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MOLECULAR CLONING AND BIOCHEMICAL CHARACTERIZATION OF RECOMBINANT LACCASE FROM *RIGIDOPORUS VINCTUS*

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Key words: laccase, *Rigidoporus vinctus*, three-dimension (3-D) structural model, ABTS [2,2'- azinobis- (3- ethylbenzothiozone-6- sulphonic acid)]

ABSTRACT

Laccases are multi-copper oxidases that widely distributed in plants and fungi. These enzymes catalyze oxidation of various compounds including phenolics and non-phenolics, and have been used in many industrial processes such as paper industry, biobleaching and bioremediation. A full length cDNA (1767 bp) encoding a putative laccase (Lac) from *Rigidoporus vinctus* was cloned by polymerase chain reaction (PCR). The coding region of RvLac encodes 517 amino acids which contained four conserved putative copper-binding regions. To further characterize the RvLac, the coding region was subcloned into an expression vector pET-20b(+) and transformed into *E. coli* BL21 (DE3) pLysS. Expression of the Lac was induced by IPTG and the recombinant His-tagged Lac was purified by Ni^{2+} -nitrilotriacetic acid Sepharose superflow column. The purified enzyme was revealed as a single band on SDS-PAGE with molecular mass of ~57 kDa. Furthermore, the enzyme activity and kinetics were determined by ABTS assay. The Michaelis constant value for ABTS is 0.07 mM. The enzyme has a half-life of 4.6 min at 75°C. The enzyme is active under a pH 2 treatment for 30 min.

I. INTRODUCTION

Laccases are multi-copper oxidases belonging to a group of polyphenol oxidases. They are widely distributed in plants and fungi (Baldrian, 2006). The enzymes catalyze oxidation of a broad range of substrates including phenolic compounds as well as non-phenolic compounds (Bourbonnais and Paice, 1990; Solomon et al., 1996; Baldrian, 2006) by a radical-catalyzed reaction mechanism (Claus, 2004). Laccases cannot oxidize non-phenolic compounds directly due to the lower redox potentials than those of phenolic compounds. However, laccases can oxidize non-phenolic compounds in the presence of small molecules (known as electron transfer mediators) that are capable of shuttling electrons between the non-phenolic compounds and the laccases (Bourbonnais and Paice, 1990). The reactive radicals (produced by laccases or laccase-mediator systems) can undergo non-enzymatic reactions such as cross-linking of monomers, degradation of polymers, and ring cleavage of aromatics (Claus, 2004).

Laccases are useful biocatalysts for diverse biotechnological applications due to its high stability, ability to catalyze the oxidation of various compounds, including: benzenediols, aminophenols, polyphenols, polyamines, and lignin-related molecules under mild reaction conditions using aerial oxygen as an oxidant (Rodríguez Couto and Toca Herrera, 2006; Widsten and Kandelbauer, 2008; Madhavi and Lele, 2009). Laccases along with laccase-mediator systems (LMS) have been used in many industrial applications such as pulp and paper industry (Widsten and Kandelbauer, 2008), textile industry (Rodríguez Couto, 2012), food industry (Minussi et al., 2002; Selinheimo et al., 2006), biosensors (Haghighi et al., 2003) and nanobiotechnology, bioremediation (e.g., clean up xenobiotics herbicide, pesticide and certain explosives in soil, waste detoxification) (Durán and Esposito, 2000), and used in cosmetics (Golz-Berner, 2004). Laccase has also been used in synthetic applications as an oxidative catalyst in green chemistry (Solomon et al., 2001; Hajdok et al., 2009; Witayakran and Ragauskas, 2009).

Rigidoporus vinctus belonging to a wood-degrading microorganism of white rot fungus is a plant pathogen that causes the death of trees. The fungus grows slowly within living tissue but once the tissue is killed, a white spongy wood decay

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rapidly develops. The fungus can spread from tree to tree by root contact. It can also be spread by movement of logs and firewood that have decay and basidiospore producing basidiomes on them. Long distance spread is probably by wind-blown spores (Hood and Dick, 1988).

Here we report the first cloning of a Lac from *Rigidoporus vinctus*, a fungal species collected at the bank of a small stream (water pH 8.17) near the entrance of Aowanda National Forest Recreation Area (N23°57′452″ E121°09′512″, H 661m), Nantou County, Taiwan. The coding region of the cDNA was introduced into an *E. coli* expression system. The functional target protein was expressed, purified, and its properties studied.

II. MATERIALS AND METHODS

1. Total RNA Preparation from *R. vinctus* and cDNA Synthesis

R. vinctus was obtained from Dr. Ka-Lai Pang (Institute of Marine Biology, National Taiwan Ocean University, Keelung 202, Taiwan). Five grams mycelium of *R. vinctus* (wet) were frozen in liquid nitrogen and ground to powder in a ceramic mortar. PolyA mRNA (8 μg) was prepared using Novagen's Straight A's mRNA Isolation System (Gibbstown, NJ, USA). Four micrograms of the mRNA were used in the 5'-RACE-Ready cDNA and 3'-RACE-Ready cDNA synthesis using Clontech's SMART RACE cDNA Amplification Kit (Mountain View, CA).

2. Isolation of Laccase cDNA

Using the *R. vinctus* 5'-RACE-Ready cDNA as a template and degenerate primer pair Lac-16 (5' CAY TGG CAY GGC YTB TTC CA 3') & Lac 17 (5' SAR RTG SMA GTC RAT GTG GC 3'), a 1.2 kb fragment was amplified by PCR. The degenerate primers were designed based on the conserved sequences of ThLac (*Trametes hirsute*, ACC43989), GlLac (*Ganoderma lucidum*, ACR24357), TvLac (*Trametes versicolor*, CAA59161), PrLac (*Phlebia radiata*, CAA36379), PgLac (*Polyporus grammocephalus*, ACR24358). The 1.2 kb fragment was subcloned and sequenced. Based on this DNA sequence, another two primers: a RvLac-4R (5' CAA GTG ACT GTG ATA CCA G 3') and a RvLac-6F (5' ATG ACC TTT CGC GCT GCA TT 3') were synthesized. The primers allowed sequence extension from 5' end of the 1.2 kb fragment by using the *R. vinctus* 5'-RACE-Ready cDNA as a template and UPM and RvLac-4R primer pair; a 0.5 kb fragment was amplified by PCR. The primers also allowed sequence extension from 3' end of the 1.2 kb fragment by using the *R. vinctus* 3'-RACE-Ready cDNA as a template and RvLac-6F and UPM primer pair; a 1.5 kb fragment was amplified by PCR. Each of the 0.5 and 1.5 kb DNA fragment was subcloned into pCR4 vector and transformed into *Escherichia coli* TOPO10. The nucleotide sequences of these inserts were determined in both strands. Sequence analysis revealed that the combined sequences covered an open reading frame of a putative RvLac cDNA (1767 bp, JN166085).

3. Bioinformatics Analysis of RvLac Sequence

The identity of the RvLac cDNA clone was verified by comparing the inferred amino acid sequence in various databases using the basic local alignment search tool (BLAST) and DELTA BLAST (Boratyn et al., 2012). Multiple alignments were constructed using ClustalW2 program. Protein secondary structure was predicted by SWISS-MODEL program and represented as α helices and β strands. A 3-D structural model of RvLac was constructed by SWISS-MODEL (Arnold et al., 2006) (http://swissmodel.expasy.org/SWISS-MODEL.html) based on the known crystal structure of *Trametes hirsuta* Lac (ThLac, PDB ID: 3PXL).

4. Subcloning of RvLac cDNA into an Expression Vector

The coding region of the RvLac cDNA was amplified using gene specific flanking primers. The 5' upstream primer contains *Eco*RI recognition site (5' GAATTC GAT GAC CTT TCG CGC TGC ATT 3') and the 3' downstream primer contains *Not* I recognition site (5' GCGGCCGC TTT GTC GCC TTC GGG CAG 3'). Using 0.2 μg of 5'-RACE-ready cDNA as a template, and 10 pmole of each 5' upstream and 3' downstream primers, a 1.5 kb fragment was amplified by PCR. The fragment was ligated into pCR4-TOPO and transformed into *E. coli*. The recombinant plasmid was isolated and digested with *Eco*RI and *Not* I. The digestion products were separated by a 0.8% agarose gel. The 1.5 kb insert DNA was gel purified and subcloned into *Eco*RI and *Not* I site of pET-20b(+) expression vector (Novagen). The recombinant DNA (pET-20b(+)-RvLac) was then transformed into *E. coli* BL21(DE3)pLysS. The recombinant protein was expressed in the *E. coli* expression system.

5. Expression and Purification of the Recombinant RvLac

The transformed *E. coli* containing the RvLac gene was grown at 37°C in 20 mL of Luria-Bertani supplemented with 55 μg/mL ampicillin and 12.5 μg/mL chloramphenicol until A_{600} reached 0.6. Protein expression was induced by the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM. The culture was incubated at 130 rpm for an additional 2 h at 25°C. The cells were harvested and soluble proteins extracted in 0.2 mL1 × PBS with glass beads for 5 times. The 1 mL extract recombinant RvLac was purified by Ni-NTA affinity chromatography (0.2 mL elution buffer for each fraction: 30% PBS containing 5% glycerol and 20-250 mM imidazole) as per the manufacture's instruction (Qiagen). The purified protein was checked by a 10% SDS-PAGE. Proteins on gel were detected by staining with Coomassie Brilliant Blue R-250. Protein concentration was determined by a Bio-Rad Protein Assay Kit (Richmond, CA) using bovine serum albumin as a reference standard.

6. Activity Assay and Kinetic Studies of the Recombinant RvLac

RvLac activity was determined by measuring 2,2'-azino-bis

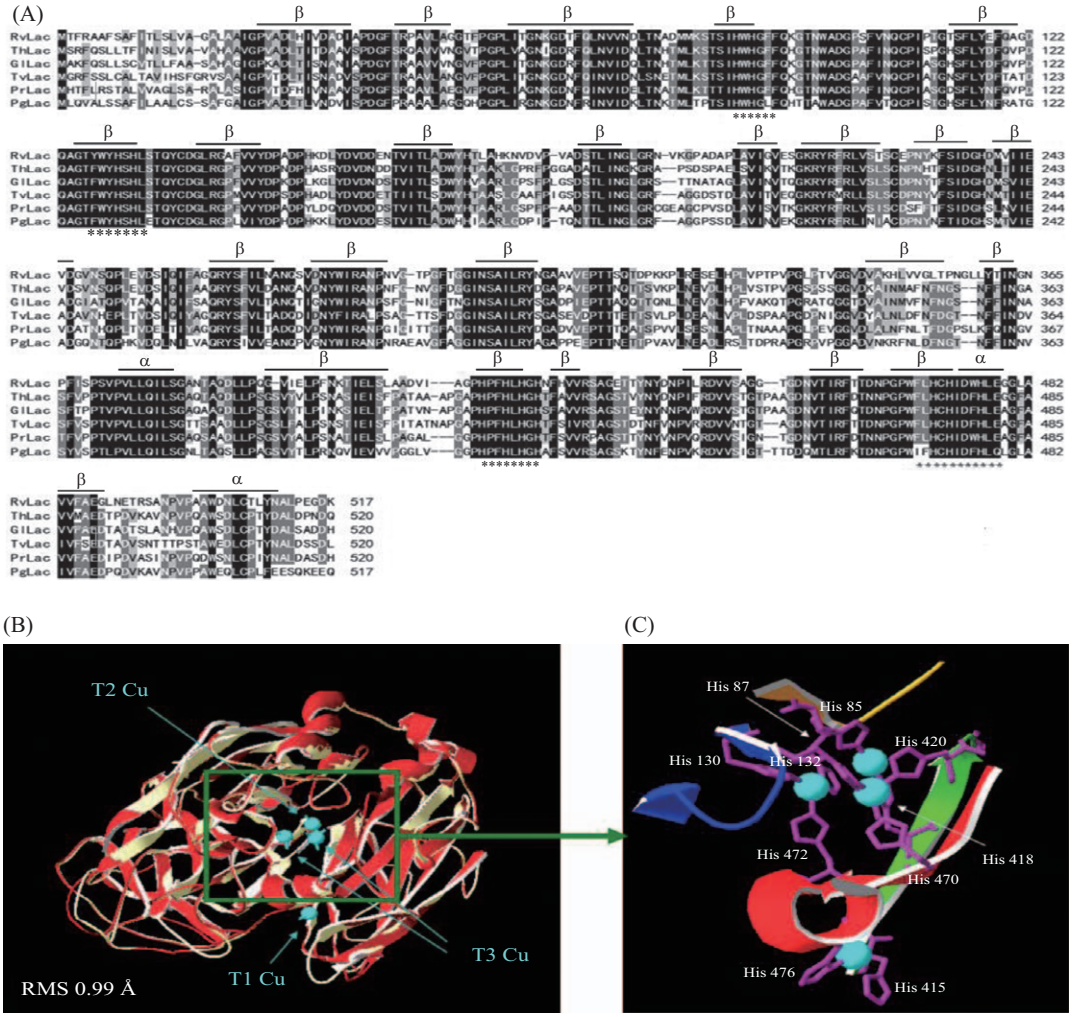

Fig. 1. Alignment of the amino acid sequences of RvLac with other organism's Lacs and 3-D structural model. (A) Sequence alignment: RvLac (this study), ThLac (*Trametes hirsute*), PrLac (*Phlebia radiata*), GlLac (*Ganoderma lucidum*), TvLac (*Trametes versicolor*), PgLac (*Polyporus grammocephalus*). Conservative replacements are shaded gray. Protein secondary structure was predicted by SWISS-MODEL program and predicted α helices and β strands are indicated. (B) A 3-D structural model of RvLac was modeled based on the known X-ray structure of *Trametes hirsuta* Lac (ThLac, PDB ID: 3PXL) via SWISS-MODEL program and was superimposed with *Cerrena maxima* (CmLac, PDB ID: 3DIV) (orange) to obtain a better structure via SPDBV_4 program. Superimposition of RvLac (white) and CmLac (orange) was shown using protein solid ribbons. The copper centers (T1Cu, T2Cu , T3Cu) are shown in blue. Putative copper binding residues (Fig. 1(C)) of RvLac are colored in light yellow (H^{85} W H^{87} G F F), deep blue (Y W Y H^{130} S H^{132} L), green (H^{415} P F H^{418} L H^{420} G H), and orange (F L H^{470} C H^{472} I D W H^{476} L E), respectively.

(3-ethylbenzothiozone-6-sulphonic acid) (ABTS) oxidation. The assay was first tested under various pH (2.0, 4.0, 6.0, 7.0, or 8.0) to find the optimal pH condition. The optimal pH appeared to be 2.0, thus most assays were performed at pH 2.0. The reaction mixture (100 μL) contained 0.1 M citric acid buffer (pH 2.0), 0.3 mM ABTS. The reaction was started by the addition of 3 μg/7 μL (0.53 μM) RvLac. The reaction was monitored by the increase in A_{420} due to the oxidation of ABTS (Lu et al., 2012).

The kinetic properties of the RvLac (3 μg/7 μL) were determined by varying the concentrations of ABTS (0.05 to 0.11 mM). The change in absorbance at 420 nm was recorded between 10 sec and 40 sec. The molar absorption coefficient of ABTS at 420 nm is 3.6×10^4 M^{-1} cm^{-1}. The K_M, V_{max} and k_{cat} were calculated from Lineweaver-Burk plots.

7. Biochemical Characterization

The stability of RvLac under various conditions was studied by assaying its ability to oxidize ABTS as described above. Aliquots of the RvLac sample were tested for: (1) *Thermal effect*. Enzyme sample (3 μg/7 μL) enzyme in 0.4% PBS containing 5% glycerol and 0.3 mM imidazole per reaction) was heated at 75°C for 2, 4, 8 or 16 min. (2) *pH effect*. The enzyme was adjusted to desired pH by adding a half volume of buffer with different pHs: 0.2 M citrate buffer (pH 2.5, or 4.0), 0.2 M Tris-HCl buffer (pH 6.0, or 8.0) or 0.2 M glycine-NaOH

buffer (pH 10.0). Each sample was incubated at the designated pH at 37°C for 30 min. After each treatment, the residual RvLac activity was tested .

III. RESULTS

1. Cloning and Characterization of a cDNA Encoding RvLac

A putative RvLac cDNA clone was identified based on its sequence homology to the published Lacs in NCBI website. The coding region of RvLac cDNA was 1551 bp that encodes a protein of 517 amino acid residues with a calculated molecular mass of 55.6 kDa (GenBank accession JN166085). Theoretical pI/Mw is 5.02/556500. Fig. 1(A) shows the optimal alignment of the amino acid sequences of RvLac with five related Lac sequences from other sources. The RvLac shares 65% homology with ThLac (*Trametes hirsute*, accession no. ACC43989), 65% with PrLac (*Phlebia radiata*, accession no. CAA36379), 64% with GlLac (*Ganoderma lucidum*, accession no. ACR24357), 60% with TvLac (*Trametes versicolor*, accession no. CAA59161), and 58% with PgLac (*Polyporus grammocephalus*, accession no. ACR24358). The N-terminal end of the protein contains 21 amino acid residues (M T F R A A F S A F I T L S L V A G A L A) as putative signal peptide identified by SignalPa and PrediSi. The secondary structure (Fig. 1(A), represented as α helices and β strands) and a 3-D structural model (Fig. 1(B)) were predicted using SWISS-MODEL program. The 3-D structural model (Fig. 1(B), white) was constructed based on the known crystal structure of *Trametes hirsuta* Lac (ThLac, PDB ID: 3PXL). The model superimposed with *Cerrena maxima* (CmLac, PDB ID: 3DIV) (orange) via the SPDBV_4 program was shown using protein solid ribbons (Fig. 1(B)). The copper centers (T1Cu, T2Cu, T3Cu) are shown in blue. Putative copper binding residues (Fig. 1(C)) of RvLac are colored in light yellow (H^{85} W H^{87} G F F), deep blue (Y W Y H^{130} S H^{132} L), green (H^{415} P F H^{418} L H^{420} G H), and orange (F L H^{470} C H^{472} I D W H^{476} L E), respectively.

2. Expression and Purification of the Recombinant RvLac

The coding region of RvLac (1,551 bp) was amplified by PCR and subcloned into a *E. coli* expression vector, pET-20b(+) as described in the Materials and methods. Positive clones were verified by DNA sequence analysis. The recombinant RvLac protein was expressed and analyzed on a 10% SDS-PAGE in the absence of a reducing agent and without boiling (Fig. 2). The recombinant RvLac was expressed as a His6-tagged fusion protein and was purified by affinity chromatography with nickel chelating Sepharose. The purified RvLac protein appears as a single band on SDS-PAGE with molecular mass of ~57 kDa (expected size of RvLac) (Fig. 2, lane 3). The Ni-NTA eluted fractions were pooled and characterized further. The yield of the purified His6-tagged RvLac was ~68 μg from 20 mL of culture. Functional RvLac was detected by activity assay as shown below.

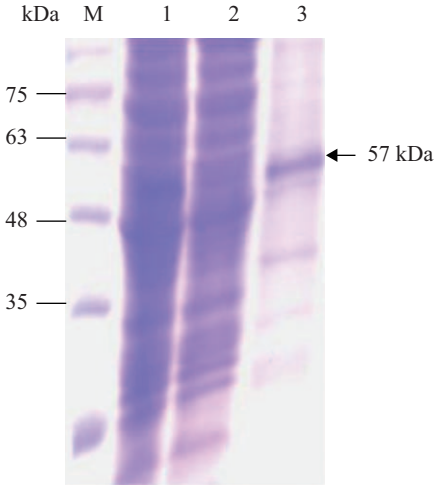

Fig. 2. Expression and purification of recombinant RvLac in *E. coli*. Fifteen μL (loading buffer without β-mercaptoethanol and without boiling) of each fraction was loaded into each lane of the 12% SDS-PAGE. Lane 1, crude extract from *E. coli* expressing RvLac; 2, flow-through proteins from the Ni-NTA column; 3, RvLac (fraction was 0.2 mL) eluted from Ni-NTA column. Molecular masses (in kDa) of standards are shown at left. Arrow indicated the target protein.

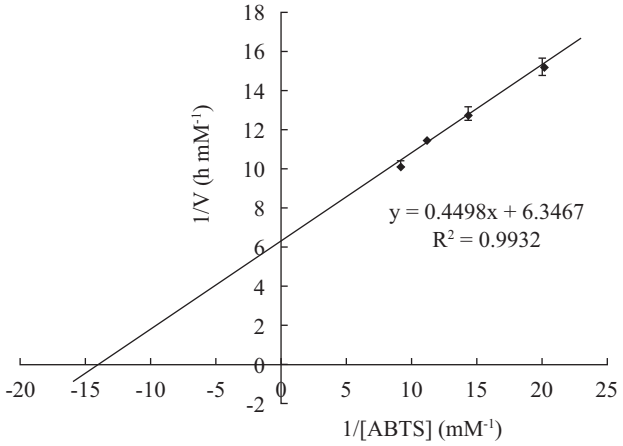

Fig. 3. Double-reciprocal plots of varying ABTS concentrations on RvLac activity. The initial rate of the enzymatic reaction was measured at 3 μg RvLac with the ABTS concentration varied from 0.05 to 0.11 mM. The K_M, V_{max} and k_{cat} calculated from the Lineweaver-Burk plots.

3. Characterization and Kinetic Studies of the Recombinant RvLac

The recombinant RvLac was used to catalyze oxidation of ABTS. Fig. 3 shows the ABTS consumption in the presence of the purified RvLac (3 μg/0.1 mL). As shown in Fig. 3, the Lineweaver-Burk plot of the velocity (1/V) against 1/[ABTS] gave the K_M = 0.07 mM, $V_{max} = 2.6 \times 10^{12}$ sec^{-1}, k_{cat} =0.084 s^{-1}.

To examine the effect of temperature on the RvLac activity, the enzyme was treated as described in the Materials and Methods and then analyzed for the residue RvLac activity.

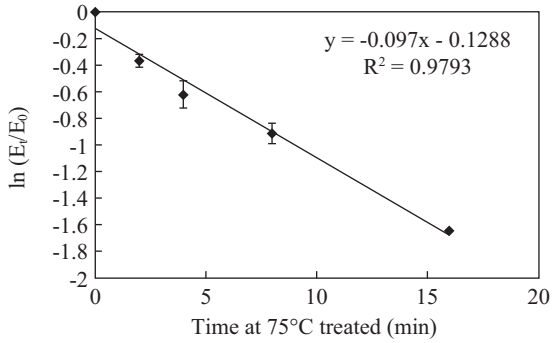

Fig. 4. Effect of temperature on the purified RvLac. The enzyme sample was heated at 75°C for various time intervals. Aliquots of the sample were taken at 0, 2, 4, 8 or 16 min and assayed for Lac activity. The thermal inactivation kinetics of RvLac activity was plotted (B). E_0 and Et are original activity and residual activity after being heated for different time intervals. Data are means of three experiments.

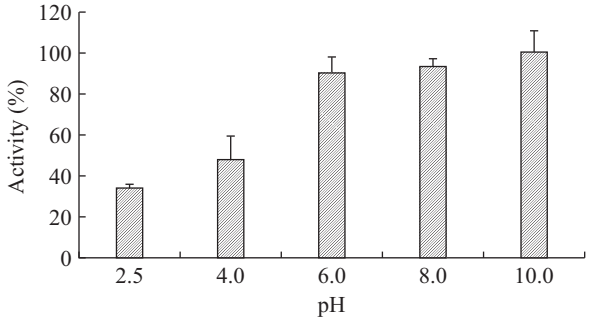

Fig. 5. The pH stability of the purified RvLac. The enzyme samples were incubated with different pH buffer at 37°C for 30 min and then assayed for Lac activity. Data are means of three experiments.

The RvLac's half-life of inactivation at 75°C was 5.8 min (Fig. 4). The RvLac activity was not effected by treating the enzyme for 30 min under pH range of 6-10 (Fig. 5). As mentioned above, pH 2 appeared to be the optimal pH for activity assay (the enzyme was stored at pH 7 and the pH was lowered to 2.0 at the time of activity assay), thus most assays were performed at pH 2.0. However, when treating the enzyme for a long period of time (i.e. 30 min) under low pH, the enzyme lost some activity (Fig. 5) presumably due to partially denaturation of the enzyme.

IV. DISCUSSION

In the present study, we have cloned, expressed, purified, and characterized a laccase from *R. vinctus*. Laccases from other organisms have been previously reported to play important roles in catalyzing the oxidation of many, particularly phenolic substances, and playing an important role in many cellular activities. The enzyme was stored at pH 7.0 and then adjusted to various for activity assay. This RvLac is most active at acidic pH like many reported fungal laccases (Baldrian, 2006). However, leaving the enzyme at low pH (ie. pH 2.5 and 4.0) for 30 min resulted in reduced activity (Fig. 5) presumably due to protein denaturation/unfolding under acidic pH. The activity was not effect by treating the enzyme for 30 min at pH range of 6-10 (Fig. 5).

The thermal stability of laccase varies greatly from strain to strain. The RvLac has a half-life of 4.6 min at 75°C. Laccase isolated from *Ganoderma lucidum* showed optimum temperature of 20-25°C and was found to be stable between 10-50°C for 4 hours (Ko et al., 2001). Laccases isolated from *Marasmius quercophilus* were found to be stable for 1 h at 60°C (Farnet et al., 2000). Laccase isolated from the spores of *Bacillus vallismortis* fmb-103 was found to be stable between 25-90°C with a maximum activity at 82°C and a half-life of 10 h at 70°C (Zhang et al., 2013). Laccases isolated from *Bacillus licheniformis* LS04 retained about 16% of the initial activity after 10 h at 80°C (Lu et al., 2012).

We compared the K_M values of the RvLac for ABTS with laccase from other organisms. As shown in Table 1, this RvLac has comparable K_M value but extremely low k_{cat} and k_{cat}/K_M. In view of the low k_{cat}, it is unlikely the enzyme will have any industrial value even though the enzyme is somewhat heat stable. However, the enzyme can be aided as a tool for structure-function studies of diverse laccases in probing amino acid residues that are important in the enzyme's activity and thermal stability.

Table 1. Kinetic analyses of RvLac and the other four Lacs. The kinetic parameters were determined as described in the Materials and Methods. The K_M value for ABTS was determined at 0.03 to 0.5 mM and 3 µg RvLac. Data represent the mean (±SE) of three separate experiments.

Protein	Substrate	K_M(mM)	k_{cat}(s^{-1})	k_{cat}/K_M
RvLac	ABTS	0.07	0.084	1.2
TvLac	ABTS	0.03	64	2461.53
ThLac	ABTS	0.07	197	2800
BpLac	ABTS	0.08	291	3637.5
PvLac	ABTS	0.20		

Values are from RvLac (this study), *Trametes versicolor* Lac (TvLac) (Jolivalt et al., 2005), *Trametes hirsuta* Lac (ThLac) (Haibo et al., 2009), *Bacillus pumilus* Lac (BpLac) (Reiss et al., 2911) and *Paraconinthyrium variabile* Lac (PvLac) (Forootanfar et al., 2011).

Abbreviations:

Lac, laccase; IPTG, isopropyl β-D-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate buffer saline.

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