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Short Communication

Oxidative dechlorination of methoxychlor by ligninolytic enzymes from white-rot fungi

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Abstract

Ligninolytic enzymes, manganese peroxidase (MnP), laccase, and lignin peroxidase (LiP), from white-rot fungi were used in an attempt to treat methoxychlor (MC), a chemical widely used as a pesticide. MnP and laccase in the presence of Tween 80 and 1-hydroxybenzotriazole (HBT), respectively, and LiP were found to degrade MC, and MnP-Tween 80 decreased MC levels by about 65% after a 24-h treatment. MC was converted into methoxychlor olefin (MCO) and 4,4′-dimethoxybenzophenone by MnP-Tween 80 or laccase-HBT treatment. These results indicate that ligninolytic enzymes from white-rot fungi can catalyze the oxidative dechlorination of MC. Moreover, a metabolite MCO was also degraded by MnP-Tween 80 or laccase-HBT treatment.

Keywords: Methoxychlor; Manganese peroxidase; Laccase; Lignin peroxidase; Dechlorination

1. Introduction

Human exposure to environmental contaminants that negatively affect the male reproductive system is increasing (Sharpe, 1993), and many of these contaminants, such as organochlorine pesticides, accumulate in the fatty tissues of the body. Methoxychlor (MC), 1,1,1-trichloro-2,2-bis(*p*-methoxyphenyl)ethane, is a newly developed pesticide that is intended as a replacement for 1,1,1-trichloro-2,2-bis(chlorophenyl)ethane (DDT), the use of which has been regulated internationally since the 1970s. MC displays both estrogenic and antiandrogenic activities in vivo and in vitro (Maness et al., 1998) and is considered to be a proestrogen that, when converted into mono- and bis-hydroxy metabolites, exhibits greater estrogenic properties than does the MC parental compound (Bulger et al., 1978). Because of the estrogenicity of MC metabolites, exposure of either the neonatal or the adult organism to high doses of MC may place the male reproductive system at risk, and MC has been reported to affect the reproductive success of male rats via decreasing sperm counts (Chapin et al., 1997).

There is a great interest in the lignin-degrading white-rot fungi and their ligninolytic enzymes, because their industrial potential for degrading recalcitrant environmental pollutants, such as polychlorinated dibenzodioxin, DDT (Bumpus et al., 1985), chlorophenols (Joshi and Gold, 1993), and polycyclic aromatic carbons (Bezalel et al., 1996; Collins et al., 1996), have become increasingly recognized. Recently, we demonstrated that ligninolytic enzymes such as manganese peroxidase (MnP) and laccase in the presence of Tween 80 and 1-hydroxybenzotriazole (HBT), respectively, are effective in removing the estrogenic activities of bisphenol A, nonylphenol (Tsutsumi et al., 2001) and steroidal hormones (Suzuki et al., 2003). Herein we present the application of MnP-Tween 80, laccase-HBT, and lignin peroxidase (LiP) to the treatment of MC and describe the dechlorination of MC without the production of hydroxy metabolites.

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2. Materials and methods

2.1. Chemicals

MC, methoxychlor olefin [1,1-dichloro-2,2-bis(*p*-methoxyphenyl)ethylene; MCO], and 4,4′-dimethoxybenzophenone (DMB) used in this study were extra-pure grade and were purchased from Wako Pure Chemicals, Sigma Chemical Company, and Tokyo Chemical Industry, respectively. All other chemicals were also extra-pure grade, obtained from commercial sources, and used without further purification.

2.2. Enzyme assay and preparation

Laccase activity was determined by monitoring the oxidation of 2,6-dimethoxyphenol (DMP) at 470 nm. The reaction mixture contained 1 mM DMP and 50 mM malonate buffer (pH 4.5). MnP activity was determined in the same manner except that the reaction mixture contained 1 mM $MnSO_4$ and 0.2 mM H_2O_2. LiP activity was determined by monitoring the oxidation of veratryl alcohol (VA) at 310 nm. The reaction mixture contained 1 mM VA, 20 mM succinate buffer (pH 3.0), and 0.2 mM H_2O_2. One katal (kat) of enzyme activity is the amount of enzyme producing 1 mol of the quinone dimer (49.3 $mM^{-1}\,cm^{-1}$) or veratraldehyde (9.3 $mM^{-1}\,cm^{-1}$) from DMP or VA, respectively, per second. Partially purified MnP was isolated from cultures of *Phanerochaete chrysosporium* (ME-446) by DEAE Sepharose CL-6B (Pharmacia) column chromatography (Wariishi et al., 1992). LiP was partially purified from cultures of *P. chrysosporium* by Mono Q HR5/5 (Pharmacia) column chromatography (Wariishi and Gold, 1990). Partially purified laccase was prepared from cultures of *Trametes versicolor* (IFO-6482) as described in our previous report (Nishida et al., 1999). Each purified ligninolytic enzyme preparation contained no other ligninolytic enzyme activities.

2.3. Enzyme treatment

For reactions with MnP, 10 ml reaction mixture consisted of 10 nkat MnP, 0.1 ml of 10 mM substrate in dimethylsulfoxide (DMSO), 1 mM $MnSO_4$, 0.1% Tween 80, 4 nkat glucose oxidase, and 2.5 mM glucose in 50 mM malonate buffer (pH 4.5). The laccase reaction mixture (10 ml) contained 10 nkat laccase, 0.1 ml of 10 mM substrate in DMSO, and 0.2 mM HBT in 50 mM malonate buffer (pH 4.5). The LiP reaction mixture (10 ml) contained 10 nkat LiP, 0.1 ml of 10 mM substrate in DMSO, 4 nkat glucose oxidase, and 2.5 mM glucose in 20 mM succinate buffer (pH 3.0). Reactions were carried out at 30 °C for 24 h. The amounts of MC, MCO, and DMB were determined by high-performance liquid chromatography (HPLC) analysis, and the identifications of the metabolites were carried out by gas chromatography–mass spectrometry (GC–MS) analysis.

2.4. Instrumentation

HPLC analyses were conducted with a Wakosil-II 5C18HG (Wako) column, isocratic elution with 80% methanol aqueous at a flow rate of 1 ml/min, and detection at 280 nm. GC–MS was carried out at 70 eV on a Shimadzu QP-5050A equipped with a 30-m fused capillary column (TC-1, Shimadzu). The oven temperature was programmed to increase from 100 to 270 °C at a rate of 10 °C/min. Products were identified by comparing their retention times on GC and comparing mass fragmentation patterns with those of authentic compounds.

3. Results and discussion

We applied the ligninolytic enzymes, 10 nkat of MnP, laccase, and LiP, to the treatment of MC. As Table 1 indicates, MnP-Tween 80 system decreased levels of MC by about 65% after a 24-h treatment and was much more effective in degrading MC than were either LiP or lac-

Table 1
Degradation of methoxychlor by ligninolytic enzymes

Enzymes	Reaction time (h)	Decrease of MC (%)	Production of metabolites (%)	
			MCO	DMB
MnP	12	42.6 ± 2.3	4.2 ± 0.5	n.d.
	24	64.9 ± 4.1	6.2 ± 0.4	trace
LiP	12	15.2 ± 1.6	n.d.	n.d.
	24	28.4 ± 2.1	n.d.	n.d.
Laccase	12	12.8 ± 2.4	0.4 ± 0.1	n.d.
	24	23.3 ± 2.6	0.7 ± 0.1	trace

n.d.; not detected.
Trace; <0.1%.
Values are averages of three experiments ± deviations.

Table 2
Mass spectra and GC retention times of enzymatic metabolites of methoxychlor

Metabolites	GC retention time (min)	Mass spectrum m/z (relative intensity, %)
MC (starting compound)	29.59	348 (0.8), 346 M^+(2.4), 274 (2.4), 228 (16.4), 227 (100), 212 (4.8), 196 (2.4), 153 (2.0), 152 (2.8)
MCO	27.31	310 (44.4), 308 M^+(68.0), 273 (26.4), 238 (100), 223 (43.2), 195 (35.2), 186 (11.2), 163 (11.6), 152 (42.4), 119 (14.4)
DMB	25.71	243 (5.2), 242 M^+(30.8), 227 (10.0), 211 (12.8), 135 (100), 107 (14.8), 92 (12.8), 77 (20.0)

case-HBT system. However, 1 nkat of each ligninolytic enzymes hardly degraded MC. The metabolites from these reaction mixtures were analyzed with the GC–MS. MnP-Tween 80 or laccase-HBT system converted MC into two products (Table 2). One of the products was identified as MCO by GC–MS from a comparison of its retention time and mass spectrum with those of an authentic compound. This result suggests that a Cl radical is eliminated from MC to yield the metabolite MCO. The other product was identified as DMB and resulted from the loss of a $^{\cdot}CCl_3$ group from MC. MCO and DMB, however, were not detected in the reaction mixture after treatment with the LiP enzyme. Although the derivatization with *N*,*O*-bis(trimethylsilyl)trifluoroacetimide or diazomethane of ethyl acetate extracts from the reaction mixtures was carried out, no derivative that included mono- and bis-hydroxy metabolites was detected in any reaction mixtures containing the ligninolytic enzymes. The amount of MCO produced in the reaction mixture containing MnP-Tween 80 or Laccase-HBT system was 6.2% or 0.7%, respectively, and trace amount of DMB was detected in the reaction mixture containing MnP-Tween 80 or laccase-HBT (Table 1). Recent in vitro studies have demonstrated that 2,2-bis(4-chlorophenyl)-1,1-dichloroethylene (DDE), which is the most stable metabolite of DDT, can act as an androgen receptor antagonist to inhibit the normal effect of androgens (Kelce et al., 1995; Chedrese and Feyles, 2001). MCO is a metabolite of MC and bears a structural resemblance to DDE, and its accumulation in the environment is, like DDE, considered undesirable. We therefore attempted to treat MCO with three ligninolytic enzymes. The levels of MCO decreased by about 15% and 5% after a 24-h treatment with MnP-Tween 80 and laccase-HBT, respectively. On the other hand, LiP did not degrade the metabolite MCO. Subsequent metabolite studies were carried out with GC–MS, but no metabolite was detected in reaction mixtures after a 24-h treatment with MnP-Tween 80, laccase-HBT, or LiP.

In the present study, we have demonstrated the oxidative dechlorination of MC by the ligninolytic enzymes MnP-Tween 80 and laccase-HBT without the production of hydroxy metabolites. MC was converted by these treatments into MCO and DMB. A possible mechanism for the dechlorination of MC by MnP/Tween 80 or laccase/HBT is shown in Fig. 1. MnP or laccase have been reported to oxidize nonphenolic compounds in the presence of unsaturated fatty acid (Tween 80) or HBT, respectively, although either enzyme alone cannot oxidize such compounds (Bao et al., 1994; Bourbonnais et al., 1998). Thus, a lipid peroxyl radical or an HBT radical is produced in the reaction mixture containing MnP or laccase, respectively. A phenylalkyl radical, which would be oxidatively produced by these radicals, could be an intermediate for the formation of both MCO and DMB. In one reaction, MCO would be formed by the cleavage of a C–Cl bond and the elimination of a Cl radical. In the other, molecular oxygen would be incorporated into the intermediate, and then DMB could be formed by the cleavage of the C–CCl_3 bond. On the other hand, no metabolite was not detected in the reaction mixture although LiP could degrade MC. The LiP oxidation of a variety of nonphenolic aromatics to their corresponding aryl cation radicals has been well established (Valli et al., 1992), and LiP oxidation of 3,4-dimethoxytoluene and 1,4-dimethoxybenzene generated to the corresponding dimeric products (Joshi and Gold, 1996). Probably, MC is oxidized to the corresponding aryl cation radical by LiP, and the dimeric products would be produced by the coupling of two cation radicals. Similar reaction probably occurs in the degradation of MCO by MnP-Tween 80 or Laccase-HBT since the formation of phenylalkyl radical in MCO is not occurred. Therefore, no metabolite including other monomeric products was detected in LiP oxidation of MC, and in MnP-Tween 80 or laccase-HBT treatment of MCO.

Levels of the metabolite MCO, which structurally resembles antiandrogenic DDE, were also decreased by MnP-Tween 80 or laccase-HBT treatment. In vivo, MC is converted to hydroxy metabolites [e.g., 1,1,1-trichloro-2,2-bis(*p*-hydroxyphenyl)ethane] that have much higher estrogenic activities than does MC (Welch et al., 1969; Bulger et al., 1978). In the present study, no hydroxylated metabolite of MC was detected in reaction mixtures after MnP-Tween 80 and laccase-HBT

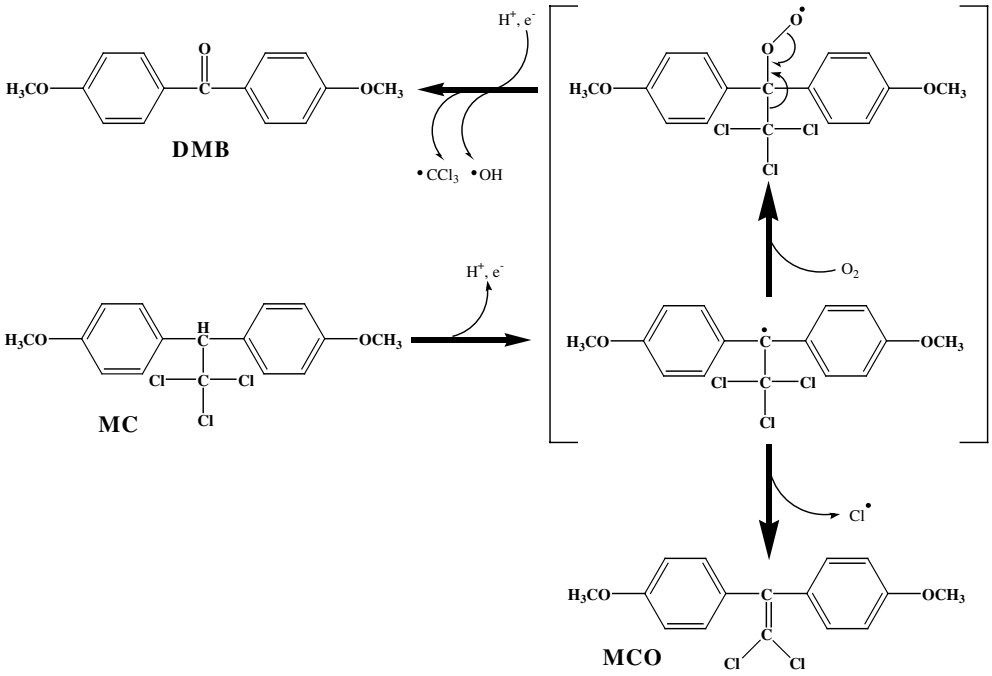

Fig. 1. Possible mechanism for dechlorination of methoxychlor (MC) by MnP-Tween 80 or laccase-HBT treatment. DMB indicates 4,4′-dimethoxybenzophenone; MCO, methoxychlor olefin.

treatments. To our knowledge, very little is actually known about the microbial or enzymatic degradation of MC. This is the first report to describe the degradation of MC and its metabolite, MCO, by the ligninolytic enzymes MnP and laccase from white-rot fungi.

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