APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1996, p. 860–864 Vol. 62, No. 3
0099-2240/96/$04.00+0


# Efficient Expression of a *Phanerochaete chrysosporium* Manganese Peroxidase Gene in *Aspergillus oryzae*

PHILIP STEWART,[1]* ROSS E. WHITWAM,[2] PHILIP J. KERSTEN,[3]
DANIEL CULLEN,[1,3] AND MING TIEN[2]

*Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706[1]; Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, Pennsylvania 16802[2]; and USDA Forest Products Laboratory, Madison, Wisconsin 53705[3]*

Received 10 November 1995/Accepted 22 December 1995

**A manganese peroxidase gene (*mnp1*) from *Phanerochaete chrysosporium* was efficiently expressed in *Aspergillus oryzae*. Expression was achieved by fusing the mature cDNA of *mnp1* with the *A. oryzae* Taka amylase promoter and secretion signal. The 3′ untranslated region of the glucoamylase gene of *Aspergillus awamori* provided the terminator. The recombinant protein (rMnP) was secreted in an active form, permitting rapid detection and purification. Physical and kinetic properties of rMnP were similar to those of the native protein. The *A. oryzae* expression system is well suited for both mechanistic and site-directed mutagenesis studies.**

The white rot basidiomycete *Phanerochaete chrysosporium* has served as a model system for investigations of lignin and organopollutant degradation (1, 9, 10, 13). System components include the $H_2O_2$-generating enzyme glyoxal oxidase, lignin peroxidases (LiPs), and manganese peroxidases (MnPs). MnPs are heme protein peroxidases which catalyze the $H_2O_2$-dependent oxidation of $Mn^{2+}$ (12, 24). MnP is oxidized by $H_2O_2$ by two electrons to generate an intermediate known as compound I. Compound I can oxidize $Mn^{2+}$ to $Mn^{3+}$ or can oxidize phenolic substrates to their corresponding radicals, and the enzyme is reduced to the one-electron-oxidized intermediate known as compound II. Compound II of MnP exhibits an absolute requirement for $Mn^{2+}$ as a reductant and upon oxidizing $Mn^{2+}$ to $Mn^{3+}$ is reduced to resting enzyme [also known as the ferric enzyme after the Fe(III) state of the heme] (21, 32, 33). $Mn^{3+}$, chelated by an organic acid, is presumed to be a diffusible oxidant, able to oxidize a wide variety of phenolic substrates (20).

Multiple isozymes of MnP and LiP are encoded by a large number of structurally related genes which are expressed under nutrient-deprived conditions (11, 19, 26). The significance of the multiplicity of isozymes is unknown, but considerable effort has focused on characterizing individual isozymes. Unfortunately, low yields and similar physical properties (e.g., pIs and molecular weights) have complicated these efforts. Efficient expression of individual isozymes in heterologous hosts would greatly facilitate investigations.

Two expression systems have been reported for MnPs, i.e., the baculovirus tissue culture system (25) and a homologous expression system in *P. chrysosporium* OGC101 (22). The baculovirus system suffers from high production costs and low yields. In the homologous system, an endogenous MnP was placed under the control of the constitutive glyceraldehyde-3-phosphate dehydrogenase promoter. MnP yields are higher than those of baculovirus, but cross-contamination by the native MnP isozymes remains a serious concern.

In the past decade, *Aspergillus* species have emerged as convenient and efficient expression systems for an array of prokaryotic and eukaryotic proteins (6, 29, 30). In particular, *Aspergillus niger* and *Aspergillus oryzae* are capable of secreting gram-per-liter quantities of heterologous proteins (7, 31). Recently, a *Coprinus cinereus* peroxidase (CiP), under the control of the Taka amylase promoter, was expressed in *A. oryzae* (2). CiP is distinctly different from the MnPs of *P. chrysosporium*, having less than 45% sequence homology at the amino acid level (5). Substrate specificity, pH optimum, and specific activity of CiP more closely resemble those of the plant peroxidases (e.g., horseradish peroxidase) than those of the extracellular peroxidases of white rot fungi (5).

Despite these differences, MnP was successfully expressed in *A. oryzae* by use of a similar construct (see Fig. 1). The recombinant MnP (rMnP) is secreted into the culture medium in an active form, making it easy to assay and purify. Expression in *A. oryzae* also eliminates the possibility of contaminating LiP or MnP isozymes.

## MATERIALS AND METHODS

**Strains and plasmids.** *P. chrysosporium* BKM-1767 was obtained from the Center for Forest Mycology Research, Forest Products Laboratory, Madison, Wis. *A. oryzae* (ATCC 11488) and *Aspergillus awamori* (NRRL 3112) were obtained from the American Type Culture Collection. The MnP cDNA was isolated from a *P. chrysosporium* λcDNA library (17) and is allelic to a previously described clone (27). pTAAMnP1 (Fig. 1) was constructed by fusing the mature MnP cDNA to a 680-bp fragment of the endogenous Taka amylase promoter and secretion signal from *A. oryzae* (2) and to a 199-bp fragment containing the glucoamylase terminator of *A. awamori* (18). Fusions were created by the PCR overlap extension technique (15) with the proofreading polymerase *Pfu* (Stratagene, La Jolla, Calif.). Plasmid pSR3 (16), containing the *amds* gene, was obtained from the Fungal Genetic Stock Center, Department of Microbiology, University of Kansas Medical Center. Plasmids were maintained in *Escherichia coli* DH5α.

**Transformations.** *A. oryzae* was transformed by a modification of the procedure of Ballance et al. (3). Flasks containing 50 ml of yeast extract-glucose medium were inoculated with $2 \times 10^8$ conidia and incubated at 37°C for 4.5 h. Germinating spores were harvested by centrifugation, washed in 0.6 M KCl, and incubated at 37°C in a mixture of 0.5% Novozyme 234 (Novo Industries, Copenhagen, Denmark), 0.5% $MgSO_4 \cdot 7H_2O$, 0.05% bovine serum albumin, and 0.6 M KCl. After incubation for 90 min, the suspension was filtered through sterile Miracloth (Calbiochem, San Diego, Calif.), washed with 0.6 M KCl, and resuspended in 1 M sorbitol containing 50 mM Tris (pH 7.4) and 50 mM $CaCl_2$ to a final concentration of $2 \times 10^8$ protoplasts per ml. pTAAMnP1 and pSR3 were cotransformed with 5 μg of each plasmid. As a negative control, pSR3 was transformed alone. Transformants were incubated at 37°C on Cove's minimal medium (8) supplemented with 10 mM acetamide as the sole nitrogen source and 20 mM CsCl to inhibit background growth. After two rounds of selection, transformants were transferred to potato dextrose agar and incubated at room

* Corresponding author. Mailing address: USDA Forest Products Laboratory, One Gifford Pinchot Dr., Madison, WI 53705. Phone: (608) 231-9464. Fax: (608) 231-9488. Electronic mail address: pstewart@students.wisc.edu.

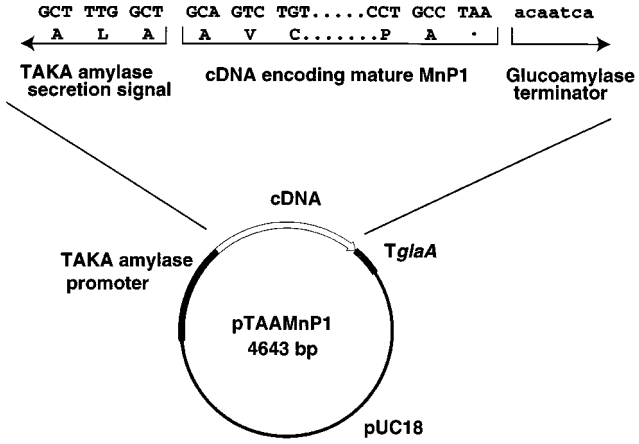


FIG. 1. Expression vector pTAAMnP1. The mature cDNA of *mnp1* was fused in frame to regulatory sequences by the PCR overlap extension technique (15). A 680-bp fragment of the Taka amylase promoter and secretion signal was PCR amplified from *A. oryzae* genomic DNA. PCR amplification of the 3′ untranslated sequence (199 bp) of the glucoamylase gene from *A. awamori* provided the terminator. Primers used to create the fusions were based on the sequences shown above and 5′-CCCGAATCGATAGAACTA-3′ and 5′-CTGTCTGGTCTTCTACAC-3′ for the Taka amylase upstream primer and glucoamylase downstream primer, respectively.

temperature. Transformation was confirmed by PCR amplification of the glucoamylase terminator from genomic DNA.

**Genomic DNA isolation for PCR.** Mycelial pellets were snap frozen in liquid nitrogen, ground in a mortar and pestle, and repeatedly extracted with phenol-chloroform. The aqueous phase was ethanol precipitated and resuspended in Tris-EDTA.

**Culture conditions.** ASP03 medium (2) was inoculated with $10^7$ spores per 500 ml and incubated at 34°C and 300 rpm for 24 h. Hemin was then added to a final concentration of 500 mg/liter, and the cultures were incubated for an additional 24 h. The cultures were harvested, and the mycelia were collected by filtration through Miracloth.

**Screening for rMnP production.** Transformants were screened by Western blot (immunoblot) analysis with polyclonal antibodies raised against *P. chrysosporium* MnPs. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with the Phast system (Pharmacia, Piscataway, N.J.) and 10 to 15% gradient gels (Phastgels; Pharmacia). Molecular weight standards were obtained from Bio-Rad Laboratories, Hercules, Calif.

**Enzyme assays.** MnP activity was measured by monitoring the oxidation of 2,6-dimethoxyphenol (Aldrich, Milwaukee, Wis.) at 469 nm (34).

**Enzyme purification.** Three liters of crude supernatant was concentrated by ultrafiltration (Minitan concentrator [Millipore, Bedford, Mass.]; 10-kDa cutoff membrane) followed by further concentration with a YM10 filter (Amicon, Beverly, Mass.) to a final volume of ~50 ml. Concentrated supernatant was dialyzed against 20 mM sodium acetate (pH 6.0) and applied to a DEAE-Biogel A (Bio-Rad) column (1.5 by 20 cm) that had been similarly equilibrated. Protein was eluted with a 50-ml wash of 0.3 M NaCl in sodium acetate (pH 6.0). After dialysis against 50 mM sodium succinate (pH 4.5), the enzyme was applied to a Cibacron Blue 3GA agarose (Sigma, St. Louis, Mo.) column equilibrated in the same buffer. The enzyme was eluted with a linear gradient of 0 to 0.4 M NaCl in column buffer (Fig. 2). Peak fractions containing MnP activity and with the greatest $A_{407}/A_{280}$ ratios were pooled, dialyzed against 10 mM sodium acetate (pH 6.0), and concentrated.

**Steady-state kinetics.** The determinations of $k_{cat}$, $K_m(H_2O_2)$, and $K_m(Mn^{2+}$-oxalate) were done with 0.5 mM sodium oxalate–10 mM sodium succinate (pH 4.5) at 29°C. The oxidation of $Mn^{2+}$ to $Mn^{3+}$ was monitored as the $A_{270}$ increase. An extinction coefficient of 5.5 $mM^{-1}$ $cm^{-1}$ was used for the $Mn^{3+}$-oxalate complex (21). $K_m(H_2O_2)$ was determined at 0.2 mM $MnSO_4$. $K_m(Mn^{2+}$-oxalate) was determined at 0.05 mM $H_2O_2$. The $k_{cat}$ was extrapolated from the $K_m(H_2O_2)$ curve. Curves were fit to the Michaelis-Menten equation by nonlinear regression. $K_m(Mn^{2+}$-malonate) was determined with 40 mM sodium malonate (pH 4.5)–0.05 mM $H_2O_2$, following $Mn^{3+}$ production at 270 nm, with an extinction coefficient of 8.5 $mM^{-1}$ $cm^{-1}$ for $Mn^{3+}$-malonate.

**Pre-steady-state kinetics.** Transient-state kinetics were performed with a three-syringe stopped-flow spectrophotometer (KinTek Instruments, State College, Pa.). The apparatus is described by Kuan et al. (21). All studies were done at 29°C. Each observed rate plotted was the average of three shots.

The rate of compound I formation, the rate of reduction of compound I to compound II, and the rate of reduction of compound II to ferric enzyme were all determined as described previously (21).

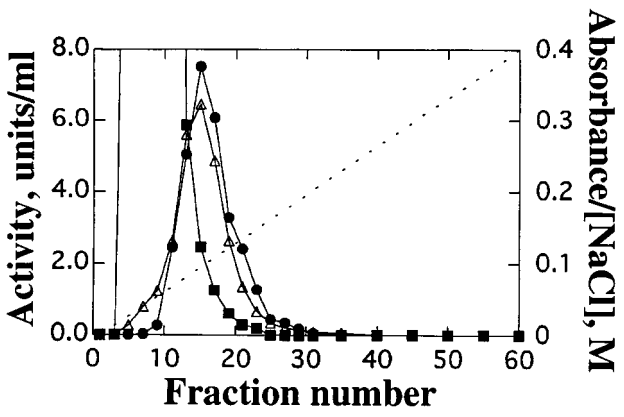


FIG. 2. Elution profile of rMnP on Cibacron Blue-agarose. The column was equilibrated in 50 mM sodium succinate (pH 4.5), and MnP activity (●) was eluted with a linear 0 to 0.4 M NaCl gradient (···) in column buffer. Other symbols: ■, $A_{280}$; △, $A_{407}$.

## RESULTS

Efficient expression of *P. chrysosporium mnp1* was obtained in *A. oryzae* transformants. Expression vector pTAAMnP1 contained the mature *mnp1* cDNA fused to the endogenous Taka amylase promoter and to the glucoamylase terminator from *A. awamori* (Fig. 1). Plasmid pSR3 confers the ability to utilize acetamide as a sole nitrogen source and was cotransformed. Fifteen putative transformants were isolated on the basis of their ability to grow on acetamide plates. Six cotransformants were confirmed by PCR amplification to possess the *A. awamori* glucoamylase terminator. Wild-type *A. oryzae* and transformants containing pSR3 only were used as negative controls. Five of the six confirmed transformants were shown by Western blot analysis to produce a protein band at the correct molecular weight (data not shown). One transformant, designated MnP-7, appeared to produce slightly more protein and was chosen for further analyses (Fig. 3).

When grown in ASP03 medium and supplemented with exogenous heme, MnP-7 produced ~0.33 μmol of activity per min per ml from culture supernatants. In the absence of Mn,

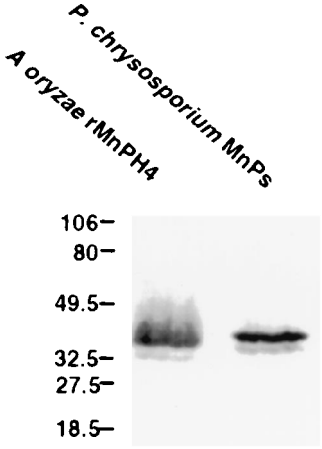


FIG. 3. Western analysis of rMnP. Purified rMnP from *A. oryzae* was compared with the extracellular fluid of *P. chrysosporium* shake cultures by Western blotting. Sizes (in kilodaltons) and positions of molecular size markers are indicated on the left.

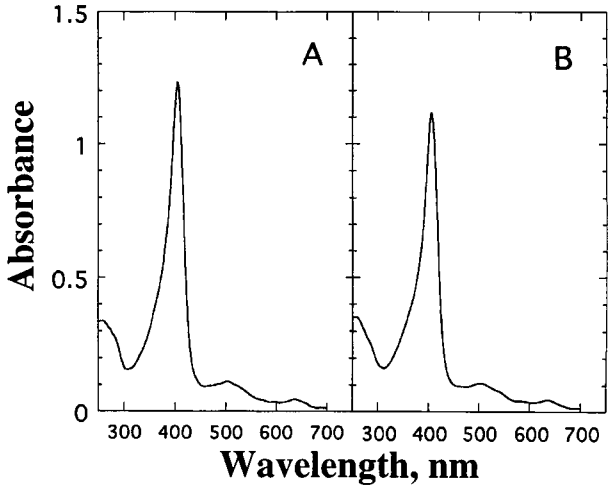


FIG. 4. Comparison of the absorption spectra of rMnP (A) with that of native H4 (B).

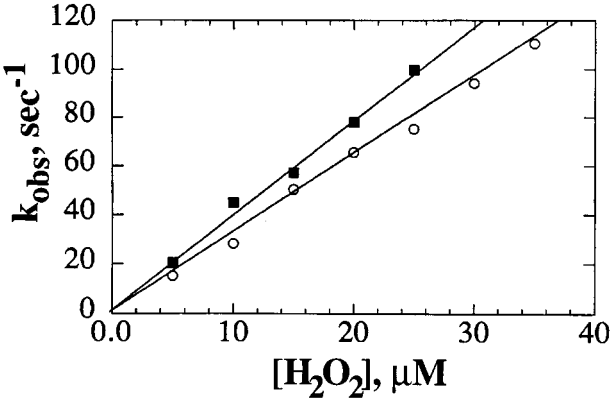


FIG. 5. Compound I formation in native and recombinant MnP at pH 4.5. Native H4 (○) or rMnP (■) was incubated with various concentrations of $H_2O_2$ in 20 mM Na tartrate (pH 4.5). Compound I formation was monitored as the decrease in $A_{397}$. Each datum point consisted of three shots in the stop-flow spectrophotometer.

no significant activity was detected. Control transformants produced no activity.

Recombinant MnP bound to both DEAE-BioGel A and to Cibacron Blue-agarose, as does the native enzyme from *P. chrysosporium*, and could be purified to homogeneity by just these two chromatography steps. Figure 2 shows the elution profile of the recombinant enzyme on the Cibacron Blue-agarose column.

A spectral trace of the purified recombinant enzyme is shown in Fig. 4A. The recombinant enzyme is spectrally identical to the native enzyme (Fig. 4B) and has an $R_z$ value ($A_{407}/A_{280}$ ratio) of 4.2. This is comparable to that of purified native enzyme.

The steady-state kinetic parameters of purified rMnP are shown in Table 1. The steady-state kinetic parameters of the recombinant enzyme are largely similar to those of purified native isozyme H4 from *P. chrysosporium*, except that the $k_{cat}$ of the recombinant enzyme is 70% of that of the native enzyme. The Michaelis constants for $H_2O_2$, $Mn^{2+}$-oxalate, and $Mn^{2+}$-malonate are all equivalent to those of the wild-type enzyme. The recombinant enzyme is expressed from an allelic variant of H4. The variation in $k_{cat}$s could be a result of sequence variations in the primary structure of the recombinant enzyme or a consequence of the heterologous expression system.

The pre-steady-state kinetic parameters of rMnP were determined and compared with those of native isozyme H4 from *P. chrysosporium*. The second-order rate constant for the formation of compound I was calculated to be $3.9 \times 10^6$ $M^{-1}$ $s^{-1}$ for rMnP and $3.2 \times 10^6$ $M^{-1}$ $s^{-1}$ for native H4 (Fig. 5).

The rate at which compound I reacts with $Mn^{2+}$ to form compound II is too high to be measured at the enzyme's optimal pH of 4.5 (21). At the nonoptimal pH 2.5, the second-order rate constant for the reaction of compound I with unchelated $Mn^{2+}$ was calculated to be $5 \times 10^4$ $M^{-1}$ $s^{-1}$ for native H4 (Fig. 6). For rMnP, the rate constant for this reaction at pH 2.5 is $1.8 \times 10^4$ $M^{-1}$ $s^{-1}$ (Fig. 6).

The reaction of compound II of MnP with chelated $Mn^{2+}$ involves at least two steps, the rapid equilibrium binding of chelated $Mn^{2+}$ to the enzyme and the oxidation of $Mn^{2+}$ to $Mn^{3+}$ (21). For native isozyme H4, the $K_d$ of $Mn^{2+}$-oxalate is $13 \pm 2$ μM, and the oxidation of $Mn^{2+}$ has a first-order rate constant of $223 \pm 6$ $s^{-1}$ (Fig. 7). Under identical conditions, compound II of rMnP has a $K_d$ of $8 \pm 3$ μM for the $Mn^{2+}$-oxalate complex and a first-order rate constant of $146 \pm 17$ $s^{-1}$ (Fig. 7).

## DISCUSSION

White rot fungi are the only known organisms capable of degrading lignin efficiently. MnP is the only lignin-depolymerizing enzyme which is ubiquitous among these organisms (14, 23). MnPs can depolymerize phenolic lignins and oxidize nonphenolic lignin via a lipid peroxidation pathway (4). MnP isozymes display significant kinetic differences and may have preferred substrates during ligninolysis (26). Efforts to characterize individual MnPs, however, are hindered by the large number of closely related isozymes and by relatively low yields.

Attempts to address these problems by heterologous expression of MnPs have had limited success (28). The most promising of these, the baculovirus system, is relatively costly and yields are low. The homologous system, in which an MnP isozyme is produced during primary metabolic growth of *P. chrysosporium* transformants, has the potential to be contaminated with other MnP and LiP isozymes.

In contrast, *Aspergillus* species have proven to be excellent hosts for the expression of heterologous proteins. Foreign DNA is stably integrated into the genome and, with appropriate signal sequences, the protein is correctly processed and

TABLE 1. Steady-state kinetic parameters of rMnP and native MnP (nMnP) isozyme H4 from *P. chrysosporium*[a]

| MnP | $k_{cat}$ ($s^{-1}$) | $K_m$($H_2O_2$) (μm) | $K_m$($Mn^{2+}$-oxalate) (μm) | $K_m$($Mn^{2+}$-malonate) (μm) |
|---|---|---|---|---|
| rMnP | 132 ± 15 | 38 ± 8 | 15 ± 5 | 17 ± 3 |
| nMnP | 191 ± 27 | 38 ± 9 | 13 ± 2 | 18 ± 2 |

[a] All assays were done at 29°C, pH 4.5, under the conditions described in Materials and Methods. Values are means ± standard deviations.

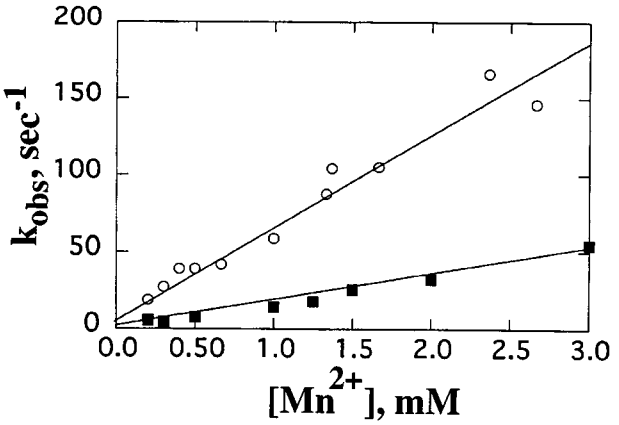


FIG. 6. Reduction of compound I of native and recombinant MnP by $Mn^{2+}$ at pH 2.5. One equivalent of $H_2O_2$ was mixed with one equivalent of either native H4 (○) or rMnP (■) to generate compound I. The solution was aged for 1 s and subsequently mixed with $Mn^{2+}$ in 20 mM tartrate (pH 2.5). Compound II formation was monitored as the increase in $A_{417}$. Each datum point consisted of three shots. Data for native H4 are from Kuan et al. (21).

secreted (6). As a recent example, another component of the lignin-degrading system of *P. chrysosporium*, glyoxal oxidase, has been efficiently expressed in *Aspergillus nidulans* (18). *A. oryzae* was reported to produce 3 g of an aspartic proteinase per liter from *Rhizomucor miehei* (7) and also to express and secrete a fully active heme-binding peroxidase from *C. cinereus* (2). Both genes were linked to the promoter and secretion signal of the highly expressed Taka amylase gene. The transcriptional terminator was provided by the 3′ untranslated region of the *A. awamori* glucoamylase gene.

By use of a similar construct, an allelic variant of MnP isozyme H4 from *P. chrysosporium* was successfully expressed in *A. oryzae*. The recombinant enzyme is secreted and does not appear to be overglycosylated as determined by its relative mobility on SDS-PAGE (Fig. 3). Without attempts to optimize yields, *A. oryzae* secreted ~5 mg of rMnP per liter, producing ~0.33 μmol/min/ml. Yields are equivalent to *P. chrysosporium* shake cultures which reflect the combined activity of multiple MnP isozymes (26). The rMnP can be purified to homogeneity in two steps (DEAE-BioGel A and Cibacron Blue-agarose) and is spectrally identical to the native H4.

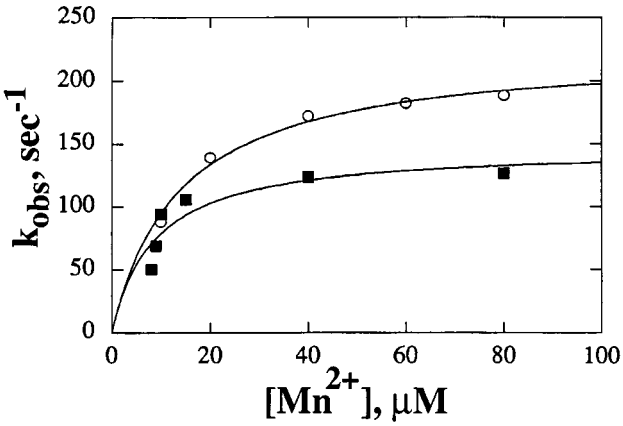


FIG. 7. Reduction of compound II of native and recombinant MnP by $Mn^{2+}$-oxalate complex at pH 4.5. One equivalent of $H_2O_2$ was mixed with one equivalent of either native H4 (○) or rMnP (■) to generate compound I. The solution was aged for 1 s and subsequently mixed with $Mn^{2+}$ in 0.5 mM oxalate–10 mM Na succinate (pH 4.5). Compound I reduction was too fast to be monitored. Compound II reduction was monitored as the decrease in $A_{424}$. Each datum point consisted of three shots. Data for native H4 are from Kuan et al. (21).

The expression of rMnP required exogenous hemin at 500 mg/liter. Lowered concentrations of hemin resulted in decreased yields (data not shown). Expression of recombinant *Coprinus* peroxidase also required the addition of high hemin levels (2).

The recombinant enzyme has a $k_{cat}$ that is 70% of that of native H4. Lower activity may be attributable to the allelic variant encoding the recombinant enzyme. Nucleotide sequence comparison of H4 (27) with its allelic variant indicates at least one amino acid difference, Ser-105→Asn-105 (data not shown). This sequence variation could conceivably alter enzyme activity. Other examples of amino acid sequence differences between *P. chrysosporium* alleles include the genes encoding LiP isozyme H8 (accession numbers M27884 and Y00262) and glyoxal oxidase (accession numbers L47286 and L47287).

The affinity of rMnP for its substrates, as indicated by its $K_m$s for $Mn^{2+}$-oxalate, $Mn^{2+}$-malonate, and $H_2O_2$, are all identical to those for the native H4, as is the $K_d$ of compound II for $Mn^{2+}$-oxalate. The reactivity of rMnP toward $H_2O_2$, as measured by the rate of compound I formation, is the same as that of native H4, within experimental error. Only the reactivity of rMnP towards $Mn^{2+}$ appears to be significantly different. Compound I reacts with $Mn^{2+}$ in rMnP at a rate that is 35% of that of the native H4. The rate at which compound II reacts with chelated $Mn^{2+}$ is thought to be the rate-limiting step in the MnP catalytic cycle (21). In rMnP, this reaction occurs at a rate that is 65% of that of the native H4, which is consistent with the rMnP having an overall steady-state rate that is 70% of that of the native H4.

The successful expression of rMnP in *A. oryzae* provides large quantities of pure, active enzyme suitable for mechanistic studies. Structure-function analyses of rMnP as well as expression of recombinant LiP in *A. oryzae* are currently being investigated.

## ACKNOWLEDGMENTS

Work was supported by U.S. Department of Energy grants DE-FG02-ER13712 and DE-FG02-87ER13690 to D. Cullen and M. Tien, respectively.

We thank Jill Gaskell, Amber Vanden Wymelenberg, and Joel Cherry for excellent technical assistance and advice.

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