JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Direct Determination of *p*-Nitrophenyl Substituent Organophosphorus Nerve Agents Using a Recombinant *Pseudomonas putida* JS444-Modified Clark Oxygen Electrode

YU LEI, PRITI MULCHANDANI, WILFRED CHEN,* AND ASHOK MULCHANDANI*

Department of Chemical and Environmental Engineering, University of California, Riverside, California 92521

A microbial biosensor for rapid, sensitive, selective, and cost-effective determination of the total content of organophosphorus nerve agents with *p*-nitrophenyl substituent is reported. The biosensor consisted of genetically engineered PNP-degrader *Pseudomonas putida* JS444 expressing organophosphorus hydrolase (OPH) on its cell surface immobilized on a dissolved oxygen electrode. Surface-expressed OPH catalyzed the hydrolysis of organophosphorus pesticides with *p*-nitrophenyl substituent such as paraoxon, methyl parathion, and parathion to release *p*-nitrophenol that was oxidized by the enzymatic machinery of *Pseudomonas putida* JS444 to carbon dioxide while consuming oxygen. The oxygen consumption was measured and correlated to the concentration of organophosphates. The sensor signal and response time were optimized with 0.086 mg dry weight of cell and operating in 50 mM pH 7.5 citrate-phosphate buffer with 50 μM $CoCl_2$ at room temperature. When operated at optimized conditions, the biosensor measured as low as 55 ppb of paraoxon, 53 ppb of methyl parathion, and 58 ppb of parathion without interference from most phenolic compounds and other commonly used pesticides, such as atrazine, coumaphos, sutan, sevin, and diazinon. The operational life of the microbial biosensor was approximately 5 days when stored in the operating buffer at 4 °C.

KEYWORDS: Organophosphorus; nerve agents; organophosphorus hydrolase; pesticides; biosensor; *Pseudomonas putida*

INTRODUCTION

Synthetic organophosphates (OPs) are among the most toxic substances known. These neurotoxic chemicals are used not only as pesticides but also as chemical warfare agents. *p*-Nitrophenyl substituent OPs (**Figure 1**), such as methyl parathion, parathion, ethyl *p*-nitrophenol thiobenzenephosphonate (EPN), and fenitrothion, are some of the most widely used pesticides in agriculture. The extensive usage of these pesticides generates large volumes of excess aqueous pesticide-containing waste. Typical pesticide concentrations in these wastes can range from 10 000 to 1 ppm, and improper disposal can be hazardous for the environment (*1*−*3*). Reuse or recycling as makeup water, or if necessary disposal, decisions have to be made on-site. This requires reliable determination of pesticides prior to reuse or during and following disposal/treatment processes.

Gas, liquid, and thin-layer chromatography coupled with different detectors, immunoassay, and enzyme biosensors based on inhibition of cholinesterase activity have been reported for monitoring OPs (*3*, *4*). Although very sensitive, these methods are time-consuming, expensive, and require skilled personnel (*5*), and are therefore unsuitable for field or on-line monitoring.

Organophosphorus hydrolase (OPH), a biological catalyst, can effectively catalyze the hydrolysis of a wide range of OP nerve agents to generate an alcohol and an acid (*6*−*8*). Unlike acetylcholinesterase (AChE), OPH takes organophosphates as substrates rather than inhibitors. Because of the advantage of multiple uses offered by biosensor based on catalytic reaction over single use by the inhibition of enzyme activity, several types of OPH-based biosensors including potentiometric, amperometric, and optical biosensors have been exploited (*9*−*11*). These OPH-based biosensors provide simple, rapid, accurate, and direct monitoring means for OP compounds; however, the lower sensitivity of potentiometric and optical biosensors and poor selectivity of amperometric biosensor over phenolic compounds limit their applications in environmental monitoring.

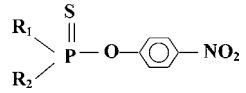

Figure 1. Chemical structure of *p*-nitrophenyl substituent organophosphates.

Pseudomonas putida JS444, isolated from *p*-nitrophenol (PNP)-contaminated waste sites, degrades PNP while releasing nitrite and consuming oxygen (*12*). Thus, correlating oxygen consumption to the concentration of PNP resulting from the OPH-catalyzed hydrolysis of OPs can provide an elegant sensing principle. Recently, functional OPH was expressed onto the cell surface of *P. putida* JS444 using an ice nucleation protein (INP) anchor. This resulted in a single microorganism that is capable

* Author to whom correspondence should be addressed [telephone (951) 827-6419; fax (951) 827-5696; e-mail adani@engr.ucr.edu].

10.1021/jf048943t CCC: $30.25
Published on Web 12/30/2004

of degrading OPs with a PNP moiety and PNP simultaneously (*13*).

In this paper, we report for the first time a microbial biosensor based on genetically engineered *P. putida* harboring both OP hydrolyzing and PNP degradation capabilities as the biological sensing element for OPs with PNP substituent. The genetically engineered *P. putida* JS444 strain displaying and anchoring OPH on the cell surface was coupled to a Clark dissolved oxygen electrode to construct a simple yet selective, sensitive, rapid, and cost-effective microbial biosensor suitable for on-field determination of organophosphates.

MATERIALS AND METHODS

Materials. Citric acid, $MgCl_2 \cdot 6H_2O$, $MgSO_4 \cdot 7H_2O$, KH_2PO_4, $Na_2HPO_4 \cdot 7H_2O$, yeast extract, and $CaCl_2$ were purchased from Fisher Scientific (Tustin, CA). PNP and paraoxon were bought from Sigma-Aldrich (Milwaukee, WI). $FeCl_3$, $ZnSO_4 \cdot 7H_2O$, and $Na_2MoO_4 \cdot 2H_2O$ were obtained from VWR (San Diego, CA). Coumaphos, diazinon, sevin, sutan, atrazine, parathion, and methyl parathion were acquired from Supelco Inc. (Bellefonte, PA). LB broth was purchased from Becton Dickinson (Sparks, MD). NH_4Cl and $CoCl_2$ were acquired from J. T. Baker (Phillipsburg, NJ).

Bacterial Strains and Growth Conditions. The details of the recombinant PNP-degrader *P. putida* JS444 expressing OPH on the cell surface and growth conditions used in this study have been described elsewhere (*13*).

Microbial Electrode Assembly. A predetermined amount of the cell suspension, based on the desired cell loading, was slowly dropped on a 25 mm diameter 0.4 μm pore size Nucleopore polycarbonate membrane (Whatman, NJ) with slight suction. The cell retaining membrane was then placed on the top of the Telfon membrane of the oxygen electrode (model YSI 5331, Yellow Springs, OH) and fixed in place by a rubber O-ring. The cells were thus immobilized (entrapped) between the two membranes.

Experiment Setup and Measurement. All measurements were made at room temperature in 4 mL of buffer with 50 μM $CoCl_2$, saturated with oxygen by bubbling air using a sparger, in a 10-mL glass cell, equipped with a magnetic stirrer (*14*). Ten to twenty microliters of a known concentration solution of organophosphate pesticide was added to the cell, and the steady-state output of the oxygen electrode (after 5 min) was measured using a digital biological oxygen monitor (model YSI 5300, Yellow Springs, OH) connected to a chart recorder.

RESULTS AND DISCUSSION

Optimization of Biosensor Operating Conditions. The response of a microbial biosensor is a function of the amount of cells immobilized on the transducer and operating buffer pH. Experiments were performed to investigate the effect of these variables on the oxygen consumed to 0.05 mM of paraoxon at room temperature.

Figure 2 shows the effect of cell loading on the response of the microbial biosensor. As expected, the response initially increased and reached a maximum at a cell loading of 0.086 mg followed by a gradual decrease. The initial increase is attributed to an increased catalytic activity of the enzymes involved in the biosensing, while the decrease at higher cell loading is due to the transport resistance of substrate and oxygen to cells embedded deeper in the immobilized layer. The trend is similar to that of other reported microbial biosensors (*15*). A cell loading of 0.086 mg dry weight was used in the subsequent work.

Buffer pH has a strong influence on the catalytic activities of OPH and the enzymes involved in PNP catabolism. As shown in **Figure 3**, the microbial biosensor response was maximal at pH 7.5. The literature on OPH enzyme and PNP degradation has shown that the optimum activities of OPH and nitrophenol oxygenase (the first enzyme involved in PNP oxidation of *P. putida* JS444) are achieved at pH of 8.5 (*9*−*11*) and 7.5−8 (*16*), respectively. The observed optimum pH of 7.5 for the microbial biosensor suggests that the PNP oxidation enzyme is the rate-limiting biosensing molecule in this biosensor for the tested organophosphate, paraoxon. The pH of 7.5 was used for subsequent studies.

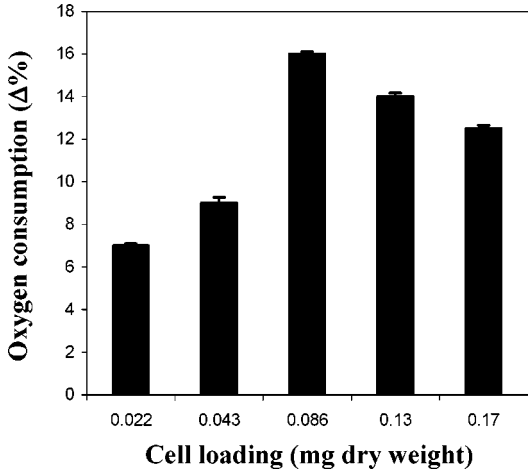

Figure 2. Effect of cell loading on biosensor response to 0.05 mM paraoxon in 50 mM pH 7.5 citrate-phosphate buffer with 50 μM $CoCl_2$ at room temperature. Data are given as mean ± 1 SD for three experiments.

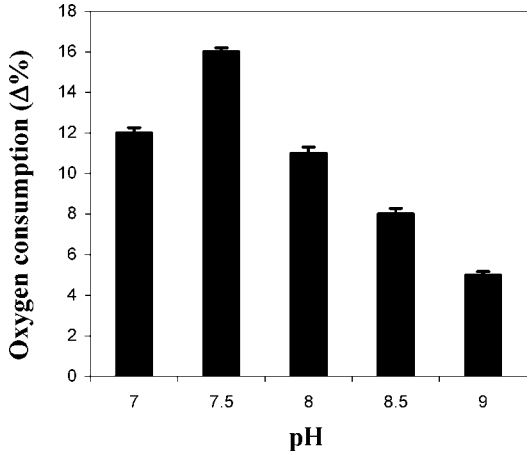

Figure 3. Effect of pH (50 mM citrate-phosphate with 50 μM $CoCl_2$) on biosensor response to 0.05 mM paraoxon with 0.086 mg cell loading at room temperature. Data are given as mean ± 1 SD for three experiments.

Performance. Table 1 tabulates the analytical characteristic of modified oxygen electrode for paraoxon, methyl parathion, and parathion derived from calibration plots shown in **Figure 4A**. The lower limits of detection (LOD), determined as 3 times the standard deviation of the signal for buffer (blank), between 53 and 58 ppb (or 0.2 μM) for the three targets, while comparable to other OPH-based biosensors (*9*), they were 1−2 orders of magnitude higher than for AChE-based biosensor, immunoassay, and gas, liquid, and thin film chromatography methods (*3*, *4*). These analytical characteristics are adequate for field and on-line monitoring of wastewaters generated during production and consumption of these pesticides. However, the applicability of the present sensor for environmental monitoring will require sample preparation involving extraction and sample concentration prior to analysis.

Because water used for reconstitution of pesticide concentrate and washing of leftovers in tanks and equipment can come from many sources, it is necessary to evaluate if there is any matrix effect of naturally occurring compounds in real samples. To

Table 1. Characteristics of Calibration Graphs for the Modified Oxygen Electrode

	in buffer				in lake water			
analyte	limit of detection (ppb)	linear range (μM)	sensitivity ($\Delta\%\ O_2\ \mu M^{-1}$)	R^2	limit of detection (ppb)	linear range (μM)	sensitivity ($\Delta\%\ O_2\ \mu M^{-1}$)	R^2
paraoxon	55	0.2–50	0.31	0.995	55	0.2–50	0.31	0.992
methyl parathion	53	0.2–50	0.31	0.997	53	0.2–50	0.30	0.995
parathion	58	0.2–50	0.31	0.995	58	0.2–50	0.31	0.998

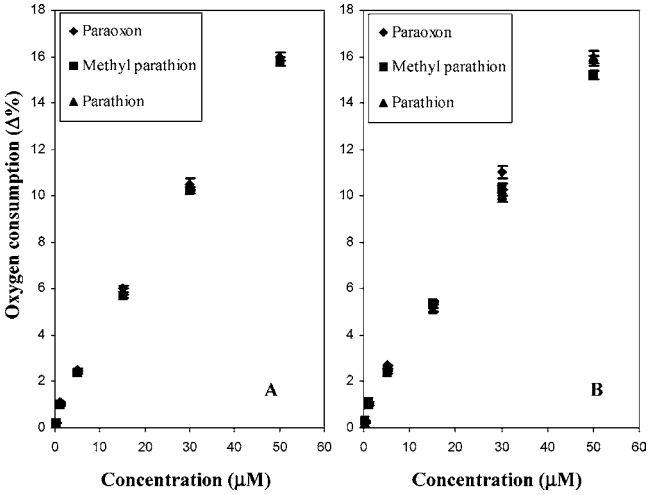

Figure 4. Calibration plot for paraoxon, methyl parathion, and parathion: (**A**) in 50 mM pH 7.5 citrate-phosphate buffer with 50 μM $CoCl_2$ and (**B**) in Lake Elsinore water filtered and adjusted to pH 7.5 and 50 μM $CoCl_2$, at room temperature, with 0.086 mg cell loading. Data are given as mean ± 1 SD for three experiments.

Table 2. Microbial Biosensor Selectivity

concentration (μM)	compound	oxygen consumption (%)
50	paraoxon	16
50	parathion	15.8
50	methyl parathion	15.8
50	coumaphos	0
50	diazinon	0
50	sutan	0
50	sevin	0
50	atrazine	0
50	2-nitrophenol	0
50	3-nitrophenol	1
50	phenol	1
50	*p*-chlorophenol	3
50	*p*-nitrophenol	16
50	3-methyl-4-nitrophenol	16
50	2,4-dinitrophenol	11
1000	glucose	13
1000	sucrose	0
1000	fructose	1
1000	galactose	1
1000	glycerol	3
1000	sodium acetate	2
1000	lactic acid	10

asses the matrix effect, the microbial biosensor was applied to measure target compounds spiked in water from Lake Elsinore (free of OPs), CA, after filtering through a 0.22 μm membrane and adjusting the pH to 7.5 and adding 50 μM $CoCl_2$. As shown in **Figure 4B** and **Table 1**, slopes of the calibration plots were similar to that observed in synthetic sample, demonstrating there was no interference from the components of Lake Elsinore, CA, and validating the applicability of the microbial biosensor for organophosphate pesticides-contaminated wastewaters.

Experiments were performed to investigate the microbial biosensor selectivity against a range of compounds. As shown in **Table 2**, there was no interference from OPs without PNP substituent (diazinon and coumaphos) and other commonly used pesticides, such as atrazine, sutan, and sevin. This is a significant benefit over AChE-based biosensors, which are unable to differentiate between organophosphates and other neurotoxic compounds (*4*) and the potentiometric OPH-based biosensors that are unable to differentiate between subclasses of OPs (*9*). The results also demonstrated there was no interference from phenolic compounds, which caused interference for OPH-based amperometric biosensors based on electrochemical oxidation of PNP (*9*−*11*). As expected, the biosensor, however, responded to *p*-nitrophenol, 2,4-dinitrophenol, and 3-methyl-4-nitrophenol, which are metabolized by the enzymatic machinery of *P. putida* JS444. Such contribution can be addressed by measuring and subtracting the response of a microbial biosensor made of wild-type *P. putida*, that is, without surface expressed OPH. The response of the wild-type *P. putida*-based microbial biosensor will also provide the concentrations of the nitrophenols that are generally present along with OPs in effluents of OP manufacturing plants and wastewater treatment facilities.

Reproducibility is an important parameter for a sensor performance. The microbial biosensor demonstrated very good reproducibility as evidenced by the low relative standard deviations ($n = 6$) of 1.51%, 1.13%, and 1.10%, respectively, for 50 μM paraoxon, methyl parathion, and parathion. Additionally, there was an excellent electrode-to-electrode reproducibility as characterized by the low relative standard deviations of 1.80%, 1.97%, and 2.02% in the response of six microbial biosensors prepared at different times using different batches of cells to 50 μM paraoxon, methyl parathion, and parathion, respectively.

To validate the microbial sensor, simulated samples (prepared by addition of OP compounds into buffer) representing OP-contaminated water were analyzed using the microbial biosensor and compared to spectrophotometric assay based on the measurement of PNP (at 412 nm) formed by the OPH-catalyzed hydrolysis of OPs. As shown in **Figure 5**, there was an excellent agreement between the two methods confirming the accuracy and reliability of the developed microbial biosensor.

Because nonspecific cellular responses to substrate(s) and intermediates of microbial catabolism can limit the selectivity of microbial-based biosensors, it is necessary to evaluate interferences from sugars and organic acids. As illustrated in **Table 2**, glycerol, sodium acetate, and sugars such as sucrose, fructose, and galactose at 20-fold higher concentrations did not interfere with the specific response of cells. However, high concentrations of glucose and lactic acid interfered. This interference is, however, not a concern as these compounds are not expected to be present in samples of interest.

The detection of paraoxon, methyl parathion, and parathion with the new engineered bacteria-based biosensor is simple, direct, single step, and rapid. The analysis time for each sample was less than 5 min, which is significantly shorter than the long incubation period required for the AChE-based biosensor (*17*, *18*). The fast response to organophosphates is attributed to the

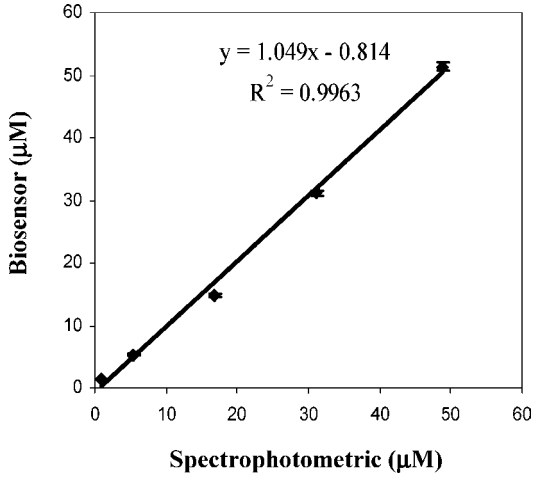

Figure 5. Accuracy of microbial biosensor. Paraoxon as target compound and operating in 50 mM pH 7.5 citrate-phosphate buffer with 50 μM $CoCl_2$ at room temperature, with 0.086 mg cell loading. Data are given as mean ± 1 SD for three experiments.

surface-expressed OPH and fast PNP degradation. When the targeted compound approaches the engineered *P. putida*, the surface-expressed OPH rapidly hydrolyzes it to produce *p*-nitrophenol, which is then rapidly degraded by the enzymatic machinery of *P. putida* JS 444.

The long-term storage and multiple-use stability of the biosensor was investigated by evaluating the response of the same biosensor to 1 μM paraoxon and storing it at 4 °C in pH 7.5, 50 mM citrate-phosphate buffer with 50 μM $CoCl_2$ when not used. The sensor demonstrated excellent operational stability as evident by no loss in the response for 35 repeated analyses over a period of 12 h. Additionally, the sensor response was stable for a period of 5 days and then dropped sharply (data not shown). Based on the literature information of more than a month stability for OPH-based enzyme biosensors (*9*), the short life-span of the microbial biosensor can be attributed to the drop of *P. putida* JS444 respiratory activity probably from the depletion of the NAD(P)H in the resting/nongrowing cells (*12*, *19*). An attempt to reactivate the sensor response by simple addition of NAD(P)H into the storage buffer was, however, unsuccessful, probably because of the inability of the whole cell to uptake NAD(P)H.

Conclusions. A whole cell biosensor using PNP-degrader *P. putida* JS444 with surface-expressed OPH integrated to a dissolved oxygen electrode for the direct measurement of the total content of organophosphate pesticides with a nitrophenyl substituent was developed. While the biosensor has been illustrated in connection with the biosensing of paraoxon, parathion, and methyl parathion, it can also be applied to fenitrothion and ethyl *p*-nitrophenol thiobenzenephosphonate (EPN) and other nitrophenyl substituent OP pesticides. This very simple and cost-effective sensor had good sensitivity, precision, accuracy, short response time, and excellent selectivity for PNP substituent organophosphorus pesticides over other organophosphorus and nonorganophosphorus pesticides and phenols. The above features should make this sensor suitable for on-site reliable determination of organophosphate-contaminated wastewaters generated by consumers and users.

ACKNOWLEDGMENT

We thank Dr. J. C. Spain of the Air Force Engineering and Service Center, Tyndall Air Force Base, FL, for providing *Pseudomonas putida* JS444.

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Received for review June 28, 2004. Revised manuscript received October 25, 2004. Accepted November 11, 2004. This work was supported by grants R82816001-0 from U.S. EPA, 99-35102-8600 from USDA, and MIPT2002J-A-139 from MIPT, and the Office for Domestic Preparedness, U.S. Department of Homeland Security. Points of view in this document are those of the author(s) and do not necessarily represent the official position of the funding agencies.

JF048943T