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Purification and biochemical characterization of two extracellular peroxidases from *Phanerochaete chrysosporium* responsible for lignin biodegradation

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ABSTRACT

Two extracellular peroxidases from *Phanerochaete chrysosporium*, namely a lignin peroxidase (LiP) and manganese peroxidase (MnP), were purified simultaneously by applying successively, ultrafiltration, ion-exchange and gel filtration chromatography. LiP and MnP have a molecular mass of 36 and 45 kDa, respectively. The optimal pHs for LiP and MnP activities were 3.0 and 4.5, respectively. Both peroxidases showed maximal activity at 30 °C and moderate thermostability. MnP activity was strongly inhibited by Fe^{2+}, Zn^{2+}, Mg^{2+} and Hg^{2+}, and enhanced by Mn^{2+}, Ca^{2+} and Cu^{2+}. LiP activity was enhanced by Ca^{2+}, Na^{+} and Co^{2+} and it was inhibited in the presence of K^{+}, Hg^{+}, Fe^{2+}, Mg^{2+} and high concentrations of Cu^{2+} and Zn^{2+}. The K_m and V_{max} for LiP toward veratryl alcohol as a substrate were 0.10 mM and 15.2 U mg^{-1}, respectively and for MnP toward Mn^{2+}, they were respectively 0.03 mM and 25.5 U mg^{-1}. The two peroxidases were also able to break down rice lignin in a small-scale solid state treatment system. Data suggest these two peroxidases may be considered as potential candidates for the development of enzyme-based technologies for lignin degradation.

1. Introduction

Lignocellulose is a macromolecular complex consisting of lignin, cellulose, and hemicellulose. Lignin is composed of heteropolymers resulting from the oxidative coupling of three *p*-hydroxycinnamyl alcohols monolignols: *p*-coumaryl, coniferyl and sinapyl alcohols. The main role of lignin is to protect the cell-wall polysaccharides against hydrolytic attack by saprophytic and pathogenic microorganisms. Therefore, the degradation of lignin is the key step to the lignocellulose transformation. The recalcitrance of lignin against biodegradation is due to its bulky and aromatic nature that limits the accessibility of microbial enzymes and decreases its biodegradability (Pérez et al., 2002; Huang et al., 2008; Vanholme et al., 2010; Hernández-Ortega et al., 2012).

At present, much attention has been focused on lignin-degrading microorganisms because of their unique ability to degrade lignin (Huang et al., 2010a). The finding that the white-rot fungus *Phanerochaete chrysosporium* can efficiently break down lignin has attracted particular attention (Hofrichter, 2002; Baldrian, 2004; Huang et al., 2010b; Feng et al., 2011). However, other fungal species may also break down lignin. *Irpex lacteus* CD2 was shown to degrade lignin extracted from corn stover (Yang et al., 2010). Lignin-degrading abilities have also been demonstrated for the four basidiomycetous fungi *Pleurotus ostreatus sensu Cooke*, *Coriolus versicolor* (L.) Quel., *Tyromyces albidus* (Schaeff.) Donk, and *Trametes gallica* Fr. (Hong et al., 2012).

Lignin biodegradation results from the action of microbial extracellular enzymes, including laccase (Lac), lignin peroxidase (LiP), manganese peroxidase (MnP) and several other peroxidases such as versatile peroxidase (VP). These enzymes are responsible for generating highly reactive and nonspecific free radicals that contribute to lignin degradation. MnP is considered to be the most common lignin-modifying peroxidase produced by almost all wood-colonizing basidiomycetes and involved in the initial attack of lignin by Mn^{3+} chelate complexes (Hofrichter, 2002; Martínez et al., 2005). LiP is the first discovered enzyme that catalyzes the partial depolymerization of methylated lignin in vitro (Tien and Kirk, 1983). Lac has been confirmed to be essential for ligninolytic

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activity in many white-rot fungi (Leonowicz et al., 2001) and it acts by oxidizing a variety of phenolic compounds and aromatic diamines (Mayer and Staples, 2002). Other enzymes such as VP, which combine features of MnP and LiP, may also play lesser roles in lignin degradation (Morgenstern et al., 2008).

Wood-rotting fungi employ ligninolytic systems that may contain one or a combination of the following enzymes, LiP, MnP and Lac according to species (Chang et al., 2012). The extracellular non-specific and non-stereoselective ligninolytic system of *P. chrysosporium* is composed of LiP and MnP, which function together with H_2O_2 producing oxidases and secondary metabolites (Singh and Pakshirajan, 2010). *P. chrysosporium* and its extracellular lignin-degrading enzymes have been investigate intensively because of their great delignifying potential (Arora et al., 2002). Of particular interest to the aims of the present study, *P. chrysosporium* offers a promising enzymatic system for biodegradation of lignin. In order to provide theoretical references for the development of a *P. chrysosporium* lignin-degrading treatment we need to better characterize each individual enzyme component of the system. This calls for efficient methods for their production, separation and purification. In previous studies, the focus was mainly on producing LiP and MnP or purifying a single enzyme of the *P. chrysosporium* ligninolytic system (Moukha et al., 1997; Ürek and Pazarlioğlu, 2004). However, simultaneous purification of the two major components of lignin-degradation system has seldom reported. Here, we report a novel process for the simultaneous purification of LiP and MnP that were produced by *P. chrysosporium* under optimal conditions. We have also determined some of the biochemical features of the purified enzymes and we have assessed their ability to break down lignin in a small scale solid state treatment system.

2. Materials and methods

2.1. Strain and chemicals

The fungus *P. chrysosporium* strain BKMF-1767 was obtained from China Center for type Culture Collection (Wuhan, China). All the chemicals used in this work were of analytical reagent grade.

2.2. Fungal culture and enzyme production

Fungal cultures were maintained on potato dextrose agar (PDA) slants at 4 °C, and then transferred to PDA plates at 30 °C for several days. The spores on the agar surface were gently scraped and suspended in sterile distilled water as spore suspension. The spore concentration was measured and adjusted to 2.0×10^6 spores per mL. *P. chrysosporium* was grown in an immobilized and non immersed liquid culture system. The culture medium was modified on the basis of the method described by Tien and Kirk (1983), which was considered advantageous for the formation of extracellular enzymes. The cultures were incubated at 30 °C on a rotary shaker at 120 rpm with a 2.5 cm-diameter throw. LiP and MnP activities peaked simultaneously on the 7th day of cultivation and gradually decreased thereafter. Therefore, the culture was harvested for purification of the extracellular enzymes on day 7.

2.3. Extracellular enzymes activity assays

LiP activity was measured as described by Tanka et al. (2009). One unit (U) of LiP activity was defined as the amount of the enzyme required to produce 1 M veratryl aldehyde from the oxidation of veratryl alcohol (VA) per minute. MnP activity was measured as described by López et al. (2007). MnP unit activity was defined as the amount of enzyme required for producing 1 M Mn^{3+} from the oxidation of Mn^{2+} per minute.

2.4. Protein determination

Protein concentration was determined using the Bradford method with bovine serum albumin (BSA) as a standard (Luo et al., 2006). The total protein concentration of the enzyme solution was estimated from a calibration curve constructed using BSA in the range from 0 to 1 mg mL^{-1}.

2.5. Extracellular enzymes purification

All the purification steps were conducted below 4 °C. A seven-day old culture (1000 mL) of *P. chrysosporium* was centrifuged for 10 min at 9000 rpm. The supernatant containing extracellular peroxidases was used as the crude enzyme preparation and it was subjected to various purification steps.

2.5.1. Ultrafiltration

The crude enzyme was concentrated 20-fold by ultrafiltration on a 10 kDa-cutoff polyethersultone membrane (Model 8400, Millipore Corporation, USA).

2.5.2. Ion-exchange chromatography

The second purification step involved a DEAE-cellulose (Sigma–Aldrich, America) column (2.6 × 30 cm), which had been equilibrated with 5 mM phosphate buffer (PB) at pH 7.2. The column was washed stepwise with 50, 150, 300, 500 mM and finally 5 mM PB containing 1 M NaCl. The flow rate was maintained at 0.5 mL min^{-1}, and a total of 50 fractions each of 2.0 mL were collected. Protein concentration was determined for each fraction from the absorbance at 280 nm. Then LiP and MnP activity were estimated as mentioned above in each fraction with high absorbance at 280 nm. The active fractions were then pooled.

2.5.3. Gel filtration

The DEAE-cellulose fraction containing the LiP and MnP activities was concentrated to 20 mL by ultra-filtration a 1000 Da-cutoff membrane and dialyzed for 4 h against 500 mL 5 mM PB (pH 7.2) at 4 °C. Then the enzyme solution was loaded onto a Sephadex G-75 (Sigma–Aldrich) column (1.6 × 20 cm) pre-equilibrated with 5 mM PB (pH 7.2) and eluted with the same buffer as the second step. Fractions of 2 mL each were collected. All the fractions with high A280 and high LiP activity were pooled and concentrated and so were those with high A280 and high MnP activity. The supernatant was collected and concentrated using a freeze dryer, stored 4 °C, and used for the characterization.

2.5.4. SDS-PAGE and analysis of the N-terminal amino acid sequence

The molecular mass of purified extracellular enzymes was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). SDS-PAGE was run on a 12% polyacrylamide gel. The molecular weights of proteins were estimated according to molecular weight standards (Sigma–Aldrich). The protein bands were sequenced using a Procise Protein Sequencer (Applied Biosystem, USA) according to the manufacturer's instruction (Endo et al., 2002).

2.6. Effect of pH on the activity and stability of LiP and MnP

The effect of pH on LiP activity was investigated by assaying LiP at various pH values (2–7) in one of the following three buffers: 50 mM glycine–HCl buffer (pH 2), 50 mM sodium tartrate buffer (pH 3–5) or 50 mM potassium phosphate buffer (pH 6.5–7). The effect of pH on MnP activity was investigated by assaying MnP at various pH values (2–7) in one of the following three buffers:

50 mM glycine–HCl buffer (pH 2–3), 50 mM butane diacid buffer (pH 3.8–6) or 50 mM potassium phosphate buffer (pH 6.5–7). The temperature of the above experiments was controlled at 25 °C.

With regard to the measurement of pH stability, both enzymes were pre-incubated in buffers of different pH values in the range of 2–7 for 24 h at 25 °C. After the desired incubation period, the residual enzymatic activities were determined under standard assay conditions.

2.7. Effect of temperature on the activity and thermal stability of LiP and MnP

LiP and MnP activities were also determined at various temperatures between 25 and 65 °C at their respective optimal pH.

The thermal stability of each purified extracellular peroxidase was determined by incubating the enzyme for 10 h under a range of temperatures. After the desired incubation periods, enzyme aliquots were withdrawn and assayed under optimal conditions to determine the residual enzymatic activities. Untreated enzymes were used as control (100%).

2.8. Effect of metallic ions on the peroxidase activities

The effects of various metallic ions, at concentrations from 5 to 30 mM, on enzyme activities were investigated by adding divalent (Mn^{2+}, Ca^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+} and Hg^{2+}), as well as monovalent (Na^{+} and K^{+}) metallic ions to the reaction mixture and incubating it for 30 min, followed by measurement of enzymes activities. The activity was expressed as a percentage of the activity level in the absence of metallic ions. Peroxidase activities measured in the absence of any metallic ions (replaced with ultrapure water) were taken as control (100%). Residual activities were measured according the activity assays.

2.9. Substrates specificities, kinetic analysis of LiP and MnP

We have determined the kinetic parameters of LiP toward its most common substrates VA, coniferyl alcohol, sinapyl alcohol and guaiacol and those of MnP toward Mn^{2+}, 2,6-Dimethoxyphenol (2,6-DMP), 2,2-azino-*bis*(3-ehtylbenzothiazolin-6-sulfnicAcid) diammonium salt (ABTS) and guaiacol. The rate of oxidation of each substrate was determined by spectrophotometry using their reported molar extinction coefficients (ε) values at their maximal absorption wavelengths. The reactions were conducted in 50 mM sodium tartrate buffer at the respective LiP and MnP optimal pH. K_m and V_{max} for the purified enzymes were calculated using the Lineweaver–Burk transformation of Michaelis–Menten equation (Dowd and Riggs, 1965) with at least six initial substrate concentrations.

2.10. Lignin biodegradation by LiP and MnP

We determined the biodegradation ability of the crude and purified enzymes preparations toward rice straw lignin in a small scale solid state treatment system. The treatment was carried out in 500 mL flasks containing 30 g of rice straw power. Each flask was stoppered and autoclaved for 30 min at 121 °C, and then 100 mL of each enzyme preparation, 20 mL hydrogen peroxide (600 mM) and 50 mL ultrapure water were added into it. The treatments were performed at 37 °C for 60 days. The humidity was maintained at the initial level (85%) for the entire treatment period. Control samples were run in parallel with deactivated LiP and MnP under the identical conditions. Three sets of experimental systems were prepared and labeled as Reactor A, Reactor B and Reactor C. An equal amount of protein (3.51 mg) was added in each reactor. Deactivated LiP and MnP were added to Reactor A. The crude enzyme preparation containing 25 U of MnP and 6.2 U of LiP was added to Reactor B and a mixture of the two purified enzymes preparations containing 421.2 U of MnP and 98.2 U of LiP was added to Reactor C. Samples collected on 10, 20, 30, 40, 50 and 60 day were used to determine lignin content. Acid detergent lignin (ADL) was determined according to the procedures outlined by Van Soest et al. (1991). Lignin content was estimated as the difference between ADL and ash content.

2.11. Statistical analysis

The results presented in this work are the mean value of three replicates, and the standard deviations were used to analyze experimental data. Statistical analyses were performed using the software package SPSS 19.0 for Windows (SPSS, Germany).

3. Results and discussion

3.1. Purification of LiP and MnP

LiP and MnP activities peaked (ca. 1.77 and 7.13 U mg^{-1}) at the 7th day of the cultivation and gradually decreased thereafter. Therefore, the culture was harvested for the purification of LiP and MnP on day 7 (Zhao et al., 2012). The cell-free supernatant obtained from 1 L of *P. chrysosporium* culture contained 35.10 mg protein. It was subjected to ultrafiltration and then to ion chromatography on a DEAE-cellulose column.

The elution pattern on the DEAE-cellulose column showed three protein peaks at 280 nm designated peak 1, peak 2 and peak 3 (Fig. 1A). Most of the LiP activity was located in peak 1 and peak 2, and the activity was higher in peak 1 than in peak 2. MnP activity was located in the same protein peaks as LiP.

The fractions with high LiP and MnP activities were pooled, concentrated and loaded onto a Sephadex G-75 column. LiP eluted in a single peak designated peak 1 and MnP eluted in a second peak designated peak 2 (Fig. 1B). Both fractions were concentrated, analyzed by SDS-PAGE and used for the following experiments.

The purification procedure is summarized in Table 1. Data reveal that LiP and MnP exhibited high specific activities of 64.57 and 212.14 U mg^{-1}, respectively. The purification factors and yield values for the purified enzymes preparations were determined as 36.69-fold and 67.67% for LiP, and 29.80-fold and 72.08% for MnP.

3.2. Molecular weights determination and N-terminal amino acid sequence of LiP and MnP

The molecular weights of the purified enzymes (MnP and LiP) was determined by SDS-PAGE to be 45 and 36 kDa, respectively (Fig. 2). The molecular weight of MnP differed slightly from that of MnP purified from the wood-degrading fungus *Trichophyton rubrum* LSK-27 (42 kDa) (Bermeka et al., 2004), and it was very close to that of MnP purified from immobilized *P. chrysosporium* (45 ± 1 kDa) (Ürek and Pazarlioğlu, 2004). Furthermore, the molecular weight of LiP obtained in this study differed significantly from those of the white-rot fungus *Phanerochaete sordida* YK-624 YK-LiP (50 kDa) (Sugiura et al., 2003) and of the lignin peroxidase from *Penicillium decumbens* P6 (46.3 kDa) (Yang et al., 2005).

A comparison of N-terminal amino acid sequences of LiP and MnP of *P. chrysosporium* to peroxidases of fungi reported in previous study is shown in Table 2. The N-terminal amino acid sequence of the purified LiP showed high similarity with LiP obtained from *Bjerkandera* sp. BOS55 and *Trametes versicolor*. And the N-terminal amino acid sequence of the purified MnP in this study was highly homologous to *P. chrysosporium* MnP2.

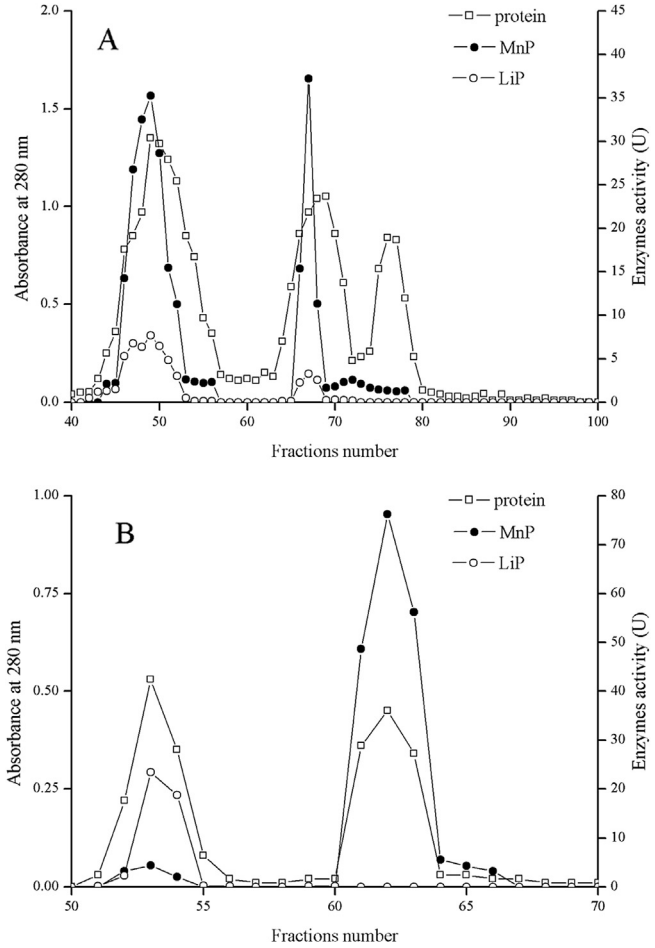

Fig. 1. Elution profile of the extracellular peroxidases from *P. chrysosporium*. (A) Ion-exchange chromatography on a DEAE-cellulose. (B) Gel filtration chromatography on a Sephadex G-75 column. Absorbance at 280 nm and enzymes activities of each fractions were monitored as described in the Materials and Methods section.

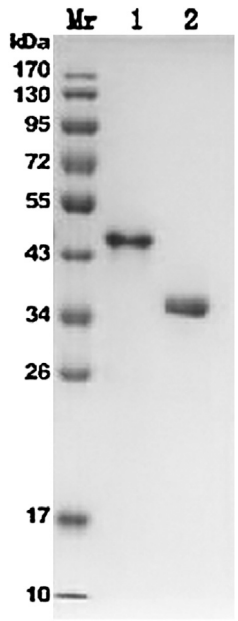

Fig. 2. SDS–PAGE of purified preparations of LiP and MnP. The chromatography was carried out on a 12% gel. Lane Mr, molecular mass markers; lane 1, MnP after all purification steps; lane 2, LiP after all purification steps.

3.3. Effect of pH on the activity and stability of LiP and MnP

pH is critical for heme stability and activity, as it impacts on the ionic form of the enzyme active site residues and thus on the binding to the heme group. The high activity exhibited by both enzymes at high pH is, in fact, a very important attribute that lends further support for their strong candidacy for potential application for lignin degradation. The effect of pH on the activity of LiP and MnP were examined over a range of pH from 2 to 7 at 25 °C. As shown in Fig. 3, MnP was active over a narrow range of pH (from 2 to 6), with an optimum pH 4.5. The optimal activity exhibited by LiP was observed at pH 3.0, which is inconsistent with that of the lignin peroxidase from *Acinetobacter calcoaceticus* NCIM 2890 (pH 1.0) (Ghodake et al., 2009) and that of P6 lignin peroxidase (pH 4.0) (Yang et al., 2005). This indicated that there were characteristic differences not only between LiP purified from *P. chrysosporium* and that from bacteria, but also between it and that from other fungi.

The pH stability profiles showed that LiP maintained 95% of its original activity at pH 3.0 after incubation for 24 h at 25 °C (Fig. 3a). MnP was stable over a slightly wider range of pH, maintaining 95–98% of its original activity at pH 3.5–4.5. MnP was optimal at pH 4.5 (Fig. 3b) which is the reported optimal pH for the enzymes of this family (Glenn and Gold, 1985; Deguchi et al., 1998).

3.4. Effect of temperature on the activity and stability of LiP and MnP

Temperature is a very important physical parameter influencing the enzyme activity (Jacob et al., 2008). The effect of temperature on LiP and MnP activities were determined at their optimal pHs. The optimum temperature for both purified enzymes was 30 °C (Fig. 4).

The thermal stability profiles revealed that LiP and MnP were highly stable at temperatures below 40 °C and 35 °C, respectively. Both enzymes were inactivated at various degrees at higher temperature (Fig. 4a and b). LiP and MnP retained more than 55% of their initial activity after 24 h incubation at 50 °C, and they maintained respectively, 38% and 8% of their maximal activity after 24 h of incubation at 65 °C (Fig. 4). The results are in accordance with the

Table 1
Purification steps of the extracellular peroxidases from *P. chrysosporium*.

Purification step	Volume (mL)	MnP activity (U)	LiP activity (U)	Total protein (mg)	Specific activity of MnP (U mg^{-1})	Specific activity of LiP (U mg^{-1})	Yield of MnP (%)	Yield of LiP (%)	Purification fold of MnP	Purification fold of LiP
Crude enzymes	1000 ± 3.50	250.15 ± 5.26	62.02 ± 1.34	35.10 ± 1.35	7.13 ± 0.32	1.77 ± 0.08	100 ± 3.25	100 ± 4.67	1.00 ± 0.04	1.00 ± 0.04
Ultrafiltration	100 ± 2.42	230.65 ± 5.02	56.23 ± 2.01	22.50 ± 1.02	10.25 ± 0.51	2.50 ± 0.01	92.20 ± 3.12	90.66 ± 3.87	1.44 ± 0.05	1.42 ± 0.04
DEAE-Cellulose	20 ± 1.54	203.85 ± 4.86	48.36 ± 1.65	5.27 ± 0.24	38.68 ± 2.86	9.18 ± 0.04	81.49 ± 3.05	77.97 ± 2.68	5.43 ± 0.18	5.21 ± 0.16
Sephadex G-75	6 ± 0.26	180.32 ± 4.92	ND	0.85 ± 0.04	212.14 ± 10.21	ND	72.08 ± 2.68	ND	29.80 ± 1.02	ND
	4 ± 0.15	ND	41.97 ± 1.75	0.65 ± 0.03	ND	64.57 ± 3.21	ND	67.67 ± 2.45	ND	36.69 ± 3.02

All data are expressed in mean value ± standard deviation. ND: not detected.

Table 2
Comparison of N-terminal amino acid sequences of LiP and MnP of *P. chrysosporium* with peroxidases of fungi reported in previous study.

Enzyme	N-terminal amino acid sequence	Reference
P. chrysosporium LiP	VACPDGVHVPTNACC	This study
P. chrysosporium MnP	AVCPDGTRVTNAACC	This study
Schizophyllum sp. F17	VTCPDGVNTATNAAA	Cheng et al., 2007
Bjerkkandra. adusta UAMH 8258 MnP	VAXPDGVNTATNAAX	Wang et al., 2002
Bjerkkandra. adusta UAMH 7308 MnP	VAXPDGVNTATNAAX	Wang et al., 2002
Bjerkandera sp. B33/3 RBP	VACPDGVNTATNAA	Moreira et al., 2005
P. chrysosporium MnP1	AVCPDGTRVSHAACC	Pease and Tien 1992
P. chrysosporium MnP2	AVCPDGTRVTNAACC	Pease and Tien 1992
P. chrysosporium MnP3	ATCPDGTKVNNACCA	Pease and Tien 1992
Bjerkandera sp. strain BOS55 LiP2 and LiP5	VACPDGRHTAINAACC NLFTVRDDI	ten Have et al., 1998
Trametes versicolor LiP	VTCPDGVNTATNAAX	Limongi et al., 1995
Trametes versicolor MnP	VAXPDGVNTATNAAX	Limongi et al., 1995

MnPs from *Lentinula edodes* (Boer et al., 2006) and *T. rubrum* LSK-27 (Bermeka et al., 2004), which were not stable at 50 °C. To sum up, LiP and MnP purified from *P. chrysosporium* in this study could be regarded as moderately thermostable enzymes, which might be a great advantage for future biotechnology and environmental applications.

3.5. Effect of metallic ions on activity of LiP and MnP

To further identify the nature of the two enzymes under investigation, the effects of a number of metallic ions on LiP and MnP activities were determined (Table 3). The findings revealed that Mn^{2+}, Ca^{2+} and Cu^{2+} enhanced the activity of MnP at all concentrations. The metallic ion exhibiting the most significant effect was Mn^{2+} which is consistent with the study by Boer et al. (2006). They showed that MnP activity was dependent upon Mn^{2+} and the substrates 2,6-DMP and ABTS were oxidized at a faster rate in the presence of Mn^{2+} than in its absence. In addition, MnP activity was enhanced in presence of low concentrations of Co^{2+}, but was slightly inhibited by higher concentrations of Co^{2+} (96%). While higher concentrations of K^{+} were observed to drive up MnP activity (116%), lower K^{+} concentrations (5 mM) did not affect

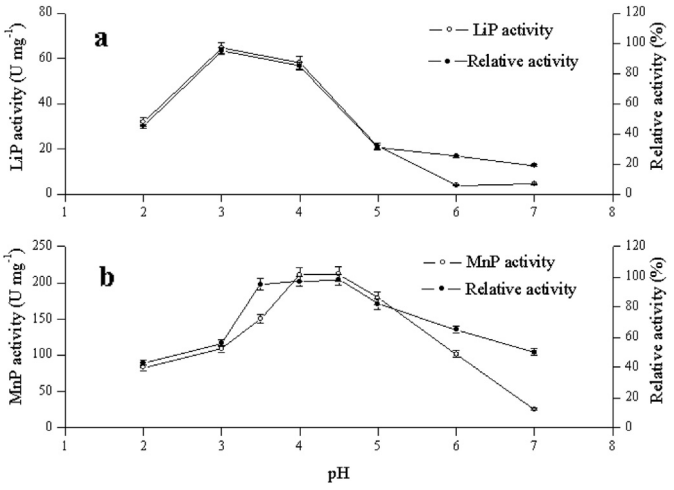

Fig. 3. Effect of pH on the activity and stability of LiP (a) and MnP (b). The results are the means of three analyses and the bars represent the standard deviation of the means.

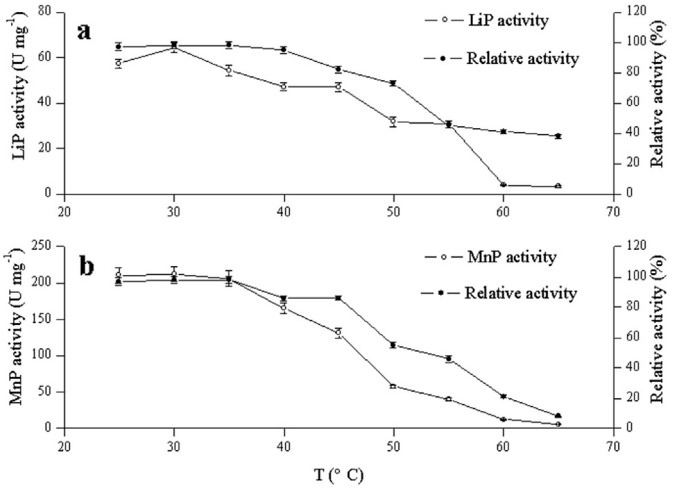

Fig. 4. Effect of temperature on the activity and stability of LiP (a) and MnP (b). The results are the means of three analyses and the bars represent the standard deviation of the means.

MnP activity (100%). On the other hand, MnP activity was strongly inhibited by Fe^{2+}, Zn^{2+}, Mg^{2+} and Hg^{2+}, and was even inactivated entirely in the presence of Hg^{2+}. Similar results were reported by Kanayama et al. (2002). LiP activity increased in the presence of Ca^{2+}, Na^{+} and Co^{2+}, while Mn^{2+} did not cause any alteration in the LiP activity. LiP activity was inhibited in the presence of K^{+}, Hg^{+}, Fe^{2+} and Mg^{2+} at all concentrations. Moreover, LiP activity was increased by Cu^{2+} and Zn^{2+} at concentrations of 5 mM and 10 mM, respectively, but it was inhibited at higher concentrations (30 mM) of Cu^{2+} and Zn^{2+}. It seems that the effect of metallic ions on the

Table 3
Effects of metal ions on the activity purified Lip and MnP from *P. chrysosporium*.

Mental ions	Concentration (mM)	Relative activity of LiP (%)	Relative activity of MnP (%)
Mn^{2+}	5	100 ± 4.3	123 ± 6.2
	10	100 ± 5.1	136 ± 6.5
	30	100 ± 4.9	145 ± 7.1
Ca^{2+}	5	112 ± 5.3	105 ± 4.8
	10	115 ± 5.4	106 ± 4.6
	30	109 ± 6.1	112 ± 5.3
Zn^{2+}	5	104 ± 4.7	86 ± 3.6
	10	105 ± 4.7	75 ± 3.1
	30	86 ± 3.8	61 ± 2.6
Co^{2+}	5	112 ± 4.8	106 ± 4.6
	10	103 ± 3.9	101 ± 5.0
	30	102 ± 4.2	96 ± 4.3
Cu^{2+}	5	113 ± 5.5	113 ± 5.3
	10	133 ± 5.9	115 ± 5.2
	30	95 ± 3.2	124 ± 5.6
Fe^{2+}	5	99 ± 3.4	85 ± 3.5
	10	98 ± 4.7	54 ± 2.3
	30	75 ± 3.2	45 ± 1.9
Mg^{2+}	5	98 ± 4.8	97 ± 4.2
	10	76 ± 2.6	55 ± 2.3
	30	72 ± 2.9	26 ± 1.2
Hg^{2+}	5	5.6 ± 0.25	ND
	10	2.4 ± 0.11	ND
	30	ND	ND
Na^{+}	5	114 ± 5.3	102 ± 5.0
	10	106 ± 4.8	100 ± 4.8
	30	105 ± 4.7	99 ± 3.8
K^{+}	5	98 ± 3.6	100 ± 4.5
	10	95 ± 3.5	108 ± 4.7
	30	92 ± 3.1	116 ± 5.2

All data are expressed in mean value ± standard deviation. ND: not detected.

Table 4
Kinetic parameters of purified LiP toward VA, coniferyl alcohol, sinapyl alcohol and guaiacol, and of purified MnP toward Mn^{2+}, 2,6-DMP, ABTS and guaiacol.

Enzyme	Substrate	Wavelength (nm)	K_m (mM)	V_{max} (U mg^{-1})	K_{cat} (s^{-1})	K_{cat}/K_m (M^{-1} s^{-1})
LiP	VA	310	0.10	15.20	8.44	8.44×10^4
	Coniferyl alcohol	265	0.45	8.54	4.74	1.05×10^4
	Sinapyl alcohol	270	0.51	3.56	1.98	3.88×10^3
	Guaiacol	465	0.35	12.42	6.90	1.97×10^4
MnP	Mn^{2+}	240	0.03	25.50	7.97	2.65×10^5
	2,6-DMP	470	0.05	20.36	6.36	1.27×10^5
	ABTS	420	0.16	12.87	4.02	2.51×10^4
	Guaiacol	465	0.42	3.65	1.14	2.71×10^3

activity of the two enzymes depends on the type and concentrations of ions used, which could provide a basis for the treatment of metal-contaminated lignocellulose waste with these enzymes.

3.6. Substrate specificities and kinetic analysis

Substrates specificities assessment revealed that LiP was able to oxidize the tested substrates VA, coniferyl alcohol, sinapyl alcohol and guaiacol, and MnP was able to oxidize Mn^{2+}, 2,6-DMP, ABTS and guaiacol. The kinetic constants of LiP and MnP with different substrates are shown in Table 4. The K_m of LiP toward VA was the lowest among the tested substrates, which indicated that LiP showed stronger affinity for VA than for other substrates. The K_m and V_{max} for MnP toward Mn^{2+} were respectively 0.03 mM and 25.50 U mg^{-1}, and the efficiency (K_{cat}) of the oxidation reactions was the highest for Mn^{2+}. In addition, LiP and MnP were considered to be non-specific to their substrates, which makes both enzymes promising for lignin degradation processes.

3.7. Biodegradation of lignin by LiP and MnP

Fig. 5 shows the degradation rate of lignin in Reactors A, B and C in the solid state treatment process. There was no degradation of lignin in Reactor A during the entire process. In the first 30 days, the lignin content decreased quickly in Reactors B and C and the rate slowed down after the 30th day. The degradation rate of lignin in Reactor C was higher than in Reactor B throughout the entire process, which indicates that the purified enzymes preparations owed stronger lignin-biodegrading efficiency than the crude preparation. Thereby, the purified enzymes showed high potential for lignin biodegradation.

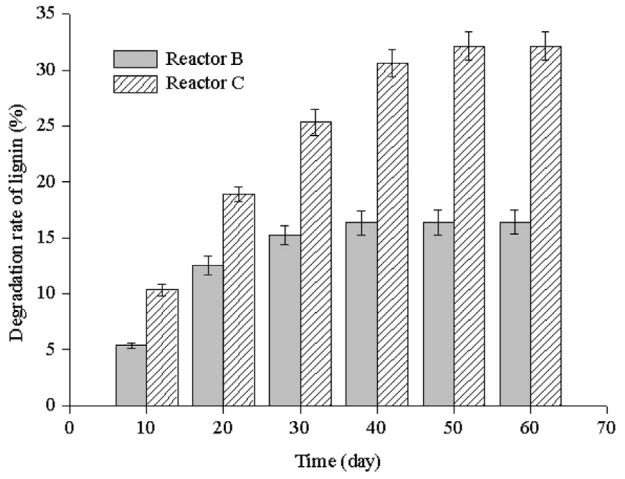

Fig. 5. Degradation rates of rice straw lignin in small scale solid state reactors. The protocol is described in the Materials and Methods section. Reactor B contained the crude enzyme preparation, Reactor C contained a mixture of the purified LiP and MnP preparations. Reactor A contained inactivated enzymes preparations and no lignin degradation was observed (the data not shown). The results are the means of three analyses and the bars represent the standard deviation of the means.

4. Conclusions

In this study, two extracellular peroxidases from *P. chrysosporium* were purified, and showed good activity and stability at their optimal pH and temperature, thus they are of interest for potential applications for lignin degradation processes. In the present report, we described a fast, simple and reproducible procedure for the simultaneous purification of LiP and MnP with a good yield of the enzymes. The present findings will help improve the understanding about the biochemical features of the two extracellular peroxidases, which could promote the application of LiP and MnP in biodegradation of lignin.

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