

Analysis of Phosphorothionate Pesticides Using a Chloroperoxidase Pretreatment and Acetylcholinesterase Biosensor Detection

CLARISSE B. S. ROEPCKE,^{*,†} SUSANNE B. MUENCH,[†] HOLGER SCHULZE,[‡]
TILL BACHMANN,[‡] ROLF D. SCHMID,[†] AND BERNHARD HAUER[†]

[†]University of Stuttgart, Institute of Technical Biochemistry, Allmandring 31, 70569 Stuttgart, Germany,
and [‡]University of Edinburgh, Chancellors Building, Division of Pathway Medicine, 49 Little France
Crescent, Edinburgh EH16 4SB, U.K., Scotland

Acetylcholinesterase (AChE) is responsible for the hydrolysis of acetylcholine in the nervous system. It is inhibited by organophosphate and carbamate pesticides. However, this enzyme is only slightly inhibited by organophosphorothionates, which makes the detection of these pesticides analytically very difficult. A new enzymatic method for the activation and detection of phosphorothionates was developed with the capability to be used directly in food samples without the need of laborious solvent extraction steps. Chloroperoxidase (CPO) from *Caldariomyces fumago* was combined with *tert*-butyl hydroperoxide and two halides. Chlorpyrifos and triazophos were completely oxidized. Fenitrothion, methidathion and parathion methyl showed conversion rates between 54 and 61%. Furthermore, the oxidized solution was submitted to an AChE biosensor assay. Chlorpyrifos spiked in organic orange juice was oxidized, where its oxon product was detected in concentrations down to 5 $\mu\text{g/L}$ (final concentration food sample: 25 $\mu\text{g/L}$). The complete duration of the method takes about 2 h.

KEYWORDS: Chloroperoxidase; acetylcholinesterase biosensor; phosphorothionates; oxidation; pesticides; juice

INTRODUCTION

Pesticides are used in agriculture in order to increase yield and control fungi, insects and weeds. Since the banning of organochlorides, organophosphates and carbamates are the classes of the most widely used insecticides, due to their high activity and relatively low persistence (1–3). The disadvantage of using these insecticides is the contamination of drinking water and food. Additionally, these pesticides are intended to kill living organisms. It was demonstrated that they show a potential dose-related chronic and acute toxicity against humans (4).

Acetylcholinesterase (AChE) (EC 3.1.1.7) is an important enzyme found in vertebrates and insects, which hydrolyzes the neurotransmitter acetylcholine in the nervous system. It is responsible for the termination of nerve impulses to the cholinergic synapses. The organophosphate and carbamate pesticides bind to the esterase active site of this enzyme and inhibit its catalytic activity. They block the active site of AChE by phosphorylation or carbamylation of the serine residue of the catalytic triad, hence preventing the termination of a nerve impulse in the postsynaptic membrane (5).

Due to excessive application in agriculture, the control of pesticides has been widely recognized as an important issue for public health. The monitoring of pesticides has increased in recent

years with most countries establishing maximum residue levels (MRL) for pesticides in food products, in order to protect consumers (6). In addition, supermarket chains and food distributors have enhanced their concerns about the pesticide levels in food due to enhanced pressure from consumers (7). Therefore, there is an urgent demand for rapid sensitive and cost-effective pesticide detection technologies.

The classical and standard assays for insecticide detection are based on gas chromatography (GC) or high performance liquid chromatography (HPLC) coupled with mass selective detectors (MSD) (8, 9). The disadvantage of these methods is that they are rather time-consuming and expensive, thus only applicable in central laboratories (10). In addition, there are a limited number of pesticides that can be identified by multiresidue methods, enhancing the possibility of false negative results (8, 9). As an alternative, AChE inhibition tests, and AChE biosensors in particular, have been repeatedly described for use in insecticide detection (11). The most common AChE biosensor design is based on the immobilization of AChE and the use of acetylthiocholine as substrate, in combination with an amperometric transducer. The reaction product thiocholine is oxidized at the working electrode (12–14).

Most of the organophosphate insecticides applied worldwide belong to the group of phosphorothionates. They are lipophilic and characterized by one thione moiety ($\text{P}=\text{S}$) and three $-\text{OR}$ groups attached to a phosphorus atom, whereas their respective

*Author to whom correspondence should be addressed. Tel: +49 71168564567. Fax: +49 71168563196. E-mail: itbcbs@itb.uni-stuttgart.de.

oxidized analogues are more polar, characterized by a double phosphorus oxygen bound ($P=O$) (15). This group of pesticides usually exhibits a reduced inhibitory effect toward AChE, due to a lower reactivity of the $P=S$ group caused by the minor electronegativity of sulfur compared to oxygen (16, 17). Despite this, the AChE inhibition of these insecticides is greatly increased *in vivo*, because they are transformed into their oxon analogues by cytochrome P-450 monooxygenase, found in living organisms (18).

Phosphorothionates are not detectable in commonly found concentrations by AChE inhibition, and therefore they are an analytical challenge. To solve this problem they have to be oxidized into their oxon analogues before the inhibition tests, in order to enhance the sensitivity and prevent suspected false negative results. According to DIN 38415-1 (Deutsches Institut für Normung), oxidations are performed using *N*-bromosuccinimide (NBS), and the excess of this reagent is destroyed by ascorbic acid (19). There are also several chemical oxidation procedures with bromine (20) or chlorine (21), which is generated in a hydrolysis reaction with sodium chloride.

Chloroperoxidase (CPO) (EC 1.11.1.10) is an enzyme produced by the marine fungus *Caldariomyces fumago*. It is a glycosylated hemoprotein containing iron(III) protoporphyrin as the prosthetic group and has a *pI* in the range 3.2–4.0 (22–27). In addition, it uses hydrogen peroxide (H_2O_2) as oxidant and does not require any cofactor. This enzyme has a broad substrate range, is easily isolated in large quantities and is relatively stable under nonoxidizing conditions. These properties make CPO one of the most attractive and promising peroxidase enzymes for synthetic applications (28). CPO and cytochrome P-450 present a number of similar physical properties as shown by electronic absorption, electron paramagnetic resonance, and Mossbauer spectroscopy. CPO, like P-450, forms a reduced + CO complex absorbing at an abnormally long wavelength (443 nm). This happens due to thiolate anion as the proximal ligand of the heme iron (29, 30). In addition, the fifth axial ligand of the catalytically active iron of CPO is, analogously to the P450 enzymes, a cysteine instead of the usual histidine. Moreover, the iron atom is more exposed than in any peroxidase known. It has aptly been named “a heme peroxidase-cytochrome P450 functional hybrid” for these reasons (24, 28).

The application of CPO to perform oxidations of organophosphorothionate pesticides, meaning the transformation of $P=S$ into $P=O$, was first described by Hernandez et al. (31). Ten organophosphorus pesticides containing the phosphorothionate group were oxidized by CPO in buffer in the presence of H_2O_2 and chloride ions. The products were identified as oxon derivatives, where the sulfur atom from the thioate group was substituted by an oxygen atom. Walz and Schwack combined a solvent extraction of the food sample with a CPO oxidation step. After that, a spectrophotometric assay with the enzyme cutinase was performed, in order to measure the inhibition of the oxidized product over this enzyme (32).

To increase the detection sensitivity of phosphorothionates toward the AChE biosensors we decided to look for an alternative pretreatment method. This method should present the following characteristics: (1) be able to transform organophosphorothionate pesticides into their oxon form; (2) be applicable in food matrixes; (3) be unable to inhibit AChE. We began by investigating the ability of the enzyme CPO to convert thionates into their corresponded oxidized analogues in a complex matrix. Organic orange juice was chosen with this purpose. The oxidation conditions were optimized; in addition the k_i values of the enzymatically oxidized products were determined. The resulting method for the detection of phosphorothionate pesticides in orange juice, combining an activation pretreatment with CPO and a detection

assay using AChE biosensors, was evaluated by the detection of chlorpyrifos as a representative of this group of pesticides.

MATERIALS AND METHODS

Reagents. All insecticides (in Pestanal quality) and Tween 20 were purchased from Riedel-de Haën (Seelze, Germany). All other reagents were of analytical grade as supplied by Sigma (Deisenhofen, Germany) or Fluka (Neu-Ulm, Germany). CPO (EC 1.11.1.10) from *Caldariomyces fumago* (suspension in 0.1 M sodium phosphate pH 4.0; concentration higher than 10,000 U/mL) was purchased from Sigma (Taufkirchen, Germany). Organic orange juice (Alnatura GmbH, Bickenbach, Germany) was bought in a local store and used as a sample for the food tests.

Solutions. Chloroperoxidase solution was prepared by dissolving 192 mg in 5 mL of acetate buffer (0.05 M, pH 4.8). Insecticide stock solutions were prepared as 1 g/L solutions in ethanol and stored at -20°C . Dilutions for sensor measurements were made daily by dilution with acetate buffer (1 M, pH 5.5) and stored at -4°C . KCl and KBr solution were prepared in acetate buffer (1 M, pH 5.5). The 7.8 mM 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) solution was prepared by dilution with phosphate buffer (50 mM, pH 7.2). Both 10 mM acetylthiocholine iodide and 10 mM acetylthiocholine chloride solutions were prepared in water. The 0.4 g/L NBS and 4.0 g/L ascorbic acid solutions were prepared in water, as well as the 5.33% (w/v) pyrogallol solution.

AChE B *Nippostrongylus brasiliensis* Expression in *Pichia pastoris*. The AChE B wild type (WT) from *Nippostrongylus brasiliensis* was expressed in a *Pichia pastoris* X33 strain (Invitrogen, Karlsruhe, Germany) transformed with a pPICZαB vector (Invitrogen, Karlsruhe, Germany) bearing the *N. brasiliensis* AChE gene (33). The strain was cultivated in a complex medium, containing 2% (w/v) peptone, 1% (w/v) yeast extract, 1% glycerol (w/v), and potassium phosphate buffer (0.1 M, pH 7.5). After 24 h incubation at 30°C and shaking at 200 rpm, this medium was exchanged for 200 mL of medium without glycerol. Induction of AChE B expression was started by the addition of 0.5% (v/v) methanol. During shaking flask expression experiments, 0.5% (v/v) methanol was added every 24 h. After 5 days, protein expression cultures were centrifuged at 5000g for 20 min, and the supernatant was used as the source of the enzyme.

Chemical Oxidation of Phosphorothionates in Buffer. 40 μL of 0.4 g/L NBS solution was added to 3.92 mL of organophosphorothionate sample solution (final NBS concentration, 4.0 mg/L) and mixed in an ultrasonic bath for 5 min. Subsequently, 40 μL of 4.0 g/L ascorbic acid solution (final ascorbic acid concentration, 40.0 mg/L) was added to remove excessive NBS by mixing in an ultrasonic bath for 5 min.

AChE Stability Test toward KCl and KBr. Both enzymes were exposed to different concentrations of KCl and KBr solutions in acetate buffer 1 M pH 5.5. The remaining activity of AChE (31) after 30 min of exposure was measured.

CPO Enzymatic Oxidation of Phosphorothionates in Food Samples for GC/MS analysis. The organophosphorothionate pesticides were dissolved in 0.4 g of organic orange juice (final concentration, 2,500 $\mu\text{g/L}$), and the mixture was preincubated with 34 U/mL CPO, acetate buffer (1 M, pH 5.5) and the halogen ion solution. The applied concentration of the halogen ions was 0.67 M for KCl and 0.1 M for KBr. The suspension was stirred for 5 min; additionally the reaction was started by adding 800 mM *tert*-butyl hydroperoxide (t-b HP) (70% aqueous solution) in the case of the KCl tests, and 100 mM in the case of the KBr tests (final amount, 4 g). The reaction was kept for 45 min under agitation with Rotamix RM1, speed 10 rpm (Elmi, Riga, Latvia).

GC/MS Analysis. The reaction solutions were extracted with 1 mL of ethyl acetate, and once more with 1 mL of ethyl acetate after 1 mL of saturated NaCl solution was added to the aqueous phase. The combined ethyl acetate phase was dried over sodium sulfate, centrifuged and concentrated with N_2 .

The analysis was performed with a Shimadzu QP 2010 gas chromatograph (GC) (Kyoto, Japan), equipped with an FS Supreme 5 column (30 m/0.25 μm , 0.25 mm), coupled with a mass spectrometry detector (MS). The following oven program was used: 100°C for 1 min, then 20°C/min to 200°C , followed by 7°C/min to 300°C , and finally 300°C for 10 min. The carrier gas employed was helium, total flow 37.7 mL/min. The MS detector mode operation was as follows: detection 40–400 m/z

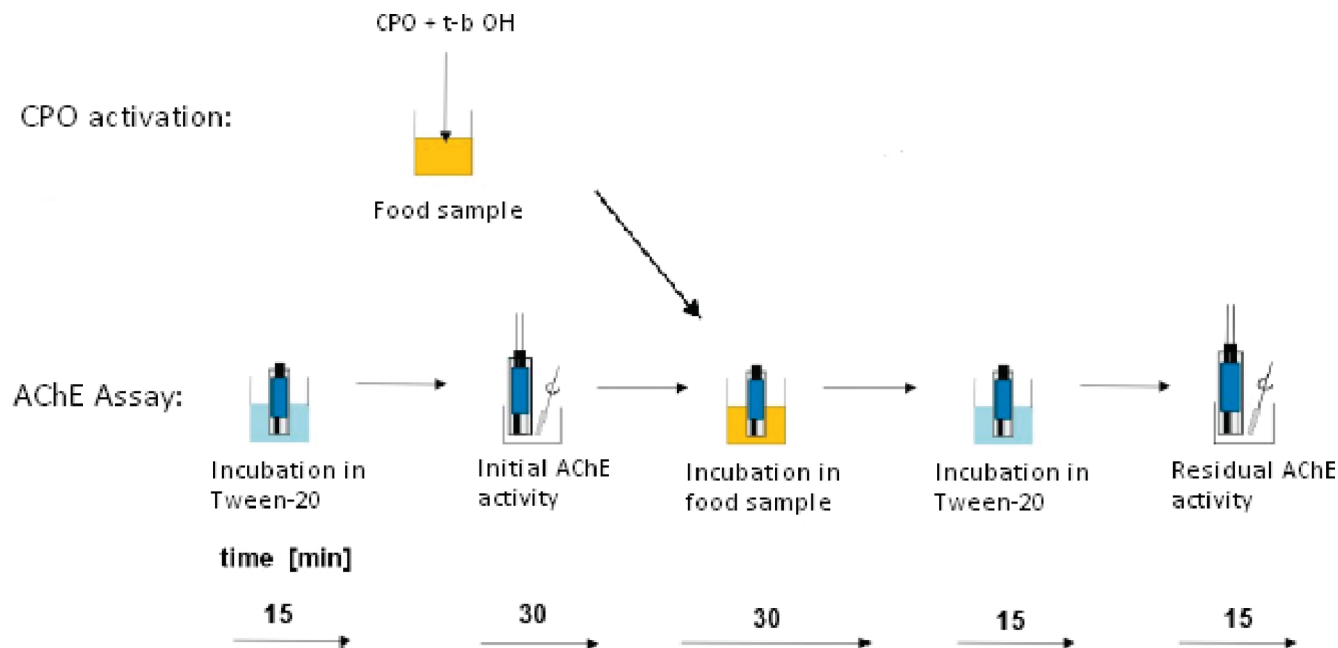


Figure 1. Flowchart of the method combining CPO pretreatment and the AChE biosensor analysis.

(5–30 min); ion source temperature 200 °C; interface temperature 250 °C. The multiplier voltage was relative to the tuning result. The injector temperature was of 250 °C.

AChE Activity Test. AChE activity was determined spectrometrically at room temperature (34). In brief, the assay containing 790 μ L of potassium phosphate buffer (50 mM, pH 7.2), 100 μ L of 7.8 mM DTNB solution and 10 μ L of 10 mM acetylthiocholine iodide solution was started by adding 10 μ L of enzyme solution. The increase of the absorption was followed at 412 nm. The volumetric AChE activity was calculated in U/mL.

CPO Activity Test. The determination of CPO activity was recorded spectrometrically at room temperature, according to McCarthy and White (35). The assay containing 800 μ L of 0.1 M phosphate buffer pH 6.0, 100 μ L of 5.33% pyrogallol solution (m/v) and 67 μ L of 0.4% H_2O_2 solution (v/v) (30% solution) was initialized by adding 33 μ L of enzyme solution. The increase of the absorption was followed at 420 nm. The volumetric CPO activity was calculated in U/mL.

Bimolecular Rate Constant. The determination of the bimolecular rate constants (k_i) was performed according to Aldridge (36). To estimate this constant, the enzyme Nb AChE WT (E) was incubated for various periods of time with different pesticide concentrations ([CX]) (with [CX] being at least 10-fold higher than [E] in potassium phosphate buffer (50 mM, pH 7.5 at 25 °C). The change of the concentration of the free enzyme [E] over time was estimated by recording the remaining activity measured as the turnover of the acetylthiocholine iodide substrate (1 mM) and followed a pseudo-first-order kinetics, $\ln[E]/[E_0] = -k_i[CX]t$ (where t represents the time of incubation, [CX] the inhibitor concentration, [E] the initial enzyme concentration, and [E] the free enzyme concentration after incubation with inhibitor). The pesticide solutions were mixed with acetate buffer (50 mM, pH 5.5), 8.5 U/mL CPO solution (in acetate buffer 50 mM, pH 5.5) and 0.1 M KCl or 0.1 M KBr (in acetate buffer 50 mM, pH 5.5). After 5 min of preincubation, 100 mM t-b HP (70% aqueous solution) was added to a final sample amount of 500 μ L. The solution was further mixed for 45 min using a Rotamix RM1, speed 10 rpm (Elmi, Riga, Latvia).

CPO Pretreatment of Food Samples for AChE Biosensor Measurement. 1.6 g as well as 3.2 g of the organic orange juice spiked with chlorpyrifos was mixed with acetate buffer (1 M, pH 5.5), 34 U/mL CPO solution and 0.1 M KBr. After 5 min of preincubation, 100 mM t-b HP (70% aqueous solution) was added (final amount of 4 g). The solution was further mixed for 45 min using a Rotamix RM1, speed 10 rpm (Elmi, Riga, Latvia). **Figure 1** illustrates the flowchart of the method combining CPO pretreatment and the biosensor test for the detection of phosphorothionates.

AChE Biosensor Measurement. Disposable biosensors with immobilized AChE were produced by screen-printing as previously described,

using a DEK 249 screen printer (DEK Ltd., Weymouth, England) (37). These transducers are formed by an Ag/AgCl reference electrode and 7,7,8,8-tetracyanoquinodimethane-graphite working electrode. The AChE B WT from *N. brasiliensis* was immobilized on the top of the working electrodes by cross-linking in glutaraldehyde vapor. All sensor experiments to determine AChE activity were carried out in a stirred buffer solution (10 mM acetate buffer, 50 mM NaCl, pH 7.5) at room temperature. The enzyme activity was determined by monitoring thiocholine formed by enzymatic hydrolysis of acetylthiocholine chloride (final concentration, 1 mM). Thiocholine was detected by oxidation at +100 mV versus Ag/AgCl. The AChE biosensor was incubated in the CPO pretreated food sample for 30 min at room temperature without stirring. Before each measurement of the AChE activity of the biosensor, the transducers were immersed in acetate buffer (10 mM, pH 7.5) containing 1% Tween 20 for 15 min.

RESULTS AND DISCUSSION

AChE Stability Test toward KCl and KBr. In order to stabilize the reaction system for both enzymes, AChE and CPO, different pHs and buffers were tested. AChE is described in the literature as having a pI of 5.3. Below this pH occurs an aggregation of the enzyme, due to changes in its conformation (38). In contrast, CPO retains its greatest oxidation activity at pH 5–6, which may produce a more favorable ionization state of the key amino acid (Glu-183) and thus reduce radical formation (39). However, this enzyme is irreversibly deactivated at pH > 6 (28). Studies in our laboratory revealed that acetate buffer was the ideal buffer to be applied in a system shared by these two enzymes. Additionally, AChE was inhibited by pHs lower than 5.5. CPO remained stable under a broad pH range (data not shown).

The stability of AChE toward different concentrations of KCl and KBr in acetate buffer (1 M pH 5.5), which are required for the CPO oxidation, can be seen in **Figure 2**. Nb WT AChE showed itself unstable in the presence of concentrations of KBr higher than 0.1 M (8.8% inhibition in the presence of 0.2 M KBr). KCl did not influence the stability of this enzyme until concentrations of 1.0 M.

GC/MS Analysis of the Products of the CPO Catalyzed Reaction. *Choice of pH and Buffer of the Reaction System.* CPO was assayed for biocatalytic oxidation of chlorpyrifos under different pHs. **Figure 3** shows that the higher the pH of the system, the

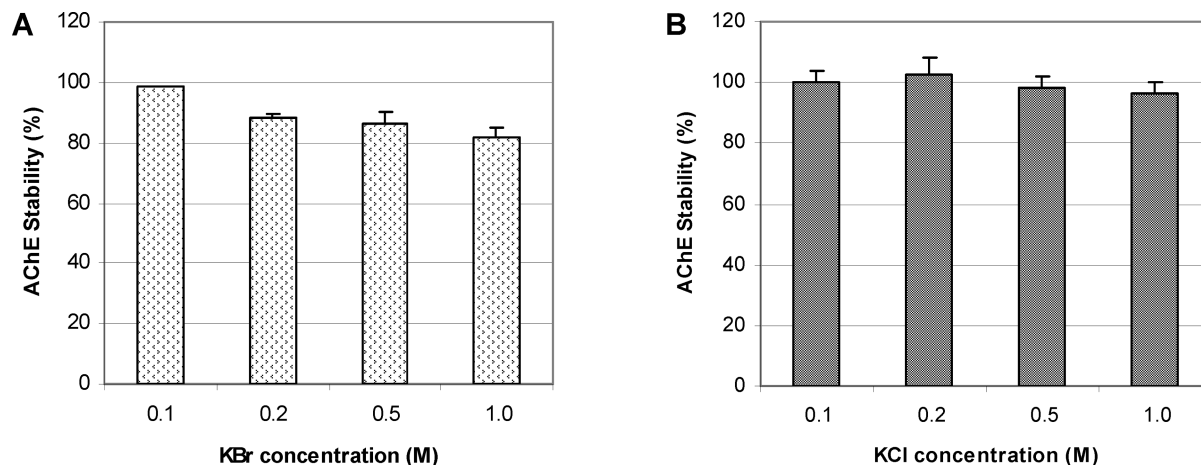


Figure 2. Stability of *N. brasiliensis* wild type AChE toward different concentrations of halogens: (A) KBr and (B) KCl.

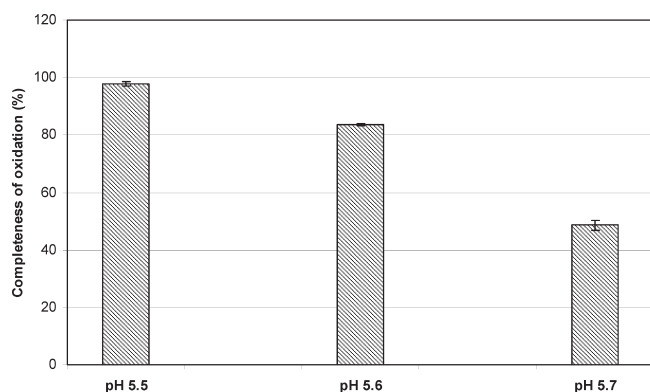


Figure 3. Completeness of oxidation of chlorpyrifos using CPO under different pHs. The reaction system was formed by CPO, t-b HP, KCl and acetate buffer 1 M ($n = 3$).

lower the product conversion rate. The oxidation reaction using pH 5.5 indicated a conversion of 100% of chlorpyrifos into chlorpyrifos oxon, while CPO could only convert about 44% of this pesticide in a system with pH 5.7. The control tests (no addition of CPO) showed the lack of oxidation of chlorpyrifos after exposition to the reaction system (data not shown). The conversion of thionates by CPO-catalyzed oxygen release from H_2O_2 at pHs lower than 4.8 was previously demonstrated (31,32). Tests using pHs lower than 5.5 were not executed, due to its inhibition activity over AChE.

Use of Different Halogen Salts and Pesticides. The oxidation of phosphorothionate pesticides increases the AChE inhibiting strength (16, 17). One way to transform phosphorothionates into their oxon form is using chemical oxidation. At the standard oxidation method for water, the DIN 38415-1 method, oxidations are performed by NBS, followed by a step where the excess of reagent is destroyed by ascorbic acid. However, this method revealed itself unsuitable for the analysis of food samples, due to a less oxidizable food matrix than water. In a previous study using an apple puree baby food spiked with 20 $\mu\text{g}/\text{kg}$ parathion, the sample was submitted to chemical oxidation, combined with the AChE biosensor assay. In this case, 20 mg/L of NBS was unable to generate an inhibition over the AChE biosensor after the 30 min incubation (40). Due to a restricted water solubility of NBS, increasing this oxidant's concentration is rather limited (32). In addition, the ascorbic acid or other antioxidants present in food can also prevent the chemical oxidation of existing thionate pesticides with NBS (40).

The enzyme CPO was chosen in order to study its ability to oxidize thionates into their oxon forms, which have a higher inhibiting influence over AChE (31, 32). To investigate whether this enzyme could transform these pesticides, we incubated CPO with orange juice samples spiked with thionate pesticides, in the presence of t-b HP and two halogen salts: KCl and KBr. The tested pesticides and their oxon forms are shown in **Figure 4**. CPO was able to activate all five organophosphorus pesticides to form oxon derivatives. The data described in **Table 1** were obtained by GC/MS analysis of the products of the five phosphorothionates submitted to the enzymatic oxidation. All pesticides produced one major degradation product, and the decrease of the molecular ions (16 m/z smaller than the original substrate) of the products suggests the oxidation of the thiophosphoryl bond ($\text{P}=\text{S}$) into phosphoryl bond ($\text{P}=\text{O}$) by mass spectroscopy (MS). This activation is similar to those performed by cytochromes P450 in *in vivo* and *in vitro* systems. Nevertheless, the major difference between both biocatalysts is that a further cleavage of oxons, which is typical of the P450-catalyzed reaction (31), was not observed with CPO. No oxidation reaction could be detected when CPO or t-b HP was alone added in the presence of the five thionate pesticides.

CPO exists in two active forms: the acidic form was described to catalyze halide-dependent, and the neutral form halide-independent, reactions. The transition between the two forms occurs between pH 3 and 5. Furthermore, the neutral form of CPO shows a broad pH optimum at around pH 5–6, where the enzyme is responsible for various halide-independent oxidation reactions (41). However, the conversion tests adding CPO with absence of chloride or bromide ions generated a low biocatalytic activity (data not shown). This data correlates to a previous report, where chloride had a great influence over a successful oxidation of phosphorothionates using CPO under a pH 4.8 (32). Halide ions (X^-) are also substrates for peroxidases; however they behave differently from conventional reduced substrates (42).

The addition of the halogen salts in the medium under a pH of 5.5 generated an important increase of the conversion rates. The results of the conversion of the five phosphorothionates are shown in **Figure 5**. The conversion rates after adding KCl and KBr were very similar, observing each pesticide individually. Chlorpyrifos and triazophos were completely oxidized by CPO into chlorpyrifos oxon and triazophos oxon, respectively. Fenitrothion, methidathion and parathion methyl were partially oxidized for addition of both types of halogen salts. It is important to point out that the concentration of KBr added to the reaction was 6-fold lower than that of KCl. Even though the

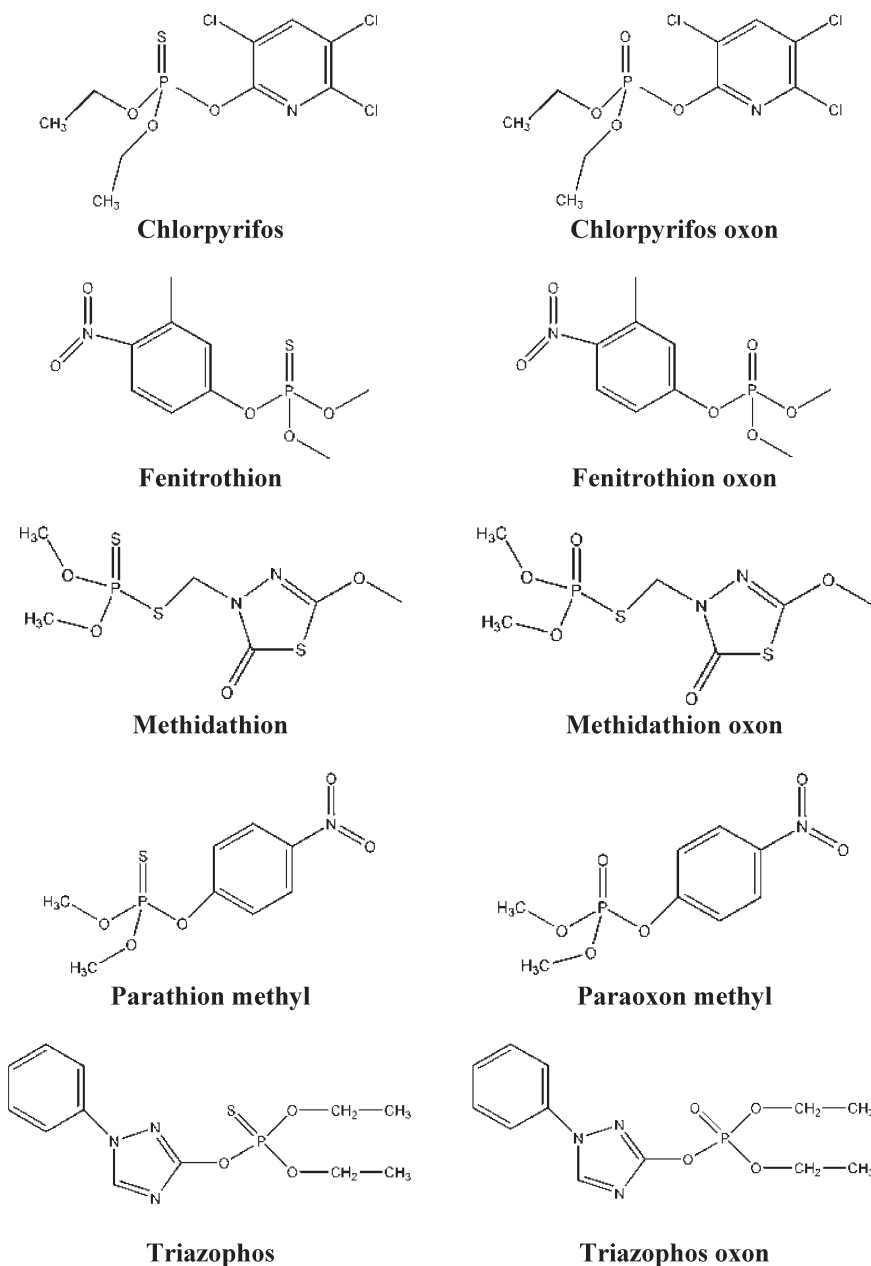


Figure 4. Organophosphorothionate insecticides used in this study and their corresponding oxon forms.

Table 1. MS Characteristics of the Product of the Oxidation of Phosphorothionate Pesticides Using CPO

pesticide	MW	mass fragments of main products (<i>m/z</i>)		
		M^+	base	other
chlorpyrifos	349	333	270	298, 272, 242, 109
fenitrothion	277	261	109	244, 79, 127
methidathion	302	286	85	145, 142, 109, 125, 229
parathion methyl	263	247	96	230, 200, 109
triazophos	313	297	161	269, 241, 188

KBr concentration added to the reaction could only reach 0.1 M due to the stability of AChE, a better conversion rate of the organophosphorothionates was obtained. The use of this same concentration (0.1 M) of KCl did not generate the oxidation of the studied organophosphorothionate pesticides. Additionally, the quantity of t-b HP was 10-fold lower in the KBr tests than in the KCl ones. One possible explanation is that bromide has a

lower redox potential than chloride, hence the level of activation of the active site is reduced. It has already been shown that CPO has a higher halogenation activity for monochlorodimedon (MCD) in the presence of bromide ions than chloride ions (22). In another study the initial activities of halohydrin formation using CPO increased 3-fold applying bromide ions, when compared to the addition of chloride ions (43). The use of KBr as a catalyst of the oxidation of phosphorothionates by CPO was described for the first time in this work.

The formation rates of the corresponding oxon forms of the studied pesticides in the presence of CPO varied from each other. Chlorpyrifos and triazophos were completely oxidized, however a complete conversion rate of the pesticides fenitrothion, methidathion and parathion methyl was not achieved. Fenitrothion was 58% converted into its oxon form for addition of both halogen salts. Further, the pesticide methidathion was 54 and 56% enzymatically oxidized in the presence of KCl and KBr, respectively. Parathion methyl was transformed into paraoxon

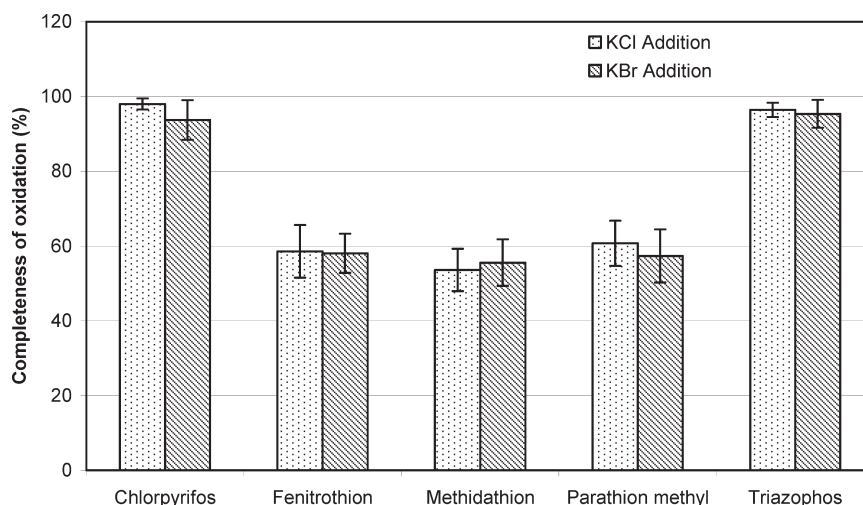


Figure 5. Completeness of oxidation of different phosphorothionate pesticides using CPO. The reaction system was formed by CPO, t-b HP and acetate buffer 1 M ($n = 3$).

Table 2. Effect of the Enzymatic Oxidation with CPO over the Bimolecular Constants (k_i) of Different Phosphorothionate Insecticides ($n=3$)

pesticide	oxon standard ^a	k_i [L/mol·min]	
		after exposure to oxidation reagents	
		KCl addn	KBr addn
chlorpyrifos	$4.92 \times 10^6 \pm 0.18 \times 10^6$	$5.47 \times 10^6 \pm 0.10 \times 10^6$	$4.87 \times 10^6 \pm 0.12 \times 10^6$
fenitrothion	$2.42 \times 10^5 \pm 0.20 \times 10^5$	$1.34 \times 10^5 \pm 0.11 \times 10^5$	$9.71 \times 10^4 \pm 0.85 \times 10^4$
methidathion	$5.30 \times 10^4 \pm 0.35 \times 10^4$	$3.15 \times 10^4 \pm 0.19 \times 10^4$	$2.58 \times 10^4 \pm 0.03 \times 10^4$
parathion methyl	$1.70 \times 10^5 \pm 0.12 \times 10^5$	$1.33 \times 10^5 \pm 0.13 \times 10^5$	$1.35 \times 10^5 \pm 0.06 \times 10^5$
triazophos	$1.02 \times 10^7 \pm 0.10 \times 10^6$	$1.19 \times 10^7 \pm 0.06 \times 10^7$	$9.36 \times 10^6 \pm 0.05 \times 10^6$

^a Obtained using chemical oxidation with NBS and vitamin C.

methyl with a conversion rate of 61% using chloride ions, and of 57% applying bromide ions.

CPO has the ability to supply several hydroperoxides and peroxy acids as electron acceptors (26, 44) in order to execute its reactions. However, preliminary tests showed that the addition of H_2O_2 to food samples generated absent or low conversion rates (data not shown). One explanation for these results could be that this peroxide reacts with antioxidants present in food samples, as for example ascorbic acid, leaving nothing remaining to react with CPO. In addition, CPO is deactivated by H_2O_2 at very low concentrations. The quantity of this peroxide in the system is critical for enzymatic oxygen transfers (45). Consequently, the addition of a higher concentration of this peroxide could compromise the activity of CPO.

In contrast, the addition of t-b HP in the tests replaced successfully H_2O_2 as an oxygen donor. Enhanced stability of CPO toward t-b HP allows its addition in high concentrations. As a result, this peroxide can be used as oxidant for the PO-catalyzed oxidation reactions (32). Additionally, the use of t-b HP is reasonable, since the *tert*-butyl alcohol, the product from t-b HP, was previously shown to exert a stabilizing effect on CPO (28, 39). However, the addition of *tert*-butyl alcohol in concentrations higher than 30% has a negative influence on CPO stability toward oxidizing conditions (28).

Determination of the Bimolecular Rate Constant (k_i) of Enzymatically Activated Phosphorothionates. We wanted to ensure that the product obtained after the enzymatic oxidation could inhibit the enzyme AChE. Therefore, the bimolecular rate constant k_i of the oxon forms resulting from the enzymatic oxidation was determined. The k_i value illustrates the sensitivity of AChE

toward pesticides. Additionally, it measures the rate of phosphorylation of this enzyme, characterizing the inhibitory capacity of the phosphothionates. The inhibition effect of the investigated pesticides toward AChE is shown in Table 2. The k_i values of the products obtained from the enzymatic oxidation were compared with the k_i values of the standard oxon form. This last one resulted from the analysis of the chemically oxidized form.

For most oxidized thionates, the deviations of k_i values from those presented by the oxon standards were low. Nevertheless, large deviations happened in the tests with addition of KBr. With the exception of chlorpyrifos, all other thionates presented k_i values after the enzymatic oxidation with KBr lower than the standard oxon. The most remarkable difference was obtained during the tests with fenitrothion, where a negative deviation of about 60% was obtained. Moreover, large deviations happened in the cases of methidathion and fenitrothion, when chloride ions were added during the tests. Comparing the k_i values, a negative deviation of about 45% was obtained for fenitrothion, and of about 41% for methidathion.

A comparison between the results obtained in the determination of the bimolecular constant and the GC/MS analysis of the products shows that a correlation between both of them can be found. The two pesticides where the oxidation reaction was most successful (chlorpyrifos and triazophos) are also the pesticides with the lowest deviation rates of the k_i values.

Phosphorothionates Conversion with CPO Pretreatment and AChE Biosensor Assay in Organic Orange Juice. The applicability of the CPO activation method combined with the detection assay using AChE biosensors for food analysis was tested. The oxidation of phosphorothionates using CPO was executed in a solution

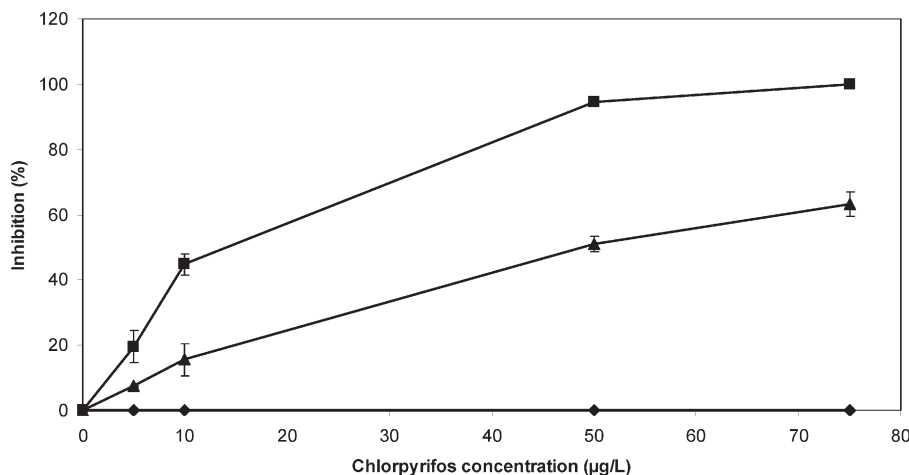


Figure 6. AChE inhibition caused by chlorpyrifos after activation with CPO, t-b HP and KBr using an amperometric AChE biosensor: (◆) no CPO addition, acetate buffer 1 M pH 5.5; (■) CPO addition, acetate buffer 1 M pH 5.5; (▲) CPO addition, organic orange juice (20%), acetate buffer 1 M pH 5.5 ($n = 3$).

with neutralized food sample. The 1 M acetate buffer was applied in order to neutralize the sample, and consequently to maintain the stability of both enzymes, CPO and AChE.

First, the stability of AChE in the reaction system without the presence of chlorpyrifos (blank) was tested. No inhibition was observed after incubation with the solution from the reaction between CPO, t-b HP and KBr. However, the blank sample of the reaction with CPO, t-b HP and KCl inhibited the Nb AChE WT immobilized on the biosensor (data not shown). Consequently, the further tests were done using bromide as halogen.

To determine whether the enzymatically oxidized product could inhibit the AChE biosensor, different concentrations of chlorpyrifos (5, 10, 50, and 75 µg/L) were exposed to the enzymatic reaction. Two conditions of the system were tested: the addition of only acetate buffer (1 M, pH 5.5), and the extra addition of 20% organic orange juice (**Figure 6**). The conversion rate of chlorpyrifos into chlorpyrifos oxon in the food sample was slightly lower than the one obtained for the solution with only acetate buffer. The application of the enzymatic method to orange juice in combination with a disposable AChE biosensor enabled detection of chlorpyrifos at concentrations down to 5 µg/L, with a final concentration of this pesticide in the orange juice sample of 25 µg/kg. The MRL established by the European Union for chlorpyrifos is 50 µg/kg food (46). Consequently, the analysis of this pesticide in a complex matrix using the chloroperoxidase pretreatment and acetylcholinesterase biosensor detection was able to detect the recommended limit by the legislation. A more diluted sample was also tested (10% organic orange juice), but this did not further enhance the assay performance. The inhibition of chlorpyrifos was proportional to its concentration from 5 to 75 µg/L in 20% organic orange juice, with a correlation coefficient of 0.9845. The reproducibility of the biosensor was estimated by determining the response of 0.1 M ATCl at five different electrodes, which were immersed in a pretreated 20% organic orange juice with 50 µg/L chlorpyrifos for 30 min. The coefficient of variation was found to be 4.53%. The intra-assay precision of the sensors was calculated by assaying one enzyme electrode for five replicate determinations, and the RSD was 1.5% at 0.1 M ATCl.

Walz and Schwack described a spectrophotometric enzyme inhibition assay using the enzyme cutinase from *Fusarium solani pisi* (EC 3.1.1.74), combining a preoxidation step using CPO from *C. fumago*, H₂O₂ and chloride (32). This method fulfilled successfully the demands to be used as a screening method. However, the food sample needed to be extracted using the QuEChERS

method, a streamlined approach that makes easier and less expensive the analysis of pesticide residues in food, before the oxidation step, in order to extract the pesticides existent in the food matrix. This process takes a longer time; in addition the use of several solvents and reagents is necessary.

Cytochrome P450 monooxygenases catalyze *in vivo* the insertion of an oxygen atom, derived from molecular oxygen, into a wide variety of organic substrates. The application of a prokaryotic cytochrome P450 mutant for the oxidation of organophosphorothionates was shown to successfully increase the sensitivity of the AChE biosensor assay (38, 47). However, the cytochrome P450 mutant needs the addition of the NAD(P)H cofactor, which demands a complex regeneration system. On the other side, the biocatalysis reaction using CPO does not require the use of a cofactor. In addition, CPO exhibits higher storage stability than the cytochrome P450 BM-3 (CYP102A1) from the soil bacterium *Bacillus megaterium*. CPO is stable for weeks at room temperature and under pH control (23). In contrast, the half-life of P450 BM-3 was characterized as being of 26 days at 4 °C in solution (48), and of 4 weeks in a sol–gel entrapped biosensor (47). Additionally, to our knowledge, this is the first time that a method for the activation and determination of phosphorothionate pesticides using a pretreatment with CPO and an AChE biosensor assay is described.

In summary, we showed here that the presented assay, applying a preoxidation step with the enzyme chloroperoxidase followed by the amperometric measurement with immobilized AChE, revealed itself as a successful method for the activation and detection of organophosphorothionate pesticides, with the capability to be used directly in food samples without the need of laborious solvent extraction steps. The duration of the whole procedure requires about 2 h.

ABBREVIATIONS USED

AChE, acetylcholinesterase; CPO, chloroperoxidase; GC, gas chromatography; H₂O₂, hydrogen peroxide; HPLC, high performance liquid chromatography; MCD, monochlorodimedon; MRL, maximum residue level; MSD, mass selective detector; NBS, *N*-bromosuccinimide; QuEChERS, quick, easy, cheap, effective, rugged, and safe; t-b HP, *tert*-butyl hydroperoxide.

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