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# Molecular characterization of cytochrome P450 catalyzing hydroxylation of benzoates from the white-rot fungus *Phanerochaete chrysosporium*

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**Abstract**

We cloned full-length cDNA (*PcCYP1f*) encoding one of the cytochrome P450s in the lignin-degrading basidiomycete *Phanerochaete chrysosporium*, which showed high homology to P450s in the CYP53 family. PcCYP1f was expressed as an active microsomal protein using the methylotrophic yeast *Pichia pastoris* expression system. Using the microsomal fraction containing PcCYP1f, a typical P450 CO-difference spectrum was obtained with absorption maximum at 448 nm. Recombinant PcCYP1f catalyzed the hydroxylation of benzoic acid into 4-hydroxybenzoic acid in the presence of NADPH and *P. chrysosporium* cytochrome P450 oxidoreductase. In contrast to other CYP53 P450s, this enzyme was shown to catalyze the hydroxylation of 3-hydroxybenzoate into 3,4-dihydroxybenzoate. Furthermore, 2- and 3-methylbenzoate were also shown to be substrates of PcCYP1f. This is the first report showing the expression of a functionally active *Phanerochaete* P450. Finally, real-time quantitative PCR analysis revealed that PcCYP1f is induced at a transcriptional level by exogenous addition of benzoic acid.


*Keywords:* Basidiomycete; Benzoate hydroxylase; Carbon monoxide-difference spectrum; Cytochrome P450; Heterologous expression; Kinetic analysis; *Phanerochaete chrysosporium*; *Pichia pastoris*; Real-time PCR; Substrate binding spectrum

White-rot fungi are capable of degrading a wide variety of recalcitrant aromatic compounds, including polymeric lignin and environmentally persistent pollutants. Extracellular ligninolytic enzymes, such as lignin and manganese peroxidases, involved in the metabolism of aromatic compounds have been extensively studied [1,2]. Intracellularly, cytochrome P450 (P450)-mediated oxygenation reactions are known to play an important role during fungal metabolism of recalcitrant xenobiotic compounds [3–10]. *Phanerochaete chrysosporium* is the most extensively studied white-rot fungus with regard to ligninolysis and xenobiotic metabolism. The gene diversity of fungal P450 was recently suggested via the whole sequence of the *P. chrysosporium* genome, where as many as 148 P450 genes have been found [11]. Recently, we utilized a series of well-characterized P450 substrates as metabolic substrates for *P. chrysosporium*, pointing out not only the gene diversity but also the functional diversity of fungal P450 molecular species. Among the substrates examined, benzoate was found to be converted by *Phanerochaete* P450(s) [12].

In the present study, we cloned *PcCYP1f*, one of the *Phanerochaete* P450 genes, and showed high homology to the P450s in the CYP53 family (benzoate-4-hydroxylase), which are known to catalyze the hydroxylation of benzoic acid at the 4-position [13–15]. PcCYP1f was expressed in the heterologous expression system using *Pichia pastoris*. In the CO-difference spectrum, recombinant PcCYP1f exhibited absorption maximum at 448 nm with no contamination of absorption at 420 nm. It was also shown to catalyze the hydroxylation of benzoic acid

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into 4-hydroxybenzoic acid in the presence of NADPH and *P. chrysosporium* cytochrome P450 reductase (PcCPR). Finally, real-time quantitative PCR analysis revealed that PcCYP1f was induced at the transcriptional level by exogenous addition of benzoic acid.

## Materials and methods

*Chemicals.* Benzoic acid was purchased from Wako Pure Chemicals. 2-, 3-, 4-Hydroxybenzoic acid, 2-, 3-methylbenzoic acid, 2-, 3-, 4-methoxybenzoic acid, 4-ethoxybenzoic acid, and 3,4-dihydroxybenzoic (protocatechuic) acid were obtained from Sigma–Aldrich. All other chemicals were of analytical grade. Deionized water was obtained from Milli Q System (Millipore).

*Culture conditions. Phanerochaete chrysosporium* (ATCC 34541) was grown from conidial inocula at 37 °C in a stationary culture (20 mL medium in a 200-mL Erlenmeyer flask) under air. The medium (pH 4.5) used in this study with 28 mM D-glucose and 1.2 mM ammonium tartrate as the carbon and nitrogen sources, respectively, was previously described [12,16]. After 2-day preincubation, benzoic acid in acetonitrile (20 μL) was added to a final concentration of 1 mM. For the control culture, only acetonitrile (20 μL) was added.

*cDNA synthesis.* After 24-h incubation in the absence or presence of benzoic acid, total RNA was isolated from *P. chrysosporium* using an RNeasy Plant Mini Kit (Qiagen). Reverse transcription (RT) was performed using oligo$(dT)_{16}$ primer and Superscript II reverse transcriptase (Invitrogen) at 42 °C for 50 min. The subsequent polymerase chain reaction (PCR) amplification was performed with the following primers: 5′-GAATTC**AAAAATGTCT**ATGGCAGTAATTGAAGCACTAACACAACTAGATTTGAAGTCGTG-3′ and 5′-GCGGCCGCTCACAGGGTCCTCCTCCTGATA-3′. Primers were designed from the *P. chrysosporium* genomic sequence of ug.1.19.1 [http://genome.jgi-psf.org/whiterot1/whiterot1.home.html]. An *Eco*RI site (underlined) was introduced upstream of the yeast consensus sequence (indicated in bold) followed by the start codon and an *Not*I site (underlined) was introduced immediately downstream of the stop codon by PCR. Four silent mutations were added to the forward primer (double underlined). PCR amplification was performed using a DNA Thermal Cycler 2400 (Perkin-Elmer) as follows: initial denaturation at 95 °C for 3 min, denaturing at 95 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 2 min for 35 cycles. PCR products were separated by electrophoresis on 1.2% agarose gel, stained with ethidium bromide, and visualized using Molecular Imager FX (Bio-Rad). Obtained cDNA was designated *PcCYP1f* and its sequence was deposited in DDBJ under Accession No. AB219059.

*Cloning and sequencing.* After purification using 1.2% agarose gel and a QIAquick gel extraction kit (Qiagen), PCR products were cloned into the pGEM-T Easy vector (Promega) and then transformed into *Escherichia coli* strain NovaBlue competent cells. Positive clones were selected by blue-white screening. Plasmids were isolated from positive clones using a QIAprep Spin Miniprep Kit (Qiagen) and sequenced with an automated DNA Sequencer (CEQ 8000; Beckman) using a DTCS Quick Start Kit (Beckman). The nucleotide and deduced amino acid sequences were analyzed using BLAST and FASTA search programs.

*Generation of recombinant P. pastoris containing PcCYP1f.* A pPICZ A vector (Invitrogen) containing the methanol-inducible AOX1 promoter for control of gene expression and encoding resistance against zeocin was used to achieve intracellular expression of heterologous genes in the *P. pastoris* wild-type strain KM71H (Invitrogen). PcCYP1f cDNA digested by *Eco*RI and *Not*I was ligated into pPICZ A treated with the same endonucleases. *E. coli* strain NovaBlue was used for transformation and propagation of recombinant plasmids. Transformation of *P. pastoris* was achieved using EasySelect *Pichia* expression kit version G (Invitrogen). The presence of *PcCYP1f* in zeocin-resistant colonies was confirmed by direct PCR of the *P. pastoris* colonies.

*Pichia pastoris cultures and preparation of microsomes.* Single colonies of *P. pastoris* were grown for 24 h at 30 °C and 300 rpm in 200 mL BMGY (1% yeast extract, 2% peptone, 0.1 M potassium phosphate (pH 6.0), 1.34% yeast nitrogen base, $4 \times 10^{-5}$% biotin, and 1% glycerol). Cells were harvested at 3000*g* for 5 min at room temperature and then inoculated in 50 mL of inducing medium (BMGY with 1% methanol instead of glycerol) in a 500-mL baffled flask. Cultures were grown for 24 h at 30 °C and 300 rpm and the cells were pelleted at 3000*g* for 5 min at room temperature and then washed once in buffer A (50 mM potassium phosphate (pH 7.4), 1 mM EDTA, 20% glycerol, 1 mM DTT, and 0.25 mM phenylmethylsulfonyl fluoride) before being resuspended in buffer A to OD 130. The cells were broken by vortexing ($10 \times 30$ s with intermediate cooling on ice) with an equal volume of acid-washed glass beads. The lysate was centrifuged at 11,000*g* for 10 min at 4 °C to remove all debris and then the resulting supernatant was centrifuged at 130,000*g* for 1 h at 4 °C to recover the microsomal pellet. Microsomes were resuspended in buffer A, stored at −80 °C, and thawed on ice immediately before use.

*Catalytic activity of PcCYP1f.* The P450 content in the microsomal fraction was determined by carbon monoxide-difference spectral analysis using an extinction coefficient of 91 $mM^{-1}\,cm^{-1}$ [17]. PcCYP1f activity was measured using a microsomal fraction of *P. pastoris* containing recombinant PcCYP1f with exogenous addition of recombinant NADPH cytochrome P450 oxidoreductase from *P. chrysosporium* (PcCPR), which was expressed in *E. coli* and purified as described previously [18].

PcCPR activity was measured as NADPH-dependent cytochrome *c* reducing activity in reaction mixtures (1 mL) containing a PcCPR solution (100 μL), cytochrome *c* (10 μM), EDTA (0.5 mM), and NADPH (30 μM) in 50 mM potassium phosphate (pH 7.4). The reaction was initiated by adding NADPH. One unit of PcCPR is defined as the amount of enzyme that is able to catalyze the reduction of cytochrome *c* at an initial rate of one pmol per min. The rate of reduction of cytochrome *c* was determined spectrophotometrically at room temperature using 21.1 $mM^{-1}\,cm^{-1}$ as the extinction coefficient for reduced minus oxidized cytochrome *c* at 550 nm [19,20]. No activity was observed when NADH was used instead of NADPH.

Activity and substrate specificity of PcCYP1f were determined in reaction mixtures (500 μL) containing 100 μM PcCYP1f, 0.4 U PcCPR, 30 μg/μL L-α-dilauroyl phosphatidylcholine, 1 mM NADPH, 3 mM glucose-6-phosphate (G6P), 0.04 U glucose-6-phosphate dehydrogenase (G6PDH), and 5 μL of a substrate solution (0–600 mM in acetonitrile) in 50 mM potassium phosphate (pH 7.4). The reaction was initiated by addition of a substrate. After incubation of the reaction mixtures at 30 °C for 15 min, 500 μL of 1 M HCl with 0.5 mM of cinnamic acid (internal standard) was added. Three types of control reactions, a zero-time control (terminated with 500 μL of 1 M HCl immediately after adding the substrate), no-NADPH control, and no-PcCYP1f control, were run parallel. The residual substrate and reaction products were analyzed by HPLC after filtration (0.45 μm) and by GCMS after extraction with 2 mL ethyl acetate (3×) and trimethylsilylation using *N*,*O*-bis(trimethylsilyl)trifluoroacetamide/pyridine (2:1, v/v). From the time course of the quantification data, the Michaelis constant ($K_m$) and the maximum velocity ($V_{max}$) were calculated.

*Real-time quantitative analysis of gene transcription.* After 2-day preincubation of *P. chrysosporium*, benzoic acid was added. Total RNA was extracted from mycelia grown for an additional 12 or 24 h in the absence or presence of benzoic acid and applied (0.5 μg) to first-strand cDNA synthesis according to the methods described above. Real-time quantitative PCR was performed using LightCycler (Roche Molecular Biochemicals) with the following gene-specific primers 5′-ATGACAGTCTGCGGAAGGTT-3′ and 5′-TTTTGCACTGCGTTCTTGTC-3′, generating a 149-bp amplicon. The PCR consisting of 2 μL of 1:32 diluted RT reaction product, 2 μL of LightCycler Fast-Start DNA Master SYBR Green I (Roche Molecular Biochemicals),