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ORIGINAL PAPER

Induction of functional cytochrome P450 and its involvement in degradation of benzoic acid by *Phanerochaete chrysosporium*

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Abstract The white rot fungus *Phanerochaete chrysosporium* has the largest cytochrome P450 contingent known to date in fungi, but the study on the function of these P450s is limited. In this study, induction of functional P450 in *P. chrysosporium* was first shown and P450-mediate degradation of benzoic acid was demonstrated in this fungus. Carbon monoxide difference spectra indicated significant induction of P450 by benzoic acid, *m*-chlorobenzoic acid, *p*-chlorobenzoic acid and *n*-hexane, and showed the effect of inducer concentration and nutrient condition on the induction of P450. The high contents of P450 in the microsomal fractions facilitated the study on the function of P450. While the *n*-hexane-induced P450 could not interact with benzoic acid, the microsomal P450 induced by benzoic acid produced type I substrate binding spectra upon the addition of benzoic acid. The benzoic acid degradation by the microsomal P450 was NADPH-dependent at a specific rate of 194 ± 14 min^{-1}, and significantly inhibited by piperonyl butoxide (a P450 inhibitor). However, inhibition of benzoic acid degradation by piperonyl butoxide was slight or not detectable in the cultures of this fungus, suggesting presumable involvement of other enzyme in benzoic acid degradation. The extracellular ligninolytic enzymes, lignin peroxidase and manganese-dependent peroxidase, were not involved in initial metabolism of benzoic acid under the test conditions.

Keywords Cytochrome P450 · Benzoic acid · White rot fungus · *Phanerochaete chrysosporium*

Abbreviations

AADH	Aryl-alcohol dehydrogenase
AALDH	Aryl-aldehyde dehydrogenase
ABT	1-Aminobenzotriazole
BA	Benzoic acid
CBA	Chlorobenzoic acid
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
PDB	Potato dextrose broth

Introduction

White rot fungus is the kind of mycelial fungi that colonize wood in nature and decompose lignin to

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cause white rotting of wood. The white rot fungus *Phanerochaete chrysosporium* has been widely studied for its ability to degrade variety of recalcitrant aromatic compounds such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), dioxins, chlorinated pesticides and some azo dyes (Bumpus 1989; Bumpus et al. 1985; Chung and Stevens 1993). While the extracellular ligninolytic enzymes have been implicated in degradation of many of the aromatic compounds by *P. chrysosporium* (Barr and Aust 1994), the important role of intracellular cytochrome P450s have also been suggested.

Cytochrome P450s are widely distributed hemoproteins involved in various steps of the biosynthesis of endogenous compounds (Jefcoate 1986; Omura 1999; Waterman et al. 1986) and in the oxidative detoxification and elimination of many hydrophobic xenobiotics including pollutants, drugs and pesticides (Omura 1999; Wislocki et al. 1980). About 150 P450 genes, which compose the largest P450 contingent known to date in fungi, have been found in *P. chrysosporium* (Doddapaneni and Yadav 2005; Martinez et al. 2004). These P450 genes of *P. chrysosporium* are classified as new members of 12 families and 23 sub-families of P450s (Doddapaneni et al. 2005a) and are all expressible under both ligninolytic and non-ligninolytic conditions (Doddapaneni and Yadav 2005). Although induction of some P450 (CYP63A1, CYP63A3 and CYP63A3) at the transcriptional level by various aromatic compounds has been observed in *P. chrysosporium* (Doddapaneni et al. 2005b; Doddapaneni and Yadav 2004), activity of induced P450 protein in this fungus has rarely been documented. Inhibition assays suggest involvement of P450s in degradation of several xenobiotics by *P. chrysosporium*, such as lindane (Mougin et al. 1996), endosulfan (Kullman and Matsumura 1996), atrazine (Mougin et al. 1997), diphenyl ether (Hiratsuka et al. 2005), dibenzyl sulfide (Van Hamme et al. 2003), nitrotoluene (Teramoto et al. 2004b) and nitrophenol (Teramoto et al. 2004a). Moreover, a series of compounds known to be P450 substrates in other organisms are degradable by *P. chrysosporium* (Matsuzaki and Wariishi 2004). However, there are limited studies that demonstrate the function of P450s directly in *P. chrysosporium*, and the study on P450-mediated benzo(a)pyrene hydroxylation in this fungus should be the only example (Masaphy et al. 1996).

Benzoic acid (BA) is widely used as plasticizer, food preservative, tobacco seasoner, flavor and perfume and antifungal agent (http://toxnet.nlm.nih.gov), and biodegradation is an important fate process of BA in environments. In microorganisms, BA plays an important role in the metabolism of various aromatic compounds. BA is an intermediate in degradation of many aromatic compounds such as toluene (Luykx et al. 2003), styrene (Braun-Lüllemann et al. 1997; Roldan-Carrillo et al. 2001), lignosulfonate (Shin and Lee 1999) and L-phenylalanine (Lapadatescu and Bonnarme 1999), and can be transformed to ring fission products (Kamada et al. 2002) or different aryl metabolites such as *p*-anisaldehyde, veratraldehyde (Mester et al. 1997) and veratryl alcohol (Harper et al. 1996; Jensen et al. 1994) in white rot fungi. Chlorobenzoic acids (CBA) are used as herbicides and are important intermediates in the metabolism of polychlorinated biphenyls (Adriaens et al. 1989; Gutenkauf et al. 1998).

Proteomic analyses of *P. chrysosporium* exposed to exogenous BA (Matsuzaki et al. 2008) has shown up-regulation of P450s (CYP63A1 and PcCYP1f). The recombinant PcCYP1f in *Pichia pastoris* has been reported to catalyze the hydroxylation of BA into 4-hydroxybenzoic acid (Matsuzaki and Wariishi 2005). However, the post-transcription regulation is complex in *P. chrysosporium*, a eukaryotic microorganism, and thus the BA degradation by P450 in this fungus is more than questionable and has not been addressed. In this study, the active P450s induced by BA and CBA were shown by classic spectrophotometric assays. The effect of nutrient condition and inducer concentration on the induction of P450 was found. Facilitated by high P450 content in substrate-induced cells, P450-mediated degradation of BA was demonstrated in microsomal fraction of *P. chrysosporium*. Furthermore, the role of P450 was also investigated during in vivo degradation of BA by *P. chrysosporium*, as well as activities of the extracellular enzymes (LiP and MnP).

Materials and methods

Microorganisms and growth media

P. 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 according to Tien and Kirk (1988), except that dimethylsuccinate was replaced by 20 mM acetate buffer. Difco potato dextrose broth (PDB, 24 g l^{-1}) was used as nutrient-rich medium, containing about 50–70 mM nitrogen.

Inocula were prepared according to Aiken and Logan (1996). Fungi grown for 6 days in a static culture flask were blended and mixed, and then a 0.5 ml aliquot of this suspension was aseptically transferred to a sterile 300-ml flask containing 100 ml of media.

Cytochrome P450 induction experiments

The fungi were incubated for 60 h at 37°C and 130 rpm in darkness. Then, BA in acetone (100 µl per flask) was added to a final concentration of 1, 2 and 5 mM, respectively. CBA was added to a final concentration of 1 mM. As controls, cultures added with acetone (100 µl per flask) and cultures without addition were set up. The mycelia were harvested after additional 1-day incubation. For induction with *n*-hexane, 200 µl per flask was added each hour to the 100-ml cultures over 6 h, and then the mycelia were harvested.

Microsomal fraction

The mycelia were harvested by centrifugation (5,000g, 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 15,000g for 15 min twice to remove cell debris, nuclei and mitochondria. Then the supernatant was centrifuged at 105,000×*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.

Carbon monoxide difference spectra

Carbon monoxide (CO) difference spectra were recorded as described before (Schenkman and Jansson 1998; 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 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.

Substrate binding spectra

Binding spectra were obtained by recording difference spectra between 350 and 500 nm after addition of BA to microsomal fractions (Jefcoate 1978). The microsomal fraction was diluted to 2 mg ml^{-1} protein with buffer B, and equally placed into two cuvettes. After a baseline spectrum was recorded, BA dissolved in 2.0 µl of dimethylsulfoxide was added to the sample cuvette to a final concentration of 5 mM, while an equal volume (2.0 µl) of dimethylsulfoxide was added to the reference cuvette. After mixing, the difference spectrum was recorded.

Degradation of benzoic acid in microsomal fractions

To investigate P450-mediated degradation of BA in the microsomal fraction, assays were carried out in reaction mixtures (1 ml) containing 0.8 mM BA, 0.2 mM NADPH in buffer B (pH 7.5), and were started by the addition of 1.3–1.4 mg of the microsomal protein. The reaction mixtures were incubated

at 30°C for 1 h. The NADPH oxidation rate was measured as described before (Faber et al. 2001; van den Brink et al. 1996) by monitoring NADPH consumption at 340 nm with interval of 2 s, and the initial rate was calculated according to linear decrease of absorbance in the first 5 min. After incubation for 1 h, the reaction was terminated by the addition of 1 ml ice-cold acetone. Then the mixture was centrifuged for 5 min at 15,000g, and the BA concentration in the supernatant was determined by HPLC (Hewlett Packard LC1100). Three types of control reactions, no-enzyme control, no-BA control and no-NADPH control, were run parallel. Moreover, the reaction mixture added with P450 inhibitor piperonyl butoxide (PB, Aldrich) and the mixture with the microsomal fraction from uninduced cells (without detectable P450) were also set up for comparison.

In vivo degradation of benzoic acid and inhibitor study

The fungi were incubated for 60 h at 37°C and 130 rpm in darkness. BA in acetone (100 µl per flask) was added into the cultures to a final concentration as indicated. The culture supernatants were aseptically sampled when flasks kept shaking, and the BA concentrations and activities of lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP) were determined. As controls, heat-killed (autoclaved) cultures with BA and cultures with no BA but acetone (100 µl per flask) were utilized. For P450 inhibitor study, PB was added to some flasks to the final concentrations of 0.4 and 2 mM, respectively, and BA was added 1 h later.

Extracellular enzyme activity

The activities of LiP and MnP were determined spectrophotometrically by the methods of Tien and Kirk (1988) and Paszczynski et al. (1988), respectively.

Benzoic acid-degrading activity of MnP was analyzed with reaction mixtures (2 ml) containing 1 mM BA, 0.2 mM $MnSO_4$, 0.1 mM H_2O_2 and culture supernatant in 50 mM tartrate buffer (pH 4.5). The culture supernatant was obtained from LN cultures grown for 6 days, and filtered (0.45 µm). The final MnP activity in the reaction mixture was more than 800 U l^{-1}, although Lip activity was less than 10 U l^{-1}. Three types of controls were used: no-enzyme control, no-H_2O_2 control, and the control with neither enzyme nor H_2O_2.

Benzoic acid determination by HPLC

BA concentration was determined by HPLC equipped with a reverse-phase column (4.6 by 250 mm, Hypersil BDS C_{18} 5 µm column). Samples were filtered (0.45 µm), directly injected into HPLC, and isocratically eluted with 5% methanol in 0.02 M ammonium acetate at 1.0 ml min^{-1}. The UV detector was set at 230 nm. BA was identical to sodium benzoate in HPLC elution profile.

Results

Spectrophotometric evidence of the induction of P450 by benzoic acid and chlorobenzoic acid

The involvement of P450s in degradation of some xenobiotics by *P. chrysosporium* has been suggested in many reports, but the occurrence of active P450 during the degradation has rarely been shown. In this work, we measured CO difference spectra of the microsomal fractions from PDB cultures of *P. chrysosporium* incubated with BA, *m*-CBA and *p*-CBA (dissolved in acetone before addition), respectively, as well as the controls added with acetone and those without addition. When the cells were incubated with BA, or *m*-CBA, or *p*-CBA, the peak at 450 nm was present in the spectra (for example Fig. 1a), indicating the presence of P450. Correspondingly, the peak was very slight in the microsomal fraction from acetone-induced cells and not observed in that from uninduced cells (Fig. 1b, c). In the spectra (Fig. 1), there were maxima at 417–420 nm, which presumably reflected the presence of P420, an inactive form of P450. The P450 and P420 contents of the microsomal fractions were calculated according to the spectra and shown in Table 1. Since both P450 and P420 contents increased upon the addition of BA and CBAs, the synthesis of P450s was induced by BA and CBAs. P420s have also been observed in cell fractions of other fungi such as *Pleurotus ostreatus* (Bezalel et al. 1997), *Aspergillus parasiticus* (Bhatnagar et al. 1982) and *Mortierella alpine* (Asperger et al. 1999), suggesting instability of these fungal P450s in cells or during isolation.

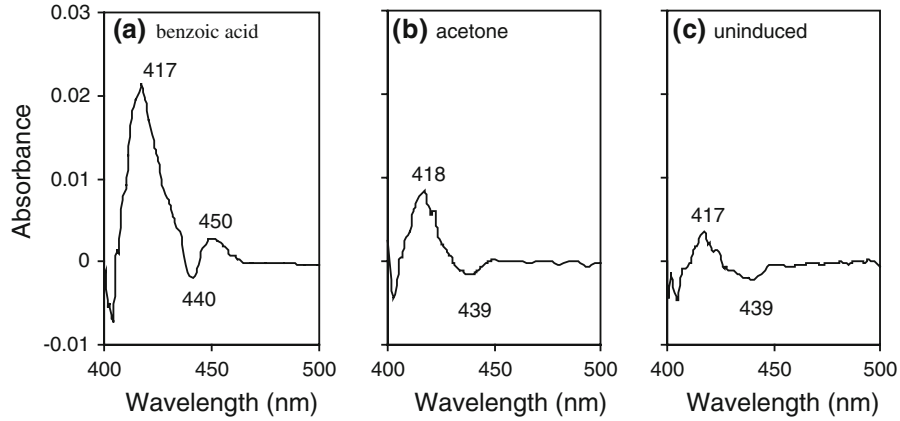

Fig. 1 Carbon monoxide difference spectra of microsomal fractions of *Phanerochaete chrysosporium* from PDB cultures. The 100-ml cultures were added with 1 mM benzoic acid (dissolved in 100 µl acetone, spectrum **a**), 100 µl acetone (spectrum **b**) and without anything (spectrum **c**), respectively; the protein concentration of every sample tested was 1 mg ml^{-1}

Effect of inducer concentration and nutrient condition on the induction of P450

In order to obtain cell fractions with higher P450 content to facilitate P450 studies, BA was added into LN cultures and PDB cultures to different concentrations as inducer. Effect of nutrient condition and BA concentration on induction of P450 and P420 was observed and shown in Table 1. Optimum results were obtained with 1 mM BA added to LN cultures, and the microsomal P450 was increased up to 62 ± 17 pmol (mg protein)$^{-1}$. This paper presented a higher P450 content of microsomal fraction from nutrient-limited cultures (LN) than that from nutrient-rich cultures (PDB), which has been not reported to date in white rot fungi. The P450 contents were not correlated with the initial concentrations of BA. The P420 contents varied corresponding to P450 contents under the test conditions. In addition, the wet weight of biomass in PDB cultures with 5 mM BA was nearly half of those in other samples, suggesting inhibition of cell growth by BA at 5 mM.

Degradation of benzoic acid by P450 in microsomal fractions

To investigate the ability of *P. chrysosporium* P450 to degrade BA, we first examined the interaction of BA with P450 by substrate binding spectra. When the microsomal fraction of 1 mM BA-induced cells from LN cultures was used, the difference spectra in the presence of BA had a maximum at around 390–394 nm and a minimum at 420–424 nm (Fig. 2a). This follows the characteristic type I pattern for molecules that interact with P450s and is related to the formation of enzyme-substrate complexes. When the microsomal fraction of 1 mM BA-induced cells from PDB cultures was used, the type I substrate binding spectrum was recorded with less intensity (Fig. 2b), corresponding to less P450 content. However, when the microsomal fraction containing about 60 pmol P450 per mg protein induced by *n*-hexane was used, substrate binding spectrum was not observed upon the addition of BA (Fig. 2c), indicating the difference between the microsomal P450s induced by hexane and by BA.

BA-degrading activity of P450 was confirmed by in vitro microsomal degradation of BA. The microsomal fraction of BA-induced cells from LN cultures was utilized and the concentration of P450 in the reaction mixture was up to 86 nM. Samples and controls were prepared as described in Materials and methods. The initial rates of NADPH consumption and degradation ratios of BA were determined and shown in Table 2. In the reaction mixtures containing P450, NADPH consumption was very slow in the absence of BA and significant (18-fold higher) upon the addition of BA. Correspondingly, BA degradation was not obvious in the reaction mixture without NADPH, and evident (15-fold higher) in the mixture with NADPH. When the mixture was added with 2 mM PB, a P450 inhibitor, BA degradation ratio and NADPH

Table 1 Cytochrome P450 and P420 contents in microsomal fractions of *Phanerochaete chrysosporium* from different cultures incubated with different inducers

Culture	Inducer	Initial concentration of inducer in the culture (mM)	P450 content in microsomal fraction (pmol mg^{-1})	P420 content in microsomal fraction (pmol mg^{-1})
PDB	–	–	ND	31 ± 6
PDB	Acetone	–	3 ± 2	78 ± 3
PDB	Benzoic acid[a]	1	34 ± 12	198 ± 48
PDB	*m*-Chlorobenzoic acid[a]	1	42 ± 5	106 ± 20
PDB	*p*-Chlorobenzoic acid[a]	1	44 ± 9	178 ± 15
PDB	Benzoic acid[a]	2	13 ± 3	111 ± 11
PDB	Benzoic acid[a]	5	38 ± 1	156 ± 11
LN	–	–	ND	41 ± 11
LN	Acetone	–	ND	73 ± 5
LN	Benzoic acid[a]	1	62 ± 17	367 ± 40

Mean ± standard deviation of the mean, $n = 3$. The cultures incubated for 60 h before addition of inducer and for another 24 h after addition, at 37°C and 130 rpm

PDB potato dextrose broth culture, *LN* low nitrogen culture, *ND* not detectable

[a] Inducer dissolved in acetone before addition

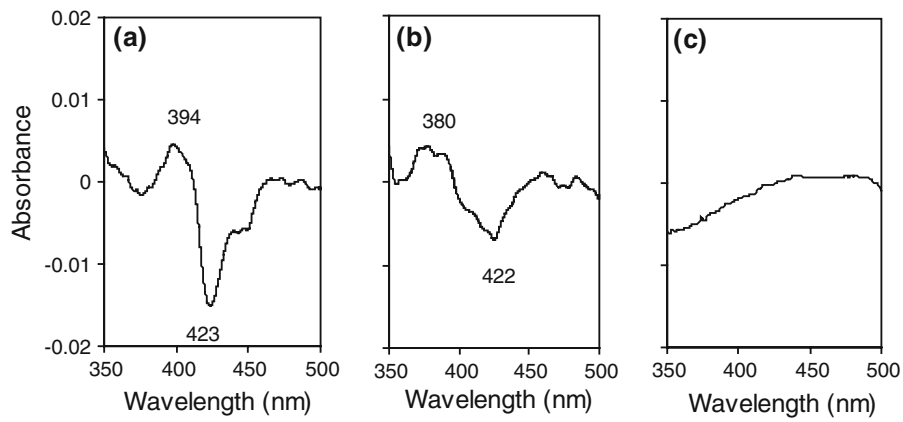

Fig. 2 Difference spectra obtained upon the addition of 5 mM benzoic acid to microsomal fractions of *Phanerochaete chrysosporium*. The microsomal fractions were isolated from LN cultures (spectrum **a**) and PDB cultures (spectrum **b**) incubated with 1 mM benzoic acid for 24 h, and PDB cultures incubated with *n*-hexane (2 µl ml^{-1} h^{-1}) for 6 h (spectrum **c**). The P450 contents of these microsomal fractions were 62, 34, 60 pmol (mg protein)$^{-1}$, respectively, and the protein concentration of every sample tested was 2 mg ml^{-1}

consumption rate were decreased by 90% and 92%, respectively. Furthermore, the reaction mixture without detectable P450 and the no-enzyme control had no significant difference in BA degradation, as well as in NADPH consumption. According to BA-dependent NADPH consumption, the specific rate of BA degradation by the microsomal P450 was up to 194 ± 14 min^{-1} (µmol NADPH oxidized per min per µmol P450).

In vivo degradation of benzoic acid and effect of P450 inhibitor

The in vivo degradation of BA was evaluated based on the disappearance of BA in culture supernatants. BA was undetectable in the controls without exogenous addition of BA throughout the experiment, and this excluded interference by endogenous BA. The decrease of BA concentrations in culture supernatants

Table 2 Initial rate of NADPH consumption and degradation ratio of benzoic acid in microsomal fractions of *Phanerochaete chrysosporium* from LN cultures

Initial concentration in reaction mixtures[a]				Initial rate of NADPH consumption (μM min^{-1})[e]	Degradation ratio of benzoic acid after 1-h incubation (%)[f]
Benzoic acid (mM)	P450 (nM)	NADPH (mM)	Piperonybutoxide (mM)		
0.8	–[b]	0.2	0	0.04 ± 0.01	0 ± 1.2
0	86 ± 23[c]	0.2	0	0.91 ± 0.05	–
0.8	86 ± 23[c]	0	0	–	1.9 ± 1.8
0.8	86 ± 23[c]	0.2	0	17.6 ± 1.2	29.4 ± 3.6
0.8	86 ± 23[c]	0.2	2	1.37 ± 0.20	3.0 ± 1.2
0.8	ND[d]	0.2	0	0.07 ± 0.04	1.6 ± 1.5

Mean ± standard deviation of the mean, $n = 3$

[a] Reaction mixtures were incubated at 30°C for 1 h

[b] The reaction mixture did not contain any enzyme (no-enzyme control)

[c] The microsomal fraction of 1 mM benzoic acid-induced cells was utilized

[d] The microsomal fraction of uninduced cells was utilized. *ND* P450 was not detectable

[e] Initial rate was calculated according to linear consumption of NADPH in the first 5 min

[f] Degradation ratio was calculated according to concentrations of residual benzoic acid in the sample and the no-enzyme control after 1-h incubation

of heat-killed controls were negligible, indicating that there was no detectable adsorption to mycelia and auto-decomposition of BA in cultures.

To investigate the role of P450 during in vivo degradation of BA, the P450 contents and degradation efficiencies of BA in LN cultures and PDB cultures, were compared (Fig. 3). When BA was added to a concentration of 1 mM, the degradation efficiency (degradation rate per gram biomass of fungus) in LN cultures was about 2-fold higher than that in PDB cultures, which correlated to the P450 content of microsomal fraction from LN cultures compared with that from PDB culture. Nonetheless, when BA was added to PDB cultures to different concentrations, there was no correlation between the degradation efficiencies and the P450 contents.

The role of P450 was also assessed by examining effect of P450 inhibitor on BA degradation. PB can significantly inhibit many P450-mediated reactions, and is expected to have no effect on the total activity of *P. chrysosporium* (Teramoto et al. 2004a). We added PB to LN cultures and PDB cultures to final concentrations of 0.4 and 2 mM before the addition of 1 mM BA. As a result, BA (1 mM) was almost exhausted in 20 h in the cultures with or without the addition of PB. Addition of PB led to very slight inhibition of in vivo degradation of BA in PDB

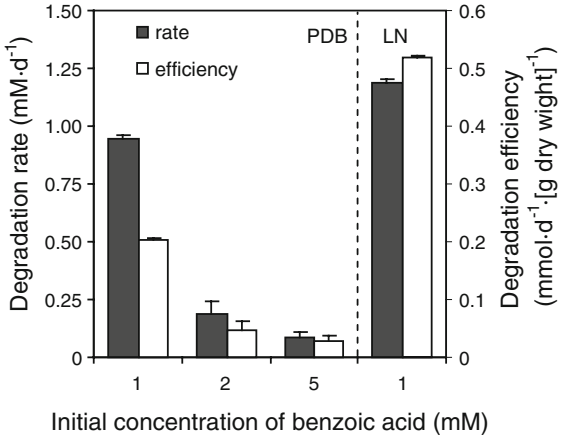

Fig. 3 Degradation rate and efficiency of benzoic acid at different initial concentration by *Phanerochaete chrysosporium* in LN cultures (LN, *right*) and PDB cultures (PDB, *left*). After preincubated for 60 h, the cultures were added with benzoic acid and incubated for 24 h at 37°C and 130 rpm. Degradation efficiency was the degradation rate divided by dry weight of the fungal biomass. *Error bars* indicate standard deviation, $n = 3$

cultures (Fig. 4a), and undetectable inhibition in LN cultures (Fig. 4b). Since BA-degrading activity of P450 was significantly inhibited by PB according to the study in microsomal fraction (as shown in Table 2), the efficient disappearance of BA in the

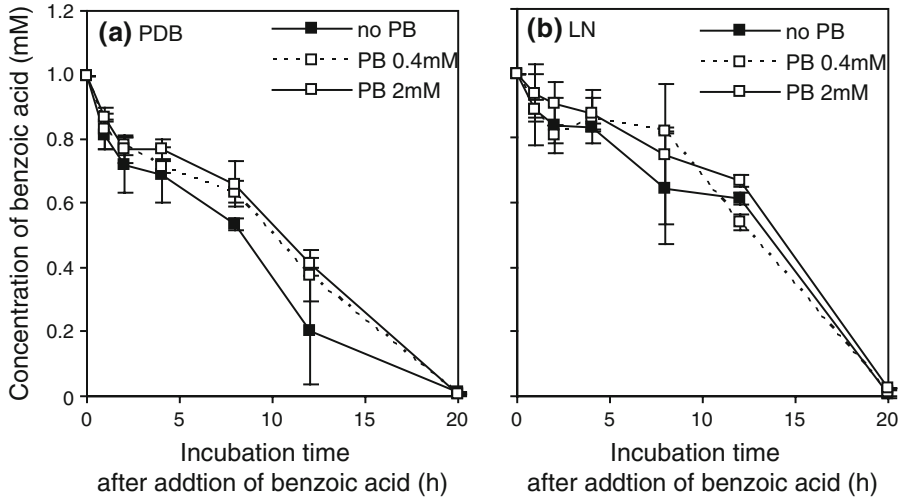

Fig. 4 Effect of P450 inhibitor piperonyl butoxide (PB) on the degradation of benzoic acid by *Phanerochaete chrysosporium* in PDB cultures (PDB, **a**) and LN cultures (LN, **b**). Benzoic acid was added after the cultures were preincubated for 60 h. The cultures were incubated at 37°C and 130 rpm. *Error bars* indicate standard deviation, $n = 3$

cultures added with PB was probably contributed to other enzyme in *P. chrysosporium*.

Role of ligninolytic enzymes in degradation of benzoic acid

The extracellular ligninolytic enzymes of *P. chrysosporium*, LiP and MnP, are involved in degradation of many aromatic compounds, such as PAHs, PCBs, azo dyes, etc. BA degradation by LiP and MnP was presumable but not evidenced. In order to investigate involvement of these enzymes in BA degradation, we determined activities of LiP and MnP during in vivo degradation of BA, and analyzed BA degradation in extracellular fraction with high activity of MnP. In the cultures incubated with BA for 24 h, LiP activity was not detectable, and MnP activity about 0–80 U l^{-1} was detected, but had no correlation with degradation efficiencies of BA. BA was added to reaction mixture with MnP activity of 815 U l^{-1}, but was not detectably degraded in both samples and controls. These results indicate that MnP and LiP were not involved in BA degradation under the test conditions.

Discussion

In the present work, we intensively studied the role of P450 in BA degradation by *P. chrysosporium*. The presence of microsomal P450 was clarified by the results of spectrophotometric assays. CO difference spectra of the microsomal fractions of BA-induced cells from LN and PDB cultures showed typical peak at 450 nm, while the peak was not present for uninduced cells and very slight for acetone-induced cells. This result indicated evidently induction of active P450 by BA, and thus evidenced that P450 is important for BA degradation by *P. chrysosporium*. In accordance with this, two P450s (CYP63A1 and PcCYP1f) have been found to be newly produced upon the addition of 1 mM BA, according to proteomic analysis of this fungus from LN cultures (Matsuzaki et al. 2008). Induction of PcCYP1f at a transcriptional level (2.4-fold) by benzoate has been observed in LN cultures (Matsuzaki and Wariishi 2005), while transcriptions of *cyp63A3* and *cyp63A1* were induced 2.73- and 1.36-fold in the presence of benzoate in nutrient-rich (malt extract) cultures (Doddapaneni et al. 2005b; Doddapaneni and Yadav 2004).

Binding of BA with microsomal P450 of *P. chrysosporium* was proved by type I pattern spectrograms. This pattern represented a shift of electrons of ferrous ions from a low to a high spin state and is one of the steps involved in the catalytic cycle of P450 (Jefcoate 1978; Sato and Omura 1978). This paper showed the typical binding spectra of microsomal fractions from both nutrient-limited (LN) and nutrient-rich (PDB) cultures, and revealed the correlation between the intensity of substrate binding spectra and the P450 contents of microsomal fractions. Moreover, BA was also added to microsomal fraction of *n*-hexane-induced cells with adequate amount of P450, but no substrate binding spectrum was found. Hexane can

induce a variety of different hydrocarbon-oxidizing microbial P450s, and has been used as multivalent inducer to screen P450s in various bacteria and yeasts. P450 of *n*-hexane-induced *Rhodococcus rhodochrous* was suggested to have versatile substrates including guaiacol and 2-ethoxyphenol (Asperger et al. 1994). In particular, our result indicated substrate specificity of P450 induced by hexane, reflecting functional diversity of P450s in *P. chrysosporium*.

It is known that fungal P450 systems are involved in oxidation of some xenobiotics, including BA, toluene, phenanthrene and benzo(a)pyrene (Bezalel et al. 1997; Faber et al. 2001; Luykx et al. 2003; Maspahy et al. 1999). All these eukaryotic P450s utilize NADPH as electron donor of the monooxygenation reactions, whereas most bacterial P450s receive electrons from NADH (Omura 1999). In the present study, BA degradation and NADPH consumption were considerable in reaction mixture containing microsomal P450 and NADPH, and was significantly inhibited by PB (a P450 inhibitor), while BA degradation was not evident in the mixtures without NADPH or sufficient P450, indicating stringent requirement for P450 and NADPH.

According to all the evidences described above, we conclude that P450 is involved in BA degradation by *P. chrysosporium* and it should be the first time that induction of functional P450 in this fungus was shown. The microsomal P450 of BA-induced cells catalyzed BA degradation at a specific rate of 194 ± 14 min^{-1}. This is similar to the value of 240 ± 30 min^{-1} estimated by Faber et al. (2001) for microsomal P450 of *Aspergillus niger*, and higher than 40 min^{-1} in the case of P450rm in *Rhodotorula minuta* (Fukuda et al. 1996).

Benzoic acid was hydroxylated only at 4-position by the P450s of *A. niger* and *R. minuta*, and the recombinant PcCYP1f of *P. chrysosporium* (Matsuzaki and Wariishi 2005). P450-mediated hydroxylation of BA at other positions has not been reported to date in fungi. Based on our results for induction of P450 by *p*-CBA, it is possible that P450 is involved in metabolism of this 4-substituted BA, which should be a type of reaction other than 4-hydroxylation. This provided an indirect evidence of P450-mdiated oxidation of BA other than 4-hydroxylation in *P. chrysosporium*. This fungus has been found to hydroxylate BA at three different positions to form 2- and 4-hydroxybenzoic acid and 3-methoxy-4-hydroxybenzoic acid, and P450s were suspected to be involved (Matsuzaki and Wariishi 2004). To confirm this, it will be necessary to characterize products of P450-mediated degradation of BA.

The effect of BA concentration and nutrient condition on active P450 contents in *P. chrysosporium* was examined for the first time, providing some information about complex regulation of P450 expression in this fungus. The correlations between P450 contents and inducer concentrations have been reported, and resulted in application of certain P450s as indicators of pollution or toxicity (Anderson et al. 1999; Chiba et al. 2002). Nonetheless, this correlation was not observed in our work (Table 1). Additionally, the induction of *cyp63A3* transcription by BA was considerable (2.73-fold over control) in nutrient-rich culture, but not detectable in nutrient-limited cultures (Doddapaneni et al. 2005b). In contrast, we found that the content of active P450 induced by BA in nutrient-limited cultures was higher than that in nutrient-rich culture. The complexity of transcriptional regulation has been shown by microarray-based global differential expression profiling of P450s in *P. chrysosporium*. All the 150 P450 genes have been found to be expressible, and tandem P450 member genes in 10 of the 16 P450 genomic clusters have showed nonassortative regulation of expression (Doddapaneni and Yadav 2005). These results lead to the interest in further research of transcriptional and post-transcriptional regulation of different P450s in *P. chrysosporium*.

Furthermore, multiple pathways of BA degradation by *P. chrysosporium* were suggested in this report. PB, a classic inhibitor of P450s, has significantly inhibited the reactions involved in metabolisms of diphenyl ether, 4-nitrophenol, 4-nitrotoluene and endosulfan in *P. chrysosporium* (Hiratsuka et al. 2005; Kullman and Matsumura 1996; Teramoto et al. 2004a, b), and inhibited the P450-mediated BA hydroxylation in rice seedlings and *Tyromyces palustris* (Kamada et al. 2002; Sawada et al. 2006). In our study, although P450 was demonstrated to be involved in BA degradation, inhibition of BA degradation by PB was slight in PDB cultures and was not detectable in LN cultures. In accordance with this, another P450 inhibitor, ABT has no effect on BA degradation by this fungus (Da-Liang et al. 2007), and the contents of microsomal P450s in BA-induced cells did not correspond to the in vivo degradation efficiencies of BA. All these suggested presumable involvement of

other enzyme in BA degradation by *P. chrysosporium*. Some enzymes are susceptible. The AADH and AALDH have been found to be significantly induced by BA, and the conversion of BA to benzaldehyde and benzyl alcohol has been proposed in LN cultures of this fungus (Matsuzaki et al. 2008). Benzoate-coenzyme A ligase or benzoate 1,2-dioxygenase is involved in BA transformation by some bacteria (Auburger and Winter 1996; Haddad et al. 2001), however, the metabolites relied on these enzymes have not been detected in *P. chrysosporium*.

The study on wastewater treatment has found correlation between the MnP specific activity and the sludge loading rates of aromatics including BA (Zhang et al. 2006), however, our results indicated that MnP were not involved in initial metabolism of BA. Under our conditions, LiP activities were not detectable and thus could not be involved in BA degradation. However, the production of hydroxyl radical by LiP has been reported by Barr and Aust (1994), and benzoate is usually used as a hydroxyl radical scavenger (Baliga et al. 1998; Ravishankar and Ramasarma 1995). Thus, the possibility cannot be excluded that LiP can catalyze BA hydroxylation. Moreover, this paper showed higher degradation efficiencies of BA and P450 contents in induced cells from LN cultures than that from PDB culture. Therefore, the reason for the correlation between MnP activity and BA degradation was probably not BA degradation by MnP, but up-regulation of BA-degrading enzymes such as P450 or LiP.

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