Flow Injection Amperometric Enzyme Biosensor for Direct Determination of Organophosphate Nerve Agents

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A flow injection amperometric biosensor for the determination of organophosphate nerve agents was developed. The biosensor incorporated an immobilized enzyme reactor that contains the enzyme organophosphorus hydrolase covalently immobilized on activated aminopropyl controlled pore glass beads and an electrochemical flow-through detector containing carbon paste working electrode, a silver/silver chloride reference electrode, and stainless steel counter electrode. The organophosphorus hydrolase catalyzed the hydrolysis of organophosphate with nitrophenyl substituent to generate *p*-nitrophenol which is then detected downstream electrochemically at the carbon paste electrode poised at 0.9 V vs the reference electrode. The amperometric response of the biosensor was linear up to 120 μM and 140 μM, with lower detection limits of 20 nM and 20 nM, for paraoxon and methyl parathion, respectively. The response was very reproducible (RSD 2%, $n = 35$) and stable for over 1 month when the immobilized enzyme column was stored at 4 °C. Each assay took ca. 2 min giving a sample throughput of 30 h^{-1}. The applicability of the biosensor to monitor paraoxon and methyl parathion in distilled water and simulated well water was demonstrated.

Introduction

Synthetic organophosphorus (OP) compounds are used widely in the agricultural industry in the U.S. and around the world as pesticides and insecticides (*1*). 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 accumulation of the neurotransmitter acetylcholine which interferes with muscular responses and in vital organs produces serious symptoms and eventually death (*2, 3*). Parathion and methyl parathion are the two widely used agricultural pesticides in the U.S. According to the latest available data, approximately 7 million pounds per year of these two pesticides were used in the U.S. (*4*). In a recently completed U.S. Geological Survey study, the widespread presence of trace amounts of these pesticides was found in the surface- and groundwaters across the U.S. (*5*). Therefore, the rapid, sensitive, selective, and reliable determination of these pesticides is essential. Additionally, analytical tools are required to monitor and control the processes currently in use or being developed to treat the large volumes of wastewater generated at both the producer and consumer levels. Current analytical techniques, such as gas and liquid chromatography, although very sensitive and reliable, have disadvantages. These techniques are time-consuming, expensive, have to be performed by a highly trained technician, and are not suitable for rapid analyses under field conditions (*6*).

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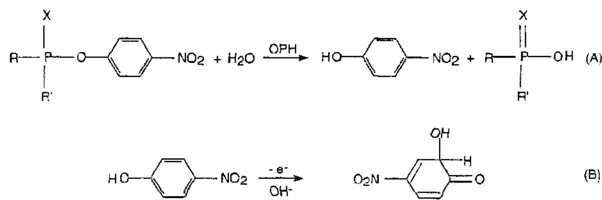

FIGURE 1. Reaction scheme for the OPH catalyzed hydrolysis of methyl parathion and paraoxon (A) followed by the electrochemical oxidation of *p*-nitrophenol (B). R and R′ are ethoxy and methoxy in paraoxon and methyl parathion, respectively, and X is O in paraoxon and S in methyl parathion.

Biosensors are ideally suited as analytical tools to provide solutions for the above problems (*7*). OP biosensors reported to-date have relied on the use of inhibition enzyme electrodes, based on the modulated activity of the cholinesterases (acetylcholinesterase, AChE, or butyrylcholinesterase, BuChE) (*6, 8, 9*). Although sensitive, these biosensors have serious limitations. They have poor selectivity (other pesticides such as carbamates and neurotoxins interfere since they also inhibit cholinestaerases) and are rather slow and tedious since the analysis involves multiple steps of reaction (measurement involves measuring initial ChE activity, incubation with inhibitor, measurement of residual activity, and regeneration which in many instances is not achieved due to irreversible inhibition) and washing. Biosensors based on catalytic reaction are superior to the inhibition type. Recently, we introduced the use of organophosphorus hydrolase (OPH)-based biosensors for direct monitoring of OPs (*10*−*16*). OPH hydrolyzes a number of OP pesticides and insecticides (e.g., paraoxon, parathion, coumaphos, diazinon) and chemical warfare agents (e.g., sarin) (*17*). The use of OPH is extremely attractive for biosensing OPs that act as substrates for the enzyme, rather than exerting an inhibitory action. Several types of OPH-based biosensors have been introduced recently, including potentiometric, optical, and thick-film screen printed amperometric devices (*10*−*16*).

In this paper, we report the development and application of a flow injection amperometric biosensor (FIAB) for the direct, sensitive, selective, rapid, and reliable determination of paraoxon and methyl parathion. The biosensor incorporates an immobilized enzyme reactor that catalyzes the hydrolysis of OPs and a carbon paste electrode that can measure *p*-nitrophenol the enzyme catalyzed hydrolysis product. The reaction and transduction scheme exploited for the reported biosensor can be represented by reactions (A) and (B) shown in Figure 1. OPH catalyzes the hydrolysis of paraoxon and methyl parathion according to reaction (A) to generate an equimolar amount of *p*-nitrophenol (PNP), which, in turn, is electrochemically oxidized (reaction B) at the carbon paste working electrode poised at a fixed potential to generate a current that is proportional to the pesticide concentration.

Experimental Section

Materials. Organophosphorus hydrolase (OPH) (7250 IU/mg protein, 15 mg protein/mL) was produced and purified

10.1021/es001773q CCC: $20.00 © 2001 American Chemical Society
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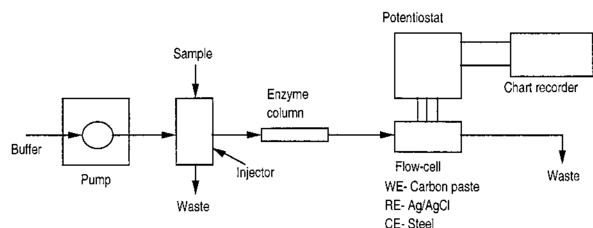

FIGURE 2. Schematic drawing of the flow injection amperometric biosensor configuration.

according to the methods described by Mulchandani et al. (*10*). Paraoxon and the aminopropyl control pore glass beads (80−120 mesh and 70 nm pore size) were obtained from Sigma Chemical Co. (St. Louis, MO). Mineral oil (white, light) was bought from Aldrich (Milwaukee, WI). Methyl parathion was purchased from Supelco Inc. (Belefonte, PA). All the other chemicals were acquired from Fisher Scientific (Tustin, CA). Double distilled water was used to prepare the solutions used in this work.

Preparation of OPH-Modified Control Pore Glass Beads Packed Column. A 250 mg sample of the aminopropyl glass beads was washed with distilled water, filtered through 0.45 μm filter, and then activated by contacting them with 3 mL of a 2.5% glutaraldehyde solution in 0.1 M, pH 8 phosphate buffer for 3 h at room temperature. The resulting orangeish-pink beads were washed thoroughly with phosphate buffer and contacted with 2 mL of 0.1 M, pH 8 phosphate containing OPH (4.5 mg/mL protein) by mixing end-over-end at 4 °C for 16 h, when 96% of the protein was immobilized. The beads were subsequently washed three times with 5 mL aliquots of phosphate buffer and then contacted with 100 mM glycine in 0.1 M, pH 8 phosphate buffer for 2 h to cap the remaining active groups. After extensive washing with the phosphate buffer the beads were then packed into a 2 mm i.d. × 5 cm long cylinder to form the immobilized enzyme column reactor.

Preparation of Carbon Paste Electrode. The carbon paste electrode was made by thoroughly hand mixing 25 mg of graphite powder with 5 mg of mineral oil to an even consistency. The paste was then packed into the two wells of a dual carbon paste electrode (MF-1004, Bioanalytical Systems Inc. (BAS), Lafayette, IN). The surface(s) of the packed electrodes were polished to a smooth shiny finish by gently rubbing them over ordinary weighing paper.

Apparatus. Figure 2 shows the schematic diagram of the flow injection amperometric biosensor (FIAB) used in this work. It consisted of a precision flow peristaltic pump (EVA Pump, Eppendorf, Madison, WI), an injection valve with a 20 μL injection loop, and a thin layer flow cell (CC-5, BAS). The thin layer flow cell consisted of the carbon paste working electrode, Ag/AgCl reference electrode, and a stainless steel counter electrode. The different components of the FIAB were connected to each other using Teflon tubing. Degassed buffer of appropriate pH supplemented with 5% methanol (added to increase the solubility of the hydrophobic OPs in the aqueous buffer) was used as the carrier buffer (pH 9.5, 0.1 M borate buffer + 0.05 mM $ZnCl_2$ + 5% methanol).

Analysis of Organophosphate Pesticides in Water. Distilled water or simulated well water (prepared by dissolving 100 mg/L $CaSO_4$; 50 mg/L $MgCl_2$; 100 mg/L $NaHCO_3$; 0.2 mg/L $FeSO_4{\cdot}7H_2O$; and 60 mg/L KNO_3) (*18*) were spiked with known amounts of the pesticide. Samples with a concentration above the detection limit were analyzed directly by the FIAB and by enzymatic assay by measuring the concentration of PNP formed at 410 nm spectrophotometrically (extinction coefficient of 17 000 M^{-1} cm^{-1}). Samples (100 mL) with a concentration below the detection limit of the sensor were extracted five times with 10 mL of chloroform. The pooled chloroform layer was then dried under vacuum in a rotary evaporator and the recovered solid dissolved in 2 mL of the carrier buffer and analyzed by FIAB.

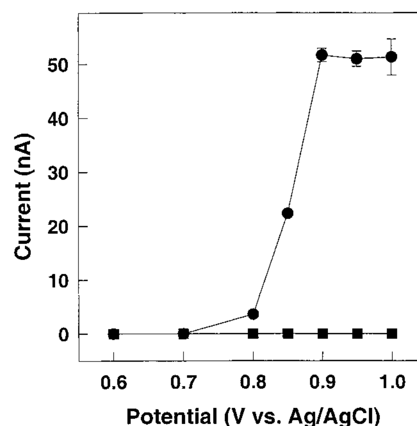

FIGURE 3. Hydrodynamic voltammogram for 1 μM (●) *p*-nitrophenol and (■) paraoxon at the carbon paste electrode in 0.1 M borate + 0.05 mM Zn^{2+} pH 9.5 buffer.

Results and Discussion

Optimization of FIAB Operational Conditions. *Divalent Metal Ion.* Organophosphorus hydrolase contains a binuclear metal ion center that is actively involved in the catalysis. The native enzyme contains two Zn^{2+} per enzyme molecule. The replacement of Zn^{2+} by Co^{2+} (incorporated in the enzyme molecule during the biosynthesis by supplementing the medium) increases the enzyme activity 2-fold (*19*). The presence of 0.05 mM Co^{2+}, however, produced a high background current and a baseline drift, which were later identified to be associated with the oxidation of Co^{2+} at the working electrode. Removal of Co^{2+} from the buffer alleviated the high background and baseline drift problem, but the current signal decreased on repeated analyses, suggesting decreasing enzymatic activity. Replacing Co^{2+} by Zn^{2+} in the buffer alleviated these problems as Zn^{2+} is not oxidized at the electrode while assisting in preserving the enzyme activity for extended periods. In subsequent studies the carrier buffers were supplemented with 0.05 mM of Zn^{2+}.

Operating Potential. Since the sensitivity of the biosensor for the pesticides of interest depends on the sensitivity toward *p*-nitrophenol (Figure 1), a hydrodynamic voltammetry study was performed to establish the appropriate electrochemical potential for its determination. Figure 3 displays the current response of the carbon paste electrode to 1 μM paraoxon and PNP at different applied potentials. The oxidation of PNP started at approximately 0.7 V (vs Ag/AgCl reference) and increased to reach a plateau at 0.9 V. Correspondingly, no paraoxon oxidation was observed in the potential range of 0.6 to 1 V. Thus, a potential of 0.9 V was selected for subsequent studies.

Buffer pH. The activity of OPH and the output current for the electrochemical oxidation of PNP are functions of the pH. Therefore, it is necessary to determine the optimum pH that will result in the maximum sensitivity of the FIAB. As shown in Figure 4, pH profiles for electrochemical oxidation of PNP and paraoxon were virtually identical. The pH optimum was however 0.5 pH unit more basic than that of the free OPH enzyme for paraoxon (*10*), suggesting a weak domination of the PNP electrooxidation in the present detection scheme. The pH 9.5 at which the signal was maximum was used for subsequent studies.

Carrier Buffer Flow Rate. Figure 5 shows the profile of the amperometric response of the FIAB as a function of the carrier buffer flow rate. The current increased with an increase in flow rate over the range feasible with the peristaltic pump

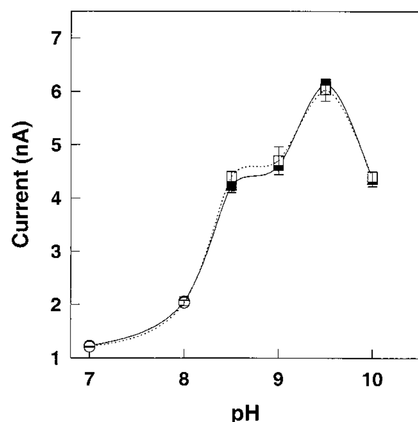

FIGURE 4. Effect of buffer [(■, □) 0.1 M borate + 0.05 mM Zn^{2+}; (●, ○) 0.1 M phosphate + 0.05 mM Zn^{2+}] pH on the response current of the FIAB for 20 μL injection of 1 μM paraoxon (filled symbols and solid line) and 1 μM *p*-nitrophenol. Operating conditions: potential 0.9 V vs Ag/AgCl; carrier flow rate 1 mL/min.

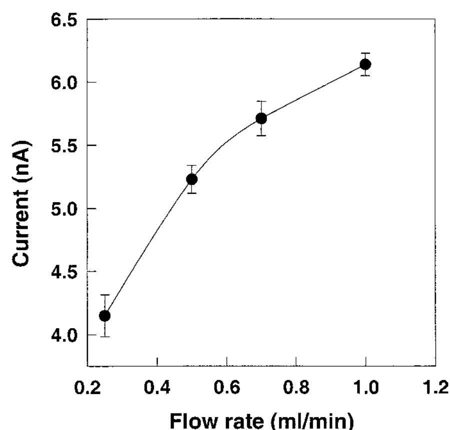

FIGURE 5. Effect of carrier buffer flow rate on the response current of the FIAB for 20 μL injection of 1 μM paraoxon. Operating conditions: potential 0.9 V vs Ag/AgCl; buffer 0.1 M borate + 0.05 mM Zn^{2+} pH 9.5.

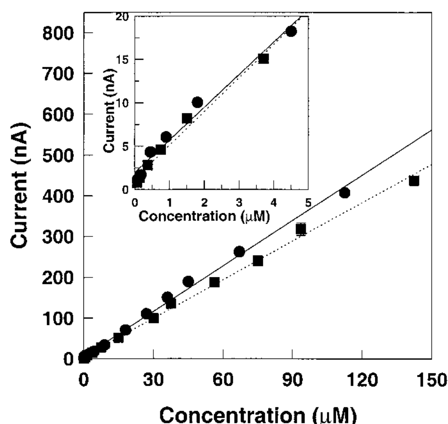

FIGURE 6. Calibration plots for (●) paraoxon and (■) methyl parathion using FIAB. Operating conditions: potential 0.9 V vs Ag/AgCl; buffer 0.1 M borate + 0.05 mM Zn^{2+} pH 9.5; carrier flow rate 1 mL/min; sample size 20 μL.

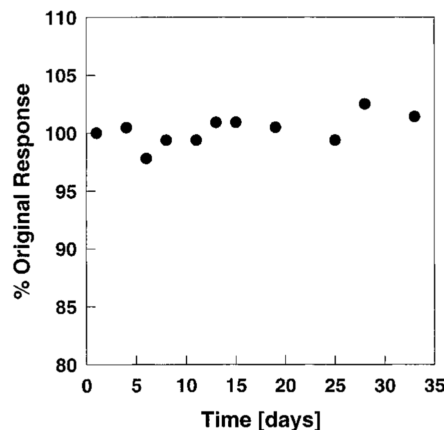

FIGURE 7. Stability of the immobilized enzyme column used in the FIAB when stored at 4 °C and used at 20 °C. Response to 20 μL injection of 1 μM paraoxon. Operating conditions: potential 0.9 V vs Ag/AgCl; carrier flow rate 1 mL/min; buffer 0.1 M pH 9.5 borate + 0.05 mM Zn^{2+}.

used in this work. Such a trend can be attributed to the improved paraoxon hydrolysis kinetics in the enzyme reactor due to improved mass transport resulting from increasing fluid velocity. A flow rate of 1 mL/min, at which the response was maximum, was used in future work.

Analytical Characterization of the FIAB. *Sensitivity and Lower Detection Limits.* Using the optimum conditions established in the above studies (pH 9.5, 0.1 M borate buffer supplemented with 0.05 mM $ZnCl_2$ and 5% methanol at a flow rate of 1 mL/min with the working electrode poised at 0.9 V), calibration plots were generated for paraoxon (parathion in nature and when ingested by humans and animals is oxidized to paraoxon which is 15 times more toxic) and methyl parathion (Figure 6). As shown, FIAB has a broad dynamic linear range, high sensitivity (3.76 nA/μM for paraoxon and 3.15 nA/μM for methyl parathion) and very good lower detection limits (20 nM for paraoxon and 20 nM for methyl parathion), estimated from the signal-to-noise characteristics (S/N = 3) for the blank. These LDL were 2 orders of magnitude lower than that achieved with OPH enzyme-based potentiometric and optical sensors and two times better than the amperometric thick-film strip electrode developed in this laboratory (*15*). The current LDL was similar or nearly an order of magnitude higher than the AChE-based biosensors (*8*).

Precision, Stability, and Response Time. The response of the FIAB was highly reproducible and stable during operation at room temperature, as demonstrated by the very low residual standard deviation of 2% for 35 repeated injections of 1 μM paraoxon over the course of 2 h.

Another salient feature of the FIAB was the direct single step measurement in approximately 2 min corresponding to a sample throughput of 30 per hour. In comparison, AChE-based biosensors require multiple steps (measurement of initial enzyme activity, incubation with sample, determination of residual activity, and regeneration of enzyme activity) and are indirect and slower responding (requires anywhere from 15 min for disposable single use type to up to 5 h) (*8, 10*).

The FIAB developed in this work demonstrated excellent storage stability (Figure 7) when the immobilized enzyme column was stored in the carrier buffer at 4 °C.

The ability to use the same enzyme column and the electrode repeatedly without regular calibration and single step rapid direct analysis are the advantages of the FIAB reported in this paper relative to the AChE-based biosensors. Additionally, these AChE-based biosensors are generally useful only for one measurement (because of irreversible inhibition of the activity).

Selectivity. Unlike AChE-based biosensors that are interfered with any compound that is an inhibitor of the AChE activity (heavy metals, carbamates, triazines, etc.), the FIAB was very selective against other widely used pesticides such as atrazine, sevin, sutan, and simazine and organophosphate

TABLE 1. Flow Injection Amperometric Biosensor Selectivity

compound	concentration, μM	current, nA ($n = 3$)
buffer	0	0
paraoxon	4.5	18.2 ± 0.2
methyl parathion	3.75	15.1 ± 0.4
sutan	5	0.6 ± 0.1
atrazine	5	0.5 ± 0.1
sevin	5	1.5 ± 0.1
simazine	5	0.5 ± 0.1
phenol	1	14.7 ± 0.2
pentachlorophenol	5	8.9 ± 0.1
diazinon	100	0

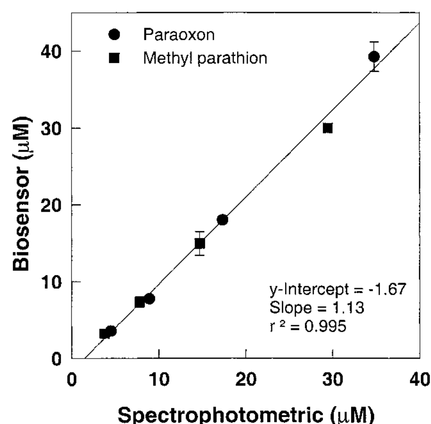

FIGURE 8. Comparison of the concentration measured by the FIAB relative to the enzyme assay technique. Operating conditions: potential 0.9 V vs Ag/AgCl; carrier flow rate 1 mL/min; buffer 0.1 M pH 9.5 borate with 0.05 mM Zn^{2+}.

insecticides such as diazinon, that do not produce PNP upon hydrolysis (Table 1). The sensor, however, was not selective against pentachlorophenol (PCP) and phenol, which, like PNP, are oxidized at the carbon paste electrode. The contribution of compounds such as PCP, phenol, and other potentially oxidizing chemicals present in environmental samples can be addressed by measuring and subtracting the response of a sample passing through a parallel column without the OPH enzyme.

The inability to determine individual OP concentration is a limitation of FIAB. However, integrating it with any chromatographic separation (HPLC or capillary electrophoresis on microfabricated chip) could facilitate determination of individual OP concentrations.

Validation of FIAB. The FIAB was validated by measuring the concentration of paraoxon and methyl parathion in distilled water and simulated well water and comparing it to the concentration measured using the enzymatic assay (based on the measurement of the PNP concentration by measuring the absorbance at 410 nm spectrophotometrically). As shown in Figure 8, there was an excellent agreement between the sensor and enzyme assay measurements. To demonstrate the applicability of the FIAB for measurements of concentrations below the detection limit, the samples were pretreated (see Experimental section). The excellent recovery and agreement of the concentration determined with the amount added (Table 2) demonstrated the accuracy and the reliability of the FIAB.

In conclusion, a flow injection amperometric biosensor for direct, one step, sensitive, selective, and rapid determination of paraoxon and methyl parathion was demonstrated. The FIAB developed in this work can also be applied to determine parathion, ethyl *p*-nitrophenyl thiobenzenephosphonate (EPN), and fenitrothion, which are also hydrolyzed by the organophosphorus hydrolase to produce *p*-nitrophenol. The above flow-injection amperometric biosensor format can potentially be easily translated from the current format to a laboratory-on-a-chip format using microfabrication technology.

TABLE 2. Validation of the FIAB

paraoxon added, nmol	measured after extraction from: distilled water, nmol	well water, nmol
2	1.9	1.7
5	4.8	4.6
10	9.6	9.7

Acknowledgments

This research was supported by grants from the USDA (99-35102-8600), the U.S. EPA (R8236663), and the National Science Foundation (9731513).

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Received for review October 16, 2000. Revised manuscript received March 13, 2001. Accepted March 27, 2001.

ES001773Q