Talanta 77 (2009) 1627–1631

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Phosphotriesterase: A complementary tool for the selective detection of two organophosphate insecticides: Chlorpyrifos and chlorfenvinfos

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ARTICLE INFO

Article history:
Received 15 July 2008
Received in revised form
26 September 2008
Accepted 29 September 2008
Available online 14 October 2008

Keywords:
Acetylcholinesterase
Phosphotriesterase
Biosensor
Chlorpyrifos
Chlorfenvinfos

ABSTRACT

This work shows the possibility of combining the high sensitivity of genetically-modified *Drosophila melanogaster* acetylcholinesterase (B394) with the ability of phosphotriesterase (PTE) to hydrolyse organophosphate compounds, in the aim of developing a biosensor selective to two insecticides of interest: chlorpyrifos and chlorfenvinfos. The studies clearly demonstrate that chlorfenvinfos is a substrate that acts as competitive inhibitor of PTE, therefore preventing the efficient hydrolysis of other pesticides, including chlorpyrifos. A bi-enzymatic sensor was designed by immobilizing both B394 and PTE in a polyvinylalcohol matrix. The sensor was shown to be able to discriminate between chlorpyrifos and chlorfenvinfos inhibitions.

1. Introduction

Pesticides are neurotoxic agents widely used for agricultural, industrial, household and warfare purposes. Neurotoxic pesticides mainly act by inhibiting acetylcholinesterase (AChE) [1], an essential enzyme that permits the transmission of electrical signals in the nervous system of most animal beings. Organophosphosphate (OP) compounds are among the most powerful neurotoxics, they are commonly used as pesticides (insecticides) and chemical warfare agents. Due to their high acute toxicity and risk towards the population, some European directives have been established to limit their presence in water and food resources. For instance, the council directive 98/83/CE concerning the quality of water for human consumption has set a maximum admissible concentration of 0.1 ppb ($\mu g\,L^{-1}$) per pesticide and 0.5 ppb for the total amount of pesticides.

Early detection of OP neurotoxins is thus important for protecting water resources and food supplies, but also for defence against terrorist activity, and for monitoring detoxification processes. Accordingly, there are growing demands for field deployable devices for reliable on-site monitoring of OP compounds.

Routine methods are based on extraction, cleanup and analysis normally performed by gas chromatography (GC) or liquid chromatography (LC) coupled to sensitive and specific detectors such as nitrogen–phosphorus detectors (NPD) [2–4], flame ionization detectors (FID) [3,4], ultraviolet detectors (UV) or diode array detectors (DAD) [5–7], although recently most methods involve the use of mass spectrometry (MS) as detector to provide ultimate confirmation [8,9]. These methods are highly sensitive and allow the determination of a large number of compounds but they are costly, long and/or complex.

Biosensors have been described for many years as being good candidates as substitutive or complementary tools to these conventional methods, as they can provide real-time qualitative information about the composition of a sample with minimum preparation. Biosensors based on the inhibition of AChE have been widely used for the detection of OP compounds. Among the numerous sensors described, some of them involve the use of recombinant acetylcholinesterases allowing to enhance dramatically the sensitivity of these devices. Such sensors have been recently described in our group for the highly sensitive detection of chlorpyrifos and chlorfenvinfos, two insecticides included since 2001 in a list of priority substances in the field of water policy (decision 2455/2001/EC) [10]. Although these biosensors show a high sensitivity, they often lack of selectivity due to the fact that they react with any cholinesterase inhibitor.

An alternative biosensing route for the detection of these insecticides involves the use of phosphotriesterase (PTE) [11]. This enzyme, also called organophosphate hydrolase (OPH), has the

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doi:10.1016/j.talanta.2008.09.050

ability of hydrolysing ester links of organophosphate compounds, thus liberating the different radicals attached to the phosphate group. Several OPH-based amperometric, potentiometric, or optical biosensors have been already described [12,13]. Recently, Deo et al. [14] have described an amperometric biosensor for organophosphate pesticides based on a carbon nanotube-modified transducer and a PTE biocatalyst. In this sensor, the detection is based on the electrochemical oxidation of *p*-nitrophenol, which is the produced by the PTE-catalysed hydrolysis of the paraoxon or methyl-parathion. However, it must be underlined that only a few insecticides including parathion, methyl-parathion and their oxidation products (paraoxon and paraoxon-methyl) can be detected by this method, as other organophosphate do not generate *p*-nitrophenol upon hydrolysis.

This work presents the development of a biosensor based on the combination of PTE and a highly sensitive recombinant acetylcholinesterase for the selective detection of organophosphate insecticides. In a first step, the activity of PTE towards the main anticholinesterase insecticides was investigated. Then, a bi-enzymatic sensor based on the co-immobilization of AChE and PTE was developed for the detection of chlorpyrifos and chlorfenvinfos.

2. Experimental

2.1. Chemicals and stock solutions

Genetically modified AChE from *Drosophila melanogaster* type B394 and PTE were produced by Protein Bio Sensor (PBS, Toulouse, France). Before immobilisation, enzymatic activities were spectrophotometrically measured using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB – Ellman's reagent) provided by Sigma. The substrate acetylthiocholine chloride (ATCh) was purchased from Sigma. A 0.1 M ATCh stock solution was daily prepared in water and stored at 4 °C.

The organophosphorus insecticides chlorpyrifos-oxon (phosphoric acid, O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) ester), chlorpyrifos-oxon-methyl (phosphoric acid, O,O-dimethyl O-(3,5,6-trichloro-2-pyridinyl) ester), dichlorvos (phosphoric acid, O,O-dimethyl O-(2,2′-dichlorovinyl) ester), paraoxon (phosphoric acid, O,O-diethyl O-(4-nitrophenyl) ester), paraoxon-methyl (phosphoric acid, O,O-dimethyl O-(4-nitrophenyl) ester) and chlorfenvinfos (phosphoric acid, 2-chloro-1-(2,4-dichlorophenyl)ethenyl diethyl ester) were purchased from Dr Ehrenstorfer (Augsburg, Germany). Stock solution of pesticides (10^{-3} M) was prepared in acetonitrile and stored at 4 °C.

Azide-unit Pendant Water-soluble Photopolymer (AWP) was provided by Toyo Gosei Kogyo Co. (Chiba, Japan).

The pastes used for screen-printing, i.e. Electrodag PE-410, 423SS and 6037SS were obtained from Acheson (Plymouth, UK). Cobalt–phtalocyanine-modified carbon paste was purchased from Gwent Electronic Materials, Ltd. (Gwent, UK). A glycerophthalic paint (Astral, France) was used as insulating layer. Transparent PVC sheets (200 mm × 100 mm × 0.5 mm) were used as printing supports.

2.2. Apparatus

Spectrophotometric measurements were performed using a Hewlett Packard diode array 8451A spectrophotometer. Amperometric measurements were carried out with a 641VA potentiostat (Metrohm, Switzerland), connected to a BD40 (Kipp & Zonen, The Netherlands) flatbed recorder.

Chromatographic determination of pesticides was carried out using a Merck L-6220 HPLC-system fitted with a Supelcosil LC18 reversed phase column (Supelco). The elution solvent used was acetonitrile/water (70:30), at a flow rate of 1 mL/min. The detection of pesticides was performed at 220 nm using a Merck L-4000 UV detector.

Screen-printed electrodes were produced using a semi-automatic DEK 248 printing machine according to a procedure previously described [15], but in a three-electrode configuration. The working electrode was a 4 mm-diameter disk, the auxiliary electrode was a 16 mm × 1.5 mm curved line and the Ag/AgCl pseudo-reference electrode was a 5 mm × 1.5 mm straight line.

2.3. Determination of PTE activity

The activity of PTE was measured using the enzyme in solution, in 10 mM phosphate buffer pH 8, using concentrations of substrate (insecticide) ranging from 10^{-6} M to 10^{-3} M. The activity was measured by two different methods, depending on the substrate used. When using paraoxon derivatives as substrate, the enzyme activity was easily monitored by spectrophotometric measurement of the paranitrophenol produced at 405 nm. Using other substrates (chlorpyrifos-oxon derivatives, dichlorvos, chlorfenvinfos, omethoate and fenamiphos), the activity was measured by HPLC coupled with a UV detector. In that case, the reaction mixture was injected after different reaction times in order to follow the hydrolysis of the pesticide.

2.4. Immobilisation of enzymes

The immobilisation of enzymes was performed by physical entrapment in a polyvinylalcohol-based photopolymer (AWP), as described in previous works [10]. AWP polymer was mixed with enzymatic solution in a ratio 70:30 (v/v). The mixture was vortex-mixed and briefly centrifuged to eliminate the foam. A volume of 3 μL was then spread onto the working electrode using a micropipette. The concentration of the initial B394 solution was adjusted in order to obtain a final enzyme loading of 1 mU/biosensor. Three types of biosensors were prepared, without PTE or based on the co-immobilization of B394 (1 mU) and PTE in different quantities (0.14 U/biosensor or 0.85 U/biosensor).

The electrodes were exposed to neon light for 3 h at 4 °C to allow the photo-polymerisation between azide groups. After drying for 48 h at 4 °C, the electrodes were ready to use.

2.5. Amperometric measurement principle

Amperometric measurements were performed in stirred solutions using a 5 mL cell. The electrodes were tested in 5 mL of 0.1 M phosphate buffer at a working potential of +100 mV versus Ag/AgCl, which corresponds to the oxidation of thiocholine, the product of the enzymatic hydrolysis of the ATCh substrate in the presence of cobalt–phtalocyanine as electron mediator. The current intensity was recorded and, after current stabilisation, 1 mM ATCh (final concentration in the cell) was injected. The time necessary to reach the plateau was 2–3 min. The measured signal corresponded to the difference of current intensity between the baseline and the plateau. The cell was washed with distilled water between measurements.

The pesticide detection was made in a three-step procedure as follows: first, the initial response of the electrode at the injection of the ATCh (1 mM) was recorded three times, then the electrode was incubated in a solution containing a known concentration of insecticide, and finally the residual response of the electrode was recorded again in the same conditions. The percentage of the inhibition was correlated with the insecticide concentration. The limit of detection was calculated as the insecticide concentration inducing a 10% decrease of the biosensor response.

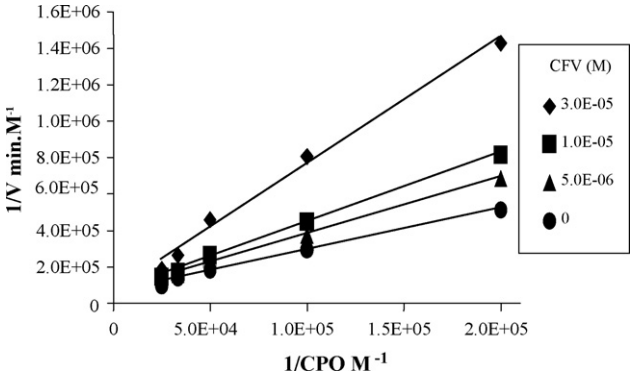

Fig. 1. $1/v$ versus 1/[CPO] plot of PTE activity in the presence of different fixed concentrations of CFV (Lineweaver–Burk representation).

3. Results and discussion

3.1. Determination of Michaelis constants (Km) of PTE enzyme for different organophosphate substrates

The activity of PTE was investigated using eight well-known organophosphate insecticides in order to determine their respective kinetic constants (Km and Vm). Parathion derivatives (ethyl and methyl) and their oxidation compounds have been widely described as PTE substrates [12,13], mainly due to the easy detection of their hydrolysis product: paranitrophenol. In order to facilitate the comparison between substrates, the maximum velocity was thus expressed throughout this work as paraoxon-ethyl-equivalent activity. The kinetic constants Km and Vm relative to paraoxon-ethyl were determined spectrophotometrically, their absolute values were 0.25 mM and 14.1 mM min^{-1}, respectively.

The relative values of the kinetic constants obtained for each pesticide are summarized in Table 1.

It clearly appears that PTE has a higher affinity for chlorpyrifos-oxon derivatives than for paraoxon compounds (lower Km), which are generally used in PTE-based sensors. Both chlorpyrifos-oxon (CPO)-ethyl and chlorfenvinfos (CFV) show a very good affinity for PTE, but even though CFV has a better affinity than CPO, its hydrolysis rate was shown to be 10,000-fold slower than CPO one. Additional "blank" assays without PTE confirmed that CFV was not chemically hydrolysed during the reaction time. The high affinity and the low degradation velocity of CFV indicate that this substrate is likely to act as a competitive inhibitor of PTE for hydrolysis of other organophosphates. Confirmation of this behaviour was obtained by carrying out kinetics of CPO hydrolysis in the presence of different fixed concentrations of CFV. As clearly shown on the Lineweaver–Burk representation ($1/v$ versus 1/[S], Fig. 1), an increase of apparent Km was observed in presence of CFV while no change occurred in the maximum rate (Vm). The replot of Km_{app} (obtained from Lineweaver–Burk plot) versus the concentration of inhibitor (CFV) allowed us to calculate the competitive inhibition constant (Ki), that was found to be equal to the Km of PTE for CFV (Ki = 0.015 mM = Km) (replot equation: $y = 2.7663x + 4 \times 10^{-5}$). Similar results were obtained using paraoxon as substrate, in that case spectrophotometric measurements allowed to determine a competitive inhibition constant Ki = 0.017 mM = Km (replot equation: $y = 15.8823x + 25 \times 10^{-5}$).

All these features confirmed that CFV acts as a competitive inhibitor of PTE.

3.2. Biosensor characterisation and kinetic study of the immobilised enzyme

The stability of the sensors needs to be evaluated in order to ensure that the decrease in the signal during inhibition measurements is due to enzyme inactivation and not to enzyme leaking. Due to the fact that the developed device is based on a bi-enzymatic system, separate assays must be performed to assess the activity of each enzyme. The operational stability of immobilized B394 acetylcholinesterase was estimated by successively measuring the response of the same enzyme electrode to 1 mM ATCh, at 100 mV versus Ag/AgCl. PTE stability was estimated by measuring the sensor response to 1 mM paraoxon using the same enzyme electrode at 700 mV versus Ag/AgCl. The sensors were washed between tests with distilled water. All three types of sensors were shown to be stable for at least 10 consecutive measurements. The biosensors based on AWP-entrapment presented a good reproducibility, with a signal maximum variation of 5%. The average response to 1 mM ATCh for $n = 5$ electrodes prepared in the same experimental conditions was 290 ± 15 nA. These reproducibility data refer to the mean value of 10 assays for each electrode.

3.3. Separate detection of CPO and CFV using the bi-enzymatic sensor

3.3.1. Optimization of incubation time

The incubation time of each sensor with various concentrations of pesticides was optimized in order to achieve the highest sensitivity of detection. It was observed that an incubation time of 10 min was sufficient to achieve the maximum inhibition rate whatever the pesticide concentration used. This incubation time was thus applied in further experiments.

3.3.2. Effect of PTE loading on AChE inhibition by CPO and CFV

The inhibition effect of CPO and CFV on sensors incorporating 1 mU of AChE and different amounts of PTE was studied using an incubation time of 10 min. Fig. 2 shows that the inhibitory effect of CPO was decreased when the amount of PTE immobilized on the electrode was increased. In each case, the limit of detection I_{10}, the I_0, I_{50}, and I_{100} were calculated corresponding to the pesticide concentration inducing 10%, 0%, 50% and 100% inhibition, respectively. These results are summarized in Table 2.

As expected from the determination of the kinetic constants of PTE, the inhibitory effect of CFV was the same whatever the concentration of PTE immobilized on the electrode, this insecticide being not hydrolysed by PTE. Consequently, the responses of a dual-sensor device incorporating B394 and B394 + PTE will be the same if the sample contains only CFV.

A different behaviour was observed when studying CPO determination, due to the rapid hydrolysis of this insecticide by PTE enzyme. For instance, no inhibition was observed for 3×10^{-8} M CPO using the bi-enzymatic sensor incorporating 0.85 U of PTE,

Table 1
Kinetic constants of PTE enzyme using eight different organophosphate insecticides (substrates). CPO = chlorpyrifos-oxon (ethyl), CFV = chlorfenvinfos.

PTE	CPO-ethyl	CPO-methyl	CFV	Dichlorvos	Paraoxon-methyl	Paraoxon-ethyl	Omethoate and Fenamiphos
Km (mM)	0.03	0.06	0.017	0.31	1.2	0.25	No hydrolysis
Relative Vm (/paraoxon)	1.8	0.25	0.0002	0.003	0.38	1	

Table 2
Characteristics of the biosensors based on the co-immobilization of B394 acetylcholinesterase (1 mU/sensor) and PTE (0, 0.14 or 0.85 paraoxon-equivalent U/sensor) for the detection of CPO and CFV.

PTE (U/sensor)	Equation	I_0 (M)	I_{10} (M)	I_{50} (M)	I_{100} (M)
CPO					
0	$I = 25.085 \ln(C) + 591.3$	5.8×10^{-11}	8.6×10^{-11}	4.3×10^{-10}	3.0×10^{-9}
0.14	$I = 17.928 \ln(C) + 376.6$	7.5×10^{-10}	1.0×10^{-9}	1.2×10^{-8}	2.0×10^{-7}
0.85	$I = 19.501 \ln(C) + 336.8$	3.2×10^{-8}	5.3×10^{-8}	4.1×10^{-7}	4.1×10^{-6}
CFV					
0 or 0.14 or 0.85	$I = 16.487 \ln(C) + 383.8$	7.7×10^{-11}	1.4×10^{-10}	2.0×10^{-9}	3.3×10^{-8}

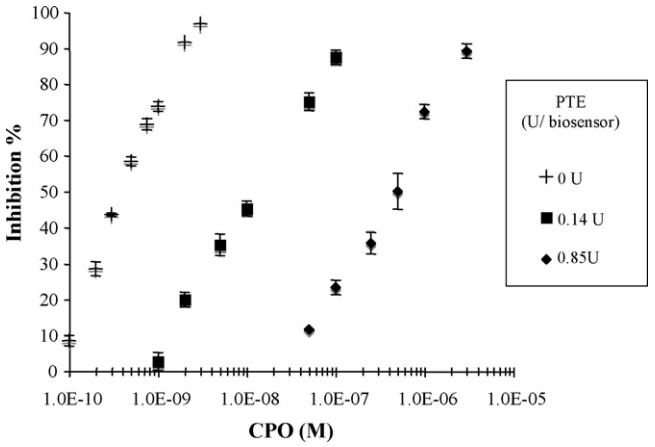

Fig. 2. Detection of CPO using a bi-enzymatic sensor incorporating 1 mU of B394 AChE and different fixed amounts of PTE.

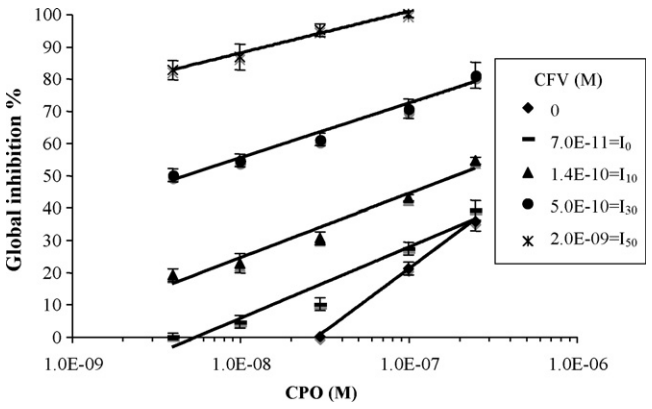

Fig. 3. Effect of CPO and CPO + CFV mixtures on the inhibition rate of a bi-enzymatic sensor incorporating 1 mU of B394 AChE and 0.85 U of PTE. Equations: CFV = 0, $I = 16.877 \ln(C) + 292.6$; CFV = 7.7×10^{-11} M (=I_0), $I = 9.587 \ln(C) + 181.4$; CFV = 1.4×10^{-10} M (=I_{10}), $I = 8.636 \ln(C) + 186.2$; CFV = 5×10^{-10} M (=I_{30}), $I = 7.372 \ln(C) + 190.8$ and CFV = 2×10^{-9} M (=I_{50}), $I = 5.622 \ln(C) + 191.1$.

while the same CPO concentration induced a total inhibition of the mono-enzymatic sensor. In such case, PTE can be considered as a discriminating tool allowing the selective detection of CPO and CFV. Globally, the combination of PTE with B394 AChE allowed determining CPO concentrations much higher than those detected using the mono-enzymatic B394-based sensor. For instance, the mono-enzymatic sensor allowed determining CPO concentrations ranging from 3×10^{-9} M to 5.8×10^{-11} M, while the bi-enzymatic sensor incorporating 0.85 U of PTE was usable from 4.1×10^{-6} M to 3.2×10^{-8} M. These features suggest the possibility of developing in the near future sensor arrays incorporating both types of sensors. Due to their complementary working ranges, such devices will allow a dramatic extension of the detectable concentration range, as well as a selective detection of CPO and CFV.

3.4. Effect of mixtures of CPO and CFV on the bi-enzymatic sensor

The bi-enzymatic sensor incorporating 1 mU of B394 AChE and 0.85 U of PTE was used in second step for the assessment of mixtures of CPO and CFV. In Fig. 3 is shown the global inhibition induced by CPO alone or mixed with CFV at concentrations of 7.7×10^{-11} M (=I_0), 1.4×10^{-10} M (=I_{10}), 5×10^{-10} M (=I_{30}) and 2×10^{-9} M (=I_{50}). It can be seen that, in the absence of CFV, PTE induced a great decrease of the inhibition rate of B394-based sensor. This feature was due to the hydrolysis of CPO by the PTE enzyme, which was only partial using high concentrations of CPO. When present, CFV induced a competitive inhibition of PTE, which in turn was unable to react with CPO. Consequently, a dramatic increase of the sensor inhibition was observed due to the non-degradation of CPO. For instance, a CPO concentration of 3×10^{-8} M was shown to induce inhibition rates of 0%, 30% and 95% using 0 M, 1.4×10^{-10} M and 2×10^{-9} M of CFV, respectively. Such effect was observed even using CFV concentrations that do not induce any inhibition of the B394 enzyme (7.7×10^{-11} M).

4. Conclusions

This work shows the possibility of combining the high sensitivity of B394 AChE with the ability of PTE to hydrolyse organophosphate compounds to develop a biosensor selective to chlorpyrifos and chlorfenvinfos. It clearly appeared in this study that chlorfenvinfos binds strongly to PTE but is not hydrolysed by this enzyme. CFV thus acts as a competitive inhibitor of PTE, therefore preventing the efficient hydrolysis of other pesticides. A bi-enzymatic sensor was designed by immobilizing both B394 AChE and PTE in a polyvinylalcohol polymer. The biosensor was used in a first step for the detection of CPO and CFV, and then for the detection of mixtures of these pesticides. In the first case, the sensor was shown to be able to discriminate between the two pesticides, since only CPO is efficiently hydrolysed by the PTE. Using pesticide mixtures, it was shown that due to the competitive inhibition of PTE by CFV, it was difficult to distinguish between inhibitions due to each compound. To become selective, this sensor must be associated with a biosensor incorporating only B394 AChE. Concretely, if both sensors give the same inhibition rate, the inhibition can be attributed to CFV. If an inhibition is observed using B394 and no inhibition occurs with the bi-enzymatic sensor, then the pesticide present is undoubtedly CPO. In between, if the two pesticides are present in the solution, a weaker inhibition is observed using the bi-enzyme sensor, but it is difficult to evaluate the part of each pesticide. In the aim of improving the selectivity of this sensor, future works will focus on the development of sensor arrays involving

different mutant cholinesterases associated or not with PTE. The possible pre-treatment of pesticide mixtures with PTE immobilized on solid-phase columns will be also investigated.

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