FULL PAPER

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Cloning of *Lentinula edodes lemnp2*, a manganese peroxidase that is secreted abundantly in sawdust medium

Abstract Manganese peroxidase (MnP), which is one of the lignin-degrading enzymes from white-rot fungi, possesses oxidative activity against phenolic compounds, making it a useful enzyme for bioremediation. A novel MnP-encoding gene (*lemnp2*) was isolated from *Lentinula edodes*. The deduced amino acid sequence showed approximately 48.8% homology to LeMnP1. The cDNA clone was approximately 1.4 kbp whereas the genomic sequence was 1.9 kbp, and comparison of the two indicated that *lemnp2* contains 13 introns. The upstream region of *lemnp2* contains putative CAAT, TATA, and metal response elements. Additionally, LeMnP2 contains conserved motifs that are observed in fungal MnPs, including 10 cysteines, a Mn-binding site, and Ca^{2+}-binding sites. The *lemnp2* transcript was identified in mycelium cultivated on sawdust medium, and the protein was secreted into the medium. MnP activity was purified from the sawdust medium as one peak during purification. Western blot analysis confirmed that LeMnP2, but not LeMnP1, was secreted into the sawdust medium. These results collectively demonstrate that LeMnP2 is the major MnP secreted into sawdust medium.

Key words *Lentinula edodes* · Lignin-degrading enzyme · Manganese peroxidase · Sawdust medium

Introduction

Lignin is one of the major components of plant cell walls, comprising 20%–30% of the cell wall in woody plants.

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Lignin is synthesized by the free radical condensation of phenolic precursors and has a heterogeneous, random, and highly branched structure. Because of the unique structure of lignin, wood cell walls are resistant to degradation by most microorganisms. White-rot basidiomycetous fungi are organisms that can secrete enzymes which can degrade lignin. Such fungi produce various isoforms of extracellular lignin-degrading enzymes, including laccase (Lcc), lignin peroxidases (LiP), and manganese peroxidases (MnP) (Gold and Alic 1993). These lignin-degrading enzymes possess oxidative activity against phenolic compounds (Cohen et al. 2002; Hofrichter 2002), which can be used for bioremediation (Boer et al. 2004; Christian et al. 2005), biobleaching (Sasaki et al. 2001), and biofuel production (Larsson et al. 2001).

MnP oxidizes Mn^{2+} to Mn^{3+}, which is a strong oxidizer, in the presence of H_2O_2. Genes encoding MnPs in the white-rot basidiomycetous fungus *Phanerochaete chrysosporium* Burds. and in several basidiomycete fungi have been cloned and characterized. Fungal MnPs and LiPs are members of the class II peroxidase family (Welinder 1992), which is further divided into four classes based on their amino acid sequence similarity (Martínez 2002). MnPs belong to two separate classes: I and II (Martínez 2002). Recently, genes of a third type of peroxidase family, the versatile peroxidases (VPs), were cloned from *Pleurotus eryngii* (DC.) Gillet (VPL1 and -2; Ruiz-Dueñas et al. 1999) and *Pleurotus ostreatus* (Jacq.) P. Kumm. (MnP2; Kamitsuji et al. 2005). VPs are enzymatically similar to both MnPs and LiPs in that they can oxidize not only Mn^{2+} but also aromatic compounds or dyes in manganese-independent reactions. The regulation of MnP genes has been well investigated in *P. chrysosporium* and has been reviewed by Gold and Alic (1993) and Martínez (2002). MnP production in fungi is regulated at the transcriptional level by nutrient nitrogen sources (Pribnow et al. 1989; Li et al. 1994). Moreover, the MnP in *P. chrysosporium* is regulated by Mn^{2+}, H_2O_2, O_2, and heat shock in N-limited cultures (Godfrey et al. 1990; Mayfield et al. 1994; Li et al. 1995). Regulatory sequences of MnP genes have also been investigated in *P. chrysosporium* and are putative

heat shock elements (HSEs) and metal response elements (MREs) in the promoter region of the MnP gene (Alic et al. 1997).

Lentinula edodes (Berk.) Pegler (Shiitake in Japanese) is one of the most cultivated mushrooms in Asia. Shiitake grown on sawdust medium blocks the secretion of lignin-degrading enzymes into the waste medium. It is reported that *L. edodes* produces strong Lcc activity in liquid cultivation (Morisaki et al. 2001; Nagai et al. 2002). Two independent Lccs have been purified (Nagai et al. 2002, 2003), and Lcc-encoding genes have been cloned (Zhao and Kwan 1999) from *L. edodes*. In addition, a MnP was purified from *L. edodes* cultures grown on a wood substrate (Forrester et al. 1990). More recently, a MnP-encoding gene, *lemnp1*, was cloned (Nagai et al. 2007). It appears that the predicted N-terminus of the deduced amino acid sequence of *lemnp1* is different from that of MnP purified by Forrester et al. (1990), suggesting that there are at least two different MnP-encoding genes in *L. edodes*. In this report, we have cloned a novel MnP gene, *lemnp2*, from *L. edodes*, and shown that LeMnP2 is the major MnP secreted into sawdust medium.

Materials and methods

Strain and culture conditions

The *L. edodes* dikaryotic strain, SR-1 (previously designated as S-1; Hirano et al. 2000), was used in all the experiments. Mycelia were maintained on 1.5% agar plates with MYPG medium containing 0.25% Bacto malt extract (Difco, Detroit, MI, USA), 0.1% Bacto yeast extract (Difco), 0.1% tryptone peptone (Difco), and 0.5% glucose. Inocula were prepared from this plate, and mycelial disks were punched out with a cork borer (7-mm diameter). For genomic DNA extraction, mycelia were cultured in MYPG liquid medium for 4 weeks at 25°C with shaking as described previously (Sakamoto et al. 2005a). For RNA extraction, the sawdust medium was prepared as described previously (Nagai et al. 2003). A membrane filter (Isopore Membrane Filter; Millipore, Billerica, MA, USA) was placed on the surface of the sawdust medium (20 g/90-mm Petri dish) before being inoculated with mycelium and placed at 25°C. RNA was extracted from mycelium on the surface of the membrane. For enzyme purification, sawdust medium was prepared as described previously (Nagai et al. 2003), and a mycelial disk was inoculated on the center of the dish (20 g sawdust medium/90-mm Petri dish), and incubated at 25°C for 16 days.

Cloning and sequencing of *lemnp2*

To clone *lemnp2*, total RNA was extracted from mycelium grown for 24 days on the surface of sawdust medium using a MasterPure Yeast RNA purification kit (EPICENTRE; Biotechnologies, Madison, WI, USA). cDNA was synthesized from the mycelial RNA using the SMART RACE cDNA Amplification kit (BD Bioscience, San Jose, CA, USA). The 3′-RACE was performed using a degenerate primer (mnp2–4U; Table 1) designed against the N-terminal amino acid sequence from the MnP purified by Forrester et al. (1990). The cDNA for the 5′-RACE was synthesized from the same RNA using a GeneRacer kit (Invitrogen, Carlsbad, CA, USA) and PCR was performed as described by Sakamoto et al. (2005b), using *lemnp2*-specific primers (Table 1) and GeneRacer primers (Invitrogen).

Mycelium was cultured for 4 weeks in liquid MYPG medium before being collected and crushed in liquid nitrogen. Genomic DNA was isolated using Isoplant (Nippon Gene, Tokyo, Japan) following the manufacturer's protocol. Genome walking was then performed with *lemnp2* genomic DNA-specific primers (see Table 1) and adaptor primers (Takara Bio, Otsu, Japan), using a genome-walking library, followed by the PCR protocol described previously (Sakamoto et al. 2005a).

Table 1. Primers used in this study

Primer name	Sequence	Strategy
mnp2–4U	GCNGTNTGYTCNGAYGG (AVCSDG)[a]	Degenerate PCR
mnp2–671L-RACE	ACAGTGAGGGGAAGAGGGGAGGACA	5′-RACE
mnp2–265L-RACE	CTGAGGCAAGGAATGGAGCGAGGTC	5′-RACE
mnp2-rtU	CAACCGGACAGCCTGCTT	Real-time PCR
mnp2-rtL	TGGAACCTCGATGCCATCA	Real-time PCR
cmnp1RT-R	TGATCCCTTCCAATTCAAGGA	Real-time PCR
cmnp1RT-F	TCCGGGACCCGCTACATC	Real-time PCR
gpd-rtU	TGTTATCTCCAATGCTTCTTGCA	Real-time PCR
gpd-rtL	CCGAATTTGTCGTGGATAACCT	Real-time PCR
gmnp 415U	GGAAAATTCCCGACTATCAC	Genome walking
3′-MnP2D GW1U	CGTGCTGACTTGATTGACTG	Genome walking
3′-MnP2D GW2U	GTTTCGCTGATGTTCAGCAA	Genome walking
5′-MnP2D GW2L	DACAAGGGGGACAATACTCG	Genome walking
5′-MnP2D GW2L	AACATAGAGCCGTCAGCACC	Genome walking
mnp2D-0001	GGTATGTTCGGACGGCACTGTT	Genome walking
mnp2D-1045L	CAAGTGGCTTCGTTGTCGGGG	Genome walking
Mnp2DGW-4U	GACTGCTCCGCTGTTGTTCC	Genome walking

PCR, polymerase chain reaction; 5′-RACE, rapid amplification of 5-cDNA ends

[a] Amino acid sequence from which the degenerate primer was designed

Measurement of MnP activity

To determine MnP activity, guaiacol ($\varepsilon = 12\,000$ M^{-1}cm^{-1}; Wako Pure Chemicals, Osaka, Japan) was used as a substrate. The reaction mixture for the standard assay contained 0.4 mM guaiacol, 0.1 mM H_2O_2, 0.2 mM $MnSO_4$, McIlvaine buffer (pH 4.0), and 10 µl enzyme solution in a total volume of 200 µl. After incubation at 30°C, the oxidation of guaiacol was detected by measuring the absorbance at 470 nm. One unit of MnP activity was defined as the amount of enzyme needed to oxidize 1 µmol guaiacol in a 200-µl reaction mixture at 30°C for 1 min.

Measurement of the *lemnp1* and *lemnp2* mRNA levels

For real-time PCR analysis, RNA was extracted from mycelium on sawdust medium inoculated after 15, 20, 25, or 30 days. Total RNA was extracted using a MasterPure Yeast RNA purification kit (EPICENTRE Biotechnologies), according to the manufacturer's instructions. Reverse transcription reactions and genomic DNA removal were performed using the QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. SYBR Premix ExTaq (Takara Bio) was used in the PCR reaction mix, and reactions were performed with a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The primer sets for each gene are listed in Table 1; glyceraldehyde-3-phosphate dehydrogenase (*gpd*; Hirano et al. 1999) was used as an internal standard and amplified using the primers gpd-rtU and gpd-rtL (see Table 1). To standardize the results, the mRNA levels of *lemnp1* and *lemnp2* were determined as a ratio of the mRNA levels of the *mnp*s and *gpd*. The amplification efficiency of *lemnp1* was significantly lower ($<10^{-4}$) than that of *lemnp2* when the mnp2-rtU and mnp2-rtL primers were employed. The amplification efficiency of *lemnp2* was significantly lower ($<10^{-4}$) than that of *lemnp1* when the cmnp1RT-R and cmnp1RT-F primers were employed.

Western blot analysis of LeMnP1 and LeMnP2

Rabbit anti-LeMnP1 and anti-LeMnP2 antibodies were generated (custom service of Takara Bio) using three peptides (MnP1: LQSDLFDEGECGEEAHES, CSPT KGGGGADGS, and CPGTGGNQGEVES; MnP2: CNVGELRLQSDFELARD, DVQQACPSPFPSFTS DRA, and RETEIPHCPNEAT) that were detected as potentially exposed epitopes on MnP1 and MnP2 by Epitope Adviser 2.1 (FQS, Fukuoka, Japan). Proteins were extracted from sawdust medium 15, 20, 25, and 30 days after inoculation. The cultivated material (mixture of mycelium and the sawdust medium) was mixed with five times its volume of extraction buffer (200 mM sodium acetate, pH 4.2), stirred for 12 h, and clarified by centrifugation (12 000 *g* for 15 min). Protein concentrations were determined using a Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin (BSA) as a standard. Samples were separated by electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes as described previously (Sakamoto et al. 2005b). LeMnP1 and LeMnP2 primary antibodies were used followed by a horseradish peroxidase (HRP)-conjugated antirabbit IgG secondary antibody (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Hybridization was visualized by using an ECL detection kit (GE Healthcare) following the manufacturer's instructions.

Purification of LeMnP2

All the purification steps were carried out at 4°C. Column chromatography was operated either with a gradient controller (AC-5900 Gradicon III; Atto, Tokyo, Japan) or with an FPLC system (GE Healthcare). The cultivated material was mixed with 100 ml 10 mM sodium phosphate buffer (PB; pH 6.0), and stirred for 12 h. The suspension was then filtered through a plastic mesh and clarified by centrifugation (12 000 *g* for 15 min). The resulting supernatant was the crude enzyme. Powdered ammonium sulfate was added to 50 ml crude enzyme to achieve 40% saturation, and the resulting precipitate was removed by centrifugation.

The supernatant was applied to a Toyopearl Butyl-650 M (Tosoh, Tokyo, Japan) column (10×100 mm) equilibrated with PB containing 40% saturated ammonium sulfate. The column was washed with the same buffer, and adsorbed proteins were eluted with a linear gradient of ammonium sulfate (80 ml, 40%-0% saturation) in PB, at a flow rate of 1 ml/min. The fractions were collected, and MnP activity was measured. Fractions containing MnP activity were dialyzed against PB and applied to a Toyopearl DEAE-650 M (Tosoh) column (10×50 mm) equilibrated with PB. After washing with PB, the adsorbed proteins were eluted with a linear concentration gradient of NaCl (40 ml, 0–0.5 M) in PB at a flow rate of 1 ml/min. The MnP active fractions were pooled and concentrated to approximately 250 µl by ultrafiltration through Centricon-30 filters (Millipore). The concentrate was then applied to a Superdex 75 HR 10/30 column (1×30 cm; GE Healthcare) equilibrated with the PB containing 100 mM NaCl. The enzyme was eluted with the same buffer at a flow rate of 250 µl/min.

Analysis of the amino acid sequence of LeMnP2

Purified LeMnP2 (100 pmol) was loaded onto a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. After electrophoresis, the protein was transferred to a PVDF membrane (Atto) by electroblotting using a semidry blotter (AE-6677 HorizBlot; Atto), and then stained with Coomassie brilliant blue (CBB). The region of the membrane containing MnP2 was excised, and the amino acid sequence was determined using an ABI Procise 491HT Protein Sequencing System (Applied Biosystems).

Results and discussion

Cloning of the *lemnp2*

Lemnp2 was cloned by 3′-RACE using cDNA that was synthesized from mycelial RNA. The design of the degenerate primer mnp2–4U (see Table 1), which was used to amplify *lemnp2*, was based on the N-terminal amino acid sequence of the MnP purified from *L. edodes* (Forrester et al. 1990) and the MnP consensus sequence. A fragment of approximately 1.1 kbp was amplified, subcloned, and sequenced. We identified a sequence similar to that of several MnP-encoding genes, and the deduced amino acid sequence was identical to the N-terminus of *L. edodes* MnP (Forrester et al. 1990). Following 5′-RACE, ~1.4 kbp of cDNA designated as *lemnp2* was cloned and sequenced. The deduced amino acid sequence of *lemnp2* (cDNA sequence of *lemnp2*; DDBJ accession no. AB306943) had a sequence identical to the N-terminus of *L. edodes* MnP (Forrester et al. 1990; Table 2), except for the third cysteine. Forrester et al. would not have detected the third cysteine by the Edman degradation sequencing method because the protein sample was not pyridilethylated; therefore, we concluded that LeMnP2 is the same as *L. edodes* MnP purified by Forrster et al. (1990). The putative amino acid sequence of *lemnp2* exhibited many of the characteristics of MnP-encoding genes. The PSORT II program (http://psort.ims. u-tokyo.ac.jp/form2.html) predicted the mature protein to be an extracellular or cell wall protein. A cleavage site was also predicted between residues 25 and 26 from putative translation start methionine (Fig. 1A). Thus, it is predicted that the putative mature protein has 350 amino acid residues, a molecular weight of 37 kDa, and a pI value of 3.98. The predicted amino acid sequence of LeMnP2 had 48.8% similarity to that of LeMnP1 (Nagai et al. 2007). Martínez

Table 2. N-terminal amino acid sequence of MnP in *Lentinula edodes*

Origin	Enzyme	N-terminal amino acid sequence	Reference
L. edodes	LeMnP2	AVCSDGT VVPDSV	This article
L. edodes	MnP	AVGSDGT VVPDSV	Forrester et al. 1990
L. edodes	LeMnP1	FACA DG VHTTSNEV	Nagai et al. 2007

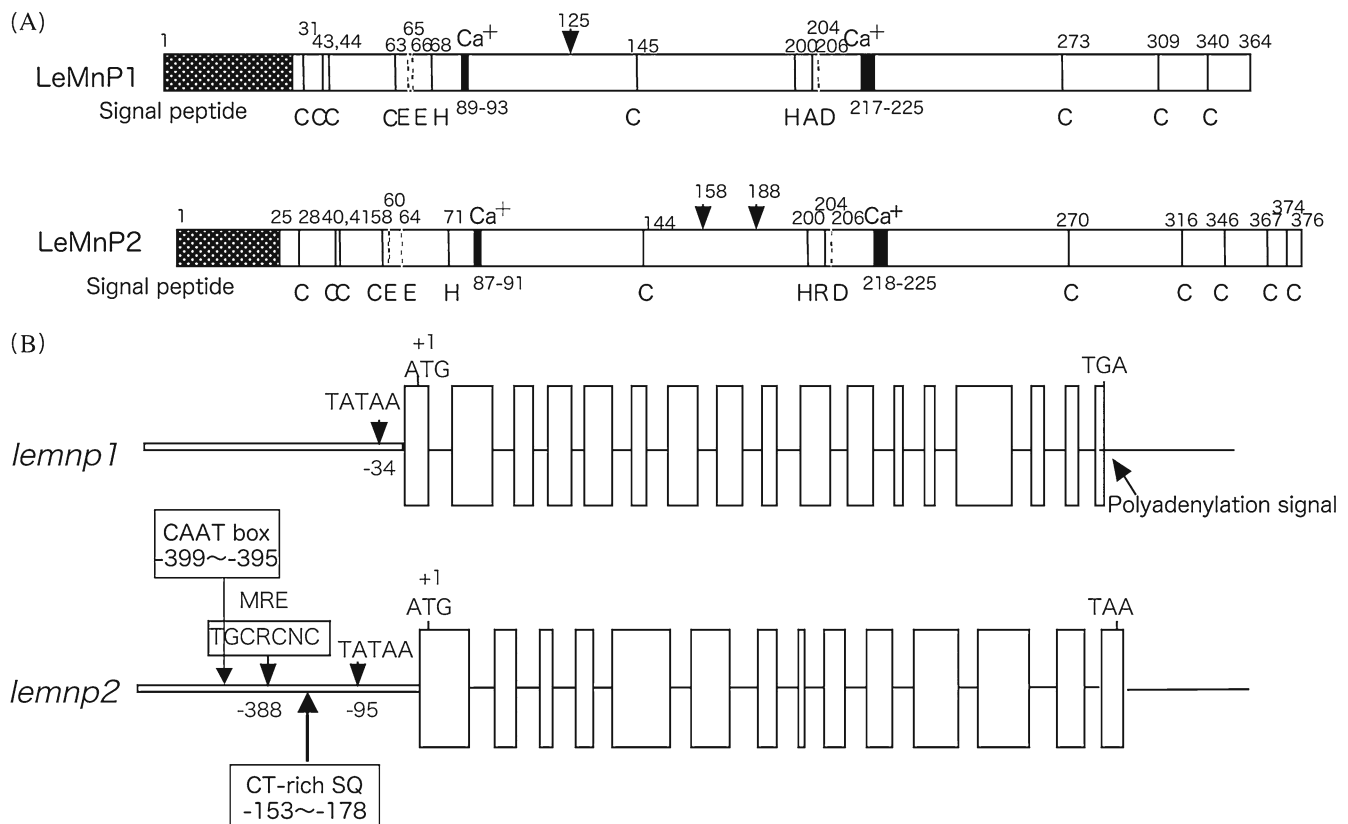Black blocks indicate amino acids identical to LeMnP2

Fig. 1. Structure of *lemnp1* and *lemnp2*. **A** Comparison of the amino acid sequences of *Lentinula edodes* LeMnP1 and LeMnP2. *A*, alanine in LeMnP1; *C*, cysteines involved in disulfide formation; *Ca⁺*, potential Ca^{2+}-binding sites; *D* and *E*, amino acids involved in manganese binding; *H*, distal and proximal histidines; *R*, arginine in LeMnP2; *arrowheads*, possible N-glycosylation site. **B** Comparison of the intron–exon structure of *L. edodes lemnp1* and *lemnp2*. *CAAT box*, CT-rich region; *MRE*, metal response element. TATA boxes are indicated by *arrowheads*

(2002) discussed the conserved amino acid residues in fungal MnPs; therefore, we compared the deduced amino acid sequence of *lemnp2* to other fungal MnPs using Clustal W (http://clustalw.ddbj.nig.ac.jp/top-j.html). The amino acid residues that are known to be involved in peroxidase function were conserved in LeMnP2 (Fig. 1A). We also identified several catalytic histidines, acidic residues, the 60th glutamic acid from the putative translation start methionine (E_{60}), E_{64} and D_{206} that are involved in binding Mn^{2+}, and R_{204}, which is required for the stable binding of Mn^{2+} (Martínez 2002). R_{204} in LeMnP2 is replaced by an alanine in LeMnP1 (see Fig. 1). R_{204} is critical for Mn^{2+} binding; therefore, Mn^{2+} oxidation activity between LeMnP1 and LeMnP2 may differ. Martínez (2002) divided the fungal peroxidases into four classes based on their amino acid sequence; according to this classification, *lemnp1* belongs to class II and *lemnp2* belongs to class I. MnPs in class I are typical manganese peroxidases whereas class II members also include VPs that have both manganese peroxidase and lignin peroxidase activities (Martínez 2002). LeMnP2 contains eight cysteine residues (in the same position as those observed in MnPs) and two additional cysteines in its C-terminus (Fig. 1A). The additional two cysteines in LeMnP2 are a conserved feature in class I MnPs (Martínez 2002) and may participate in stabilizing the Mn-binding site (Sundaramoorthy et al. 1994). These data collectively suggest that LeMnP1 and LeMnp2 have different oxidative activities for Mn^{2+}.

The structure of the *lemnp2*

The genomic DNA fragment of *lemnp2* was amplified from genomic DNA using primers designed against its cDNA sequence (genome sequence of *lemnp2*; DDBJ accession no. AB306944). The coding regions of the genomic fragments were compared to the cDNA sequence, and 13 introns were identified in *lemnp2* (see Fig. 1B). Except for the second intron, these introns have 5′- (GTRNGT) and 3′- (YAG) splice site consensus sequences (Gurr et al. 1987). We sequenced ~1.3 kb upstream of the putative translational start site and identified the start site for transcription at −65 nt by comparing it to that of the cDNA fragment. In filamentous fungi, a number of transcription initiation sites may occur at, or immediately downstream of, CT-rich sequences (Gurr et al. 1987). In the 5′-flanking region of *lemnp2*, a CT-rich sequence was observed between −153 and −178 nt, a consensus TATA box was located at −95 to −91 nt (Fig. 1B), and a CAAT box was located between −399 and −395 nt from the putative translation start codon. A metal response element (MRE) motif (TGCRCNC) was observed between −379 and −373 nt (Fig. 1B). However, heat shock elements (HSE) and xenobiotic responsive elements (XRE) that have been previously observed in several *mnp* promoters (Martínez 2002) were not found in the 5′-flanking region of *lemnp2*. We also sequenced ~0.6 kb downstream from the ORF and identified a polyadenylation site 204 nt from the putative translational stop codon. There were no consensus sequences for a polyadenylation signal from higher eukaryotes (AATAAAA) in the *lemnp2* gene. This observation is in agreement with previous reports demonstrating that such consensus sequences are lacking in most yeasts and filamentous fungi (Ballance 1990).

The mRNA, protein, and activity of MnPs in sawdust medium

Nagai et al. (2007) reported that *lemnp1* is an MnP-encoding gene from *L. edodes*; therefore, we compared the transcription and secretion of MnP-encoding genes. Nagai et al. (2002) revealed that MnP activity was not detectable in liquid culture and that *lemnp1* and *lemnp2* mRNAs were rare (data not shown). We cultivated *L. edodes* on sawdust medium and measured the MnP activity at 15, 20, 25, and 30 days after inoculation using guaiacol as a substrate. MnP activity was not detected on days 15, 20, and 30, but was observed 25 days after inoculation (0.012 units/mg protein). We also investigated the mRNA levels of *lemnp1* and *lemnp2* in mycelia that were grown on sawdust medium. On days 15 and 20 the mRNA levels of *lemnp1* were higher than those of *lemnp2* (Fig. 2A), but the MnP activity on these days was undetectable, suggesting that the mRNA levels of *lemnp1* do not correlate with MnP activity. *lemnp2* was strongly expressed on day 25 and immediately decreased 30 days after inoculation (Fig. 2A). This peak in *lemnp2* mRNA correlates with the MnP activity secreted into sawdust medium. The differential transcription patterns between *lemnp1* and *lemnp2* suggest that there are differences in the regulatory sequences of their promoter regions. One example of this difference is that the promoter region of *lemnp2* has an MRE whereas that of *lemnp1* does not (see Fig. 1B). However, the inducer(s) of *lemnp1* and *lemnp2* are still unclear; therefore, regulation of these genes should be carefully investigated in the future.

We performed Western blot analysis on protein extracted from sawdust medium using an α-LeMnP2 antibody. We found that LeMnP2 was only present in the medium on day 25 (see Fig. 2B), which was the day on which we observed the highest level of MnP activity. We did not observe any positive bands on blots probed with an α-LeMnP1 antibody (data not shown), suggesting that *lemnp1* is transcribed but not translated or secreted into culture medium. These data suggest that LeMnp2 is primarily responsible for MnP activity in the sawdust medium.

Characterization of MnP secreted in the sawdust medium

To confirm that MnP was secreted into the sawdust medium, we purified MnP from the medium. During all the purification steps, we only observed a single peak for MnP activity (data not shown), suggesting that *L. edodes* predominantly produces only one MnP in sawdust medium. After gel filtration using a Superdex 75 column, only one protein band was present on the SDS-PAGE gel when the MnP activity peak fraction was loaded (Fig. 3). The molecular weight of

Fig. 2. Transcription and translation levels of *lemnp1* and *lemnp2* on sawdust medium at day 15, day 20, day 25, and day 30 after inoculation. **A** Expression levels of *lemnp1* and *lemnp2* in mycelium grown on sawdust medium. Data represent means and standard deviations of three independent experiments. *y*-axis represents the ratio between the transcription of *mnp*s and *gpd*. **B** Western blot analysis of LeMnP2 secreted into sawdust medium
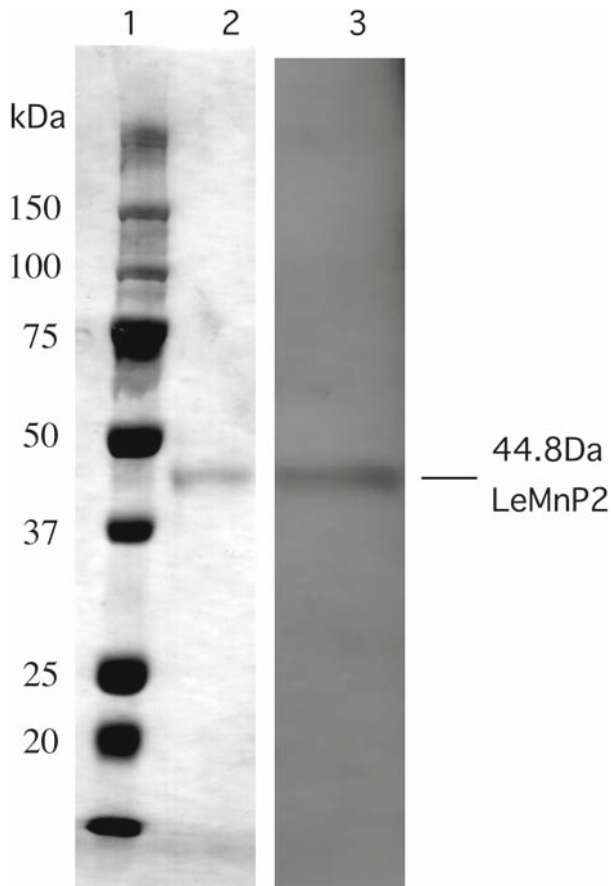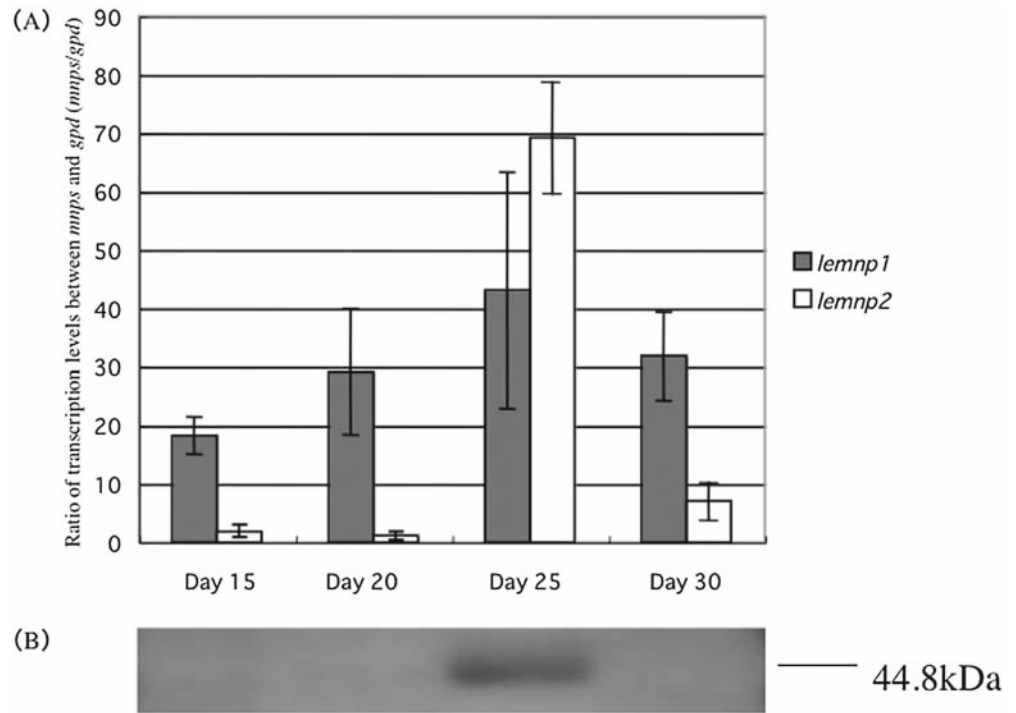

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis of purified LeMnP2. *Lane 1*, molecular size marker; *lane 2*, SDS-PAGE and Coomassie brilliant blue (CBB) staining of LeMnP2; *lane 3*, Western blot analysis of LeMnP2 using α-LeMnP2

LeMnP2 was estimated to be 48 kDa by gel filtration and 44.8 kDa by SDS-PAGE. These results suggest that the enzyme is active in its monomeric form. The N-terminal amino acid sequence of this protein was consistent with that of the deduced amino acid sequence of *lemnp2* (we could not detect the third cysteine by the Edman degradation sequencing method because the protein sample was not pyridilethylated) and with that of the previously purified MnP from *L. edodes* (Forester et al. 1990; see Table 2). We could not identify any amino acid peaks other than the deduced amino acid sequence of *lemnp2* by the Edman degradation sequencing method. The purified MnP reacted with an α-LeMnP2 antibody (see Fig. 3) but not with the α-LeMnP1 antibody. Collectively, these data suggest that *L. edodes* predominantly secretes LeMnP2, but not LeMnP1, into the sawdust medium.

We could not detect LeMnP1 in sawdust cultures by Western blot analysis using an α-LeMnp1 antibody. Additionally, we failed to detect LeMnP1 at any stage or in any tissue (including the fruiting body) by Western blot analysis (data not shown). Heterologously expressed LeMnP1 in *Escherichia coli* reacted with the LeMnP1 antibody (data not shown), suggesting that if LeMnP1 is secreted in the sawdust medium, we were unable to detect it. It is possible that *lemnp1* is transcribed but not translated or secreted into culture medium. Another possibility is that the α-LeMnP1 antibody could not detect native LeMnP1 because it might be glycosylated (Martínez 2002). Only a single MnP activity peak was observed throughout our purification steps (see Fig. 3), making this possibility unlikely. However, LeMnP1 might have different enzymatic properties to LeMnP2, which could explain why we were unable to detect LeMnP1 enzyme activity.

In this article, we report isolating and characterizing a novel manganese peroxidase-encoding gene, *lemnp2*, from *L. edodes* and purifying the protein encoded by this gene. Moreover, we found that LeMnP2 is the major MnP secreted by *L. edodes* into sawdust medium. We also observed MnP activity and abundant LeMnP2 secretion in a sawdust medium block used for mushroom production (data not shown); therefore, LeMnP2 can be collected from waste sawdust media for Shiitake production. The molecular and biochemical data reported here provide valuable information about lignin-degrading enzymes in white-rot fungi and may be useful for future industrial applications.

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