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Microsomal transformation of organophosphorus pesticides by white rot fungi

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Abstract

The enzymatic mechanism for the transformation of organophosphorus pesticides (OPPs) by different white-rot fungi strains was studied. With the exception of *Ganoderma applanatum* 8168, all strains from a collection of 17 different fungi cultures were able to deplete parathion. Three strains showing the highest activities were selected for further studies: *Bjerkandera adusta* 8258, *Pleurotus ostreatus* 7989 and *Phanerochaete chrysosporium* 3641. These strains depleted 50 to 96% of terbufos, azinphos-methyl, phosmet and tribufos after four-days exposure to the pesticides. In order to identify the cellular localization of the transformation activity, the extracellular and microsomal fractions of *Pleurotus ostreatus* 7989 were evaluated *in vitro*. While the activities of ligninolytic enzymes (lignin peroxidase, manganese peroxidase and laccase) were detected in the extracellular fraction, no enzymatic modification of any of the five pesticides tested could be found, suggesting the intracellular origin of the transformation activity. In accordance with this observation the microsomal fraction was found able to transform three OPPs with the following rates: 10 μmol mg prot^{-1} h^{-1} for phosmet, 5.7 μmol mg prot^{-1} h^{-1} for terbufos, and 2.2 μmol mg prot^{-1} h^{-1} for azinphos-methyl. The products from these reactions and from the transformation of trichlorfon and malathion, were identified by mass-spectrometry. These results, supported by specific inhibition experiments and the stringent requirement for NADPH during the *in vitro* assays suggest the involvement of a cytochrome P450.

Introduction

Organophosphorus and carbamate pesticides are widely used in agricultural and residential applications as insecticides, herbicides, fungicides and rodenticides. OPPs are esters of phosphoric acid in some cases containing thioether groups (Figure 1). There are also amides, fluor and cyanophosphoric compounds (Abdelsalm 1987). This family of chemicals replaced the organochlorine pesticides banned for use in the United States since the 1970s. Unlike organochlorine pesticides, which are persistent in the environment and cause biological damage as they accumulate in an organism over time, OPPs and carbamate pesticides are short-lived in the environment and fast-acting on their 'target pest'. Direct mortality of wildlife from organochlorine pesticides is uncommon (Hayes et al. 1971); however, mortality is the primary documented effect on non-target wildlife from OPPs and carbamate pesticides (Grue et al. 1983), since their toxicity is not specific. In addition to birds, which appear to be the most sensitive class of animals affected by these compounds, OPPs are the most likely pesticides to be involved in acute human poisonings (Hart 1993). After the Food Quality Protection Act of 1996, the US Environmental Protection Agency put forty OPPs in the highest priority group and placed severe restrictions upon the use of three of them: chlorpyrifos, azinphos-methyl and methyl-parathion (Hileman 2000). OPPs and carbamate pesticides primarily affect the nervous system by inhibiting acetylcholinesterase whose main function is the break down of the neurotransmitter acetylcholine. When acetylcholinesterase is inhibited, acetylcholine accumulates leading to an increase of

the nerve impulse transmission then to nerve exhaustion and, ultimately, to general failure of the nervous system. The respiratory muscles are the most critical group affected and respiratory paralysis is often the immediate cause of death (Hart 1993).

Some OPPs are transformed by bacteria, such as *Altermonas*, *Bacillus Pseudomonas*, and *Flavobacterium*, harboring the organophosphorus hydrolase or organophosphorus acid anhydrolase activities involved in the cleavage of P—O bonds (Munnecke 1976). However, these enzymes showed a limited capacity to cleave the thioether bond present in several OPPs (Munnecke 1976) (Figure 1). On the other hand, the ligninolytic fungus *Phanerochaete chrysosporium* has been shown to be able to mineralize chlorpyrifos, fonofos, and terbufos, and the contribution of extracellular ligninolytic enzymes has been suggested (Bumpus et al. 1993). In a previous work we demonstrated that chloroperoxidase from the fungus *Caldariomyces fumago* was able to oxidize 7 of 10 OPPs assayed, although no oxidation was detected when other heme proteins such as lignin peroxidase, horseradish peroxidase or cytochrome c were used (Hernandez et al. 1998). A fungal laccase produced by *Pleurotus ostreatus* has been reported as able to perform the oxidative degradation of two nerve agents, VX and RVX, which are organophosphorus compounds containing thioether bonds and structurally similar to OPPs (Amitai et al. 1998). However, this oxidation was performed in a mediator-assisted reaction.

In this work, we present the identification of the enzymatic system involved in the transformation of OPPs by white rot fungi and the transformation products of five OPPs, recalcitrant to the bacterial organophosphorus hydrolase and acid anhydrolase activities.

Materials and methods

Chemicals

Veratryl alcohol, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS), and sodium malonate were purchased from Aldrich (Milwaukee, WI). HPLC-grade organic solvents were from Fisher Scientific (Springfield, NJ). Organophosphorus pesticides: azinphos-methyl, tribufos, phosmet, malathion, trichlorfon, parathion and terbufos were obtained from Ultra Scientific (North Kingstown, RI). Buffer salts were obtained from J.T. Baker (Phillipsburg, NJ.). Glucose, yeast extract, potato dextrose agar (PDA) and malt extract were purchased from Difco Laboratories (Detroit, MI). Glycerol, Electric eel acetylcholinesterase, Miconazole (1-[2,4-dichloro-β-([2,4-dichlorobenzyl]oxy)-phenethyl]imidazole nitrate salt), Metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone) and 1-aminobenzotriazole were obtained from Sigma-Aldrich Co. (St. Louis, MI).

Organisms and culture media

Bjerkandera adusta 4312, 7308, 8258; *Pleurotus ostreatus* 7964, 7972, 7980, 7988, 7989 and 7992; *Phanerochaete chrysosporium* 3641, 4521 and 3642; *Sporotrichum pulverulentum* 4521; *Coriolopsis gallica* 8260; *Ganoderma. Applanatum* 8168 and *Trametes versicolor* 8272 were obtained from the University of Alberta Mold Herbarium (Edmonton, Canada). *Pleurotus ostreatus* IE8 was obtained from the Ecology Institute (Xalapa, Mexico). *Phanerochaete chrysosporium* ATCC 24725 was obtained from the American Type Culture Collection (Manassas, MD). All fungi were maintained on PDA plates. Inocula were prepared by homogenizing 1 cm^2 of mycelium from a colony grown on a PDA in 50 ml of GMY medium (Pickard et al. 1999), using a Sorvall Omnimixer (Norwalk, CN) for 10 s. After 3 days incubation in an orbital shaker at 28 °C and 200 rpm, the culture was homogenized again, and approximately 2 mg dry weight aliquots were used for inoculation of 100 ml of cereal-bran medium (Pickard et al. 1999) in 500 ml flasks. The cultures were incubated at 28 °C and 200 rpm in an orbital shaker for 4 days.

Enzyme assays

Extracellular enzymes were obtained from two different media: GMY medium (non ligninolytic) and cereal-bran media (ligninolytic) (Pickard et al. 1999). Lignin peroxidase was measured according Tien & Kirk (1988) following the H_2O_2-dependent oxidation of veratryl alcohol to veratraldehyde at 25 °C. Reaction mixtures contained 4 mM veratryl alcohol in 40 mM succinate buffer pH 3 and the reaction was started by the addition of 0.4 mM H_2O_2. Absorbance was monitored at 310 nm ($\epsilon_{210} = 9300\ M^{-1}\ cm^{-1}$ for veratraldehyde). Manganese peroxidase activity was estimated by the formation of oxidized manganic-malonate complex in the presence of H_2O_2 at 25 °C (Wariishi & Gold 1992). The reaction mixture contained 1 mM manganous sulfate in 50 mM malonate buffer pH 4.5 and 5–50 μl of enzyme extract. Reactions were started by the addition of H_2O_2 to a final

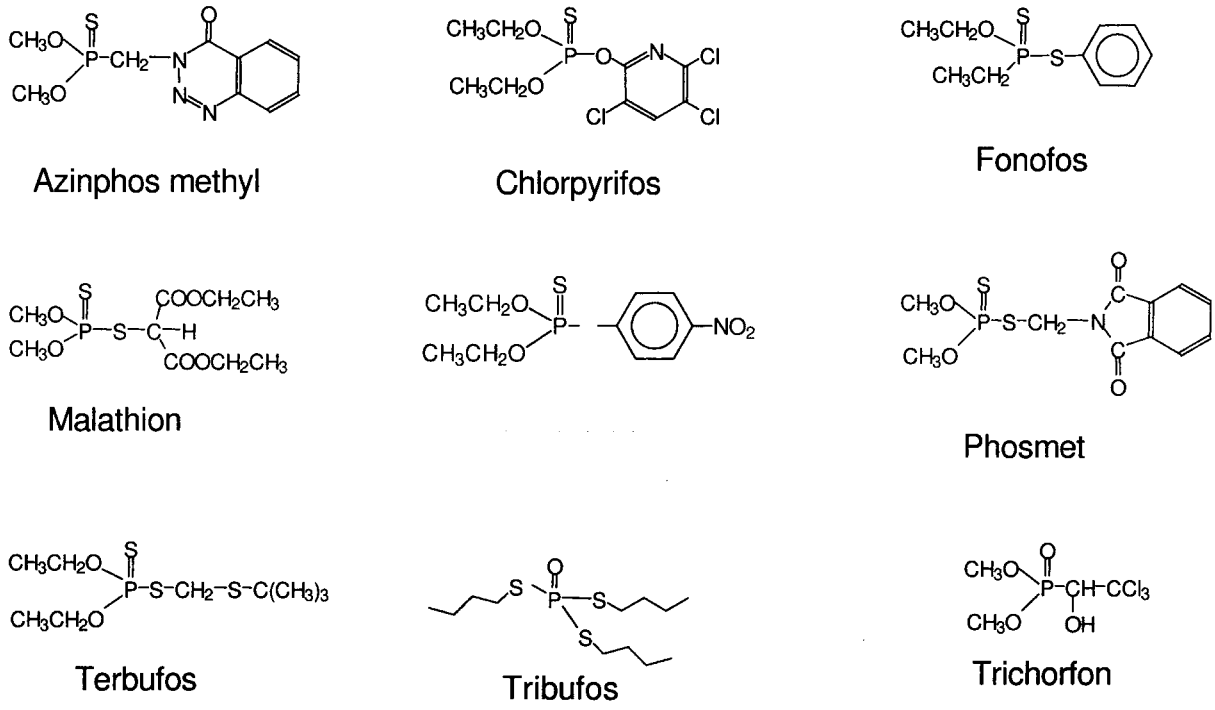

Figure 1. Structure of different organophosphorus pesticides.

concentration of 0.1 mM and monitored at 270 nm (ϵ_{270} = 11590 M^{-1} cm^{-1} for manganic-malonate complex). Laccase activity was determined by the oxidation of ABTS at 25 °C (Woolfenden & Wilson 1982). The reaction mixture contained 1 mM ABTS in 100 mM sodium acetate buffer pH 4.5 with 5–50 μl of enzyme extract. The oxidation was followed at 436 nm (ϵ_{436} = 29300 M^{-1} cm^{-1} for ABTS). For OPPs transformation assays, the extracellular media were concentrated by ultrafiltration on an Amicon cell with a 10,000 Da cutoff membrane.

In vivo *transformation experiments*

Fifty ml of cereal-bran medium in 250 ml flask were inoculated and incubated for 2 days at 28 °C and 200 rpm. Then, OPP was added to 20 mM final concentration, and the cultures incubated four more days. Then, 50 ml tetrahydrofuran (THF) were added to the culture, the mixture was centrifuged, and the supernatant analyzed by high performance liquid chromatography (HPLC, Perkin-Elmer, series 200) equipped with a 100 × 2.1 mm ODS Hypersil 5 μm column (Hewlett Packard). Samples were isocratically eluted with acetonitrile: water (45 : 55) at 0.3 ml/min and the pesticide concentration was monitored with a diode array detector (Perkin-Elmer, model 235C). Three series of control cultures were carried out: abiotic, autoclaved (killed) biomass, and time zero extractions. No significant pesticide disappearance was detected in any of these controls. Pesticide transformation was estimated as the peak disappearance and quantified using a standard curve.

Microsomal fraction

Fungal biomass (100 g wet weight) was resuspended in 250 ml of 20 mM Tris-HCl buffer pH 7.0 containing 1 mM EDTA, 0.5 mM DTT, 10% glycerol and homogenized with four 30 s pulses in a Sorvall Omnimixer. The mixture was then homogenized in a Milton Gauli homogenizer at 3,500 psi. The microsomal fraction was isolated by differential centrifugation as described by Cinti et al. (1972). The biomass homogenate is centrifuged first at 600 g for 5 min to remove cell debris, then the supernatant is centrifuged at 12,000 g for 10 min. Addition of 8 mM $CaCl_2$ to the supernatant allows complete sedimentation of microsomes at 27,000 g for 15 min. The microsomal pellet was then washed two times with 20 mM tris-HCl buffer containing 8 mM $CaCl_2$. Enzymatic markers were assayed to determine the purity of the micro-

Table 1. Catalytic activities of enzyme markers for purity of microsomal preparation

	Activity of marker enzymes (U/mg protein)[a]				
	Catalase	β-Glucosidase	Alkaline phosphatase	NADPH-cytochrome c reductase	Cytochrome c oxidase
Homogenate	1349.7 ± 3.5	13.2 ± 2.0	10.0 ± 2.0	12.4 ± 1.6	79.7 ± 2.5
Microsomal fraction	91.3 ± 3.2	ND[b]	ND	21.0 ± 1.1	ND

[a]Activity unit is defined as μmol of substrate transformed per minute.
[b]Not detected.

somal preparations (Table 1). Most of endoplasmic reticulum marker (NADPH-cytochrome c reductase) (Lake 1987) was found in the microsomal preparation. No lysosome (β-glucosidase) (Boehringer Mannheim 1975), plasmatic membrane (alkaline phosphatase) (Boehringer Mannheim 1975), nor mitochondrial (cytochrome oxidase) (Storrie & Madden 1990) markers were detected in the microsomal preparations. Only very low catalase activity (peroxisome marker) (Boehringer Mannheim 1975) was detected, probably due to marginal catalase activity of the hemeprotein cytochrome P450. The microsomal preparations were frozen for subsequent use.

In vitro *transformation experiments*

Enzymatic transformation of OPPs was assayed with the microsomal fractions. Reactions were carried out in 2 ml reaction mixtures containing 0.2 mM organophosphorus pesticide and 0.4 mM β-NADPH in 100 mM phosphate buffer pH 5.0. The reaction was started by the addition of 0.2 to 0.3 mg of the microsomal protein. Pesticide transformation was monitored by HPLC and spectrophotometrically by NADPH oxidation at 340 nm. Control experiments were carried out in the absence of microsomes or without NAPH addition or with thermoinactivated microsomes, and no significant pesticide disappearance was detected. The transformation rates are expressed in μmol pesticide per mg of protein per hour.

For product analyses, 10 ml reaction mixtures were prepared containing 0.2 mM pesticide, 0.4 mM NADPH, and 1 to 1.4 mg of microsomal protein. After 24 h reaction, the mixture was lyophilized and extracted with methanol. Under these conditions 80% or more of the substrate is transformed (monitored by HPLC), assuring high products concentration. The organic extract was reduced with nitrogen flow and analyzed by gas chromatography-mass spectrometry (GC-MS) technique. The GC-MS analyses were carried out in a Agilent Technologies GC chromatograph (model 6890 N) coupled to a mass spectrometry detector (model 5973 N), equipped with a 30.0 m $\times$ 250 μm $\times$ 0.25 μm, HP-5MS 5% phenylmethyl siloxone capillary column. The temperature program started at 50 °C for 1 min, then to 250 °C at 15 °C/min, and held for 5 min.

Inhibition of biotransformation of OPPs

The enzymatic transformation of OPPs by the microsomal fraction was assayed in the presence of cytochrome P450 inhibitors. Reactions were carried out in 2 ml reaction mixtures containing 0.2 mM phosmet pesticide, 0.4 mM β-NADPH in 100 mM phosphate buffer pH 5.0, and 0.1 mM, 1.0 mM or 5 mM of the cytochrome P450 inhibitors (miconazole, 1-aminobenzotriazole or metyrapone). The reaction was started by the addition of 0.2–0.3 mg of microsomal protein and monitored by HPLC.

Acetylcholinesterase inhibition

The toxicities of the pesticides and their products after microsomal transformation were estimated as the inhibition of acetylcholinesterase activity. The acetylcholinesterase activity was determined using the Michel method (Hawkins & Knittle 1972) in a medium containing 16 μg/ml of enzyme with or without 1 μM pesticide. Pesticide microsomal transformations were carried out as mentioned above and monitored by HPLC until the substrate was completely transformed. Then, the reaction mixture was lyophilized and a portion, equivalent to 1 μM in substrate basis, was added to the acetylcholinesterase reaction mixture.

Table 2. Parathion depletion by 18 strains of white rot fungi after 96 h culture[a]

Fungi	Pesticide depletion (%)
B. adusta 4312	49.0 ± 4.2
B. adusta 7308	49.0 ± 5.5
B. adusta 8258	**78.3 ± 3.6**
G. applanatum 8168	ND
P. ostreatus 7964	44.5 ± 4.1
P. ostreatus 7980	44.5 ± 4.7
P. ostreatus 798	18.4 ± 4.0
P. ostreatus 7989	**96.9 ± 2.9**
P. ostreatus 7972	32.3 ± 3.1
P. ostreatus 7992	26.0 ± 2.5
P. ostreatus IE8	48.5 ± 2.3
P. chrysosporium FC-322	10.5 ± 3.0
P. chrysosporium 3641	**55.0 ± 2.1**
P. chrysosporium 3642	28.6 ± 2.5
P. chrysosporium ATCC 24725	30.0 ± 2.0
S. pulverulentem 4521	7.7 ± 3.2
C. gallica 8260	28.5 ± 2.5
T. versicolor 8272	9.5 ± 3.4

[a] The initial pesticide concentration was 20 mM.
ND: no detected.

Results

Parathion depletion by 18 different strains of lignolytic fungi was determined after 96 h-incubation in cereal-bran medium. This was an initial and semiquantitative screening of the fungal strains in order to select three strains to be studied. All strains, except *Ganoderma applanatum* 8168, were able to deplete parathion to different extents (Table 2). The three strains showing the highest activities were selected for further studies: *Bjerkandera adusta* 8258, *Pleurotus ostreatus* 7989 and *Phanerochaete chrysosporium* 3641. These strains depleted parathion, terbufos, azinphos-methyl, phosmet and tribufos, and their specific rates of pesticide depletion are shown in Table 3. All the OPPs tested were depleted by the three fungi, even those containing thioether bonds (Figure 1). In general, *B. adusta* 8258 showed the highest rates for all five pesticides, with the exception of terbufos, which was depleted faster by *P. ostreatus* 7989. The supernatants form the fungal cultures with parathion were extracted and analyzed by GC-MS. In all three strains, paraoxon was detected as metabolite. Fungi grown on non-ligninolytic medium were also able to deplete OPPs, but in a less extent, mainly due to a slower fungal growth.

The extracellular ligninolytic enzymes lignin peroxidase, manganese peroxidase and laccase were produced by cultures of *B. adusta* 8258, *P. ostreatus* 7989 and *P. chrysosporium* 3641 in ligninolytic and non-ligninolytic media. The highest laccase (250 μmol min^{-1} mg protein^{-1}) and manganese peroxidase (100 μmol min^{-1} protein^{-1}) activities were found in *P. ostreatus* 7989 cultures under ligninolytic conditions, while no laccase activity could be detected in *P. chrysosporium* 3641. *B. adusta* cultivated in ligninolytic medium showed the highest lignin peroxidase activity (112 μmol min^{-1} protein^{-1}), while no lignin peroxidase activity could be detected in *P. ostreatus* 7989 cultures. Concentrated extracellular media were evaluated for the enzymatic modification of OPPs, under the conditions described for each enzyme in the Materials and Methods section. No activity on pesticides could be found in any of the extracts under the different conditions assayed. In addition, commercial preparations of lignin peroxidase and manganese peroxidase from *Phanerochaete chrysosporium* did not catalyze the oxidation of OPPs. Purified preparations of fungal laccases from *Pleurotus ostreatus*, *Trametes versicolor* and *Coriolopsis gallica* (Tinoco et al. 2000) assayed either in the presence of ABTS or 1-hydroxybenzotriazole (HBT) showed no oxidation of any organophosphorus pesticide.

Microsomal fractions were obtained from *Pleurotus ostreatus* 7989 and assayed for the NADPH-dependent transformation of OPPs. The purity of the microsomal preparation was tested with enzymatic markers (Table 1). The microsomal fraction was able to transform the pesticides tested with transformation rates of 10 ± 1.2 μmol mg prot^{-1} h^{-1} for phosmet, 5.7 ± 0.95 μmol mg prot^{-1} h^{-1} for terbufos, and 2.2 ± 0.7 μmol mg prot^{-1} h^{-1} for azinphos-methyl. Pesticide transformation by the microsomal fraction required the addition of NADPH, suggesting that cytochrome P450 activity may be involved. NADH was able to act as a cofactor in the reaction, and no NADPH oxidation was detected in the absence of OPPs.

The transformation products from the *in vitro* microsomal reactions with different OPPs were isolated and their structures were determined by GC-MS (Table 4 and Figure 2). The compound identification was performed by matching with standard mass spectra (Hites 1990; Davies & Frearson 1997) and by comparing with other reported spectra from metabolic products of OPPs (Lin et al. 1980). The products for azinphos-methyl detected on the GC-MS analysis (retention times in

Table 3. Specific depletion rates (μmol g'dry wt^{-1} h^{-1}) of five OPPs by three white-rot fungi strains. OPPs were added to 20 mM final concentration to two days old cultures and incubated four more days. The amount of depleted pesticide was then measured and related to fungal dry biomass and culture time (96 h)

Fungi	Pesticides				
	Parathion	Azinphos methyl	Phosmet	Terbufos	Tribufos
B. adusta 8258	217 ± 10	223 ± 8	220 ± 2	23 ± 9	84 ± 8
P. ostreatus 7989	169 ± 4	106 ± 6	103 ± 4	59 ± 5	45 ± 5
P. chrysosporium 3641	145 ± 6	203 ± 8	45 ± 3	17 ± 7	25 ± 2

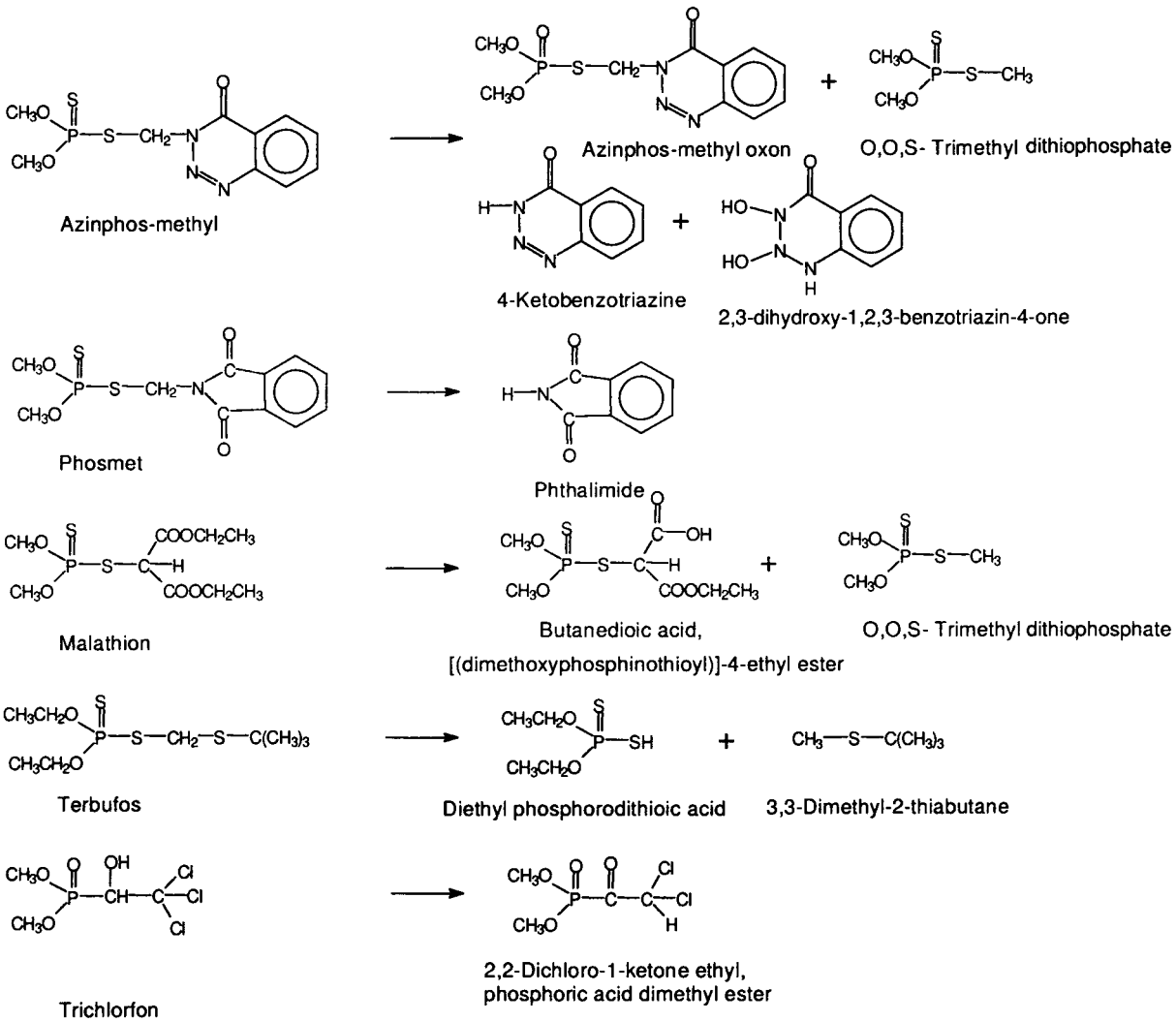

Figure 2. Structure of the identified products from microsomal transformation of OPPs.

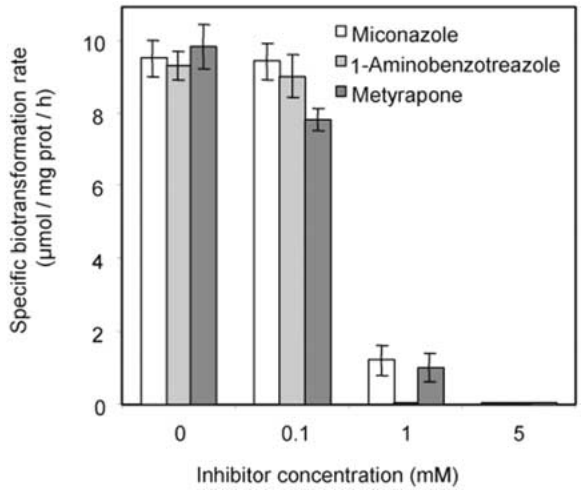

Figure 3. Effect of cytochrome P450 inhibitors on the transformation of phosmet pesticide by microsomal fraction from *P. ostreatus* 7989.

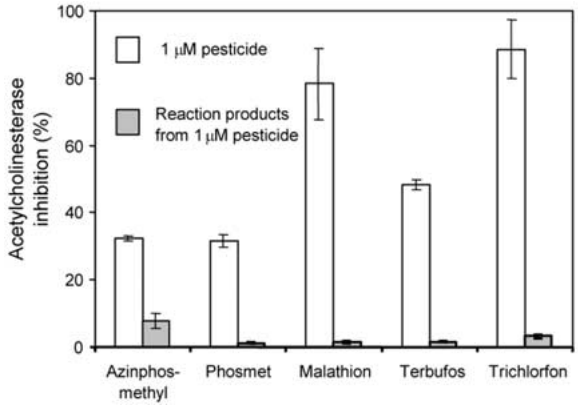

Figure 4. Acetylcholinesterase inhibition by 1 μM of pesticides and their equivalent products from microsomal transformation.

parentheses) were O,O,S-trimethyl dithiophosphate (6.74 min); 4-ketobenzotriazine (10.19 min); 2,3-dihydroxy-1,2,3-benzotrazin-4-one (10.90 min); and azinphos-methyl oxon (16.78 min), while the substrate azinphos methyl showed a retention time of 18.96 min under our analysis conditions. The microsomal reaction on malathion produced butanedioic acid, [2-(dimethoxyphosphothionyl)-4-ethyl ester]; and O,O,S-trimethyl dithiophosphate. Terbufos was transformed to 3,3-dimethyl-2-thiabutane and diethyl phosphorodithioic acid. Finally, 2,2-dichloro-1-ketone ethyl and phosphoric acid dimethyl ester, and phthalimide were identified from reactions with trichlorfon and phosmet, respectively (Table 4). The chemical structures of these products are shown in (Figure 2).

Inhibition experiments were performed upon the enzymatic transformation of phosmet. Miconazole, 1-aminobenzotriazole and metyrapone, all known cytochrome P450 inhibitors (Bossche & Koymans 1998), were used in concentrations up to 5 mM. Each of the three inhibitors was able to stop the microsomal transformation of OPP (Figure 3). In addition, reaction mixtures containing microsomes previously bubbled with carbon monoxide showed no catalytic activity.

Toxicity before and after microsomal transformation of five different OPPs was determined as the inhibition of the acetylcholinesterase activity (Figure 4). In all the cases, the microsomal transformation of pesticides significantly reduced their capacity to inhibit acetylcholinesterase activity. No substrate was detected after microsomal transformation, thus the remaining inhibition capacity of the reaction mixture could be attributed to the presence of oxon derivatives, as shown for azinphos-methyl.

Discussion

The capacity of ligninolytic fungi for OPP metabolism seems to be widely distributed (Table 2). The molecular structures of selected OPPs are diverse; trichlorfon is a phosphate, parathion a phosphorothioate, and tribufos a phosphorotrithioate, and azinphos methyl, phosmet, malathion, and terbufos are phosphorodithioates. For most of these compounds, the presence of a thioether bond renders the pesticide recalcitrant to bacterial transformation via the organophosphorus hydrolase activity (Amitai et al. 1998; Munnecke 1976), although they can be transformed by the liver enzymatic system (Lin et al. 1980). Thus, for these kinds of pesticides, fungal biotransformation seems to be a more promising alternative than bacterial transformation.

Amitai et al. (1998) conducted the oxidative degradation of two nerve agents, VX (*o*-ethyl *S*-[*N,N*-disopropylaminoethyl]methylphosphothiolate) and-RVX (*o*-isobutyl *S*-[*N,N*-diethylaminoethyl]methylphosphothiolate), with a fungal laccase. These compounds share their chemical structure and toxic mode of action with several of the OPPs tested here. Under our conditions extracellular concentrates from *P. ostreatus* cultures and purified enzymatic preparations from different sources were unable to modify any of the OPPs tested. The extracellular peroxidases from white rot fungus were also unable to transform

Table 4. Mass spectral data of the products from the biocatalytic tranformation of OPPs by microsomal fraction of *P. ostreatus* 7989

Pesticide	Products	Mass spectral ions (*m/z*)[a]
Azinphos-methyl	4-Ketobenzotriazine	147 **[M^+]** (100), 104 [M]-CONH (55), 76 C_6H_4 (55), 50 (22)
	Azinphos-methyl oxon	301 **[M^+]** (1), 161 (12), 160 $C_8H_6N_3O$ (100), 133 (10), 132 $C_7H_4N_2O$ (87), 109 $C_2H_6O_3P$ (23), 105 (25), 104 $C_6H_4N_2$ (22), 77 C_6H_5 (66), 76 C_6H_4 (24), 51 (10), 50 (12)
	O,O,S-Trimethyl dithiophosphate	172 **[M^+]** (100), 126 [M]-SCH_2 (15), 125 [M]-SCH_3 (56), 109 (15), 93 [M]-CH_3PS_2 (53), 79 [M]-C_3H_9OS (16), 63 [M]-$C_3H_9S_2$ (20)
	2,3-dihydroxy-1,2,3-benzotriazine-4-one	181 **[M^+]** (37), 134 [M]-NHNHOH (100), 116 (13), 106 C_6H_4NO (10), 104 C_6H_4CO (6), 91 C_6H_4NH (11), 77 C_6H_5 (23)
Phosmet	Phthalimide	148 [M]+H (10), 147 **[M^+]** (100), 104 [M]-CONH (56), 103 [M]-$CONH_2$ (26), 76 C_6H_4 (57), 75 C_6H_3 (10), 74 C_6H_2 (13), 50 (20)
Trichlorfon	2,2-Dichloro,1-ketone ethyl, phosphoric acid dimethyl ester	224 (2), 222 (3), 220 **[M^+]** (5), 187 (10), 185 (26), 145 (10), 109 (100), 79 (17)
Malathion	Butanedioic acid, [(dimethoxyphosphino-thioyl)]-4-ethyl ester	302 **[M^+]** (2), 159 [M]-$C_4H_8O_3PS$ (13), 158 [M]-$C_6H_8O_4$ (81), 157 [M]-$C_6H_9O_4$ (11), 147 [M]-$C_4H_{12}O_2PS$ (22), 145 [M]-$C_2H_6O_2PS_2$ (47), 143 [M]-$C_7H_{11}O_4$ (100), 132 [M]-$C_4H_{11}O_3PS$ (13), 125 [M]-$C_6H_9O_4S$ (88), 93 [M]-$C_7H_{13}O_5S$ (70), 87 [M]-$C_4H_8O_4PS_2$ (10), 79[M]-$C_8H_{15}O_5S$ (15), 55 (15)
	O,O,S-Trimethyl dithiophosphate	172 **[M^+]** (100), 141 [M]-OCH_3 (10), 125 [M]-SCH_3 (70), 109 (22), 93 [M]-CH_3PS_2 (73), 79 [M]-C_3H_9OS (22), 63 [M]-$C_3H_9S_2$ (22)
Terbufos	3,3-Dimethyl-2-thiabutane	104 **[M^+]** (38), 89 [M]-CH_3 (10), 57 C_4H_9 (100), 56 C_4H_8 (12)
	Diethyl phosphoro-dithioic acid	186 **[M^+]** (100), 169 [M]-CH_5 (5), 153 [M]-SH (14), 142 [M]-C_3H_8 (18), 137 [M]-CH_5S (20), 121 [M]-HS_2 (55), 109 [M]-C_2H_5OS (25), 97 H_2PS_2 (58), 65 H_2PS (25)

[a] Values in parenthesis are relative intensities (in percentage). **[M^+]**, molecular ion.

the insecticide lindane (Mougin et al. 1996), and the herbicide S-triazine (Moungin et al. 1997). The oxidation of the nerve agents reported by Amitai et al. (1998) was performed in the presence of ABTS as a mediator. It is possible that the oxidation was catalyzed by the ABTS radical cation instead of being performed enzymatically. Nevertheless, under our conditions, laccases from different sources were also unable to oxidize diverse OPPs, even in the presence of two different radical mediators.

The intracellular nature of the enzymatic system involved in OPP transformation was clearly demonstrated by the microsomal location of the activity (Figure 2 and Table 4). Mougin et al. (1996, 1997) were the first to suggest that cytochrome P450 system is involved in the pesticide degradation in white rot fungi. Transformation of azinphos-methyl was achieved either through the cleavage of the S—C bond or through oxidative desulfuration. Further dealkylation of 2-methyleneazide benzaldehyde or methylation of the azinphos-methyl oxon yielded 4-ketobenzotriazine and O,O,S-trimethyl dithiophosphate, respectively. These products are similar to those observed after the bacterial transformation of azinphos methyl (Engelhardt et al. 1981; Engelhardt & Wallnöfer 1983). In the case of terbufos, cleavage of the S—C bond took place, yielding 3,3-dimethyl-2-thiobutane and O,O-diethyl phosphorodithioic acid as products. A similar cleavage pattern has been reported for other OPPs using rat liver microsomes (Casida & Fukunanga 1968). Only one product from the phosmet transformation could be identified, phthalimide, indicating the occurrence of a C—N bond cleavage. However, the other expected product, O,O-dimethyl phosphorodithioic acid was not detected. Malathion was enzymatically converted to O,O,S-trimethyl phosphorodithioic acid and butanedioic acid (dimethoxyphosphinothioyl)-4-ethyl

ester. At least two different reactions seemed to be involved, the cleavage of the S—C bond as observed for the transformation of azinphos methyl, and hydrolysis of one of the ethyl ester bonds, with the concomitant release of one ethanol molecule. Transformation of trichlorfon yielded only one product, 2,2-dichloro-1-ketone ethyl dimethylphosphate. Oxidative dehalogenation of trichlorfon probably occurred by the deprotonation of the hydroxyl group and subsequent ketone and hydrogen chloride formation. Dehalogenated product from trichlorfon metabolization was also reported by Eto (1974), using dog and rabbit liver homogenates, and it was also detected after alkaline hydrolysis (data not shown).

In general, *P. ostreatus* 7989 microsomal preparations catalyzed the transformation of OPPs at rates ranging from 2.2 to 10 μmol per mg of protein per hour. These rates are significantly higher than those estimated by Butler & Murray (1997) for human liver microsomes (0.1 to 1.1 μmol per mg of protein per hour). From *P. ostreatus 7989* cells, 9.8 mg of microsomal protein per g of dry biomass could be obtained, and thus the microsomal activity may account for 95% of both phosmet and terbufos depletion, and 20% of the azinphos depletion in whole cultures (Table 3). Microsomal OPPs transformation presented a stringent requirement for NADPH or NADH and was strongly inhibited by miconazole, 1-aminobenzotriazole and metyrapone (Figure 3), suggesting the participation of a cytochrome P450 enzymatic system. Microsomal preparations from rat liver containing cytochrome P450 have been shown to be able to transform the phosphorothioate moieties of OPPs to phosphate groups (Ma & Chambers 1994; Whitehouse & Ecobichon 1975). These preparations were also able to detoxify OPPs by dearylation, yielding *p*-nitrophenol from parathion (Ma & Chambers 1994). Paraoxon, diethylphosphorothioic acid, diethylphosphoric acid and *p*-nitrophenol were produced by a NADPH-reconstituted cytochrome P450 system from rat liver (Yoshihara & Neal 1977).

We conclude so far that ligninolytic fungi were able to deplete *in vivo* several OPPs including phosphorodithioates and phosphorotrithioates, which are recalcitrant to the bacterial organophosphorus hydrolase. No oxidation of OPPs could be detected with extracellular ligninolytic peroxidases and laccases. On the other hand, fungal microsomal preparations transformed *in vitro* OPPs in a NADPH-dependent reaction. Inhibition experiments suggested the participation of the enzymatic system cytochrome P450 in the oxidation of organophosphorus pesticides by ligninolytic fungi. The reaction products from *in vitro* microsomal transformation of five organophosphorus pesticides were determined by GC-MS and showed that cleavage of N—C and S—C bonds may occur. Finally, microsomal transformation significantly reduced the toxicity of pesticides as estimated by their capacity to inhibit acetylcholinesterase activity (Figure 4).

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