Homologous Expression of Recombinant Manganese Peroxidase in *Phanerochaete chrysosporium*

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The promoter region of the glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) was used to drive expression of *mnpl*, the gene encoding Mn peroxidase isozyme I, in primary metabolic cultures of *Phanerochaete chrysosporium*. A 1,100-bp fragment of the *P. chrysosporium gpd* promoter region was fused upstream of the *mnpl* gene to construct plasmid pAGM1, which contained the *Schizosaccharomyces pombe ade5* gene as a selectable marker. pAGM1 was used to transform a *P. chrysosporium ade1* auxotroph to prototrophy. Ade* transformants were screened for peroxidase activity on a solid medium containing high carbon and high nitrogen (2% glucose and 24 mM NH₄ tartrate) and o-anisidine as the peroxidase substrate. Several transformants that expressed high peroxidase activities were purified and analyzed further in liquid cultures. Recombinant Mn peroxidase (rMnP) was expressed and secreted by transformant cultures on day 2 under primary metabolic growth conditions (high carbon and high nitrogen), whereas endogenous wild-type *mnp* genes were not expressed under these conditions. Expression of rMnP was not influenced by the level of Mn in the culture medium, as previously observed for the wild-type Mn peroxidase (wtMnP). The amount of active rMnP expressed and secreted in this system was comparable to the amount of enzyme expressed by the wild-type strain under ligninolytic conditions. rMnP was purified to homogeneity by using DEAE-Sepharose chromatography. Blue Agarose chromatography, and Mono Q column chromatography. The Mn and absorption spectrum of rMnP were essentially identical to the Mn and absorption spectrum of wtMnP, indicating that heme insertion, folding, and secretion were normal. The steady-state kinetic values for the oxidation of Mn(II) and 2,6-dimethoxyphenol by rMnP and wtMnP also were very similar. This system is suitable for generating site-directed mutants of Mn peroxidase.

The white rot basidiomycete *Phanerochaete chrysosporium* has been the focus of numerous studies on the degradation of lignin (7, 14, 24) and aromatic pollutants (6, 16, 23). Two families of extracellular peroxidases, manganese peroxidase (MnP) and lignin peroxidase (LiP), along with an extracellular H₂O₂-generating system, are thought to be the major components of this organism's extracellular lignin-degrading system (13, 14, 24). Significantly, both LiP and MnP depolymerize synthetic lignins in vitro (17, 45). Furthermore, MnP has been detected in cultures of essentially all white rot fungi which efficiently degrade lignin (19, 32, 35). MnP has been purified and extensively characterized (10, 13, 14, 26, 27, 33, 44). This enzyme oxidizes Mn(II) to Mn(III). Mn(III), complexed with an organic acid chelator such as oxalate, which is secreted by *P. chrysosporium*, oxidizes the substrates either directly, in the case of lignin and phenolic lignin model compounds (43, 45), or possibly through the mediation of organic radicals (31, 47). MnP occurs as a series of isozymes that are encoded by a family of genes, and the sequences of cDNA and genomic clones that encode two MnP isozymes have been determined previously (12, 13, 29). The results of these studies, as well as spectroscopic and kinetic studies (4, 26, 30), showed that the heme environment of MnP is similar to that of other plant and fungal peroxidases (9). The crystal structure of LiP has been described (36, 37), and studies to determine the crystal structure of MnP are in progress (42).

Efficient expression systems for recombinant MnP (rMnP) and LiP are required for structure-function studies performed with these enzymes. To our knowledge, successful expression of MnP or LiP in heterologous prokaryotic or eukaryotic microbial systems has not been achieved. Although heterologous expression of these proteins has been achieved in the baculovirus system (22, 34), the baculovirus system has several disadvantages, including a relatively high cost compared with microbial systems, a relatively low yield of recombinant protein, and an apparent requirement for exogenous heme for optimal expression. In *P. chrysosporium*, the various isozymes of MnP and LiP are expressed only during the secondary metabolic (idiopathic) stage of growth, which is triggered by depletion of nutrient nitrogen (13, 24). Previously, we demonstrated that *mnp* gene transcription is regulated by both nutrient nitrogen and Mn ion levels (5, 13, 38). In this paper we describe the first homologous expression system for MnP, in which the coding region of the *mnpl* gene was placed under the control of the *P. chrysosporium* glyceraldehyde-3-phosphate dehydrogenase (*gpd*) primary metabolic promoter.

MATERIALS AND METHODS

Organisms. *P. chrysosporium* wild-type strain OGC101, auxotrophic strain OGC107-1 (Adel), and prototrophic transformants were maintained as described previously (1). *Escherichia coli* XL1-Blue and DH5αF’ were used for subcloning plasmids.

Cloning and sequencing the *P. chrysosporium gpd* gene. Plaque lifts from a *P. chrysosporium* λEMBL3 genomic library (12) were probed with a 1.8-kb Scal fragment from the *Aspergillus nidulans gpdA* gene in plasmid pAN5-22 (39).
FIG. 1. Construction of MnP expression vector. (A) Junction of gpd promoter-mpn1 coding region. gpd 5′ UTR, 5′ untranslated region of the gpd gene; oligo, synthetic oligonucleotide linker which spans nucleotide positions 4 through 38 of the mpn1 coding region. (B) Restriction map of pAGM1 containing the adeS gene and the gpd promoter fused to the mpn1 coding region. The restriction sites in parentheses are not present in pAGM1.

Heterologous hybridizations were carried out at 28°C with washing at 34°C as previously described (3). Fourteen positive clones were analyzed by Southern blotting of restriction digests to select a clone which appeared to include the entire P. chrysosporium gpd gene. A 5.8-kb PstI fragment identified by further Southern analysis was subcloned into BluescriptII SK+ (Stratagene). Using exonuclease III to generate overlapping deletion subclones (20), we sequenced 3.2 kb of the clone by the dyeoxy method (41), using a Sequenase 2.0 kit (U.S. Biochemicals) and α-32P-labeled dATP (NEN-Dupont). A 1.8-kb SvuI fragment containing 1.06 kb of the gpd promoter region was subcloned into the SmaI site of BluescriptII SK+.

Construction of pAGM1. A 1.06-kb XbaI-NspI fragment containing the gpd promoter, including the ATG translation initiation codon at the 3′ end, was used in a three-way ligation with an NspI-NotI 35-bp synthetic linker (Oligos Etc., Inc.) with 5′ and 3′ overhangs on the noncoding strand and BluescriptII SK+ digested with XbaI and NotI. The 35-bp linker was identical to nucleotides 4 through 38 of the mpn1 coding region (Fig. 1A). The XbaI-NotI insert fragment, which contained the gpd promoter and linker, and a 3.6-kb NotI-EcoRI fragment of the mpn1 genomic clone (12), including the entire mpn1 coding region from nucleotide 39, were used in a second three-way ligation with the pOG118 shuttle vector containing the Schizosaccharomyces pombe adeS gene as a selectable marker (11) to give pAGM1 (Fig. 1B).

P. chrysosporium transformations. Protoplasts of P. chrysosporium Ade1 were transformed with 2 μg of EcoRI-linearized pOG118 or pAGM1 as described previously (1, 3). Prototrophic transformants were transferred to minimal medium and purified by isolating single basidiospores as previously described (1–3).

Screening for expression of rMnP isozyme 1 (rMnP1). pAGM1 transformants were inoculated onto petri plates containing 10 ml of high-carbon, high-nitrogen medium (standard basal medium) (25) containing 0.2% typtone, 2% glucose, 24 mM ammonium tartrate, 20 mM sodium 2,2-dimethyl succinate (DMS) buffer (pH 4.8), 1.8% agar, and the peroxidase substrate o-anisidine (2 mM). The plates were incubated at 37°C for 48 h and then were flooded at room temperature with 1 ml of a solution containing 2 mM MnSO4 and 0.5 U of glucose oxidase in 100 mM sodium malonate buffer (pH 4.5).

Production of rMnP1. pAGM1 transformants were grown from conidial inocula at 38°C in 20-ml stationary cultures in 250-ml Erlenmeyer flasks for 2 days. The medium which we used was as described elsewhere (25), except that it was supplemented with 2% glucose, 12 mM ammonium tartrate, and 20 mM DMS (pH 4.5). The mycelial mat from one flask was homogenized for 20 s in a blender and was used to inoculate a 2-liter Erlenmeyer flask containing 1 liter of the medium described above, except that in the large cultures the pH was adjusted to pH 6.5 and sodium succinate was used instead of DMS. Cultures were grown at 38°C on a rotary shaker at 200 rpm for 60 h.

Purification of rMnP1. The filtrate obtained from six 1-liter cultures was concentrated to ~200 ml and was dialyzed against 20 mM sodium acetate (pH 6.0) at 4°C by using a hollow fiber filter system (10,000-molecular-weight cutoff, Amicon).

DEAE-Sepharose chromatography. The concentrate was applied to a DEAE-Sepharose CL-6B column (2.5 by 20 cm) equilibrated with 50 mM sodium acetate buffer (pH 6.0). The protein was eluted at 4°C with a linear sodium succinate gradient (total volume, 400 ml) in which the pH decreased from 6.0 to 4.0 in 30 mM sodium succinate. The fractions containing MnP activity were concentrated by membrane ultrafiltration.

Blue Agarose chromatography. The DEAE-Sepharose fraction was applied to a Cibacron Blue 3GA Agarose column (1.0 by 25 cm) equilibrated with 10 mM sodium succinate buffer (pH 4.5) at 4°C. The MnP was eluted at 4°C with a linear NaCl gradient (0 to 0.5 M). Fractions containing MnP activity were concentrated and desalted by membrane ultrafiltration.

Mono Q chromatography. The Blue Agarose fraction was applied to a Mono Q HR 5/5 column (Pharmacia) equilibrated with 10 mM sodium acetate (pH 6.0) in a fast protein liquid chromatography (FPLC) system. The protein was eluted at room temperature with a linear two-phase gradient (0.01 to 0.1 M sodium acetate [pH 6.0], followed by 0.1 to 0.3 M sodium acetate). Active MnP fractions were pooled and desalted with a Centricron microconcentrator (Amicon).

SDS-PAGE. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed by using a 12% Tris–glycine gel system (28) and a MiniProtean II apparatus (Bio-Rad); the gels were stained with Coomassie blue (15).

Spectroscopic procedures and enzyme assays. Enzyme absorption spectra were determined with a Shimadzu model UV-260 spectrophotometer at room temperature by using a cuvette with a 1-cm light path. MnP activity was determined by monitoring the formation of Mn(III) malonate at 270 nm as described previously (10, 46). The reaction mixtures (1 ml) contained MnP (0.5 μg/ml), MnSO4 (0.025 to 0.5 mM), and H2O2 (0.02 to 0.1 mM) in 50 mM sodium malonate (pH 4.5). Oxidation of 2,6-dimethoxyphenol (2,6-DMP) by MnP was monitored by measuring quinone dimer formation at 469 nm as described previously (46).

Chemicals. Glucose oxidase, DEAE-Sepharose CL-6B, and Cibacron Blue 3GA Agarose were obtained from Sigma.
Molecular biology reagents were obtained from New England Biolabs and U.S. Biochemicals. o-Anisidine (2-methoxyaniline), H₂O₂, and 2,6-DMP were obtained from Aldrich. The concentration of the H₂O₂ stock solution was determined as described previously (8). 2,6-DMP was purified by recrystallization before it was used. All other chemicals were reagent grade chemicals.

RESULTS

Expression of rMnP1. The 82 Ade⁺ transformants obtained with pAGM1 were screened on plates by the o-anisidine assay. Although the levels of expression in individual transformants varied considerably, all but one of the pAGM1 transformants were positive for MnP activity under primary metabolic conditions, whereas the Ade1 auxotroph and three different Ade⁺ transformants obtained with pOG118 were negative under these conditions. Three of the pAGM1 transformants that expressed the highest levels of rMnP activity in this initial screening assay were purified by isolating single basidiospores (2) and were analyzed further in liquid cultures.

The time courses for the appearance of MnP activity in nitrogen-sufficient stationary cultures of purified pAGM1 transformants, wild-type strain GOCl01, the Ade1 auxotroph, and Ade⁺ pOG118 transformants are shown in Fig. 2. Extracellular MnP activity was detected only in the cultures of strains transformed with pAGM1. The MnP activity reached a maximum level after 60 h and decreased thereafter. No new activity peak appeared on day 5 or 6 in any of the cultures; this is the period during which endogenous MnP is expressed in low-nitrogen cultures (5, 11). These observations indicated that the activity observed in the transformants was due to rMnP. Furthermore, when transformant 15 was grown in high-carbon-, high-nitrogen-containing medium in the absence of exogenous Mn, the extracellular MnP activity levels were similar to those found in cultures containing Mn (data not shown). The maximum MnP activity for Mn(II) oxidation observed in transformant 15 cultures was ~2 μmol·min⁻¹·ml⁻¹. Although this value is approximately 30% of the level of MnP activity expressed under nitrogen-limited conditions by wild-type strain OGC101, it is approximately twofold higher than the levels of MnP activity expressed in nitrogen-limited cultures of either the Ade1 auxotroph or pOG118 transformants (data not shown). The level of total intracellular MnP activity in transformant 15 cultures was ~0.04% of the level of extracellular activity.

The influences of several culture parameters on the expression of rMnP were examined. The effect of pH on rMnP expression was examined in high-nitrogen-containing stationary cultures with DMS as the buffer. As shown in Fig. 3, an initial culture medium pH of 6.5 resulted in a twofold increase in the maximum MnP activity compared with the activity at an initial pH of 4.5. Increasing the pH to more than 6.5 likewise resulted in a decrease in the amount of rMnP expressed. The maximum level of rMnP activity observed with either phosphate or acetate buffer was approximately two-thirds of the maximum level of activity observed with DMS buffer. Furthermore, rMnP protein was efficiently expressed without addition of exogenous hemin to the culture medium. Addition of Tween 80 (0.1%, vol/vol) to shaking cultures increased extracellular MnP activity approximately 1.5-fold, whereas varying the concentrations of nutrients such as glucose (0.1 to 2%) and
ion-exchange chromatography on DEAE-Sepharose, Cibachron Blue Agarose, and Mono Q columns. One major peak of rMnP activity eluted from both the DEAE-Sepharose (Fig. 4A) and the Blue Agarose columns. The RZ value (A_{405}/A_{280}) of the pooled Blue Agarose fractions was ~4.0. In order to purify the enzyme further, the Blue Agarose fraction was applied to a Mono Q column. A typical elution profile obtained for rMnP with a Mono Q column is shown in Fig. 4B. Several peaks were detected at 405 nm, including a major peak that had the same retention time as wild-type MnP (wtMnP). The MnP activity profile correlated with the A_{405} profile (data not shown). The RZ value, as well as the SDS-PAGE results, suggested that the level of purity of the recombinant protein was ~95%; the RZ value obtained for homogeneous wild-type enzyme was 6.0 (10, 44). The major Mono Q peak fractions were pooled, concentrated, and subjected to SDS-PAGE (Fig. 5). We observed one intense band, similar to the wild-type enzyme band, at an M_r of ~46,000. The specific activity of the purified rMnP1 for oxidation of Mn(II) was 341.3 μmol/min/mg (Table 1). The specific activity of the enzyme in crude extracellular filtrates was 16.1 μmol/min/mg. The enzyme was purified 21.2-fold in this study.

The absorption spectrum of purified rMnP1 (Fig. 6) had a Soret maximum at 406 nm and visible bands at 502 and 632 nm. The shapes and intensities of these spectral bands were identical to the shapes and intensities of wtMnP isozyme 1 (wtMnP1) bands (Fig. 6) (10, 44), suggesting that rMnP1 and wtMnP1 have similar heme environments. Like the wild-type enzyme, rMnP oxidized Mn(II) to Mn(III), and the oxidation of 2,6-DMP by rMnP was strictly dependent on both Mn(II) and H_2O_2 (Table 1). Furthermore, the specific activity of purified rMnP was similar to the specific activity of wtMnP1 when either Mn(II) or 2,6-DMP was the substrate (Table 1).

Under steady-state conditions, linear Lineweaver-Burk plots were obtained for 1/v versus 1/[Mn(II)] and 1/v versus 1/[H_2O_2] over a range of substrate concentrations (data not shown). Most importantly, the K_m, V_max, and k_cat (V_max/[E])

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**TABLE 1. Reactions of rMnP1 and wtMnP1**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Mn(II)</th>
<th>With Mn(II) and H_2O_2</th>
<th>Without Mn(II)</th>
<th>Without H_2O_2</th>
</tr>
</thead>
<tbody>
<tr>
<td>rMnP1</td>
<td>341.3</td>
<td>158.3</td>
<td>N*</td>
<td>N</td>
</tr>
<tr>
<td>wtMnP1</td>
<td>332.2</td>
<td>161.1</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

* MnP activity was determined in 50 mM sodium malonate (pH 4.5) as described in the text. Mn(III) malonate formation was monitored at 270 nm as described in the text and previously (10, 46). 2,6-DMP oxidation was monitored at 469 nm for the formation of a quinone dimer (46).

* N, negligible.
has been published previously (36, 37) and that of MnP isoenzyme 1 is currently under investigation (42), heterologous expression of LiP and MnP has not been achieved in a microbial host. Although these enzymes have been expressed in the baculovirus system (22, 34), this system is not well suited for cost-effective production of the large quantities of enzyme needed for structural analysis. Previously, we developed a DNA transformation system for *P. chrysosporium* which was based on complementation of auxotrophic mutants by heterologous or homologous biosynthetic genes (1, 3). We recently utilized this system to study the expression of a reporter gene under the control of the *mnp* gene promoter (11). In this paper we describe homologous expression of *mnp*1 under the control of the *gpd* promoter. This system allows production of rMnP under primary metabolic conditions when the endogenous *mnp* genes are not expressed, facilitating purification of the recombinant protein.

The *P. chrysosporium* *gpd* gene was cloned from a λEMBL3 library of wild-type strain OGC101. The nucleotide sequence of this gene, which will be published elsewhere, is similar to the recently published *gpd* sequence of wild-type strain ME-446 (18). The *gpdA* promoter from *Aspergillus nidulans* has been used for high-level constitutive expression of intracellular and extracellular homologous and heterologous proteins in that organism (40). In this study we found that the *P. chrysosporium* *gpd* promoter region can direct transcription of the *mnp1* coding region in the presence of high carbon and nitrogen when the endogenous *mnp* genes are not expressed.

Almost all of the Ade+ transformants obtained with pAGM1 produced MnP under high-carbon, high-nitrogen conditions, as determined by o-anisidine screening and enzyme assays. None of the pOG18 transformants nor the wild-type or Ade auxotrophic strains produced MnP under these conditions (Fig. 2). This indicates that the pAGM1 transformants expressed MnP1 under control of the constitutive *gpd* promoter. The maximum level of MnP activity was observed in pH 6.5 cultures (Fig. 3), whereas the level of endogenous MnP activity was maximal in pH 4.5 cultures. Furthermore, we have shown that expression of endogenous MnP protein in *P. chrysosporium* requires the presence of Mn ions (5, 11, 13), and the results of *mnp*1 promoter reporter studies have shown that the Mn requirement resides in the *mnp1* promoter region (11). However, pAGM1 transformants produce MnP in the absence of Mn (data not shown). Finally, using PAGM1, we recently constructed mutant *mnp* genes by site-directed mutagenesis. When *P. chrysosporium* is transformed with plasmids containing the mutant genes, only mutant proteins are expressed; no wild-type proteins are observed (27a). All of these results indicate that the rMnP1 in pAGM1 transformants is produced under the control of the constitutive *gpd* promoter. The data also indicate that any additional requirements for production of active MnP, including the insertion of heme and protein secretion, must be available during primary as well as secondary metabolism and that any cis-acting factors that restrict transcription of endogenous *mnp* genes to secondary metabolism must reside in the *mnp* promoter regions.

Although most Ade+ transformants obtained with pAGM1 gave some level of positive reaction in the o-anisidine screening test, indicating that rMnP was expressed, individual transformants varied considerably. Our previous results have indicated that integration of transforming DNA in *P. chrysosporium* is predominately ectopic, with single or multiple plasmid copies integrating at various chromosomal locations (1, 3, 13). The results described in this paper suggest that although the position of pAGM1 integration may affect the level of rMnP expression, there may not be specific chromo-
somal sites that determine expression during primary or secondary metabolism. 

rMnP was readily purified by a combination of anion-exchange chromatography and Blue Agarose column chromatography (Fig. 4A). FPLC with a Mono Q column resulted in separation of several protein peaks (Fig. 4B). These multiple peaks may have arisen because of alternative posttranscriptional modification, such as glycosylation. The major Mono Q column protein peak was examined further. The results of an SDS-PAGE analysis suggested that rMnP has a Mₙ of 46,000, a value which is nearly identical to the Mₙ of wtMnP (Fig. 5). wtMnP and rMnP also had identical UV-visible spectral features (Fig. 6), suggesting that the environment and orientation of the heme in the two enzymes are similar. Significantly, heme insertion appears to be normal, and exogenous heme is not required for expression of this recombinant heme protein. This contrasts with the baculovirus system, in which exogenous heme is apparently required for MnP expression (34). The addition of exogenous heme can lead to adventitious binding of extra heme to protein (unpublished data). Like wtMnP, rMnP is able to oxidize Mn(II) to Mn(III), and the 2,6-DMP oxidation activity of rMnP depends on both Mn(II) and H₂O₂ (Table 1). Furthermore, rMnP exhibits Kₘ, Vₘₐₓ, and kₐₚ values for Mn(II) and H₂O₂ that are very similar to the values obtained for wtMnP (Table 2), suggesting that the substrate binding and catalytic efficiency of wtMnP and rMnP are the same. The level of rMnP activity produced in either stationary or agitated cultures is about 30% of the level of total MnP activity produced by multiple mnp genes in heterokaryotic wild-type strain OGC101 (a derivative of BKM-F-1767) under ligninolytic conditions and about twice the level of activity produced by the Ade₁ parental homokaryon. This suggests that the P. chrysosporium gpd promoter is more efficient than the combined multiple mnp promoters present in strain Ade₁. Alternatively, the higher rMnP yield may reflect increased metabolic activity during primary metabolism compared with idiopathic metabolism. Most importantly, this expression system enables efficient purification of a single rMnP isozyme.

To our knowledge, this is the first report of efficient protein expression in P. chrysosporium. Whereas we previously demonstrated the ability of a secondary metabolic promoter (mnp1) to direct expression of a primary metabolic gene (ara1) (11), in this study we demonstrate that a secondary metabolic gene can be expressed under the control of a primary metabolic promoter. The expression system described above will enable us to generate site-directed MnP mutants for structure-function studies. Similar studies on the homologous expression of recombinant LiP are planned.

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