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ORIGINAL ARTICLE

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Isolation of cDNA and genomic fragments encoding the major manganese peroxidase isozyme from the white rot basidiomycete *Pleurotus ostreatus*

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Abstract We have isolated the cDNA and genomic sequences encoding the major isozyme of manganese peroxidase, MnP3, from the white rot basidiomycete *Pleurotus ostreatus* strain IS1. The gene *mnp3* is interrupted by 10 introns and encodes a mature protein of 357 amino acid residues with a 26-amino-acid signal peptide. The amino acid residues known to be involved in peroxidase function and those that form the Mn-binding site in the *Panerochaete chrysosporium* MnP isozyme are conserved in MnP3. Comparison of the deduced primary structure of MnP3 with those of other peroxidases from various white rot fungi suggested that MnPs from *P. ostreatus* and *Trametes versicolor* belong to a subgroup that is more similar to the lignin peroxidases than MnPs from *P. chrysosporium* or *Ceriporiopsis subvermispora*.

Key words Lignin degradation · White rot fungi · Ligninolytic enzyme · Edible mushroom · Heme protein

Introduction

The white rot basidiomycetes degrade plant cell wall lignin extensively and rapidly. Four classes of extracellular enzymes – lignin peroxidases (LiPs), manganese peroxidases (MnPs), laccases, and the H_2O_2-generating enzymes glyoxal oxidases – have been implicated in the degradation of lignin by *Phanerochaete chrysosporium*. MnPs, which are H_2O_2-requiring heme glycoprotein enzymes,[1,2] oxidize Mn(II) to Mn(III). Using transient-state kinetics, it was suggested that the substrate of MnP is Mn(II), forming a complex with an organic dicarboxylic acid such as oxalate, rather than Mn(II) itself.[2,3] The substrate oxidized by MnP then oxidizes phenolic substituents of lignin[2,3] and possibly nonphenolic substituents via specific mediator molecules such as a peroxidized lipid.[4] Generaly, MnPs occur as a series of isozymes encoded by a family of genes.[5] Many MnP isozymes have been purified, and their genes were cloned from various white rot basidiomycetes including *Trametes versicolor*,[6] *Ceriporiopsis subvermispora*,[7] and *P. chrysosporium*.[8] X-ray crystallographic and site-directed mutagenesis studies have defined the Mn-binding site in the *P. chrysosporium* MnP1 (H3) isozyme.[9–11]

Pleurotus ostreatus has been reported to secrete a series of MnP isozymes into the culture medium, whereas no Lip activity was observed under various culture conditions. In this organism, MnPs are considered to be the key enzymes in the lignin degradation system.[12] Cloning of genes encoding the MnP isozymes would permit us to determine their primary structures and provide clues to their evolutional relations and individual contributions to lignin biodegradation. One of the *mnp* genes from *P. ostreatus* strain IFO 36160 has been isolated and sequenced.[13] In our recent experiments, another strain, IS1, has been shown to secrete at least three isoforms of MnP at high levels into the liquid culture medium. The major isoenzyme, MnP3, was purified and its N-terminal amino acid sequence determined (unpublished data). Comparison of the sequence with that of the previously cloned MnP from the IFO 30160 strain suggested that these two MnPs are not allelic forms and are encoded by distinct genes. We report here cloning of the cDNA and the structual gene fragments of *P. ostreatus* MnP3, with the aim of understanding its structure, function, and evolutional relations with other MnPs or LiPs secreted by various white rot basidiomycetes.

Materials and methods

Strains, media, and plasmid

Pleurotus ostreatus IS1 is a dikaryotic strain selected as a good producer of MnP isozymes from our laboratory stocks. This strain was grown in potato dextrose agar (Difco) for maintenance. To prepare mRNA for reverse

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transcriptase-polymerase chain reaction (RT-PCR), this strain was grown in glucose-peptone medium (glucose 2%, polypeptone 0.5%, yeast extract 0.2%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%) supplemented with 0.5 mM $MnSO_4$ and hot water-extracted wheat bran extract (175 g of wheat bran was autoclaved with 500 ml of water for 20 min at 120°C and centrifuged at 10000 rpm for 10 min. The recovered supernatant was used as the supplement for 1 liter of medium). *Escherichia coli* JM109 [*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ (lac-proAB)*/F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)] was used for routine recombinant DNA experiments that required a bacterial host and grown in Luria-Bertani medium. The pGEM-T vector (Promega) was used for cloning PCR products.

mRNA extraction

Pleurotus ostreatus strain IS1 was cultured for 11 days at 28°C. Total RNA was prepared from the mycelia using the RNA Isolation Kit (Stratagene), and poly (A)$^+$ RNA was purified with Oligotex-dT30 (Daiichi Pure Chemicals).

Cloning of cDNA fragments

For cloning of the 3′ end, cDNA was synthesized using reverse transcriptase (RAV-2) (Takara Biochemicals) and a dT17adapter (5′-GACTCGAGTCGACATCGATTTTT-TTTTTTTTTTTT-3′). Then the rapid amplification of cDNA ends (RACE) for the 3′ end[14] was carried out using TaKaRa Ex Taq polymerase (Takara Biochemicals) and the dT17adapter and POMNP (5′-GCNAAYGCNGCN-TGYTGYGT-3′), which has a sequence corresponding to the determined N-terminal end of the purified MnP3, as primers. Each PCR cycle consisted of template denaturation at 95°C for 40 s, primer annealing at 55°C for 1 min, and DNA extension at 72°C for 3 min. After 40 cycles, an additional extension step of 15 min at 72°C was included. All the PCR-amplified fragments were analyzed on 1.2% agarose gels and extracted using Qiaex II (Qiagen), followed by cloning into pGEM-T.

For cloning of the 5′ end, cDNA was synthesized using the primer MnPcDNA (5′-GCAGTGAGAGCTGGG-AAGGG-3′) using reverse transcriptase (RAV-2) followed by addition of a poly(A)-tail at the 5′ end by terminal deoxynucleotidyl transferase (Toyobo). Subsequently, 5′RACE was performed using dT17 and 5RACE2 (5′-CTCTGCAGTGGATTGGGGAC-3′) as primers. The PCR conditions were the same as those used for 3′RACE. Analysis and cloning of the amplified fragments were also carried out. The location and direction of the each primer molecule are indicated in Fig. 1.

Cloning the genomic sequence

The structural gene was amplified using the primers MnP3G1 (5′-CTCGTCTCTTCAACTCCAGC-3′) and MnP3G2 (5′-GTTGATGAAAGGAAAGTGACGC-3′) (Fig. 1), with genomic DNA extracted from *P. ostreatus* IS1

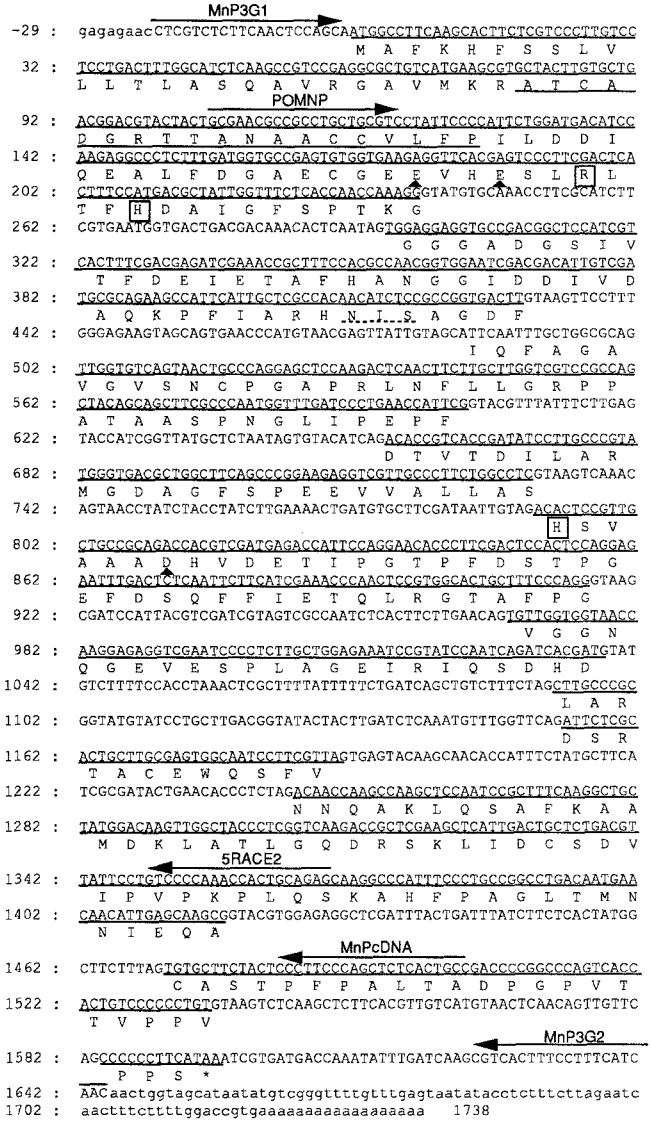

Fig. 1. Nucleotide sequence of the cDNA and structural gene encoding *Pleurotus ostreatus* MnP3. The deduced amino acid sequences are shown below the corresponding nucleotide sequence. *Arrows* indicate the positions and directions of the oligonucleotide primers used for the polymerase chain reaction (PCR). The *underlined* amino acid sequence was confirmed by amino acid sequencing of the N-terminal domain of the purified enzyme. The amino acids indicated by *arrowheads* are putative residues constituting the Mn-binding site. The conserved amino acid residues responsible for the peroxidase function are indicated by *boxes*. The amino acid sequence marked by a *dashed underline* is the putative *N*-glycosylation site

as the template. PCR was performed for 30 cycles of template denaturation at 95°C for 1 min, primer annealing at 50°C for 1 min, and DNA extension at 72°C for 2 min. Analysis and cloning of the amplified fragment were also carried out.

Nucleotide sequence analysis

The nucleotide sequences of the cloned fragments were analyzed using an ABI 377 DNA sequencer by the dye-terminator method. The determined cDNA and structural

gene sequences appear in DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession numbers AB011546 and AB016519, respectively.

Results

Structure of *mnp3* coding sequence

Sequence analysis indicated that the isolated structural gene *mnp3* contains a coding sequence of 1074 nucleotides interrupted by 10 introns; it encodes a protein of 358 amino acid residues (Fig. 1). The number of introns and some of their positions in *mnp3* are different from those of the previously cloned *P. ostreatus* MnP.[13] All the 5′RACE products isolated from independent experiments initiated −29 nucleotides upstream of the first ATG codon, suggesting that this was the start site for *mnp3* transcription. The open reading frame ended with a TAA terminator codon, followed by 121 nucleotides in the 3′ untranslated region of the cDNA. Typical polyadenylation signals were not present, as is sometimes the case with basidiomycete genes.

Deduced amino acid sequence of MnP3

The predicted amino acid sequence (Fig. 1) contains a 26-amino-acid signal peptide sequence followed by a sequence identical to the determined N-terminal sequence of the purified MnP3 isozyme (unpublished data). The signal peptide sequence contained specific motifs for removal by a signal peptidase and Kex2-related endpeptidase.[15] The amino acid residues known to be involved in peroxidase functions, (i.e., the distal His and Arg, and the proximal His) were conserved in MnP3. The residues that constitute the Mn-binding site of *P. chrysosporium* MnP isozyme[10,11] were also conserved (Fig. 1). The calculated molecular weight of the unprocessed and processed protein were 37400 and 34600, respectively. The estimated Mr of the purified MnP3 isozyme was 42kDa (unpublished data), and this difference was most likely due to glycosylation of the protein.

Discussion

Overall sequence homology analysis (Table 1) revealed that MnP3 had a higher degree of homology to *Trametes versicolor* MP2, and the previously cloned *Pleurotus ostreatus* MnP (67.4% and 64.6%) had medium homology to LiPs from various species (59.3%–59.9%),[6,18–22] and had a low level of homology to MnPs from *P. chrysosporium*[8,16,17] and *C. subvermispora* (45.6%–54.5%).[7] The unrooted phylogenetic tree based on the distances among amino acid sequences of LiPs and MnPs (Fig. 2) indicated that MnP3 has diverged far from MnPs of *Phanerochaete*

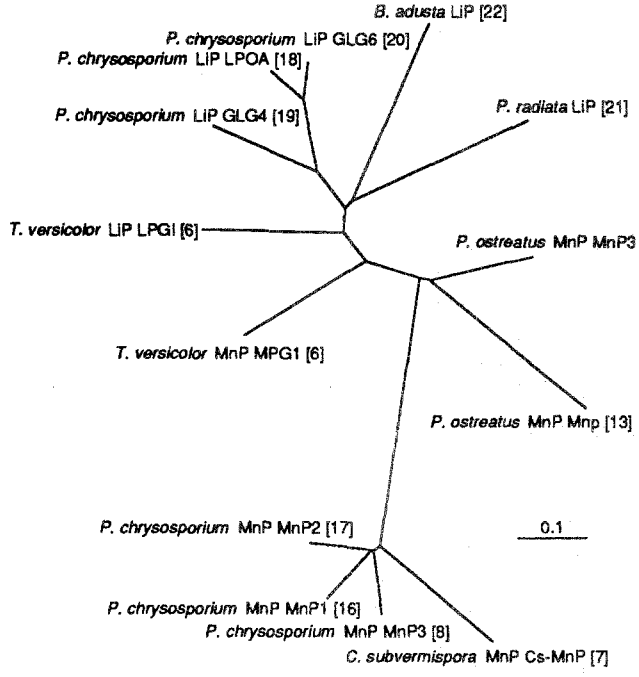

Fig. 2. Unrooted phylogenetic tree based on the distances among primary peptide sequences of LiPs and MnPs from various white rot basidiomycetes. Reference numbers are *bracketed*. The tree was generated by the neighbor-joining method, using CLUSTAL W[23]

Table 1. Percent identity of amino acid sequences among known lignin and manganese peroxidases

Genus/species	Ref.	Sequence	1	2	3	4	5	6	7	8	9	10	11	12	13
P. ostreatus MnP MnP3	This work	1		64.8	67.4	45.6	54.3	54.5	54.4	59.3	59.5	59.9	55.2	58.4	56.5
P. ostreatus MnP Mnp	13	2			55.4	44.6	49.0	48.7	48.4	51.6	55.6	52.7	50.7	52.2	53.5
T. versicolor MnP MPG1	6	3				42.1	48.0	49.6	49.5	57.8	61.7	59.5	58.0	69.0	57.6
C. subvermispora MnP Cs-MnP	7	4					69.2	69.2	71.3	40.6	39.4	42.4	40.7	40.4	38.4
P. chrysosporium MnP MnP1	16	5						83.2	81.0	47.2	45.5	48.5	46.4	45.9	41.9
P. chrysosporium MnP MnP2	17	6							81.5	46.2	46.9	47.6	44.7	47.2	42.3
P. chrysosporium MnP MnP3	8	7								46.4	46.8	46.2	46.0	45.2	42.3
P. chrysosporium LiP LPOA	18	8									72.1	87.9	61.9	63.2	61.1
P. chrysosporium LiP GLG4	19	9										72.4	58.7	65.1	61.4
P. chrysosporium LiP GLG6	20	10											61.5	61.6	60.0
P. radiata LiP	21	11												62.0	59.6
T. versicolor LiP LPG I	6	12													61.4
B. adusta LiP	22	13													

MnP, manganese peroxidase; Lip, lignin peroxidase

chrysosporium and *Ceriporiopsis subvermispora* and is rather close to a group characterized by LiPs and *T. versicolor* MnP.

Three other structual characteristics were found to be consistent with the phylogenetic tree. It has been reported that the positions of 10 cysteine residues involved in disulfide bond formation to maintain the tertiary structure of the enzyme are conserved in the MnPs from *P. chrysosporium* and *C. subvermispora*.[7,9] The last two of these cysteine residues are present in the extended C-terminal tail. *P. ostreatus* MnPs lack the extended C-terminal tail and hence also the last two cysteine residues[13] as well as the LiPs and *T. versicolor* MP2.[6] MnPs from *P. chrysosporium* and *C. subvermispora* have 7–10 amino acid residues inserted around residue 248 (*P. chrysosporium* MnP3 numbers),[7,8,16,17] which is not present in the amino acid sequences of the LiPs or *T. versicolor* MP2.[6] Neither of the *P. ostreatus* MnPs contain this insertion sequence (Fig. 1).[13] It has been demonstrated that *P. chrysosporium* MnP1 has an *N*-glycosylation site at Asn131,[9] whereas *T. versicolor* MP2 and LPG1 have such sites at a different position, Asn103 (numbered according to MP2).[24] The deduced amino acid sequences of both *P. ostreatus* MnPs contained one putative *N*-glycosylation site at positions corresponding to those of the *T. versicolor* peroxidases (Fig. 1).[13]

These results suggested that *P. ostrestus* MnPs and *T. versicolor* MP2 possess characteristics intermediate to those of LiPs and MnPs from *P. chrysosporium*. Because some but not all of the introns share their positions in these genes, it is possible that these fungal peroxidase genes have developed from a common ancestor gene. It seems that in *P. chrysosporium* peroxidases have gained more specialized properties during evolution than those in *T. versicolor* or *P. ostreatus*. In this context, it is of interest to determine how each enzyme has obtained, or even lost, its specificity for various substrates. Further analysis of the fungal peroxidases, including determination of the three-dimensional structure or site-directed mutagenesis of the substrate binding sites, will provide some insight into the evolution of these molecules.

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