Biodegradation of Fenthion by *Phanerochaete chrysosporium*

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ABSTRACT

Fenthion was metabolised by the white-rot fungus *Phanerochaete chrysosporium* in liquid culture when applied to the limit of its solubility in aqueous media (7.2 µM). Using GC-NPD, no evidence for either adsorption to or accumulation in intra-cellular compartments was obtained and after 5 days culture, less than 1% of fenthion remained in the extracellular medium. Fenthion did not inhibit spore germination at the concentrations supplied, but appeared to inhibit the rate of spread of the fungus on solid media. Fenthion was oxidised by lignin peroxidase (LiP), but enzyme turnover was dependent on the concentration of H₂O₂ supplied, with inhibition accompanying the formation of the inactive intermediate Compound III at an H₂O₂ concentration equal to the $k_m$ of LiP for H₂O₂ (c. 0.4 mM). Available evidence suggests that fenthion was metabolised via the fungal ligninolytic system and it represented the reducing substrate for LiP Compound I to return to the native state.

INTRODUCTION

Fenthion is an organophosphorus pesticide (Figure 1) used to control bird pests in agricultural systems. In particular, it is used against the Red-billed Quelea, *Quelea quelea*, a major pest of cereal crops in semi-arid areas of sub-Saharan Africa. For instance, during the 1997/98 season more than 5000 l of a commercial formulation (Queletox R) were sprayed at 7 l/ha to protect wheat, sorghum, millet and sunflower crops in South Africa (Geertsema, 1998). After application, fenthion and toxic degradation products, particularly the sulphone and sulfoxide derivatives for example, can be persistent, depending on environmental conditions (Minelli et al., 1996; Rotunno et al., 1997; Lacorte et al., 1997). In addition, off-target drift for 3 km was detected in samples taken at a height of 6 m 20 h after application and at 9 m after 64 h (van der Walt et al., 1998; van der Walt, 2000 or see page 91). Also, birds that have collected a lethal dose of fenthion may be able to disperse and contaminate other sites up to 30 km distant, resulting in secondary contamination. Both primary and secondary contamination have been detected in non-target birds such as raptors (van der Walt et al., 1998) and recent reports suggest that fenthion persists
Figure 1 Chemical structure of fenthion, the active ingredient of numerous organophosphorus pesticides, including Tiguvon and Queletox.

in the soil for at least 54 days after spraying (van der Walt, 1999). So, control methods which are less damaging to the environment are urgently sought, with remediation of contaminated sites being a priority.

White-rot fungi are micro-organisms which colonise wood and are capable of mineralising polymeric lignins (for a review see Kirk and Farrell, 1987). Phanerochaete chrysosporium is the best characterised wood-rotter (for reviews, see Gold and Alic, 1993; Reddy and D'Souza, 1994), but there are other efficient lignin-degrading fungi including Pleurotus spp. (Orth et al., 1993), Trametes spp. (Vares and Hatakka, 1997), Panaeolus spp. (Heinzkill et al., 1998) and Phlebia radiata (Niku-Paavola et al., 1988). *P. chrysosporium* can attack lignins as well as numerous xenobiotics (see for instance Haemmerli et al., 1986; Bumpus, 1989; Lin et al., 1990; Paszcznksi and Crawford, 1991; Stahl and Aust, 1993; Yadav and Reddy, 1993; Yadav et al., 1995), using an extra-cellular ligninolytic system, which lacks specificity with respect to the nature of the aromatic substrate and enables the fungus to modify recalcitrant organic polymers oxidatively. Initial extra-cellular oxidation of lignin sub-structures can be carried out by two microbial peroxidases, lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP). Both enzymes use H$_2$O$_2$ as the oxidant to catalyse one-electron oxidation reactions of aromatic substrates, but require distinct redox mediators for completion of the catalytic cycle (for LiP, see Harvey et al., 1986; Hammel and Moen, 1991; Goodwin et al., 1995; Candeias and Harvey, 1995; for MnP, see Glenn et al., 1986; Paszczynski et al., 1986). With respect to MnPs, the oxidation of non-phenolic substrates is mediated by lipid peroxyl radicals (Jensen et al., 1996). Also extra-cellular laccases, produced by many white-rot fungi including *P. chrysosporium* (Srinivasan et al., 1995), may be indirectly involved in the oxidation of non-phenolic lignin sub-structures by a mechanism of redox mediation by oxidisable substrates such as ABTS (Bourbonnais and Paice, 1990). Low-molecular weight compounds produced by the initial extra-cellular oxidation of polymeric aromatic substrates can then be intra-cellularly metabolised and mineralised. In the case of xenobiotics such as 2,4-dinitrotoluene and 2,4,5-trichlorophenol, the multi-step catabolic pathways were shown to involve cycles of intra-cellular reductions and extra-cellular oxidations (Valli et al., 1992; Joshi and Gold, 1993), terminating in intra-cellular oxidative ring cleavage prior to complete mineralisation (Rieble et al., 1994). Because of their catabolic versatility, white-rot fungi including *P. chrysosporium* hold potential in practical applications as bio-remediation agents (see for instance Aust, 1990; Lamar et al., 1990; Lamar and Dietrich, 1990; Morgan et al., 1993; Holroyd and Caunt, 1997).

In the case of *P. chrysosporium*, LiPs can represent the only extra-cellular oxidative enzymes involved in ligninolysis (Leisola et al., 1985). The catalytic cycle of LiP, which is typical for the family of peroxidase enzymes (Banci, 1997), involves an initial two-electron oxidation with H$_2$O$_2$ to yield Compound I, which is reduced back to the native state in two steps of single-electron reduction via the intermediate Compound II.

In this paper we report on the metabolism of fenthion by liquid cultures of *P. chrysosporium* maintained on cellulose, under conditions that favour the production of LiP as the
Biodegradation of Fenthion by *Phanerochaete chrysosporium*

only detectable ligninolytic extra-cellular oxidative enzyme (Zacchi *et al*., 2000) and on the oxidation of fenthion by LiP.

**MATERIALS AND METHODS**

Tiguvon, a commercial formulation containing fenthion as the active ingredient, was utilised for initial tests, incorporated as an emulsion into agar plates. For analytical determinations, pure fenthion (98% min.) was purchased from the Institute of Organic Industrial Chemistry, Poland.

*Phanerochaete chrysosporium*, strain BKM-F-1767 (ATCC 24725), was maintained in 300 ml medical flat bottles, containing 50 ml of 2% (w/v) malt extract agar, that were incubated for 2 weeks at 37°C. Both liquid and solid-phase cultures were grown as follows.

**Liquid cultures:** 600 ml growth medium in 2-l Erlenmeyer flasks closed with foam stoppers was inoculated with a spore inoculum of $2\times10^7$ spores and incubated under a continuous agitation regime (130 rpm, 2.5 cm cycle) at 37°C. A non-limiting nutrient N regime (24 mM) was used according to Tien and Kirk (1988), with the following modifications: microcrystalline cellulose (Avicel PH-101, from Fluka Chemika) was used as the C-source; veratryl alcohol (VA) was included in the medium composition, to a final concentration of 1.5 mM. The pH was adjusted to 4.5 with 10 M NaOH. All chemicals were purchased from Sigma, unless otherwise stated. A purified LiP preparation (48 µM; 39.6 U/mg protein; Rz = 2) was obtained from a 5 day-old culture’s broth of *P. chrysosporium*, after concentration of the medium and purification of the enzyme according to Tien and Kirk (1988). One unit of LiP was defined as that amount of enzyme able to form 1 µmole/min of veratraldehyde using $\varepsilon = 9.3$/mM/cm (Tien and Kirk, 1984).

**Solid phase cultures:** The solid medium (2% w/v agar) contained per litre: 0.5 g MgSO$_4$$\cdot$7H$_2$O, 0.6 g KH$_2$PO$_4$, 0.4 g K$_2$HPO$_4$, 0.5 g (NH$_4$)$_2$SO$_4$, 0.1 g sodium deoxycholate, 1 mg thiamine hydrochloride and 1.5 mM VA, 0.4% (w/v) cellulose with or without 0.02% (w/v) Poly R-478. Petri dishes were inoculated by a loop of spores placed in the centre of the plates.

**Analytical Methods**

Extra-cellular LiP activity was measured using veratryl alcohol as substrate according to Tien and Kirk (1984). Fungal growth in liquid cultures was estimated by measuring mycelium dry weights after thorough washing with distilled water to remove extra-cellular-attached polysaccharides, and dried at 90°C for 24 h.

HPLC analyses were performed using a C8 12 cm column (Merck) connected to a Kauner ERC pump 64 with a Varian 9050 UV-Vis. detector. The mobile phase was methanol: H$_2$O (8:2). Potassium phosphate buffer (20 mM, pH 2.75) was used for incubating LiP (0.1 nmol) with veratryl alcohol and H$_2$O$_2$ (both 250 nmol), and fenthion (7.2 nmol-maximum solubility in water). Spectroscopic analyses of the reaction between LiP and fenthion were carried out using a HP8452A Diode Array spectrometer. Gas chromatography was performed using a Perkin Elmer 8310 gas chromatograph, with a DB5 capillary column (30 mm x 0.25 mm, 0.25 µm film thickness) and nitrogen and phosphorus detection (GC-NPD); oven temperature was programmed between 200–250°C, at a ramp rate of 10°C/min; injection temperature 230°C; detector temperature 300°C; column flow rate 50 ml/min of N$_2$. For analysis of fenthion in the extra-cellular medium, 20–300 ml samples were extracted 4–5 times with dichloromethane, clarified with anhydrous Na$_2$SO$_4$ then evaporated to dryness and taken up in 3 ml of methanol. Pellets of mycelia (3–4 g) were suspended
in 100 ml dichloromethane and homogenised with an Ultra-Turrax T25 homogeniser 3
times for 20-s periods. The resultant homogenate was filtered through a column of anhy-
drous Na$_2$SO$_4$ to remove water and then evaporated to dryness. Samples were taken up in
5 ml acetone for analysis.

RESULTS

Tiguvon, a commercial preparation of fenthion in an oil base, was investigated for its
inhibitory effects on the growth of *P. chrysosporium* and expression of LiP. Agar plates
containing cellulose as the C-source and increasing concentration of Tiguvon (Figure 2)
were inoculated in the centre with a spore suspension of *P. chrysosporium*, using a sterile
point. Tiguvon inhibited the rate of spread of the fungus across the plate surface, with a
linear dose response curve up to 50 ppm (Figure 2).

Tiguvon had no effect on the fungal ability to degrade and bleach the polymeric dye Poly
R when this was simultaneously incorporated into the agar matrix. This dye is degraded
by the ligninolytic system of *P. chrysosporium*, via a mechanism involving LiP and the
fungal secondary metabolite VA acting as a redox mediator (Candeias and Harvey, 1995).

When pure fenthion was supplied at a final concentration of c. 7.2 $\mu$M to liquid cultures of
*P. chrysosporium*, the biomass yield (Figure 3) was comparable to that obtained from control
cultures where no fenthion had been added (data not shown). Furthermore, fenthion did

![Figure 2](image1.png)

**Figure 2** Dose-response curve of fenthion assayed with *P. chrysosporium* maintained on 2% agar
plates to which Tiguvon was incorporated over the concentration range shown. The diameter of
colonies relative to control plates without Tiguvon gave a measure of percentage inhibition.

![Figure 3](image2.png)

**Figure 3** Growth of *P. chrysosporium* in liquid culture in the presence of 7.2 $\mu$M fenthion added
at the time of inoculation. Data points are means of three replicates, error bars indicate standard
deviations.
Biodegradation of Fenthion by *Phanerochaete chrysosporium*

Figure 4 Production of LiP by liquid cultures of *P. chrysosporium* (a) without fenthion; (b) to which fenthion (7.2 µM final concentration in the culture broth) was added at the time of inoculation; (c) on the second day of growth; and (d) on the third day of growth.

not inhibit the production of LiP detected in the extra-cellular medium (Figure 4). By contrast, the addition of fenthion stimulated the synthesis of higher titres of LiP than measured in its absence (Figure 4).

In order to assess whether fenthion was degraded by *P. chrysosporium* in liquid cultures, samples were analysed by GC-NPD. When fenthion (elution time 5.92 min, see Figure 5a) was added to cultures at the time of inoculation, two fenthion-derived metabolites were detectable on the third day of growth, prior to the appearance of LiP. Based on their elution times (6.43 and 6.59; Figure 5c) and by comparison with data on known substances, they were tentatively identified as fenthion sulphoxide and fenthion sulphone. (The organism was exposed to fenthion and material with phosphorus was observed, presumably derived from the parent fenthion.) However, by the fourth day of growth when LiP was detected in the culture medium, no peaks with elution times of 6.43 and 6.59 could be detected and only a residual amount of the original sample was evident (Figure 5d). After 5 days the amount of fenthion detectable represented less than 1% of the original sample (data not shown). Furthermore, neither fenthion nor its degradation products could be detected in fungal pellets of mycelia that were extracted with dichloromethane after 5 days of growth.

To explore whether LiP itself was capable of oxidising fenthion, the enzyme was purified from culture media and its ability to oxidise fenthion investigated. Figure 6a shows the UV-visible spectrum of LiP (absorbance maximum at 408 nm; Renganathan and Gold, 1986) in the presence of 5 equivalents of fenthion (7.2 nmol; absorbance maximum at 250 nm).
L. Zacchi et al.

Figure 5  GC-NPD analysis of (a) fenthion and extracts concentrated from the culture broth of liquid cultures of *P. chrysosporium*. 20-ml samples were extracted with dichloromethane and taken up in 3 ml methanol for analysis. Culture broth was extracted after 3 days growth of *P. chrysosporium* in (b) the absence and (c) presence of 7.2 µM fenthion; in (d), samples were extracted after 5 days growth.

On addition of 7 equivalents of H$_2$O$_2$, the absorbance maximum at 408 nm (native state of LiP) shifted to 412 nm (native state and oxidised intermediate compound II; Renganathan and Gold, 1986) and then returned to 408 nm, indicating turnover of LiP with fenthion as reductant. Fenthion was oxidised in the course of the reaction as judged by the decrease in absorbance at 250 nm, as well as by HPLC analysis (data not shown). However, when H$_2$O$_2$ was supplied in an amount equal to the $k_m$ of LiP for H$_2$O$_2$ (c. 0.4 mM), the formation of oxidised fenthion was not stoichiometric to the amount of H$_2$O$_2$ supplied, as judged by HPLC analysis of the products of the reaction. Analysis by UV-visible spectrometry showed a shift in the absorbance maximum for LiP to 420 nm followed by a decrease in value, indicative of the formation of the inactive enzyme intermediate, Compound III (Renganathan and Gold, 1986). To obviate this problem, VA was included in the reaction mixture. However, whilst able to prevent the formation of the inactive intermediate compound III by supplying an increased level of reducing equivalents, VA did not serve as a redox mediator in these reactions, rather, it inhibited the oxidation of fenthion. Figure 5b shows that when veratryl alcohol was supplied in an amount equimolar to fenthion (7.2 nmol) the absorbance at 250 nm did not decrease, rather, the absorbance at 310 nm increased corresponding to the formation of veratraldehyde from the oxidation of veratryl alcohol.

**DISCUSSION**

In the presence of fenthion, liquid cultures of *P. chrysosporium* not only produced higher titres of LiP but also transformed the pesticide, possibly into fenthion sulphoxide and
Biodegradation of Fenthion by *Phanerochaete chrysosporium*

Figure 6  Absorbance spectra for the reaction of LiP (1.5 nmol) with 7.2 nmol fenthion and 20 nmol H$_2$O$_2$ in 20 mM potassium phosphate buffer pH 2.75 in (a) the absence and (b) presence of 50 nmol veratryl alcohol.

sulphone derivatives prior to complete mineralisation. These latter fenthion derivatives were identified in the extra-cellular medium during the primary growth phase of the fungus, consequently the possibility that fenthion, when added at the outset of growth, was initially degraded by abiotic means cannot be ruled out. However, the rapid disappearance of both fenthion and derivatives at the time of LiP appearance supports the view that fenthion was metabolised during fungal secondary metabolism via the ligninolytic system centred on LiP expression. This is further supported by the fact that purified LiP was able to accept fenthion as a reductant, but only at low levels of H$_2$O$_2$. At high levels of H$_2$O$_2$, evidence for enzyme inactivation was found. LiP is known amongst peroxidases to be sensitive to H$_2$O$_2$ (Wariishi and Gold, 1990). It readily forms the catalytic dead-end enzyme intermediate Compound III by reaction of the active intermediate Compound II with H$_2$O$_2$, when substrates other than dimethoxylated aromatics are supplied as reductants (phenols or mono-methoxylated aromatics of high redox potential, for example). It can also be driven into Compound III when the concentration of reductant relative to H$_2$O$_2$ supplied is low. In the case of fenthion, the low solubility of this compound in an aqueous medium may have contributed to enzyme inactivation at the higher H$_2$O$_2$ levels. The problem of enzyme inactivation during catalysis can be circumvented by addition of veratryl alcohol, which both stabilises Compound II against reaction with H$_2$O$_2$ as well as serving as a redox mediator. Surprisingly, no evidence for redox mediation with fenthion by veratryl alcohol was obtained at the concentrations of the reactants used. This may simply reflect the low solubility of fenthion in aqueous medium, but the possibility of fenthion having a high
redox potential relative to veratryl alcohol radical cations cannot be ruled out. Alternatively, there may be no kinetic pathway between reactants.

Some evidence for growth inhibition by Tiguvon incorporated in agar plates was obtained, but it is unclear whether this was due to fenthion itself, given that in liquid cultures fungal growth, measured in terms of biomass yield, was not affected by the presence of the pesticide. Fenthion was not inhibitory to the germination of spores, differently from, for example, crystal violet, which inhibited the germination of spores of *P. chrysosporium* grown under similar conditions (Bumpus and Brock, 1988). The capacity of fungal spores to germinate in the presence of the xenobiotic may be exploited in practical applications using direct spore inocula, rather than growing the fungus on wood chips or other material prior to incorporation into contaminated soil (see, for example, Holroyd and Caunt, 1997). Furthermore, *P. chrysosporium* appeared to metabolise, rather than adsorb or accumulate, fenthion into intra-cellular compartments, paving the way for optimising degradation methods with this fungus.

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**REFERENCES**


