Fluorescence-based sensing of \(p\)-nitrophenol and \(p\)-nitrophenyl substituent organophosphates

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Abstract

A novel detection method for organophosphate neurotoxins has been described, based on the fluorescence quenching of a Coumarin derivative. These dyes are similar in structure to some organophosphates (OPs), and they fluoresce in the blue–green region of the spectra. This methodology has been utilized for the detection of organophosphates whose hydrolysis product is \(p\)-nitrophenol by using an enzyme, organophosphorus hydrolase (OPH). Coumarin1 in the presence of \(p\)-nitrophenol results in a quenching of fluorescence, providing a direct measure of the concentration of \(p\)-nitrophenol present in the sample. The decrease in fluorescence intensity is proportional to the paraoxon concentration in the range of \(7.0 \times 10^{-7} \text{--} 1.7 \times 10^{-4} \text{ M}\). The specificity of this sensing application for \(p\)-nitrophenyl substituent OPs has also been demonstrated. OPs are a class of synthetic organic pesticides which generally have a short residual life and can cause numerous acute and chronic health effects. They have been an integral part of the agricultural industry for the past several decades due to their target specificities and selectable toxicities. The toxic nature of these compounds can be attributed to the species–specific inhibition of acetylcholinesterase (AChE), an important enzyme responsible for the regeneration of neural synaptic function. In addition to their wide agricultural and urban usage, they have also been exploited for the development of neurological chemical warfare agents. Currently available technologies for OP detection include sol–gel thin films, screen printed electrodes, acoustic patterning, gas chromatography–mass spectrometry, and various other intricate techniques that have limited field applicabilities. This optically-based approach promises much simpler and more direct detection capabilities.

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1. Introduction

Each year organophosphorus compounds (OPs) poison thousands of humans throughout the world, causing hundreds of deaths \[1\]. This class of compounds has achieved enormous commercial success as a key component in the arsenal of agricultural pesticides and herbicides, and is currently an integral element of modern agriculture. Accordingly, they are used to control moths, ants, cockroaches, termites, fruit flies and similar insects, fleas, locusts, caterpillars and ticks, to name a few. These compounds do not bioaccumulate due to their rapid break down in the environment. For this reason, they are preferred over organochlorides for insecticide/pesticide use. Even though OPs are generally considered safer than the organochlorides, they are highly toxic to humans and other mammals and may be carcinogenic \[2\]. For example, parathion has an oral LD\(_{50}\) in rats of about 2–30 mg kg\(^{-1}\) which is more toxic than dichloro-diphenyl-trichloroethane (DDT) (oral LD\(_{50}\) in rats = 87 mg kg\(^{-1}\)) \[3\]. Approximately 60 million pounds of OP pesticides are applied to 38 million acres of U.S. agricultural crops annually \[4\]. Nonagricultural use accounts for about 17 million pounds per year, which includes usage such as malathion in the manufacture of flea powders, dichlorvos for polychlorovinyl resin pet collars and pest strips \[5\]. The amount of OP pesticide usage as a percentage of total insecticide usage has increased from 58% in 1980 to 70%
in 2001. \( p \)-Nitrophenyl (\( p \)-NP)-substituent organophosphates, which include ethyl parathion, methyl parathion, paraoxon, and fenitrothion, are more extensively used than their non-\( p \)-NP counterparts [6]. Malathion and diazinon (\( p \)-NP-substituent OPs) were ranked number one and three, respectively, as the most commonly used organophosphate insecticides active ingredients by EPA [7]. The extensive usage of these pesticides generates large volumes of pesticide-containing waste. Typical pesticide concentrations can range from 1 to 10,000 ppm and improper disposal can be hazardous to the environment [8]. Data compiled by the National Water Quality Assessment Program (NAWQA) provides a very good summary of the occurrence and concentrations of all pesticides samples (including organophosphates) collected from streams, ground water sites, agricultural areas, urban areas and major aquifers [9]. Another study conducted by NAWQA, National Stream Quality Accounting Network (NASQAN) and U.S. Geological Survey (USGS) in U.S. from 1992 to 97 evaluated the occurrence and distribution of 11 OPs in surface and ground water samples. The analysis concluded that OPs (specifically diazinon, chlorpyrifos and malathion) were detected more often and at higher concentrations in surface water than in ground water. Diazinon was the most commonly detected OP in surface water with percent detections ranging from 22 to 100\% of samples and chlorpyrifos and malathion from 0 to 91\% and 0 to 43\%, respectively. Chlorpyrifos was the most widely distributed OP in surface water with concentrations ranging from 0 to 0.3 \( \mu \)g L\(^{-1} \) [10]. \( p \)-Nitrophenol, the degradation product of some of the OPs, is corrosive and acutely toxic by oral route [11]. This class of compounds has also been found as environmental contaminants in fresh water and in the atmosphere, acute exposure to which causes headaches, nausea and cyanosis [12]. Therefore, detection of these compounds is a matter of environmental and health concern.

The neurotoxic OPs work by inhibiting cholinesterases (ChE), enzymes of the nervous system important for nerve transmission. The enzymes are inhibited by binding the OP compound which, upon hydrolysis, leaves a stable, phosphorylated and largely unreactive enzyme. This inhibition results in the accumulation of acetylcholine [13] at the neuron/neuron and neuron/muscle (neuromuscular) junctions or synapses, causing headache, rapid twitching of voluntary muscles and abdominal cramps [14,15]. The severity of the symptoms depends on the degree of acetylcholinesterase inhibition, and the more severe effects include muscle paralysis which can lead to severe difficulty in breathing, and eventually death by respiratory failure. In addition to ChE, OPs inhibit a number of other enzymes, including plasma pseudocholinesterase, neuropathy target esterase (NTE), A-esterases (tissue esterases capable of hydrolyzing OP esters) and carboxy-esterases, as well as other esterases and proteases [16–18].

In addition to the acute exposure symptoms, long-term exposure to low doses may lead to the development of cancer, genetic diseases, and other dangerous effects [13,19–21]. Some OP neurotoxins are associated with an organophosphate-induced delayed neurotoxicity (OPIDN) in humans and susceptible species [22,23]. OPIDN is characterized by distal degeneration of sensory and motor axons that occurs after a delay of about 8–21 days following OP poisoning [24]. Recovery from these complex and poorly understood diseases is usually poor and there is no specific treatment.

One of the most important aspects for minimizing the potential hazard to humans and the environment is monitoring of these pesticide residues. Previously reported sensors for organophosphate detection have been based on sol–gel thin films and attenuated total reflection infrared (ATR-IR) spectroscopy [25], AChE-based biosensors [26–29], lateral field excited acoustic sensor with polymer coating [30], solid phase microextraction with tin-oxide gas sensor [31], capillary column gas chromatography using nitrogen–phosphorus and electron capture [32], carbon paste electrochemical transducer [33], gas chromatography–mass spectrometry [34], high performance liquid chromatography [35,36], fluorescence [37,38], potentiometric ion-selective electrode [39,40], pH-sensitive field-effect transistor [41] and amperometric microbial detection [42,43]. All these detection methodologies have their own advantages and disadvantages. Gas and liquid chromatographic methods are the most common analytical methods currently in use and, although complicated technologies, they have been shown to be quite sensitive [8].

Fluorescence-based methods can be used to detect very low concentrations of an analyte or a molecule of interest. Fluorescent probes enable researchers to detect individual components of complex systems, such as living cells, with exceptional sensitivity and selectivity. Coumarin compounds are derivatives of 1,2-benzopyrone which display high fluorescence quantum yield in the blue–green region upon photoexcitation. Previously, the fluorescence properties of coumarin deriva-
tives combined with substrates have been used to investigate enzymatic process [44]. In a recent study the fluorescence properties of 7-isothiocyanato-4-methylcoumarin have been utilized by Orbulescu et al. for development of a highly sensitive biosensor for the detection of low concentrations of paraaxon [45].

The present study reports the development of a new sensor for the direct detection of \( p \)-nitrophenol and \( p \)-nitrophenol-substituent organophosphorus neurotoxins. In this system, detection is based on the fluorescence quenching of coumarin1, competitive inhibitor of an OP-hydrolyzing enzyme, organophosphorus hydrolase (OPH, E.C. 3.1.8.1). OPH is a 72 kDa homodimeric enzyme which catalyzes the hydrolysis of the P–O, P–S, P–F, and P–CN bonds of OP-neurotoxins. This makes OPH a suitable recognition element for the detection of these substrates. OPH operates near the limits of diffusion with some substrates and can be altered to enhance its specificity towards a variety of OP-substrates [46–50]. The kinetic evaluation of OPH with the fluorescence compounds coumarin and coumarin1 determined these compounds to be competitive inhibitors of the enzyme. While Orbulescu et al. [45] focused on the surface characterization of the OPH-based biosensor, this current study concentrated upon the sensing element, OPH, and the potential of using an untethered dye as the reporter. In addition, our analysis demonstrated the applicability of this method beyond paraaxon to other \( p \)-nitrophenol OPs.
2. Experimental procedure

2.1. Materials

The enzyme, organophosphorus hydrolase, was purified as previously described [51]. Paraoxon was purchased from Chem-Service, Inc. (West Chester, PA) and coumarin dyes were obtained from Sigma (Aldrich, St. Louis, MO) and were diluted in ethanol for use. Water used for preparation of aqueous solutions was from a Millipore Direct-Q Water system (resistivity, 18 MΩ cm−2).

2.2. Instrumentation

All fluorescence intensities were measured using a Photon Technology International (PTI) Quantum Master Fluorescence spectrophotometer equipped with a xenon lamp. The excitation and emission spectra were evaluated for both dyes; buffer without coumarin dye was used as a control. Coumarin and coumarin1 were first prepared in 95% ethanol and then at the required concentrations as solutions in 20 mM 2-(N-cyclohexylamino)ethane sulfonic acid (CHES), pH 9.0. The total reaction volume for the fluorescent assays was 3 ml.

2.3. Procedures

The fluorescent compounds coumarin and coumarin1 were selected for evaluation based on their structural similarity with some organophosphates (Fig. 1). A one molar solution of both coumarin and coumarin1 were made in 95% ethanol. The solutions were stored at room temperature and used within 1–4 days. Both compounds were stable under these conditions. Due to their limited solubility in aqueous solutions, all kinetic assays with these compounds were done in 1% ethanol. Control assays without coumarin dye was used as a control. Coumarin and coumarin1 were first prepared in 95% ethanol and then at the required concentrations as solutions in 20 mM 2-(N-cyclohexylamino)ethane sulfonic acid (CHES), pH 9.0. The total reaction volume for the fluorescent assays was 3 ml.

Measurements of ΔA min−1 were converted to μmol product per second using the molar extinction coefficient for the product, p-nitrophenol, of 17,000 M−1 cm−1. The Kᵢ values for coumarin and coumarin1 were 4.7 and 0.3 mM, respectively.

3. Results and discussion

Kinetic evaluation determined that both coumarin and coumarin1 behave as competitive inhibitors of OPH; however, comparison of the emission spectra relative to the buffer control demonstrated that the coumarin emission peak coincided with the Raman peak of water. Since the emission peak of coumarin1 was distinct, it was selected as the fluorescent reporter for sensor development. The excitation and emission peaks of coumarin1 were observed at 343 and 465 nm, respectively.

3.1. Fluorescence measurements for p-nitrophenol

The fluorescence intensity of coumarin1 was measured following the addition of varying concentrations of p-nitrophenol. As expected, the system showed fluorescence quenching with increasing concentration of pNP (Fig. 2a). A calibration graph was obtained for p-nitrophenol and the minimum detection limit was found to be 1.8 μM (Fig. 2b). When the fluorescence changes of pNP in the absence of OPH was tested (Fig. 2c), the system showed a similar response as in the presence of OPH. This demonstrated that OPH is not required for detection of p-nitrophenol alone.

3.2. Fluorescence measurements for paraoxon

As paraoxon is hydrolyzed, a chromogenic, p-nitrophenol (pNP) and a colorless product, diethyl phosphate are released. The hydrolytic generation of p-nitrophenol leads to quenching of the coumarin1 fluorescence. The relative concentrations of coumarin1 and OPH were standardized for all assays at a 1:1 molar ratio. If the amount of coumarin1 was in excess in solution, the background fluorescence reduced the detection limit for the OP compound, as demonstrated in Fig. 3. The fluorescence intensity of coumarin1 was measured and used as a background signal level. OPH was added and the intensity of fluorescence was measured again (FI₀). Equimolar concentration of OPH and coumarin1 were used for all the experiments.

Fig. 1. (a) Coumarin1, the fluorescent compound and competitive inhibitor selected as the Reporter in this study. (b) Coumaphos, an OP insecticide commonly used for control of a wide variety of livestock insects.
Control experiments were performed to monitor for fluorescence intensity changes in the absence of OPH (Fig. 4a). Paraoxon was added in a concentration series and fluorescence intensities (FIₙ) were again measured (Fig. 4b). Relative fluorescence intensity change, RFI, was calculated as:

\[
RFI = \frac{FI_0 - FI_n}{FI_0}, \quad \text{where } n = 1, 2, 3 \ldots \ n
\]

RFI represents the ratio of change of fluorescence in the presence of paraoxon to the fluorescence in the absence of paraoxon. Approximately 15% change in the RFI was observed in the absence of OPH, while in the presence of OPH 80% RFI was observed in the fluorescence intensity of coumarin1 at the highest concentration of PX. The changes in the absence of OPH are likely due to auto hydrolysis of PX. A calibration curve for paraoxon is presented in Fig. 5 in which the mean relative fluorescence intensity (RFI) is plotted against the paraoxon concentration. The minimum paraoxon detected was 0.7 µM, which corresponds to less than 2% fluorescence quenching. Good linearity was observed at paraoxon concentrations up to 8 µM. Experiments were performed to determine if there were any
Fig. 4. (a) Fluorescence of coumarin1 at varying paraoxon concentrations in the absence of OPH. 20 mM CHES buffer, pH 9.0. \([\text{Coumarin1}] = 2.33 \times 10^{-8} \text{M}\). PX concentration from \(0.4 \times 10^{-6}\) to \(0.173 \times 10^{-3} \text{M}\); coumarin1 was excited at 343 nm. (i) C1, (ii) 0.4 \(\mu\text{M}\), (iii) 0.7 \(\mu\text{M}\), (iv) 1.8 \(\mu\text{M}\), (v) 3.5 \(\mu\text{M}\), (vi) 7 \(\mu\text{M}\), (vii) 18 \(\mu\text{M}\), (viii) 35 \(\mu\text{M}\), (ix) 87 \(\mu\text{M}\), and (x) 173 \(\mu\text{M}\). (b) Fluorescence of coumarin1 at varying paraoxon concentrations in the presence of OPH. 20 mM CHES buffer, pH 9.0. \([\text{Coumarin1}] = 2.33 \times 10^{-8} \text{M}\). PX concentration from \(0.4 \times 10^{-6}\) to \(0.173 \times 10^{-3} \text{M}\); coumarin1 was excited at 343 nm. \(\text{Coumarin1:OPH} = 1:1\). (i) C1, (ii) C1 + WT, (iii) 0.4 \(\mu\text{M}\), (iv) 0.7 \(\mu\text{M}\), (v) 1.8 \(\mu\text{M}\), (vi) 3.5 \(\mu\text{M}\), (vii) 7 \(\mu\text{M}\), (viii) 18 \(\mu\text{M}\), (ix) 35 \(\mu\text{M}\), (x) 87 \(\mu\text{M}\), and (xi) 173 \(\mu\text{M}\).

Fig. 5. Calibration curve with paraoxon. Mean relative fluorescence intensity change (RFI) is plotted as a function of added paraoxon (PX) concentration. 20 mM CHES buffer, pH 9.0, coumarin1 concentration \(2.33 \times 10^{-8} \text{M}\), 1:1 coumarin1:OPH.

Fig. 6. Selectivity of the methodology. (a) Fluorescence of coumarin1 at diisopropyl fluorophosphate (DFP) concentrations from \(1.65 \times 10^{-6}\) to \(0.165 \times 10^{-3} \text{M}\). (i) C1, (ii) C1 + WT, (iii) 1.65 \(\mu\text{M}\), (iv) 3.3 \(\mu\text{M}\), (v) 6.6 \(\mu\text{M}\), (vi) 16.5 \(\mu\text{M}\), (vii) 33 \(\mu\text{M}\), (viii) 49.6 \(\mu\text{M}\), (ix) 82.8 \(\mu\text{M}\), and (x) 165 \(\mu\text{M}\). (b) Fluorescence of coumarin1 at varying malathion concentrations from \(0.4 \times 10^{-6}\) to \(0.087 \times 10^{-3} \text{M}\). (i) C1, (ii) C1 + WT, (iii) 0.4 \(\mu\text{M}\), (iv) 0.7 \(\mu\text{M}\), (v) 1.8 \(\mu\text{M}\), (vi) 3.5 \(\mu\text{M}\), (vii) 7 \(\mu\text{M}\), (viii) 18 \(\mu\text{M}\), (ix) 35 \(\mu\text{M}\), and (x) 87 \(\mu\text{M}\). Both OPs were tested in the presence of OPH–coumarin1, in 20 mM CHES buffer, pH 9.0. \([\text{Coumarin1}] = 2.33 \times 10^{-8} \text{M}\). Coumarin1 was excited at 343 nm. Coumarin1:OPH = 1:1.

Changes in fluorescence intensity associated with diethyl phosphate (DeP), the second product of paraoxon degradation. The calibration curve was repeated, with addition of varying concentrations of DeP. No changes in fluorescence were observed for DeP (Data not shown).

3.3. Analytical procedure and evaluation

The principle of this method was validated with parathion, a \(p\)-nitrophenyl substituent organophosphorus compound. The detection methodology was as described for the paraoxon studies, fluorescence intensities at varying concentrations of parathion were measured and a calibration curve generated from the measurements. The minimum parathion concentration detected was 0.7 \(\mu\text{M}\), and linearity \((Y = 0.0069 + 0.00209X, R = 0.98575)\) was observed at concentrations up to 143 \(\mu\text{M}\). These results provided evidence that this detection method will work for other nitrophenyl substituent organophosphates.
To determine if the detection was selective toward nitrophenyl substituent organophosphates as it is designed to be, non-pNP producing OPs like malathion and DFP were tested. Using the same detection methodology, the fluorescence intensity was measured (Fig. 6a and b) and the relative fluorescence intensities were calculated. No evidence of quenching was observed at 7 μM DFP and 7 μM malathion but at the same concentration, PX gave around 11% quenching in fluorescence.

4. Conclusions

The results presented here report the development of an alternative method for the detection of p-nitrophenyl-substituted organophosphates using the fluorescence changes of a competitive inhibitor, coumarin1. Coumarin1 in the presence of p-nitrophenol leads to fluorescence quenching which is suspected to be due to fluorescence resonance energy transfer (FRET). The energy transfer efficiency for the coumarin1 and pNP pair for the ratio of 1:1 concentration was found to be 50% [52]. This approach allows for the development of a simple, cost-effective and easy methodology for detection of p-nitrophenol and, by coupling with OPH, for detection of pNP-substituent organophosphates. It has been illustrated that this concept can be developed as a biosensor of paraoxon and parathion, it can also be applied to other nitrophenyl substituted OP pesticides like methyl parathion and fenitrothion. Further studies are underway to better characterize the critical components for sensitivity that include type of coumarin, binding characteristics of coumarin to the enzyme, and surface attachment methods.

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