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ORIGINAL PAPER

Novel evidence of cytochrome P450-catalyzed oxidation of phenanthrene in *Phanerochaete chrysosporium* under ligninolytic conditions

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Abstract The presence of cytochrome P450 and P450-mediated phenanthrene oxidation in the white rot fungus *Phanerochaete chrysosporium* under ligninolytic condition was first demonstrated in this study. The carbon monoxide difference spectra indicated induction of P450 (130 pmol mg^{-1} in the microsomal fraction) by phenanthrene. The microsomal P450 degraded phenanthrene with a NADPH-dependent activity of 0.44 ± 0.02 min^{-1}. One of major detectable metabolites of phenanthrene in the ligninolytic cultures and microsomal fractions was identified as phenanthrene *trans*-9,10-dihydrodiol. Piperonyl butoxide, a P450 inhibitor which had no effect on manganese peroxidase activity, significantly inhibited phenanthrene degradation and the trans-9,10-dihydrodiol formation in both intact cultures and microsomal fractions. Furthermore, phenanthrene was also efficiently degraded by the extracellular fraction with high manganese peroxidase activity. These results indicate important roles of both manganese peroxidase and cytochrome P450 in phenanthrene metabolism by ligninolytic *P. chrysosporium*.

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Abbreviations

ABTS	2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate)
HPLC	High performance liquid chromatography
LiP	Lignin peroxidase
LN	Low nitrogen
MnP	Manganese-dependent peroxidase
P420	Cytochrome P420
P450	Cytochrome P450
PAHs	Polycyclic aromatic hydrocarbons
PB	Piperonyl butoxide
PDA	Potato dextrose agar

Introduction

Polycyclic aromatic hydrocarbons (PAHs), many of which are known to be toxic, mutagenic and/or carcinogenic (Mallick et al. 2007; Marston et al. 2001; Mastrangelo et al. 1996; Xue and Warshawsky 2005), are commonly released by the incomplete combustion of organic compounds, and persist in the environment, and usually accumulate in living organisms. Phenanthrene has been shown to be toxic to marine diatoms (Kusk 1981), gastropods (Pipe and

Moore 1986a), mussels (Pipe and Moore 1986b), crustaceans (Colores et al. 2007), and fish (Black et al. 1983; Sutherland et al. 1991). Moreover, it has both a "bay-region" and a "K-region" and is often used as a model substrate for studies on the metabolism of carcinogenic PAHs. Cytochrome P450s, a super family of hemoproteins involved in metabolisms of PAH and many other xenobiotics (Sato and Omura 1978), catalyzed initial oxidation of phenanthrene in animals (Jacob et al. 1996; Korytko et al. 2000; Shou et al. 1994). The oxidative products are hydrated by epoxide hydrolase to the corresponding *trans*-dihydrodiols or isomerized to phenols (Bruice et al. 1976; Cerniglia and Heitkamp 1989; Sutherland et al. 1991). Formation of the *trans*-dihydrodiols and phenols has also been found in most cases of phenanthrene metabolism by fungi (Casillas et al. 1996; Cerniglia et al. 1989; Sutherland et al. 1993), although involvement of fungal P450s was only demonstrated in a few species (Bezalel et al. 1997).

White rot fungi are a group of mycelial fungi that colonize wood in nature and decompose lignin to cause white rotting of wood. One of the most studied white rot fungi, *Phanerochaete chrysosporium*, can mineralize a wide variety of PAHs and other significant environmental pollutants (Bumpus et al. 1985; Bumpus and Aust 1987; Hammel et al. 1986, 1992), and thus has been investigated extensively for possible use in the bioremediation of PAHs (Brodkorb and Legge 1992; Bumpus 1989; Dhawale et al. 1992; Morgan et al. 1991; Zheng and Obbard 2002). In the ligninolytic cultures of this fungus, it was reported that 9,10-oxidation and ring cleavage to form 2,2′-diphenic acid (DPA) was a major fate of phenanthrene. This novel pathway led to more efficient mineralization (5.6–6.4% in 14 days) than that in nonligninolytic cultures (0.11–0.22% in 14 days) in which *P. chrysosporium* degrades phenanthrene to form the dihydrodiols and phenanthrols (Hammel et al. 1992). The extracellular ligninolytic enzymes, manganese peroxidase (MnP) and laccase, can support the oxidization of phenanthrene to DPA without requirement of H_2O_2, and 9,10-phenanthrenequinone was expected to be the intermediate product (Bohmer et al. 1998; Moen and Hammel 1994; Pickard et al. 1999). In contrast, another ligninolytic enzyme, lignin peroxidase (LiP) can not oxidize phenanthrene, although it is able to oxidize many PAHs that have ionization potentials less than about 7.6 eV (Hammel et al. 1986). However, LiP can catalyze oxidation of 9-phenanthrol to form 9,10-phenanthrenequinone (Tatarko and Bumpus 1994).

Phanerochaete chrysosporium has more than 150 P450 genes, which is the largest P450 contingent known to date in fungi (Doddapaneni and Yadav 2005; Martinez et al. 2004). Although the function of these P450s in ligninolytic *P. chrysosporium* has rarely been demonstrated, the P450s were suggested to be involved in degradation of several organopollutants including some PAHs (Hiratsuka et al. 2005; Kullman and Matsumura 1996; Mougin et al. 1996; Mougin et al. 1997; Teramoto et al. 2004a, b; Yadav et al. 2006), and P450-mediated benzo[*a*]pyrene hydroxylation was found in microsomal fractions from nonligninolytic *P. chrysosporium* (Masaphy et al. 1996). Nonetheless, ligninolytic metabolism of phenanthrene was proposed to be not initiated by the classical P450 monooxygenase, because phenanthrene *trans*-9,10-dihydrodiol, which is the principal phenanthrene metabolite in nonligninolytic *P. chrysosporium* cultures, was proved to be not formed in the ligninolytic cultures (Hammel et al. 1992). In this paper, on the contrast, P450-catalyzed oxidation of phenanthrene to form phenanthrene *trans*-9,10-dihydrodiol was demonstrated to be present and play an important role in phenanthrene metabolism by *P. chrysosporium* under ligninolytic condition.

Materials and methods

Organisms and culture conditions

Phanerochaete chrysosporium strain BKM-F-1767 (ATCC 24725) was maintained on potato dextrose agar (PDA). Low nitrogen (LN) medium with 2.4 mM nitrogen was prepared as described by Tien and Kirk (Tien and Kirk 1988), except that dimethylsuccinate was replaced by 20 mM acetate buffer.

Inocula were prepared according to the method of Aiken and Logan (Aiken and Logan 1996). Fungi grown for 6 days in a static culture flask were blended and mixed, and then a 0.1 ml aliquot of this suspension was aseptically transferred to a sterile 250 ml flask containing 25 ml of media. The fungi were incubated at 37°C and 130 rpm.

After incubated for 3 days, the cultures had relative high MnP activity and obvious LiP activity, and thus were ligninolytic. Then, 0.5 mg of phenanthrene

dissolved in 25 µl of dimethyl formamide was added to each flask. Two types of controls were prepared, one with sterile culture and phenanthrene and the other with intact culture but no phenanthrene. The fungal biomass of the sterile controls was approximately equivalent to that in the experimental cultures. All the flasks were incubated at 37°C in darkness (to prevent photooxidation of the phenanthrene) with shaking at 130 rpm on a rotary shaker. The cultures were routinely examined by microscopy to ensure the absence of bacterial contamination.

Extraction and detection of phenanthrene and the metabolites

Extraction

The flasks were incubation for some time (stated in the Results) after addition of phenanthrene. Before extraction, 0.5 mg pyrene was added as an internal standard to determine the extraction efficiency. For determination of phenanthrene, the cultures were extracted by two methods. One used 5 volumes of ethyl acetate after homogenization by Ultra-Turrax (Fluko, FA25, Germany), and the other included addition of 5 volumes of methanol and ultrasonication for 40 min (sealed). The extracts were both filtered through 0.45 µm-pore-size filter and then directly analyzed by high-performance liquid chromatography (HPLC). The ratio of degraded phenanthrene was calculated by comparing with the results of the sterile controls which were extracted in the same way.

For detection of the metabolites, the ethyl acetate extracts were dried over anhydrous sodium sulfate and concentrated with a rotary vacuum evaporator at 35°C. The aqueous phase was acidified to pH 2.5 with HCl and then extracted by ethyl acetate and concentrated in the same manner. The residues were redissolved in methanol for HPLC or derivatized for gas chromatography-mass spectrometry (GC–MS), and then compared with residues from controls.

HPLC analysis

Reverse phase HPLC analysis of metabolites was performed with a Hewlett Packard 1100 series liquid chromatography (Hewlett Packard, Palo Alto, CA, USA) equipped with a reverse phase C_{18} column (Hypersil, Thermo Corp., Waltham, MA, USA), 5 µm (250 mm × 4.6 mm). The column was eluted with a linear gradient of 30–70% methanol in water over a period of 30 min after an initial delay of 1 min, at a flow rate of 1.2 ml min^{-1} (Nordqvist et al. 1981; Pangrekar et al. 2003). The UV detector was set at both 254 and 270 nm to detect metabolites. UV absorbance spectra were determined with the diode array spectrophotometer (Hewlett Packard G1315A) and compared to those of phenanthrene metabolites published previously (Bao and Yang 1991; Bezalel et al. 1996; Cerniglia et al. 1989; Grasselli and Ritchey 1975; Jerina et al. 1976; Moody et al. 2001; Sutherland et al. 1990, 1991).

Purification

The fractions (0.5–1.1 ml) of peaks represented metabolites were collected during HPLC analysis, and were characterized by HPLC in the same manner to ensure that every fraction exhibited a unique peak. This procedure was repeated five times to obtain a sufficient amount of metabolites for analysis. The collected fractions were extracted into ethyl acetate, dried over anhydrous sodium sulfate, and concentrated under a stream of nitrogen. Before analysis by GC–MS, the residuals were acetylated with acetic anhydride in pyridine.

GC–MS analysis

Before analysis by GC–MS, the neutral extracts were acetylated with acetic anhydride in pyridine and the acidic extracts were methylated with diazomethane (5). GC–MS analysis of derivatized metabolites was performed on a DSQ GC–MS (Thermo Corp., Waltham, MA, USA), equipped with a quadrupole mass filter and a VF-5MS (Varian, Palo Alto, CA, USA) capillary column (30 m × 0.25 mm × 0.25 µm). Analyses were performed in the electron ionization mode with electron energy of 70 eV. The injector temperature was 200°C. The column was held at 50°C for 2 min, and programmed to rise to 300°C at 10°C min^{-1}, after which the temperature was held isothermally at 300°C for 3 min.

Preparation of extracellular and microsomal fractions

The fungi were incubated as described above. To prepare extracellular fractions, some flasks were

incubated for 17 days after addition of phenanthrene, and the culture supernatants were periodically sampled during the incubation. The samples were centrifuged at 9,000*g* for 10 min and filtered through 0.2 μm-pore-size filter to obtain extracellular fractions.

To prepare microsomal fractions for P450 study, some flasks were incubated for 24 h after addition of phenanthrene dissolved in dimethyl formamide. No-induced and dimethyl formamide-induced cultures were set up as controls for analysis of P450 induced by phenanthrene. The mycelia were harvested by centrifugation (5000*g*, 15 min, twice) and the fungal pellets were washed extensively with 0.1 M sodium phosphate buffer (pH 7.5, ice-cold) containing 10 mM EDTA. The microsomal fraction was then prepared according to Masaphy et al. (1996). The pellets were resuspended in 0.1 M sodium phosphate buffer (called buffer B, pH 7.5, ice-cold) containing 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF and 20% (v/v) glycerol. Then the cells were disrupted by glass beads (0.4–0.6 mm, 1 g mycelium in 1 ml buffer B with 1 g glass beads) using a dismembrator (Sartorius, Mikro-Dismembrator S, Germany) over a period of 2 min with 20-s bursts followed by 20-s cooling on ice. The biomass homogenate was centrifuged at 15000*g* for 15 min twice to remove cell debris, nuclei and mitochondria. Then the supernatant was centrifuged at 105000*g* for 90 min to pellet the microsomal fraction. After washed twice with buffer B, the microsomal fraction was suspended in buffer B and stored at −80°C.

Enzyme activity assay

Cytochrome P450 determination

The P450 contents of microsomal fractions were determined by carbon monoxide (CO) difference spectra as described previously (Daliang et al. 2007; Omura and Sato 1964). Firstly, the total protein concentrations of the microsomal fractions were determined by the Bradford method (Bradford 1976). The sample was diluted to about 1 mg ml^{-1} protein with buffer B and then added with 1 mM KCN. Subsequently, the sample was dispensed equally into two cuvettes (1 ml per cuvette) and a baseline spectrum was spectrophotometrically recorded in the range of 400–500 nm (Shimadzu, UV-2401PC, Japan). One cuvette was then gently gassed with CO at a rate of 3 ml min^{-1} for 40 s, and the other one was gassed with N_2 to the same extent. An equal volume (10 μl) of sodium dithionite solution (400 mg ml^{-1}) was accurately added into each cuvette, and then the difference spectrum was recorded. The concentrations of P450 and P420 (an inactive form of P450) were calculated using the extinction coefficients, $\varepsilon_{450-490}$ value of 91 mM^{-1} cm^{-1} and $\varepsilon_{420-490}$ value of 110 mM^{-1} cm^{-1} respectively (Omura and Sato 1964). The P450 and P420 contents in microsomal fractions were expressed in pmol P450 per mg of protein.

Microsomal transformation of phenanthrene

The 1-ml assay solution contained 3 mM $MgCl_2$, 1 mM NADPH, 5 mg BSA, microsomal fractions (1–1.4 mg of protein) and phenanthrene in 100 mM potassium phosphate buffer (pH 7.5). Two types of control reactions, no-NADPH controls and sterile controls, were run parallel. The reaction mixtures were incubated at 37°C overnight, supplemented with pyrene as internal standard, extracted with ethyl acetate, dried over anhydrous sodium sulfate, and concentrated under a stream of nitrogen. The residues were redissolved in methanol for HPLC and compared with residues from controls. Phenanthrene and its metabolites were analyzed by reverse phase HPLC. The metabolite was purified by HPLC, acetylated with acetic anhydride in pyridine and then analyzed by GC–MS. The HPLC analysis, purification and GC–MS analysis were the same as described above. The inhibition of cytochrome P450 activity was determined with a P450 inhibitor, piperonyl butoxide (PB, Aldrich). The decrease in the amounts of phenanthrene degraded and phenanthrene *trans*-9,10-dihydrodiol produced was measured by HPLC.

Ligninolytic enzymes determination

The activities of LiP, MnP and lacass were determined spectrophotometrically by the method of Tien and Kirk (1988), Paszczynski et al. (1988) and Niku-Paavola et al. (1988). LiP activity in extracellular fractions was assayed with 3,4-dimethoxybenzyl alcohol as the substrate. MnP activity was assayed with Mn^{2+} as substrate. Laccase activity was assayed with 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) as substrate. One unit (U) of enzyme oxidizes 1 μmol of the substrate per min in the presence of H_2O_2.

Degradation of phenanthrene in extracellular fraction

Phenanthrene-degrading activity of MnP was analyzed with 2-ml reaction mixtures. Three types of mixtures were set: the first one contained 0.2 mM $MnSO_4$, 0.1% Tween 80, extracellular fractions and phenanthrene (20 mg l^{-1}) in 50 mM tartrate buffer (pH 4.5), the second one had the same compositions plus 0.1 mM H_2O_2, and the third one had 0.1 mM H_2O_2 and 0.2 g (wet weight) sterile mycelia in addition. The mixtures with sterile extracellular fractions were used as controls. The extracellular fractions tested for phenanthrene-degrading activity were from 4 days old and 2 days old cultures, with MnP activity of higher than 1000 U l^{-1} and lower than 150 U l^{-1} respectively.

Cytochrome P450 inhibitor experiments

The LN cultures were incubated for 3 days at 37°C and 130 rpm in the dark, and then PB was added to a final concentration of 2 mM. After another 60-min incubation, the cultures were added with phenanthrene (20 mg l^{-1}), incubated for another 7 days and extracted as described above. Degradation rate of phenanthrene and formation of phenanthrene metabolites were analyzed by HPLC and compared to those in cultures without PB. The assays were all performed in triplicate and the mean values and standard deviations of the data were presented.

Results

Degradation efficiency and metabolites of phenanthrene in ligninolytic cultures

In the LN cultures of *P. chrysosporium* incubated for 3 days, the MnP and LiP activities were more than 1320 U l^{-1} and 91 U l^{-1} respectively, indicating the cultures were ligninolytic. Laccase activity was not detectable in the LN cultures in our work.

Subsequently, the ligninolytic cultures were added with 20 mg l^{-1} phenanthrene, incubated for 1, 7 or 20 days, and then extracted by two methods. The recoveries of the internal standard pyrene from all samples and recoveries of phenanthrene from sterile controls were more than 90% when the cultures were extracted with methanol and ultrasonicated simultaneously. When the cultures were extracted with ethyl acetate after homogenization, the recoveries were only 40 $\sim$ 70% and the data of parallel samples were of higher standard deviations. This indicated the former method could extract more phenanthrene adsorbed by the mycelia, and was thus suitable to determine the amount of phenanthrene in the cultures to calculate degradation rates. The latter method was more feasible for metabolites analysis, since it is necessary to separate the extract from the aqueous fraction before concentration.

In the LN cultures, the fungus was able to degrade 6.7 $\pm$ 1.7% of the added phenanthrene in 1 day (7.5 $\pm$ 1.9 μM day^{-1}), 24.8 $\pm$ 0.3% in 7 days (3.98 $\pm$ 0.05 μM day^{-1} on average), and 47.8 $\pm$ 4.4% in 20 days (2.68 $\pm$ 0.25 μM day^{-1} on average). The average degradation efficiency was 6.5 $\pm$ 1.6 μmol day^{-1} g^{-1} (μmol per day per gram dry weight of biomass) in 1 day, 2.57 $\pm$ 0.03 μmol day^{-1} g^{-1} in 7 days, and 1.74 $\pm$ 0.16 μmol day^{-1} g^{-1} in 20 days.

After concentration of the ethyl acetate extracts from the cultures incubated with phenanthrene for 7 days, the metabolites were analyzed by comparing the HPLC elution profiles (Fig. 1a) with those of concentrated extracts from the control cultures without phenanthrene (Fig. 1b). A major detectable metabolite (compound I) was eluted at 18.2 min. Its UV absorption spectrum with λ_{max} of 269 nm (Fig. 1a inset) was similar to that of phenanthrene *trans*-9,10-dihydrodiol (Bezalel et al. 1996; Sutherland et al. 1991), but differed from that of the *cis*-9,10-dihydrodiol with λ_{max} of 278 nm (Moody et al. 2001). Compound I was then purified by HPLC, acetylated and analyzed by GC–MS. The MS spectrum presented the diacetylated derivative at *m/z* 296, fragment ions at *m/z* 236, *m/z* 194, and *m/z* 165 (Fig. 2), corresponding to the respective sequential losses of acetic acid, ketene, and CHO, which is identical to those of diacetylated phenanthrene 9,10-dihydrodiol (Bezalel et al. 1996; Hammel et al. 1992). The relative abundances of major mass ions of acetylated compound I were compared with those of acetylated phenanthrene *trans*-9, 10-dihydrodiol and *cis*-9,10-dihydrodiol reported previously (Bezalel et al. 1996; Hammel et al. 1992). According to these results of HPLC–UV and GC–MS, we identified compound I as phenanthrene *trans*-9, 10-dihydrodiol. Compound I was also detected in the cultures incubated with phenanthrene for 1 day, and its

concentration was about 21% of that in the cultures incubated with phenanthrene for 7 days.

In the HPLC profile, another three metabolites (II, III, IV) were eluted at 20.4, 23.3 and 29.8 min (Fig. 1a). The retention times of II and IV were identical to those of 9-phenanthryl β-D-glucopyranoside and 9-phenanthrol which were found in extract of nonligninolytic cultures (data not published). But the UV spectra of II and IV were both ambiguous despite partially similar to those of 9-phenanthrol and the glucoside conjugate. The UV spectrum of III had a peak at 269 nm. These three compounds were not formed in sufficient quantities for further isolation or structural characterization, and compound I seems to be the principle metabolite of phenanthrene in neutral extract. However, the possibility of formation of other metabolites could not be excluded.

Role of cytochrome P450 monooxygenase in metabolism of phenanthrene

The formation of phenanthrene *trans*-9,10-dihydrodiol suggested that the initial hydroxylation was mediated by P450 enzymes. To demonstrate the existence of P450 during degradation of phenanthrene by *P. chrysosporium*, reduced CO difference spectrum of microsomal fraction from phenanthrene-induced cells were recorded. The spectrum of the microsomal fraction showed peaks at 450 nm (Fig. 3a), indicating the occurrence of P450, the contents of which were 130 ± 22 pmol mg^{-1}. The spectrum of the microsomal fraction had major peaks at 420 nm which were attributed to P420. Because P450 was very slight in pure solvent-induced cells (Fig. 3b) and not observed in no-induced cells (Fig. 3c), the synthesis of P450 was expected to be induced by addition of phenanthrene to the LN cultures.

The microsomal fraction with active P450 was utilized for in vitro degradation of phenanthrene. The metabolites were characterized by HPLC and UV spectroscopy (Fig. 4). The major detectable metabolite (I_m) had an identical UV spectrum (λ_{max} 210 nm, 269 nm, the same as the UV spectrum in Fig. 1) and HPLC retention time to phenanthrene *trans*-9,10-dihydrodiol. The compound I_m was then purified by HPLC, acetylated and analyzed by GC–MS. The MS spectrum of acetylated compound I_m, which was almost the same as that of acetylated compound *I*, also showed a molecular ion at *m/z* 296 and fragment ions at

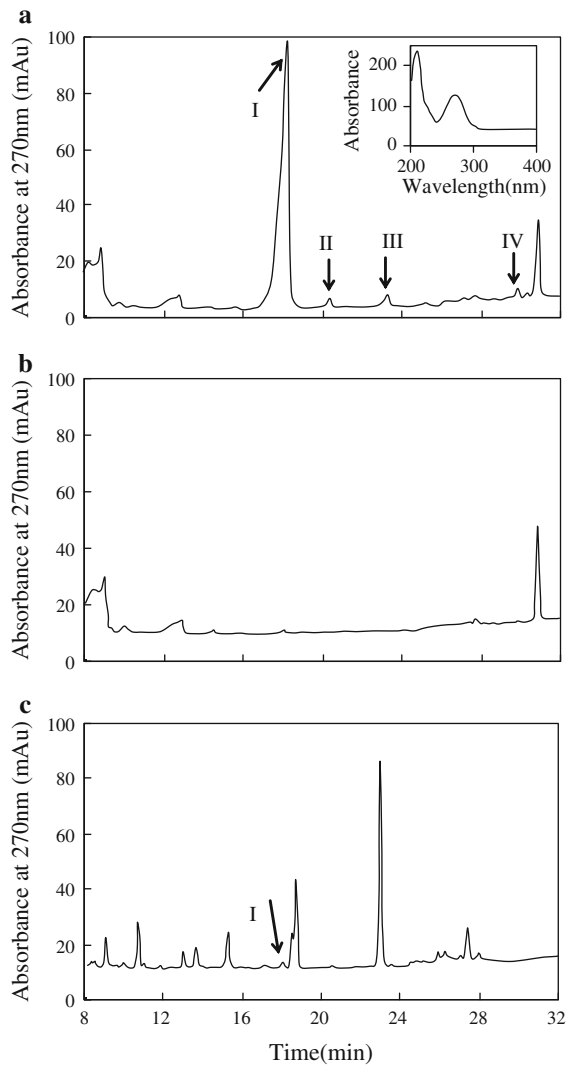

Fig. 1 HPLC elution profiles of neutral extracts of ligninolytic LN cultures of *Phanerochaete chrysosporium*. After preincubated for 3 days, the 25-ml LN cultures were added with 20 mg l^{-1} phenanthrene (dissolved in 25 µl dimethyl formamide, (**a**), with only 25 µl dimethyl formamide (**b**), or with 20 mg l^{-1} phenanthrene and 2 mM piperonyl butoxide (**c**). These cultures were then incubated for 7 days at 37°C and 130 rpm before phenanthrene and the metabolites were extracted and analyzed by HPLC. Inset, UV spectrum of metabolite I from ligninolytic LN cultures of *Phanerochaete chrysosporium*

m/z 236, *m/z* 194, *m/z* 178, *m/z* 165, *m/z* 152 and *m/z* 76, with relative abundance of 0.2, 7.2, 100, 6.3, 30.4, 7.6, and 2.5 respectively. Accordingly, the metabolite I_m was also identified as phenanthrene *trans*-9,10-dihydrodiol.

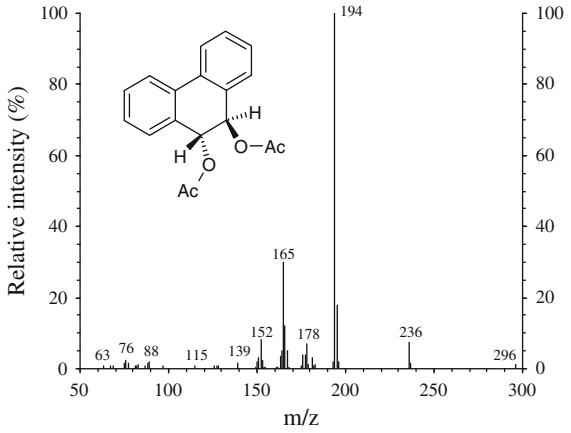

Fig. 2 Mass spectrum of the acetylated derivative of phenanthrene *trans*-9,10-dihydrodiol (compound I indicated in Fig. 1) produced from phenanthrene by *Phanerochaete chrysosporium*. After preincubated for 3 days, the LN cultures were added with 20 mg l^{-1} phenanthrene and incubated for 7 days at 37°C and 130 rpm. The metabolite was extracted, purified by HPLC, acetylated and analyzed by GC–MS

In the reaction mixture with microsomal P450 and NADPH, the degradation rate of phenanthrene was 57 ± 3 nM min^{-1} on average. The rate was 7 ± 9 nM min^{-1} in the controls without addition of NADPH, while it was 0 ± 8 nM min^{-1} in the microsomal fractions without detectable P450. Moreover, no peak at 18–19 min was appeared in the HPLC elution profiles of extracts from these controls. Accordingly, the specific activity of P450-catalyzed oxidation of phenanthrene was 0.44 ± 0.02 min^{-1} on average under the test conditions. This activity was similar to that of microsomal P450 from *Pleurotus ostreatus* which also oxidized phenanthrene to the *trans*-9,10-dihydrodiol (Bezalel et al. 1997).

The role of P450 was also investigated by the P450 inhibitor assay for both in vivo and in vitro metabolism of phenanthrene (Figs. 1c and 4b). Piperonyl butoxide (PB), a P450 inhibitor which can significantly inhibit many P450-mediated reactions, was used at 2 mM for inhibition assay. In the microsomal fractions, PB inhibited the phenanthrene degradation and metabolite I_m formation by similar ratios, 84 ± 2% and 84 ± 3%. This indicated that P450 was essential for metabolism of phenanthrene to form the *trans*-9,10-dihydrodiol. In the intact cultures incubated with phenanthrene for 7 days, PB led to decrease of phenanthrene degradation and phenanthrene trans-9,10-dihydrodiol formation by different ratios, 53 ± 14% and 95 ± 1% respectively. This suggested the important role of P450-catalyzed oxidation and involvement of other enzyme in the initial metabolism of phenanthrene by the intact fungi.

Furthermore, some compounds were newly produced upon addition of PB into the cultures and the microsomal fractions incubated with phenanthrene (Fig. 1c and 4b). The UV spectra of these compounds were quite different from those of phenanthrene metabolites reported (Bao and Yang 1991; Bezalel et al. 1996; Cerniglia et al. 1989; Grasselli and Ritchey 1975; Jerina et al. 1976; Moody et al. 2001; Sutherland et al. 1990, 1991), and the spectra of compounds eluted

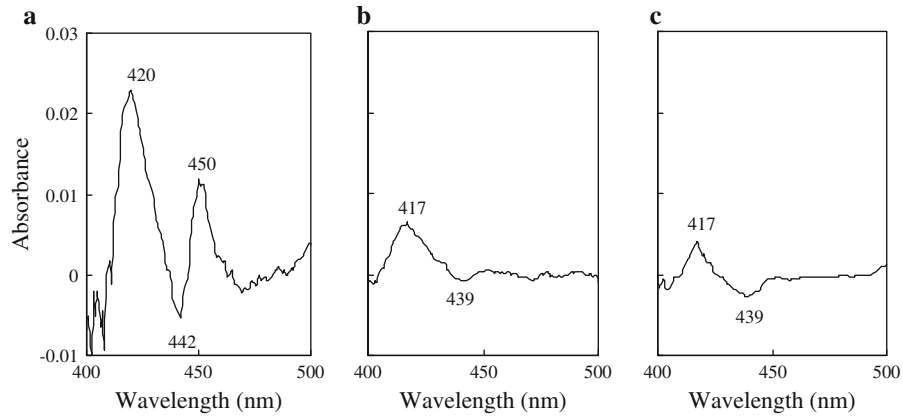

Fig. 3 Carbon monoxide difference spectra of microsomal fractions of *Phanerochaete chrysosporium* from ligninolytic LN cultures. The 25-ml cultures were added with 20 mg l^{-1} phenanthrene (dissolved in 25 µl dimethyl formamide, spectrum a), 25 µl dimethyl formamide (spectrum b) and without anything (spectrum c), respectively; the protein concentration of every sample tested was 1 mg ml^{-1}

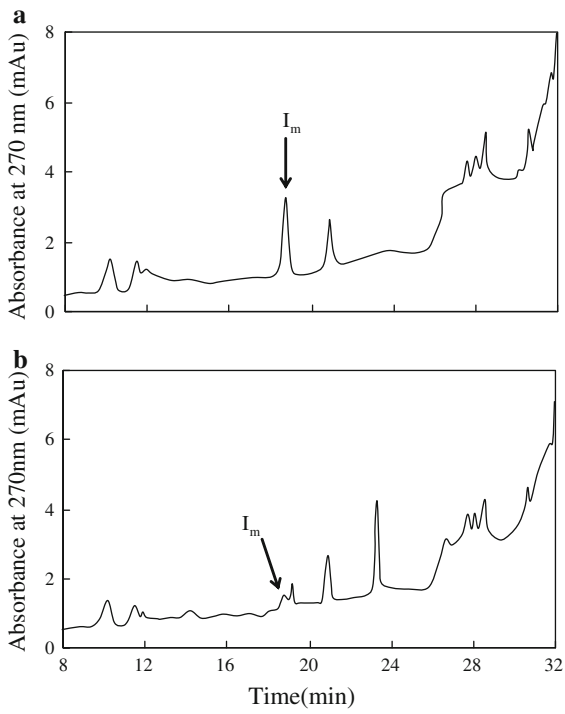

Fig. 4 HPLC elution profiles of neutral extracts from reaction mixture of phenanthrene and microsomal fractions of phenanthrene-induced *Phanerochaete chrysosporium* in ligninolytic LN cultures. The 1-ml reaction mixtures containing the microsomal fractions with 130 nmol P450 were added with 20 mg l^{-1} phenanthrene (**a**) or with 20 mg l^{-1} phenanthrene and 2 mM piperonyl butoxide (**b**)

at 10.5, 18.5, 22.8 and 27.2 min had peaks at the same wavelengths as those of PB (λ_{max} of 240, 290 nm). Accordingly, these compounds may be formed from PB and produced by the fungus and microsomal enzymes respectively.

Degradation of phenanthrene by MnP

Moen and Hammel have reported that MnP can oxidize phenanthrene to DPA in LN culture of *P. chrysosporium* (Moen and Hammel 1994). In accordance with this, we also detected DPA in the cultures (data not shown). Moreover, phenanthrene degradation was observed in extracellular fractions with a MnP activity of 1114 U l^{-1}, but not detectable in those with MnP acivity less than 150 U l^{-1}. The mixtures with high MnP activity can degrade phenanthrene without addition of H_2O_2, at the rate of 1.76 ± 0.02 nM min^{-1} on average during the 7-day reaction. The rate was up to 2.15 ± 0.11 nM min^{-1} in the mixtures added with H_2O_2, and decreased to 1.42 ± 0.08 nM min^{-1} in presence of sterile mycelia and H_2O_2. Compared with these mixtures, the intact cultures had higher MnP activity ranging from 1100 to 2200 U l^{-1} (Fig. 5a, controls), and degraded phenanthrene at the rate of 2.76 ± 0.03 nM min^{-1} on average, during the 7-day incubation with phenanthrene. This result evidenced the important role of MnP in phenanthrene degradation by the fungus.

Either MnP or LiP was not inhibited upon the addition of 2 mM PB (Fig. 5). In the cultures added with PB, MnP activity was still 1100 to 2400 U l^{-1} during the 7-day incubation (Fig. 5a, PB 2 mM), but the rate of phenanthrene degradation decreased to 1.30 ± 0.39 nM min^{-1}. This indicated that the oxidation supported by MnP should not be the only major fate of phenanthrene in the cultures.

Discussion

In this study, we investigated phenanthrene metabolism by ligninolytic *P. chrysosporium* with special interest in the role of P450. Phenanthrene *trans*-dihydrodiols are generally formed from phenanthrene by the successive activities of P450 monooxygenase and epoxide hydrolase in animals (Jacob et al. 1996; Korytko et al. 2000; Shou et al. 1994), while the *cis*-dihydrodiols are characteristic of bacterial dioxygenase-mediated metabolism. Our result indicated that ligninolytic *P. chrysosporium* and the microsomal fraction were capable of metabolizing phenanthrene to phenanthrene *trans*-9,10-dihydrodiol, suggesting oxidative function of P450 in phenanthrene metabolism.

Spectral detection of P450 in both cytosolic and microsomal fractions also provided strong evidence that P450 is important for phenanthrene oxidation by *P. chrysosporium*. It has been reported that phenanthrene could induce the expression of three CYP63 genes (*pc*-1, *pc*-2, *pc*-3, *pc*-4, *pc*-6) in nutrient-rich culture of this fungus (Doddapaneni and Yadav 2004; Doddapaneni et al. 2005; Subramanian and Yadav 2008; Yadav et al. 2006). Our results indicated significant induction of P450 by phenanthrene in the nutrient-limited (LN) cultures of *P. chrysosporium*.

It is known that some fungal P450 systems are involved in oxidation of xenobiotics, including toluene, phenanthrene and benzo[*a*]pyrene (Bezalel et al. 1997; Luykx et al. 2003; Maspahy et al. 1999). All

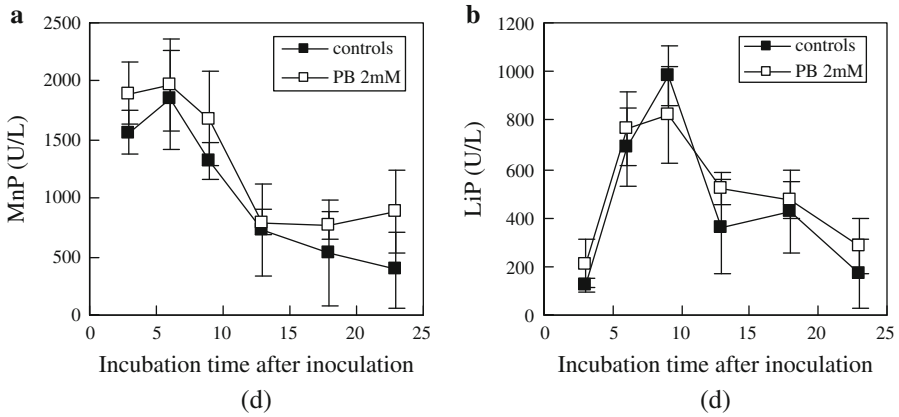

Fig. 5 Effect of piperonyl butoxide (PB) on manganese peroxidase activity (MnP) and lignin peroxidase activity (LiP) in LN cultures of *Phanerochaete chrysosporium*. After incubated for 3 days, the LN cultures were added with 20 mg l^{-1} phenanthrene (*-filled square-* controls) or with 20 mg l^{-1} phenanthrene and 2 mM piperonyl butoxide (*-open sqaure-* PB 2 mM). *Error bars* indicate standard deviation obtained from triplicate flasks

these eukaryotic P450s utilize NADPH as the electron donor of the monooxygenation reactions, whereas most bacterial P450s receive electrons from NADH (Omura 1999). In our study, the microsomal transformation of phenanthrene to the *trans*-dihydrodiol presented stringent requirement for P450 and NADPH, which demonstrated in vitro P450-mediated oxidation of phenanthrene.

The P450 mechanism of oxidation was further supported by the results of P450 inhibition assay. PB, a well-known inhibitor of cytochrome P450s, has inhibited degradation of phenanthrene and naphthalene in *Phlebia lindtneri* (Mori et al. 2003), as well as hydroxylation of benzo[*a*]pyrene in *Pleurotus pulmonarius* (Masaphy et al. 1998, 1999). In our study, PB significantly inhibited phenanthrene degradation and the *trans*-9,10-dihydrodiol formation in both microsomal fractions and intact cultures of *P. chrysosporium*. Collectively, the evidences presented in this paper demonstrated that P450 is involved in metabolism of phenanthrene to form phenanthrene *trans*-9,10-dihydrodiol in ligninolytic *P. chrysosporium*.

P450s mediating oxidations of phenanthrene have also been identified in *Cunninghamella elegans* (Cerniglia and Yang 1984; Cerniglia et al. 1989) and *P. ostreatus* (Bezalel et al. 1996), and there is obvious interspecies variability in the regio- and stereoselectivity of these P450s and epoxide hydrolases involved in phenanthrene metabolism. *C. elegans* produces mainly the *trans*-1,2-dihydrodiol with small amounts of the *trans*-3,4- and *trans*-9,10-dihydrodiols, while *P. ostreatus* produces *trans*-9R,10R-dihydrodiol. *P. chrysosporium* has been reported to produces *trans*-9S,10S-dihydrodiol and *trans*-3R,4R-dihydrodiol under nonligninolytic conditions (Sutherland et al. 1993). Further analysis by circular dichroism spectrum is needed to elucidate the stereoselectivity of the P450 and epoxide hydrolases in ligninolytic *P. chrysosporium*.

Besides MnP, other enzymes involved in phenanthrene metabolism, including P450s, seem to be affected by nutrient condition in different ways. The *trans*-9,10-dihydrodiol has been found to be the principal phenanthrene metabolite in nonligninolytic (nutrient-rich) cultures of *P. chrysosporium* ATCC 34541, in which *trans*-3,4-dihydrodiol, phenanthrols and phenanthrol conjugates has also been detected (Sutherland et al. 1991). We obtained similar result with nonligninolytic *P. chrysosporium* ATCC 24725, the strain used in the present work. Phenanthrene *trans*-3,4-dihydrodiol, 3-, 4-, and 9-phenanthrol and 9-phenanthryl β-D-glucopyranoside were all detectable, and their absorbance at 254 nm were all comparable to that of the *trans*-9,10-dihydrodiol (data not published). In contrast, these metabolites were not accumulated in ligninolytic (nutrient-limited) cultures, except the *trans*-9,10-dihydrodiol. The P450 oxidizing PAH at "bay-region" may be down-regulated during ligninolytic metabolism, while the P450 oxidizing at "K-region" was not significantly affected. This leads to the interest in further research on regulation of different P450s and other enzymes involved in PAH metabolism by *P. chrysosporium*.

Interestingly, it has been proposed that phenanthrene 9,10-dihydrodiol was not obviously produced from phenanthrene during ligninolytic metabolism by *P. chrysosporium* (Hammel et al. 1992). Additionally, DPA was reported to be the major metabolite in ligninolytic *P. chrysosporium* (Hammel et al. 1992), and MnP was found to support the oxidation of phenanthrene to DPA (Moen and Hammel 1994). But in our study under almost the same conditions, P450-catalyzed oxidation was found to be another major fate of phenanthrene, because the 9,10-dihydrodiol was evidently formed, and when PB inhibited P450 activity and had no effect on MnP activity, degradation rate of phenanthrene decreased by 53%. This contradiction must be due to the difference on concentration of phenanthrene added to cultures. In the study reported by Hammel et al. (Hammel et al. 1992), the concentration of phenanthrene added was 2 μmol l^{-1}, which is 56-fold lower than that in our study. Moreover, we could not detect P450 in cells from cultures added with 2 μmol l^{-1} phenanthrene (data not shown), thus the P450 might not be induced or not be enough to catalyze detectable oxidation of phenanthrene. These showed effect of substrate concentration on enzymatic mechanism involved in organopollutant degradation by the white rot fungus.

The possible occurrences of some other reactions in phenanthrene metabolism were also suggested in this work. It is known that phenanthrene oxides are unstable in aqueous solution and can either isomerize to phenols or be hydrated (Bruice et al. 1976; Cerniglia and Heitkamp 1989). 9-Phenanthrol and 9-phenanthryl *β*-D-glucopyranoside, which have been detected in nonligninolytic cultures of *P. chrysosporium* (Sutherland et al. 1991), was probably produced according to HPLC analyses in this work. More conclusive analyses

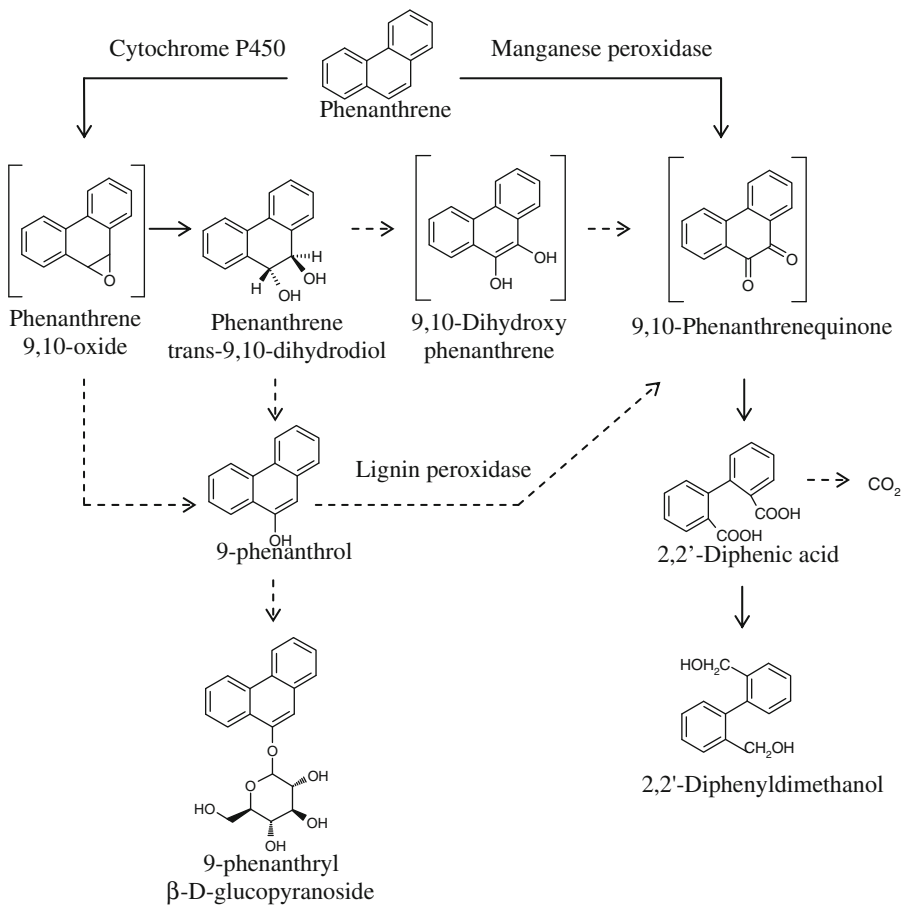

Fig. 6 Proposed pathway for the metabolism of phenanthrene in the ligninolytic culture of *Phanerochaete chrysosporium*. The *dashed arrows* indicate reactions which probably occur and produce trace metabolites. The intermediates shown in brackets are not identified

are still required for identification of these two metabolites. Moreover, the LiP activity was ranged from 91 to 986 U l^{-1} in the cultures (Fig. 5). Although LiPs can not oxidize phenanthrene (Hammel et al. 1986), LiP H8 from *P. chrysosporium* was reported to be able to oxidize 9-phenanthrol to phenanthrene-9, 10-quinone which could be rapidly cleaved to DPA (Tatarko and Bumpus 1994). It was also reported that trace quantities of DPA could be detected after addition of phenanthrene *trans*-9,10-dihydrodiol to ligninolytic cultures (Hammel et al. 1992). According to Hammel et al., 2,2′-Diphenyldimethanol can be produced from DPA in LN cultures of *P. chrysosporium*. Consequently, a proposed pathway for phenanthrene degradation by ligninolytic *P. chrysosporium* is presented in Fig. 6, including the probable reactions (shown as dashed arrows).

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