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Increased paraoxon detection by acetylcholinesterase inactivation with ionic liquid additives

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Abstract

This is the first study using ionic liquids (ILs) as additive in the aqueous solvent medium for detection of paraoxon by acetylcholinesterase inhibition method. A systematic comparison of various ILs with organic solvents has been made. The aqueous buffer solution containing ionic liquid ethylpyridinium hexafluorophosphate $[EtPy]^+[PF_6]^-$ has been found to give the best results. The inhibition kinetic follows the first order model. Ionic liquids modified aqueous solutions show the potential to provide a promising and effective medium in detection of paraoxon with acetylcholinesterase.
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Keywords: Paraoxon; Acetylcholinesterase; Ionic liquids; Pyridinium; Imidazolium

1. Introduction

Organophosphates are among the most toxic substances, and have been commonly used as pesticides, insecticides and chemical warfare agents. Paraoxon is the oxidation-metabolized product of parathion, which has been considered as one of the most acutely toxic pesticide. The toxicity of paraoxon includes DNA damage, chromosomal aberration, and malignant transformation [1–5]. The acute toxicity effect of OP is due to the inhibition of acetylcholinesterase (AChE), which occurs in the central nervous system of most animals, including humans. Exposure to organophosphates results in acetylcholine accumulation, which can interfere with muscular responses and the function of vital organs [6,7]. Degradation of organophosphate has been a subject of considerable attention. For example, biotransformation of various organophosphorous compounds in different conditions [8,9], uses of live biocatalysts for organophosphate detoxification [10], developments of detection methods including gas, liquid and thin-layer chromatography with different detectors, and various types of spectroscopy [11,12]. Pesticide detection is usually performed in aqueous solution. However, these compounds have low solubility in water and high solubility in organic solvents. Therefore, organic solvents are often used to dissolve organophosphate and improve detection procedure. Currently, there is growing interest on the development of biosensors for detection [13–15]. Enzymatic analysis is now often used for detection of organophosphates for its simple procedure and high selectivity. However, depending on the nature and amount of organic solvent, enzyme activity can be affected dramatically.

Lately, ionic liquids (ILs) have gained much attention [16–20]. Due to their many attractive features, such as low vapor pressure, stability at room temperature, chemical and thermal stability, non-flammability, non-toxicity, etc., these liquids have been considered as effective and environmental friendly, 'green' solvents [21–23]. The ILs have been investigated as reaction media in many organic and organometallic syntheses [24–26], in polymerization of methyl methacrylate [27], as solvents for extraction [28], multiphase bioprocess operations [29], and electrolytes in electrochemistry [30]. More recently, ionic liquids have also been used in the studies of enzymatic systems and are proved to be highly effective

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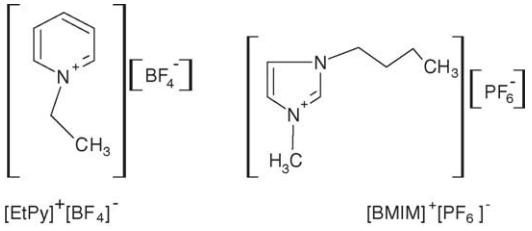

Fig. 1. Structures of $[EtPy]^+[BF_4]^-$ and $[BMIM]^+[PF_6]^-$

reaction medium [31–34]. Inspired by our earlier studies on biocatalysis in ionic liquids, we embarked on investigating the effect of ILs in the detection of paraoxon. The ionic liquids used in this study are shown in Fig. 1.

The aim of this work is to study the effect of ionic liquid on detection of paraoxon by AChE inhibition. A comparison of the activity of enzyme AChE in ionic liquids and organic solvents has been performed. The results obtained are promising and would be important in developing AChE based sensor for the detection of organophosphates.

2. Experimental

2.1. Materials and enzymes

The enzyme acetylcholineserase (EC 3.1.1.7, from bovine erythrocytes) was purchased from Sigma. Paraoxon, 5,5′-dithiobis (2-benzoic acid), acetylthiocholine iodide, and sodium phosphate buffer (pH 7.2, 0.02 M) were purchased from Aldrich.

The organic solvents, acetone, cyclohexane, and butanone, were of analytical grade and from Aldrich. Enzyme solution 1.25 unit/ml was prepared. One unit will hydrolyze 1 μmol of acetylcholine to choline and acetate per min at pH 8.0 at 37 °C.

2.2. Ionic liquid synthesis

2.2.1. Synthesis of $[EtPy]^+[BF_4]^-$ and $[EtPy]^+[CF_3COO]^-$

These ionic liquids were synthesized according to the method reported earlier, and tested by FTIR and NMR [35].

A general synthetic procedure could be described as followings: trifluoroacetic acid or tetrafluoroboric acid (0.2 mol) were slowly added to a stirred slurry of silver(I) oxide (0.1 mol) and distilled water (50 ml). To avoid photodegradation of silver(I) oxide, the reaction mixture was fully covered with aluminum foil. The reaction mixture was stirred continuously until the reaction was complete, which was indicated by the formation of a solution. A solution of *N*-ethyl-pyridinium bromide (0.2 mol) was added to the reaction mixture. As reaction took place and ILs formed, a yellow precipitate of silver(I) bromide started to be observed. The mixture was stirred at room temperature for a certain time until no more precipitate formed. The precipitate of silver(I) bromide was filtered off, and then the solvent was removed by rotary evaporation under vacuum, at about 65 °C. The resulting ionic liquids were put in an oven overnight at 65 °C to remove the moisture (yield 92%).

2.2.2. Synthesis of $[BMIM]^+[PF_6]^-$ and $[EtPy]^+[PF_6]^-$.

Representative procedure: Synthesis and purification were carried out following the literature method [36]. $[EtPy]^+[PF_6]^-$ was prepared by modifying Carda–Broch's method as below. *N*-ethyl-pyridinium bromide (37.6 g, 0.2 mol) was dissolved in 100 ml of distilled water. Hexafluorophosphoric acid (34.5 ml, 0.24 mol, 60%) was slowly added and the stirred for 3 h. As a result, precipitate of silver bromide was formed. The salt was filtered off, and washed with distilled water till the pH of the filtrate was about 5.5. The solvent was removed by rotary evaporation under vacuum at about 65 °C. The resulting ionic liquids were put in an oven overnight, at 65 °C, to remove the moisture (yield 34%).

2.3. Residual AChE activity measurement

10^{-5} M paraoxon was prepared from 10^{-3} M paraoxon stock solution (in pure butanone) and diluted 100-fold in distilled water. In this experiment, the final concentration of paraoxon in the cell was 10^{-7} M, while the amount of butanone was negligible and had no appreciable effect on acetylcholinesterase activity [37].

The residual activity was measured following the procedure described by Kim et al. [38]. First, 33 μl of 10^{-5} M paraoxon was added to 1 ml of 0.25 mM 5,5′-dithiobis(2-nitrobenzoic acid) in pH 7.2 phosphate buffer (because a higher pH causes hydrolysis of paraoxon). One hundred microlitres of the desired solvent was added, followed by of 33 μl of enzyme, and the mixture was incubated at 37 °C for specified time. Following incubation 33 μl of 20 mM acetylthiocholine iodide in phosphate buffer was added, allowed to react for 3 min and the absorbance was recorded at 405 nm. The reference solution containing all of the above materials except enzyme was used to eliminate non-enzymatic hydrolysis. Product inhibitions are negligible at this low concentration.

This procedure is based on the coupling of the following reactions [39]:

$$\text{acetylthiocholine} \xrightarrow{\text{enzyme}} \text{thiocholine} + \text{acetate}$$

$$\text{thiocholine} + \text{dithiobisnitrobenzoate} \rightarrow \text{yellow color}$$

2.4. Kinetic parameter calculation

The acetylcholinesterase inactivation by paraoxon is followed by the scheme below [37]:

$$E + I \overset{K\text{i}}{\rightleftarrows} \text{EI} \xrightarrow{k\text{i}} \text{EI}'$$

where E is the initial enzyme activity, I is the inactivator concentration, EI is the reversible enzyme–inactivator complex, EI′ is the irreversible complex. K_i is the equilibrium constant and k_i is the rate constant.The relationship between decrease of enzyme activity and time could be described as:

$$\frac{dE}{dt} = -\frac{k_i I}{I + K_i} E, \quad K_{obs} = \frac{k_i I}{I + K_i}$$

where t is the incubation time, K_{obs} is the apparent inhibition constant. When $I \ll K_i$,

$$K_{obs} = \frac{k_i}{K_i} I$$

k_i/K_i is considered as the efficiency of inactivation.

3. Results and discussion

3.1. Determination of optimum solvent content

In order to determine the effective concentration of ionic liquid, a series of solutions with different concentrations of ionic liquid were prepared and tested for enzyme activity. Initially, the enzyme activity was measured before and after the addition of paraoxon using ionic liquid $[EtPy]^+[BF_4]^-$.

The optimized IL content is the one in which high enzyme activity is preserved and also that permits best inactivation by paraoxon. Therefore, a set of different IL concentration was tested. As Fig. 2 shows, best results were obtained with 2.5% of ionic liquid. Interestingly, enzyme activity could be maintained at lower concentration of ionic liquid and the activity increased with the content of ionic liquid up to 2.5%. However, the activity decreased significantly on increasing the IL content further. This could be because enzyme structure may be modified in the presence of small amount of organic solvent as reported by Mionetto et al. [40]. However, at higher concentration of ionic liquids the enzyme activity may be quenched, and toxic effect may predominate.

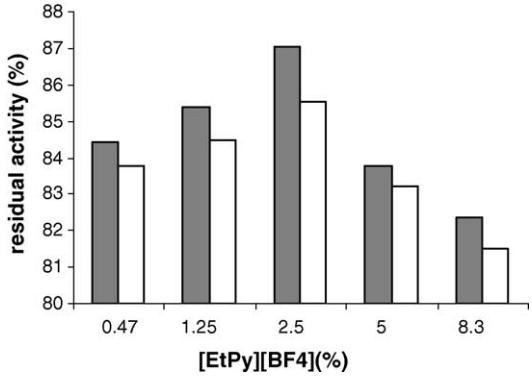

Fig. 2. Ionic liquid content: percentage of residual acetylcholinesterase activity in the presence of different concentration of ionic liquid $[EtPy]^+[BF_4]^-$, compared to acetylcholinesterase activity in pH 7.2 phosphate buffer after 0.5 h incubation at 37 °C. (■) Without paraoxon; (□) with 10^{-7} M paraoxon.

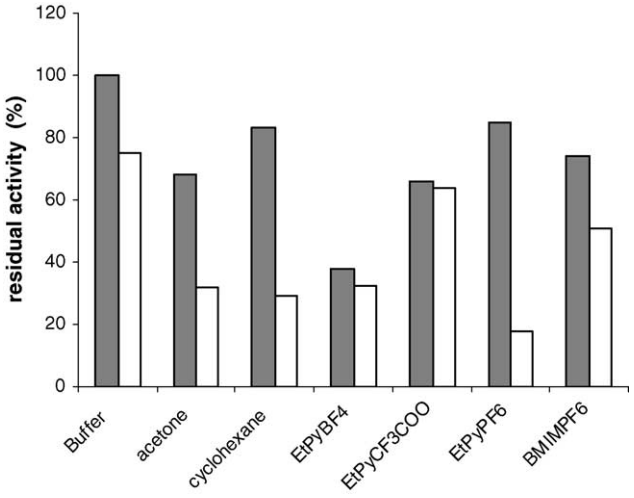

Fig. 3. Solvent effect: percentage of residual acetylcholinesterase activity in the presence of different solvents (2.5% content, except that $[EtPy]^+[PF_6]^-$ is 0.125 mM), compared to acetylcholinesterase activity in pH 7.2 phosphate buffer after 1 h incubation, 37 °C. (■) Without paraoxon; (□) with 10^{-7} M paraoxon.

3.2. Solvent selection

In the study reported earlier [37], cyclohexane has been found as good solvent for paraoxon detection (aqueous solution containing 5% cyclohexane permit 100% inactivation by paraoxon). Since high concentration of solvent may course a dramatic lose of enzyme activity, we used 2.5% content as middle course to compare buffer, cyclohexane and acetone with various ionic liquids.

The results are shown in Fig. 3. The ionic liquid $[EtPy][PF_6]$ is partially soluble in water and a 2.5% (v/v) addition of its saturated solution gives 0.125 mM final concentration in detection medium. This study shows that 0.125 mM $[EtPy]^+[PF_6]^-$ aqueous solution is the most suitable medium, in which enzyme activity of about 84% is maintained before adding paraoxon. After inhibition the enzyme activity decreased 67%. Also it was observed that enzyme can maintain better activity in hydrophobic solvent than in hydrophilic solvent. This could be because a hydrophilic solvent could strip water from enzyme, thereby causing denaturization [41]. Although buffer can maintain high enzyme activity, the inactivation is not significant.

3.3. Kinetic study

As described in Section 2.4, the efficiency of a solvent could be measured from rate constants and equilibrium constant for the reaction. Fig. 4 shows the kinetics of acetylcholinesterase inhibition by various concentrations of paraoxon in 0.125 mM $[EtPy]^+[PF_6]^-$ solution.

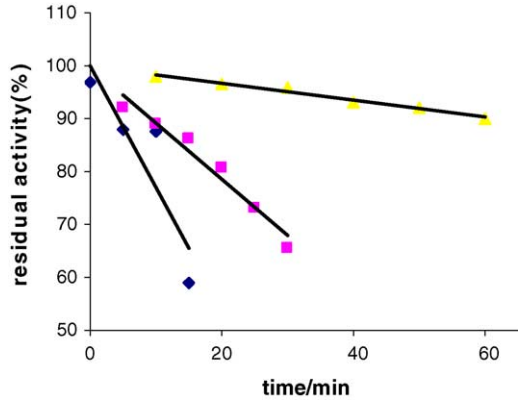

Fig. 4. Kinetic of acetylcholinesterase inhibited by paraoxon. (▲) 2.75×10^{-9}M; (■) 2.75×10^{-8}M; (♦) 2.75×10^{-7}M in the solution containing 0.125 mM $[EtPy]^+[PF_6]^-$ at 37 °C.

Table 1
Comparison of k_i/K_i in different solvents

Solvent	Buffer	Cyclohexane	$(EtPy)^+(PF_6)^-$
Content	–	2.5% (v/v)	0.125 mM
k_i/K_i (min^{-1} μM^{-1})	0.02	0.37	0.44
$\frac{k_i/K_i\,(\text{solvent})}{k_i/K_i\,(\text{buffer})}$	–	18.5	22

In order to compare with traditional detection medium, similar kinetic studies were carried out in buffer solution and 2.5% (v/v) cyclohexane separately. The slope of these curves, K_{obs}, when plotted against paraoxon concentration gives k_i/K_i the efficiency of inactivation. Results of this study are shown in Table 1.

As data shows the inhibitions in 2.5% cyclohexane, and 0.125 mM$[EtPy]^+[PF_6]^-$ are 18.5-fold and 22-fold more efficient than in buffer. These results suggest that ionic liquid modified solution could be used to enhance the detection of paraoxon.

4. Conclusions

This is the first systematic study employing ionic liquids in the detection of organophosphate paraoxon. Results show that the inhibition kinetic of acetylcholinesterase follows the first order model. The inhibition efficiency in 0.125 mM $[EtPy]^+[PF_6]^-$ is 22-fold than it is in buffer. Further investigation on the application of this solvent system for various organophosphates and mechanism are underway.

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