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Full Length Research Paper

Purification and characterization of three laccase isozymes from the white rot fungus *Trametes* sp. HS-03

Weiyun Guo[1], Zhaoyang Yao[2], Chenyan Zhou[1], Duan Li[1], Hongli Chen[1], Qiang Shao[3], Zongyi Li[3] and Huigen Feng[1]*

[1]Department of Life Science and Technology, Xinxiang Medical University, Xinxiang 453003, People's Republic of China.
[2]School of Basic Medicine, Xinxiang Medical University, Xinxiang 453003, People's Republic of China.
[3]College of Life Science, Henan Normal University, Xinxiang 453007, People's Republic of China.

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Three laccase isozymes (LacI, LacII and LacIII) were isolated from the culture supernatant solution of *Trametes* sp. HS-03. Diethylaminoethyl (DEAE)-sepharose fast flow anion exchange chromatography and Sephadex G-100 size-exclusion chromatography was performed to achieve electrophoretic homogeneity. The molecular masses (64.2, 60.7 and 38.9 kDa), isoelectric points [pIs (7.3, 4.7 and 3.5), and N-terminal amino acid sequences (G-I-G-P-V, A-I-G-P-T and S-I-G-P-V) were found to be different for the three laccase isozymes. LacI and II have similar thermostability, while LacIII showed better thermostability. LacIII also showed optimal activity at 80°C, with a half-life of 125 min at 70°C. The p*I*-value of LacI and the molecular mass of LacIII differ significantly from previously described fungal laccases.

Key words: *Trametes* sp. HS-03, laccase isozymes, purification, characterization.

INTRODUCTION

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) form part of a larger group of multicopper oxidases that oxidize a wide variety of substrates with concomitant reduction of molecular oxygen to water (Thurston, 1994). Laccases are distributed widely in nature and found in higher plants, fungi, insects and bacteria (Montazer et al., 2009). However, laccases of fungal origin are the most well studied, particularly those found in white-rot fungi. Due to their interesting catalytic properties, laccases have broad potential in many industrial and environmental applications, including paper processing and the biotransformation of environmental pollutants such as pesticides, industrial dyes and effluents (Champagne and Ramsay, 2010; Hadibarata et al., 2012). They are used in the food industry, particularly in tea and coffee fermentation, and in vinification processes to improve wine quality by removing fermentation inhibitors so as to increase yield of ethanol (Baldrian, 2006). They have also been used in drug analysis, and even as a tool for medical diagnostics (Rodriguez and Toca, 2006).

It is well known that white-rot fungi secrete a variety of oxidative enzymes, including lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Isikhuemhen and Mikiashvili, 2009; Kim et al., 2012). These enzymes are polymorphic, and different isoforms with different properties are produced depending on environmental conditions and the fungal species (Baldrian, 2006). Although there have been many papers dealing with laccase-producing fungi, *Trametes* sp. is one of the best studied white-rot fungi, and is known to secrete several laccase isoforms. In this study, three laccase isozymes generated from *Trametes* sp. HS-03 were purified and characterized. These isozymes showed a number of different properties from previously reported laccases. The study therefore may be helpful to exploit the capabilities of laccases.

*Corresponding author. E-mail: xinmigwy@163.com or fenghuigen@xxmu.edu.cn. Tel: +86 373 3029889. Fax: +86 373 3029887.

Abbreviations: ABTS, 2,2'-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid); **SDS-PAGE,** sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **IEF–PAGE,** isoelectric focusing gel; **pIs,** isoelectric points.

MATERIALS AND METHODS

2,2'-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was purchased from Sigma. The chromatography media used [diethylaminoethyl (DEAE)-Sepharose fast flow and Sephadex G-100] were from Amersham Pharmacia Biotech. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) calibration proteins were from New England Biolabs and Bio-Rad. All other chemicals used were of analytical grade.

Microorganism and culture condition

Trametes sp. HS-03 isolated from a pulp mill wastewater effluent in Xinxiang ,Henan, China was inoculated on potato dextrose agar plates for 5 days at 28°C. Five mycelial agar blocks of 0.5 cm diameter taken from the growing fungal colonies were used as the inoculum in liquid cultures medium. The liquid culture medium contained: 1% (w/v) glucose, 0.7% (w/v) yeast extract, 0.2% (w/v) KH_2PO_4, 0.5% (w/v) $MgSO_4{\cdot}7H_2O$, 0.1% (w/v) $CaCl_2{\cdot}2H_2O$ and trace elements solution containing N-hydroxyacetanilide and thiamine-HCl. The cultures were incubated in 500 ml Erlenmeyer flask containing 100 ml liquid culture medium with constant shaking (150 rpm) at 28°C. The 5-day-old cultures was filtered and centrifuged at 20,000 *g* for 20 min, and then the supernatant solutions were collected as the crude enzyme.

Enzyme assays and protein analysis

Laccase activity was detected by measuring the absorbance increase at 420 nm (ε_{420}=36,000 M^{-1} cm^{-1}) during the oxidation of 0.5 mM ABTS in 100 mM citrate-phosphate buffer, (pH) 5.0 at 25°C. One unit (U) of laccase activity was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol ABTS per minute under the assay conditions. Protein concentrations were determined by the method of Bradford using bovine serum albumin as standard. All of the measurements were performed in triplicate. Purified proteins were submitted to Shanghai GeneCore BioTechnologies Co. Ltd. for N-terminal sequence analysis using automated Edman degradation and subjected for wavelength scan (300 to 700 nm) on TU-1900 UV/Vis spectrophotometer (Beijing, China). The copper content was determined by a Z-5000 Atomic absorption spectrometer (Hitachi Ltd., Tokyo, Japan).

Laccase purification

All operations described below were performed at 4°C. The crude enzyme was concentrated by ultrafiltration on an Amicon PM-10 membrane, dialyzed (10 kDa cut off) overnight against 10 mM sodium phosphate buffer (pH 6.0) and applied to a DEAE-Sepharose Fast Flow anion exchange chromatography column (2.5 cm × 20 cm), equilibrated with the dialyzed buffer. The column was washed with the equilibration buffer until the A_{280} reading was less than 0.02, and the bound proteins were subsequently eluted from the column using an increasing linear gradient of 0 to 0.6 M NaCl (400 ml) at a flow rate of 0.5 ml/min. Each fraction containing laccase activity was further purified by loading onto a Sephadex G-100 size-exclusion chromatography column (1.2 cm × 100 cm) equilibrated with 10 mM sodium phosphate buffer (pH 6.0) at a flow rate of 0.4 ml/min. Each enzyme was pooled and stored at 4°C.

Biochemical characterization of purified laccases

SDS-PAGE was used to determine protein purity and the molecular mass of the purified enzymes under denaturing conditions using a 10% (w/v) acrylamide gel. Protein was stained with Coomassie Brilliant Blue R-250. The isoelectric points of the laccase isozymes were determined using a Bio-Rad Model 111 Mini IEF cell with a 5% polyacrylamide gel and ampholyte (pH 3 to 10). The isoelectric points of the isozymes were determined by comparison with protein standard markers between pH 4.45 and 9.6 by silver staining. The optimal pHs of the laccase isozymes were studied from pH 2.0 to 9.0. To determine the pH stability, isozymes were kept at 25°C for 4 h in different buffers and the residual laccase activity was determined under standard assay conditions using ABTS as substrate. The optimal temperature for the laccase reaction was assayed from 25 to 100°C at 5°C intervals. Thermostability of the laccase isozymes was investigated by incubating laccase isozymes from 25 to 100°C. All experiments were performed three times, and the measurements were highly reproducible.

RESULTS

Purification of laccase isozymes

DEAE-Sepharose fast flow anion-exchange chromatography successfully separated the supernatant solutions of 5 days' cultures of *Trametes* sp. HS-03 into three distinct peaks with laccase activity, and the resulting fractions were named LacI, LacII and LacIII (Figure 1). The first peak (LacI) was partially eluted during washing of the column with the equilibration buffer, which suggested that LacI was poorly adsorbed on DEAE-Sepharose Fast Flow. A salt gradient resolved two peaks further (LacII and LacIII) that featured laccase activity. These were eluted at NaCl concentrations of approximately 0.09 and 0.28 M, respectively. The three active fractions were collected separately and further purified to homogeneity by means of Sephadex G-100 size-exclusion chromatography. At the end of the purification process, the LacI, LacII and LacIII laccase fractions had been purified 31.7-, 192- and 9.6-fold, with an overall yield of 11.8, 39 and 1.2%, respectively (Table. 1). The apparent homogeneity of the three laccase isozymes was monitored on SDS-PAGE (Figure 2). The relative molecular masses of LacI, LacII and LacIII were approximately 64.2, 60.7 and 38.9 kDa. Analytical isoelectric focusing established pI values of 7.3, 4.7 and 3.5 for LacI, LacII and LacIII, respectively (Figure 3).

The three laccase isozymes were observed as single bands by both SDS-PAGE and IEF-PAGE, suggesting that the purified laccase isozymes were present as monomeric proteins. The N-terminal amino acid sequences were found to be G-I-G-P-V for LacI, A-I-G-P-T for LacII and S-I-G-P-V for LacIII, which were similar to that of other fungal laccases (D'Souza-Ticlo et al., 2009; Schmidt et al., 2011). LacI, LacII and LacIII contained 4.0, 3.9 and 2.5 mol of copper per mol of protein, respectively. The UV–visible spectra of LacI and LacII showed typical characteristics of blue multicopper oxidases, including a peak at around 600 nm and a shoulder at 330 nm (Uthandi et al., 2010), while no absorbance of Lac was detected near 600 nm. These confirmed the authenticity

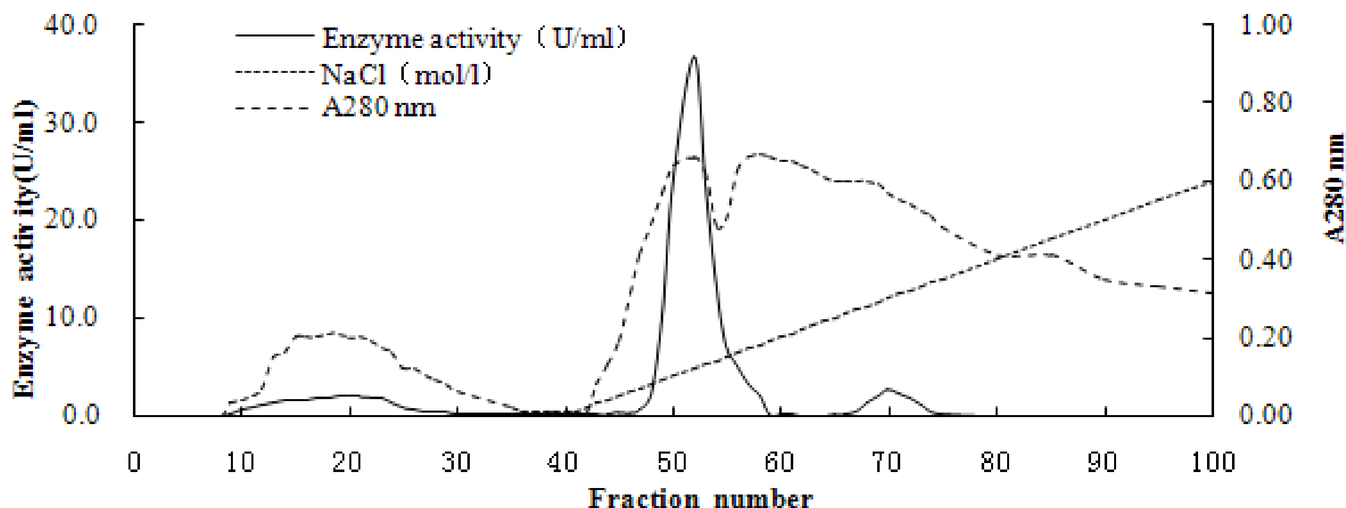

Figure 1. DEAE-sepharose fast flow elution profile of proteins secreted by *Trametes* sp. HS-03, showing three distinct fractions (LacI, LacII and LacIII)

Table 1. Summary of purification of laccase isozymes from *Trametes* sp. HS-03[a].

Purification step	Fraction	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor (fold)
Crude enzyme		1285	530	2.4	100	1
ultrafiltration		1157	351	3.3	90	1.4
DEAE-sepharose fast flow chromatography	LacI	154	18	8.6	12	3.6
	LacII	765	35	22	60	9.2
	LacIII	71	30	2.4	5.5	1
Sephadex G-100 chromatography	LacI	151	2	76	11.8	31.7
	LacII	507	1.1	461	39	192
	LacIII	16	0.7	23	1.2	9.6

[a] All purification procedures were performed at 4°C in sodium phosphate buffer (10 mM, pH 6.0) (buffer A). The crude enzyme was dialyzed overnight and applied to a DEAE-sepharose fast flow anion exchange chromatography column equilibrated with buffer A. LacI was poorly adsorbed on DEAE-Sepharose eluted during washing of the column with buffer A. The column was subsequently eluted using an increasing gradient of 0 to 0.6 M NaCl at a flow rate of 0.5 ml/min. LacII and LacIII were eluted at NaCl concentrations of approximately 0.09 and 0.28 M, respectively. LacI, LacII and LacIII were collected separately and further purified to homogeneity by loading onto a Sephadex G-100 size-exclusion chromatography column (1.2 cm × 100 cm) equilibrated with buffer A at a flow rate of 0.4 ml/min.

of the enzyme preparation and allowed confident identification.

Biochemical characterization of purified laccases

The optimal pHs for most fungal laccases are 2.0 to 5.5 for ABTS substrate. Figure 4a shows that the optimal pHs with ABTS were 3.5 for LacI, 4.0 to 4.5 for LacII and 2.5 to 3.0 for LacIII. These enzymes therefore fell within the normal range of fungal laccases for this property. LacI, LacII, and LacIII had good stability at pHs of 2.5 to 4.5, 3.5 to 5.5 and 2.0 to 4.5, respectively (Figure 4b), after incubation for 4 h. However, at pH values higher than 7.0, all the laccase isozymes lost their activity very rapidly. The optimal temperatures with ABTS were 40, 50 and 80°C, respectively (Figure 5a). The activities of LacI and LacII were stable below 50°C, decreased rapidly at approaching 60°C, and over 70°C they lost virtually all activity. However, LacIII retained 80% of its initial activity after incubation at 80°C for 30 min (Figure 5b).

The thermostability of LacIII was investigated further by measuring the residual enzyme activity after incubation between 40 and 90°C at 10°C intervals. As shown in Figure 6, LacIII retained high levels of activity at high temperatures, with 80 and 60% of the initial activity remaining after incubation at 60 and 70°C for 2 h, respectively, although LacIII became rapidly less stable at

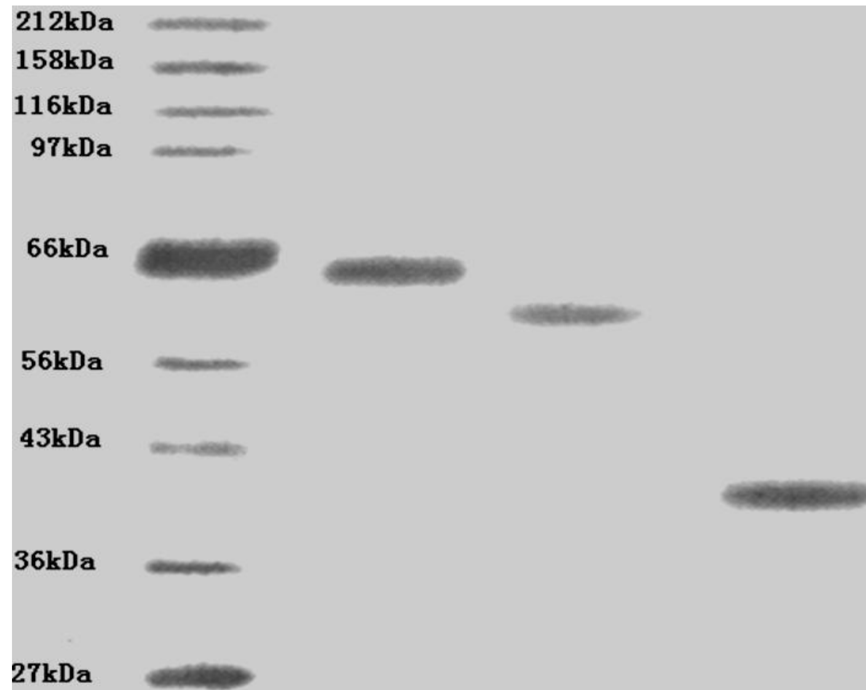

Figure 2. SDS-PAGE (10% polyacrylamide gel) of purified laccase isozymes from *Trametes* sp. HS-03. Lane 1, Molecular weight markers with indicated molecular masses in kDa on the left; lane 2, purified LacI; lane 3, purified LacII; lane 4, purified LacIII.

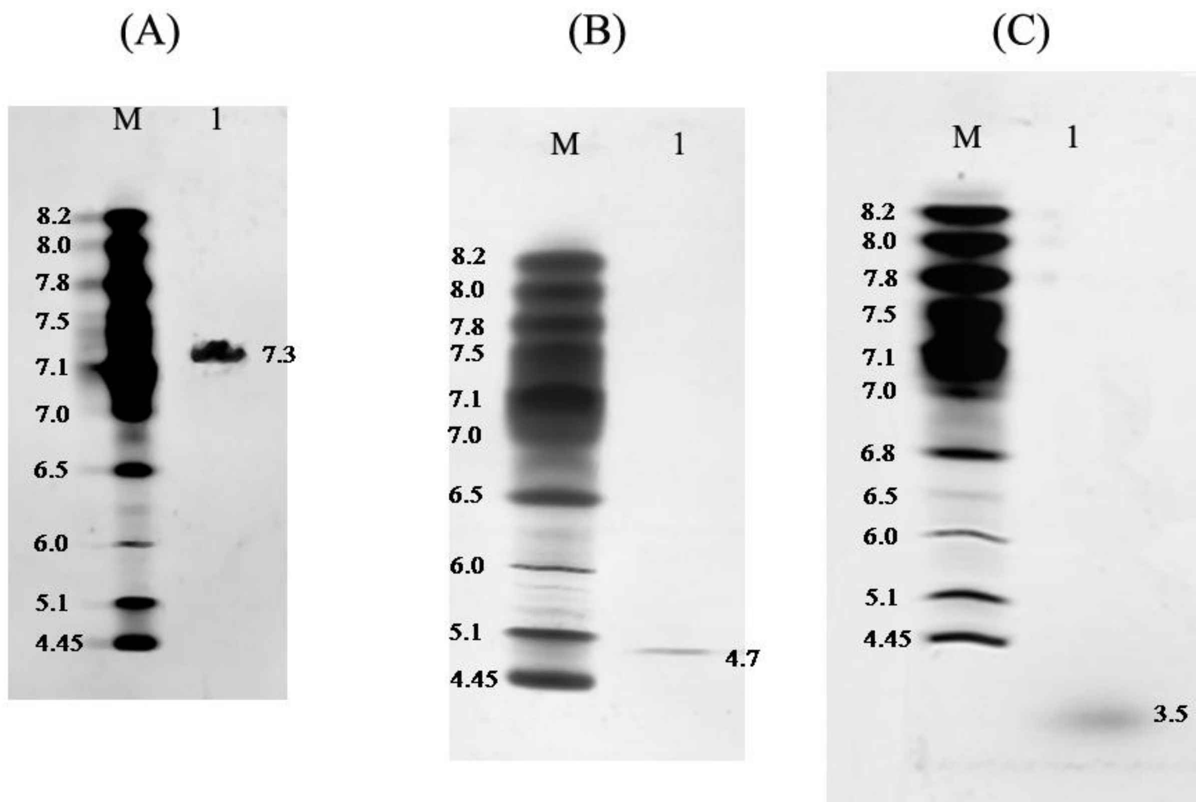

Figure 3. IEF of purified laccases. A: Lane 1, standard protein markers of different pI value; lane 2, purified LacI. B: Lane 1: standard protein markers of different pI value; lane 2, purified LacII. C: Lane 1: standard protein markers of different pI value; lane 2, purified LacIII.

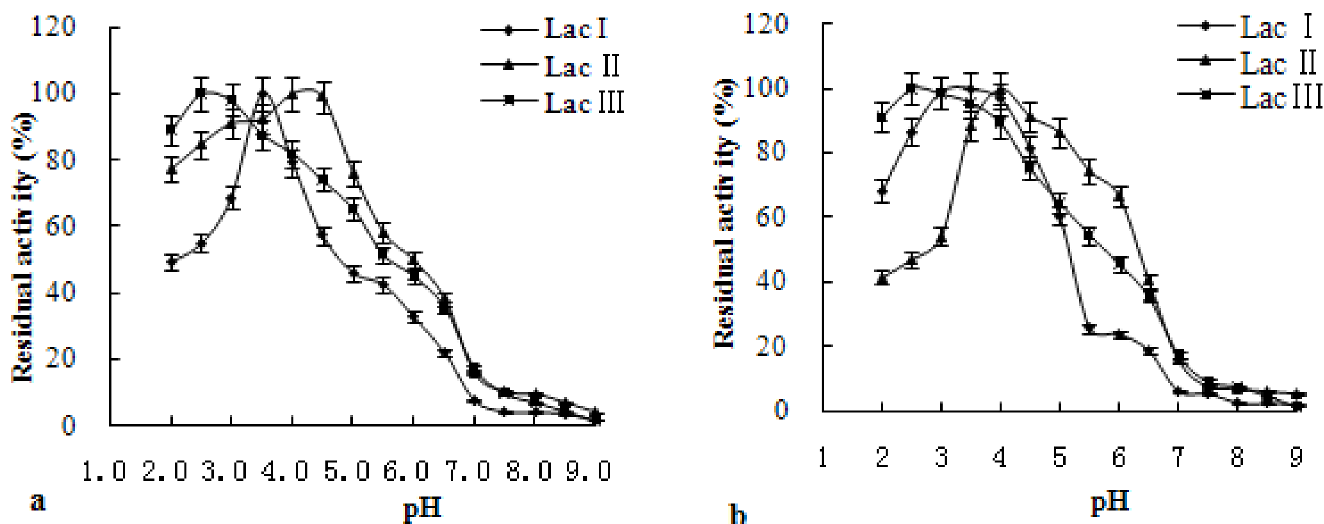

Figure 4. Effect of pH on the activities of the laccase isozymes from *Trametes* sp. HS-03. (a) Optimal pH for LacI (100% = 73.78 U/mg), LacII (100% = 457.48 U/mg) and LacIII (100% = 21.72 U/mg); b, pH stability for LacI, LacII and LacIII. The initial activities were determined at each pH value and defined as 100% (LacI: 100% = 74.37 U/mg, LacII: 100% = 459.65 U/mg, LacIII: 100% = 21.96 U/mg).

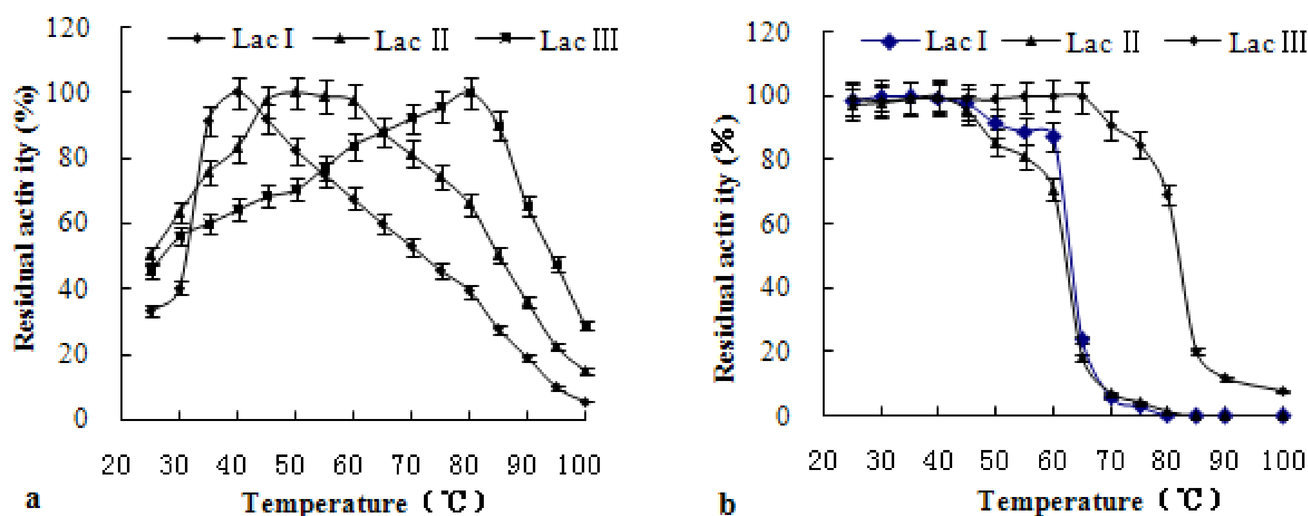

Figure 5. Effect of temperature on the activities of laccase isozymes. (a) Optimal temperature for LacI (100% = 73.84 U/mg), LacII (100% = 458.69 U/mg) and LacIII (100% = 22.38 U/mg). b, thermal stability of LacI, LacII and LacIII. The initial activities were defined as 100% (LacI: 100% = 74.62 U/mg, LacII: 100% = 459.18 U/mg, LacIII: 100% = 22.67 U/mg).

90°C. Moreover, the half-life (t 1/2) of LacIII was 125 min at 70°C.

DISCUSSION

In our study, three laccase isozymes of LacI, LacII and LacIII are excreted into the extracellular space by *T. versicolor* HS-03. These three isozymes were obtained in purified form by two-step chromatography using DEAE-Sepharose Fast Flow anion exchange chromatography followed by Sephadex G-100 size-exclusion chromatography. This two-step procedure allows effective and easy purification of large volumes of this culture, and should therefore allow the ready preparation of laccases on scales appropriate for industrial application. The overall yield of LacIII was only 1.2%, however, and this would need to be improved for industrial applications.

Some of the physicochemical characteristics of these three laccases are somewhat different to those of most laccases reported previously. The pIs of fungal laccases vary widely, from 2.9 to 6.9 (Li et al., 2010).Though the

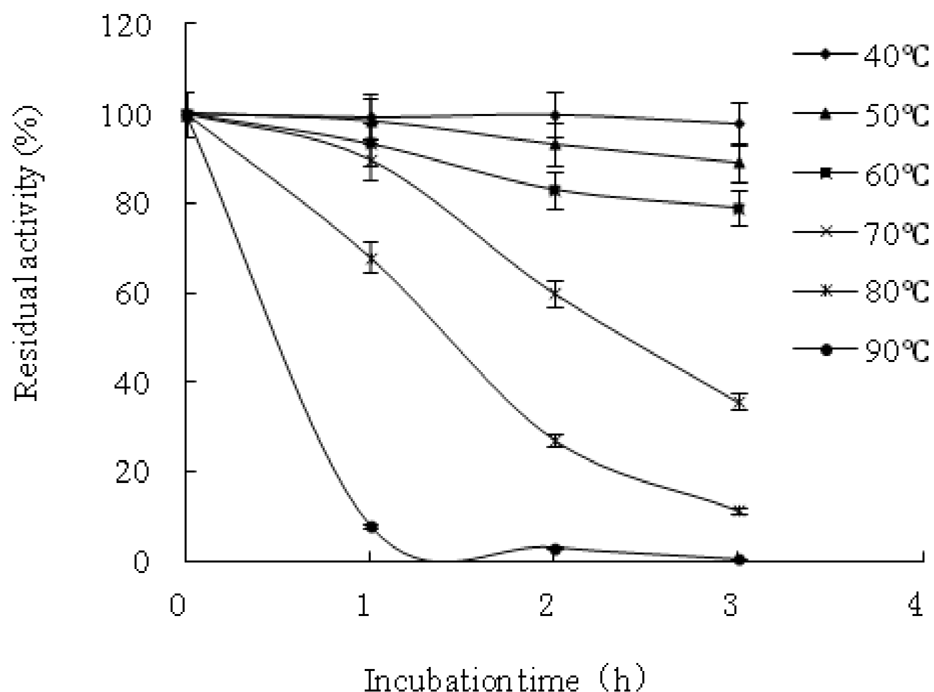

Figure 6. Effect of temperature on the thermal stability of LacIII. The initial activity was defined as 100% (LacI: 100% = 74.82 U/mg, LacII: 100% = 460.85 U/mg, LacIII: 100% = 21.76 U/mg).

pIs of LacII and LacIII were found to be 4.7 and 3.5 and these enzymes are more similar to other reported fungal laccases, the pI of LacI was found to be 7.3, which is therefore unusually high for fungal laccases. It is, however, lower than the pI of a laccase isolated from the white-rot fungus *Coriolus hirsutus*, which displayed a pI of 7.4 (Shin and Lee, 2000). In addition, LacIII has an apparent molecular mass of only 38.9 kDa. This is much lower than the molecular mass of typical fungal laccase, which generally have molecular masses of 60 to 90 kDa (Baldrian, 2006; Rogalski and Janusz, 2010). The molecular masses of LacI and LacII observed were similar to those of most other fungal laccases.

The typical optimal temperature range of catalytic activity for laccases is 30 to 55°C. Unexpectedly, LacIII maintained its activity up to 80°C. This optimal temperature is significantly higher than those of other thermostable fungal laccases (Wu et al., 2010). However, the unique oxidative thermostable laccase isolated from *Trametes hirsuta* was thermostable at 85°C (Zhang et al., 2009), and a laccase isolated from the thermophile *Thermus thermophilus* HB27 was thermostable at over 92°C (Miyazaki, 2005). The activity of fungal laccases usually drops suddenly above 60°C (Baldrian, 2006) whereas LacIII retains the majority of its activity in thermostability assays, with 80 and 60% initial activity remaining after incubation at 60 and 70°C for 2 h, respectively. The typical half-life of a fungal laccase is less than 1 h at 70°C and below 10 min at 80°C (Sadhasivam et al., 2008). More significantly, LacIII has a half-life of 125 min at 70°C, which is higher than the thermostable metal-tolerant laccase from a marine-derived fungus with the half-life of 90 min at 70°C (D'Souza-Ticlo et al., 2009), but less than laccases from *Pycnoporus sanguineus* (SCC 108), which have a half-life of 170 min at 75°C (Litthauer et al., 2007). The thermostability of LacIII suggests that it may have potential use of high process temperatures including the biotransformation of various industrial effluents such as treatment of textile mill wastewater and black liquor from paper and pulp industry, and further research is required.

In summary, three laccase isozymes were obtained by a straight-forward two-step chromatography procedure from the extracellular fluid of *Trametes* sp. HS-03. These three laccases featured some significantly different physicochemical characteristics to those of most other laccases reported previously. The results provide evidence for the important variety of laccases produced by *Trametes* sp. HS-03, and these isozymes are likely to have specialized niches of application. The molecular origin of these differences, however, remains to be determined. Furthermore, because of the high levels of laccase produced by *Trametes versicolor* HS-03 grown on very simple media and the ease of its induction, the laccase preparations obtained from this organism are expected to have broad utility in various biotechnological applications. Thus, this is the preliminary step towards investigation of the role of these enzymes from *Trametes* sp. HS-03.

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