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Amperometric microbial biosensor for direct determination of organophosphate pesticides using recombinant microorganism with surface expressed organophosphorus hydrolase

Priti Mulchandani [a], Wilfred Chen [a,*], Ashok Mulchandani [a,*], Joseph Wang [b,*], Liang Chen [b]

[a] *Department of Chemical and Environmental Engineering, University of California, Riverside, CA 92521, USA*
[b] *Department of Chemistry and Biochemistry, New Mexico State University, Las Cruces, NM 88003, USA*

Abstract

An amperometric microbial biosensor for the direct measurement of organophosphate nerve agents is described. The sensor is based on a carbon paste electrode containing genetically engineered cells expressing organophosphorus hydrolase (OPH) on the cell surface. OPH catalyzes the hydrolysis of organophosphorus pesticides with *p*-nitrophenyl substituent such as paraoxon, parathion and methyl parathion to *p*-nitrophenol. The later is detected anodically at the carbon transducer with the oxidation current being proportional to the nerve-agent concentration. The sensor sensitivity was optimized with respect to the buffer pH and loading of cells immobilized using paraoxon as substrate. The best sensitivity was obtained using a sensor constructed with 10 mg of wet cell weight per 100 mg of carbon paste and operating in pH 8.5 buffer. Using these conditions, the biosensor was used to measure as low as 0.2 μM paraoxon and 1 μM methyl parathion with very good sensitivity, excellent selectivity and reproducibility. The microbial biosensor had excellent storage stability, retaining 100% of its original activity when stored at 4 °C for up to 45 days.

Keywords: Organophosphate; Organophosphorus hydrolase; Recombinant microorganism

1. Introduction

Neurotoxic organophosphate (OP) compounds have found wide applications as pesticides and insecticides in agriculture and as chemical warfare agents in military practice (FAO, 1989; USDA 1992; Compton, 1988). In view of the growing public environmental and security concerns, the need for effective detection devices has intensified. Current analytical techniques such as chromatography (gas, liquid and thin layer), immunoassay and enzyme biosensors based on inhibition of cholinesterase activity, although very sensitive, have disadvantages (Sherma, 1995).

Organophosphorus hydrolase (OPH) hydrolyzes a wide range of OP nerve agents (Donarski et al., 1989; Dumas et al. 1989a,b; Dumas et al., 1990) and the use of OPH is extremely attractive for the biosensing of OPs that act as substrates for the enzyme, rather than inhibitors. Because of the superiority offered by biosensors based on catalytic reaction over the inhibition type, our group has exploited the use of OPH for developing potentiometric, optical and amperometric biosensors based on purified enzyme or whole cells. (Mulchandani et al., 1998a,b, 1999a,b,c; Roger et al., 1999; Wang et al., 1999).

The present biosensor relies on the hydrolysis of the OP compounds by the OPH displayed and anchored on the cell surface followed by the amperometric detection of the generated *p*-nitrophenol. Biosensors based on similar transduction principles using purified OPH immobilized on thick-film screen printed carbon electrode (Mulchandani et al., 1999c) and in a Nafion film covering the surface of a carbon paste electrode (Wang et al., 1999), were recently demonstrated. These biosensors required purified OPH, a laborious, time consuming and costly effort.

* Corresponding authors. Tel.: +1-909-787-2473; fax: +1-909-787-3188.
E-mail address: wilfred@engr.ucr.edu (W. Chen).

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Instead of purified enzymes, whole cells can be used. Cells anchoring and displaying the specific enzyme on the cell surface offer the advantage of eliminating the diffusional mass transport resistance due to the cell walls enveloping the biocatalyst. Earlier, we employed the Lpp-OmpA anchor system to display OPH on the cell surface of *Escherichia coli* (Richins et al., 1997). Recently, using a new anchor system based on the ice nucleation protein from *Pseudomonas syringae* INA5, functional OPH was displayed on the surface of *Moraxella* sp. (Shimazu et al., 2001). The OPH activity for this new cell line was 80-fold higher than in *E. coli* and thus offers a potential for greatly improved biosensor performance. This paper reports development, characterization and evaluation of an amperometric microbial biosensor for sensitive, selective, rapid and direct determination of OP pesticides. Carbon paste modified with whole cells of genetically engineered *Moraxella* sp. anchoring and displaying OPH on its cell surface, was used as working electrode in a three electrode electrochemical cell. Oxidation current of the *p*-nitrophenol generated by the hydrolysis of OP pesticides such as paraoxon and methyl parathion, is correlated to the analyte concentration. The advantages of incorporating whole cells into carbon-paste transducers were described earlier (Wang and Lin, 1988; Gorton, 1995)

2. Materials and methods

2.1. Reagents

Paraoxon and methyl parathion were acquired from Supelco Inc. (Bellefonte, PA). All other chemicals were purchased from Fisher Scientific (Tustin, CA). All the solutions were made in distilled deionized water.

2.2. Bacterial strains and plasmids

Moraxella sp. isolated from activated sludge by selective enrichment with PNP (Spain et al., 1979) expressing OPH on the cell surface was used. Plasmid pINCOP was used to express INPNC-OPH on the cell surface. Expression of OPH was tightly regulated by a *tac* promoter (Shimazu et al., 2001).

2.3. Growth conditions

Strains bearing plasmids were grown in 50 ml of Luria-Bertani media supplemented with kanamyacin to a final concentration of 50 μg/ml in 250 ml flasks in an Innova 4000 shaker (New Brunswick Scientific, NJ) with vigorous aeration (300 rpm) at 30 °C. Bacteria harboring expression vectors were grown to an $OD_{600} = 0.4$ (attained after 3–4 h) before induction with 1 mM IPTG. 1 mM $CoCl_2$ was added to the culture 24 h after induction. After 48 h of growth, cells were harvested by centrifugation at $5000 \times g$ for 10 min, washed with pH 8.0, 50 mM citrate-phosphate + 0.05 mM $ZnCl_2$ buffer (henceforth designated as buffer A) twice, resuspended in buffer A and stored at 4 °C for 24 h. Appropriate volume of cell suspension (corresponding to the desired cell weight) was then dried at 25 °C under vacuum and mixed in carbon paste.

2.4. Electrode fabrication

The carbon paste of working electrode was prepared by mixing appropriate weight of cells with carbon paste containing 75% (w/w) graphite powder and 25% (w/w) mineral oil. The whole cell modified carbon paste was subsequently packed firmly into the electrode cavity (3 mm diameter × 1 mm deep) of a Kel-F sleeve (Bioanalytical System Inc., Lafayette, IN) and polished to a smooth shiny finish by gently rubbing over an ordinary weighing paper.

2.5. Apparatus

Amperometric measurements were performed using a Bioanalytical Systems (BAS) voltammetric analyzer (Model LC-4C) coupled to a chart recorder (Model BD112, Kipp and Zonen, Holland). All experiments were conducted using a 3-electrode electrochemical cell (30 ml volume with a working volume of 10 ml) inside a Faraday cage (BAS, Model C2 cell stand), with a Ag/AgCl reference electrode (BAS, MF 2063), and a platinum wire auxiliary electrode (BAS, MF 1032). A small magnetic stirrer and bar provided the convective transport.

2.6. Procedure

All experiments were performed by applying a potential of +0.9 V to the working electrode vs. the Ag/AgCl reference and allowing the transient current to decay prior to measurements. Five to ten microliters of OP compound, dissolved in pure methanol, was added to the cell and the steady-state current was recorded.

3. Results and discussion

3.1. Optimization of sensor performance

Different parameters influencing sensitivity of the amperometric microbial biosensor were optimized using paraoxon. Sensors were prepared using varying amounts of the genetically engineered *Moraxella* spp. displaying and anchoring OPH on cell surface to determine the effect of cell loading. As shown in Fig. 1, the

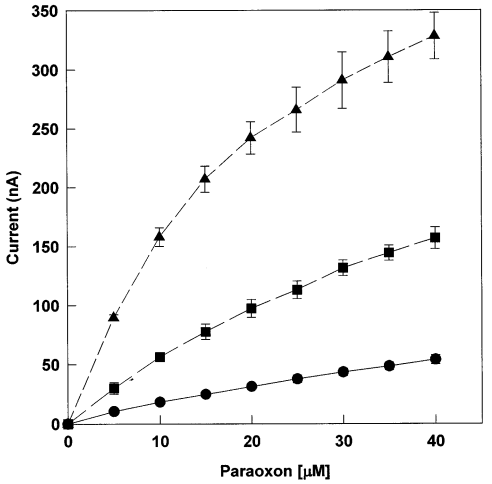

Fig. 1. Effect of cell loading in carbon paste upon paraoxon calibration curves. (●) 2.5 (■) 5 and (▲) 10 mg of wet cell weight per 100 mg of carbon paste. Response of the sensor to 5 μM of paraoxon at +0.9 V in a stirred citrate-phosphate buffer (0.05 M, pH 8) at room temperature. Data are given as mean ± S.D. for four experiments.

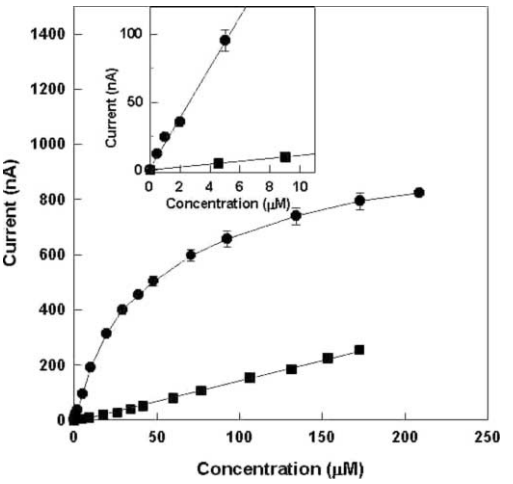

Fig. 3. Calibration plots for paraoxon (●) and methyl parathion (■) using microbial biosensor prepared with 10 mg of wet cell weight per 100 mg of carbon paste and measured in pH 8.5 citrate-phosphate buffer. Other conditions, as in Fig. 1. Data are given as mean ± S.D. for four experiments.

sensor sensitivity was a direct function of the cell amount incorporated in the carbon paste. Increasing the cell loading beyond 10 mg, however, was not beneficial since the background current was rather large and the signal was very noisy. A 10 mg of wet cell weight per 100 mg of carbon paste was used for subsequent experiments.

The effect of the buffer pH on the response of the sensor was evaluated between 7.0 and 9.0. As shown in Fig. 2, the highest sensitivity and linear range was achieved at pH 8.5. This optimum was 0.5 pH units below the optimum for the free enzyme (Mulchandani et al., 1999a). This effect may be attributed either to the effect of the cell walls on which the enzyme is anchored and displayed or the influence of the carbon matrix in which the cells were immobilized. All subsequent sensor measurements were performed at pH 8.5.

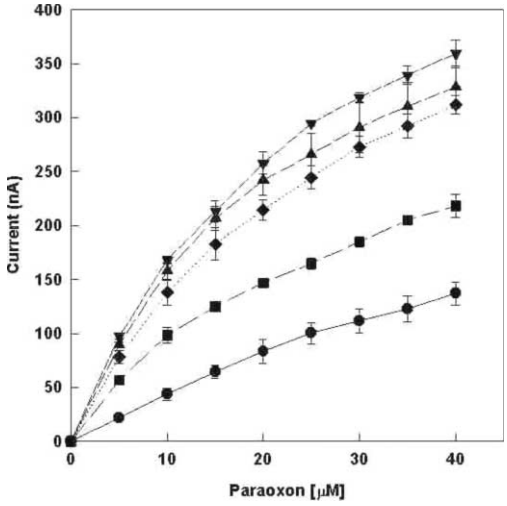

Fig. 2. Effect of buffer pH upon paraoxon calibration curves. (●) pH 7, (■) 7.5, (▲) 8, (▼) 8.5 and (◆) pH 9, 0.05 M borate buffer. Other conditions, as in Fig. 1. Data are given as mean ± S.D. for four experiments.

3.2. Analytical characterization of sensor

Calibration plots for paraoxon and methyl parathion are shown in Fig. 3. The amperometric microbial biosensor has a broad dynamic linear range (up to 40 μM for paraoxon and 175 μM for methyl parathion), high sensitivity (12.24 ± 0.39 nA/μM, $r^2 = 0.98$ for paraoxon and 1.49 ± 0.02 nA/μM, $r^2 = 0.998$ for methyl parathion, for three independent calibrations) and very good lower detection limits (200 nM for paraoxon and 1 μM for methyl parathion). These LDL, estimated from the signal-to-noise characteristics ($S/N = 3$) of the response to blank, are an order of magnitude lower than the potentiometric and optical microbial sensors and similar to the OPH-based amperometric thick-film strip electrode developed in this laboratory. This improved LDL can be attributed to significantly higher activity of OPH on *Moraxella* sp. compared to *E. coli* used in previous studies and also inherent higher sensitivity of amperometric measurement. The current LDL is an order of magnitude higher than for the AChE-based biosensors.

Selectivity is an important criterion for any analytical tool. Non-specific cellular responses generally limit the microbial biosensor selectivity. Table 1 shows the response of the amperometric microbial biosensor to various chemicals commonly encountered either in cell culturing or analyte samples. Carbon and energy sources (glucose, sucrose, lactose and glycerol) and

metabolic products (acetic and lactic acids) did not interfere with the sensor response even at 5 mM concentration, approximately three orders of magnitude higher than the analyte. Pyruvic acid, a metabolite, however, did interfere. A further investigation into the possible cause of this interference indicated that the signal was resulting from the electrooxidation of the pyruvic acid at the cell-free carbon paste electrode. This contribution can be easily addressed by measuring and subtracting the response of a cell-free electrode. Other herbicides and insecticides (sutan, sevin, atrazine and simazine) did not show any interference. However, because the transduction is based on the oxidation of *p*-nitrophenol, phenol did cause (an expected) interference. Once again, like pyruvic acid, these interferences can be addressed by measuring and subtracting the current from a cell-free electrode.

The response of the amperometric microbial biosensor was highly reproducible as demonstrated by the very low residual standard deviation of 4% for 10 repeated injection of 5 μM paraoxon. Additionally, the low standard deviation for the slopes of the line fitted to the linear range of the calibration plot data ($n = 3$) in Fig. 3, for both paraoxon and methyl parathion, demonstrate the very good reproducibility of the sensor.

The amperometric microbial biosensor developed in this work demonstrated excellent storage stability for more than 45 days (Fig. 4) when the complete microbial sensor assembly was stored in buffer A at 4 °C in between analysis.

The ability to use the same amperometric microbial biosensor repeatedly without regular calibration and single step rapid analysis are the advantages of the amperometric microbial biosensor reported in this paper over the AChE-based biosensors which are generally useful for one measurement, require multiple step and are slower responding.

Table 1
Selectivity of amperometric microbial biosensor

Compound	Concentration (mM)	Current (nA)
Glucose	5	0
Sucrose	5	0
Lactose	5	0
Glycerol	5	0
Sodium acetate	5	0
Lactic acid	5	0
Pyruvic acid	5	19.4
Sevin	0.005	0
Sutan	0.005	0
Atrazine	0.005	0
Simazine	0.005	0
Phenol	0.001	50.7

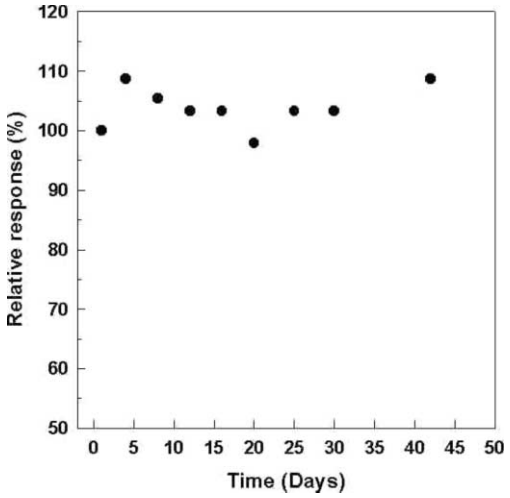

Fig. 4. Amperometric microbial biosensor long-term stability when stored at 4 °C in between measurements. Other operating conditions similar to Fig. 3.

Another salient feature of the amperometric microbial biosensor is the very short response time (2 min) necessary for analysis.

4. Conclusions

In conclusion, an amperometric microbial biosensor suitable for determination of OP pesticides was developed. Compared to biosensors based on cholinesterase inhibition, the use of organophosphorus triester hydrolyzing enzyme, OPH, allows the direct and selective determination and does not require multiple steps. Use of the cells anchoring and displaying the enzyme on the cell surface eliminates the costly, time consuming and laborious protocol for enzyme purification and alleviates the mass transport resistance due to the cell walls experienced when the cells expressing the enzyme in the cytoplasm or periplasm are used. The biosensor exhibited excellent stability and very good reproducibility. The configuration developed in this work lends itself to adaptation in flow-injection systems for the determination of effluent streams from pesticides production and pesticide detoxification/degradation facilities.

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