Enzyme Biosensor for Determination of Organophosphates

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Received 2 June 1998; revised 5 August 1998; accepted 9 September 1998

Abstract: A potentiometric enzyme biosensor for the direct measurement of organophosphate pesticides was developed. The basic element of this enzyme biosensor was a pH electrode modified with an immobilized organophosphorus hydrolase layer formed by cross-linking OPH with bovine serum albumin and glutaraldehyde. Organophosphorus hydrolase catalyzes the hydrolysis of organophosporus pesticides to release protons, the concentration of which is proportional to the amount of hydrolyzed substrate. The organophosphate enzyme biosensor had a response time of less than 3 min, a useful operating range of 0.002–0.4 mM for paraoxon and parathion, and a long-term stability of over a month when stored in pH 8.5, 1 mM HEPES + 100 mM NaCl buffer at 4 °C. The effects of various parameters, buffer concentration, pH, temperature and enzyme loading are described. © 1998 John Wiley & Sons, Inc. Field Analyt Chem Technol 2: 363–369, 1998

Keywords: organophosphates; nerve agents; enzyme biosensor; organophosphorus hydrolase; paraoxon; parathion

Introduction

Synthetic organophosphates (OP), amongst the most toxic substances known [1, 2], are widely used as pesticides, insecticides, and chemical warfare agents [3–5]. A large volume of wastewaters contaminated with these neurotoxic compounds, generated at both the producer and consumer levels, requires treatment before being released in the environment [6]. Because currently available abiotic treatment methods, chemical hydrolysis, and incineration are considered inadequate, biotic methods based on enzymes and microorganisms are being investigated in many research laboratories [6–11]. The successful use of any laboratory-developed technology for detoxification of the organophosphate neurotoxins will require analytical tools for monitoring concentrations of these neurotoxins.

Biosensors based on acetylcholinesterase (AChE) inhibition have been an active research area over the last decade [12–22]. Although sensitive and useful as single-use disposable sensors for environmental monitoring, biosensors based on AChE inhibition have several limitations for on-line process monitoring applications. First of all, these biosensors have extensive and tedious protocols that require long incubation with inhibitors prior to analysis for good sensitivity, as well as treatment with pyridine-2-aldoxime after analysis to partially regenerate/recover the enzyme activity, which is inhibited irreversibly by OP [13, 15, 21]. Second, because 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 that may be required for monitoring detoxification processes.

Organophosphorus hydrolase (OPH), a biological catalyst, has been shown to effectively hydrolyze a range of organophosphate esters, pesticides such as parathion, coumaphos and acephate, and chemical warfare agents such as soman, sarin, VX, and tabun [23–28]. The catalytic hydrolysis of each molecule of these compounds releases two protons, the measurement and correlation of which to the OP concentration forms the basis of a potentiometric enzyme electrode. The detection scheme based on monitoring the OPH-catalyzed hydrolysis products of OPs offers the advantages of simpler, more direct, and quicker measurements of only organophosphorus–type nerve agents over that based on the inhibition of acetylcholinesterase activity, which re-

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Contract grant sponsor: U. S. EPA
Contract grant number: R8236663-01-0

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sponds to all types of nerve agents. Additionally, the OPH-based biosensor has the potential of quantifying individual organophosphate pesticides when used as a detector in conjunction with an HPLC system for chromatographic separation.

Recently, potentiometric biosensors for the direct determination of organophosphates were reported [29]. These sensors consisted of OPH-expressing recombinant *Escherichia coli* cells cryoimmobilized by entrapment in poly(vinyl)alcohol gel. The immobilized cells were either suspended in a batch reactor with a pH electrode or packed in a column and placed upstream of a flow cell with a pH electrode. The long response time, attributable to the various mass transfer resistances present in the system, and the need of special equipment for cryoimmobilization of the cells are the limitations of the reported biosensing systems.

This article describes the construction of a simple potentiometric enzyme biosensor for the direct, sensitive, selective, and rapid determination of organophosphate nerve agents, with the use of OPH immobilized via cross linking on the surface of a pH electrode that can be potentially used for on-line monitoring of detoxification processes.

Materials And Methods

Reagents

HEPES, yeast extract, tryptone, ammonium sulfate, potassium monobasic phosphate, potassium dibasic phosphate, cobalt chloride, glutaraldehyde, isopropanol, and glycerol were purchased from Fisher Scientific (Tustin, CA). Bovine serum albumin, polyethyleneimine, lysozyme, and PMSF were obtained from Sigma Chemical Company (St. Louis, MO). Paraoxon, parathion, sevin, sutan, atrazine, and simazine were acquired from Supelco Inc. (Bellefonte, PA). Dialysis membrane was purchased from Spectrum Medical industries, Inc. (Los Angeles, CA). Chromatography packing materials, Sephadex G-150 and DEAE-Sephadex A-50, were obtained from Pharmacia Biotech (Uppsala, Sweden). All the solutions were made in distilled deionized water.

OPH Production and Purification

OPH was purified from recombinant *E. coli* carrying plasmid pJK33 (obtained from Dr. Jeffrey Karns, USDA, Beltsville, MD) according to the reported protocol [30].

Enzyme Biosensor Construction

A 10-μl aliquot from a 14-μl mixture, prepared by mixing 2 μl of 10% bovine serum albumin, 2 μl of 2.5% glutaraldehyde (a bifunctional protein cross-linker) and 10 μl of buffer containing different amounts of OPH, was spread on the surface of the pH electrode (Accumet, Model 13-620-289, Fisher Scientific, Tustin, CA) held upside down in a clamp and allowed to dry for 30–40 min. The gel was subsequently covered with a 12–14-kDa molecular weight cut-off dialysis membrane, which was held in place by an O-ring, and washed thoroughly with buffer (pH 8.5, 1 mM HEPES plus 100 mM NaCl and 50 mM $CoCl_2$) to remove any excess glutaraldehyde. To study the effect of enzyme amount on the biosensor response, pH electrodes were modified with a constant amount of protein to ensure that there was no variation in the properties of the resulting enzyme membrane by maintaining a constant total protein concentration of 2%, with the use of bovine serum albumin.

Experimental Setup and Measurement

All measurements were made in 5 ml of an appropriate buffer, thermostated to the desired temperature, in a 10-ml working volume jacketed glass cell, equipped with a magnetic stirrer. The temperature of the liquid in the cell was controlled by circulating water in the cell jacket with the use of a circulating water bath (Model 1160, VWR Scientific, San Francisco, CA). Next 5–10 μl of OP nerve agent, dissolved in pure methanol, was added to the cell, and the changes in potential, that is pH, were recorded with a pH/ion analyzer (Model 255, Corning Science Products, Corning, NY) connected to a flatbed chart recorder (Model BD112, Kipp and Zonen, Holland).

Results and Discussion

Effect of Various Parameters

The response of OPH-modified enzyme biosensor was a strong inverse function of the buffer concentration (Figure 1). The magnitude of the response, the lower detection limit, and the response time (data not shown) of the biosensor were better in the weak buffer. Such behavior can be attributed to the fact that when a concentrated buffer is employed, more of the basic buffer ions permeate through the dialysis membrane and the enzyme layer and neutralize part of enzymatically generated hydrogen ions. Thus, the hydrogen-ion concentration at the glass-sensing surface is lowered, which produces a smaller response. Because an objective of this work was to develop a rapid and sensitive biosensor for organophosphate pesticides, 1 mM buffer was selected for subsequent investigations. Because it was rather difficult to work with the 1 mM buffer, 100 mM sodium chloride was added to it. Addition of this neutral salt made the buffer easier to work with without affecting the enzyme electrode response characteristic (data not shown).

The response of the enzyme biosensor was a strong function of the starting pH. This profile mirrored the pH profile for the free enzyme. The lowering of the response at high and low pH can be attributed to the reduced activity of the enzyme under these conditions. A starting pH of 8.5, which gave the maximum sensitivity, lowest response time, and largest dynamic range was used subsequently.

The operating temperature of the enzyme biosensor affected its response to OP. The response increased with temperature, reached a maximum at 45 °C and then decreased.

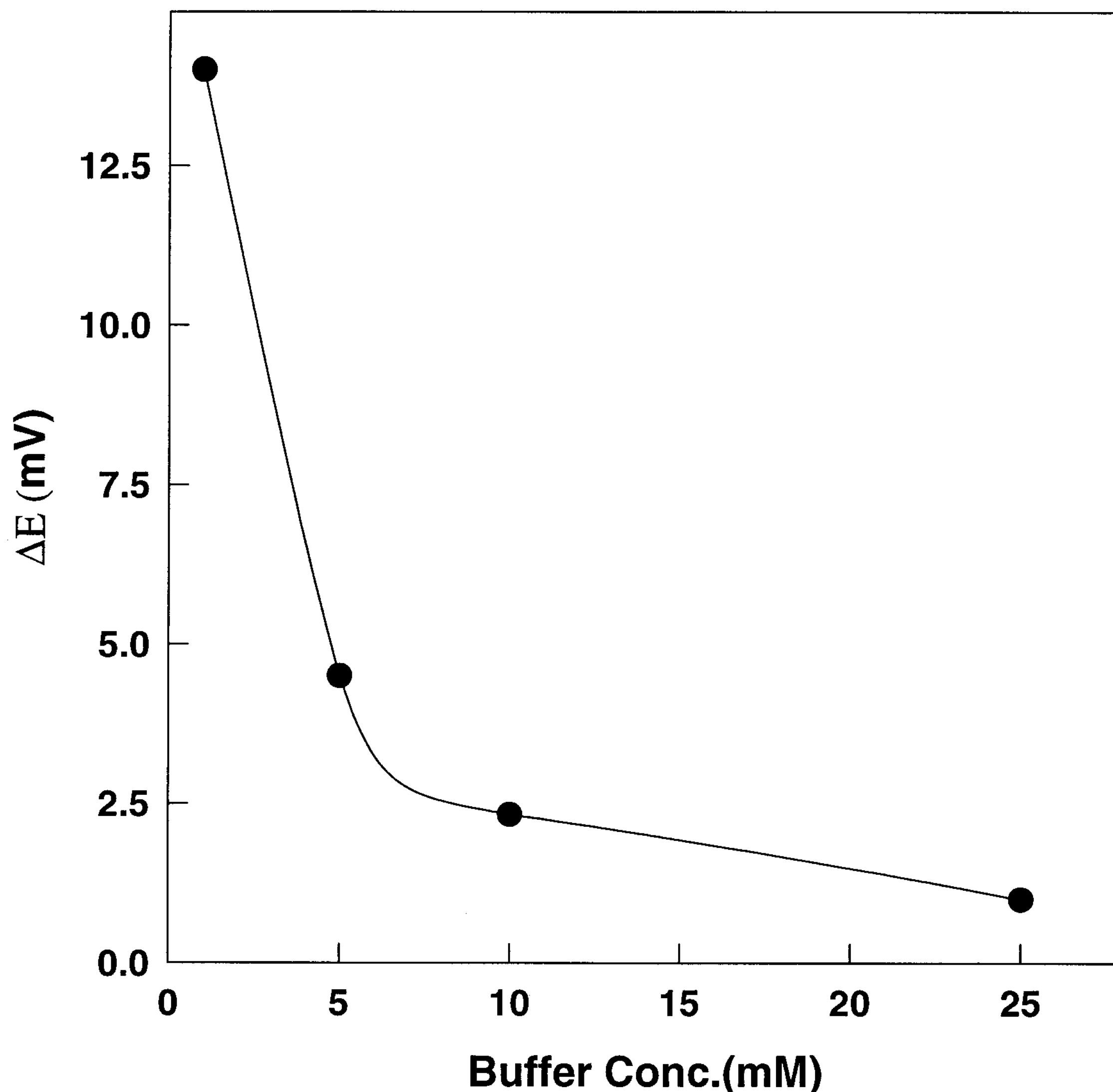

FIG. 1. Effect of buffer concentration on the response of the enzyme electrode to 0.1 mM paraoxon in pH 8.5 HEPES buffer with 0.05 mM $CoCl_2$ at 20 °C. Enzyme amount: 775 IU.

This temperature profile was similar to that reported for the free enzyme [31]. Although 45 °C was determined to be the optimum temperature for the enzyme biosensor operation, subsequent experiments were still performed at room temperature, 20 °C. This was done in order to overcome the problem of excessive evaporative losses during the course of the experiment and ease of operations.

Figure 2 shows the effect of enzyme units (amount) used in the preparation of the enzyme biosensor on its response. The biosensor response increased with the amount of OPH used in the preparation, and subsequently reached a plateau. This result was in agreement with the observations reported for other enzyme-modified electrodes [32, 33]. The observed phenomenon can be explained as follows. Initially, the enzyme reaction rate is the rate controlling step, and increasing amount of the enzyme results in increased sensitivity and shorter response time. However, in the plateau region mass transport becomes the rate-controlling step, and no further increase in sensitivity or decrease in response time can be obtained. The mass transfer controlling region is preferred for an enzyme electrode, because in this region the response of the electrode becomes relatively insensitive to changes in enzyme activity and thus prolongs the biosensor operational and storage lifetimes [34]. The OPH loading of 500 units was therefore selected for all subsequent experiments.

Analytical Characteristics

The OPH-based enzyme biosensor involves a simple single-step direct measurement. The time-resolved signal changes of the OPH-modified enzyme biosensor caused by the addition of paraoxon is shown in Figure 3. The steady-state values of the potential change were reached in less than 3 min. This response time was a slight improvement over

FIG. 2. Effect of enzyme protein (organophosphorus hydrolase specific activity 77.5 IU (μg^{-1}) loading on the response of the enzyme electrode to 0.1 mM paraoxon in pH 8.5, 1 mM HEPES + 100 mM NaCl + 0.05 mM $CoCl_2$ at 20 °C.

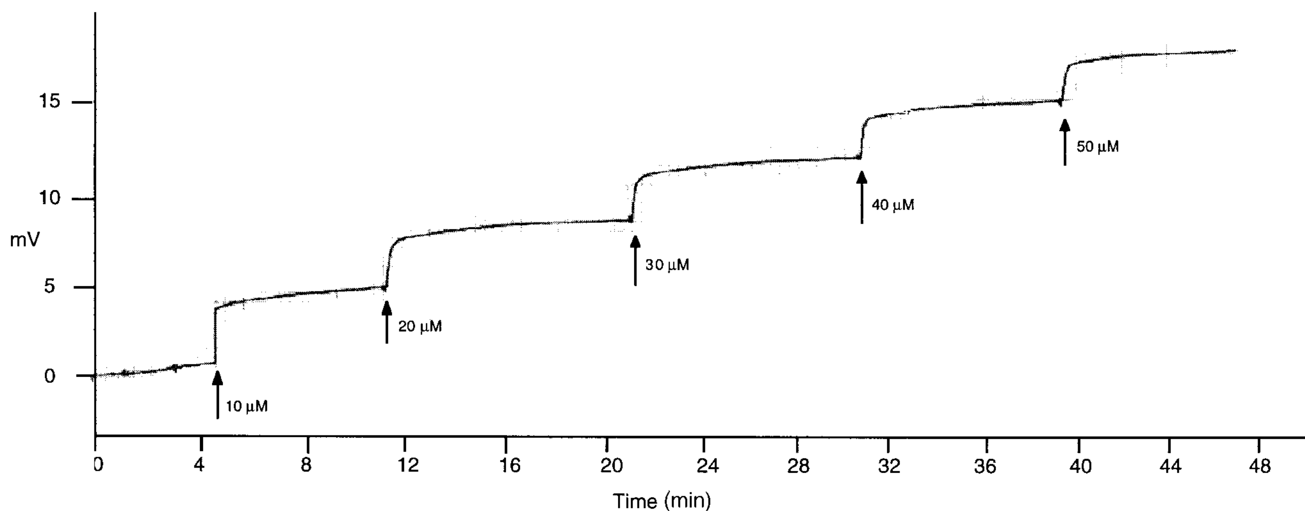

FIG. 3. Time-resolved response curve of enzyme electrode to additions of 10 μM paraoxon. Conditions: 1 mM HEPES + 100 mM NaCl + 0.05 mM $CoCl_2$, pH 8.5, 20 °C; 500 IU of enzyme immobilized.

the OPH-based microbial biosensor [29]. AChE-based biosensors, in contrast, involve multiple steps (measurement of the initial AChE activity, incubation with the analyte sample, determination of the inhibited enzyme activity, and regeneration of enzyme activity) and indirect measurement. They require anywhere from 15 min to 5 h [13, 15, 18, 19, 22]. (The short response time biosensors are of the disposable type, where the enzyme reactivation step is excluded; hence they are unsuitable for multiple-use on-line process monitoring.)

Figure 4 shows the calibration plots for paraoxon and parathion obtained with the use of the potentiometric enzyme biosensor. The lower detection limits (three times the standard deviation of the response obtained for a blank) of the present OPH-modified enzyme biosensor for paraoxon and parathion are 2 μM. These detection limits are comparable to the OPH-based microbial biosensor [29]. However, the detection limits are 1–3 orders of magnitude higher than for AChE-based biosensors [12, 16–18, 20, 22]. This will therefore limit the applicability of the present sensor for environmental monitoring to off-line analysis. For any such application of the present biosensor, off-line sample preparation involving solvent extraction and concentration will be necessary. The present enzyme biosensor, however, would be ideal for (1) on-line monitoring of detoxification processes for treatment of wastewater generated during production and consumption of the organophosphate-based pesticides and insecticides and disposal of organophosphate-based nerve agents, and (2) selectively (please see later) monitoring only the organophosphate-based pesticides/neurotoxins.

Reproducibility in manufacturing biosensors can be a serious problem, especially if manual preparation steps are involved. In a test series, four OPH-modified enzyme biosensors were prepared with the use of 500 IU of OPH and tested in 1 mM HEPES with 100 mM NaCl and 0.05 M $CoCl_2$, pH 8.5 at 20 °C to 0.1 mM paraoxon. The low relative standard deviation of 5% in the response of four enzyme electrodes demonstrated an excellent reproducibility from

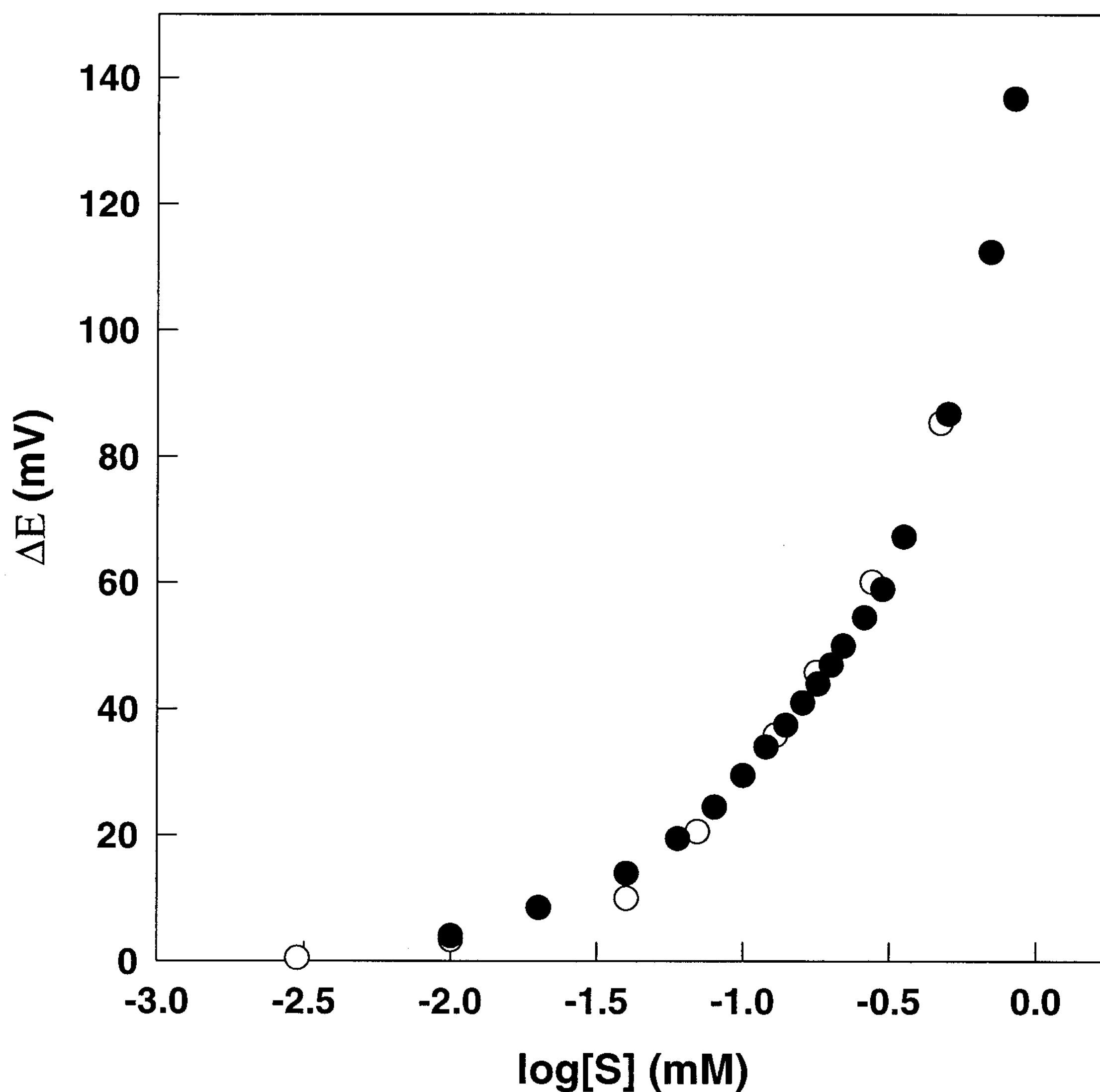

FIG. 4. Calibration plots for organophosphates (open circles) paraoxon, (solid circles) parathion: Conditions: 1 mM HEPES + 100 mM NaCl + 0.05 mM $CoCl_2$, pH 8.5, 20 °C; 500 IU of enzyme immobilized.

enzyme electrode to enzyme electrode. The OPH-modified enzyme electrode also demonstrated a high precision of analysis for paraoxon (RSD = 4.23%, n = 9) and parathion (RSD = 3.54%, n = 7).

The OPH-modified enzyme biosensor had a very high selectivity for organophosphates. Other widely used pesticides, such as atrazine, sutan, sevin, and simazine, at concentrations of 10 times the lower detection limits of paraoxon and parathion (20 μM) did not interfere (data not shown). This is a significant benefit over the AChE-based biosensors, especially for on-line monitoring of detoxification processes.

The long-term storage and the multipleuse stability of the enzyme biosensor was investigated by evaluating the response of the same biosensor to paraoxon with repeated storage at 4 °C in pH 8.5, 1 mM HEPES with 100 mM sodium chloride and 0.05 mM $CoCl_2$ buffer. The enzyme biosensor was very stable, retaining over 95% of its original response, for the 1-month period during which it was used a total of 20 times. These results are in agreement with the OPH-based microbial biosensor developed by Rainina, Efremenko, Varfolomeyev, Simonian, and Wild [29]. In comparison, AChE-based biosensors were unlike OPH in that AChE is inactivated every time the biosensor is exposed to neurotoxins [13, 15, 21] and loses as much as 40% of the original response after as few as three repeated uses [13, 15].

In conclusion, an OPH-modified potentiometric enzyme biosensor for the direct, rapid, and selective measurement of organophosphate nerve agents was developed. The sensor had excellent stability, precision, accuracy, selectivity for organophosphate over other neurotoxins, and short response time. These features make it a potential analytical tool ideal for long-term monitoring of chemical and biological detoxification processes.

Acknowledgments

This work was supported by a grant from the U. S. EPA (R8236663-01-0). We thank Dr. J. S. Karns of the USDA for providing *E. coli* strain carrying plasmid pJK33.

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