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Expression of the Lignin Peroxidase H2 Gene from *Phanerochaete chrysosporium* in *Escherichia coli*

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The DNA sequence for the extracellular lignin peroxidase isozyme H2 from *Phanerochaete chrysosporium*, obtained from cDNA clone λML-6, was synthesized by PCR and successfully expressed in *Escherichia coli* under control of the T7 promoter. The portion of the cDNA encoding the signal peptide, not found in the mature native enzyme, was not included. Recombinated lignin peroxidase H2 (rLiPH2) was produced in inclusion bodies in an inactive form. Active enzyme was obtained by refolding with glutathione-mediated oxidation in a medium containing urea, Ca^{2+}, and hemin. The recombinant enzyme had spectral characteristics and kinetic properties identical to that of native enzyme isolated from *P. chrysosporium*. Surprisingly, rLiPH2, like the native enzyme, also exhibited some manganese peroxidase activity.

Key Words: **white-rot fungus; lignin; recombinant lignin peroxidase; manganese peroxidase.**

The white-rot fungus *Phanerochaete chrysosporium* is capable of degrading lignin and a wide variety of organopollutants (1–4). In response to nutrient deprivation, the fungus secretes two types of heme-containing peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP) (5). The LiP isozymes (H1, H2, H6, H7, H8 and H10) can directly oxidize a variety of organic substrates (6,7). On the other hand, MnP isozymes (H3, H4, H5 and H9) catalyze the oxidation of Mn^{2+} to Mn^{3+} which functions as a redox mediator to oxidize a wide variety of organic molecules (8–9). Both LiP and MnP are involved in lignin and xenobiotic degradation by this fungus (4,5,10).

Although the high resolution X-ray crystal structure of LiP has been solved (11), important biophysical questions relating to its ability to oxidize highly electropositive chemicals remain to be answered (12). LiP is the only peroxidase known to date which can directly oxidize highly electropositive xenobiotics and lignin. However, the substrate binding site for veratryl alcohol (a natural substrate for the enzyme) and lignin has not been identified (11,13). The answers to these questions and other structure-function relationships for LiP would be greatly facilitated by the availability of recombinant enzyme variants.

LiP is one of the few peroxidases which has not been successfully recombinantly expressed. Heterologous expression in *E. coli* of genes encoding the mature peptide of LiPH8 from *P. chrysosporium* (14,15) and a similar enzyme from *Tremetes versicolor* (16) has been attempted. Although the protein was expressed at a high level, it was expressed in an inactive form as an inclusion body and attempts to refold the enzyme were essentially unsuccessful (14–16). Heterologous expressions of LiPH8 (17) and H2 (18) in baculovirus expression systems were attempted and low levels of active peroxidases were detected in the growth medium. In addition to the high cost and low enzyme production, enzymes with only partial activity (60% for H8 and 77% for H2) were obtained. These expression systems are not suitable for many studies on structure-function relationships of these enzyme.

Here we report the expression of recombinant LiPH2 (rLiPH2) in *E. coli* and the successful refolding of rLiPH2 from inclusion bodies to yield active enzyme.

MATERIALS AND METHODS

Chemicals. Hydrogen peroxide, hemin, dithiothreitol (DTT), oxidized glutathione (GSSG), ampicillin, chloramphenicol, Sephadex G75-50, and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Sigma Chemical Co (St. Louis, MO). Veratryl alcohol and calcium chloride were purchased from Aldrich Chemical Co (Milwaukee, WI). Oligonucleotides, PR5H2 (AACATATGCCGAACCTCGACAAGCG) and HR3H2 (ATAGAGAATTCTATGTTGGGGGACGGCGGC) for 5′ end and 3′ primers, respectively, were synthesized

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Abbreviations used: LiP, lignin peroxidase; rLiPH2, recombinant lignin peroxidase isozyme H2; MnP, manganese peroxidase; DTT, dithiothreitol; GSSG, oxidized glutathione; IPTG, isopropyl-β-D-thiogalactoside.

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by the Macromolecular Resources of Colorado State University. λML-6 containing LiPH2 cDNA (19) was generously provided by Dr. M. Tien, Department of Molecular and Cell Biology, Pennsylvania State University, University Park, PA.

Generation of the engineered LiPH2 gene by PCR. Amplification of the mature region of the LiPH2 gene was achieved by PCR using *Pfu* DNA polymerase, which has 3′-5′ proof-reading activity (Stratagene, La Jolla, CA). Reaction conditions were as follows: 12 ng of plasmid λML-6 DNA, 125 ng each of oligonucleotides PR5H2 and PR3H2, 250 μM of each dNTP, 10 μl of 10x *Pfu* DNA polymerase buffer, and 2.5 units of cloned *Pfu* DNA polymerase in a final volume of 100 μl. Initial denaturation at 95°C for 4 min was followed by 30 thermal cycles of denaturation (30 seconds at 95°C), annealing (45 seconds at 55°C) and extension (4 min at 72°C) using a programmable GeneAmp System 9600 thermal cycler (Perkin-Elmer, Emeryville, CA). The product was electrophoretically analyzed using 0.8% agarose gel electrophoresis. After purification using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA), the DNA sequence was further confirmed using an ABI Prism 377 DNA Sequencer (PE/Applied Biosystems, Foster City, CA) by the Biotechnology Center Service Laboratory, Utah State University.

Cloning the engineered cDNA gene into the E. coli expression vector pET21a(+). The commercially available ampicillin-resistant *E. coli* expression vector pET21a(+) (Novagen, Madison, WI) contains the IPTG-inducible T7 promoter. NdeI and EcoRI are two available restriction sites in the multiple cloning region. In order to clone the gene of interest into pET21a(+), two primers, PR5H2 and PR3H2, corresponding to two ends of the mature LiPH2 gene, were flanked with NdeI and EcoRI sequences, respectively. The resulting pET21a-LiPH2 construct was transformed into BL21(DE3)pLysS cells (Novagen, Madison, WI), which allowed IPTG-induced expression of the rLiPH2 protein without the leader sequence.

Expression and refolding of recombinant LiPH2. Overnight cultures (25 ml) of *E. coli* BL21(DE3)pLysS containing the plasmid pET21a-LiPH2 were inoculated into 1 L Luria-Bertani (LB) medium containing 75 μg/mL ampicillin and 34 μg/ml chloramphenicol. The cultures were grown at 37°C and 200 rpm to an absorbance of 0.6 at 600 nm. IPTG was added to a final concentration of 1 mM and the cultures were further incubated at 37°C for 4 hours. After 4 hours the bacteria were harvested by centrifuging at 4°C and 3000×g for 5 minutes. The cell pellets from 1 L cultures, unless stated otherwise, were resuspended and washed twice in 50 mL of buffer A (2 mM EDTA and 2 mM DTT in 20 mM Tris-HCl, pH 8.0). The washed cell pellets were resuspended in 50 mL buffer A and frozen overnight at −70°C. After thawing, 10 μM PMSF (in isopropanol), 2 mg/ml lysozyme, 1% Triton X-100, 0.1 mg/mL DNase, and 0.1 mg/ml RNase (final concentrations) were each added to the resuspended solution. The cells were incubated on ice for 30 minutes and then subjected to 3 cycles of freezing/thawing in liquid nitrogen and a 42°C water bath. The lysed cells were centrifuged at 16000×g for 30 minutes at 4°C. The cell membranes were removed by washing the pellets three times with buffer A containing 1% (v/v) Triton X-100. The purified pellets containing the rLiPH2 inclusion bodies were resuspended in 100 mL of buffer A containing 8 M urea and 2 mM DTT and incubated at 4°C for 4 hours. The denatured-reduced inclusion bodies were diluted with refolding solution under anaerobic conditions. The final refolding solution contained 10 mM $CaCl_2$, 0.7 mM GSSG, and 8 μM hemin in 20 mM Tris-HCl buffer (pH 8.0). Refolding took place in the dark and at room temperature for 24 hours.

Purification. The refolded mixture was extensively dialyzed against 10 mM sodium acetate buffer, pH 6.0, centrifuged at 16000×g for 30 min and the supernant concentrated in an Amicon 8400 concentrator using a YM-10 membrane (Amicon, Beverly, MA). The concentrated samples were applied to a DEAE-cellulose ion-exchange column (2×20 cm) and eluted with a 10 to 400 mM sodium chloride gradient. The appropriate fractions were pooled, concentrated, and applied to a two-step size-exclusion column consisting of Biogel P-30 (1×10 cm) (Bio-Rad, Richmond, CA) and Sephadex G-75 (1×30 cm) with the Biogel P-30 on the top of the column. Fractions with highest activity and absorbance at 408 nm were pooled.

Characterization. The rLiPH2 was analyzed on a denaturing 12% Ready Gel (Bio-Rad, Richmond, CA) using a Bio-Rad mini-protein II electrophoresis cell to determine identity and purity. The gels were stained with Coomassie brilliant blue. Absorption spectra were recorded on a UV-2101PC spectrophotometer (Shimadzu, Columbia, MD) at room temperature with a slit width of 0.5 nm and a scan speed of 18 nm/s. Lignin peroxidase activity was measured as the oxidation of veratryl alcohol (VA) to veratryl aldehyde with an increased absorbance at 310 nm (7). Reaction mixtures contained 50 mM sodium tartrate, pH 2.5, 2.0 mM VA, 40 nM rLiPH2, and 500 μM H_2O_2. Reactions were initiated by addition of H_2O_2. An extinction coefficient of 9300 M^{-1} cm^{-1} for veratryl aldehyde (7) was used for calculation of enzyme turnover number. Manganese peroxidase activity was measured as the oxidation of Mn^{2+} to Mn^{3+} by following the formation of Mn^{3+}-malonate complex at 270 nm (20). Reaction mixtures contained 200 mM sodium phosphate, pH 3.0, 1.2 mM Mn^{2+}, 50 mM sodium malonate, 40 nM rLiPH2, and 100 μM H_2O_2. An extinction coefficient of 8500 $M^{-1}cm^{-1}$ for Mn^{3+}-malonate complex was used for calculation of enzyme turnover number (20).

RESULTS AND DISCUSSION

Construction of pET21a (+) with the cDNA gene encoding LiPH2. rLiPH2 was expressed without the 21 amino acid leader sequence. To remove the leader sequence, oligonucleotide-directed, site-specific deletion was performed by PCR. The mature LiPH2 DNA was subcloned into the pET21a (+) expression vector. The oligonucleotide PR5H2 corresponding to the first 6 amino acids of mature LiPH2 was synthesized with a NdeI endonuclease restriction site (CATATG), of which ATG is the initiation codon. The resulting construct, pET21a-LiPH2, shown in Figure 1, was transformed into BL21(DE3)pLysS cells according to the commercial transformation protocol (Novagen, WI).

Expression of rLiPH2 in E. coli. The pET expression system was developed for the cloning and expression of a wide variety of genes from both prokaryotic and eukaryotic sources (21). In this system, expression is controlled by bacteriophage T7 transcription and translation signals, and is induced by a source of T7 RNA polymerase in the host cell, which is regulated by IPTG. To express rLiPH2, the transformant was grown in LB medium and IPTG (1 mM) was added for induction of protein expression when the absorbance of culture reached 0.6 at 600 nm. After 4 hours induction, the cultures were harvested, centrifuged and lysed by freezing-thawing. The cell supernant and pellets were separated and subjected to SDS-PAGE analysis. Expression of rLiPH2 was confirmed and most of rLiPH2 was found in the cell pellets (Figure 2), indicating that rLiPH2 was produced as inclusion bodies. The migration of rLiPH2 in SDS-PAGE was similar to that of native LiPH2 (Figure 2), indicating that the molecular weight of rLiPH2, was about to be 38 kDa, which corre-

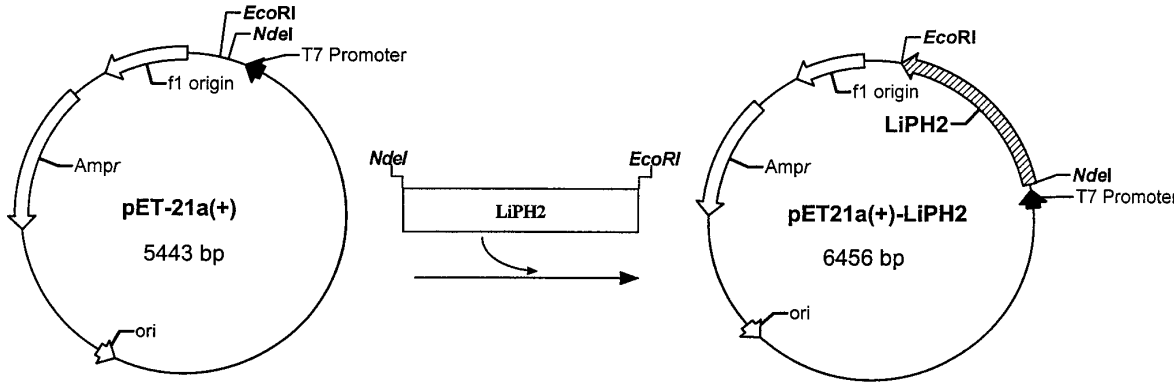

FIG. 1. Construction of an expression vector containing the coding sequence for LiPH2. The cloning sites of pET21a (+) were NdeI and EcoRI. The LiPH2 gene with NdeI and EcoRI at the 5′ and 3′ were obtained following amplification using the designed primers as described in Materials and Methods. Following the endonuclease restriction enzyme reaction, the cDNA gene was ligated into expression vector pET21a(+). The first codon of the mature LiP holoenzyme is adjacent to the start codon of the vector NdeI restriction site.

sponded to that predicted from the deduced amino acid sequence of the mature LiPH2 gene.

Refolding and purification of rLiPH2. rLiPH2 was expressed as an inactive form in inclusion bodies, similar to that reported for recombinant LiPH8 (14,15), MnPH4 (22), and horseradish peroxidase (HRP) (23), however only minimal (ie, ~1%) of active LiP has been reported (15). Active recombinant MnPH4 and HRP were obtained by *in vitro* refolding (22,23). The overall structures, active site, and calcium binding sites in LiP are very similar to those in MnP and HRP (24). We recently reported that calcium plays a crucial role in the stability of LiP and calcium was required to reactivate thermally inactivated LiPH8 (25). Additionally, room temperature was the best temperature for reactivating thermally inactivated LiPH8 (25). Therefore, the conditions for refolding rLiPH2 were the procedures for refolding MnP and HRP modified by including the optimal conditions for the reactivation of thermally inactivated LiPH8. The inclusion bodies were solubilized in 8 M urea and 2 mM DTT and then rapidly diluted with refolding solution. The solution was incubated at room temperature for 24 hours, and then extensively dialyzed against 10 mM sodium acetate buffer, pH 6.0. The samples were centrifuged and the supernant concentrated and purified by DEAE anion-exchange column followed by size-exclusion chromatography. Fractions with highest enzyme activity were pooled and subjected to SDS-PAGE analysis. The purified rLiPH2 was found to be near homogenous by SDS-PAGE and the molecular weight was again about 38 kDa. The total amount of active enzyme was approximately 3.4 mg/ L culture.

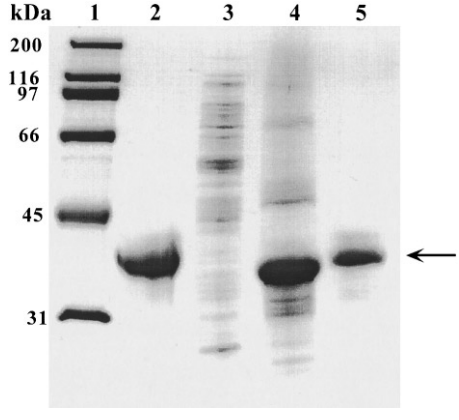

FIG. 2. SDS-PAGE of rLiPH2. All samples with loading buffer were heated at 95°C for 10 minutes before loading into the gel. The gel was run at 200 volt and stained with Coomassie brilliant blue. Samples are: Lane 1, molecular weight marker; lane 2, native LiPH2; lane 3, supernant after centrifugation of lysed cells; lane 4, washed inclusion bodies; lane 5, purified rLiPH2.

Spectral and kinetic properties of rLiPH2. The absorption spectrum of rLiPH2 exhibited absorption maximum at 408, 500, and 634 nm (Figure 3A), which were identical to those of native LiPH2 isolated from *P. chrysosporium* (Figure 3B). The typical absorption spectrum suggests that rLiPH2 is pentacoordinate, high spin heme protein (26). The Rz value (A_{408}/A_{280} ratio) for rLiPH2, shown in Figure 3A, was found to be 3.9 which was even higher than that of purified native LiPH2 (3.2) (Figure 3B), which suggests that rLiPH2 is pure, active and suitable for kinetic studies. Indeed, the purified rLiPH2 had veratryl alcohol oxidase activity which absolutely depended on both VA and H_2O_2 (Figure 4). The turnover number at pH 2.5 for rLiPH2 was calculated to be 39 s^{-1} which is slightly higher than that of LiPH2 (30 s^{-1}) isolated from *P. chrysosporium*. Surprisingly, rLiPH2 also exhibited Mn^{2+} oxidase activity with a turnover number of 14 s^{-1}. Although this is much lower than that of native MnPH4 (140 s^{-1}), it does demonstrate that LiPH2 has both lignin peroxi-

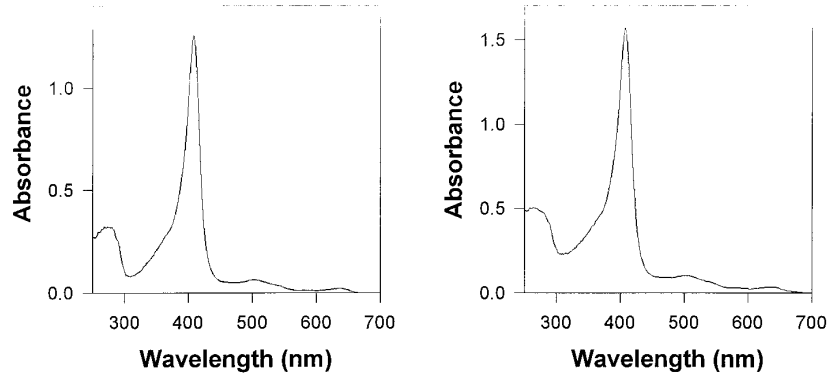

FIG. 3. Absorption spectra of purified rLiPH2 (7.5 μM) (A) and native LiPH2 (10 μM) (B). The spectra were recorded in 10 mM sodium acetate buffer, pH 6.0, at room temperature.

dase and manganese peroxidase activities as previously reported for native enzyme (27) and the manganese peroxidase activity of native LiPH2 was most likely not due to contamination (28). However, the pH optimum (pH 3.0) of MnP activity of rLiPH2 was different from that of native MnP (pH 4.5).

Successful expression and reconstitution of recombinant LiP has been pursued by many researchers for more than 10 years. This success will be invaluable in a variety of mechanistic studies and also greatly facilitate future studies on structure-function relationships for this enzyme using site-directed mutagenesis. We have already shown here that rLiPH2 exhibited both LiP and MnP activities and there is no reason to expect that the MnP activity of rLiPH2 is due to contamination. Experiments on the mechanism for the MnP activity of rLiPH2 are underway.

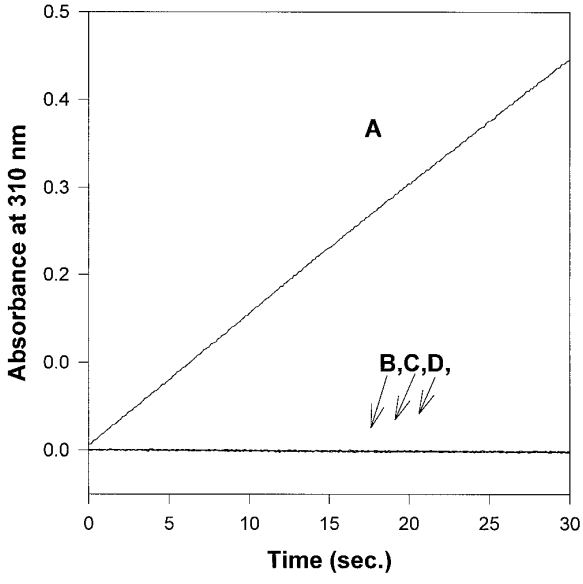

FIG. 4. Time course of veratryl alcohol oxidation by rLiPH2. The reaction conditions were described in Materials and Methods. Reaction mixture contained: A, all required reagents; B, without VA; C, without H_2O_2; D, without rLiPH2.

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