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Review

Biosensors for direct determination of organophosphate pesticides

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Abstract

Direct, selective, rapid and simple determination of organophosphate pesticides has been achieved by integrating organophosphorus hydrolase with electrochemical and opitical transducers. Organophosphorus hydrolase catalyzes the hydrolysis of a wide range of organophosphate compounds, releasing an acid and an alcohol that can be detected directly. This article reviews development, characterization and applications of organophosphorus hydrolase-based potentiometric, amperometric and optical biosensors. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Organophosphorus (OP) compounds are used widely in the agriculture industry around the world as pesticides and insecticides. These neurotoxic compounds, which are structurally similar to the nerve gases soman and sarin, irreversibly inhibit the enzyme acetylcholinesterase, essential for the functioning of the central nervous system in humans and insects, resulting in the buildup of the neurotransmitter acetylcholine which interferes with muscular responses and in vital organs produce serious symptoms and eventually death (Donarski et al., 1989; Chapalamadugu and Chaudhry, 1992; FAO, 1989; USDA, 1992; Compton, 1988).

OP pesticides and insecticides are used extensively by farmers in India. In the 1995–1996 financial year, in excess of 2200 t of methyl parathion (MP), an organophosphate, alone was produced in India (http://www.pan-uk.org/actives/methylpa.htm). In a recently completed US Geological Survey study, a widespread presence of trace amounts of these pesticides was found in surface and ground waters across the US (Gilliom et al., 1999). A similar presence of the deadly OPs could be expected in the water resources across India. Therefore, rapid, sensitive, selective and reliable determination of OPs is necessary in order to take immediate necessary action. Additionally, analytical tools to properly monitor and control any treatment process that may be adopted to treat the large volumes of wastewaters generated at both the producer and consumer levels will be necessary. Current analytical techniques such as gas and liquid chromatography (Sherma, 1993; Yao et al., 1991), are very sensitive and reliable, but cannot be carried out infield. These techniques are time consuming, expensive and have to be performed by highly trained technicians.

Biological methods such as, immunoassays and inhibition of cholinesterase activity, for OP determination have also been reported (Sherma, 1993). Despite the promise of immunoassay techniques, since these methods require long analysis time (1–2 h) and extensive sample handling (large number of washing steps), they are unsuitable for on-line monitoring of detoxification processes.

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Biosensing analytical devices, based on the acetylcholine esterase (AChE) inhibition test, using AChE modified amperometric transducers (measuring thiocholine and *p*-aminophenol produced by hydrolysis of butyrylthiocholine and *p*-aminophenyl acetate, respectively, or hydrogen peroxide generated as a result of the oxidation of choline produced from acetylcholine hydrolysis in the presence of choline oxidase) have been reported (Palchetti et al., 1997; Diehl-Faxon et al., 1996; La Rosa et al., 1994; Martorell et al., 1994; Skladal, 1991; Marty et al., 1992; Palleschi et al., 1992; Skladal and Mascini, 1992; Mionetto et al., 1994; Trojanowicz and Hitchman, 1996). Potentiometric transducers (measuring the pH change as a result of acetic acid production) have also been reported (Kumaran and Tranh-Minh, 1992; Tran-Minh et al., 1990; Chuna Bastos et al., 1991; Kumaran and Morita, 1995; Dzyadevich et al., 1994) as has the use of fiber optic technology (monitoring the pH change using a fluorescein label attached to AChE or dextran, or chemiluminescence) (Rogers et al., 1991; Hobel and Polster, 1992; Garcia de Maria et al., 1994; Moris et al., 1995). Although sensitive, biosensors based on AChE inhibition have limitations: (1) since AChE is inhibited by neurotoxins which include not only OP pesticides but also carbamate pesticides and many other compounds, these analytical tools, are not selective and cannot be used for quantitation of either an individual or a class of pesticides which may be required to monitor detoxification processes, for example, detoxification of OP pesticides. (2) These protocols involve multiple steps requiring measurement of the uninhibited activity of AChE, followed by incubation of the sensor with the analyte sample for 10–15 min (and even longer for good sensitivity) and the measurement of the AChE again to determine the degree of inhibition. A final step of reactivation/regeneration, which in many cases is partial and in some cases not possible due to irreversible inhibition, is necessary if the electrode has to be reused.

Organophosphorus hydrolase (OPH) is an organophosphotriester hydrolyzing enzyme first discovered in soil microorganisms *Pseudomonas diminuta* MG and *Flavobacterium* spp. The enzyme has a broad substrate specificity and is able to hydrolyze a number of OP pesticides such as paraoxon, parathion, coumaphos, diazinon, dursban, methyl parathion, etc., and chemical warfare agents, sarin and soman (Munnecke, 1980; Dumas et al., 1989a,b, 1990).

As shown in Fig. 1, OPH catalyzed hydrolysis of OP compounds generates two protons as a result of the cleavage of the P–O, P–F, P–S or P–CN bonds and an alcohol, which in many cases is chromophoric and/or electroactive.

The above enzyme reaction can be combined with a variety of transduction schemes to construct simple biosensors for the direct and rapid determination of OPs. For example, OPH can be combined with potentiometric transducers such as a pH electrode or a field effect transistor, or a pH indicator dye to quantify protons produced that can be then correlated to the OP concentration. Similarly, OPH can be combined with an optical transducer to monitor the production of chromophoric products such as *p*-nitrophenol produced during the hydrolysis of OPs such as paraoxon, parathion, methyl parathion, or chlorferon from the hydrolysis product of coumaphos. OPH can be integrated with an amperometric transducer to monitor the oxidation or reduction current of the hydrolysis products. We have successfully combined OPH with the above transduction devices to construct a variety of enzyme biosensors. This review presents an overview of these different biosensors.

2. Potentiometric OPH-based enzyme electrode

The basic element of this very simple enzyme electrode was a pH electrode modified with an immobilized purified organophosphorus hydrolase (OPH) layer formed by cross-linking OPH with bovine serum albumin and glutaraldehyde. Other components of the system included a pH meter, a measuring cell placed on a magnetic stirrer for mixing and a chart recorder (Fig. 2). The sensor signal and response time were optimized with respect to the buffer pH, ionic concentration of buffer, temperature, and units of OPH immobilized using paraoxon as substrate. The best sensitivity and response times were obtained using a sensor constructed with 500 IU of OPH and operating in pH 8.5, 1 mM HEPES buffer supplemented with 100 mM NaCl and 0.05 mM cobalt chloride at 20°C. Operating under these conditions, the biosensor was able to detect as low as 2 μM of paraoxon, ethyl parathion, methyl parathion and diazinon with very good accuracy and selectivity (other non-organophosphate pesticides such as simazine, triazine, atrazine, sevin and sutan, did not interfere) in approximately 2 min. The biosensor was completely stable for at least 1 month when stored in pH 8.5, 1 mM HEPES + 100 mM NaCl buffer at 4°C. This biosensor was used to measure paraoxon, parathion and methyl parathion in simulated samples

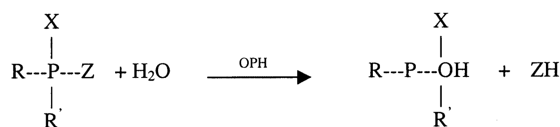


Fig. 1. Reaction scheme for the OPH catalyzed hydrolysis of Ops. X is oxygen or sulfur, R is an alkoxy group ranging in size from methoxy to butoxy, R′ is an alkoxy or phenyl group and Z is a phenoxy group, a thiol moiety, a cyanide or a fluorine group.

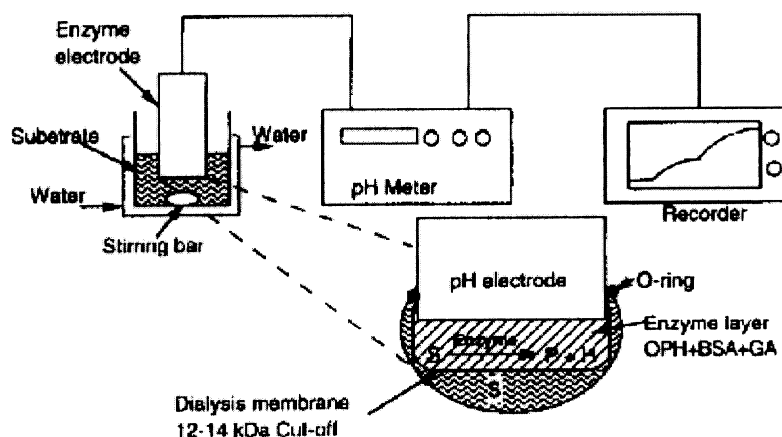


Fig. 2. Schematic of potentiometric OPH-based biosensor.

and showed a high correlation ($r^2 = 0.998$) to a spectrophotometric enzymatic assay (Mulchandani et al., 1999a).

3. Optical OPH-based biosensors

Two different optical based biosensors were constructed. The first based on the measurement of the decrease in the fluorescence intensity of the fluorescein isothiocyanate (FITC), which is covalently immobilized to the enzyme. The method employed poly(methyl methacrylate) beads to which the FITC-labeled enzyme was adsorbed. Analytes were then measured using a commercially available microbead fluorescence analyzer (KinExA fluorescence analyzer, Sapidyne Inc., Boise, ID, USA). Fig. 3 shows the schematic of the biosensor. This assay was based on a substrate-dependent change in pH at the local vicinity of the enzyme. Similar to OPH-modified pH electrode biosensor, the sensitivity of this biosensor was an inverse function of the ionic strength of the assay buffer. The dynamic concentration range for the assay extended from 25 to 400 μM for paraoxon with a detection limit of 8 μM. Organophosphorus insecticides measured using this technique included parathion, methylparathion, dursban, fensulfothion, crotoxyphos, diazinon, mevinphos, dichlorvos, and coumaphos. The technique was used to measure coumaphos in biodegradation samples of cattle dip wastes and showed a high correlation ($r^2 = 0.998$) to a HPLC method (Roger et al., 1999).

In another fiber-optic biosensor format, the analysis was based on the relationship between the amount of OP hydrolyzed and the amount of chromophoric product formed (quantified by measuring the absorbance at the λ_{max} of the product) by the enzyme catalyzed hydroysis. The fiber-optic setup (Fig. 4) used in the study consisted of a 75-W xenon arc lamp light source housed in PowerArc lamp housing (b), powered by a constant voltage dc lamp power (a) with a monochromator (c) attached to one arm of the bifurcated fiber-optic bundle, set at a desired cutoff wavelength (400 nm for *p*-nitrophenol, hydrolysis product of paraoxon and parathion, and 348 nm for chlorferon, hydrolysis product of coumaphos), a 0.5-m bifurcated quartz fiber-optic bundle (d), a second monochromator (e), set at the same wavelength as the first one, attached to the other arm of the bifurcated fiber bundle, a photomultiplier detection system (f), and a strip chart recorder (g). OPH was immobilized on a nylon microporous membrane by cross-linking with glutaraldehyde in the presence of bovine serum albumin and was attached to the open end of a plastic tube by an O-ring to the optical fiber bundle at a distance of 0.8 mm (path length for absorbance) from the common end of Y-shaped bifurcated optical fiber bundle.

Operating at optimized analytical conditions (pH 9, 30°C and 123 IU of immobilized OPH), the biosensor was able to detect as low as 2 μM of paraoxon and parathion and 5 μM of coumaphos, selectively without interference from carbamates and triazines in less than 2 min. The biosensor response had excellent reproducibility and the immobilized enzyme was stable for more than 1 month when stored at 4°C in the analytical buffer (Mulchandani et al., 1999b).

An advantage of the present sensor over the two previous biosensors that were based on the pH change, is the use of higher ionic strength buffer in the analysis. This allows the enzyme to function at its maximum activity over the complete duration/range of the assay rather than just at the start. Additionally, the use of higher ionic strength buffer precludes the need to adjust the sample pH to that of the analytical buffer.

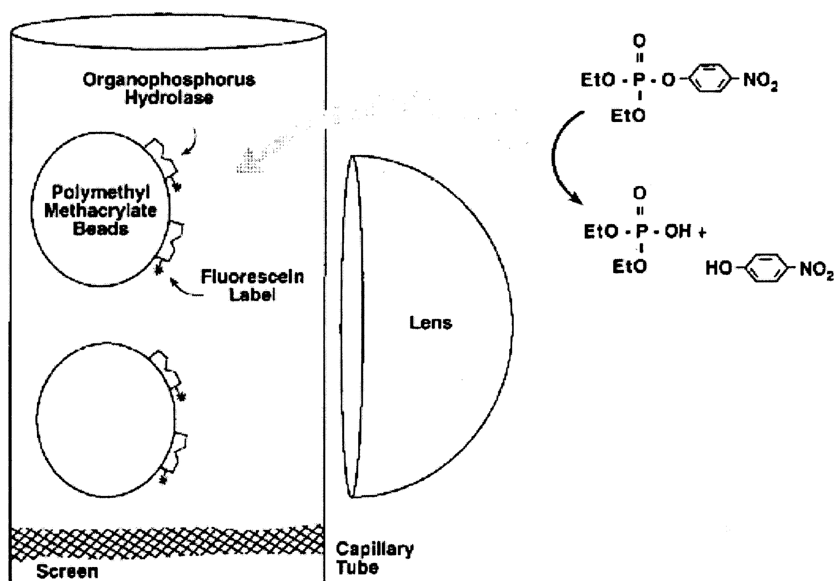
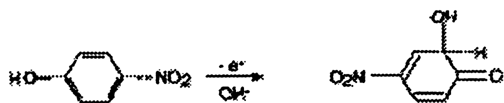


Fig. 3. Schematic of the OP assay in the KinExA fluorescence analyzer.

4. Amperometric biosensors

Hydrolysis of certain OP pesticides, such as parathion, methyl parathion, paraoxon, EPN and fenitrothion, generate *p*-nitrophenol (PNP), which is electroactive. PNP can be electrochemically oxidized at the anode according to the following reaction.



The oxidation current, measured at a fixed potential using a potentiostat, is directly proportional to the concentration of PNP formed. We have constructed two kinds of amperometric enzyme electrodes for OP determination. In the first format (Fig. 5), screen printed thick-film carbon electrodes were modified by OPH that was deposited on the electrode in Nafion film.

Hydrodynamic voltammetry investigations identified the oxidation potential of PNP to be 0.85 V versus Ag/AgCl reference electrode and 1080 IU of OPH to be optimum for the biosensor. The amperometric signals were linearly proportional to the concentration of the hydrolyzed paraoxon and methyl parathion substrates up to 40 and 5 μM, showing detection limits of 9×10^{-8} and 7×10^{-8} M, respectively. Such detection limits were substantially lower compared to the (2–5) $\times10^{-6}$ M values for OPH-based potentiometric and fiber-optic devices. The high sensitivity was coupled to a faster and simplified operation with the potential for field-deployment (Mulchandani et al., 1999c).

In another format, we constructed a remote OPH-based amperometric biosensor. The remote electrochemical sensor consisted of a PVC housing tube connected to a 16-m-long shielded cable via three-pin environmentally sealed rubber connectors. The assembly included the OPH-modified carbon-paste working electrode, a Ag/AgCl reference electrode (BAS, Model RE-4) and a platinum wire counter electrode. Two female coupling connectors, fixed with epoxy in the PVC tube, served for mounting the working and reference electrodes; brass screws, located within these connectors, provided the electrical contact. The carbon paste electrode was prepared by thoroughly hand mixing 120 mg mineral oil and 180 mg graphite powder. The resulting paste was packed firmly into the electrode cavity (3 mm diameter, 1 mm depth) of a 4-cm-long Teflon® sleeve. Electrical contact (to the inner part of the paste) was established via a stainless steel screw, contacting the brass screw within the female connector on the PVC housing. The paste surface was smoothed on a weighing paper. The enzyme OPH was immobilized by casting a 10-μl droplet, containing 5 μl (of 108

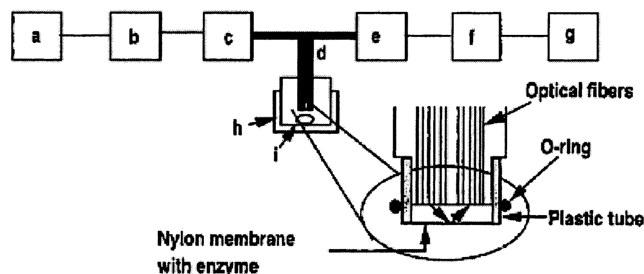


Fig. 4. Schematic of OPH-modified fiber-optic biosensor.

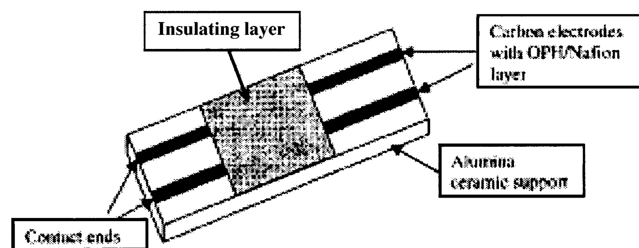


Fig. 5. Schematic of OPH-modified screen printed thick-film electrode.

IU/ml) OPH and 5 μl Nafion® (in 1% ethanol) onto the carbon surface, and allowing the solvent to evaporate.

Operating in chronoamperometric mode (from open circuit to +0.85 V), the response of the biosensor was linear in the range of 4.6–46 μM for paraoxon and up to 5 μM for methyl parathion. The lower detection limits of the biosensor for paraoxon and methyl parathion was 0.9 μM and 0.4 μM, respectively. An important characteristic of a remote sensor was that its rapid response to sudden concentration changes with no carry over between samples (Fig. 6). High stability Fig. 7 was another important characteristic of the in situ sensor. (Wang et al., 1999).

In conclusion, we have developed a number of OPH-based biosensors that can determine organophosphate pesticides selectively, rapidly, and directly in a single-step. Because the detection involves the conversion of the substrate to a product, as against inhibition in the case of AChE-based biosensors, the biosensors are reusable. Some of the formats reported in this review can potentially be deployed in the field for on-site and in-situ measurements. The detection limit of OPH-based biosensors could be further improved by either lowering the enzyme K_M or increasing the bimolecular rate constant (Carr and Bowers, 1980; Eddowes, 1990). Advances in enzyme engineering have made these goals potentially achievable. One such example of site-directed mutagenesis of OPH in order to improve the rate of hydrolysis of the chemical warfare agent, soman, was recently reported (Lai et al., 1996; Mason et al., 1997).

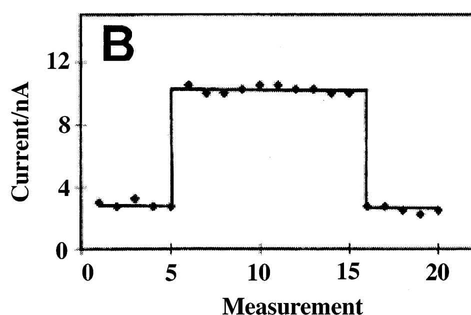


Fig. 6. Response of the remote biosensor to changes in methyl parathion concentration from 4.6 μM to 23 μM and back to 4.6 μM.

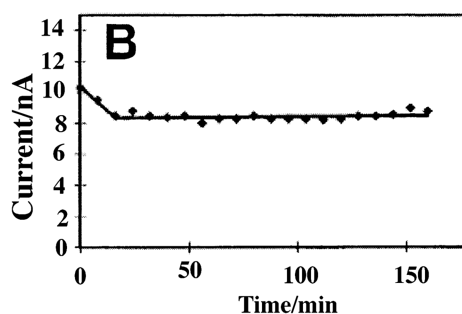


Fig. 7. Stability of the OPH-modified remote electrochemical sensor response to 7.5 μM methyl parathion.

We are currently working to improve the k_{cat}/K_M of the OPH for methyl parathion, coumaphos and chlorpyrifos.

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