PUB: 01/2012

Anal. Bioanal. Electrochem., Vol. 4, No. 1, 2012, 1 - 16

Analytical & Bioanalytical Electrochemistry

Full Paper

Detection of Organophosphorous Pesticides Using a Monoenzyme Biosensor: A Voltammetric Study

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Received: 31 December 2011 / Accepted: 25 February 2012 / Published online: 28 February 2012

Abstract- Acetylcholinesterase (AChE) was an important cholinesterase enzyme present in the living organisms, which is responsible for transmission of impulses through synaptic clefts by oxidation of acetylcholine to choline. Acetylcholinesterase enzyme was immobilized through silica sol–gel process on the surface of carbon paste electrode which was used to fabricate monoenzyme biosensor. It is a rapid, simple and sensitive biosensor used for determination of two organophosphorous pesticides monocrotophos and phosphamidon in 0.1 M phosphate buffer and in 0.1 M KCl. Acetylthiocholine chloride (ASChCl) was used as substrate by enzymatic hydrolysis it gives thiocholine which undergone electrochemical oxidation and produces anodic current around at 0.60 V *vs.* saturated calomel electrode. The effect of scan rate, pH, enzyme loading and substrate concentration on the biosensor response was studied. The biosensor provided a high sensitivity, large linear concentration range from 50–900 ppb, 0.1–1.25 ppm for monocrotophos and phosphamidon. The detection limits were found to be 45 ppb, 0.06 ppm for monocrotophos and phosphamidon respectively. The results showed the optimum conditions for pH, substrate concentration, and incubation time were at room temperature, pH 7.0, 1 mM, 4 and 3 min for monocrotophos and phosphamidon respectively.

Keywords- Acetylthiocholine Chloride, Immobilization, Acetylcholinesterase, Monocrotophos , Phosphamidon

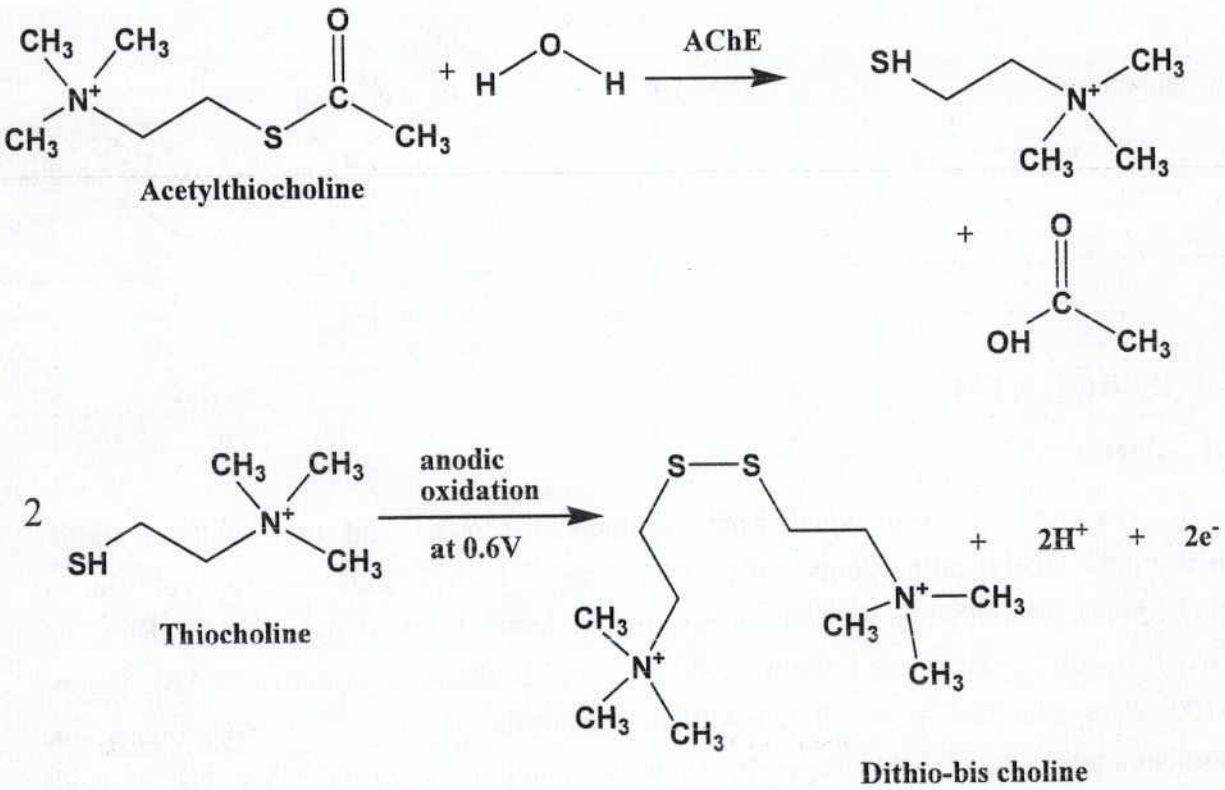
1. INTRODUCTION

Organophosphorous pesticides are widely used in agriculture and their properties provide numerous benefits in terms of production and quality increase [1]. Their presence in water and food poses a potential hazard to human health [2]. Hence fast and economically viable methods are required for their detection in the environment and in agro food products [3]. Many methods have been developed in the last few years for the determination of pesticides. The analysis of pesticides was usually carried out by gas and liquid chromatography with a selective element detector [4, 5]. Nevertheless, these procedures are expensive and frequently require laborious, complex, time consuming, sample treatment such as extraction of pesticides, extract cleaning, solvent substitution and clean up steps. Furthermore these approaches do not improve our understanding of the natural processes governing chemical species behaviour, their transport, bioavailability, and their long term impact on aquatic systems. The stability of samples during long–term storage was questionable, as there are subject to various biological, chemical and physical effects [6]. Finally, the analysis was usually performed in a specialized laboratory by skilled personnel and was not suitable for in situ application. These issues turn out to be a major problem when rapid and sensitive measurements are needed in order to take the necessary corrective actions in a timely approach. Accordingly a rapid, unfailing and simple sensor for detecting pesticides continues to be an issue of interest in electro analytical research [7, 8].

Biosensors for detection and quantification of pollutants have attracted extraordinary interest in recent years, because of its role in the development of highly sensitive, selective, chemical analysis, low cost and short analysis time associated with these devices. Biosensors based on the inhibition of acetylcholinesterase have been used for the detection of pesticides in different samples [9–11]. Different types of immobilization methods are available, among them, sol–gel immobilization can be preferred through the usage of silicate materials for the entrapment of a biological moiety which was introduced in the mid of 1950s [12] but importance was not realized at that time. The entrapment of proteins into alkoxysilane–derived silicate glasses via the sol–gel method has been reported [13]. This pioneering work was greatly recognized by this group when the technique was applied independently to the doping of transparent silica glasses with alkaline phosphatase, chitinase, aspartase and β-glucosidase and copper–zinc superoxide dismutase, cytochrome C and myoglobin entrapped in tetramethyl orthosilicate [14]. Tetraethyl orthosilicate derived sol–gel monolith doped with glucose oxidase was used as a recognition element in a flow injection analytical system [15]. The chemistry of sol–gel processing generally involves the hydrolysis of alkoxysilane or alkyl silicate or alkoxy mettalate or a mixture of these, in the presence of acid or base catalysis to form hydroxy derivatives. A cascade of condensation reactions gives rises to soluble, colloidal clear sols and ultimately phase separated polymers, which produce the final

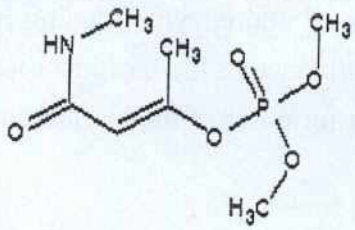
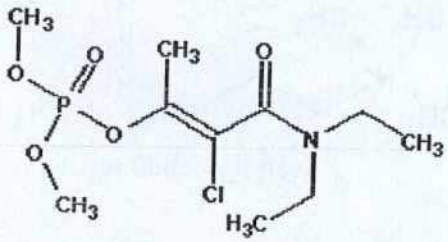
matrices in various configurations such as monoliths, shelts, granulates, micro particles, thick and thin films.

Accordingly, in the present work studies on the immobilization of acetylcholinesterase enzyme onto carbon paste electrode was carried out. Experimental parameters such as the scan rate, pH and enzyme loading have been investigated to evaluate the conditions for the best performance of the biosensor towards the determination of pesticides. The structural and molecular formulae of the monocrotophos and phosphamidon were shown in Table 1.



Scheme 1. The mechanism of the enzyme catalyzed reaction

Table 1. The structural and molecular formulae of monocrotophos and phosphamidon pesticides

Pesticide	Molecular formula	Structure
Monocrotophos	$C_7H_{14}NO_5P$	
Phosphamidon	$C_{10}H_{19}ClNO_5P$	

2. EXPERIMENTAL

2.1. Materials

All chemicals were obtained from commercial sources and used without further purification. Acetylcholinesterase (E.C.3.1.1.7,type–VI–s/1.5 mg, electric eel source, 500U/1.5mg), acetylthiocholine chloride were purchased from Sigma–Aldrich chemicals, USA. Tetraethyl orthosilicate (TEOS), cetyl trimethyl ammonium bromide (CTAB), Triton–X-100 were obtained from Sigma–Aldrich chemicals co. USA. Monocrotophos and phosphamidon were obtained from Accustandard solutions company, USA. The pesticide stock solution was prepared by dissolving in acetone (GR grade) solution. The graphite fine powder was procured from Lobo chemie and silicon oil (Himedia). The acetone (GR grade) was obtained from Merk Specialities Pvt. Ltd. Phosphate buffer solution was prepared by mixing appropriate quantity of 0.1 M aqueous sodium dihydrogen phosphate monohydrate and 0.1 M aqueous disodium hydrogen phosphate. All the chemicals were of analytical grade and aqueous solutions were prepared with double distilled water. The enzyme stock solutions were stored at –20 °C. All stock and working solutions of chemicals were stored at – 4 °C.

2.2. Apparatus

The electrochemical experiments were carried out using a model CH–660C (CH Instruments, USA). All the experiments were carried out in a conventional three electrode electrochemical cell. The electrode system contained a working electrode which

was an enzyme modified carbon paste electrode, a platinum wire as a counter electrode and saturated calomel electrode as reference electrode. All the experiments were carried out at room temperature 25±2 °C.

2.3. Preparation of bare carbon paste electrode

The bare carbon paste electrode was prepared by hand mixing of 70% graphite powder with 30% silicon oil in an agate mortar to produce a homogenous carbon paste. The paste was packed into the cavity of homemade PVC (3 mm in diameter) and then smoothed on a weighing paper. The electrical contact was provided by a copper wire connected to the paste in the end of the tube [16-18].

2.4. Preparation of silica sol–gel solution and enzyme modified carbon paste electrode

A homogenous TEOS silica sol was prepared by mixing 2 ml of TEOS, 1 ml of H_2O, 50 µl of 0.1 M HCl, 25 µl of 10% triton–X-100. In this solvent less hydro sol procedure, TEOS was added to distilled water and stirred magnetically to form a gray two phase dispersion at room temperature. When the solution gets acidified, the dispersion transforms to clear solution with in 30 min. The solution can be stored for several months when refrigerated at –20 °C.

The 5 µl of stock sol–gel solution was vortexed with 45 µl of phosphate buffer containing 0.5 U of enzyme stock solution. The 4 µl of the enzyme sol was spread on the electrode surface. This film was allowed to polymerize at room temperature for 3–5 min. This electrode was gently washed with phosphate buffer (pH 7.0) and was used for further experimental procedure [19]. The 0.04 U of enzyme was immobilization on the electrode surface.

3. RESULTS AND DISCUSSION

3.1. Cyclic voltammetric studies

The cyclic voltammogram (CV) of the sensor in the presence and absence of 1 mM substrate in phosphate buffer (pH 7.0) at a scan rate of 10 mVs^{-1} is shown in Fig. 1.

In the absence of substrate, the fabricated biosensor working electrode doesn't show any response and only a small background current was observed in CV. When substrate was added to the buffered solution anodic peak was observed at 0.60 V. The reaction mechanism was shown in scheme.1. The investigated assay of AChE activity from the plot of current versus acetylthiocholine chloride concentration has been shown in Fig. 2. The apparent Michaelis–Menten constant (K_m^{app}) was thus estimated to be 500 µM using the Lineweaver–Burk plot of 1/I *vs.* 1/[acetylthiocholinechloride], where 'I' represents catalytic current of the analyte. The relatively low K_m^{app} value indicates the high affinity of the enzyme towards the substrate .

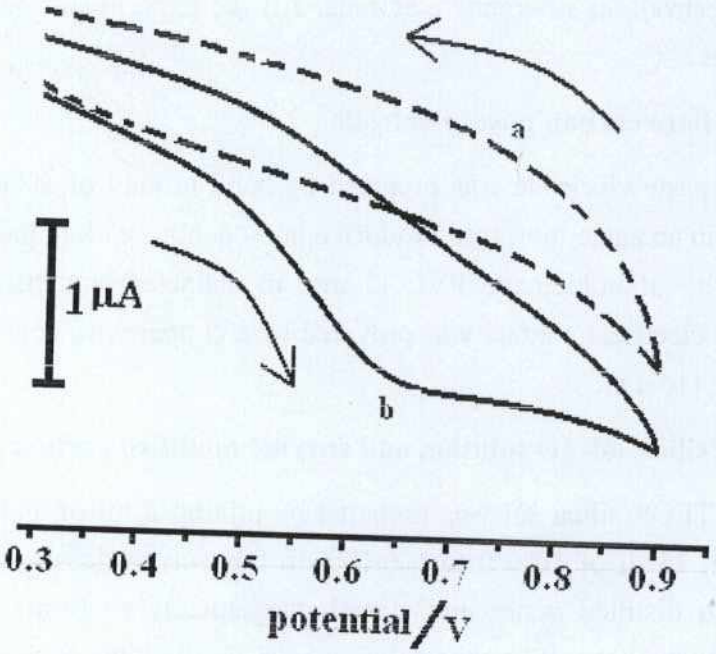


Fig. 1. Cyclic voltammogram of enzyme electrode in 0.1 M phosphate buffer, pH 7.0 and 0.1 M KCl (a) without substrate (dashed line) (b) with 1 mM substrate (solid line)

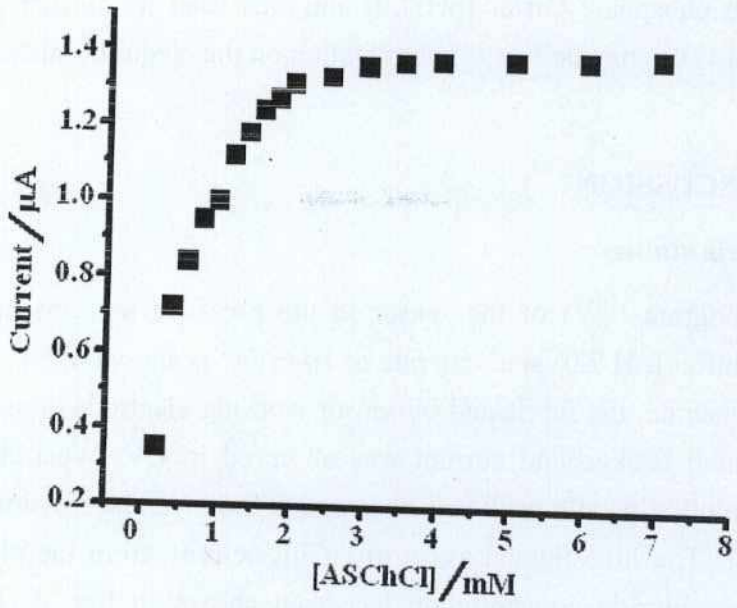


Fig. 2. Calibration graph for acetylthiocholine chloride obtained with AChE immobilized sensor in 0.1 M phosphate buffer / 0.1 M KCl at pH 7.0

3.2. Effect of scan rate and pH

The effect of scan rate on the peak current at the enzyme modified carbon paste electrode in 0.1 M KCl was investigated in the presence of 1 mM acetylthiocholine chloride. As shown in the Fig. 3 the anodic peak current increases linearly with the square root of scan rate in the range 5 to 50 mVs^{-1}. The correlation coefficient was 0.9918, which indicate the electrode reaction was diffusion controlled process [16].

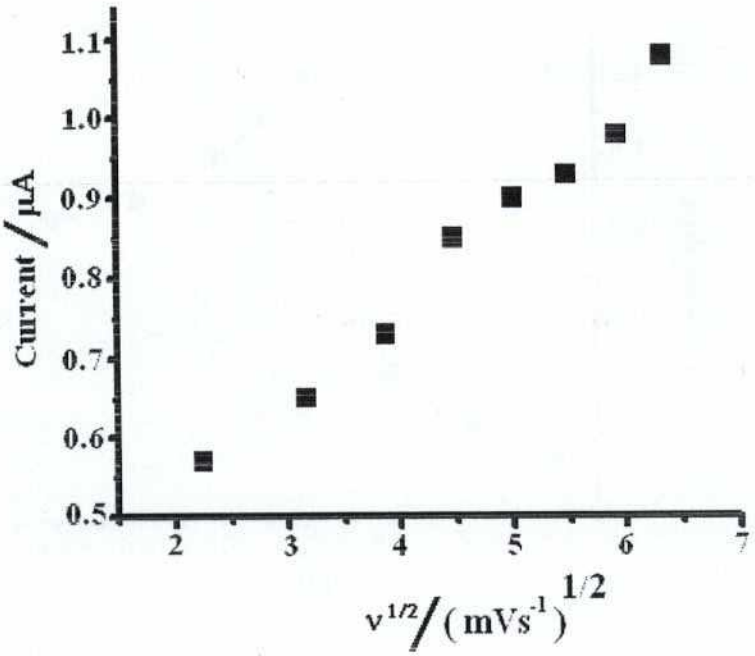


Fig .3. The enzyme electrode response by varying square root of scan rate for 1 mM ASChCl in 0.1 M phosphate buffer, pH 7.0 and 0.1 M KCl

The electrochemical response of enzyme modified carbon paste electrode towards 1 mM acetylthiocholinechloride was generally pH dependent Fig. 4(A). As the pH of the solution increases the response of the biosensor increases until attaining a physiological pH 7.0 and from there onwards the response of the biosensor decreases. The anodic peak potentials of acetylthiocholine chloride shifted from 0.68 to 0.51 V with respect to the pH from 5.5 to 8.5. The potential diagram was constructed by plotting the graph of anodic peak potential E_{pa} *vs.* pH of the solution. The enzyme electrode shows maximum sensitivity at pH 7.0 Fig. 4(B). The pH dependence of oxidation peak potentials of substrate, E_{pa}=0.9752+0.055pH (r=0.9947).The graph has a good linearity with a slope of 55 mVs^{-1}, this behavior nearly obeys the Nernst equation for equal number of protons and electrons transfer reaction [20, 21]. All experiments including inhibition studies were carried out at pH 7.0.

3.3. Effect of enzyme concentration

The effect of enzyme concentration on peak current response with 1 mM substrate was shown in Fig. 5. When the enzyme concentration is increased from 0.03 to 0.3 U in sol–gel matrix entrapped onto the carbon paste electrode, it was noticed that peak current increases up on increase in the enzyme concentration. The rate of enzyme catalyzed reaction was dependent on the amount of enzyme immobilized. The highest sensitivity towards the inhibitors was found with electrode containing low enzyme loading [22].

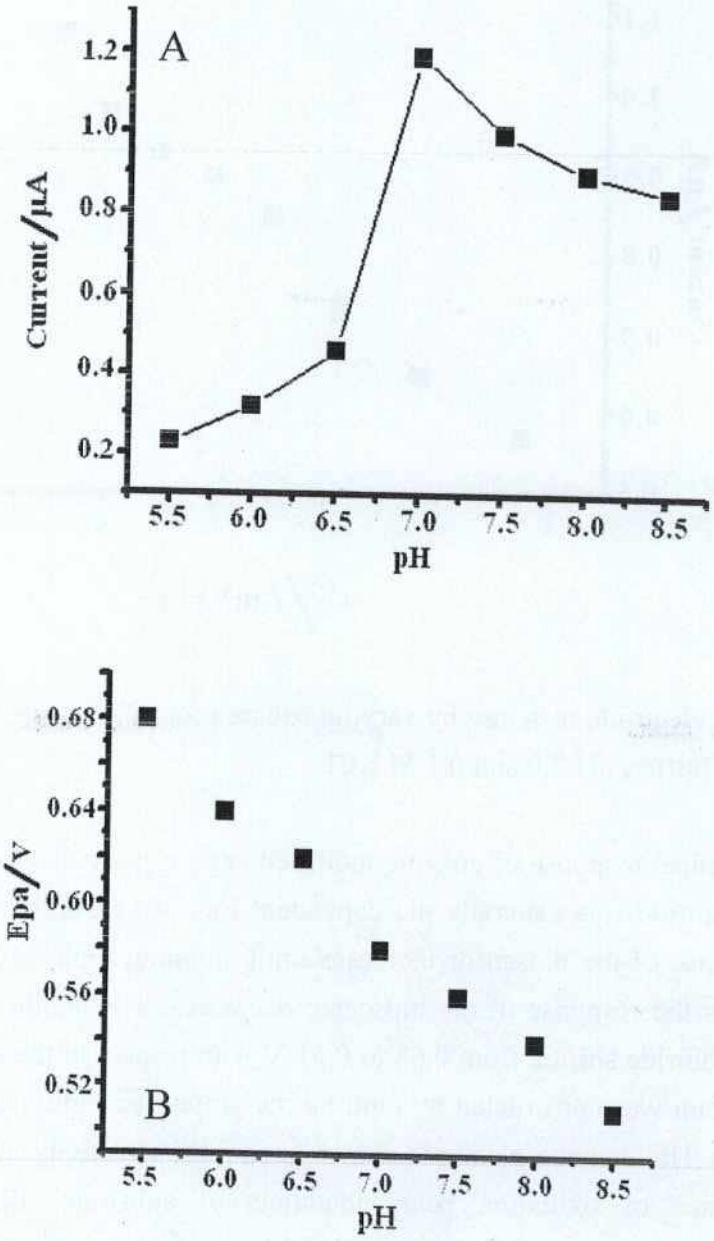


Fig. 4. (A) Effect of pH on the enzyme electrode response (B) Plot of Epa *vs.* pH to 1 mM ASChCl

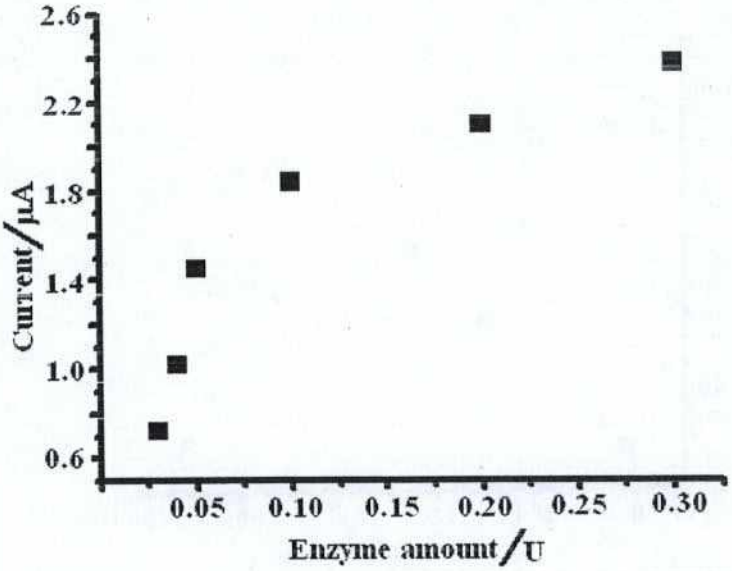


Fig. 5. The effect of enzyme loading into sol–gel matrix on the response of biosensor system

3.4. Detection of organophosphate pesticides

The biosensor was used for the detection of different organophosphorous pesticides such as monocrotophos and phosphamidon. The sensor has been used to carry out inhibition studies by incubation with pesticide solution to determine lower detection limits. To obtain an inhibition plot the percentage inhibition method was followed. The electrode response was first measured in 0.1 M phosphate buffer, 0.1 M KCl, pH 7.0 in presence of 1 mM acetylthiocholine chloride this value corresponds to (Ii) the current before inhibition. The electrode was washed with same buffer and incubated for 4 and 3 min with monocrotophos and phosphamidon pesticide solution at a known concentration. The second value corresponds to (IF), which was the current after inhibition. The inhibition percentage and percentage of residual enzyme activity were calculated as follows [23].

Inhibition % (I %) = [(Ii–IF) / Ii] X 100 (1)

Residual enzyme activity % (REA %) = [IF / Ii] X 100 (2)

The organophosphorous compounds are known to inhibit the activity of AChE and in the presence of monocrotophos and phosphamidon the rate of thiocholine production was reduced. Quantitative analyses of individual pesticides were carried out according to the above procedure. Calibration plots based on the dependence of the % inhibition on concentration were linear and shown in Fig.6 A&B for monocrotophos and phosphamidon respectively.

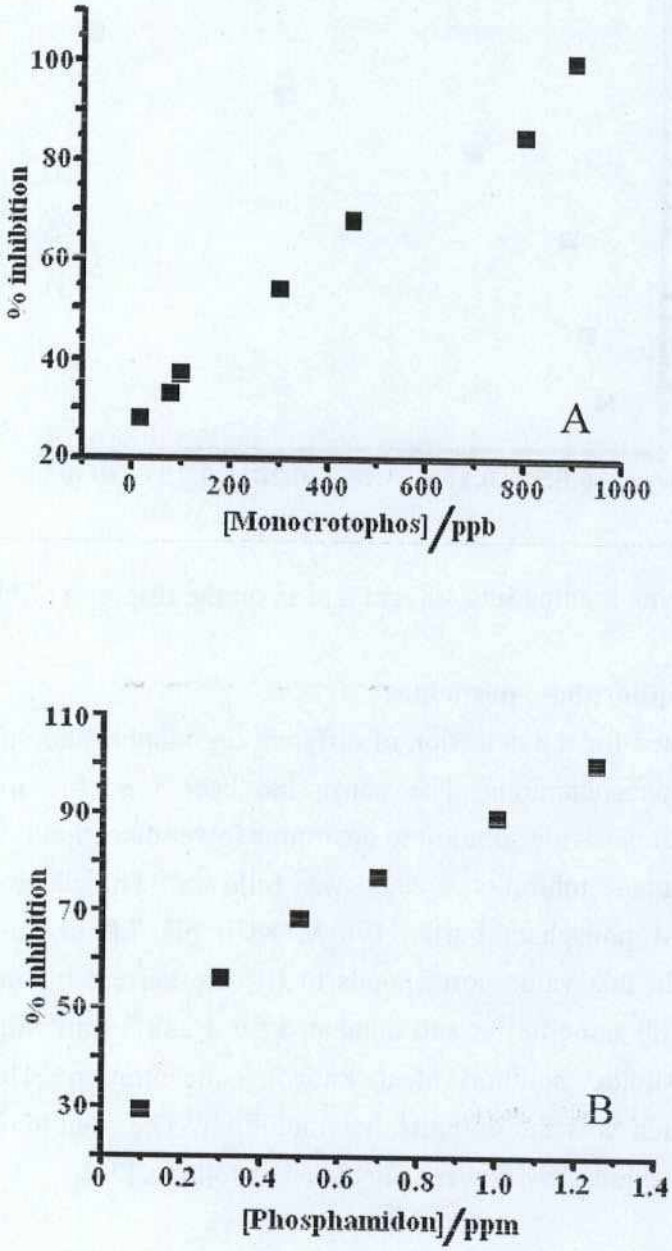


Fig. 6. Inhibition plots of (A) Monocrotophos after 4 min incubation time (B) Phosphamidon after 3 min incubation time in 0.1 M phosphate buffer at pH 7.0, 0.1 M KCl

The detection limit (DL) and quantification limit (QL) values were found to be 45 ppb, 0.06 ppm and 151 ppb, 0.2 ppm for monocrotophos and phosphamidon respectively. It was seen that the lowest DL value was achieved with monocrotophos; this clearly indicates that the monocrotophos was more toxic than phosphamidon. The behavior of enzyme activity within the concentration range of 50–900 ppb and 0.1–1.25 ppm at different incubation times were shown in Fig.7 A&B for monocrotophos and phosphamidon respectively.

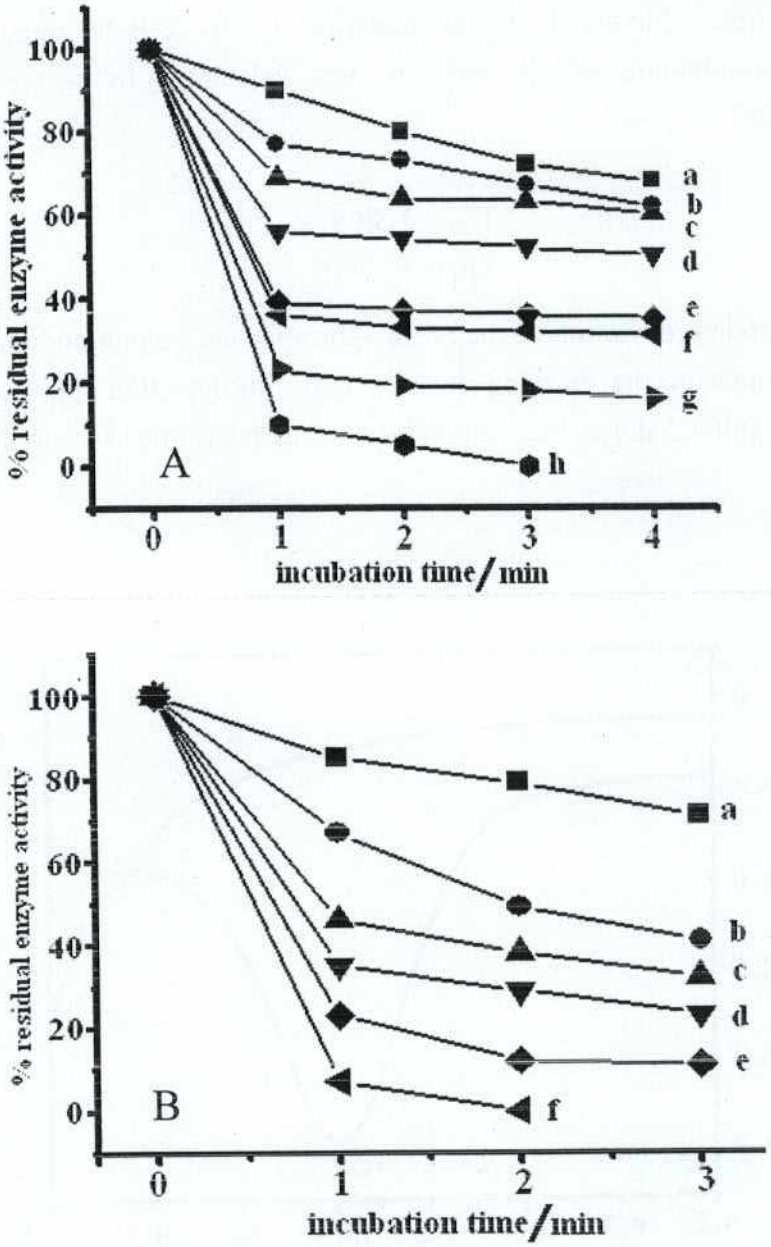


Fig. 7. Effect of incubation time in various inhibitor concentrations on the residual enzyme activity in 0.1 M phosphate buffer / KCl, pH 7.0 (A)For monocrotophos (a) 50 ppb (b) 80 ppb (c) 100 ppb (d) 150 ppb (e) 300 ppb (f) 450 ppb (g) 800 ppb (h) 900 ppb(B) For phosphamidon (a) 0.1 ppm (b) 0.3 ppm (c) 0.5 ppm (d) 0.7 ppm (e) 1.0 ppm (g) 1.25 ppm

It reveals that the level of inhibition of enzyme increases with increase in incubation time and as well as increase in concentration of pesticides. A complete inhibition was observed at shorter incubation times of 4&3 min for monocrotophos and phosphoamidon of concentrations 900 ppb&1.25 ppm respectively. The inhibition effect of pesticides on the immobilized enzyme was studied by employing differential pulse voltammetry (DPV) Fig. 8.

When the concentration of pesticides increases the residual enzyme activity decreases with respect to different time intervals. This was shown in Fig. 9A & B for monocrotophos and phosphamidon. Determination of DL and QL was calculated by using the following expression [16, 24–26].

$$DL = 3\ Sb/S \quad (3)$$

$$QL = 10\ Sb/S \quad (4)$$

Where 'S_b' is standard deviation of the mean values for ten voltammograms of the blank solution, S is the slope of the working curve, DL is the detection limit, and QL is the quantification limit. Table 2 shows the various parameters determined for monocrotophos and phosphamidon.

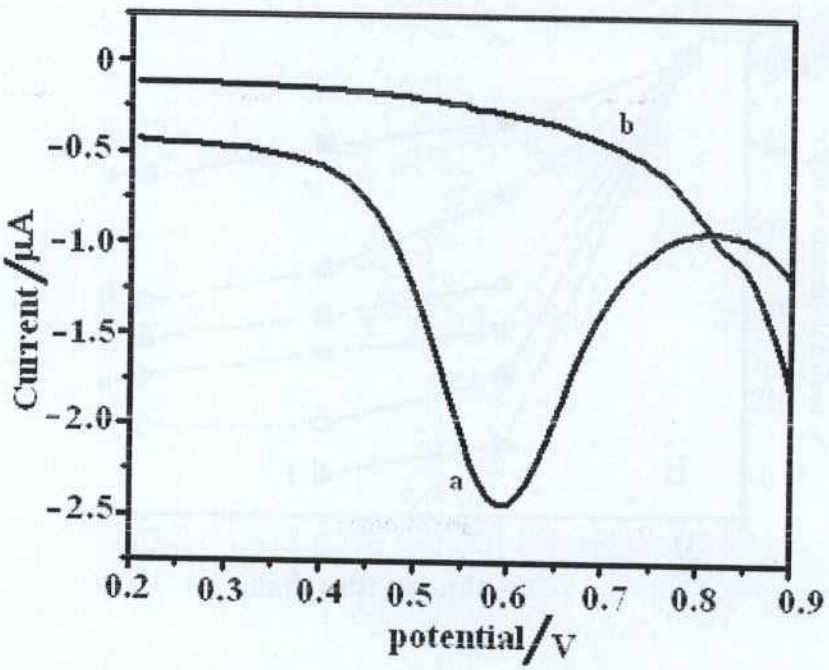


Fig. 8. Differential pulse voltammogram of (a) substrate alone (b) with 900 ppb monocrotophos/1.25 ppm phosphoamidon pesticide solutions

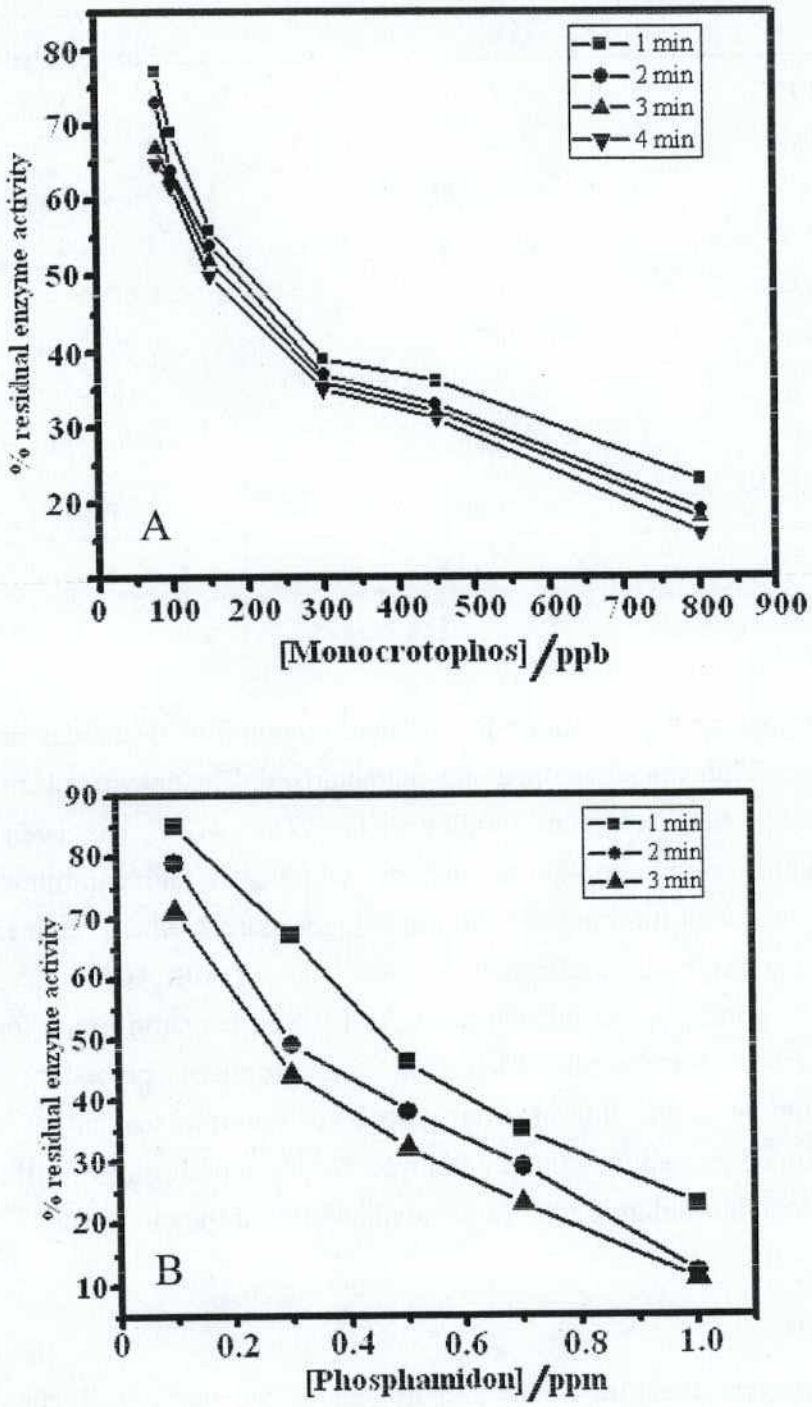


Fig. 9. The variation of residual enzyme activity with different inhibitor concentrations with time in 0.1 M phosphate buffer / KCl (A) Monocrotophos (B) Phosphamidon

Table 2. The various parameters determined for monocrotophos and phosphamidon

Parameters	Monocrotophos	Phosphamidon
Response time (min)	1	1
Incubation time (min)	4	3
Linear range	50 – 900 ppb	0.1 – 1.25 ppm
Intercept of calibration curve	28.63	33.52
Slope of calibration curve	0.0772	0.0566
Correlation coefficient	0.9926	0.9671
Standard deviation (S.D.)	3.6703	7.1844
Detection limit (DL)	45 ppb	0.06 ppm
Quantification limit (QL)	151 ppb	0.2 ppm

4. CONCLUSIONS

The work describes a new biosensor for the determination of pesticides monocrotophos and phosphamidon, which was developed and characterized. The enzyme electrode provided good linearity to the pesticide concentration range. The AChE has been successfully immobilized through a simple sol–gel technology, which was taking minimal preparation time. The enzyme was well immobilized with in sol–gel matrix, which retained satisfactory enzymatic catalytic activities. The advantage of this biosensor with AChE enzymes provided a high sensitivity, ease of preparation, selectivity and lower detection limits for the analysis of organophosphorous compounds. Moreover, the proposed procedure for enzyme immobilization could be extended to other enzymes as it is simple, fast and very efficient. In addition, the developed procedure can be employed for the monitoring of different types of organic pollutants and thus enlarging the future applicability of the biosensor.

Acknowledgements

The authors are very thankful to the Department of Science and Technology (DST), Government of India, New Delhi for the funding given through project no SR/FT/CS - 025/2009.

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