Mycoscience (2007) 48:125–130
DOI 10.1007/s10267-006-0334-4

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Isolation and characterization of the gene encoding a manganese peroxidase from *Lentinula edodes*

Received: May 11, 2006 / Accepted: October 15, 2006

Abstract A genomic DNA sequence and cDNA encoding a putative manganese peroxidase were isolated from the white-rot basidiomycete *Lentinula edodes*. The gene, called *lemnp1*, consists of a 1985-bp open reading frame interrupted by 16 introns and was flanked by an upstream region having putative CAAT, TATA, and heat shock elements and by a downstream region having polyadenylation signals. The *lemnp1* gene encodes a protein of 364 amino acids that shows high sequence homology to manganese peroxidases of other basidiomycetes. The deduced N-terminal amino acid sequence is different from the *L. edodes* manganese peroxidase reported previously.

Key words Bioremediation · Gene sequence · *Lentinula edodes* · Manganese peroxidase

White-rot fungi produce various isoforms of extracellular lignin-degrading enzymes, including laccase, lignin peroxidase (LiP), and manganese peroxidase (MnP) (Gold and Alic 1993). These enzymes have attracted wide attention because of their ability to degrade environmentally persistent xenobiotics and endocrine-disrupting chemicals such as dioxins or bisphenol A (Cristian et al. 2005; Boer et al. 2004). White-rot fungi secrete many kinds of MnPs and LiPs during the vegetative mycelial growth stage (Gold and Alic 1993). Generally, MnPs and LiPs occur as a series of isozymes encoded by a family of genes; many MnP and LiP isozymes have been purified, and their encoding genes have been cloned from various white-rot basidiomycetes (Martínez 2002). Basidiomycete MnPs and LiPs are heme-containing class II fungal peroxidases (Welinder 1992). LiP oxidizes the nonphenolic unit of lignin to produce a cation radical on the aromatic ring. MnP removes an electron from the phenol moiety of lignin to generate a phenoxy radical. Fundamental to the catalytic activity of MnP is the oxidation of Mn^{2+} to Mn^{3+} in the presence of H_2O_2. Mn^{3+} is stabilized by organic chelators and can then oxidize phenolic substrates. These two types of peroxidases share a high degree of primary sequence homology; the catalytic site amino acid residues (the distal histidine and arginine, and the proximal histidine) are conserved, as are eight or ten cysteine residues involved in disulfide bond formation (Gold and Alic 1993). In addition, the manganese-binding sites are conserved in MnPs (Irie et al. 2000). Recently, a third type of peroxidase, named versatile peroxidase (VP), was isolated and cloned from *Pleurotus eryngii* (DC.) Quél (VPL1 and -2; Ruiz-Dueñas et al. 1999) and *P. ostreatus* (Jacq.: Fr.) Kummer (MnP2; Kamitsuji et al. 2005). VPs show characteristics of both MnP and LiP; they can oxidize Mn^{2+}, but can also oxidize aromatic compounds or dyes in manganese-independent reactions.

Lentinula edodes (Berkely) Pegler is an important edible mushroom in Japan, more commonly known as the shiitake mushroom. The culture filtrates of this fungus exhibit strong laccase activity, and we have reported the purification of two laccases from *L. edodes* cultures (Nagai et al. 2004). In contrast, a recent report indicated that *L. edodes* has no LiP and only weak MnP activity (Morisaki et al. 2001). Forrester et al. (1990) reported that *L. edodes* produced only one MnP in solid-state cultures using oak wood as the substrate. The use of the MnPs produced by this readily available, edible fungus has clear advantages for environmental bioremediation: the waste culture after mushroom harvesting can be used as a source of the enzyme, and the enzyme produced by *L. edodes* would be nontoxic to humans. In this study, we isolated an *mnp* gene from *L. edodes*, with a view toward using MnP for environmental bioremediation.

A commercial dikaryotic strain of *L. edodes* H600 (Hokken, Tochigi, Japan) was used in all experiments. For genomic DNA and mRNA extraction, mycelia were

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Table 1. Primers used in this study

Primer name	Sequence	Strategy
TFHDA-U	ACNTTYCAYGAYGCNAT	Degenerate PCR
NCPGAP-L	GGNGCNCCNGGRCARTT	Degenerate PCR
5′RACE1	AAACGTATTTCTCCCGGCACTGGAGATTC	5′RACE, probe construction
5′RACE2	TGAGCATCCACAATATCATCGATACCAC	5′RACE
3′RACE1	GCGGACGGTTCCATTATAGCGTTTTCTG	3′RACE, probe construction
3′RACE2	GTGGTATCGATGATATTGTGGATGCTCA	3′RACE
MnATGU	CAATGTTTTTTATCGGACTTCTCAGTAC	Full-length cDNA
MnTGAL	TCCAATTCAAGGATCACGATGGTGGGCTG	Full-length cDNA
5′GSP1	GTTTCGATGTCAGAAAACGCTATAATGGAA	Genome-walking
5′GSP2	GGGTACACATAAAATGAAGAAAAGTAAT	Genome-walking
3′GSP1	TGCAGTAGGTGAGTTATTCCATTGCTCTAA	Genome-walking
3′GSP2	TTCCTGATCTTCTCAATTTCTGCAATAC	Genome-walking
gmnpU	CCTGCTCCTCCTGTACCATCTGGACACAAA	Full-length genome
gmnpL	CCTGCCAGGCTATTAATCAAACTTTTGAT	Full-length genome

cultured in 0.25× MYPG liquid 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 by the method of Sakamoto et al. (2005). To obtain a DNA fragment encoding part of the MnP from *L. edodes*, degenerate sense and antisense primers (TFHDA-U and NCPGAP-L; listed in Table 1) were synthesized based on two amino acid sequences, TFHDAI and NCP-GAP, which are highly conserved in MnP-encoding genes in basidiomycetes. Degenerate polymerase chain reaction (PCR) was performed for 30 cycles with ExTaq DNA polymerase (Takara Bio, Shiga, Japan) after genomic DNA was denatured at 94°C for 2 min. Each cycle consisted of 30 s at 94°C, 1 min at 55°C, and 2 min at 72°C. A 440-bp fragment, named c9-5, was amplified by the PCR, electrophoresed on a 1% (w/v) agarose gel, extracted from the gel, purified using SUPREC-01 (Takara Bio), ligated into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA), and sequenced. The nucleotide sequences of the cloned fragments were analyzed by using an ABI 3100 capillary sequencer (Applied Biosystems, Foster City, CA, USA) and the GENETYX Mac version 12 software (GENETYX, Tokyo, Japan). The nucleotide sequence of c9-5 showed 54.4% homology to *mnp3* of *P. ostreatus* (DDBJ accession number AB016519).

Total RNA was isolated from *L. edodes* mycelia using a FastRNA Pro Red kit (Qbiogene, Carlsbad, CA, USA) according to the method of Sakamoto et al. (2005). cDNA was synthesized from total RNA using the SMART PCR cDNA amplification kit (Clontech, San Jose, CA, USA). RACE (rapid amplification of cDNA ends) PCR was performed using specific primers (5′RACE1, 5′RACE2, 3′RACE1, and 3′RACE2; see Table 1) designed from the sequence of the c9-5 fragment, the SMART universal primer A mix, and the nested universal primer A (Clontech). The PCR conditions were the same as described above. Bands amplified by nested PCR were purified, subcloned, and sequenced. 5′-RACE PCR produced a 370-bp band. The sequence of this product initiated at –22 nucleotides upstream from the deduced start codon, suggesting that transcription starts from this position. 3′-RACE PCR produced two bands (1079 and 1062 bp) having different poly A signals at 259 and 276 nucleotides downstream from the stop codon. Finally, the full-length open reading frame (ORF) was amplified by PCR using two specific primers, MnATGU and MnTGAL (Table 1), the Phusion DNA polymerase (FINEZYME, Espoo, Finland), and total cDNA synthesized from RNA extracted from the mycelia as template. The amplified fragment was subcloned and sequenced. This cDNA included a 1095-bp ORF, which encodes 364 amino acids. We named this gene *lemnp1* and the encoded enzyme LeMnP1.

The genomic DNA including *lemnp1* was cloned by genome-walking using a Universal Genome Walker kit (Clontech) following the method of Sakamoto et al. (2005). Genome-walking PCR was performed using specific primers (5′GSP1, 5′GSP2, 3′GSP1, and 3′GSP2; Table 1) designed from the sequence of c9-5, the adaptor primers (Clontech), and the genome-walking library as template. The PCR conditions were the same as described above. The nested PCRs produced several positive bands. The longest positive bands were obtained using a *Stu*I library for the regions both upstream (1.8 kbp) and downstream (2.5 kbp) from c9-5. These fragments were subcloned and sequenced. The PCR to amplify the full length genomic DNA including the ORF of the *lemnp1* gene was performed using two specific primers, gmnpU and gmnpL (Table 1), the Phusion DNA polymerase (FINEZYME), and genomic DNA as template. A 4460-bp fragment was obtained by this PCR. Finally, this fragment was subcloned and sequenced. A comparison of the genomic DNA and the cDNA showed that the *lemnp1* gene consisted of a 1985-bp ORF interrupted by 16 introns (Fig. 1). The nucleotide sequence data reported in this article were submitted to the DDBJ/EMBL/GenBank nucleotide sequence database with the accession number AB241061. To our knowledge, this is the first report of the isolation of an *mnp* gene from *L. edodes*.

To construct a probe for Southern blotting, a genomic DNA fragment of the *lemnp1* gene was amplified by PCR using two primers, 5′RACE1 and 3′RACE1 (see Table 1), and the PCR conditions described above. The amplified fragment was purified and labeled using an ECL direct nucleic acid labeling kit (GE Healthcare Bio-Sciences, Uppsala, Sweden). Southern blot analysis was performed by

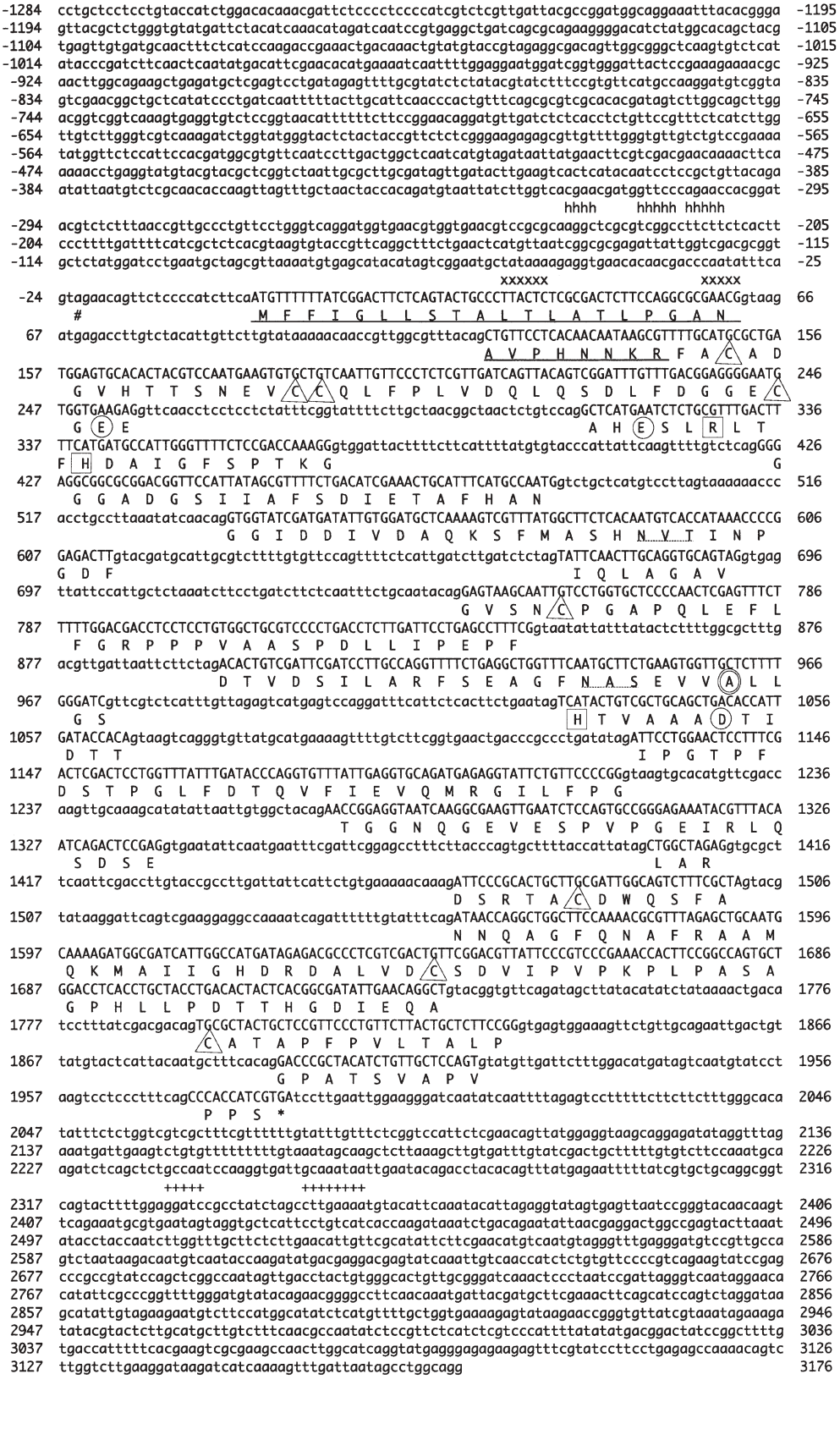

Fig. 1. The nucleotide sequence of the *lemnp1* gene. The deduced amino acid sequence is shown *below* the nucleotide sequence. *Capital letters* correspond to the deduced open reading frame. The transcription initiation site (#), putative stop codon (*), TATA box and CAAT motif (*x*), putative HSE (*h*), and putative polyadenylation signal (+) are marked. *Open boxes* show the catalytic site amino acids mediating peroxidase function. The amino acid residues indicated by *open circles* are residues constituting the putative Mn-binding site. *Open triangles* indicate cysteine residues involved in disulfide bonds. Ala195 is marked by a *double circle*. Putative signal peptides are *underlined*. The amino acid sequences marked by *dashed underlines* are putative *N*-glycosylation sites

the method of Hirano et al. (1999). The genomic DNA of *L. edodes* was digested with *Bam*HI, *Bgl*II, *Stu*I, *Xba*I, *Xho*I, or *Stu*I and *Xba*I, electrophoresed in a 0.75% agarose gel, and transferred to a nylon membrane (Hybond-N^+; GE Healthcare Bio-Sciences). Hybridization and detection were performed using the ECL detection kit (GE Healthcare Bio-Sciences), according to the manufacturer's instructions. Southern hybridization showed the expected positive bands (Fig. 2). This result suggested that *lemnp1* is a single-copy gene.

Amino acid residues known to be involved in peroxidase function, Arg72, His76, and His200 (Martínez 2002), were conserved in LeMnP1. The residues that constitute the Mn-binding sites of the *Phanerochaete chrysosporium* Burds MnP isozymes (Alic et al. 1997) and the *Agaricus bisporus* (Lange.) Imbach MnP (Lankinen et al. 2005) were also conserved in LeMnP1 (Glu65, Glu69, and Asp206). Also, eight cysteine residues involved in disulfide bond formation (Gold and Alic 1993) were conserved in LeMnP1 at positions corresponding to the cysteines in other MnP isozymes. A search for a compatible three-dimensional (3-D) molecular model for LeMnP1 using the LIBRA system (DDBJ service, http://www.ddbj.nig.ac.jp/search/libra_i-j.html), suggested that it resembles the structure of *P. chrysosporium* MnP1 (Sundaramoorthy et al. 1994) or LiPH8 (Blodig et al. 2001) with a high degree of similarity. Additionally, LeMnP1 has two putative *N*-glycosylation sites at Asn125 and Asn189. Asn125 corresponds to the glycosylation site of *Pleurotus ostreatus* MnPs (Irie et al. 2000) and *Phlebia radiata* Fr.: Fr. MnPs (Hildén et al. 2005). These results suggest that LeMnP1 is a fungal class II peroxidase family member and possibly exhibits manganese peroxidase activity.

An unrooted phylogenetic tree based on the distances among amino acid sequences of LiPs and MnPs was generated by the neighbor-joining method using CLUSTAL W (http://align.genome.jp/). The result (Fig. 3) indicated that LeMnP1 is closely related to a group of genes characterized by *P. ostreatus* MnPs (DDBJ, U21878, AJ243977, and AB016519) and *P. eryngii* VPs (AF007221, AF007222) and is divergent from the MnPs of *P. chrysosporium* (J04624, J04980, and U70998) and *Ceriporiopsis subvermispora* (Pilát) Gilbertson and Ryvarden (AF036254 and AF161585).

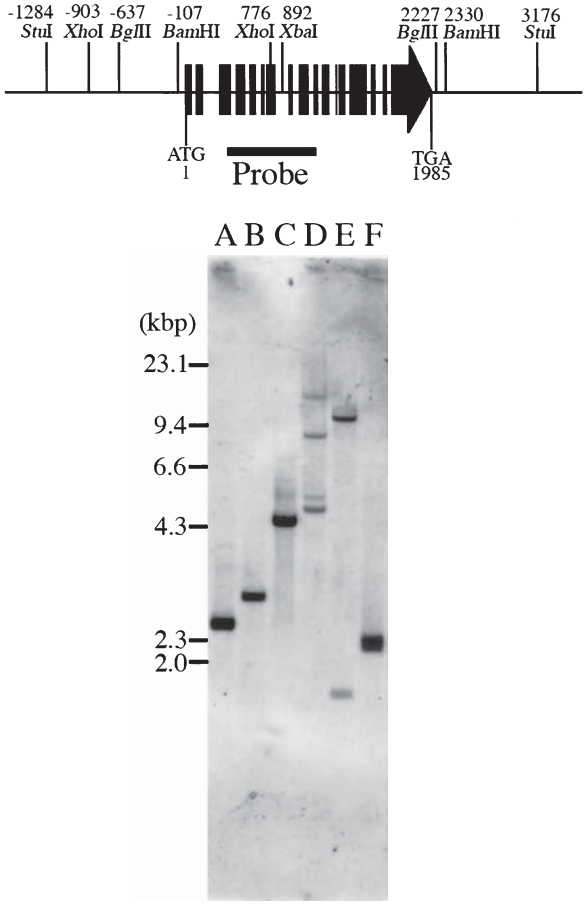

Fig. 2. Southern blot analysis of genomic DNA with a genomic map of the *lemnp1* gene. Genomic DNA was digested with *Bam*HI (*A*), *Bgl*II (*B*), *Stu*I (*C*), *Xba*I (*D*), *Xho*I (*E*), or *Stu*I and *Xba*I (*F*). The *boxes* indicate exons, and the *numbers* represent the position relative to the translation start codon

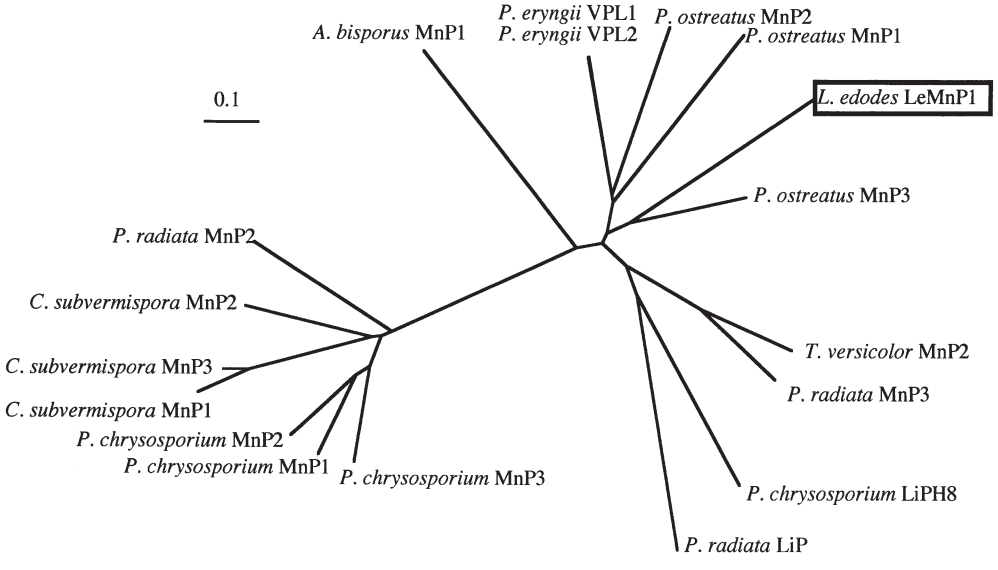

Fig. 3. An unrooted phylogenetic tree based on the distance among primary peptide sequences of lignin peroxidases (LiPs) and manganese peroxidases (MnPs) from various basidiomycetes. The LeMnP1 of *Lentinula edodes* is *boxed*. DDBJ accession numbers are given in the text, with the exception of *Trametes versicolor* (L.: Fr.) Pilát MnP2 (AF102515), *Phanerochaete chrysosporium* LiPH8 (M27401), and *Phlebia radiata* LiP (AY743218)

Amino acid sequence homology analysis against MnPs of basidiomycetes revealed that LeMnP1 showed a high degree of homology to *P. ostreatus* MnP3 (69.4%; AB016519) and *P. radiata* MnP3 (66.1%; AJ310930) and a low degree of homology to *P. chrysosporium* MnP1 (50.0%; J04624) and MnP2 (48.2%; J04980). *P. ostreatus* MnP2 (Cohen et al. 2001), and *P. eryngii* VPL1 and VPL2 (Ruiz-Dueñas et al. 1999), which are closely related to LeMnP1 in the unrooted phylogenetic tree, are reported to have VP activity. Kamitsuji et al. (2005) and Hildén et al. (2005) reported that a tryptophan residue exposed on the surface of the enzyme (W197 of *P. ostreatus* MnP2, or W194 of *P. eryngii* VPL1, –2) is necessary for VP activity, because tryptophan forms a suitable binding site for polymeric substrates. Considering that the corresponding amino acid residue of LeMnP1 is substituted to alanine (Ala195, Fig. 1), LeMnP1 may not have VP activity.

The hydrophobic nature of the first 28 amino acids of LeMnP1 and the presence of the dipeptide Lys27–Arg28, which are characteristics of the processing site of the KEX2 protease in *Saccharomyces cerevisiae* Gasp. (Fuller et al. 1989), suggested that this 28-amino-acid sequence is the signal peptide (see Fig. 1). Thus, the N-terminal amino acid sequence of mature LeMnP1 was deduced to be FACADGVHTTSNE. This sequence shows high homology to MnP3 of *P. ostreatus* (Irie et al. 2000), but it is different from that of the previously reported MnP from *L. edodes*, AVCSDGTVVPDS (Forrester et al. 1990). Kamitsuji et al. (2004) have reported that the production of MnP isozymes by *P. ostreatus* was controlled by culture conditions (nitrogen concentration) at the transcriptional level. In *P. chrysosporium*, the expression of the MnP gene can be regulated by Mn, nitrogen concentration, and heat shock (Martínez 2002). Considering that *L. edodes* was previously reported to produce only one MnP in oak wood culture (Forrester et al. 1990), LeMnP1 may be an isozyme of *L. edodes* repressed by the environmental conditions employed in the previous study. Although the cDNA of *lemnp1* was cloned from the total RNA of the mycelia cultured in MYPG liquid culture, MnP activity was not detected under this condition (Nagai et al. 2002). This result suggests that *lemnp1* might be repressed in the liquid culture. The promoter region of *lemnp1* contains the CAAT motif (CAAT, from –34 nt to –31 nt) and a TATA box (TATAAAA, from –59 nt to –53 nt) slightly upstream from the putative transcription start position (see Fig. 1). Heat shock elements (HSE) consist of arrays of the 5-bp unit NGAAN arranged as inverted repeats (Fernandes et al. 1994). An HSE-like sequence was detected in *lemnp1*, but this sequence contains some extra nucleotides within the inverted repeats (from –321 nt to –302 nt; Fig. 1). No putative metal response element was detected in *lemnp1*.

Continuing investigations into the regulation of *lemnp1* gene expression are currently underway, as well as studies to isolate genes encoding isozymes of MnPs. Methods to transform *L. edodes* have already been described (Sato et al. 1998), and future studies will include the overexpression of *lemnp1* in *L. edodes* to facilitate environmental bioremediation using the edible mushroom *L. edodes*.

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