	99.9 ± 0.5
	2.5	95.2 ± 0.5
	5	89.2 ± 1.3
TGA	0.05	97.4 ± 1.4
	0.5	73.6 ± 1.2
	2.5	7.4 ± 0.8

[a] Residual activities (%) were measured using ABTS as the substrate after adding each inhibitor to the assay mixture to reach the final inhibitor concentrations. The values are presented as the mean ± SD of triplicate tests.

Table 3
The kinetic parameters of rLac1.[a]

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})
ABTS	103.9 ± 6.5	65.6 ± 4.7	0.63 ± 0.03
DMP	276.7 ± 25.0	38.5 ± 1.8	0.14 ± 0.02
Guaiacol	1263.4 ± 147.9	5.0 ± 0.4	0.0039 ± 0.0002

[a] The molecular weight of rLac1 was 63,700 Da, which was used to calculate the k_{cat} (s^{-1}) values. All of the values were calculated by the linear regression (correlation coefficient ≥ 0.99) of double reciprocal plots, $1/v$ vs. $1/[S]$, from every set of triplicate measurements.

In summary, this study reports the full-length laccase gene from *G. fornicatum* and expression in *P. pastoris*. The laccase of *G. fornicatum* is a novel yellow laccase with the characteristics of thermal and pH stability as well as organic solvent-tolerance. Although the structure of the active site appears conserved in most of the fungal laccases, these enzymes vary significantly in their properties. It is proposed that glycosylation of proteins plays important roles in the biochemical properties of the proteins. To better understand the structure-function relationship of rLac1, the three-dimensional structure of this enzyme is being solved.

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