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Biosensor for Direct Determination of Fenitrothion and EPN Using Recombinant *Pseudomonas putida* JS444 With Surface-Expressed Organophosphorous Hydrolase. 2. Modified Carbon Paste Electrode

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Abstract

A whole cell–based amperometric biosensor for highly selective, sensitive, rapid, and cost-effective determination of the organophosphate pesticides fenitrothion and ethyl *p*-nitrophenol thiobenzenephosphonate (EPN) is discussed. The biosensor comprised genetically engineered *p*-nitrophenol (PNP)-degrading bacteria *Pseudomonas putida* JS444 anchoring and displaying organophosphorous hydrolase (OPH) on its cell surface as biological sensing element and carbon paste electrode as the amperometric transducer. Surface-expressed OPH catalyzed the hydrolysis of organophosphorous pesticides such as fenitrothion and EPN to release PNP and 3-methyl-4-nitrophenol, respectively, which were subsequently degraded by the enzymatic machinery of *P. putida* JS444 through electrochemically active intermediates to the TCA cycle. The electrooxidization current of the intermediates was measured and correlated to the concentration of organophosphates. Operating at optimum conditions, 0.086 mg dry wt of cell operating at 600 mV of applied potential (vs Ag/AgCl reference) in 50 m*M* citrate-phosphate buffer, pH 7.5, with 50 μ*M* $CoCl_2$ at room temperature, the biosensor measured as low as 1.4 ppb of fenitrothion and 1.6 ppb of EPN. There was no interference from phenolic compounds, carbamate pesticides, triazine

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herbicides, or organophosphate pesticides without nitrophenyl substituent. The service life of the biosensor and the applicability to lake water were also demonstrated.

Index Entries: Organophosphorous hydrolase; amperometric biosensor; fenitrothion; ethyl *p*-nitrophenol thiobenzenephosphonate; *Pseudomonas putida*.

Introduction

Because of their efficacy, low cost, and widespread availability, organophosphate pesticides, such as fenitrothion and ethyl *p*-nitrophenol thiobenzenephosphonate (EPN), are widely applied as pesticides and insecticides in many countries *(1)*. Their toxicity to humans and the ecosystem and their widespread contamination of soil, sediments, groundwater, and food continue to be a concern today; consequently, there is a growing interest in their rapid, sensitive, selective, and reliable detection and determination for the protection of our food and water supply and the environment *(2,3)*. Laboratory-based methods, such as gas chromatography (GC), liquid chromatography (LC), and capillary electrophoresis *(1,4–8)*, and bioanalytical methods, such as immunoassay *(9–12)*, cholinesterase biosensor *(13–16)*, and alkaline phosphatase–based biosensor *(17)*, are commonly used for the assay of organophosphates. Although very sensitive, they are unsuitable for rapid and repeated measurements required for field application *(18,19)*.

Organophosphorous hydrolase (OPH), isolated from natural soil microorganisms *Pseudomonas diminuta* MG and *Flavobacterium* sp., has been shown to hydrolyze a wide range of organophosphorous pesticides *(20,21)*. Owing to the reversibility compared to inhibition, potentiometric, optical, and amperometric biosensor *(22–33)* based on purified OPH and hybrid biosensor *(34)* using a natural *p*-nitrophenol (PNP)–degrading microorganism with OPH together have been exploited to mainly monitor paraoxon, methyl parathion, and parathion. However, either the detection limit (potentiometric and optical biosensor) or selectivity (amperometric biosensor) or the difficulty of purifying OPH limits their further application *(22–34)*.

To overcome the aforementioned drawbacks, efforts have been made to concomitantly express OPH onto the cell surface of *Pseudomonas putida* JS444, a native PNP-degrader from soil, using the INPNC surface anchor, resulting in the engineered single bacterium that can rapidly degrade organophosphate pesticides as well as PNP through electroactive intermediates while consuming oxygen *(35)*.

Using the genetically engineered *P. putida* JS444, a whole-cell biosensor for the direct measurement of organophosphate pesticides with nitrophenyl substituent was developed with modified Clark dissolved oxygen electrode *(36,37)*. This very simple and low-cost sensor, which correlated the oxygen consumption to the organophosphate concentration, had excellent precision and accuracy; a short response time; and excellent

selectivity for paraoxon, methyl parathion, parathion, fenitrothion, and ethyl *p*-nitrophenol thiobenzenephosphonate (EPN) over the other organophosphates and pesticides. These features make it a potential analytical tool ideal for on-line monitoring. However, the relatively low sensitivity limits its further application for environmental samples.

Recently, we reported an amperometric microbial biosensor using genetically engineered *P. putida* JS444 for the direct measurement of paraoxon, methyl parathion, and parathion *(38)*. This biosensor improves the sensitivity for monitoring environmental samples. In the present study, we extended the application of this amperometric biosensor to other organophosphates with nitrophenyl substituent such as fenitrothion and EPN under the optimum operating conditions described before *(38)*. The novel microbial amperometric biosensor provides rapid, sensitive, cost-effective, accurate detection of fenitrothion and EPN.

Materials and Methods

Bacterial Strain and Growth Conditions

All chemical reagents and the construction of the recombinant PNP-degrader *P. putida* JS444 with surface-expressed OPH and its growth conditions used have been described elsewhere *(35)*. All solutions were prepared in distilled deionized water.

Assembly of Microbial Biosensor

Carbon paste was prepared by mixing 75% (w/w) graphite powder and 25% (w/w) mineral oil. The paste was subsequently packed firmly into the electrode cavity (3 mm in diameter and 1 mm deep) of a Kel-F sleeve (Bioanalytical System, Lafayette, IN) and polished to a smooth, shiny finish by gently rubbing over a weighing paper to make the carbon paste electrode.

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, Florham Park, NJ) with slight suction. The cell retaining membrane was then placed on top of the carbon paste electrode and held in place with a rubber O-ring. The electrode was kept at 4°C until use.

Experiment Setup and Measurement

Amperometric measurements were performed using a Bioanalytical Systems (BAS) voltammetric analyzer (Model LC-4C) coupled to a chart recorder (Model BD112; Kipp and Zonen, Delft, Holland). All experiments were conducted using a three-electrode electrochemical cell (10-mL volume with a 4-mL working volume) inside a Faraday cage (BAS, Model C2 cell stand) with an Ag/AgCl reference electrode (BAS; MF 2063) and a platinum wire auxiliary electrode (BAS; MF 1032).

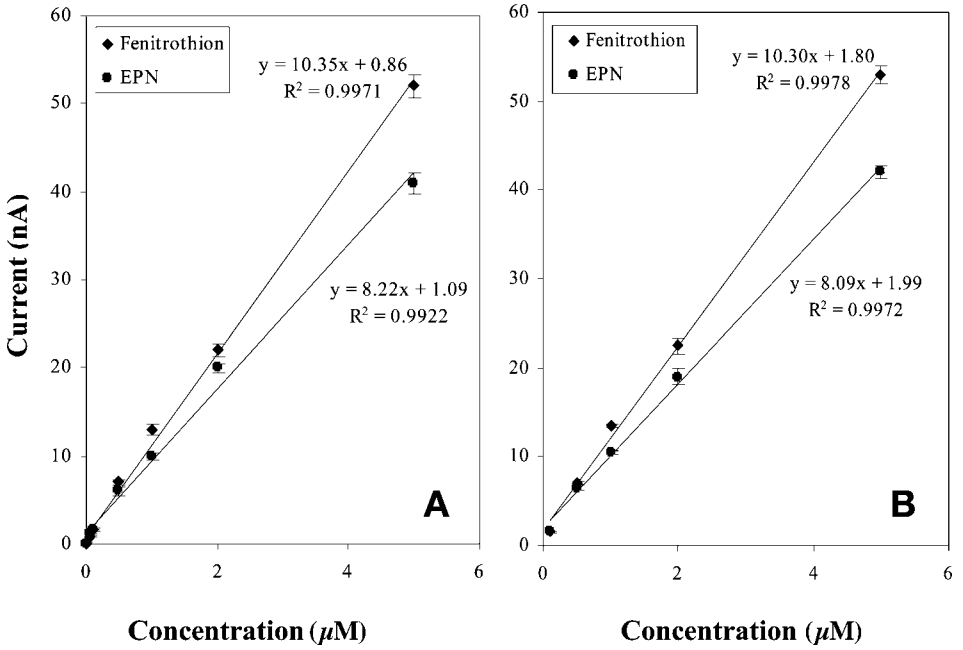

Fig. 1. Calibration plots for fenitrothion and EPN. Operating conditions were as follows: **(A)** in 50 m*M* citrate-phosphate buffer, pH 7.5, with 50 μ*M* $CoCl_2$; and **(B)** in water from Lake Elsinore filtered and adjusted to pH 7.5 and 50 μ*M* $CoCl_2$ at room temperature with a cell loading of 0.086 mg and applying +0.6 V to a working electrode vs the Ag/AgCl reference. Data are given as the mean ± 1 SD for three experiments.

All measurements were performed under our reported optimized conditions with an applied potential of +0.6 V (vs Ag/AgCl reference electrode); 0.086 mg dry wt of cell; and operating in 50 m*M* citrate-phosphate buffer, pH 7.5, with 50 μ*M* $CoCl_2$ at room temperature *(38)*. A stirred solution was employed to provide convective transport.

Results and Discussion

Analytical Characteristics

Figure 1A shows the dependence of biosensor response to different concentrations of fenitrothion and EPN in operating buffer. The plots were linear up to 5 μ*M*, with sensitivities of 10.35 and 8.22 nA/μ*M* for fenitrothion and EPN, respectively. The lower limits of detection (LODs), determined as three times the standard deviation of the signal for buffer (blank), were 1.4 ppb of fenitrothion and 1.6 ppb of EPN. The lower LOD for fenitrothion is 1 to 2 orders lower than those of cholinesterase- or alkaline phosphatase–based biosensors *(14–17)* and OPH-based biosensor *(33)*, but no such number is available for EPN in these enzyme biosensors *(14–17)* for comparison. The lower LODs are also 20- to 100-fold lower than those of the microbial biosensor based on dissolved oxygen consumption using the same *P. putida*

engineered cells *(37)*. More important, these detection limits are comparable with those for immunoassay *(9–12)* and GC and LC methods *(1,4–8)*. This should make the biosensor an ideal analytical tool for sensitive detection of organophosphates in the environment without any sample preconcentration.

To evaluate the matrix effect of naturally occurring compounds in real samples, the microbial biosensor was applied to measure target compounds spiked in lake water from Lake Elsinore, CA, after the lake water was filtered through a 0.22-μm membrane and adjusted to pH 7.5 and 50 μ*M* $CoCl_2$. As shown in Fig. 1B, slopes of the calibration plots in lake water were similar to those observed in a synthetic sample (Fig. 1A), thus demonstrating that there was no interference from the components of Lake Elsinore, CA, and validating the applicability of the microbial biosensor for environmental sample.

The response of the amperometric microbial biosensor is very reproducible, as demonstrated by the low relative SDs ($n = 4$) of 5.9 and 4.6% for 1 μ*M* fenitrothion and EPN, respectively. Additionally, there is an excellent electrode-to-electrode reproducibility, as characterized by the low relative SDs of 4.0 and 4.8% in the response of three microbial biosensors prepared at different times using different batches of cells to 1 μ*M* fenitrothion and EPN, respectively.

The excellent agreement (Fig. 2) between the fenitrothion or EPN concentrations measured by the biosensor and spectrophotometric assay based on the measurement of PNP (at 412 nm) formed by the OPH-catalyzed hydrolysis of organophosphates demonstrates the high accuracy and reliability of the developed microbial biosensor.

The novel microbial biosensor provides a simple, direct, single-step, and rapid method for the detection of fenitrothion and EPN. The analysis time for each sample was <5 min, which is significantly shorter than the hours required for GC and LC and immunoassays. The biosensor also shows excellent precision and selectivity, as described in our previous report *(38)*. When stored in operating buffer at 4°C, the sensor response was stable for a period of 5 d. The rapid drop of the respiratory activity after 5 d is hypothesized to be a result of the depletion of the NAD(P)H in the resting/nongrowing cells *(34,36–39)*. The addition of NAD(P)H to operating buffer in order to revive the cell activity was unsuccessful, probably owing to the inability of the cells to uptake NAD(P)H.

Conclusion

An amperometric microbial biosensor using genetically engineered *P. putida* JS444 was developed for the detection of fenitrothion and EPN. The expression of OPH on the surface of *P. putida* JS444, allows single bacteria that can rapidly degrade organophosphate pesticides as well as PNP through benzoquinone, hydroquinone, maleylacetate, and β-ketoadipate to tricarboxylic acid intermediates while releasing nitrite and consuming

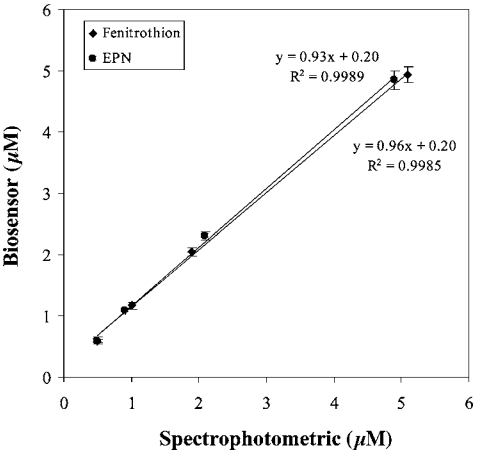

Fig. 2. Accuracy of microbial biosensor. Fenitrothion and EPN were the target compounds operating in 50 m*M* citrate-phosphate buffer, pH 7.5, with 50 μ*M* $CoCl_2$ at room temperature with a cell loading of 0.086 mg. Data are given as the mean ± 1 SD for three experiments.

oxygen while alleviating the need for isolation and purification of enzyme, as is the case with enzyme-based sensors and enzyme-microbial hybrid biosensor, thereby reducing the overall cost. This very simple and low-cost sensor had excellent sensitivity, precision, and accuracy; a short response time; as well as excellent selectivity for organophosphorous pesticides with nitrophenyl substituent such as fenitrothion and EPN over carbamate pesticides, triazine herbicides, and organophosphate pesticides without nitrophenyl substituent. These features make this sensor a potential analytical tool ideal for on-line monitoring of effluents from chemical-processing facilities producing and using organophosphate pesticides with nitrophenol substituent and environmental samples without any preconcentration.

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