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Characterization of Manganese Peroxidases from the Hyperlignolytic Fungus IZU-154

MASAAKI MATSUBARA, JUN SUZUKI, TETSUYA DEGUCHI, MASAHIKO MIURA, AND YOSHIHISA KITAOKA*

Biotechnology Research Section, Chemical, Polymer, Bio-Technology Laboratory, Kobe Steel, Ltd. Kobe 651-22, Japan

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Four isozymes of manganese peroxidase (MnP) were identified in the culture fluid of the hyperlignolytic fungus IZU-154 under nitrogen starvation conditions. One of them was purified and characterized kinetically. The specific activity and k_{cat}/K_m value of the MnP from IZU-154 were 1.6 times higher than those of the MnP from a typical lignin-degrading fungus, *Phanerochaete chrysosporium*. Two cDNAs encoding MnP isozymes from IZU-154 were isolated. The coding sequence of the two cDNAs, IZ-MnP1 cDNA and IZ-MnP2 cDNA, were 1,152 (384 amino acids) and 1,155 (385 amino acids) bp in length, respectively. They exhibit 96.2% identity at the nucleotide level and 95.1% identity at the amino acid level. Southern blot analysis indicated that two MnP isozyme genes exist in IZU-154 genomic DNA. The primary structures of two MnPs from IZU-154 were similar to those of MnPs from *P. chrysosporium*. The amino acid sequences including the important residues identified in MnPs from *P. chrysosporium*, such as the manganese-binding residues, the calcium-binding residues, the disulfide bonds, and the N-glycosylation site, were conserved in the two deduced IZ-MnPs. However, several discrepancies were found in the context around the distal histidine residue between MnP from IZU-154 and MnP from *P. chrysosporium*, which likely led to the difference in the kinetic parameters for MnP function.

The white-rot fungus *Phanerochaete chrysosporium* secretes two families of hemeprotein peroxidase, lignin peroxidase (LiP) and manganese peroxidase (MnP) (11, 21, 23, 42). These peroxidases plus H_2O_2-generating enzymes are produced during secondary metabolism in response to nitrogen or carbon starvation (4, 5, 17, 20, 21), and are thought to be major components in the lignin biodegradation system (14, 51).

The structure and mechanism of MnPs, in addition to those of LiPs, have been studied extensively (10, 13, 26). MnP is a heme glycoprotein with a molecular weight of approximately 46,000 and occurs as a family of isozymes. MnP production is induced by Mn(II) (1), and the oxidation of substrates by MnP is also dependent on Mn(II) (10, 13, 33). The catalytic cycle of MnP is similar to that of LiP and horseradish peroxidase (13, 39, 43, 48). The native MnP is oxidized by H_2O_2 to a highly reactive two-electron oxidized state, compound I. Compound I returns to its resting state after two separate one-electron reductions by Mn(II), with compound II as an intermediate. The Mn(III) produced by the enzymatic oxidation of Mn(II) can oxidize lignin, lignin derivatives, and other phenolic substrates (10, 13, 45, 50, 51). Organic acids such as malate and tartrate stimulate the catalytic activity of MnP by chelating Mn(III) (9, 45, 49, 50, 52).

MnP isozymes are encoded by several different genes in *P. chrysosporium*. Several cDNAs (MnP1, MnP2a, and MnP2b [38] and MP-1 [34]) and genomic DNAs (MnP1 [12] and MnP2 [28]) of MnP isozymes have been isolated and characterized. Recently, the crystal structure of MnP from *P. chrysosporium* has been reported (41). The analysis showed that the overall structure of MnP is closely similar to that of LiP and that MnP has two structural calcium ions and two *N*-acetylglucosamine residues. Moreover, a manganese-binding site was identified in the crystal structure.

* Corresponding author. Mailing address: Biotechnology Research Section, Chemical, Polymer, Bio-Technology Laboratory, Kobe Steel, Ltd., 5-5, Takatsuka-dai 1-chome, Nishi-ku, Kobe 651-22, Japan. Phone: 81-78-992-5733. Fax: 81-78-993-2035.

MnP has been also found in the culture broth of other white-rot fungi (15, 32, 36). The white-rot fungus IZU-154 has great lignolytic activity and selectivity toward wood lignin (31). As an industrial application of this fungus, biomechanical pulping (19), biobleaching of kraft pulp (7, 8), and decolorization of kraft bleaching effluents (25) have been demonstrated. IZU-154 produces MnP as a major extracellular protein and secretes very little LiP under lignolytic conditions. IZU-154 under nitrogen-limited condition and its MnP enzyme can also degrade recalcitrant high-molecular-mass compounds, such as nylon (3) and melanin (18). Significant shifting of the molecular weight distributions of nylon and melanin to lower molecular weights were observed, and this resulted in the apparent degradation of nylon and decolorization of melanin.

In our previous study, IZU-154 has shown significantly superior lignin-degrading activity both in the degradation of lignin model compounds (31) and in a study of biobleaching with untreated kraft pulp (7) compared with other lignin-degrading fungi, i.e., *P. chrysosporium* and *Coriolus versicolor*. However, MnP, a major component in lignin biodegradation, was produced almost equally in IZU-154 and *P. chrysosporium*. Thus, the difference in lignin-degrading activities of IZU-154 and other fungi have not yet been defined. In this paper, we evaluate the enzymatic property and the primary structure of MnP from IZU-154 in comparison with those of MnP from *P. chrysosporium*.

MATERIALS AND METHODS

Organism. The wild-type strain of fungus IZU-154, which has been reported previously (31), and *P. chrysosporium* ME446 were used in this study. IZU-154 was deposited as the strain name of NK-1148 under the accession number FERM BP-1859 with the National Institute of Bioscience and Human Technology of the Ministry of Industry and Technology, Ibaraki, Japan. Since secondary mycelia were observed and the sexual cycle was not observed in our previous study, the fungus IZU-154 belongs to the family *Deuteromycotina. Escherichia coli* DH5α (GIBCO-BRL) was used for DNA manipulation.

Culture conditions. IZU-154 was grown in a 50-ml low-nitrogen medium in a 300-ml flask at 30°C. For large-scale cultivation, this organism was grown in a 5-liter low-nitrogen medium in a 10-liter jar fermentor with 1 vol/vol/min aeration and 120 rpm of agitation at 30°C. The low-nitrogen medium contained (per

TABLE 1. Catalytic properties of MnPs from IZU-154 and *P. chrysosporium*

MnP source	Sp act for 2,6-DMP oxidation (nkat/mg)	Kinetic parameters for Mn(II) oxidation[a]		
		K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)
IZU-154	345.9	44.7	161.0	3.60
P. chrysosporium	221.7	53.7	124.9	2.33

[a] Reactions were carried out in 50 mM sodium malate (pH 4.5). Apparent K_m and k_{cat} values for Mn(II) were determined by using 0.1 mM H_2O_2.

liter) 10 g of glucose, 0.2 g of diammonium tartrate, 2 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$, 1 mg of thiamine · HCl, 0.5 g of Tween 80, 1 ml of trace elements solution, 0.2 mmol of $MnSO_4$, and 20 mmol of sodium tartrate (pH 4.5). The trace elements solution contained (per liter) 1.5 g of sodium nitriloacetate, 3 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of $MnSO_4 \cdot H_2O$, 1 g of NaCl, 0.1 g of $FeSO_4 \cdot 7H_2O$, 0.1 g of $CoSO_4$, 82 mg of $CaCl_2$, 0.1 g of $ZnSO_4$, 10 mg of $CuSO_4 \cdot 5H_2O$, 10 mg of $AlK(SO_4)_2$, 10 mg of H_3BO_3, and 10 mg of $NaMoO_4$.

Assay of MnP activity. MnP activity was measured by 2,6-dimethoxyphenol (2,6-DMP) oxidation (52). The reaction mixture contained 50 mM sodium malate (pH 4.5), 0.5 mM $MnSO_4$, 1 mM 2,6-DMP, and 0.1 mM H_2O_2, and the reaction was monitored by measuring the A_{469}. One katal of MnP activity is defined as the amount of MnP that forms 1 mol of quinone dimer per s at 30°C.

Purification of MnP isozymes. One liter of each culture broth of IZU-154 and *P. chrysosporium* was concentrated on an ultrafiltration membrane to approximately 10 ml and subjected to gel filtration chromatography on a Superdex 75 column (HiLoad 26/60; Pharmacia) in buffer A (20 mM sodium acetate, 0.1 M NaCl [pH 4.5]) at a flow rate of 2 ml/min. An obviously dominant peak containing MnP activity was pooled and then desalted by dialysis and concentrated by membrane ultrafiltration.

To observe the number of isozymes, a portion of the preparation was applied to an isoelectric focusing gel Ampholine PAG plate (Pharmacia) according to the manufacturer's instructions.

To obtain purified isozyme, the pooled fractions from gel filtration were further purified by two consecutive ion-exchange chromatographies on a Mono-Q HR5/5 column (Pharmacia). The first chromatograph was applied with a gradient of 10 to 300 mM Na-acetate (pH 5.0) at a flow rate of 0.5 ml/min for 1 h. The second chromatograph was applied with a gradient of 50 to 100 mM sodium acetate (pH 5.0) at 0.5 ml/min for 1 h. The purity of the MnP isozyme was monitored by isoelectric focusing gel electrophoresis.

Determination of kinetic constants. Kinetic constants of purified MnPs from IZU-154 and from *P. chrysosporium* for oxidation of Mn(II) to Mn(III) were measured. A preliminary experiment to determine the optimum concentration of enzymes was performed with buffer B (50 mM malate, 200 μM $MnSO_4$, 100 μM H_2O_2 [pH 4.5]) with various concentration of enzymes (0.10 to 1.7 μg/ml) at 30°C for 30 s. A_{290} was measured to calculate the Mn(III)-malate chelate product. The initial rate in the oxidation of Mn(II) at a concentration of 25 to 1,500 μM in buffer C (50 mM malate, 100 μM H_2O_2 [pH 4.5]) was measured with 0.58 and 0.85 μg/ml of MnP from IZU-154 and *P. chrysosporium*, respectively. These data were analyzed on Michaelis-Menten plots, and the kinetic constants shown in Table 1 were determined by successive applications of the simplex algorithm (30) and the algorithm of Marquardt (27) for nonlinear least-squares fitting of a hyperbolic curve to the experimental data.

Isolation of poly(A) RNA. Mycelia were isolated from the culture broth of IZU-154, in which MnP activity had just begun to be detected. The mycelial pellets were then immediately frozen in liquid nitrogen and ground into a fine powder with mortar and pestle. The cell powder was suspended in guanidium thiocyanate denaturing solution (2), the suspension was treated with phenol and chloroform, and then total RNA was precipitated by isopropanol. Poly(A) RNA was isolated from total RNA by chromatography on oligo(dT)-cellulose.

Construction of a cDNA library. The cDNA library was constructed with the Superscript plasmid system for cDNA synthesis and plasmid cloning (GIBCO-BRL). The cDNA synthesized from poly(A) RNA was ligated to the *Sal*I-*Not*I fragment of pSPORT1, and then *E. coli* DH5α was transformed with the ligation products.

Preparation of DNA probe for isolation of MnP cDNA. Two DNA primers were synthesized, the nucleotide sequences of which were deduced from the amino acid sequences around two conserved histidine residues, i.e., distal histidine and proximal histidine, in *P. chrysosporium*. The nucleotide sequences of the distal and proximal histidine primers were 5′-ATCCGCCTCACCTTCCACGA-3′ and 5′-GCGACGGAGTGGGAGGCGAG-3′, respectively.

To obtain the fragments of MnP cDNA, reverse transcription-PCR of poly(A) RNA from IZU-154 was performed with the distal and proximal histidine primers. PCR products (approximately 400-bp fragments) were isolated and then labeled with digoxigenin by using the DIG DNA Labeling kit (Boehringer Mannheim).

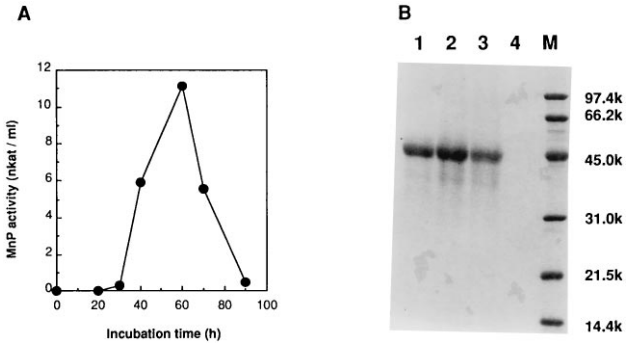

FIG. 1. (A) Profile of MnP expression in a liquid culture of IZU-154. An aliquot of culture broth was subjected to MnP activity assaying as described in Materials and Methods. The same culture broth at 40, 60, 70, and 90 h of incubation was removed and concentrated with acetone. (B) The precipitated protein was analyzed on SDS-PAGE as described by Laemmli (24). Lanes 1 to 4, 40, 60, 70, and 90 h of incubation, respectively; lane M, molecular weight marker.

Screening of MnP cDNA clones. Colony screening of the cDNA library was performed with the digoxigenin-labeled probe. The blots were hybridized overnight at 65°C and then washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) for 5 min at 65°C and twice with 0.1× SSC–0.1% SDS for 30 min at room temperature. The positive clones were detected by enzyme-linked immunosorbent assaying with the DIG Luminescent Detection kit (Boehringer Mannheim).

DNA sequencing. Overlapping deletion mutants of MnP cDNA were constructed by the exonuclease III method (16) and were sequenced in both directions by the dideoxy method (40).

Southern blot analysis. Genomic DNA of IZU-154 was prepared from frozen cells by phenol and chloroform extraction. Digests of genomic DNA by several restriction enzymes were hybridized with labeled IZ-MnP1 cDNA and were detected by the DIG Luminescent Detection kit.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the following accession numbers: D83010 for IZ-MnP1 and D83011 for IZ-MnP2.

RESULTS

Expression of MnP during growth. Aliquots of the extracellular fluid were removed at various times, and MnP activity for 2,6-DMP oxidation was measured. As shown in Fig. 1A, MnP activity was first detected on day 2. The activity increased to a maximum on day 3 and then gradually declined to a low level by day 4. A portion of culture broth was also concentrated by acetone precipitation and was applied to SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 1B). A strong band with a molecular weight of 43,000 was detected in the broth on day 2, in accordance with the enzyme activity, and the intensity of this protein band was synchronized with the profile of MnP activity. The protein with a molecular weight of 43,000 was proved to have MnP activity by staining for MnP activity (data not shown). Furthermore, it was observed that MnP was a strikingly abundant protein compared with others in the extracellular fluid.

Separation of MnP isozymes. From the nitrogen-limited culture broth of IZU-154, MnP was purified by gel filtration to a single band by SDS-PAGE. With this preparation, the purity of MnP is approximately 80% according to the RZ value (A_{406}/A_{280}). Then, the MnP isozyme was separated on an isoelectric focusing gel and stained with Coomassie blue. As shown in Fig. 2, four separate bands were observed. All four bands were positive by staining for MnP activity and by staining for glycosylation (data not shown). The pI values of the four MnPs were 5.1, 4.9, 4.5, and 3.7, respectively. There are two major isozymes with pI values of 4.9 and 5.1, and the others were

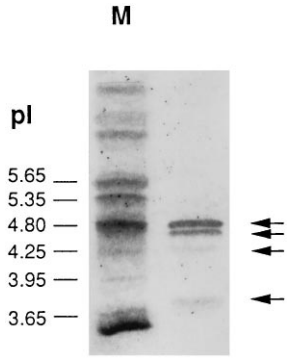

FIG. 2. Analysis of MnP isozymes on isoelectric focusing gel electrophoresis. Purified MnP isozymes were separated on an isoelectric focusing gel Ampholine PAG plate (Pharmacia) with a pH range of 4.0 to 6.0 according to the manufacturer's instructions and were stained with Coomassie brilliant blue. Arrows on the right indicate the migration of MnP isozymes. Lane M, pI markers.

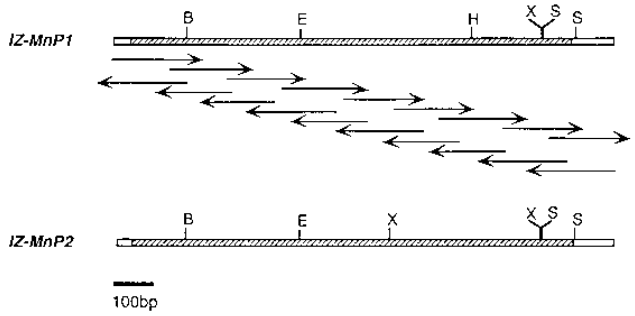

FIG. 3. Restriction maps and sequencing strategies of IZ-MnP1 and IZ-MnP2 cDNAs. Arrows indicate the directions and extents of the determined sequence of IZ-MnP1 cDNA. IZ-MnP2 cDNA was sequenced by a similar strategy. The hatched areas represent the coding regions. Abbreviations for restriction enzymes: B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; S, *Sac*I; X, *Xho*I.

minor. The isozyme with a pI of 4.9 was further purified by ion-exchange chromatography. The RZ value (A_{406}/A_{280}) of the purified enzyme solution was 5.7.

Kinetics of the MnP reactions. The major MnP isozymes of IZU-154 (pI, 4.9) and *P. chrysosporium* (pI, 4.9) were purified by ion-exchange chromatography to electrophoretic homogeneity on an isoelectric focusing gel. To compare the functional differences between MnP from IZU-154 and from *P. chrysosporium*, the purified MnPs were analyzed kinetically. For the determination of the kinetical parameters of MnP, we employed the Mn(II) oxidation assay, and the reaction was monitored by measuring the Mn(III) product chelated with malate. The 2,6-DMP oxidation which is routinely used for MnP activity assay is not a direct reflection of MnP activity, because the oxidation of 2,6-DMP is catalyzed by Mn(III) which is generated by the oxidation of Mn(II). The kinetic parameters for MnPs from IZU-154 and *P. chrysosporium* are shown in Table 1. The kinetic parameters for MnP from *P. chrysosporium* were thought to be almost equivalent to the reported data (29) when several differences in the assay condition were considered. The specific activity for 2,6-DMP oxidation and the k_{cat}/K_m value for Mn(II) oxidation of IZU-154 MnP are both approximately 1.6 times higher than those for *P. chrysosporium* MnP.

MnP cDNAs encoding two MnP isozymes. Approximately 30,000 cDNA clones were screened with the partial fragment of MnP cDNA, which was the reverse transcription-PCR product of poly(A) RNA from IZU-154, by using the primers described in Materials and Methods, and eight positive clones were isolated by the screening. These cDNA clones were separated into two groups according to restriction enzyme mapping and the partial nucleotide sequences. Two representative cDNAs (IZ-MnP1 and IZ-MnP2) were used for further analysis. A set of deletion mutants of the two MnP cDNAs were sequenced in both strands by the strategy shown in Fig. 3.

The nucleotide sequences and the deduced amino acid sequences of IZ-MnP1 cDNA and IZ-MnP2 cDNA are shown in Fig. 4. The coding sequences of IZ-MnP1 cDNA and IZ-MnP2 cDNA are 1,152 (384 amino acids [aa]) and 1,155 (385 aa) bp, respectively. These two cDNAs exhibit 96.2% identity at the nucleotide level and 95.1% identity at the amino acid level. The homologies between IZ-MnP1 cDNA and *P. chrysosporium* MnP1 cDNA (38) are 72.5% at the nucleotide level and 79.0% at the amino acid level.

Comparison of primary structures of IZ-MnP1 and IZ-MnP2 with MnPs from *P. chrysosporium*. The deduced amino acid sequences of IZ-MnP1 and IZ-MnP2 were aligned with two MnPs from *P. chrysosporium*, i.e., phamp1a (34) and phamnp (38) (Fig. 5). The MnPs from IZU-154 possess four residues extended at the C terminus in comparison with MnPs from *P. chrysosporium*. When IZ-MnP1 was compared with IZ-MnP2, most of the amino acid conversion was found in the predicted leader sequence (24 aa from first Met), and, moreover, conserved context could not be found in the leader sequences of four MnPs. However, there are several segments whose long consecutive sequences are highly conserved among the mature protein regions of four MnPs, whereas most of these conserved domains are located in the N-terminal halves of MnPs.

Southern blot analysis of genomic DNA. Southern hybridization of genomic DNA was carried out to determine the number of MnP isozyme genes. Genomic DNA prepared from IZU-154 was digested with *Eco*RI and other restriction enzymes and probed with IZ-MnP1 cDNA. As shown in Fig. 6, hybridization to four (*Eco*RI [lane 1]) and two (*Kpn*I [lane 2]) DNA fragments was observed. Because both IZ-MnP1 and IZ-MnP2 cDNAs include a single *Eco*RI site (Fig. 3), the corresponding genes are expected to be digested with *Eco*RI in at least one site in the coding sequence. Therefore, two genes are thought to be separated into at least four bands by digestion with *Eco*RI. In both IZ-MnP1 and IZ-MnP2 cDNA, a *Kpn*I site was not found and the digest of genomic DNA with *Kpn*I gave two distinct hybridization bands. The results of hybridization indicate that two copies of DNA encoding MnP isozymes exist in IZU-154 genomic DNA.

DISCUSSION

The white-rot fungus IZU-154 has great lignolytic activity compared with other white-rot fungi (31) and can be applied to the biobleaching process of kraft pulp (7, 8). IZU-154 produces MnP efficiently in regular shaking culture, and MnP activity is first detected on day 2 (Fig. 1). On the other hand, *P. chrysosporium* needs to be flushed with oxygen gas during secondary metabolism to produce MnP efficiently (21), and it takes 2 to 3 days to start producing MnP. IZU-154 is, therefore, thought to be suitable for MnP production compared with *P. chrysosporium*. The fact that IZU-154 can highly degrade lignin and produce negligible amounts of LiP indicates that lignin degradation by IZU-154 is caused by MnP and that LiP is not necessary for lignin degradation. Actually, biobleaching of kraft pulp was successfully performed by purified MnP with the H_2O_2-generating system (18).

In the present study, two cDNAs encoding two MnP

IZ-MnP1

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-42                                                  CTTCTCTGGTTCTATCACACCCACCCGGCACTTCCTGCACCA

   1 ATGGCCTTCACATCTCTCCTCTCTCTTGTCGCCCTCGCCGCGGTTGCACGCGCTGCCCCCGCCGCCGAAACCGCTGTGTGTCCCGACGGCACTCGCGTCA
     M  A  F  T  S  L  L  S  L  V  A  L  A  A  V  A  R  A  A  P  A  A  E  T  A  V  C  P  D  G  T  R  V  S

 101 GTAACTCTGCTTGCTGCGCTTTTATCCCTCTGGCGCAAGATCTTCAGGCGACTGTGTTCCAGAATGACTGTGGTGAGGATGCACACGAGGTTATCCGCCT
       N  S  A  C  C  A  F  I  P  L  A  Q  D  L  Q  A  T  V  F  Q  N  D  C  G  E  D  A  H  E  V  I  R  L

 201 CATCTTCCACGACGCTGTGGCCATCTCTCGCAGCAAGGGCCCTTCTGCCGGTGGAGGGGCTGACGGCTCGATGCTCCTGTTCCCGACCGTCGAGCCGCTC
      I  F [H] D  A  V  A  I  S  R  S  K  G  P  S  A  G  G  G  A  D  G  S  M  L  L  F  P  T  V  E  P  L

 301 TTTGCTGCTAATAACGGCATCGACGACTCGGTCAACAATCTCATCCCCTTCCTGGCTAAGCACCCTGTTTCAGCTGCGGATCTTGTTCAGTTCGCTGGTG
     F  A  A  N  N  G  I  D  D  S  V  N  N  L  I  P  F  L  A  K  H  P  V  S  A  A  D  L  V  Q  F  A  G  A

 401 CTGTCGCGCTCAGCAACTGCCCCGGCGCTCCCCGCCTGGAATTCTTGGCCGGTCGCCCCAACCACACCATTCCTGCCATTGATGGCTTGGTTCCAGAGCC
       V  A  L  S  N  C  P  G  A  P  R  L  E  F  L  A  G  R  P  N  H  T  I  P  A  I  D  G  L  V  P  E  P

 501 TCAGGACGACGTGACCACTATCCTCGCGCGATTCGAAGATGCGGGAGGATTCACCCCCTTCGAAGTGGTCTCTCTCCTCGCCTCGCACTCCGTCGCTCGC
      Q  D  D  V  T  T  I  L  A  R  F  E  D  A  G  G  F  T  P  F  E  V  V  S  L  L  A  S [H] S  V  A  R

 601 GCCGACAAGGTCGATGAGACCATTGATGCTGCTCCCTTTGACTCCACTCCCTTCACCTTTGACACCCAGGTCTTCCTTGAGGTTCTCCTCAAGGGAACCG
     A  D  K  V  D  E  T  I  D  A  A  P  F  D  S  T  P  F  T  F  D  T  Q  V  F  L  E  V  L  L  K  G  T  G

 701 GCTTCCCCGGATTAAGCAACAACACCGGCGAAGTCGCTTCTCCCCTCCCCAAGGGCAGCGGCAACGACACTGGCGAGATGCGTCTCCAGTCCGACTTCGC
       F  P  G  L  S  N  N  T  G  E  V  A  S  P  L  P  K  G  S  G  N  D  T  G  E  M  R  L  Q  S  D  F  A

 801 TCTCGCCCGCGACTCGCGCACAGCTTGCTTCTGGCAGGGTTTCGTCAATCAACAGGAGTTTATGGCTGCCAGCTTCAAGTCTGCTGTCGCCAAGCTTGCC
      L  A  R  D  S  R  T  A  C  F  W  Q  G  F  V  N  Q  Q  E  F  M  A  A  S  F  K  S  A  V  A  K  L  A

 901 GTGCTCGGGCACAACCGCGACGACCTGATCGACTGCTCCGAGGTCGTCCCTGTCCCCAAGCCCGCCGTCAAGAAGCCTGCGAGCTTCCCGGCCACCACCA
     V  L  G  H  N  R  D  D  L  I  D  C  S  E  V  V  P  V  P  K  P  A  V  K  K  P  A  S  F  P  A  T  T  S

1001 GCGCCAAGGACCTCGAGCTCAATTGCAACTCGCAGAAGTTCCCGACTCTCACTGTTGACCAGGGCGCCACCCAGAGTCTGATCCCCCACTGCTCGAACGG
       A  K  D  L  E  L  N  C  N  S  Q  K  F  P  T  L  T  V  D  Q  G  A  T  Q  S  L  I  P  H  C  S  N  G

1101 TGGACAGAATTGCCCAGCTGTCCAGTTCGACGGTCCCTCTCAGGCTTCCTCTTGAGCTCGTTTTTTATCCTATATGTACCATTTGTGATCAAATTAGAGG
      G  Q  N  C  P  A  V  Q  F  D  G  P  S  Q  A  S  S  *

1201 CTATTCACACACGACATGTATGCAGCGGCTCCGAATTGATTGGTTGCACATCT(A)n
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IZ-MnP2

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-30                                                             TCTATCACACCCACCCGGCACTTCCTGACA

   1 ATGGCCTTACATCTCTCCTCTCTCTTGTCGGCCTCGCCGCGGTTGCACCGCGCTGCCCCCGCCGCGGAAACCGCTGTGTGTTTCGACGGCACTCGCGTCA
     M  A  L  H  L  S  S  L  L  S  A  S  P  R  L  H  R  A  A  P  A  A  E  T  A  V  C  F  D  G  T  R  V  S

 101 GCAACTCTGCTTGCTGTGCTTTTATCCCTCTGGCGCAAGATCTTCAGGCGACTGTGTTCCAGAATGACTGTGGTGAGGATGCACACGAGGTTATCCGCCT
       N  S  A  C  C  A  F  I  P  L  A  Q  D  L  Q  A  T  V  F  Q  N  D  C  G  E  D  A  H  E  V  I  R  L

 201 CATCTTCCACGACGCGTGGCACATCTCTCGCAGCAAGGGCCCTTCCGCCGGTGGAGGGGCTGACGGCTCGATGCTACTGTTCCCGACCGTCGAGCCGCTC
      I  F [H] D  A  W  H  I  S  R  S  K  G  P  S  A  G  G  G  A  D  G  S  M  L  L  F  P  T  V  E  P  L

 301 TTTGCTGCTAATAACGGCATTGACGACTCGGTCAACAATCTCATCCCCTTCCTGGCTAAGCACCCTGTTTCAGCTGCGGATCTGGTCCAGTTCGCTGGTG
     F  A  A  N  N  G  I  D  D  S  V  N  N  L  I  P  F  L  A  K  H  P  V  S  A  A  D  L  V  Q  F  A  G  A

 401 CTGTCGCGCTCAGCAACTGCCCCGGCGCTCCCCGCCTGGAATTCTTGGACGGTCGCCCCAACCACACCATTCCTGCCATTGATGGCTTGGTTCCAGAGCC
       V  A  L  S  N  C  P  G  A  P  R  L  E  F  L  D  G  R  P  N  H  T  I  P  A  I  D  G  L  V  P  E  P

 501 TCAGGAGGACGACGTGACCACTATCCTCGCGCGATTCGAAGATGCGGGAGGATTCACCCCCTTCGAAGTGGTCTCTCTCCTCGCCTCGCACTCCGTCGCT
      Q  E  D  D  V  T  T  I  L  A  R  F  E  D  A  G  G  F  T  P  F  E  V  V  S  L  L  A  S [H] S  V  A

 601 CGCGCCGACAAGGTCGATGAGACCATTGATGCTGCTCCCTTTGACTCCACTCCCTTCACCTTCGACACCCAGGTCTTCCTCGAGGTTCTCCTCAAGGGAA
     R  A  D  K  V  D  E  T  I  D  A  A  P  F  D  S  T  P  F  T  F  D  T  Q  V  F  L  E  V  L  L  K  G  T

 701 CCGGCTTCCCCGGATTAAGCAACAACACCGGCGAAGTTGCCTCTCCCCTCCCCAAGGGCAGCGGCAACGACACTGGCGAGATGCGTCTCCAGTCCGACTT
       G  F  P  G  L  S  N  N  T  G  E  V  A  S  P  L  P  K  G  S  G  N  D  T  G  E  M  R  L  Q  S  D  F

 801 CGCTCTCGCCCGCGACTCGCGCACAGCTTGCTTCTGGCAGGGTTTCGTCAATGAACAGGAGTTCATGGCTGCCAGCTTCAAGTCTGCTGTCGCCAAGCTC
      A  L  A  R  D  S  R  T  A  C  F  W  Q  G  F  V  N  E  Q  E  F  M  A  A  S  F  K  S  A  V  A  K  L

 901 GCCGTGCTCGGGCACAACCGCGACGACCTGATCGACTGCTCCGAGGTCGTCCCTGTCCCCAAGCCCGCCGTCAACAAGCCTGCGAGCTTTCCGGCCACCA
     A  V  L  G  H  N  R  D  D  L  I  D  C  S  E  V  V  P  V  P  K  P  A  V  N  K  P  A  S  F  P  A  T  T

1001 CCAGCGCCAAAGACCTCGAGCTCAACTGCAACTCGCAGAAGTTCCCGACTCTCACTGTTGACCAGGGTGCCACCCAGAGTTTGATTCCCCACTGCTCGAA
       S  A  K  D  L  E  L  N  C  N  S  Q  K  F  P  T  L  T  V  D  Q  G  A  T  Q  S  L  I  P  H  C  S  N

1101 CGGTGGACAGAATTGCCCAGCTGTCCAGTTCGACGGTCCCTCTCAGGCTTCCTCTTGAGCTCGTTTTTAATACTATATGTACCATTTATGATCGCATTAG
      G  G  Q  N  C  P  A  V  Q  F  D  G  P  S  Q  A  S  S  *

1201 AGGCTATTCACACACGGCATGTATGCAGCGGCTCCGGCTTTGTTTGGTGCCCATC(A)n
```

FIG. 4. Nucleotide and deduced amino acid sequences of IZ-MnP1 and IZ-MnP2 cDNAs. Distal and proximal histidine residues are boxed. Regions which can be hybridized with the distal and proximal histidine primers used for PCR are indicated by horizontal arrows. Vertical arrows, putative cleavage sites of IZ-MnP1 and IZ-MnP2 proteins.

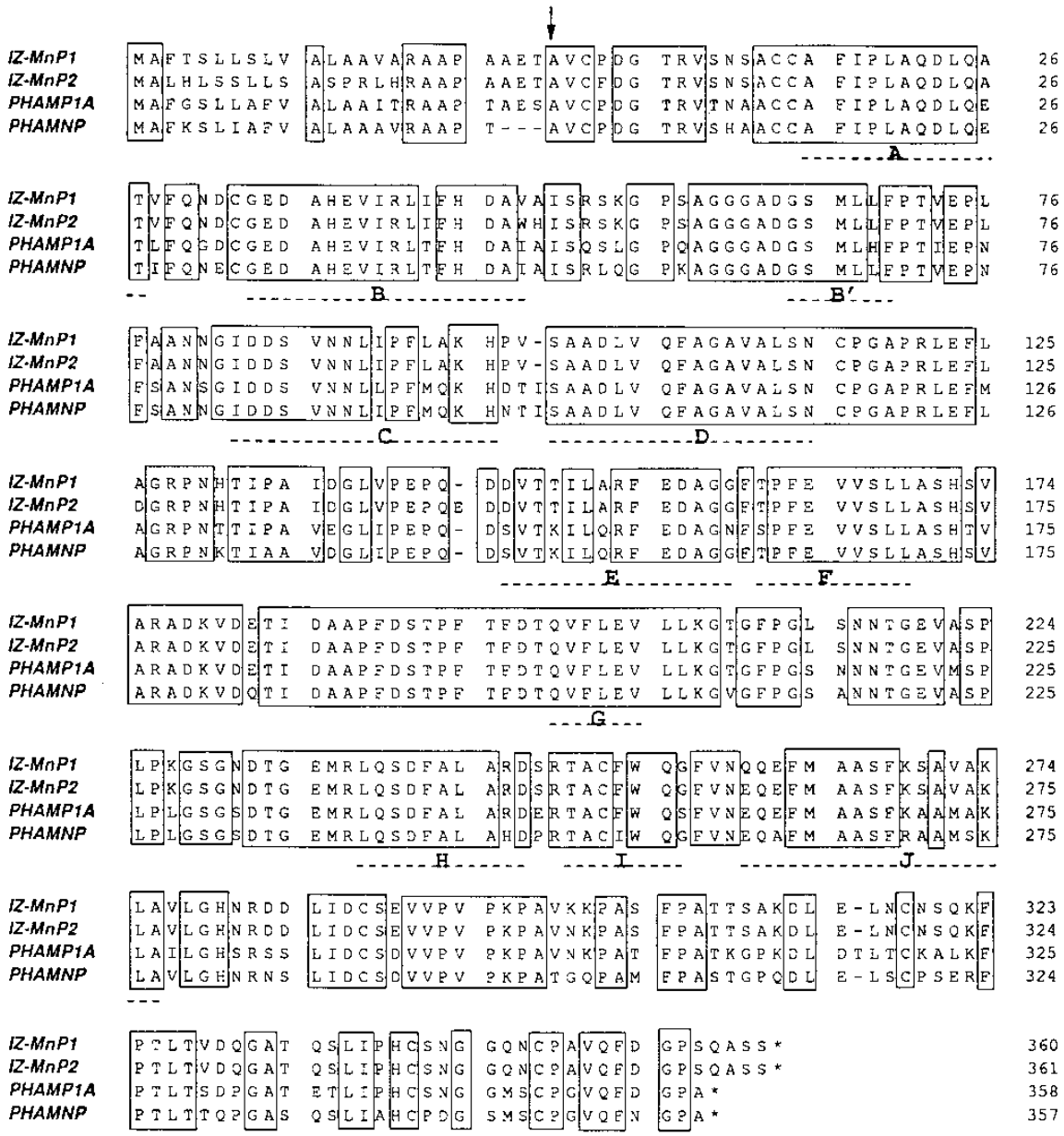

FIG. 5. Alignment of the primary structures of IZ-MnPs and *P. chrysosporium* MnPs. Four amino acid sequences are aligned to give maximum matching. Amino acid sequences for comparison used here were phamp1a and phamnp; both are *P. chrysosporium* MnPs (34, 38). Boxed sequences, residues conserved among four MnPs; broken lines below the sequence, predicted locations of helices in *P. chrysosporium* MnP (41); vertical arrow at the top, the putative N terminus of the mature MnP, which is numbered 1.

isozymes (IZ-MnP1 and IZ-MnP2) from IZU-154 were isolated and characterized. The molecular weight of MnP determined from SDS-PAGE was approximately 43,000 (Fig. 1), whereas the calculated molecular weights of IZ-MnP1 and IZ-MnP2 from amino acid sequences were 37,721 and 38,084, respectively. This difference may be due to a posttranslational glycosylation, since MnP isozymes were observed to be glycosylated. Such differences were also reported for MnP (34, 38) and LiP (44) from *P. chrysosporium*. There are three potential N-glycosylation sites at Asn-76, Asn-131, and Asn-217 in the MnP sequence of *P. chrysosporium*. Crystal structure analysis showed that, of these residues, only Asn-131 actually binds to carbohydrate (41). The corresponding asparagine residue, Asn-130 (in IZ-MnP numbering), is found in both IZ-MnP1 and IZ-MnP2. The amino acid sequence including Asn-130 follows the general rule of the N-glycosylation site, Asn-X-Ser/Thr (22).

Because MnP of IZU-154 is an extracellular protein, a leader sequence at the N terminus was expected. The first 24 aa of the two IZ-MnPs which possess characteristics of signal peptides (47) are thought to be the leader sequence. The putative N-terminal region of mature enzyme is highly homologous to MnP from *P. chrysosporium*, and it supports the same processing site of MnPs. IZ-MnP1 and IZ-MnP2 cDNAs en-

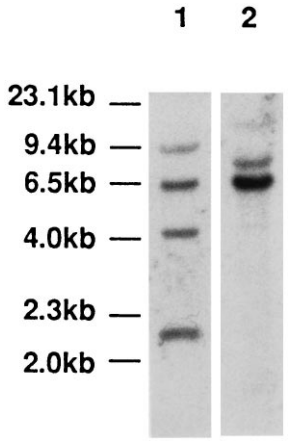

FIG. 6. Southern hybridization of IZU-154 genomic DNA with IZ-MnP1 cDNA. Digests of IZU-154 genomic DNA by restriction endonucleases *Eco*RI (lane 1) and *Kpn*I (lane 2) were separated by electrophoresis. The blot was hybridized with labeled IZ-MnP1 cDNA as described in Materials and Methods. Size standards are given at the left.

code 384- and 385-aa proteins, respectively; therefore, the mature proteins preceded by 24-aa leader peptides consist of 360 (37,721 Da) and 361 (38,084 Da) aa, respectively.

In the alignment of four MnP primary structures, several identical segments were found. These domains were conserved in the two fungi, which indicates that these domains likely constitute the essential structure for MnP function. According to crystal structure, there are 10 major helices and one minor helix in MnP from *P. chrysosporium*, all of which are also found in LiP (41). All 10 cysteine residues which participate in five disulfide bonds in *P. chrysosporium* MnP are conserved in IZ-MnPs. The long-conserved segments among four MnPs are mostly in accordance with the residues forming helices. Helices A, B′, D, F to G, and H (in *P. chrysosporium* MnP numbering) are almost conserved. The fact that there are several conversions in helices C, E, I, and J means that these helices may not participate in forming essential structure, whereas the precise function of these helices was unknown. Actually, these helices are located apart from the active center in the crystal structure (41). Moreover, there are quite large differences between *P. chrysosporium* MnPs and IZ-MnPs in the long loop structure in the C-terminal region, and the functional role of this region has not yet been defined.

Two histidine residues conserved among many peroxidases are essential for peroxidase activity. One of the histidine residues, distal histidine, is related to the cleavage of H_2O_2 for formation of compound I, and the other histidine, proximal histidine, is the axial ligand of heme (6, 37). These two histidine residues are located in helices B and F in *P. chrysosporium*, and these residues were also found in IZ-MnP1 (His-46 and His-172) and IZ-MnP2 (His-46 and His-173). The sequence around the proximal histidine residue was identical among the four MnPs. However, the residues close to the distal histidine residue contain several conversions between *P. chrysosporium* MnPs and IZ-MnPs (e.g., Ile-44, Val-49, and Ala-50 in IZ-MnP1). These differences likely contribute to the difference in the kinetical parameters of MnP from each organism.

The Mn(II), the substrate of MnP, is hexacoordinated to the carboxylate oxygens of Glu-35, Glu-39, Asp-179, a heme propionate oxygen, and two water oxygens (41). The two glutamate residues are in helix B, and Asp-179 is in the loop between helices F and G. These residues and the context around them are conserved among the four MnPs compared. MnP also has two structural calcium ions, like other fungal peroxidases (41). The calcium-binding residues, Asp-47, Gly-62, Ser-66, Asp-64, Ser-174, Asp-191, Thr-193, Thr-196, and Asp-198, are located in helix B′ and in the loop between helices F and G. The residues constituting calcium ligands and the contexts around these residues are also identical among the four MnPs.

In conclusion, the comparison of four MnPs found that there are differences in the distal histidine region, whereas the other functional domains, i.e., calcium- and manganese-binding regions and disulfide bonds, are conserved. The long loop structure at the C terminus included several amino acid conversions; however, its contribution to the difference in the functional parameters of MnP was not defined.

Four isozymes of MnP were found in the culture fluid of *P. chrysosporium* (21), and the N-terminal sequences for three of them were determined (35). Three sequences were significantly similar to each other, whereas there were differences in the sequences. This shows that there are at least three isozyme genes encoding MnPs in *P. chrysosporium*. In this study, we isolated two MnP cDNAs of IZU-154, and Southern blot analysis indicated that two copies of the MnP gene exist in IZU-154 (Fig. 6). However, by using isoelectric focusing, at least four MnP proteins were found in the culture fluid of IZU-154 (Fig. 2). Two isozymes were dominant proteins, and the rest were minor bands. From these findings, it was suggested that these proteins are possibly the products of two MnP isozyme genes, and hence by the difference of posttranslational modification, four MnP proteins were generated. This can be confirmed by determination of the N-terminal amino acid sequences of the four MnP proteins.

In this paper, we showed that the deduced products of MnP cDNAs from IZU-154 and *P. chrysosporium* are significantly similar and that the kinetic parameters of MnP for oxidation of 2,6-DMP and Mn(II) are also similar when those for IZ-MnP are compared with those for *P. chrysosporium* MnP. Moreover, IZU-154 can produce only slightly higher amounts of MnP than *P. chrysosporium* under optimal conditions for each organism (data not shown). Thus, the difference in the lignin-degrading activities of each fungus cannot be explained by the functional role of MnP. On the other hand, additional features of IZU-154 have been defined. Previously, the abilities of IZU-154 and *P. chrysosporium* to degrade lignin were determined with ^{14}C-labeled high-molecular-mass lignin model compound (DHP-lignin) (46). IZU-154 could degrade it to $^{14}CO_2$ through a low-molecular-mass compound and exhibited little activity for repolymerization, while *P. chrysosporium* repolymerized the produced low-molecular-mass compound to a high-molecular-mass compound. Therefore, the degree of complete degradation by *P. chrysosporium* was lower than that by IZU-154. These observations are not attributed to MnP function and indicate that a repolymerization of the degraded lignin occurred during fungal treatment. Hence, lignolytic ability is not dependent only on MnP even in IZU-154, whereas many other enzyme systems are thought to be involved and are likely to be more invaluable in this process.

MnP from IZU-154 can degrade a variety of polymers such as lignin (18), nylon (3), and melanin (18). Mn(III) generated by MnP possibly migrates into polymer molecules and initiates nonspecific oxidation. The radical produced by the oxidation can trigger the ensuing radical degradation. MnP can be applied to industrial processes, such as modification and degradation of polymers and xenobiotic compounds, bioremediation, and decolorization of wastewater. Efficient production of MnP by overexpression is necessary to use MnP for industrial

purposes, and evolutionary insight that structural comparison offers is essential to generate an efficient and stable enzyme.

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REFERENCES

1. **Brown, J. A., J. K. Glenn, and M. H. Gold.** 1990. Manganese regulates the expression of manganese peroxidase by *Phanerochaete chrysosporium*. J. Bacteriol. **172:**3125–3130.
2. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
3. **Deguchi, T.** Unpublished data.
4. **Faison, B. D., and T. K. Kirk.** 1985. Factors involved in the regulation of a ligninase activity in *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **49:**299–304.
5. **Fenn, P., and T. K. Kirk.** 1981. Relationship of nitrogen to the onset and suppression of lignolytic activity and secondary metabolism in *Phanerochaete chrysosporium*. Arch. Microbiol. **130:**59–65.
6. **Finzel, B. C., T. L. Poulos, and J. Kraut.** 1984. Crystal structure of yeast cytochrome c peroxidase refined at 1.7Å resolution. J. Biol. Chem. **259:** 13027–13036.
7. **Fujita, K., R. Kondo, K. Sakai, Y. Kashino, T. Nishida, and Y. Takahara.** 1991. Biobleaching of kraft pulp using white-rot fungus IZU-154. Tappi J. **74:**123–127.
8. **Fujita, K., R. Kondo, K. Sakai, Y. Kashino, T. Nishida, and Y. Takahara.** 1993. Biobleaching of softwood kraft pulp with white-rot fungus IZU-154. Tappi J. **76:**81–84.
9. **Glenn, J. K., L. Akileswaran, and M. H. Gold.** 1986. Mn(II) oxidation is the principal function of the extracellular Mn peroxidase from *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **251:**688–696.
10. **Glenn, J. K., and M. H. Gold.** 1985. Purification and characterization of an extracellular Mn(II)-dependent peroxidase from the lignin-degrading basidiomycete, *Phanerochaete chrysosporium.* Arch. Biochem. Biophys. **242:**329–341.
11. **Glenn, J. K., M. A. Morgan, M. B. Mayfield, M. Kuwahara, and M. H. Gold.** 1983. An extracellular H_2O_2-requiring enzyme preparation involved in lignin biodegradation by the white rot basidiomycete *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. **114:**1077–1083.
12. **Godfrey, B. J., M. B. Mayfield, J. A. Brown, and M. H. Gold.** 1990. Characterization of a gene encoding a manganese peroxidase from *Phanerochaete chrysosporium*. Gene **93:**119–124.
13. **Gold, M. H., H. Wariishi, and K. Valli.** 1989. Extracellular peroxidase involved in lignin degradation by the white rot basidiomycete *Phanerochaete chrysosporium*. ACS Symp. Ser. **389:**127–140.
14. **Hammel, K. E., and M. A. Moen.** 1991. Depolymerization of a synthetic lignin in vitro by lignin peroxidase. Enzyme Microb. Technol. **13:**15–18.
15. **Hatakka, A.** 1994. Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. FEMS Microbiol. Rev. **13:**125–135.
16. **Henikoff, S.** 1987. Unidirectional digestion with exonuclease III in DNA sequence analysis. Methods Enzymol. **155:**156–165.
17. **Jeffries, T. W., T. K. Kirk, and S. Choi.** 1981. Nutritional regulation of lignin degradation by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **42:** 290–296.
18. **Kashino, Y., M. Matsubara, and S. Yamada.** Unpublished data.
19. **Kashino, Y., T. Nishida, Y. Takahara, K. Fujita, R. Kondo, and K. Sakai.** 1993. Biomechanical pulping using white-rot fungus IZU-154. Tappi J. **76:** 167–171.
20. **Keyser, P., T. K. Kirk, and J. G. Zeikus.** 1978. Lignolytic enzyme system of *Phanerochaete chrysosporium*: synthesized in the absence of lignin in response to nitrogen starvation. J. Bacteriol. **135:**790–797.
21. **Kirk, T. K., and R. L. Farrell.** 1987. Enzymatic "combustion": the microbial degradation of lignin. Annu. Rev. Microbiol. **41:**465–505.
22. **Kornfeld, R., and S. Kornfeld.** 1985. Assembly of asparagine-linked oligosaccharides. Annu. Rev. Biochem. **54:**631–664.
23. **Kuwahara, M., J. K. Glenn, M. A. Morgan, and M. H. Gold.** 1984. Separation and characterization of two extracellular H_2O_2-dependent oxidases from lignolytic cultures of *Phanerochaete chrysosporium*. FEBS Lett. **169:** 247–250.
24. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
25. **Lee, S.-H., R. Kondo, K. Sakai, T. Nishida, and Y. Takahara.** 1993. Treatment of kraft bleaching effluents by lignin-degrading fungi I: decolorization of kraft bleaching effluents by the lignin-degrading fungus IZU-154. Mokuzai Gakkaishi **39:**470–478.
26. **Leisola, M. S. A., B. Kozulic, F. Meussdoerffer, and A. Fiechter.** 1987. Homology among multiple extracellular peroxidases from *Phanerochaete chrysosporium*. J. Biol. Chem. **262:**419–424.
27. **Marquardt, D. W.** 1963. An algorithm for least-squares estimation of nonlinear parameters. J. Soc. Ind. Appl. Math. **11:**432–441.
28. **Mayfield, M. B., B. J. Godfrey, and M. H. Gold.** 1994. Characterization of the MnP2 gene encoding manganese peroxidase isozyme 2 from the basidiomycete *Phanerochaete chrysosporium*. Gene **142:**231–235.
29. **Mayfield, M. B., K. Kishi, M. Alic, and M. H. Gold.** 1994. Homologous expression of recombinant manganese peroxidase in *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **60:**4303–4309.
30. **Nelder, J. A., and R. Mead.** 1965. A simplex method for function minimization. Computer J. **7:**308–313.
31. **Nishida, T., Y. Kashino, A. Mimura, and Y. Takahara.** 1988. Lignin biodegradation by wood-rotting fungi I: screening of lignin-degrading fungi. Mokuzai Gakkaishi **34:**530–536.
32. **Orth, A. B., D. J. Royse, and M. Tien.** 1993. Ubiquity of lignin-degrading peroxidases among various white- and brown-rot fungi. Appl. Environ. Microbiol. **59:**4017–4023.
33. **Paszczynski, A., V. B. Huynh, and R. L. Crawford.** 1986. Comparison of ligninase-I and peroxidase-M2 from the white rot-fungus *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **244:**750–765.
34. **Pease, E. A., A. Andrawis, and M. Tien.** 1989. Manganese-dependent peroxidase from *Phanerochaete chrysosporium*: primary structure deduced from cDNA sequence. J. Biol. Chem. **264:**13531–13535.
35. **Pease, E. A., and M. Tien.** 1992. Heterogeneity and regulation of manganese peroxidases from *Phanerochaete chrysosporium*. J. Bacteriol. **174:**3532–3540.
36. **Perie, F. H., and M. H. Gold.** 1991. Manganese regulation of manganese peroxidase expression and lignin degradation by the white-rot fungus *Dichromitus squalens*. Appl. Environ. Microbiol. **57:**2240–2245.
37. **Poulos, T. L., and J. Kraut.** 1980. The stereochemistry of peroxidase catalysis. J. Biol. Chem. **255:**8199–8205.
38. **Pribnow, D., M. B. Mayfield, V. J. Nipper, J. A. Brown, and M. H. Gold.** 1989. Characterization of a cDNA encoding a manganese peroxidase, from the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. J. Biol. Chem. **264:**5036–5040.
39. **Renganathan, V., and M. H. Gold.** 1986. Spectral characterization of the oxidized states of lignin peroxidase, an extracellular heme enzyme from the white rot basidiomycete *Phanerochaete chrysosporium*. Biochemistry **25:** 1626–1631.
40. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
41. **Sundaramoorthy, M., K. Kishi, M. H. Gold, and T. L. Poulos.** 1994. The crystal structure of manganese peroxidase from *Phanerochaete chrysosporium* at 2.06-Å resolution. J. Biol. Chem. **269:**32759–32767.
42. **Tien, M., and T. K. Kirk.** 1983. Lignin-degrading enzyme from the hymenomycete *Phanerochaete chrysosporium* burds. Science **221:**661–663.
43. **Tien, M., T. K. Kirk, C. Bull, and J. A. Fee.** 1986. Steady-state and transient-state kinetic studies on the oxidation of 3,4-dimethoxybenzyl alcohol catalyzed by the ligninase of *Phanerochaete chrysosporium*. J. Biol. Chem. **261:** 1687–1693.
44. **Tien, M., and C.-P. D. Tu.** 1987. Cloning and sequencing of a cDNA for a ligninase from *Phanerochaete chrysosporium*. Nature (London) **326:**520–523.
45. **Tuor, U., H. Wariishi, H. E. Schoemaker, and M. H. Gold.** 1992. Oxidation of phenolic arylglycerol-β-aryl ether lignin model compounds by manganese peroxidase from *Phanerochaete chrysosporium*: oxidative cleavage of an α-carbonyl model compound. Biochemistry **31:**4986–4995.
46. **Uezono, Y., and T. Deguchi.** Unpublished data.
47. **von Heijne, G.** 1985. Signal sequences: the limits of variation. J. Mol. Biol. **184:**99–105.
48. **Wariishi, H., L. Akileswaran, and M. H. Gold.** 1988. Manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*: spectral characterization of the oxidized states and the catalytic cycle. Biochemistry **27:**5365–5370.
49. **Wariishi, H., H. B. Dunford, I. D. MacDonald, and M. H. Gold.** 1989. Manganese peroxidase from the lignin-degrading basidiomycete *Phanerochaete chrysosporium*: transient-state kinetics and reaction mechanism. J. Biol. Chem. **264:**3335–3340.
50. **Wariishi, H., K. Valli, and M. H. Gold.** 1989. Oxidative cleavage of aphenolic diarylpropane lignin model dimer by manganese peroxidase from *Phanerochaete chrysosporium*. Biochemistry **28:**6017–6023.
51. **Wariishi, H., K. Valli, and M. H. Gold.** 1991. *In vitro* depolymerization of lignin by manganese peroxidase of *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. **176:**269–275.
52. **Wariishi, H., K. Valli, and M. H. Gold.** 1992. Manganese(II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*: kinetic mechanism and role of chelators. J. Biol. Chem. **267:**23688–23695.