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Mechanism for Oxidation of High-Molecular-Weight Substrates by a Fungal Versatile Peroxidase, MnP2[∇]

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Unlike general peroxidases, *Pleurotus ostreatus* MnP2 was reported to have a unique property of direct oxidation of high-molecular-weight compounds, such as Poly R-478 and RNase A. To elucidate the mechanism for oxidation of polymeric substrates by MnP2, a series of mutant enzymes were produced by using a homologous gene expression system, and their reactivities were characterized. A mutant enzyme with an Ala substituting for an exposing Trp (W170A) drastically lost oxidation activity for veratryl alcohol (VA), Poly R-478, and RNase A, whereas the kinetic properties for Mn²⁺ and H₂O₂ were substantially unchanged. These results demonstrated that, in addition to VA, the high-molecular-weight substrates are directly oxidized by MnP2 at W170. Moreover, in the mutants Q266F and V166/168L, amino acid substitution(s) around W170 resulted in a decreased activity only for the high-molecular-weight substrates. These results, along with the three-dimensional modeling of the mutants, suggested that the mutations caused a steric hindrance to access of the polymeric substrates to W170. Another mutant, R263N, contained a newly generated N glycosylation site and showed a higher molecular mass in sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. Interestingly, the R263N mutant exhibited an increased reactivity with VA and high-molecular-weight substrates. The existence of an additional carbohydrate modification and the catalytic properties in this mutant are discussed. This is the first study of a direct mechanism for oxidation of high-molecular-weight substrates by a fungal peroxidase using a homologous gene expression system.

Pleurotus ostreatus, known as the oyster mushroom, is a white rot basidiomycete that degrades plant cell wall lignin effectively (33). It secretes a series of isozymes of extracellular oxidizing enzymes belonging to manganese peroxidase (MnP) (2, 10) and laccase (11, 28), which are thought to play a key role in lignin biodegradation (5). However, the expressions of MnP isozymes are regulated differentially (6, 7), and their physiological roles seem to be different from each other (15). MnP oxidizes efficiently Mn²⁺ to Mn³⁺ at a defined recognition site known as the Mn²⁺-binding site, and Mn³⁺ can act as a diffusible mediator (43). Normally, MnP does not oxidize nonphenolic aromatic compounds like veratryl alcohol (VA), which is a typical substrate for lignin peroxidase (LiP), the best-studied ligninolytic peroxidase secreted by the white-rot fungus *Phanerochaete chrysosporium* (32). *P. ostreatus* MnP2 (~43 kDa) has a wide substrate specificity, since it oxidizes VA (17). Peroxidases with a similar substrate specificity have also been reported in *Pleurotus eryngii* (24), *Bjerkandera adusta* (25, 27), and *Lepista irina* (44). These enzymes are known as versatile peroxidase (VP) or hybrid peroxidase, because they have properties of both LiP and MnP (22).

Most interestingly, *P. ostreatus* MnP2 oxidizes directly even high-molecular-mass compounds such as RNase A (~13.7 kDa) and a polymeric azo dye, Poly R-478 (40 to 100 kDa) (17), a property which has not been reported in the other fungal peroxidases. In contrast, *P. chrysosporium* LiP oxidizes Poly R-478 (12), RNase A (36), and ferricytochrome *c* (35)

only when VA was concomitantly present in the reaction mixture. In *P. chrysosporium* LiP isozyme H8 (PcLiPH8) a point mutational analysis demonstrated that an exposed tryptophan residue (W171) was crucial for VA oxidation (9, 37). It was suggested that oxidized VA formed a cation radical complex with the enzyme (18) and that this enzyme-cation radical complex oxidized RNase A (36).

The corresponding tryptophan residue is conserved among LiP and also VP isozymes (22). A point mutation analysis of *P. eryngii* VPL (PeVPL) demonstrated that the tryptophan (W164) is essential for the oxidation of low-molecular-weight substrates such as VA and Reactive Black 5 (*M_r* = 991), although the mutant enzymes were produced by an *Escherichia coli* expression system (31) in which the active enzymes were prepared as a fraction of an in vitro refolded polypeptide in the presence of heme (30). *P. ostreatus* MnP2 also has the corresponding tryptophan (W170), which may suggest that MnP2 oxidizes VA at this residue. However, how MnP2 directly oxidizes the high-molecular-weight substrates in the absence of the redox mediators remains to be elucidated.

The gene encoding MnP2 was cloned (10), and a recombinant *mnp2* gene under the control of the *P. ostreatus* *sd11* promoter was successfully introduced into wild-type *P. ostreatus* by genetic transformation (39). The isolated transformants included recombinant strains with enhanced Poly R-478-decolorizing and benzo[*a*]pyrene-removing activities, demonstrating their high potential as biocatalysts in the remediation of polluted environments (39). Moreover, cultural conditions for exclusive production of the recombinant MnP2 without concomitant expression of the endogenous *mnp2* were developed (38). Unlike heterologous expression systems, it is expected that a homologous expression system reflects the phys-

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TABLE 1. Primers used for construction of pIpM2g derivatives encoding MnP2 variants

Mutant	Primer	Nucleotide sequence ^a
W170A	FW-W170	CTGCTTTCCTCGTGAGCTCTC TTCGGCAT
	RV-W170A	<u>GGCGACGACTTCAACGGT</u> GCTG
V166/168L	FW-W170	CTGCTTTCCTCGTGAGCTCTC TTCGGCAT
	RV-V166/168L	CCAGACGAGCTCGAGGGTGC TGAATCCAGC
E249D	FW-E249D	GACTGGCAGTCTATGGTCAG TAAGTACTACGTTCCCGT
	RV-E249	GCATGCACTCCTGGAGTCTG TAGTTGA
E249Q	FW-E249Q	CAATGGCAGTCCATGGTCAG TAAG
	RV-E249	GCATGCACTCCTGGAGTCTG TAGTTGA
R263N or D	FW-R263NorD	(A/G)ACTTCACCCAAGTCATG AGGAAAGCCT
	RV-R263	GTTCTGAATCTTCGGCATGT TGTC
Q266F	FW-Q266F	CGCTTCACCTTCGTTATGAGG AAGCTTCTCTCCTC
	RV-R263	GTTCTGAATCTTCGGCATGT TGTC

^a Underlined triplets contain a base substitution(s) (in boldface) to cause the desired amino acid change in each variant.

iological conditions during the enzyme production process of the original organism, including carbohydrate modification, signal peptide processing, secretion, and maintenance in the culture filtrate, and that expressed enzymes may have the same structural and catalytic properties as the native ones. Actually, the purified recombinant MnP2 showed properties identical to those of native MnP2 in terms of electrophoresis, spectroscopic analysis, and reactivity for Mn^{2+} , H_2O_2 , VA, and RNase A (38).

In this report, we utilized the homologous expression system to produce MnP2 variants with desired amino acid substitutions and characterized their catalytic properties with various substrates.

MATERIALS AND METHODS

Strains and culture conditions. The *P. ostreatus* dikaryotic strain 261 (ATCC 66376) was used as a host strain throughout the present study. Recombinant *P. ostreatus* to produce MnP2 variants was isolated as described below. Strains were grown on a 3.9% potato dextrose agar (Nissui Co., Tokyo, Japan) plate for maintenance.

E. coli JM109 [*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac-proAB)/F' (traD36 proAB⁺ lacI^q lacZΔM15)*] (41) was used as a host bacterium for standard recombinant DNA constructions and grown on Luria-Bertani medium.

Production and purification of the recombinant MnP2 variants. The expression plasmid pIpM2g contains the genomic sequence of *P. ostreatus mnp2* (39) and was used to construct mutant *mnp2* genes. Mutant gene constructs encoding MnP2 variants with desired amino acid substitutions were constructed by inverse PCR amplification of pIpM2g using primers containing mismatch base pairs (Table 1), followed by self-ligation. Seven pIpM2g derivatives encoding mutant MnP2 variants, W170A, V166/168L, E249D, E249Q, R263D, R263N, and Q266F, were constructed, and the base substitutions were verified by nucleotide sequencing.

Recombinant strains to produce MnP2 variants were selected from *P. ostreatus* transformants as follows. Carboxin-resistant marker plasmid pTM1 (13) and one of the pIpM2g derivatives were concomitantly introduced into *P. ostreatus* protoplasts using the polyethyleneglycol-CaCl₂ method described previously (39).

Then, 8 to 34 carboxin-resistant transformants were isolated for each mutant construct and 8 to 12 fast-growing strains were selected from the carboxin-resistant transformants for each mutant and screened for MnP productivity. MnP productivity was evaluated by measuring Mn^{2+} -dependent guaiacol-oxidizing activity (19). One unit of MnP was defined as the amount of enzyme that increased the A_{465} by 1.0 min⁻¹, and this definition was used as an indication of the enzyme amount in the following experiments. MnP productivity in filtrate from an agitated culture of the transformants on PGY medium supplemented with hot-water extract of wheat bran, in which the recombinant MnP2 was exclusively expressed and no endogenous MnP isozymes were produced (38), was evaluated. Among the transformants tested, the recombinant strain with the highest MnP productivity was chosen and used to overexpress each mutant MnP2 variant. The enzymes were purified from the culture filtrate to homogeneity by a purification protocol described in a previous report (16). Native MnP3 was also purified from stationary cultures of wild-type *P. ostreatus* (16) and used as a control.

Analysis of carbohydrate modification. To analyze additional modification of R263N by N-glycans, purified R263N and wild-type MnP2 were denatured in boiled water, followed by digestion with glycopeptidase F (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. Digested and intact enzymes, as a control, were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Catalytic analysis of the MnP2 variants. Kinetic parameters of the wild-type and recombinant MnP2 enzymes for Mn^{2+} , H_2O_2 , and VA were determined as described previously (34): the kinetic constants for Mn^{2+} peroxidase activity were calculated by the formation of Mn^{3+} -tartrate complex in sodium tartrate buffer (pH 5.0) by measuring absorbance at 238 nm; the kinetic constants for VA oxidation activity were calculated by the measure of veratryl aldehyde in sodium tartrate buffer (pH 3.0) by measuring the absorbance at 310 nm; the K_m and V_{max} for the enzyme-oxidizing substrate H_2O_2 were also obtained in sodium tartrate buffer (pH 5.0) by measuring Mn^{2+} oxidation. Measurements were carried out by using a U-2001 UV-VIS spectrophotometer (Hitachi, Tokyo, Japan) at 25°C.

Oxidation of RNase A (Sigma-Aldrich, Detroit, MI) was performed by using basically the same protocol as described previously (17). The reaction was initiated by adding 60 μM H_2O_2 to a 50 mM sodium tartrate buffer solution (pH 3.0, 1.0 ml) containing 3.0 U of each MnP2 variant and 10 μM RNase A. As a control, reaction mixtures missing RNase A were also prepared. Fluorescence due to coupling of the tyrosine residues of RNase A was monitored by a fluorescence meter (Shimadzu, Kyoto, Japan) with an excitation wavelength of 315 nm at 25°C. Emission spectra were recorded at 410 nm (1).

Decolorization of Poly R-478 (Sigma-Aldrich, St. Louis, MO) by recombinant MnP2 variants was performed in a solution containing 0.02% Poly R-478, 3.0 U of each recombinant MnP2, and 50 mM sodium tartrate buffer (pH 3.0, 1.0 ml). The reaction was initiated by adding 200 μM H_2O_2 at 25°C (see Fig. 3B). Decolorization of Poly R-478 was assayed at $A_{520/350}$ (17). In the experiment with stepwise addition of H_2O_2 , reactions were initiated by addition of 0.1 mM H_2O_2 to the reaction mixture. Then, 0.1 mM H_2O_2 was added repeatedly every 10 min.

Self-reduction of oxidized MnP2 variants. Transient-state kinetic measurements were conducted by using a U-2001 UV-VIS spectrophotometer at 25°C. Compound I formation was verified by absorbance at 407, 532, and 647 nm on mixing 2.0 U of the resting-state enzyme with 1 eq of H_2O_2 in 50 μl of 20 mM sodium succinate buffer (pH 4.5). Successive self-reductions to compound II and, furthermore, to the resting state were monitored by changes in absorbance at 418, 529, and 558 nm and at 407, 502, and 637 nm, respectively (17).

Enzyme activities of wild-type MnP2 and R263N at different pHs. To compare the influence of acidic pH on the catalytic activity levels of each enzyme, wild-type MnP2 and R263N were preincubated in 50 mM sodium tartrate buffer (pH 3.0) for 30, 60, 90, and 120 min, followed by measurement of the reactivity with Mn^{2+} , VA, and Poly R-478. Reaction conditions for Poly R-478 were as described above. The VA oxidation was carried out with 1.0 U of enzyme added to 0.1 mM H_2O_2 –1.6 mM VA in sodium tartrate buffer (pH 3.0, 1.0 ml). The Mn^{2+} oxidation was carried out with 0.5 U of enzyme added to 0.1 mM H_2O_2 –0.2 mM $MnSO_4$ in sodium tartrate buffer (pH 4.5, 1.0 ml). To study the pH optimum of wild-type MnP2 and R263N reactivity for Mn^{2+} , VA, and Poly R-478, the enzyme reactivity for each substrate was measured in 50 mM sodium tartrate buffer (pH 2.0 to 5.0). The relative activity of the enzyme for each substrate was evaluated by taking the maximum reactivity as 100%.

Molecular modeling. Three-dimensional (3D) models of wild-type and point-mutated MnP2s were built by homology modeling with CPHmodels (21) using an amino acid sequence deduced from the *mnp2* genomic sequence (GenBank accession no. AJ243977). The root mean square deviation (RMSD) of pairwise Cα atoms of the input structures was calculated by using FATCAT pairwise alignment (42).

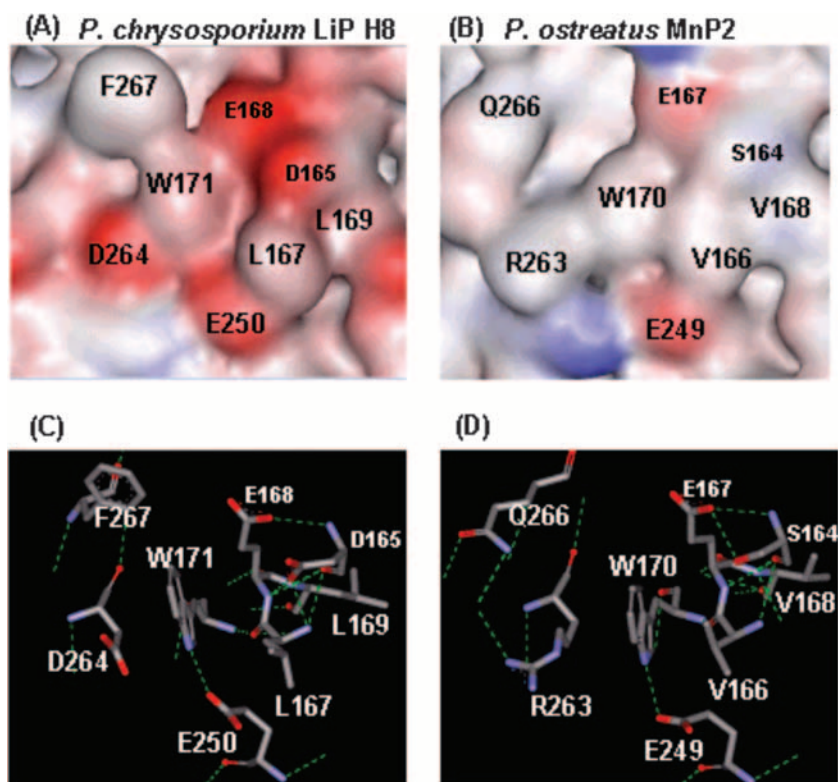


FIG. 1. Comparison of the microenvironment surrounding the exposed Trp in PcLiPH8 (A and C) and *P. ostreatus* MnP2 (B and D). The surrounding environments of Trp171 in the PcLiPH8 crystal structure from the PDB code 1B82 (A and C) and those of Trp170 in the *P. ostreatus* MnP2 3D model (B and D) obtained by homology modeling with PeVPL (PDB code 2BOQ) as a template are exhibited. The RMSD of pairwise C α atoms of the *P. ostreatus* MnP2 (VP) and PeVPL was 0.43 Å. The superficial structures are represented in panels A and B; the red and blue colors indicate negative and positive electrostatic potentials, respectively. The molecular structures are shown in panels C and D; the red and blue modules indicate oxygen and nitrogen, respectively, and the dashed green line indicates an H bond.

RESULTS

Design of the MnP2 variants with an amino acid substitution(s). To elucidate the contribution of the amino acid residues to the different reactivities of MnP2 and PcLiPH8, enzyme surface conformations in the vicinity of the exposed Trp were compared. In PcLiPH8, W171 is surrounded by acidic amino acid residues (D165, E168, E250, and D264), which are thought to stabilize the VA-enzyme cation radical complex by anionic charge (Fig. 1A). On the other hand, the anionic charge of these residues can cause electrostatic repulsion between LiP and the substrates, which might be one reason for VA-dependent polymer oxidation in LiP. In the corresponding region, *P. ostreatus* MnP2 possesses only two acidic amino acid residues, E167 and E249 (Fig. 1B). Furthermore, E249 seems to interact with the indole ring of W170 via a hydrogen bond (Fig. 1D), as demonstrated in PcLiPH8 by crystallographic analysis (Fig. 1C). In Fig. 1, it is also shown that F267 and L167 in PcLiPH8 protrude from the surface compared to Q266 and V166 in MnP2. It is possible that LiP requires the binding of VA to overcome the probable steric hindrance between the redox site and polymeric compounds.

Seven mutant MnP2 variants were designed to elucidate the contribution of each amino acid residue to the different functional properties between LiP and MnP2. They included the following: W170A (W170 converted to Ala); R263D and

R263N, introducing the corresponding acidic amino acid of PcLiPH8 (D264) or its neutral derivative Asn; E249Q and E249D to see the contribution of the proposed hydrogen bond between E249 and the indole ring of W170; and V166/168L and Q266F to imitate the conformation in the vicinity of W171 of PcLiPH8 (L167/169 and F267).

Production of the recombinant MnP2 variants in *P. ostreatus*. Expression plasmids with the appropriate base substitution(s) were constructed and introduced into the wild-type *P. ostreatus* strain by cotransformation with a carboxin-resistant marker plasmid (13). Carboxin-resistant transformants were isolated and approximately 10 fast-growing isolates for each mutant were subjected to screening for Mn²⁺-dependent guaiacol-oxidizing activity using a culture condition in which no endogenous MnP isozymes were expressed (39). For wild-type MnP2 and the four variants (W170A, R263N, Q266F, and V166/168L), significant MnP activity was observed in most of the transformants tested, indicating successful cotransformation and expression of the recombinant *mnp2* in these isolates. On the other hand, no MnP activity was detected for the remaining MnP2 variants (E249D, E249Q, and R263D), even though 12 transformants for each were subjected to the screening for MnP productivity. These results strongly suggested that an active MnP enzyme was not produced for these mutants in *P. ostreatus*.

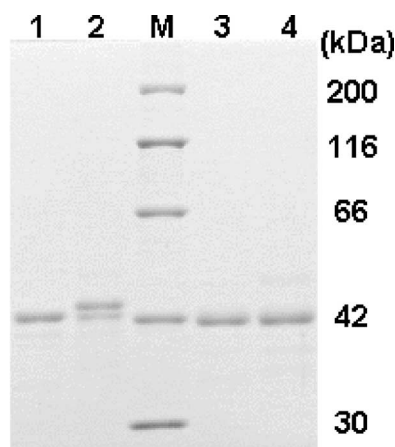


FIG. 2. SDS-PAGE analysis before and after deglycosylation. Wild-type MnP2 (lanes 1 and 3) and R263N (lanes 2 and 4), with (lanes 3 and 4) or without (lanes 1 and 2) *N*-glycosidase treatment, were subjected to SDS-PAGE analysis. The sizes of the molecular mass standards (lane M) are indicated on the right side. The cleavage of *N*-glycans from the purified enzymes was carried out by glycopeptidase F under denaturing conditions (pH 8.6) at 37°C for 20 h.

Transformants with the highest productivity of each MnP2 variant were selected, and recombinant enzymes were produced and purified to homogeneity using a previously described protocol (16). In SDS-PAGE and isoelectric focusing analyses, three variants—W170A, Q266F, and V166/168L—showed mobilities identical to that of wild-type MnP2 (data not shown), suggesting that each variant was processed and modified similarly to the native enzyme.

Interestingly, R263N showed slightly slower mobility in SDS-PAGE (Fig. 2) and an acidic pI value in isoelectric focusing analyses (data not shown), indicating that this variant had a larger molecular weight and different isoelectric properties compared to wild-type MnP2. In R263N, there was a newly generated stretch of amino acids, Asn263-Phe264-Thr265, which corresponded to the consensus sequence for the *N*-glycosylation site, Asn-X-Thr or Ser (where X is any amino acid except Pro) (4). It is conceivable that R263N contained an additional carbohydrate chain attached to Asn263. To confirm the additional *N* glycosylation, purified R263N, as well as wild-type MnP2 as a control, was heat denatured and subjected to digestion with glycopeptidase F, which cleaves GluNAc-Asn bonds, followed by SDS-PAGE analysis (Fig. 2). After digestion with glycopeptidase F, the wild-type and mutant MnP2 showed the same electrophoresis pattern with an increased

mobility, demonstrating the existence of the additional carbohydrate modification in R263N. The UV-VIS spectral analysis of R263N, as well as of W170A, Q266F, and V166/168L, showed shapes and intensities at 407, 502, and 637 nm identical to those of wild-type MnP2 (data not shown), suggesting that the introduced amino acid substitutions did not cause significant changes in the heme environment in these variants.

Catalytic properties for low-molecular-weight substrates. Under steady-state conditions, Lineweaver-Burk plots were obtained for $1/v$ versus $1/[Mn^{2+}]$, $1/v$ versus $1/[H_2O_2]$, and $1/v$ versus $1/[VA]$ for each MnP2 variant. The determined K_m and V_{max} values are summarized in Table 2. It was demonstrated that all of the MnP2 variants tested showed kinetic properties for H_2O_2 almost identical to those of wild-type MnP2, indicating that the amino acid substitutions make no significant changes in terms of access or reactivity of H_2O_2 to the heme moiety. These results are consistent with the results of UV-VIS spectral analyses of the heme environment described above. Similar results were also obtained in K_m values for Mn^{2+} , whereas variants showed nearly doubled V_{max} values for Mn^{2+} compared to the wild type. Although the molecular mechanism of the observed increase in V_{max} values for Mn^{2+} remains to be elucidated, the equal amounts of the enzymes with respect to Mn^{2+} -oxidizing activity were used in the following experiments (see Materials and Methods).

As for W170A, oxidation of VA was not observed when 2.0 U of enzyme was used for 1 to 25 mM VA (Table 2). Under the same conditions, wild-type and other variant MnP2s significantly oxidized VA to veratryl aldehyde. From these results, it was demonstrated that MnP2 oxidized VA substantially at W170, which is consistent with the results obtained for PcLiP (9, 37) and PeVPL (31). With respect to the surrounding environment of W170, the catalytic efficiencies (V_{max}/K_m) for VA were almost unchanged in the V166/168L and Q266F variants, suggesting that these amino acid substitutions had little effect on the oxidation of low-molecular-weight aromatic substrates at W170. On the other hand, R263N exhibited >3-fold the V_{max} value for VA compared to wild-type MnP2. Increased catalytic activities of R263N for polymers were also observed in the following experiments and are discussed below.

Oxidation of high-molecular-weight substrates. RNase A-oxidizing activity was monitored by fluorescence spectroscopy to detect dihydroxyphenyl formation between RNase A molecules (Fig. 3A). W170A lost substantial oxidizing activity for RNase A. Q266F exhibited less than half the RNase A-oxidizing activity of wild-type MnP2. On the other hand, V166/168L and R263N showed slightly lower and higher RNase A-oxidizing

TABLE 2. Steady-state kinetic parameters for low-molecular-weight compounds of MnP2 variants^a

MnP2 wild type or variant	Avg K_m (μ M) $\pm$ SD			Avg V_{max} (μ mol/min/mg) $\pm$ SD		
	Mn^{2+}	H_2O_2	VA	Mn^{2+}	H_2O_2	VA
Wild type	22.3 $\pm$ 2.8	10.3 $\pm$ 0.2	5,080 $\pm$ 222	43.1 $\pm$ 2.6	50.8 $\pm$ 5.9	4.4 $\pm$ 0.1
W170A	21.0 $\pm$ 2.9	9.9 $\pm$ 0.8	ND	96.9 $\pm$ 0.3	45.6 $\pm$ 4.1	ND
R263N	22.3 $\pm$ 2.2	9.8 $\pm$ 1.0	5,059 $\pm$ 274	98.1 $\pm$ 8.4	41.1 $\pm$ 1.0	14.1 $\pm$ 0.7
Q266F	25.9 $\pm$ 1.4	9.3 $\pm$ 0.6	2,322 $\pm$ 221	89.4 $\pm$ 4.4	43.8 $\pm$ 4.8	2.5 $\pm$ 0.5
V166/168L	26.6 $\pm$ 4.7	7.0 $\pm$ 0.4	4,361 $\pm$ 339	74.8 $\pm$ 6.7	31.1 $\pm$ 3.3	3.6 $\pm$ 0.4

^a The kinetic parameters of the wild-type and recombinant MnP2 enzymes for Mn^{2+} , H_2O_2 , and VA were determined as described previously (16, 34, 38). ND, not detected. The averages were determined from three independent experiments.

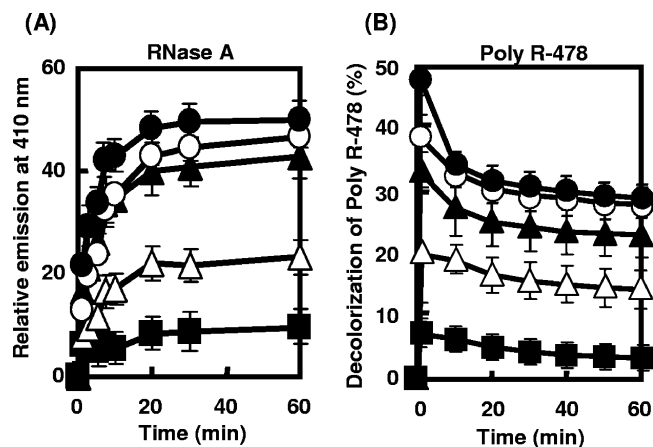


FIG. 3. Oxidation activity of high-molecular-weight substrates by the MnP2 variants. (A) Oxidation of RNase A was evaluated by measuring the relative emission at 410 nm with an excitation wavelength of 315 nm. Reaction mixtures consisted of each variant enzyme (3.0 U/ml) and RNase A (100 μ M) in 50 mM sodium tartrate buffer (pH 3.0). Reactions were initiated by the addition of H_2O_2 (60 μ M). (B) Decolorization of Poly R-478 was assayed at $A_{520/350}$. Reaction mixtures consisted of each enzyme (3.0 U/ml) and 0.02% Poly R-478, in 50 mM sodium tartrate buffer (pH 3.0). Reactions were initiated by the addition of 0.2 mM H_2O_2 . Symbols: $\circ$, wild-type MnP2; $\blacksquare$, W170A; $\bullet$, R263N; $\triangle$, Q266F; $\blacktriangle$, V166/168L. The averages and standard deviations were determined from three independent experiments.

activities, respectively, than that of wild-type MnP2. Decolorizing activity for the polymeric dye Poly R-478 was monitored with each variant enzyme (Fig. 3B). In this experiment, after the red color of the solution faded rapidly in the beginning, gradual recolorization of the reaction solution was observed, possibly as a consequence of a series of reactions triggered by the initial oxidation. Changes in the Poly R-478-decolorizing activity among variants were similar to those obtained with RNase A-oxidizing activity. From these results, it was demonstrated that W170 played a crucial role in oxidation of the high-molecular-weight substrates by MnP2. It is of interest that Q266F exhibited less than half the activities compared to wild-type MnP2 for both RNase A and Poly R-478, whereas it showed almost unchanged catalytic efficiency for VA (Table 2). It is likely that a steric hindrance generated by the amino acid substitutions (Gln266 to Phe) inhibited access of the polymeric substrates to W170.

Unique reaction properties of R263N. When 0.1 mM H_2O_2 was added repeatedly every 10 min, R263N decolorized Poly R-478 until the third addition of H_2O_2 ; the decolorizations of Poly R-478 in the first, second, third, and fourth cycles were $18.9\% \pm 3.8\%$, $34.5\% \pm 2.5\%$, $47.2\% \pm 0.6\%$, and $47.6\% \pm 1.0\%$, respectively. On the other hand, wild-type MnP2 reduced its activity in the second cycle, and no further decolorization was observed after the third cycle; the decolorizations of Poly R-478 in the first, second, third, and fourth cycles were $18.2\% \pm 5.0\%$, $24.9\% \pm 3.1\%$, $23.9\% \pm 1.5\%$, and $23.6\% \pm 0.5\%$, respectively. The final decolorization of Poly R-478 by R263N reached up to twice that produced by wild-type MnP2. The other three variants showed no remaining activity after the second cycle (data not shown). To investigate the reason for the persistent activity of R263N, wild-type MnP2 and R263N

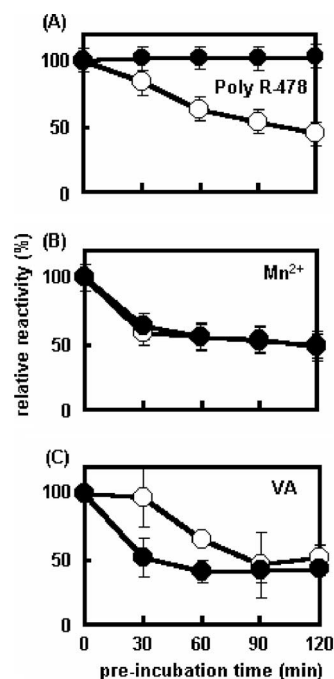


FIG. 4. Effect of preincubation at pH 3.0 on the reactivity with different substrates in wild-type MnP2 and R263N. Wild-type MnP2 and R263N were preincubated in 50 mM sodium tartrate buffer (pH 3.0) for 30, 60, 90, or 120 min, followed by measurement of the reactivity with Poly R-478 (A), Mn^{2+} (B), and VA (C). The relative activity of the enzyme for each substrate was evaluated by taking the maximum reactivity as 100%. Symbols: $\circ$, wild-type MnP2; $\bullet$, R263N. The averages and standard deviations were determined from three independent experiments.

were preincubated at pH 3.0, followed by measurement of the reactivity with various substrates in the appropriate pH conditions (see Materials and Methods). When the remaining activity was monitored with Poly R-478 as a substrate, R263N exhibited stable decolorizing activity for 120 min, but wild-type MnP2 showed a linear decrease in its activity (Fig. 4A). It is possible that the high stability of R263N at pH 3.0 accounts for the persistence of its Poly R-478-decolorizing activity in the experiment with stepwise addition of H_2O_2 . In contrast, when Mn^{2+} and VA were used as a substrate, the enzyme activity decreased not only in wild-type MnP2 but also in R263N depending on the preincubation time (Fig. 4B and C). These observations are intriguing because the preincubation at pH 3.0 led to different inactivation effects on R263N in a substrate-dependent manner.

In the following experiment, pH optima of R263N and wild-type MnP2 for different substrates were surveyed (Fig. 5). Interestingly, R263N and wild-type MnP2 showed different profiles of relative activity when Poly R-478 was used as a substrate: the reactivity of R263N with the polymeric substrate remained high at pH 3.5 to 5.0. In contrast, the two enzymes exhibited almost identical profiles to the relative oxidizing activity for Mn^{2+} and VA (Fig. 5B and C), suggesting that recognition of these substrates was not influenced by the mutation. This hypothesis is also supported by the fact that K_m values for Mn^{2+} and VA were unchanged in R263N (Table 2).

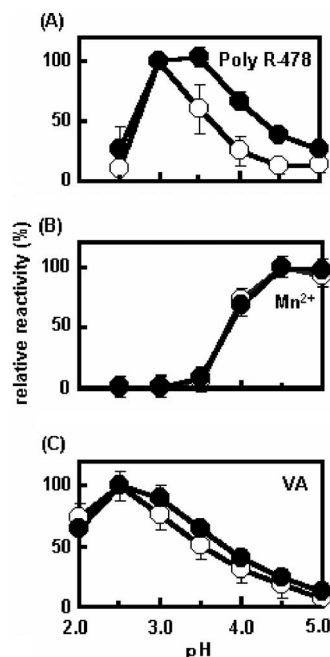


FIG. 5. pH optima of wild-type MnP2 and R263N for different substrates. The pH optima of reactivity for Poly R-478 (A), Mn^{2+} (B), and VA (C) were surveyed by using wild-type MnP2 and R263N in 50 mM sodium tartrate buffer (pH 2.0 to 5.0). The relative activity of the enzyme for each substrate was evaluated by taking the maximum reactivity as 100%. Symbols: ○, wild-type MnP2; ●, R263N. The averages and standard deviations were determined from three independent experiments.

Spontaneous decay of compounds I and II in the variants. We monitored self-reduction of H_2O_2 -activated MnP2 variants spectroscopically (Fig. 6). It was demonstrated that compound I of wild-type MnP2 was reduced to compound II, followed by further reduction to the resting state. The reduction of compound II was inhibited by a substitution of W170 to Ala (W170A). It was demonstrated that W170 is indispensable for the self-reduction of compound II in MnP2. Inhibition of spontaneous reduction of compound II was also observed for *P. ostreatus* MnP3, which lacks the corresponding Trp residue. These results are consistent with the results observed in the site-directed mutagenesis analysis of heterologously expressed PeVPL (31). In the remaining MnP2 variants, the self-reduction rates were decreased, despite the presence of W170 (Fig. 6). Especially, in V166/168L, the reduction was very slow, and the decay of compound II to the resting state was extremely inefficient. These results suggested that self-reduction rates may be affected by conformational changes in the microenvironment of W170. However, it is noteworthy that there seems to be little consistency between the rate of spontaneous decay of compound I (Fig. 6) and the catalytic activity of the mutant enzymes for the polymeric substrates (Fig. 3).

DISCUSSION

Lignin-degrading systems in white rot fungi have been studied in association with increasing expectation for their application in the pretreatment of lignocellulosic materials (23) or the degradation of environmental pollutants (26). Extracellu-

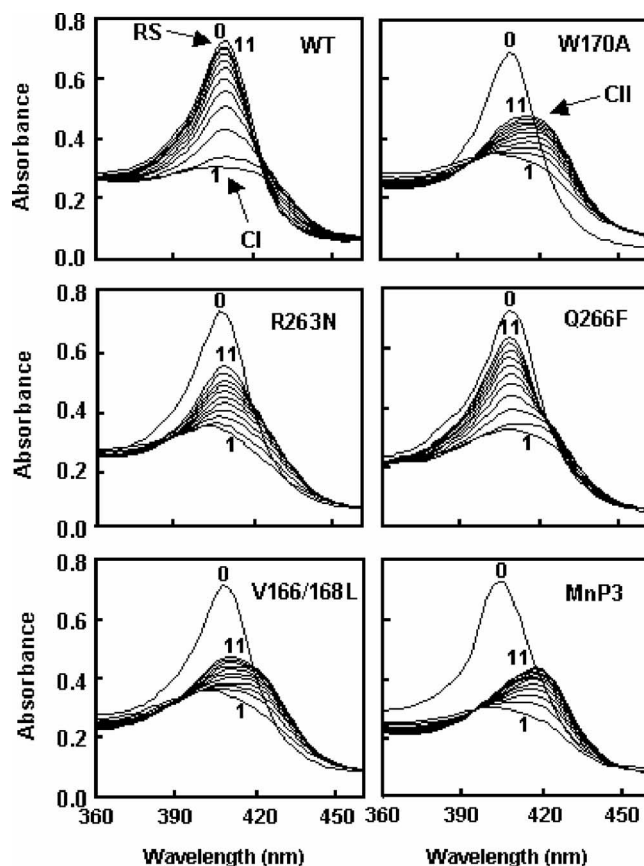


FIG. 6. Spontaneous reduction of the compound I form of MnP2 variants and MnP3. UV spectra for compound I formation and its spontaneous decay in the absence of a substrate were measured. Compound I was obtained by adding one equivalent of H_2O_2 to the reaction mixture, and its self-reduction was monitored. Increases in absorbance traces at 418 and 407 nm show reduction to compound II and a resting enzyme, respectively. Traces 1 to 11 correspond to 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 min, respectively, and trace 0 indicates the spectrum before H_2O_2 addition (resting state). The typical spectra for the resting state (RS), compound I (CI), and compound II (CII) are indicated in the figure.

lar oxidizing enzymes are considered to trigger a chain reaction of radical oxidations that leads to the decomposition of high-molecular-weight lignin (14). Various fungal peroxidases with different substrate spectra were characterized (3, 20, 27), and their oxidation mechanisms were studied using native and artificially expressed recombinant enzymes (8, 30, 38). In the present study, we investigated the oxidation mechanism for high-molecular-weight substrates by MnP2, using mutant enzymes produced by recombinant *P. ostreatus* strains because a homologous expression system has the native protein synthesis, modification, and secretion pathway of the original organism.

Seven MnP2 variants, namely, W170A, E249D, E249Q, R263N, R263D, Q266F, and V166/168L, were tested for expression by the recombinant *P. ostreatus* strains. However, the extracellular MnP activity of three mutants—E249D, E249Q, and R263D—was not detected, even though 12 recombinant strains were screened for each mutant. These mutants were designed to change charge environments in the surrounding region of W170. Taking the fact that other mutants were suc-

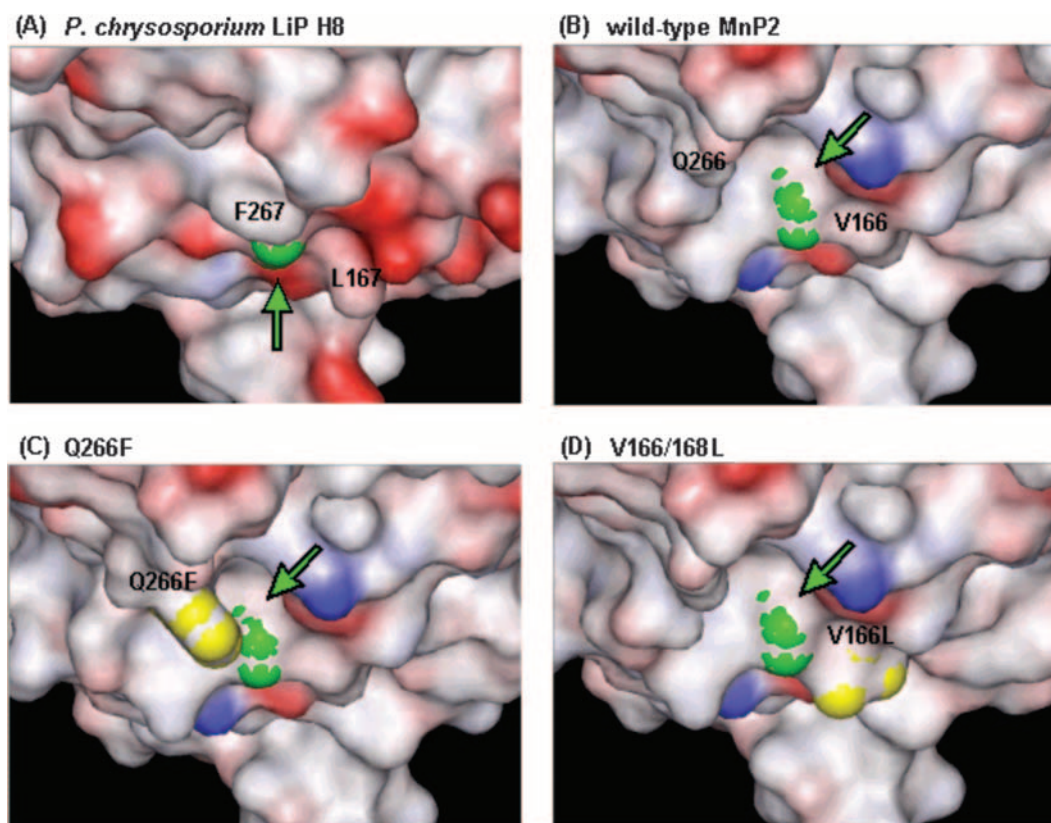


FIG. 7. Surface conformation around the redox active Trp residue in PcLiPH8 and *P. ostreatus* MnP2 variants. Views from the upper side of the redox active Trp residue in PcLiPH8 and *P. ostreatus* MnP2 variants are displayed. The surface of Trp residue is colored green and indicated by an arrow. The yellow-colored residues indicate the introduced amino acid substitution (Q266F and V166L). V168L was buried inside and was invisible from the surface. The 3D structure of PcLiPH8 was from PDB code 1B82, and those of *P. ostreatus* MnP2 variants were obtained by homology modeling with PeVPL (PDB code 2BOQ) as a template. The RMSDs of pairwise C α atoms of Q266F and PeVPL and of V166/168L and PeVPL were 0.45 and 0.44 Å, respectively. The red and blue colors indicate negative and positive electrostatic potentials, respectively.

cessfully expressed in the parallel experiments into account, it is plausible that *P. ostreatus* failed to express an active enzyme for these mutants. The reason for the unsuccessful expression is unclear, but it is likely that substitutions of charged amino acids may cause conformational changes leading to misfolding of the enzyme. It is noteworthy that the amino acid substitution of R263 to Asp, but not to Asn, seemed to inhibit the expression of active MnP2 by the recombinant *P. ostreatus*. In R263N, the existence of an additional N-glycan was demonstrated by digestion with glycopeptidase F (Fig. 2). These results are in good agreement with the positive effect of N-glycosyl modification on protein folding (29). Interestingly, Pérez-Boada et al. reported that a substitution of the corresponding R257 to an acidic amino acid conferred some LiP-type catalytic properties to PeVPL using a reconstituted enzyme fraction consisted of a refolded polypeptide expressed by recombinant *E. coli* and heme (31).

By substituting W170 to Ala, the oxidation activity of MnP2 was drastically decreased for VA but not for Mn²⁺ (Table 2). It is noteworthy that, when an excess amount of W170A and VA was used (10.0 U of W170A and 25 mM VA), the absorbance at 310 nm slightly increased, indicating the possible generation of veratryl aldehyde. For the polymeric substrates RNase A and Poly R-478, slight oxidation by W170A was

observed (Fig. 3). It was suggested that MnP2 may have an alternative oxidation site(s), although the reactivity is negligible. From these observations, we conclude that W170 is the substantial redox site for VA and high-molecular-weight substrates in MnP2, which is consistent with the previous findings that the oxidizing activity for VA and Poly R-478, but not for Mn²⁺, was lost by chemical modification of the purified MnP2 with *N*-bromosuccinimide (17).

While MnP2 directly oxidized Poly R-478 and RNase A (17), LiP formed enzyme-cation radical complexes with VA to oxidize them (18). It is likely that the different surrounding environments of the redox active center reflect the dependence on or independence from VA in the oxidation of these compounds by the two peroxidases. When steric features in the surrounding region of the exposed Trp are compared between MnP2 and PcLiPH8 (Fig. 1), it is clear that F267 and L167 in PcLiPH8 protrude from the enzyme surface compared to Q266 and V166 in MnP2. In the mutants Q266F and V166/168L, it was demonstrated that oxidizing activities for RNase A and Poly R-478 were decreased (Fig. 3), whereas the catalytic efficiency for the low-molecular-weight compounds was decreased (Table 2). From these results, it is suggested that the amino acid substitutions had an inhibitory action on polymer oxidation through steric hindrance. This hypothesis is sup-

ported by 3D modeling of the MnP2 variants (Fig. 7). On the other hand, the effect of the acidic residues in the surrounding region of PcLIPH8 on the reactivity with the polymeric compounds remains unclear, because substitutions of the corresponding amino acids resulted in unsuccessful expression of the mutant MnP2 in *P. ostreatus*.

R263N exhibited slightly higher reactivity the polymeric substrates than wild-type MnP2 (Fig. 3). When Poly R-478 was used as a substrate, increased enzyme stability at pH 3.0 (Fig. 4) and higher reactivity at a wider pH range (Fig. 5) were observed compared to wild-type MnP2. In contrast, with respect to Mn^{2+} - and VA-oxidizing activity, neither the stability at pH 3.0 (Fig. 4) nor the profile of oxidizing activity over the corresponding pH range (Fig. 5) was changed by the mutation. It is likely that the additional N-glycan was attached at the introduced Asn (N263) and that it may have an effect on interaction of the enzyme with the polymeric substrates. To prove this hypothesis, it is effective to compare catalytic properties of R263N with or without the N-glycans. Cleavage of the N-glycans from the purified R263N, as well as wild-type MnP2, by glyco-N-peptidases was tried, but denaturing pretreatment of the enzymes was essential (data not shown). Utilization of heterologously expressed R263N in the *E. coli* system could be an alternative clue to approach this issue. In the present situation, the possible function of the additional N-glycan in the activity of R263N remains to be elucidated. However, it is expected that, with its higher catalytic activity and the wider pH stability, R263N can be used for various bioprocesses, for example, as an immobilized enzyme in bioreactors for the decomposition of recalcitrant environmental pollutants (40).

In conclusion, it was demonstrated that *P. ostreatus* MnP2 oxidizes high-molecular-weight substrates, as well as VA, at the exposing Trp residue (W170) and that a relatively open space in the vicinity of W170 contributes to easier access of the polymeric compounds. In contrast, the corresponding redox active center (W171) of PcLIPH8 is embedded by the surrounding amino acid residues, which may cause steric hindrance for access of bulky molecules, and so complex formation of the enzyme-VA cation radical is a prerequisite to oxidize polymeric compounds such as Poly R-478 and RNase A.

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REFERENCES

- Amado, R., R. Aeschbach, and H. Neukom. 1984. Dityrosine: in vitro production and characterization. *Methods Enzymol.* **107**:377–388.
- Asada, Y., A. Watanabe, T. Irie, T. Nakayama, and M. Kuwahara. 1995. Structures of genomic and complementary DNAs coding for *Pleurotus ostreatus* manganese(II) peroxidase. *Biochim. Biophys. Acta* **1251**:205–209.
- Cai, D., and M. Tien. 1993. Lignin-degrading peroxidases of *Phanerochaete chrysosporium*. *J. Biotechnol.* **30**:79–90.
- Christlet, T. H., M. Biswas, and K. Veluraja. 1999. A database analysis of potential glycosylating Asn-X-Ser/Thr consensus sequences. *Acta Crystallogr. D Biol. Crystallogr.* **55**:1414–1420.
- Cohen, R., L. Persky, and Y. Hadar. 2002. Biotechnological applications and potential of wood-degrading mushrooms of the genus *Pleurotus*. *Appl. Microbiol. Biotechnol.* **58**:582–594.
- Cohen, R., O. Yarden, and Y. Hadar. 2002. Lignocellulose affects Mn^{2+} regulation of peroxidase transcript levels in solid-state cultures of *Pleurotus ostreatus*. *Appl. Environ. Microbiol.* **68**:3156–3158.
- Cohen, R., O. Yarden, and Y. Hadar. 2001. Transcript and activity levels of different *Pleurotus ostreatus* peroxidases are differentially affected by Mn^{2+} . *Environ. Microbiol.* **3**:312–322.
- Conesa, A., P. J. Punt, and C. A. M. J. J. van den Hondel. 2002. Fungal peroxidases: molecular aspects and applications. *J. Biotechnol.* **93**:143–158.
- Doyle, W. A., W. Blodig, N. C. Veitch, K. Piontek, and A. T. Smith. 1998. Two substrate interaction sites in lignin peroxidase revealed by site-directed mutagenesis. *Biochemistry* **37**:15097–15105.
- Giardina, P., G. Palmieri, B. Fontanella, V. Riviaccio, and G. Sannia. 2000. Manganese peroxidase isoenzymes produced by *Pleurotus ostreatus* grown on wood sawdust. *Arch. Biochem. Biophys.* **376**:171–179.
- Giardina, P., G. Palmieri, A. Scaloni, B. Fontanella, V. Faraco, G. Cennamo, and G. Sannia. 1999. Protein and gene structure of a blue laccase from *Pleurotus ostreatus*. *Biochem. J.* **341**:655–663.
- Harvey, P. J., L. P. Candeias, P. J. King, J. M. Palmer, and R. Wever. 1995. Lignin peroxidase catalysis: reaction with veratryl alcohol and a polymeric dye. *Poly R. Biochem. Soc. Trans.* **23**:340S.
- Honda, Y., T. Matsuyama, T. Irie, T. Watanabe, and M. Kuwahara. 2000. Carboxin resistance transformation of the homobasidiomycete fungus *Pleurotus ostreatus*. *Curr. Genet.* **37**:209–212.
- Joseleau, J. P., S. Gharibian, J. Comtat, A. Lefebvre, and K. Ruel. 1994. Indirect involvement of lignolytic enzyme-systems in cell-wall degradation. *FEMS Microbiol. Rev.* **13**:255–264.
- Kamitsuiji, H., Y. Honda, T. Watanabe, and M. Kuwahara. 2005. Mn^{2+} is dispensable for the production of active MnP2 by *Pleurotus ostreatus*. *Biochem. Biophys. Res. Commun.* **327**:871–876.
- Kamitsuiji, H., Y. Honda, T. Watanabe, and M. Kuwahara. 2004. Production and induction of manganese peroxidase isozymes in a white-rot fungus *Pleurotus ostreatus*. *Appl. Microbiol. Biotechnol.* **65**:287–294.
- Kamitsuiji, H., T. Watanabe, Y. Honda, and M. Kuwahara. 2005. Direct oxidation of polymeric substrates by multifunctional manganese peroxidase isoenzyme from *Pleurotus ostreatus* without redox mediators. *Biochem. J.* **386**:387–393.
- Khindaria, A., G. Nie, and S. D. Aust. 1997. Detection and characterization of the lignin peroxidase compound II-veratryl alcohol cation radical complex. *Biochemistry* **36**:14181–14185.
- Kofujita, H., Y. Asada, and M. Kuwahara. 1991. Arkil-aryl cleavage of phenolic β -O-4 lignin substructure model compound by Mn-peroxidase isolated from *Pleurotus ostreatus*. *Mokuzai Gakkaishi* **37**:555–561.
- Kuan, I. C., K. A. Johnson, and M. Tien. 1993. Kinetic analysis of manganese peroxidase: the reaction with manganese complexes. *J. Biol. Chem.* **268**:20064–20070.
- Lund, O., K. Frimand, J. Gorodkin, H. Bohr, J. Bohr, J. Hansen, and S. Brunak. 1997. Protein distance constraints predicted by neural networks and probability density functions. *Protein Eng.* **10**:1241–1248.
- Martínez, A. T. 2002. Molecular biology and structure-function of lignin-degrading heme peroxidases. *Enzyme Microb. Technol.* **30**:425–444.
- Martínez, A. T., M. Speranza, F. J. Ruiz-Dueñas, P. Ferreira, S. Camarero, F. Guillén, M. J. Martínez, A. Gutierrez, and J. C. del Río. 2005. Biodegradation of lignocelluloses: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. *Int. Microbiol.* **8**:195–204.
- Martínez, M. J., F. J. Ruiz-Dueñas, F. Guillén, and A. T. Martínez. 1996. Purification and catalytic properties of two manganese peroxidase isoenzymes from *Pleurotus eryngii*. *Eur. J. Biochem.* **237**:424–432.
- Mester, T., and J. A. Field. 1998. Characterization of a novel manganese peroxidase-lignin peroxidase hybrid isozyme produced by *Bjerkandera* species strain BOS55 in the absence of manganese. *J. Biol. Chem.* **273**:15412–15417.
- Novotný, C., K. Svobodová, P. Erbanová, T. Cajthaml, A. Kasinath, E. Lang, and V. Šásek. 2004. Ligninolytic fungi in bioremediation: extracellular enzyme production and degradation rate. *Soil Biol. Biochem.* **36**:1545–1551.
- Palma, C., A. T. Martínez, J. M. Lema, and M. J. Martínez. 2000. Different fungal manganese-oxidizing peroxidases: a comparison between *Bjerkandera* sp. and *Phanerochaete chrysosporium*. *J. Biotechnol.* **77**:235–245.
- Palmieri, G., P. Giardina, C. Bianco, A. Scaloni, A. Capasso, and G. Sannia. 1997. A novel white laccase from *Pleurotus ostreatus*. *J. Biol. Chem.* **272**:31301–31307.
- Parodi, A. J. 2000. Role of N-oligosaccharide endoplasmic reticulum processing reactions in glycoprotein folding and degradation. *Biochem. J.* **348**:1–13.
- Pérez-Boada, M., W. A. Doyle, F. J. Ruiz-Dueñas, M. J. Martínez, A. T. Martínez, and A. T. Smith. 2002. Expression of *Pleurotus eryngii* versatile peroxidase in *Escherichia coli* and optimization of in vitro folding. *Enzyme Microb. Technol.* **30**:518–524.
- Pérez-Boada, M., F. J. Ruiz-Dueñas, R. Pogni, R. Basosi, T. Choinowski, M. J. Martínez, K. Piontek, and A. T. Martínez. 2005. Versatile peroxidase oxidation of high redox potential aromatic compounds: site-directed mutagenesis, spectroscopic, and crystallographic investigation of three long-range electron transfer pathways. *J. Mol. Biol.* **354**:385–402.
- Reddy, C. A., and T. M. D'Souza. 1994. Physiology and molecular biology of the lignin peroxidases of *Phanerochaete chrysosporium*. *FEMS Microbiol. Rev.* **13**:137–152.
- Sarikaya, A., and M. R. Ladisch. 1997. Mechanism and potential applications of bio-ligninolytic systems in a CELSS. *Appl. Biochem. Biotechnol.* **62**:131–149.

34. Sarkar, S., A. T. Martínez, and M. J. Martínez. 1997. Biochemical and molecular characterization of a manganese peroxidase isoenzyme from *Pleurotus ostreatus*. *Biochim. Biophys. Acta* **1339**:23–30.
35. Sheng, D., and M. H. Gold. 1998. Irreversible oxidation of ferricytochrome *c* by lignin peroxidase. *Biochemistry* **37**:2029–2036.
36. Sheng, D., and M. H. Gold. 1999. Oxidative polymerization of ribonuclease A by lignin peroxidase from *Phanerochaete chrysosporium*: role of veratryl alcohol in polymer oxidation. *Eur. J. Biochem.* **259**:626–634.
37. Sollewijn Gelpke, M. D., J. Lee, and M. H. Gold. 2002. Lignin peroxidase oxidation of veratryl alcohol: effects of the mutants H82A, Q222A, W171A, and F267L. *Biochemistry* **41**:3498–3506.
38. Tsukihara, T., Y. Honda, R. Sakai, T. Watanabe, and T. Watanabe. 2006. Exclusive overproduction of recombinant versatile peroxidase MnP2 by genetically modified white rot fungus, *Pleurotus ostreatus*. *J. Biotechnol.* **126**:431–439.
39. Tsukihara, T., Y. Honda, T. Watanabe, and T. Watanabe. 2006. Molecular breeding of white rot fungus *Pleurotus ostreatus* by homologous expression of its versatile peroxidase MnP2. *Appl. Microbiol. Biotechnol.* **71**:114–120.
40. Wesenberg, D., I. Kyriakides, and S. N. Agathos. 2003. White-rot fungi and their enzymes for the treatment of industrial dye effluents. *Biotechnol. Adv.* **22**:161–187.
41. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**:103–119.
42. Ye, Y., and A. Godzik. 2003. Flexible structure alignment by chaining aligned fragment pairs allowing twists. *Bioinformatics* **19**:ii246–ii255.
43. Youngs, H. L., M. D. Sollewijn Gelpke, D. Li, M. Sundaramoorthy, and M. H. Gold. 2001. The role of Glu39 in MnII binding and oxidation by manganese peroxidase from *Phanerochaete chrysosporium*. *Biochemistry* **40**:2243–2250.
44. Zorn, H., S. Langhoff, M. Scheibner, M. Nimtz, and R. G. Berger. 2003. A peroxidase from *Lepista irina* cleaves β,β -carotene to flavor compounds. *Biol. Chem.* **384**:1049–1056.