Oxidative biodegradation of phosphorothiolates by fungal laccase

G. Amitai[a,*], R. Adani[a], G. Sod-Moriah[a], I. Rabinovitz[a], A. Vincze[a], H. Leader[a], B. Chefetz[b], L. Leibovitz-Persky[b], D. Friesem[b], Y. Hadar[b]

[a]*Department of Pharmacology, Israel Institute for Biological Research, P.O. Box 19, Ness Ziona 74100, Israel*
[b]*Faculty of Agriculture, Hebrew University, Rehovot 76100, Israel*

Received 17 August 1998; received in revised form 9 September 1998

Abstract Organophosphorus (OP) insecticides and nerve agents that contain P-S bond are relatively more resistant to enzymatic hydrolysis. Purified phenol oxidase (laccase) from the white rot fungus *Pleurotus ostreatus* (*Po*) together with the mediator 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) displayed complete and rapid oxidative degradation of the nerve agents VX and Russian VX (RVX) and the insecticide analog diisopropyl-Amiton with specific activity: k_{sp} = 2200, 667 and 1833 nmol min^{-1} mg^{-1}, respectively (pH 7.4, 37°C). A molar ratio of 1:20 for OP/ABTS and 0.05 M phosphate at pH 7.4 provided the highest degradation rate of VX and RVX. The thermostable laccase purified from the fungus *Chaetomium thermophilium* (*Ct*) in the presence of ABTS caused a 52-fold slower degradation of VX with k_{sp} = 42 nmol min^{-1} mg^{-1}. The enzymatic biodegradation products were identified by ^{31}P-NMR and GC/MS analysis.

Key words: Enzymatic oxidation; Laccase; 2,2′-Azinobis(3-ethylbenzthiazoline-6-sulfonate); Phosphorothiolate; *O*-Ethyl *S*-[*N*,*N*-diisopropylaminoethyl]methylphosphonothiolate; *O*-Isobutyl *S*-[*N*,*N*-diethylaminoethyl]methylphosphonothiolate; Diisopropyl-Amiton; *Pleurotus ostreatus*

1. Introduction

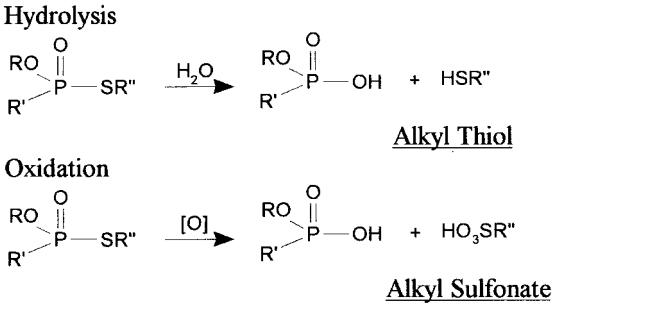
Enzymatic degradation of organophosphorus (OP) insecticides and nerve agents is carried out either by OP acid hydrolases (OPH) (EC 3.1.8.1) or OP acid anhydrolase (OPAA, EC 3.1.8.2) [1]. Certain OPHs show selectivity toward the insecticide parathion and particularly toward its oxo metabolite *O*,*O*-diethyl *p*-nitrophenyl phosphate (paraoxon). OPH was isolated either from bacterial or mammalian origins and catalyzes the breakage of the P-O bond of paraoxon with a bimolecular rate constant of 5×10^7 M^{-1} s^{-1} [1]. OPH enzymes with high paraoxonase activity were purified from *Pseudomonas diminuta* MG, *Pseudomonas* sp. and *Flavobacterium* bacterial species ATCC 27551 [2,3] and also from human plasma [4]. OPAA isolated from the thermophilic bacteria *Altermonas* sp. JD6.5 displayed high hydrolytic activity toward the nerve agent soman that contains a P-F bond in its chemical structure [5,6]. However, it was observed that the P-S bond in certain phosphorothiolates (e.g. amiton and VX) is usually more resistant to OPH enzymatic hydrolysis. Since VX is a persistent nerve agent and its decontamination is critical for survival it became a challenge to find an enzymatic system for its environmentally-safe degradation. It was previously noted that certain phosphorothiolate nerve agents such as VX could rapidly be degraded by chemical oxidation of the P-S bond using various peroxides such as hydrogen peroxide [7] and monomagnesium perphthalate [8]. As shown in Scheme 1, the oxidative hydrolysis of phosphonothiolates such as VX leads to the formation of *O*-ethyl methylphosphonic acid and *N*,*N*-dialkylaminoethanesulfonate as compared to the corresponding alkylthiol formed via the hydrolysis pathway. The alkylthiol hydrolytic product is notorious for its mercaptane smell and moderate toxicity. Thus, the oxidative pathway forms non-toxic and more environmentally benign degradation products.

Therefore, it is conceivable that enzymatic oxidative breakdown of the P-S bond in certain phosphorothiolates (e.g. VX), rather than hydrolysis, will eventually lead to more efficient detoxification. The degradative properties of phenol oxidase (laccase), lignin peroxidase and Mn peroxidases isolated from various white rot fungi have been extensively studied [9–11]. These enzymes are applicable for biodegradation of lignin as a more environmentally safe degradation method than the chlorine-based bleaching process. Laccase isolated from various fungi such as *Trametes versicolor*, and *Pleurotus ostreatus* degrade lignin efficiently using various mediators such as 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS), 1-hydroxy benztriazole (HOBT) and several phenothiazines such as promazine and chlorpromazine [12]. The combination of fungal laccase with certain mediators was proved to be active in delignification of kraft pulp and created considerable interest in the paper industry [13]. Since oxidative biodegradation of lignin proceeds without the formation of a direct Michaelis complex between laccase and the substrate but rather by using a diffusible oxidizing mediator, the scope of laccase substrates may be expanded. Indeed, we have successfully employed fungal laccase together with several mediators for degradation of toxic phosphorothiolates. We report here the rapid oxidative degradation of *O*,*O*-diethyl *S*-[*N*,*N*-diisopropylaminoethyl]phosphorothiolate (DiPr-Amiton), and the nerve agents *O*-ethyl *S*-[*N*,*N*-diisopropylaminoethyl]methylphosphonothiolate (VX) and *O*-isobutyl *S*-[*N*,*N*-diethylaminoethyl]-methylphosphonothiolate (RVX) by laccase purified from *Pleurotus ostreatus* using ABTS as a mediator. This approach offers an important advantage over existing biocatalysts that require precise protein engineering of the catalytic site and overcomes the problem of low binding affinity of certain OP substrates.

*Corresponding author. Fax: (972) (8) 9381559.
E-mail: amitai@iibr.gov.il

Abbreviations: OP, organophosphates; VX, *O*-ethyl *S*-[*N*,*N*-diisopropylaminoethyl]methylphosphonothiolate; RVX, *O*-isobutyl *S*-[*N*,*N*-diethylaminoethyl]methylphosphonothiolate; DiPr-Amiton, *O*,*O*-diethyl *S*-[*N*,*N*-diisopropylaminoethyl]phosphorothiolate; ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate); *Po*, *Pleurotus ostreatus*; AChE, acetylcholinesterase

PII: S0014-5793(98)01300-3



VX: R= C_2H_5, R'= CH_3, R''= $CH_2CH_2N(iPr)_2$
RVX: R= iso-C_4H_9, R'= CH_3, R''= $CH_2CH_2N(Et)_2$
DiPr Amiton: R= C_2H_5, R'= OC_2H_5, R''= $CH_2CH_2N(iPr)_2$

Scheme 1. Degradation pathways of VX, RVX and DiPr-Amiton by hydrolysis and oxidation.

2. Materials and methods

2.1. OP substrates

The OP compounds *O*-ethyl *S*-[*N*,*N*-diisopropyl aminoethyl]methylphosphonothiolate (VX), *O*-isobutyl *S*-[*N*,*N*-diethylaminoethyl]methylphosphonothiolate (RVX) and *O*,*O*-diethyl *S*-[*N*,*N*-diisopropylaminoethyl]phosphorothiolate (DiPr-Amiton) were synthesized according to previously described procedures [14,15]. The structure and purity of the OP compounds were elucidated by ^{1}H- and ^{31}P-NMR and GC/MS analysis.

2.2. Growth of fungus and purification of Pleurotus ostreatus laccase

2.2.1. Culture of organisms: Pleurotus ostreatus (Po) 'Florida' F6. The fungus was grown in a basidomycete synthetic medium (BSM) that contained the following per liter of culture: glucose (5 g), K_2HPO_4 (1 g), L-asparagine (0.6 g), KCl (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), yeast extract (Difco, 0.2 g), $ZnNO_3$ (0.003 g), $CuSO_4 \cdot 5H_2O$ (0.003 g), $Ca(NO_3)_2 \cdot 4H_2O$ (0.006 g) at pH 5.5. Stock cultures were maintained on 2% agar BSM. A typical preparation consisted of 20 discs (5 mm diameter) in 250 Erlenmeyer flasks containing 60 ml of liquid BSM. After 5 days of growth, the mycelium was homogenized by Ultra-Turrax (TP18/10, IKI-WERK, Staufen, Germany) for 15 s. Shaken cultures were cultivated (40 ml) in 500-ml Erlenmeyer flasks

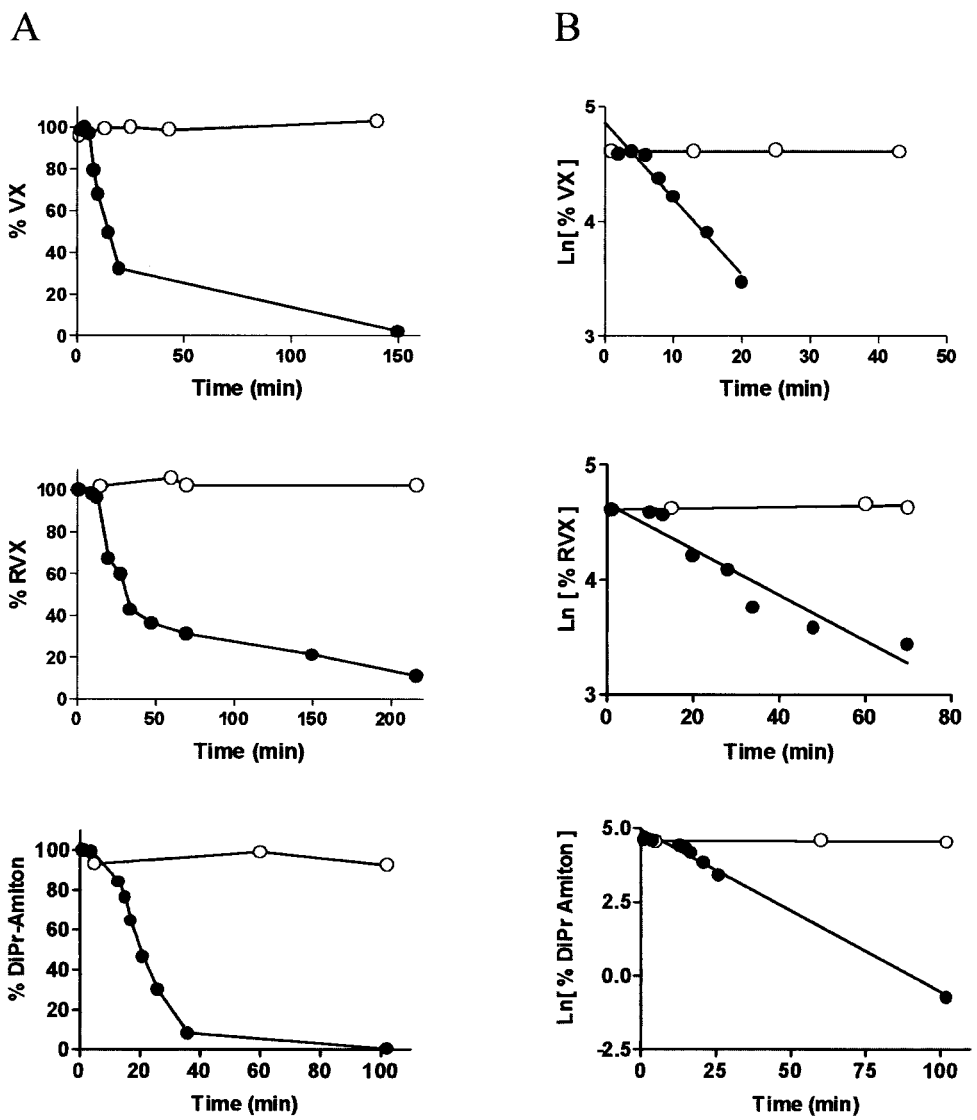


Fig. 1. Degradation kinetics of phosphorothiolates by *Pleurotus ostreatus* laccase in the presence of ABTS ([OP] = 10 μM, [ABTS] = 200 μM, pH = 7.4, 50 mM phosphate, 37°C). A: Time-course of OP enzymatic degradation. B: Ln[%OP] as a function of degradation time. Enzyme degradation (●); buffer control (○).

containing 200 ml liquid BSM. All of the flasks were incubated for 2 days at 28°C in the dark in a rotary shaker at 120 rpm and then were used (12 ml) for inoculation in 250-ml Erlenmeyer flasks containing 60 ml of liquid BSM.

2.2.2. Protein purification. After 8 days of growth the extracellular medium was collected by filtration through glass wool followed by 0.45-μm filters. The fluids were concentrated 50-fold relative to the original concentration, using an Amicon 10K membrane filter and dialyzed against 10 mM sodium acetate buffer, pH 6. The proteins were separated on FPLC AKTA explorer (Pharmacia Biotech, Sweden). The concentrated extracellular medium was loaded onto a Hi-Trap Q anion exchange column (HiTrap Q 5-ml column, Pharmacia Biotech) previously equilibrated with 10 mM sodium acetate, pH 6. After washing with 6 column volumes (cv) of 10 mM sodium acetate buffer, the enzyme was eluted by two linear NaCl gradients, 0–0.3 M in 30 cv; 0.3–1.0 M in 10 cv. Enzyme activity was monitored using ABTS as a substrate and the active fractions were collected, concentrated and loaded onto a gel filtration column (Superdex 200HR 10/30, Pharmacia Biotech). Prior to elution, the column was equilibrated with 10 mM sodium acetate buffer, pH 6. After elution, the active fractions were collected. This fraction showed a single band on PAGE (10% acrylamide) as visualized by silver stain. Laccase from *Chaetomium thermophilium* was purified according to Chefetz et al. [16].

2.3. Enzyme activity

Laccase activity was monitored using ABTS as a substrate. The reaction mixture contained 0.05 ml of enzyme, 0.1 ml of ABTS (5 mM), 0.1 M phosphate buffer (pH 6) and was incubated for 3 min at 30°C. Absorbance at 436 nm was recorded.

2.4. Enzymatic assay for degradation kinetics of OP substrates

The enzymatic degradation mixture contained 50 μl of OP substrate at a final concentration of 10 μM (stock solutions of VX, RVX or DiPr-Amiton at 0.2 mM concentration in citrate buffer, pH 5.6), 20 μl of ABTS (10 mM) and 20 μl of purified *Pleurotus ostreatus* laccase (6 μg/ml) in 1 ml of phosphate buffer (50 mM, pH 7.4) incubated at 37°C. Three control solutions that were monitored parallel to the degradation solution contained the OP substrate in the presence of the following reagents: control 1: phosphate buffer; control 2: phosphate buffer with ABTS; and control 3: phosphate buffer with laccase. Degradation of OP compounds in all buffer control solutions was less than 10% at the time enzymatic degradation was complete. The residual OP level was monitored by 5×10^3-fold dilution of the degradation solution for measuring the initial rate of inhibition of fetal bovine serum acetylcholinesterase (FBS-AChE) for 5 min at 25°C. The activity of FBS-AChE was determined using the Ellman method [17].

2.5. GC/MS analysis

Samples of solutions after enzymatic degradation were evaporated to dryness using Speed Vac centrifuge under reduced pressure. The solid residue was dissolved in 0.1 ml 5 mM HCl and 0.25 ml of chloroform/methanol (9:1) mixture was added. Methylation was performed with excess diazomethane in ether. Organic phase aliquots of 1 μl were injected to the GC injector. GC/MS analysis was performed using VG model 70VSEQ mass spectrometer directly interfaced to a GC model HP 5890. Chemical ionization (CI) gas was isobutane. GC conditions: HP5 column, 30 m length, 0.22 mm i.d., 1 μm film, head pressure: 55kPa, and temperature programming: 40°C for 1 min, heating rate: 10°C/min up to 280°C and remains at 280°C for 10 min.

Table 1
First order rate constants (k_{obs}, min^{-1}) and specific activity values (k_{sp}, nmol min^{-1} mg^{-1}) of VX, RVX and DiPr-Amiton degradation by fungal laccase

Source of laccase (substrate)	k_{obs} (min^{-1})	k_{sp} (nmol min^{-1} mg^{-1})
Pleurotus ostreatus (VX)	0.066	2200
Pleurotus ostreatus (Amiton)	0.055	1833
Pleurotus ostreatus (RVX)	0.020	667
Chaetomium thermophilium (VX)	0.0013	42

k_{obs} values were calculated for 6 μg/ml purified laccase, [OP] = 10 μM, [ABTS] = 200 μM, pH = 7.4, 50 mM phosphate, 37°C.

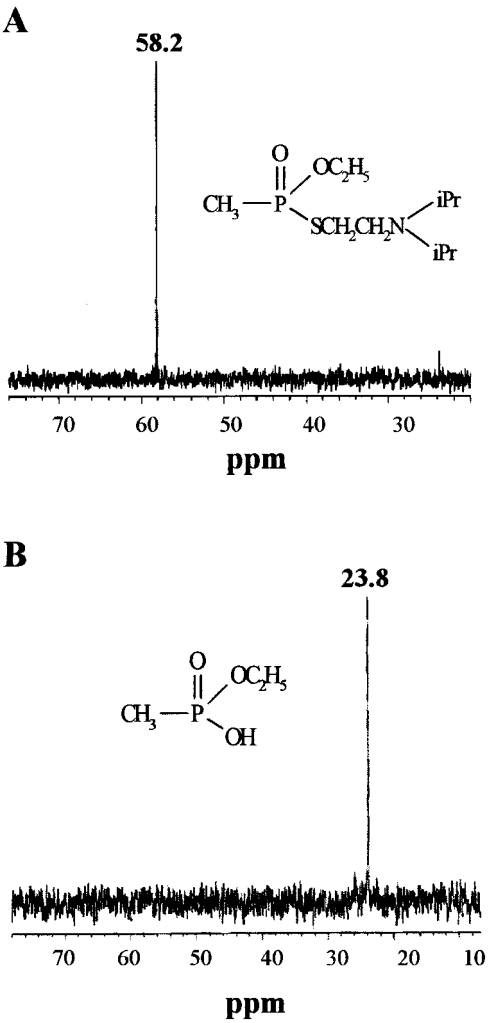


Fig. 2. ^{31}P-NMR spectra of VX before (A) and after (B) oxidative biodegradation in ammonium bicarbonate (50 mM, pH = 7.4, 25°C).

2.6. NMR spectra

NMR spectra were recorded with a GN 300WB NMR instrument (General Electric, USA) at 121.65 MHz for ^{31}P and 300.0 MHz for ^{1}H. The NMR spectra were determined for purity and structure elucidation of the synthesized phosphorothiolates and for the analysis of degradation products of VX. Degradation of 0.5 mM VX was performed in ammonium bicarbonate buffer 0.05 M, pH 7.5, 25°C, using *Pleurotus ostreatus* laccase and 5 mM ABTS. ^{31}P-NMR spectra of VX were recorded with ABTS before addition of laccase and after 6 h incubation with laccase and ABTS at 25°C. The spectra were performed with a pulse delay of 2 s using proton decoupler mode and trimethylphosphate 1% in benzene as an external standard. D_2O was added to the aqueous degradation solution for locking the deuterium signal.

3. Results and discussion

The time-course of enzymatic degradation of VX, RVX and DiPr-Amiton by purified *Pleurotus ostreatus* laccase in the presence of ABTS as a mediator is described in Fig. 1A. Fig. 1B presents the semilogarithmic plots of Ln (%OP) as a function of time. These linear curves were used for the calculation of first order rate constants of OP degradation k_{obs} (min^{-1}). The k_{obs} values for VX, RVX and DiPr-Amiton are: 6.6, 5.5 and 2.0×10^{-2} min^{-1}, respectively, and the cor-

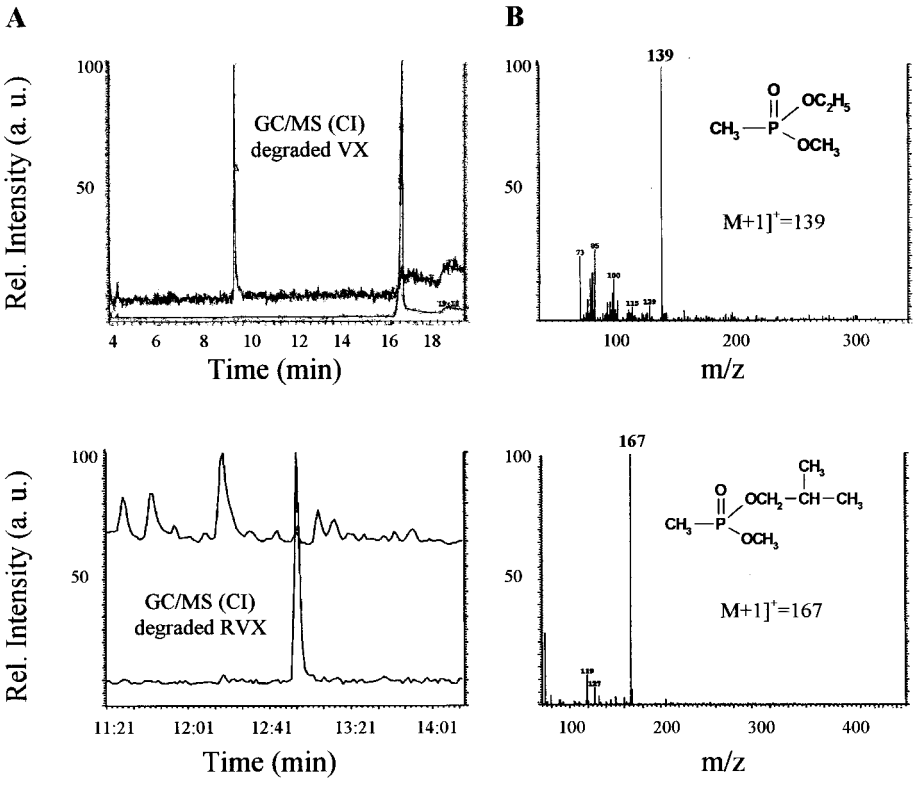


Fig. 3. GC/MS total ion current (TIC) and mass chromatograms (A) and mass spectra (B) obtained after degradation of VX (upper chart) and RVX (lower chart) by *Pleurotus ostreatus* laccase.

responding specific activity values (k_{sp}) are: 2200, 1833 and 667 nmole min^{-1} mg^{-1}, respectively (Table 1). As shown in Fig. 1A, the oxidative enzymatic degradation of all phosphorothiolates proceeds to completion. Complete degradation of both optical isomers of VX was confirmed by ^{31}P-NMR spectrum following 6 h incubation of 0.5 mM VX with laccase/ABTS in ammonium bicarbonate buffer (0.05 M, pH 7.5) (Fig. 2). The ^{31}P-NMR signal of VX at 58.2 ppm (Fig. 2A) disappeared completely after oxidative biodegradation and a new signal, assigned to *O*-ethyl methylphosphonic acid is formed at 23.8 ppm (Fig. 2B). The mass chromatograms and mass spectra obtained from the GC/MS analysis of VX and RVX are presented in Fig. 3A and B, respectively. The formation of *O*-ethyl methylphosphonic acid and *O*-isobutyl methylphosphonic acid is displayed in the mass chromatogram and mass spectra of the methyl esters of these degradation products of VX and RVX following methylation by diazomethane (see Section 2). The mass spectrum of the compound eluting with a retention time of 17:07 min (see total ion current, TIC in upper part of Fig. 3A) of degraded VX has an M+1]$^+$ of $m/z = 139$ calculated to be the quasimolecular ion of *O*-ethyl *O*-methyl methylphosphonate (Fig. 3B, upper part where M is the molecular ion). The mass spectrum of the compound eluting with a retention time of 12:55 min (see TIC in lower part of Fig. 3A) of degraded RVX has an M+1]$^+$ of $m/z = 167$ calculated to be the quasimolecular ion of *O*-isobutyl *O*-methyl methylphosphonate (Fig. 3B, lower part). The appearance of the molecular ion peaks of methyl esters of the corresponding *O*-alkyl methylphosphonic acids together with the absence of the molecular ions of VX and RVX and of their corresponding *N*,*N*-dialkylaminoethane thiols in the GC/MS analysis after degradation (Fig. 3) indicate complete oxidative biodegradation of both optical isomers of these OP compounds by the laccase/ABTS mixture.

The dependence of degradation rate of VX, RVX and DiPr-Amiton on pH, buffer and ABTS concentration are described in Fig. 4A, B and C, respectively. The optimal pH for degradation of VX and RVX is 7.4 as compared to DiPr-Amiton that is degraded more rapidly at pH 8. The most rapid degradation rate of VX and RVX is obtained at a concentration of 0.05 M phosphate whereas the rate of DiPr-Amiton degradation is higher at 0.2 M phosphate. The optimal molar ratio of ABTS/OP for VX and RVX degradation is 1:20 whereas the rate of DiPr-Amiton degradation reaches its maximum at a molar ratio of 1:10.

The oxidative degradation of VX, RVX and DiPr-Amiton by *Po* laccase together with ABTS as mediator (Table 1) displays relatively rapid reaction rates that are unprecedented so far for enzymatic degradation of phosphorothiolates. For instance, the calculated $t_{1/2}$ for VX degradation by *Po* laccase (1 mg/ml) with ABTS is 5 s. It is pertinent to note that the cobalt metallo-enzyme form of recombinant *Pseudomonas diminuta* OPH displayed hydrolytic activity toward VX and RVX [18]. Furthermore, a very slow (14–16 days) and only partial degradation of the P-S containing insecticides Fonofos and Terbufos was caused by nitrogen-limited cultures of the fungus *Phanerochaete chrysosporium* [19]. This white rot fungus caused slow mineralization of Fonofos and Terbufos only to a level of 12.2 and 26.6%, respectively [19]. The exact mechanism of oxidative degradation of phosphorothiolate by laccase/mediator is as yet unknown and it is assumed that the sulfur atom is oxidized followed by cleavage of the P-S bond [20]. The nitrogen atom at β position to the carbon bound to the sulfur atom may also play an important role in the enzy-

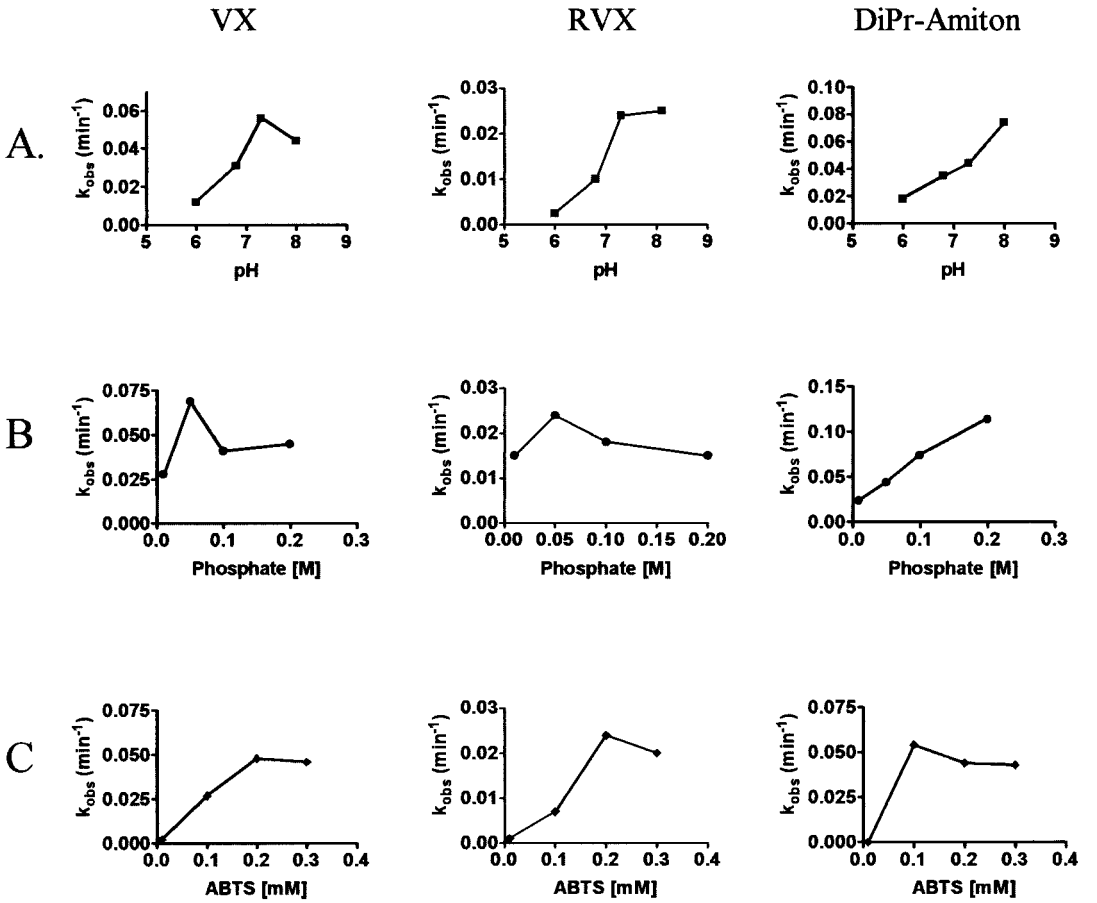


Fig. 4. Dependence of VX, RVX and DiPr-Amiton degradation rate by *Pleurotus ostreatus* laccase on pH, phosphate and ABTS concentration ([OP] = 10 μM, 37°C). A: k_{obs} (min^{-1}) as a function of pH (ABTS 0.2 mM, phosphate 50 mM). B: k_{obs} (min^{-1}) as a function of phosphate concentration (ABTS 0.2 mM, pH = 7.4). C: k_{obs} (min^{-1}) as a function of ABTS concentration (pH = 7.4, phosphate 50 mM).

matic oxidation reaction. One possible pathway is the formation of an *N*-oxide intermediate in the *N*,*N*-dialkyl aminoethyl moiety at alkaline pH that may affect the cleavage of the P-S bond [20]. The formation of *N*-oxide derivative of *N*,*N*-dialkylaminoethylsulfonate moiety was not confirmed as yet probably due to its low volatility that causes difficulties in GC separation. We have shown here that oxidative biodegradation of both optical enantiomers of VX and RVX by laccase/ABTS was verified by the enzymatic AChE assay, GC/MS and ^{31}P-NMR analysis. It was previously noted by Hall et al. [21] that the P(−) enantiomer of VX is 6.4-fold more toxic than its P(+) antipode. However, in contrast to other chiral OP compounds where the P(+) enantiomer is far less toxic than its P(−) counterpart, the P(+) isomer of VX is still very toxic (LD_{50} = 0.21 μmol/kg, subcutaneous in rats) and the apparent bimolecular rate constants of AChE inhibition in vitro by these isomers are similar (4.5×10^7 and 2.8×10^6 M^{-1} min^{-1}, respectively) [21]. Therefore, oxidative biodegradation of both optical enantiomers of VX and RVX by laccase/ABTS is important, since it leads to complete detoxification of these toxic nerve agents.

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