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Journal of Molecular Catalysis B: Enzymatic 5 (1998) 423–428

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Involvement of 3,4-dichlorophenol hydroxylase in degradation of 3,4-dichlorophenol by the white rot fungus *Phanerochaete chrysosporium*

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Received 26 September 1997; accepted 3 December 1997

Abstract

Phanerochaete chrysosporium was able to grow in a C-limited medium containing 3,4-dichlorophenol. In comparison with cultures without 3,4-dichlorophenol, we observed about 30% reduction of the biomass together with the lack of lignin peroxidase and manganese peroxidase production. A weak dechlorination was observed but only methylated dichloroaromatic products such as 3,4-dichloroanisole, 4,5-dichloroguaiacol, and 4,5-dichloroveratrole were identified as chlorinated metabolites showing that methylation, but also hydroxylation reactions occurred. To confirm this hydroxylation reaction we have studied the degradation of 3,4-dichlorophenol, and of several chlorinated metabolites by mycelial pellets of *P. chrysosporium* lacking peroxidase activity. Under these conditions the degradation and the dechlorination of 3,4-dichlorophenol increased strongly and the amount of chloride recovered was 80% of the stoichiometric amount recoverable. We observed similar results with 4,5-dichlorocatechol. An NADPH-dependent 3,4-dichlorophenol hydroxylase was detected in cell free extracts and 4,5-dichlorocatechol was identified as hydroxylation product.

Keywords: 3,4-Dichlorophenol; Hydroxylase; *Phanerochaete chrysosporium*; Lignin peroxidase; Degradation

1. Introduction

The ability of the lignin degrading fungus *Phanerochaete chrysosporium* to degrade and mineralize a wide variety of environmentally persistent organic pollutants has generated interest in using this organism in the treatment of hazardous material [1,2]. The breakdown of most organopollutants by *P. chrysosporium* is closely linked with ligninolytic metabolism and it is generally thought that lignin peroxidase (LiP) and manganese peroxidase (MnP) whose normal function is lignin degradation also catalyze xenobiotic oxidations [3].

However several organopollutants degraded by this fungus [4–6] are not LiP or MnP substrates and it seems clear that other peroxidases independent mechanisms must exist for their

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1381-1177/98/$19.00
PII: S1381-1177(98)00056-3

initial oxidation. Moreover, a number of chloroaromatic pollutants [7,8] and polychlorinated biphenyls [9] were shown to be extensively degraded by *P. chrysosporium* in nutrient rich media in which LiP and MnP are not known to be produced.

In order to effectively use this fungus for remediation of hazardous wastes, it is essential to elucidate the metabolic pathways responsible for xenobiotic degradation and to characterize the major enzymes involved and their expression conditions. In this study we have shown that 3,4-dichlorophenol (3,4-DCP (I)) was dechlorinated by mycelial pellets of *P. chrysosporium* lacking peroxidase activity. In these conditions the first step of the 3,4-DCP degradation was an hydroxylation reaction of 3,4-DCP to 4,5-dichlorocatechol (II) catalyzed by an NADPH-dependent hydroxylase.

2. Experimental

2.1. Chemicals, strain and culture conditions

Chloroaromatic products were purchased from Sigma-Aldrich Chimie (St. Quentin Fallavier, France) and from Helix Biotech (Richmond, Canada). *P. chrysosporium* (ATCC 24725) was grown in agitated liquid medium at 32°C, under air atmosphere. The culture medium was composed of 3 g/l glycerol, 2.4 g/l ammonium tartrate, 2 g/l KH_2PO_4, 0.5 g/l $MgSO_4$,$7H_2O$, 0.108 g/l $Ca(CH_3COO)_2$, 0.5 g/l Tween 80, 1 g/l of commercial phospholipids (NAT 80) supplied by Nattermann Phospholipid, 0.34 g/l veratryl alcohol, 0.86 mg/l thiamine disulfide, $3H_2O$, 30 mg/l $MnSO_4$, H_2O, 0.27 mg/l $Fe_2(SO_4)_3$, 6 mg/l $FeSO_4$, $7H_2O$, 0.7 mg/l $ZnSO_4$, $7H_2O$, and 0.07 mg/l $CuSO_4$, $7H_2O$. A 10-mM acetate solution, pH 4.5, was used as buffer. The following culture volumes were employed: 50 ml/250 ml or 1000 ml/6000 ml (medium/Erlenmeyer flasks). Agitation rates were 160 and 140 rpm respectively. A suspension of conidia in 0.1% Tween 80 served as inoculum to obtain a final concentration of 10^5 conidia/ml. LiP and MnP activities were measured with veratryl alcohol and vanillylacetone respectively as substrates [10].

2.2. Metabolism of 3,4-DCP

3,4-DCP in methanol was added to culture medium at a final concentration of 250 μM. To follow the degradation of 3,4-DCP the mycelium was recovered after various times of incubation by filtration on a Büchner funnel. The supernatant was acidified with H_2SO_4. The pellets were ground in sand and both the mat and extracellular medium were extracted three times with ethyl acetate. The total organic fraction was washed with water, dried over anhydrous magnesium sulfate and evaporated to dryness under reduced pressure. Products were dissolved in methanol and analyzed by high performance liquid chromatography (HPLC) or gas chromatography-mass spectrometry (GCMS).

2.3. Chloroaromatic products degradation by P. chrysosporium mycelium

P. chrysosporium mycelium was produced in culture medium (1000 ml) containing 3,4-DCP (250 μM) in order to repress peroxidase expression. After 7 days of incubation, the mycelium was recovered by filtration and washed with 10 mM acetate buffer pH 4.5. The pellets were resuspended in the same buffer. The experiments started with the introduction of the chloroaromatic product at a final concentration of 250 μM. Every day, aqueous samples from the flasks were centrifuged 10 min at 5000 rpm and organic compounds were quantified using HPLC.

2.4. Analytical methods

HPLC analysis was conducted with an HPLC-System 400 (Kontron Instruments) equipped with a C18 reversed-phase Ultrasphere 5 μm ODS 0.46 $\times$ 15 cm column. The mobile

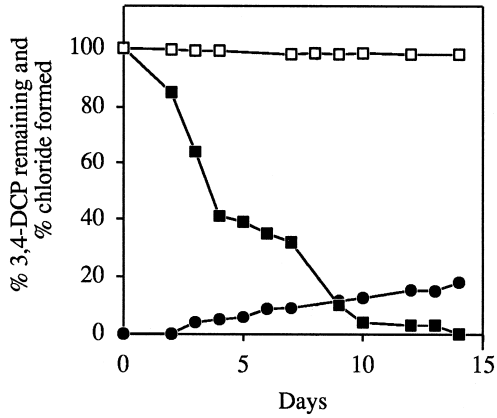

Fig. 1. Degradation of 3,4-DCP added to *P. chrysosporium* cultures on day 0. ■ 3,4-DCP. ● chloride ion. □ abiotic degradation of 3,4-DCP in culture medium without fungus. Values are the means of duplicate experiments. The percent error of the mean for 3,4-DCP and chloride ion determinations were less than 10%.

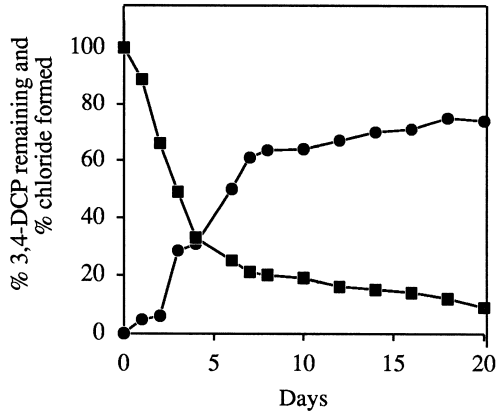

Fig. 2. Degradation of 3,4-DCP by 7-day-old washed mycelium grown in a culture medium containing 3,4-DCP. ■ 3,4-DCP. ● chloride ion. Values are means of triplicate experiments. The percent error of the mean for 3,4-DCP and chloride ion determinations were less than 12%.

phase gradient consisted of methanol-water acidified with 4 mM phosphoric acid (starting at 20/80 for 5 min and following a linear gradient to 100% methanol over 35 min). The flow rate throughout the gradient was 1 ml min^{-1}. The UV detector was set at 230 and 290 nm. The concentration of chloride ion in the samples was measured with a specific electrode (model 96-17B Orion) or with a colorimetric method using mercuric thiocyanate [11]. GCMS was performed at 70 eV on a VG 70/250 SEQ mass spectrometer fitted with an HP5890 gas chromatograph and a 50 m Ultra 1 column (Hewlett Packard). The gas chromatograph temperature was programmed from 50 to 340°C with a temperature gradient of 10° min^{-1}.

2.5. Preparation of cell-free extract and 3,4-DCP hydroxylase assay

A 7-g mycelium mat obtained as described above was resuspended in 14 ml of 50 mM sodium phosphate buffer pH 7.0 containing 0.004% (w/v) PMSF and 1 mM EDTA and was subjected to sonication in pulse mode using Branson Sonifier 450 for 18 min at 80 W. The sonicate was centrifuged at $15\,000 \times g$ for 20 min at 4°C in a Sigma 2K15 centrifuge to remove unbroken cells, and then ultracentrifuged at $120\,000 \times g$ for 90 min in a Centrikon T-1045 (Kontron Instruments) to remove particulate material. 3,4-DCP hydroxylase activity was assayed by measuring the quantity of 4,5-dichlorocatechol formed. The reaction mix-

Table 1
Mass spectra of fungal metabolites

Substrate or metabolite	Mass spectrum m/z (relative intensity)
3,4-DCP	162(100), 135 (7.2), 99 (40), 63 (34.5), 37 (14.5)
3,4-dichloroanisole	176 (100), 161 (40), 133 (52.7), 111 (9), 97 (5), 74 (12.7), 63 (25.4), 37 (7.2)
4,5-dichloroguaiacol	192 (90.4), 177 (100), 149 (62), 133 (9.5), 113 (21.4), 97 (14.3), 85 (31), 73 (9.5), 62 (19), 50 (26), 28 (14.3)
4,5-dichloroveratrole	206 (100), 191 (71.4), 163 (33.3), 145 (19), 128 (52.4), 113 (38), 99 (40.4), 85 (16.6), 73 (9.5), 62 (19), 50 (23.8), 28 (23)

Products identified from the *P. chrysosporium* metabolism of 3,4-DCP. 3,4-DCP was added to culture medium before inoculation. Cultures were incubated for 5 days and extracted. Then products were analyzed as described in the text.

Table 2
Degradation of chloroaromatic products by *P. chrysosporium* mycelium lacking peroxidase activity

Substrates	Degradation %[a]	Chloride ion %[b]
3,4-DCP	80	64
4,5-dichlorocatechol	100	78
4,5-dichloroguaiacol	80	14
4,5-dichloroveratrole	15	6

Substrates were used at 250 μM. Results are expressed in percentage of substrate degradation[a] and chloride ion[b] released after 7 days of incubation.

ture (1 ml) contained 40 μM FAD, NADPH generating system consisting of 250 μM glucose-6-phosphate, 250 μM $NADP^+$ and 0,3 U/ml glucose-6-phosphate dehydrogenase, 200 μl crude extract in 67 mM MOPS buffer, pH 7.0. The reaction was initiated by addition of 250 μM 3,4-DCP and conducted for 30 min at 37°C. The reaction was stopped by trichloroacetic acid addition. The solution was centrifuged 5 min at 5000 rpm and then analyzed by HPLC on a C18 reversed phase Lichrospher 5 μm 0.46×25 cm column with a mobile phase consisting of 60% A (acetonitrile/water/acetic acid 20/76/4), 40% B (acetonitrile/acetic acid 96/4) during 2 min followed by a linear gradient to 100% B over 8 min. Flow rate was 0.9 ml min^{-1} and UV detector was set at 290 nm. One unit of 3,4-DCP hydroxylase activity was defined as the amount of enzyme required for the formation of 1 μmol of 4,5-dichlorocatechol per minute.

3. Results and discussion

Under the culture conditions the formation of regular pellets was observed and high levels of LiP and MnP (1000 and 800 IU/l) were obtained. As other chlorophenols [12], 3,4-DCP was oxidized by purified LiP and when the pollutant was added to cultures after appearance of peroxidases, a similar pathway as the one observed for the degradation of 2,4-dichlorophenol [13] was responsible, at least in part, for the dechlorination of 3,4-DCP (results not shown).

However *P. chrysosporium* was able to grow on the culture medium containing 250 μM 3,4-DCP. In comparison with cultures without 3,4-DCP, we observed circa 30% reduction of the biomass together with the lack of LiP and MnP production. Although the complete disappearance of 3,4-DCP was achieved after about 2 weeks, only a weak amount of chloride, corresponding approximately to 15% of the starting material was recovered (Fig. 1). Furthermore the single chlorinated products detected by GCMS in ethyl acetate extracts were methylated dichloroaromatic products such as 3,4-dichloroanisole (III), 4,5-dichloroguaiacol (IV) and 4,5-dichloroveratrole (V) (Table 1). The methylation of phenols by *P. chrysosporium* has often been observed during the degradation of various aromatics [13–15] and the formation of 3,4-dichloroanisole which was rapidly volatilized under our agitated culture conditions is the major pathway of biotransformation of 3,4-DCP during the fungus growth phase. However, the formation of 4,5-dichloroguaiacol and 4,5-dichloroveratrole suggests that a hydroxylation reaction of 3,4-DCP to 4,5-dichlorocatechol, which is subsequently methylated to 4,5-dichloroguaiacol and 4,5-dichloroveratrole, competed with the direct methylation of 3,4-DCP.

To confirm this hydroxylation reaction we have studied the degradation of 3,4-DCP and of several chlorinated metabolites by washed pellets obtained from *P. chrysosporium* cultures

Table 3
Components required for 3,4-DCP hydroxylase activity

Omission from the complete system	Addition	Activity %
None	–	100
3,4-DCP	–	0
Protein extract	–	0
NADPH generating system	–	0.5
NADPH generating system	NADPH (250 μm)	94.5
FAD	–	64

The complete assay system contained FAD, NADPH generating system and 3,4-DCP as described in Section 2.

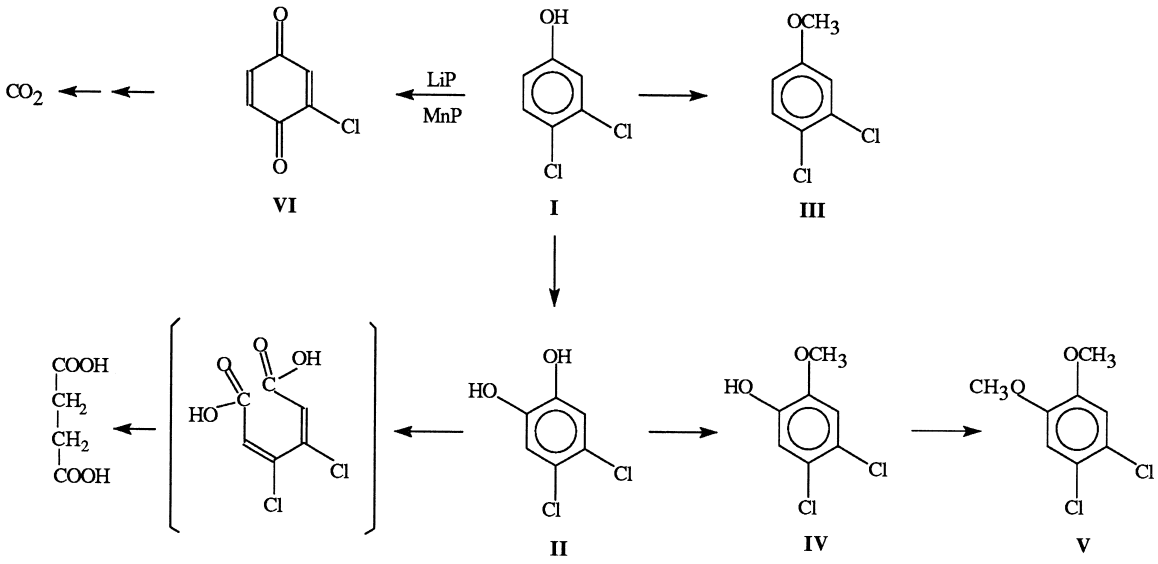

Fig. 3. Proposed metabolic pathways for 3,4-DCP degradation by *P. chrysosporium*. I: 3,4-dichlorophenol; II: 4,5-dichlorocatechol; III: 3,4-dichloroanisole; IV: 4,5-dichloroguaiacol; V: 4,5-dichloroveratrole; VI: 2-chloro-1,4-benzoquinone.

supplemented with 3,4-DCP to repress the peroxidases production and produce the hydroxylation enzyme. Under these conditions 3,4-DCP was strongly degraded and dechlorinated (Fig. 2). The amount of chloride recovered was 80% of the stoichiometric amount recoverable. As previously observed no monochloroaromatic product was detected by GCMS in ethyl acetate extracts. Similar results were observed with 4,5-dichlorocatechol. In contrast, 4,5-dichloroguaiacol was methylated under these conditions to produce 4,5-dichloroveratrole which itself was not readily metabolized by the fungus (Table 2). These results show that the methylation reactions are not strategic steps in the observed dechlorination of 3,4-DCP.

After mycelium sonication and ultracentrifugation of the sonicate, an NADPH-dependent 3,4-DCP hydroxylase activity was measured in the cell soluble fraction (Table 3). 4,5-dichlorocatechol was identified in the samples as a hydroxylation product by comparing its retention time by HPLC with an authentic standard. The 3,4-DCP hydroxylase from *P. chrysosporium* a dimer of 129 kDa has been partially purified (results not shown) and its characterization is actually in progress in our laboratory.

These results and the absence of monochloroaromatic degradation products suggest a pathway involving an hydroxylation of 3,4-DCP to form 4,5-dichlorocatechol (Fig. 3), followed by a cleavage of the aromatic ring and elimination of chlorine from the aliphatic intermediates as observed with bacteria [16]. In conclusion, this study shows that the first step of 3,4-DCP degradation may occur by oxidation, methylation or hydroxylation reactions (Fig. 3), confirming the high enzymatic potential of *P. chrysosporium* for the degradation of xenobiotics. However the efficiency of the dechlorination is tightly depending on expression conditions of these different enzymatic activities.

Acknowledgements

Our research was supported in part by the Conseil Régional d'Aquitaine and the Centre Technique du Papier (Grenoble).

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