An enzyme based biosensor for the direct determination of diisopropyl fluorophosphate

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Received 27 October 1998; received in revised form 25 January 1999; accepted 1 February 1999

Abstract

The ability of organophosphate hydrolase (OPH, EC 3.1.8.1) from \textit{Pseudomonas diminuta} to hydrolyze diisopropyl fluorophosphate (DFP) was used to develop a biosensor for the direct determination of DFP. A fluoride sensitive ion-selective electrode was exploited as the physical transducer for a batch-mode biosensor, and OPH enzyme immobilized on silica gel was used as a biological recognition element. The correlation between DFP concentration and hydrolyzed fluoride extended over a concentration range of $2.5 \times 10^{-5}$–$5 \times 10^{-3}$ M. The influence of MeOH on DFP hydrolysis by immobilized OPH was greatly reduced from that of the soluble enzyme, and the effect of paraoxon on DFP detection in mixed samples was investigated. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Biosensor; Organophosphate hydrolase; Organophosphorus neurotoxins; Diisopropyl fluorophosphate; DFP detection

1. Introduction

Diisopropyl fluorophosphate (DFP) is a phosphono-fluoridate neurotoxin similar to the chemical warfare agents (CW agents) sarin and soman with significantly reduced toxicity:

\[
\begin{array}{ccc}
\text{DFP} & \text{sarin} & \text{soman} \\
\text{\textit{iPrO}} & \text{\textit{iPrO}} & \text{CH}_3 \\
\text{\textit{iPrO}} & \text{\textit{iPrO}} & \text{CH}_3 \\
\end{array}
\]

The structural similarity with the CW agents makes DFP an attractive analog for the development of determination and destruction technologies for CW agents.

All of these neurotoxins are capable of irreversibly inhibiting acetylcholinesterase (AChE) – the key enzyme in the maintenance of nerve impulse transmission [1]. This AChE-inhibition effect has been used to create several enzyme-based biosensors for various neurotoxins, including DFP [2–5]. Despite the high sensitivity of AChE-based biosensors, such devices are limited in specificity because cholinesterases may be inhibited by a wide variety of toxins from heavy metals to proteins. By using the capability of organophosphate hydrolase (OPH) to hydrolyze organophosphorus (OP) neurotoxins, it has been pos-
sible to develop an OPH-based biosensor for the direct detection of OP neurotoxins [6], and OPH can be used to uniquely distinguish between organophosphate and carbamate insecticides [7].

The enzymatic hydrolysis of DFP by DFPase (paraoxonase) from mammalian blood serum was first shown in 1946 [8]. Subsequently, a variety of organophosphorus acid anhydrolases (OPAAs; variably termed somanases, sarinases, DFPases, paraoxonases, and/or phosphotriesterases depending on the substrates investigated) were identified (EC 3.1.8). Among these enzymes, organophosphorus hydrolase (OPH, phosphotriesterase EC.3.1.8.1) from Pseudomonas diminuta was uniquely found to be able to hydrolyze various phosphonofluoridates such as DFP, soman, and sarin as well as an extremely wide range of neurotoxins containing P–O, P–F, P–S and P–CN bonds (Table 1). None of the other OPAAs reported to date have such capacity.

The present study describes a new application of OPH in the development of a biosensor for the direct determination of DFP. The analysis is based on the reaction:

The enzymatic hydrolysis of DFP by OPH generates a single fluoride ion for each hydrolytic turnover. The quantity of DFP hydrolyzed corresponds with the quantity of fluoride released, and this stoichiometric relationship permitted the creation of a potentiometric biosensor for the detection of DFP.

Table 1
Organophosphate hydrolase kinetic capacities for various OP neurotoxins

<table>
<thead>
<tr>
<th>Hydrolyzed bonds</th>
<th>Examples</th>
<th>k_{cat} (s^{-1})</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>P–O</td>
<td>Parathion, coumaphos</td>
<td>150–5000</td>
<td>[9]</td>
</tr>
<tr>
<td>P–F</td>
<td>DFP, soman, sarin</td>
<td>1.0–290</td>
<td>[10]</td>
</tr>
<tr>
<td>P–S</td>
<td>Acephate, VX, malathion</td>
<td>0.01–3</td>
<td>[11]</td>
</tr>
<tr>
<td>P–CN</td>
<td>Tabun</td>
<td>77</td>
<td>[12]</td>
</tr>
</tbody>
</table>

2. Experimental

2.1. Reagents

Diethyl p-nitrophenyl phosphate (paraoxon), DFP, aminopropyltriethoxysilane and glutaraldehyde were obtained from Sigma Chemicals (USA); HEPES, HEPES sodium salt, sodium chloride, glycerol, glycine, and potassium phosphate (monobasic and dibasic) were obtained from Fisher Products (USA). Total Ionic Strength Adjustment Buffer TISAB III and Fluoride standard solution (0.1 mol/l) were obtained from Orion (USA).

2.2. Enzyme and immobilization

OPH was isolated from a recombinant E. Coli strain expressing opd from Pseudomonas diminuta in our laboratory; this recombinant strain produced the native enzyme lacking the removed leader sequence without substituting any additional amino acids at the 5'-terminal end of the protein. This enzyme had extremely high specific activity for paraoxon (18 000 U/mg) and it was purified according to methods described previously [13]. The purified enzyme was immobilized on silica gel particles, which were activated in several steps performed in an ultrasonic bath. Gel particles were cleaned by washing with 1 N HCl, rinsed, then placed into a 20% H₂O₂ solution, and subsequently silanized in an aqueous 10% aminopropyltriethoxysilane solution for 6 h at 85°C and thoroughly rinsed. The bi-functional reagent glutaraldehyde (10% aqueous solution) was applied to the particles for 2 h; the particles were thoroughly rinsed, and 1 ml of protein in 10 mM phosphate buffer was added to 1 g (w/w) activated particles. The immobilization was carried out at 4°C for 12 h with gentle stirring. The resulting biocatalyst was washed with 100 mM K-phosphate buffer, pH 8.3 and stored at 4°C. The specific activity of biocatalyst was 0.026 U/mg of particles.

2.3. Apparatus and procedures

A batch-mode measurement unit, fitted for F⁻ ion-selective electrode, was developed at the Yerevan Physics Institute (Armenia); the general scheme of the analytical system is shown in Fig. 1. The inte-
grated system consisted of a Plexiglas temperature-controlled measurement cell (1) with an inside volume of 5 ml, containing a magnetic stirrer (2). The combination F⁻ electrode (3) (Orion, Model 96-09) was inserted through a Plexiglas stopper (4) which sealed the measurement cell. An entry port (5) in the stopper permitted the injection of enzyme and substrates as well as allowing for the removal of excess liquid from the measurement cell. The signal was detected by a digital pH-meter (Corning Ion Analyzer 350) (6) and registered by chart recorder (7), and computer interface (8) complete the functional components of the unit.

In performing the assays, the temperature-controlled (25 ± 8°C) measurement cell was filled with standard buffer (50 mM Hepes Buffer with 500 mM KCl, pH 7.2) or substrate solutions, and the F⁻ electrode–stopper assembly was carefully plugged into the system to prevent ingestion of air bubbles into the measurement cell. The F⁻ electrode was calibrated with sodium fluoride standard solutions ranging from 10 to 1000 μM fluoride using both TISAB (total ionic strength adjuster buffer) and standard buffer. The signal of each sample was recorded for 3 min to ensure baseline stability (less than ± 3 mV). In order to insure that any potentially absorbed fluoride was removed from the surface of electrode, the stir bar, and reaction chamber, the entire system was rinsed between all calibration readings and reaction assays with reaction buffer containing 20% methanol (v/v) followed by a thorough rinsing with methanol-free reaction buffer.

Enzymatic reactions were initiated by injecting OPH into the reaction chamber, which had been previously filled with a substrate solution. The resulting signal (in millivolts) was recorded for every 4 s and converted to μM concentration of fluoride released by fitting to the standard curve. Semilogarithmic plots of fluoride concentration versus signal in millivolts could be fit to the equation:

$$y = a + b \log [F^-]$$

in which [F⁻] is the concentration of fluoride in μM, a and b are constants, and y is the signal in millivolts. Rearranging to solve for [F⁻] reconstructs the formula to

$$10^{(y-a)/b} = [F^-]$$

which was used in KaleidaGraph to convert the recorded signal to fluoride concentration in the reaction vessel at any time point. The slope (dF/s) of the line obtained by plotting fluoride concentration against time was employed in the calculation of initial reaction velocity. The non-enzymatic hydrolysis of

![Fig. 1. The batch-mode manifold of biosensor. The components of the system include a Plexiglas temperature-controlled measurement cell (1) containing magnetic stirrer (2), the fluoride combination electrode (3) is sealed into the measurement cell with a Plexiglas stopper (4). Samples are injected into the entry port (5), and digital pH-meter (6), chart recorder (7), and computer interface (8) complete the functional components of the unit.](image-url)
DFP under the assay conditions was negligible over the time required for the assay. Reaction velocities were calculated by dividing the μmol of product that appeared per second by the μmol of enzyme included in the reaction volume. Plotting concentration of substrate versus reaction velocity provided a data set, which could be fit directly to the Michaelis–Menten equation [14]:

\[ v = \frac{V_{max} [S]}{K_m + [S]} \]

or to the modified equation which incorporates substrate inhibition [14]:

\[ v = \frac{V_{max} [S]}{K_m + [S](1 + [S]/K_i)} \]

in which \( v \) is the velocity, \( V_{max} \) the maximal velocity, \( [S] \) the concentration of substrate, \( K_m \) the Michaelis constant, and \( K_i \) is the inhibition constant. Biosensor analyses with immobilized enzyme utilized the same procedures as described for the soluble enzyme.

3. Results and discussion

3.1. Kinetics of DFP hydrolysis

DFP is a much smaller substrate than either paraoxon or phosphonate; the leaving group of the molecule is a fluoride ion rather than para-nitrophenol; and the phosphoric acid derivative has isopropyl groups instead of the ethanyl moieties. The solubility limit of DFP permitted substrate saturation assays only to 7 mM DFP without the addition of methanol or other organic solvents (Fig. 2). DFP saturation curves were fit with the standard Michaelis–Menten equation, and a \( K_m \) value of approximately 0.6 mM was estimated for soluble enzyme.

3.2. Influence of organic solvent on DFPase activity

Because many organophosphorus neurotoxins have poor solubility in aqueous solutions, some organic co-solvents (e.g. methanol) may be used to increase the dissolved OP concentration. In this case the catalytic activity of enzyme could be affected by these organic additives, and it is necessary to investigate the behavior of the enzyme in the presence of organic solvent. While the paraoxonase activity of both soluble and immobilized OPH was unaffected by the addition of up to 20% methanol: methanol was identified as a competitive inhibitor with \( K_i = 0.64 \) M [15]. Increasing methanol concentrations did affect activity of soluble enzyme in the hydrolysis of DFP (Fig. 3) as methanol concentrations greater than 10% significantly inhibited the soluble enzyme (greater than five-fold) and 20% methanol inhibited the rates of DFP hydrolysis.
by more than five-fold. The immobilized enzyme was much more tolerant of methanol, and concentrations up to 20% methanol had no effect on biocatalyst activity, thus allowing the use of the OPH biosensor in various extraction applications which might require or result in the presence of organic liquids.

3.3. Optimization of biocatalyst content

The rate of DFP hydrolysis by soluble enzyme was proportional to the amount of added OPH, and the specific activity of immobilized biocatalyst was 0.026 U/mg of gel. To optimize the analysis, the dependence of reaction velocity on the total wet weight of the biocatalyst was investigated. Fig. 4 shows that the reaction rate began to reach saturation with 1 g of biocatalyst (w/w); it is important to work at saturated conditions to maintain high reproducibility of the biosensor.

3.4. The effect of pH

It was shown that a single catalytic group with a pK_a=6.14 must be ionized for hydrolytic activity. There was clearly a dependence of the kinetic parameters on pH, which was maximal for the enzyme from pH 7–10; the activity steadily decreased at pH values below 7.0. Fig. 5 summarizes the effect of pH on DFP biosensor response. There are moderate changes in sensor response in pH range from 6.5 to 9, whereas the response of the biosensor sharply decreased when pH dropped to less than 6.5.

3.5. DFP concentration measurements

Kinetic measurements with immobilized enzyme are shown in Fig. 6. In order to obtain a baseline with different concentrations of DFP, the immobilized enzyme was initially omitted from the reaction vessel. After baseline was recorded, the biocatalyst was introduced into the measurement chamber and the kinetic responses were determined. The initial kinetics were used to calculate the reaction rate which showed linear correlation between DFP concentration and the rate of fluoride released over a concentration range of $2.5 \times 10^{-5}$–$5 \times 10^{-3}$ M.

3.6. DFP detection in the mixed samples

It has been extensively documented that recombinant OPH is capable of hydrolyzing a broad spectrum of organophosphate neurotoxins resulting in the cleavage of P–O, P–F, P–S, and P–CN bonds [8–10,16–22]. The best substrate for the recombinant OPH used in these studies was paraoxon with $k_{cat}$ value greater than 15 000 s$^{-1}$, and DFP had hydrolytic activities of
DFP has been shown to be a competitive inhibitor of paraoxonase activity with the inhibition constant equal 0.32 mM [9]. However, there are no data relative to DFP activity in the mixed solutions of neurotoxins which might influence catalytic efficiency by the inhibition or activation of the biocatalyst itself. Thus, DFPase activity was investigated in the presence of different concentrations of paraoxon, and the rate of DFP activity for soluble enzyme increased up to 20% at concentrations of paraoxon up to 0.2 mM. Further increase in the paraoxon concentration led to slight decreases in activity (Fig. 7). The same behavior was observed...
observed for immobilized enzyme, and it appeared that the paraoxon hydrolysis product, p-nitrophenol (p-NPh), may be responsible for that effect.

3.7. Long-term stability of biosensor

The stability of immobilized OPH is significantly higher than for soluble enzyme and provided accurate DFP measurements for at least six months (Fig. 6). The biocatalyst functions under room temperature and was stored in the 10 mM phosphate buffered saline buffer at pH 7.2 in the refrigerator (8°C).

These studies have led to the development of a new type of enzyme-based sensor for organophosphothioates such as DFP, which is directly applicable to the detection of the CW agents, soman, and sarin.

Fig. 6. Calibration curves for DFP; circle – fresh immobilized enzyme, squares – after six months. 50 mM HEPES buffer with 500 mM KCl, pH 7.2, 25°C. Left – full curves, right – linear ranges of the calibration curve, \( f(x) = -0.000111 + 0.004159x \), \( R = 0.99849 \); \( f(x) = 0.000061 + 0.0017863x \), \( R = 0.99936 \).

Fig. 7. DFPase activity for soluble and immobilized enzyme in the presence of different concentration paraoxon (circles) and p-nitrophenol (triangles). DFP concentration 5 mM. Squares – immobilized enzyme.
Acknowledgements

This work was supported partially by Grant from CRDF (The Civilian Research and Development Foundation (RPI-359), and Sandia National Laboratories – Livermore (Contract #402632).

We thank Dr. Janet Grimsley for providing enzyme used in this study and helpful discussions, and Dr. Melinda Wales for help in data processing and evaluations.

References
