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Purification and Characterization of Manganese Peroxidase of the White-Rot Fungus *Irpex lacteus*

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The production of manganese peroxidase (MnP) by *Irpex lacteus*, purified to electrophoretic homogeneity by acetone precipitation, HiPrep Q and HiPrep Sephacryl S-200 chromatography, was shown to correlate with the decolorization of textile industry wastewater. The MnP was purified 11.0-fold, with an overall yield of 24.3%. The molecular mass of the native enzyme, as determined by gel filtration chromatography, was about 53 kDa. The enzyme was shown to have a molecular mass of 53.2 and 38.3 kDa on SDS-PAGE and MALDI-TOF mass spectrometry, respectively, and an isoelectric point of about 3.7. The enzyme was optimally active at pH 6.0 and between 30 and 40°C. The enzyme efficiently catalyzed the decolorization of various artificial dyes and oxidized Mn (II) to Mn (III) in the presence of H_2O_2. The absorption spectrum of the enzyme exhibited maxima at 407, 500, and 640 nm. The amino acid sequence of the three tryptic peptides was analyzed by ESI Q-TOF MS/MS spectrometry, and showed low similarity to those of the extracellular peroxidases of other white-rot basidiomycetes.

Key words: decolorization, *Irpex lacteus*, manganese peroxidase, white-rot fungi

The white-rot fungus, *Irpex lacteus,* has been studied in connection with lignin biodegradation (Capelari and Zadražil, 1997), biodegradation of polycyclic aromatic hydrocarbons (PAHs) (Kim and Song, 2000; Novotný *et al.*, 2000), and the decolorization of textile industry wastewaters (Shin, 2004). A good capacity for decolorization of synthetic dyes by *I. lacteus* has been demonstrated in stationary liquid cultures and a packed-bed bioreactor (Novotný *et al.*, 2000; Kasinath *et al.*, 2003). The fungus was also able to degrade an anthraquinone dye in contaminated soil (Bhatt *et al.*, 2000). From inhibitory studies, it was suggested that manganese peroxidase (MnP; EC 1.11.1.13) played a major role in the decolorization of synthetic dyes by *I. lacteus* (Kasinath *et al.*, 2003).

MnP is a glycosylated heme-containing enzyme produced by many ligninolytic fungi, including *Phanerochaete chrysosporium* (Glenn and Gold, 1985; Wariishi *et al.*, 1989), *Phlebia radiata* (Hofrichter *et al.*, 1999; Van-Aken *et al.*, 1999), *Nematoloma frowardii* (Schneegaβ *et al.*, 1997; Hofrichter *et al.*, 1999), *Pleurotus eryngii* (Caramelo *et al.*, 1999), and *Bjerkandera adusta* (Mester and Field, 1997; Mester and Field, 1998; Wang *et al.*, 2002). MnPs from white rot fungi have been used in studies of the biodegradation of lignin (Michel *et al.*, 1991; Paice *et al.*, 1993; Hilden *et al.*, 2000), PAHs (Watanabe *et al.*, 2000), humic acids (Zeigenhagen and Hofrichter, 1998), synthetic dyes (Heinfling *et al.*, 1998; Swamy and Ramsay, 1999), and polychlorinated biphenyls (Beaudette *et al.*, 1998). During the catalytic cycle, the active center of the MnP is oxidized by H_2O_2. Reduction to the resting enzyme is achieved by two successive one-electron transfers; thereby, oxidizing Mn (II) to Mn (III). This is facilitated by fungal organic acids on chelation of the highly reactive Mn (III) state (Wariishi *et al.*, 1992; Kuan *et al.*, 1993; Kuan and Tien, 1993; Kishi *et al.*, 1994; Timofeevski and Aust, 1997; Banci *et al.*, 1998). However, the purification and characterization of the MnP produced from *I. lacteus* remain to be elucidated, which are the purposes of this study.

Materials and Methods

Organism and culture conditions

The *I. lacteus* strain KR 35W was provided by Prof. Kyu-Jung Kim (Kangnung National University). The strain was maintained on MGPY (1% malt extract, 1% glucose, 0.5% peptone, and 0.5% yeast extract) agar slants at 4°C. The fungal inocula were prepared in 250-ml Erlenmeyer

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flasks, containing 100 ml of MGPY medium, for 7 days. Four mycelial agar discs (0.9 cm) were obtained from a fresh MGPY agar culture for use as the inocula. The liquid inocula were gently homogenized, and used at a 10% (v/v) dilution. The stationary cultures were performed in 250-ml Erlenmeyer flasks, containing 20 ml of textile industry wastewater, at 28°C, as described in the literature (Shin, 2004).

Enzyme assay and purification

The laccase activity was determined via the oxidation of 2,2'-azino-bis(3-ethylbenzthiazoline)-6-sulfonate (ABTS) (Wolfenden and Wilson, 1982). The lignin peroxidase (LiP) activity was assayed as the method described by Tien and Kirk (1984), using veratryl alcohol as substrate. The MnP activity was assayed by measuring the oxidation of Mn (II) to Mn (III) at 270 nm, as described previously (Wariishi *et al.*, 1989). The assay mixture contained 1.0 mM $MnSO_4$ in 50 mM sodium malonate (pH 4.5), and the reaction was initiated by the addition of 0.2 mM H_2O_2. One enzyme unit was defined as the amount of enzyme required to oxidize 1 μmol of substrate per min, under the assay conditions.

The culture fluid was used for enzyme purification after filtration through a Whatman No. 1 filter paper. Cold acetone (-10°C) was added to give 65% saturation, and the precipitated proteins collected. After removing the acetone, the precipitate was dissolved in 20 mM sodium phosphate buffer, pH 8.0. The sample was applied to a HiPrep™ 16/10 Q XL column, which was equilibrated with the same buffer. The enzyme fractions were eluted with a linear concentration gradient of 0 - 1.0 M NaCl in the same buffer, at a flow rate of 5 ml/min. The fractions containing MnP activity were combined and concentrated. Gel filtration was performed using a HiPrep 26/60 Sephacryl S-200 column eluted with the same buffer, at a flow rate of 50 ml/h. The homogeneity of the enzyme was confirmed by SDS-PAGE and the protein concentration determined by a modified method of Lowry *et al.* (1951), using bovine serum albumin as a standard protein.

Enzyme characterization

The M_r of the native enzyme was estimated by gel filtration chromatography on a Superose 12 HR 10/30 column mounted in the Pharmacia FPLC system. Standard proteins of known molecular masses were used to calibrate the column. The M_r of the enzyme was also estimated by MALDI-TOF mass spectrometry and SDS-PAGE on a 12.5% polyacrylamide gel. The isoelectric point of the enzyme was determined on an isoelectric focusing gel (IEF-PAGE), with a pH gradient from 3 to 10 (125 by 65 mm, 0.4 mm thick; Bio-Rad, USA), by comparison of the purified enzyme with protein standard markers. Coomassie brilliant blue G-250 was used for staining, according to the method of Neuhoff *et al.* (1988).

MS characterization

The purified protein was excised from the gel and digested with trypsin, as described by Shevchenko *et al.* (1996). The resulting peptide mixture was analyzed by MALDI-MS and ESI Q-TOF MS/MS, using a 4700 Proteomic Analyzer (Applied Biosystems, USA). The protein was first identified by searching the NCBI nonredundant database, using the MASCOT Peptide Mass Fingerprint software (Matrixscience, London) (Mann and Wilm, 1994). The MALDI mass spectra of the tryptic peptide mixtures were searched from all entries in the database. For de novo sequence interpretation of the ESI tandem mass spectra, the PreSeq routine (a part of the Masslynx 3.5 software, Micromass) was used. All complete or partial sequences were compared with those of the proteins from a MASCOT or BLAST similarity search (www.ncbi.nlm.nih.gov/BLAST/) to obtain the identifications with higher confidence.

Results and Discussion

Production and purification of MnP

Of the enzymes tested, the LiP activity was negligible and that of the laccase was not related to the decolorization (Fig. 1), indicating that these enzymes play only a small role in the decolorization of dyestuffs in the wastewater. The MnP activity of the culture filtrates was shown to correlate with the decolorization of textile industry wastewater (Fig. 1). After three days, the decolorization rate rapid increased, and removed 90% of the color in 6 days. The MnP activity reached a maximum after 7 days, indicating that the MnP may be involved in the decolorization of the textile industry wastewater. After harvesting of the culture on day 7, the culture filtrate was treated with cold acetone. The enzyme was purified to homogeneity from

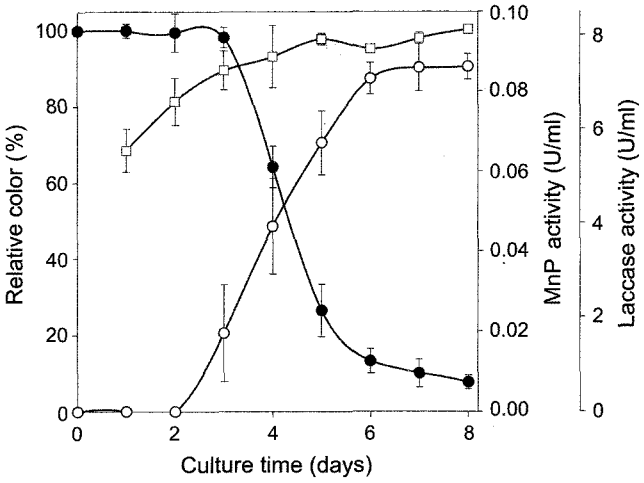

Fig. 1. Time courses of the extracellular MnP activity (○), laccase activity (□), and decolorization of textile industry effluent (●). The decolorization rate was determined by measuring the absorbance at 600 nm.

Table 1. Purification of MnP from the culture filtrate of *I. lacteus*

Step	Activity (U)	Protein (mg)	Sp. activity (U/mg)	Yield (%)	Purification (fold)
Culture filtrate	42.4	111	0.38	100	1
Acetone treatment	34.6	26.6	1.30	81.6	3.4
HiPrep Q	17.5	7.60	2.30	41.3	6.1
HiPrep S-200	10.3	2.45	4.20	24.3	11.0

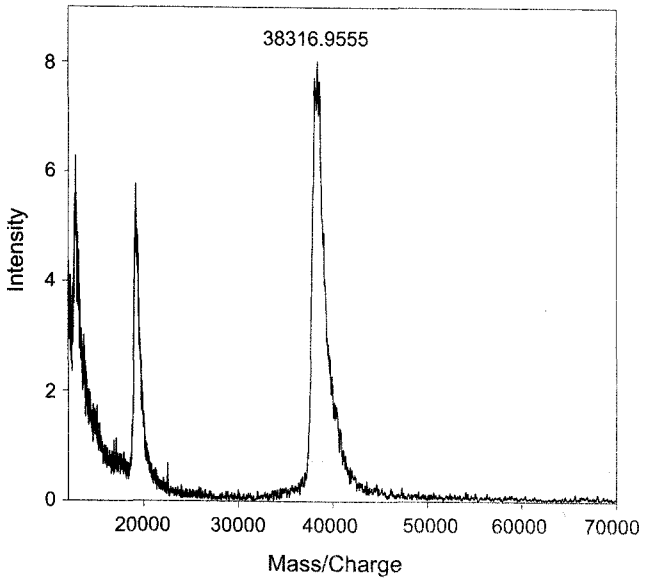

Fig. 2. MALDI-TOF mass spectrum of the native MnP purified from *I. lacteus*. The MnP peak is at 38,316.95 Da.

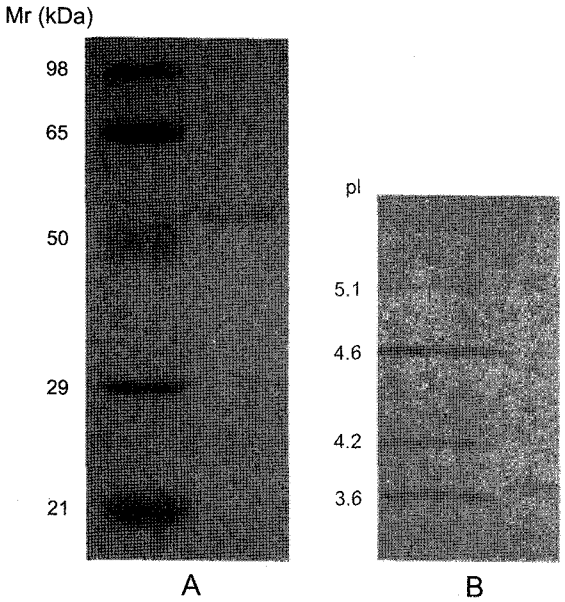

Fig. 3. SDS-PAGE (A) and isoelectric focusing (B) of the purified MnP from *I. lacteus*. Molecular mass markers and *p*I standards are shown on the left of each gel.

the culture filtrate of *I. lacteus*, as summarized in Table 1. Three steps were required for the purification, and by the end of the process, the enzyme had been purified 11.0-fold, with a yield of 24.3%.

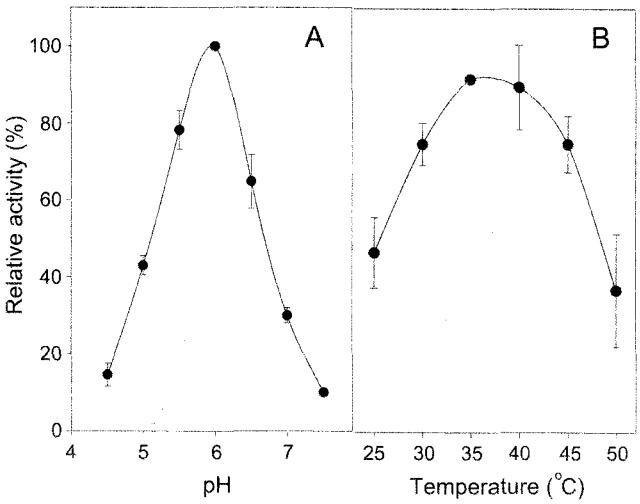

Fig. 4. Effect of pH (A) and temperature (B) on the MnP activity. The enzyme activity was assayed at various pHs and temperatures under the standard assay conditions.

Molecular mass and pI

The apparent molecular mass of the purified enzyme was about 53 kDa, as determined by gel filtration chromatography on Superose 12 HR 10/30. The molecular mass of the purified enzyme was estimated to be 38.3 and 53.2 kDa by MALDI-TOF mass spectrometry (Fig. 2) and SDS-PAGE (Fig. 3A), respectively. Since the MnP is a glycoprotein, and the carbohydrate content of the enzyme affected by its migration in gel filtration chromatography and SDS-PAGE, mass spectrometry is a more accurate reflection of the true molecular mass. Similar results have also been reported for the MnP of *B. adusta* (Wang *et al.*, 2002) and the novel peroxidase of *P. eryngii* (Ruiz-Duenas *et al.*, 1999). These data indicate that the enzyme contains about 28% total carbohydrate and is monomeric, similar to those described for other fungal MnPs. Most are monomeric proteins, with molecular masses between 43 and 46.5 kDa (Matsubara *et al.*, 1996; Schneegaß *et al.*, 1997; Palma *et al.*, 2000; Wang *et al.*, 2002). The isoelectric point of the enzyme was 3.7 (Fig. 3B), which is a usual feature among fungal MnPs, which exhibit acidic isoelectric points (Matsubara *et al.*, 1996; Schneegaß *et al.*, 1997; Palma *et al.*, 2000; Wang *et al.*, 2002).

Effect of pH and temperature

The effect of pH on the enzyme activity was examined

using pH values ranging from 4.5 to 7.5. From the bell-shaped pH profile shown in Fig. 4A, the optimum pH for the enzyme activity was estimated to be around 6.0.

As shown in Fig. 4B, the optimal temperature for the enzyme activity was about 35°C, with activity over the broad range of temperatures tested.

Catalytic properties

Numerous dyes were decolorized by the MnP of *I. lacteus* in the presence of Mn and H_2O_2 (Table 2). These dyes differed in their chemical structures and ease of decolorization. The azo dye, methyl orange, was the most rapidly decolorized. Significant decolorization of different groups of dyes has previously been observed in liquid cultures of *I. lacteus* (Novotný *et al.*, 2001), suggesting that MnP plays a major role in the decolorization of dyes (Kasinath *et al.*, 2003). In the presence of H_2O_2, the enzyme readily oxidized Mn (II), as measured by the formation of Mn (III)-malonate (Fig. 5). The spectrum of the Mn (III)-malonate formed (λ_{max} = 267 nm) closely resembles that previously shown upon the oxidation of Mn (II) to Mn (III) by the MnP of *P. chrysosporium* (Glenn and Gold, 1985). This suggests that the enzyme produces Mn (III) from the oxidation of Mn (II), which subsequently oxidizes the substrates.

Table 2. Decolorization of various dyes by MnP of *I. lacteus*

Dye[a]	Class	λ_{max} (nm)	ΔABS/min/ mg protein
Bromophenol blue	triphenylmethane	592	0.19
Methyl orange	azo	477	0.90
Poly R-478	polymeric	520	0.05
RBBR	anthraquinone	592	0.17
Methylene blue	heterocyclic	664	0.03

[a]The concentration of each dyes was 20 mg/l.

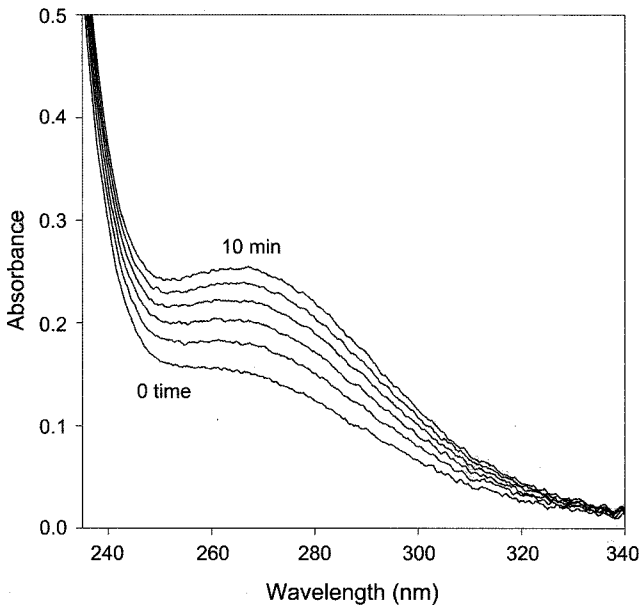

Fig. 5. Production of Mn (III)-malonate from the oxidation of Mn (II) by the MnP. The reaction mixture contained 0.5 mM $MnSO_4$, 0.5 mM Na-malonate (pH 4.5) and the enzyme. The reaction was initiated by the addition of 0.2 mM H_2O_2, and the spectra recorded every 2 min.

Spectroscopic properties

The absorption spectrum of the purified enzyme showed maxima at about 407, 500, and 640 nm (Fig. 6). The addition of H_2O_2 to the enzyme solution resulted in a red shift from 407 and 500 to 409 and 525 nm, respectively; the absorbance also decreased. These spectral characteristics are very similar to those of MnP from *P. chrysosporium* (Glenn and Gold, 1985), suggesting that the enzyme is a heme protein with iron protoporphyrin IX as the prosthetic group.

MS characteristics

The MALDI-TOF MS spectra of the tryptic peptides of the enzyme are shown in Fig. 7. The peptide mass fingerprint of the enzyme was that of a non-homologous fungal peroxidase from a Mascot database search. Three peptides, *m/z* 1039.6, 1631.9, and 1854.0, were further analyzed with ESI Q-TOF MS/MS spectrometry. The amino acid sequences of each peptide were LSILGHDER, VTPEPFDSVTAQNAR, and GNQADVQSLPR, with the Swiss-Prot accession number of P83918. The amino acid sequence of the peptide with $[M+H]^+$ = 1039.6 showed 78% similarity to those of the versatile peroxidase

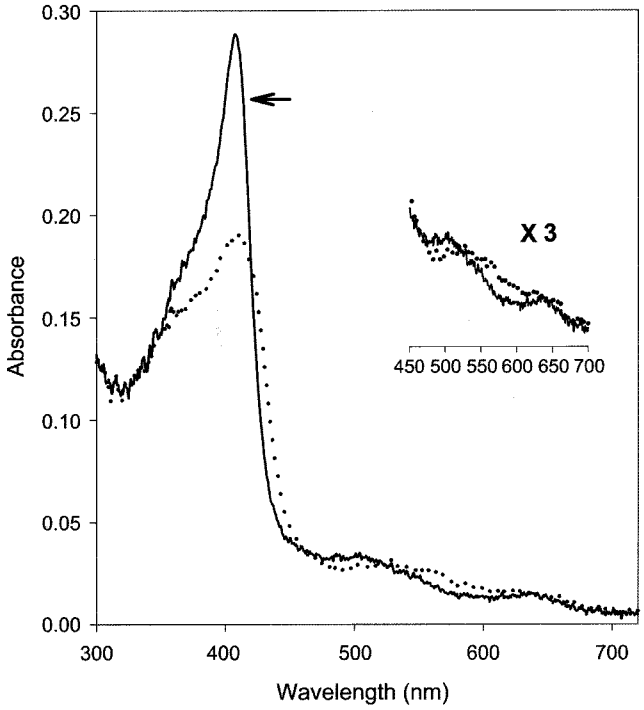

Fig. 6. Absorption spectra of the *I. lacteus* MnP. The oxidized form was obtained by the addition of 0.1 mM H_2O_2 to the reduced from (arrow). The enlarged spectrum over the wavelength region indicated is shown. H_2O_2, and the spectra recorded every 2 min.

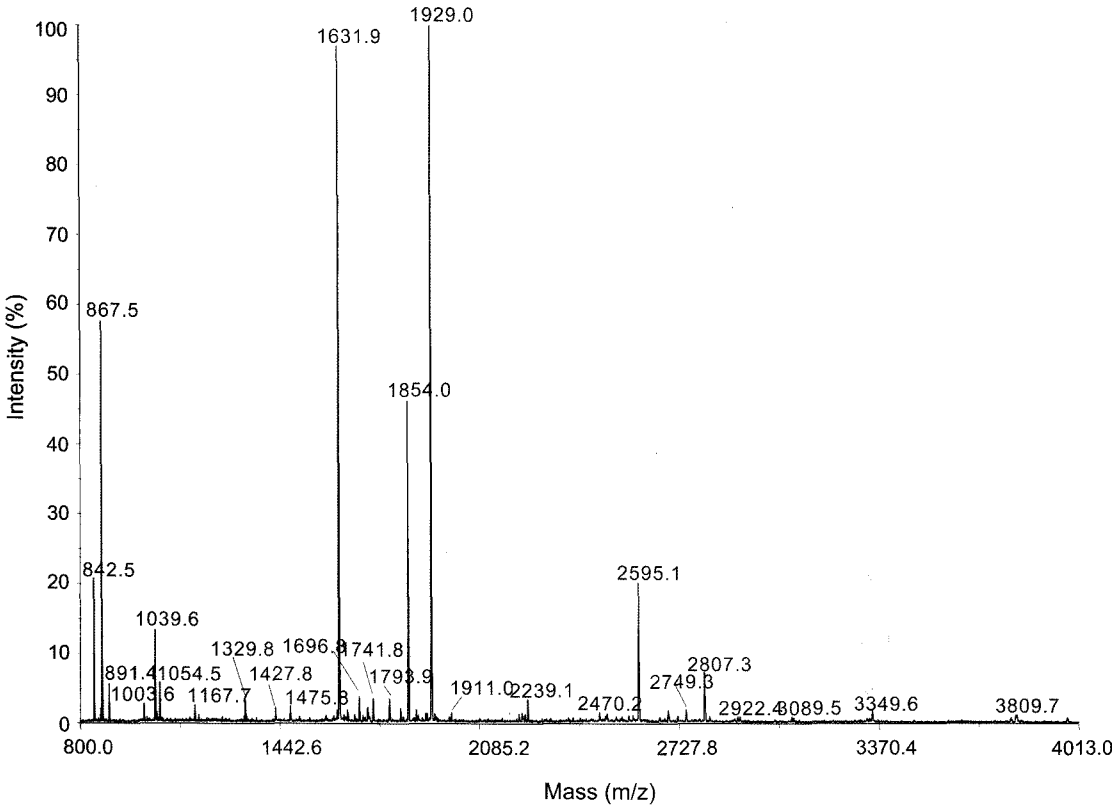

Fig. 7. MALDI-TOF MS spectrum of a tryptic digest from the MnP of *I. lacteus*. Three peptide fragments, *m/z* 1039.6, 1631.9, and 1854.0, were further analyzed with ESI Q-TOF MS/MS spectrometry.

Table 3. Identification of tryptic peptides from *I. lacteus* MnP

$[M+H]^+$	Amino acid sequences	Similar sequences in the database of the NCBI (Accession No.[a])	Similarity (%)
1039.6	LSILGHDER	^{300}LSILGHDLT (AY217015)	78
		^{271}LSILGHDLT (AF490538)	78
		^{298}LAILGHNRR (AY677128)	67
1631.9	VTPEPFDSVTAQNAR	^{170}LVPEPFDSVTKILAR (AY677128)	67
		^{171}LVPEPFDSVTDILNR (AY217015)	60
		^{142}LVPEPFDSVTDILNR (AF490538)	60
		^{163}LIPEPFDTVTDILAR (AB011546)	60
		^{167}LVPEPFDSVDTILAR (AJ515245)	60
		^{167}LVPEPFDSVDSILAR (AF007224)	60
1854.0	GNQADVQSLPR	^{282}GNQAKLQSAFR (AY677128)	64
		^{248}GNQGEVESPLA (AY217015)	45
		^{219}GNQGEVESPLA (AF490538)	45
		^{239}GNQGEVESPLA (AB011546)	45
		^{243}DNKGEAQSPLQ (AJ515245)	27
		^{243}DNKGEAQSPLQ (AF007224)	27

[a]AY677128: MnP isozyme precursor of *T. versicolor*; AY217015: RBPa precursor of *Bjerkandera* sp. B33/3; AF490538: RBP of *Bjerkandera* sp. B33/3; AB011546: MnP of *Pleurotus ostreatus*; AJ515245: putative versatile peroxidase precursor of *Lepista irina*; AF007224: versatile peroxidase VPL2 precursor of *P. eryngii*.

precursor (RBPa, AY217015) and versatile peroxidase (VP) of *Bjerkandera* sp. B33/3 (RBP, AA049485). The peptide with $[M+H]^+$ = 1631.9 showed the highest similarity (67%) to that of the MnP isozyme precursor of *T. versicolor* (AY677128). With the peptide with $[M+H]^+$ = 1854.0, no significant similarity (27 to 64%) was found with any other white rot fungal peroxidase (Table 3).

The amino acid sequences of the tryptic peptides of the purified MnP produced by *I. lacteus* were shown to have little similarity to those of other fungal MnPs and there-

fore, the molecular biological properties of the enzyme will require further studies.

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