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Choline oxidase as a selective recognition element for determination of paraoxon

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ABSTRACT

A simple mono-enzyme biosensor has been developed for specific determination of paraoxon (POX). In this biosensor, choline oxidase (ChOx) was introduced and used as a new recognition element. Besides, by application of Prussian blue (PB), as an electron mediator, the necessity of applying oxygen or hydrogen peroxide sensor, as the internal transducer, was abated. The method was based on PB chemically modified screen-printed electrode coupled with ChOx for detection of POX as inhibitor. The concentration of H_2O_2 produced by ChOx was electrochemically determined by the PB modified electrode at −50 mV versus the internal screen-printed Ag pseudo-reference electrode. The decrease in current caused by the addition of inhibitor was used for evaluation of POX concentration. The experimental parameters such as the quantity of enzyme, substrate concentration and incubation time were optimized for ChOx inhibition. At the incubation time of 5 min, the biosensor response was linear from 0.1 to 1 μM of POX with a detection limit of 0.1 μM.

1. Introduction

Amongst the highly toxic substances known, organophosphorus (OP) compounds, have come into widespread use as pesticides, insecticides or chemical warfare agents (Donarski et al., 1989; Chapalamadugu and Chaudhry, 1992; Compton, 1988; FAO, 1989; USDA, 1992). Therefore, scientific interest has been increased to control spread of these compounds to the environment and food chain. Traditionally, the content of pesticides has been determined using gas and liquid chromatography with various detection techniques (Lacorte and Barcelo, 1994; Minelli et al., 1996; Lacorte et al., 1996). In spite of their sensitivity, these methods are time-consuming, expensive and require tedious sample pretreatment (Lacorte and Barcelo, 1994). Biosensors can be a viable alternative to classical methods because they are selective, sensitive, less expensive and do not require sample pretreatment (Karube and Nomura, 2000).

The majority of biosensors for OP compounds are based on specific inhibition of acetyl/butryl-cholinesterase (AChE/BChE) as the recognition component (Kousba et al., 2004; Padilla, 1995). Usually the OP biosensors could be categorized in bi- and mono-enzyme biosensors. In both systems, OP determination is performed by kinetic measurements of the initial velocity of inhibited AChE.

In bi-enzyme biosensors, after incubation of AChE with an OP compound, the kinetic measurements are performed by amperometric determination of H_2O_2 as shown by Scheme 1A and reactions (I) and (II):

$$\text{Acetylcholine} + H_2O \xrightarrow{\text{AChE}} \text{Acetic acid} + \text{Choline} \quad \text{(I)}$$

$$\text{Choline} + 2O_2 \xrightarrow{\text{ChOx}} \text{Betaine} + 2H_2O_2 \quad \text{(II)}$$

OP abates the biocatalytic production of choline and subsequently reduces the cathodic current produced by H_2O_2. The reduction in cathodic current is proportional to the OP compound concentration (Jaffrezic-Renault, 2001; Zhang et al., 2001). Optimization of these bi-enzyme biosensors is often complicated due to the problems raised by conjugation of two enzymatic processes such as the difference in optimum pH or specific activity (Suprun et al., 2005).

In mono-enzyme biosensors, as shown in Scheme 1B and reaction (III), usually a synthetic substrate, e.g. acetylthiocholine (ATCh) is used (Skladal, 1996; Hart et al., 1997; Skladal et al., 1997; Albareda-Sirvent et al., 2001). In this system, due to specific inhibition of AChE by OP compound, the biocatalytic production of thiocholine (TCh) is abated and subsequently the electric current produced by the electroactive product is declined. The reduction in

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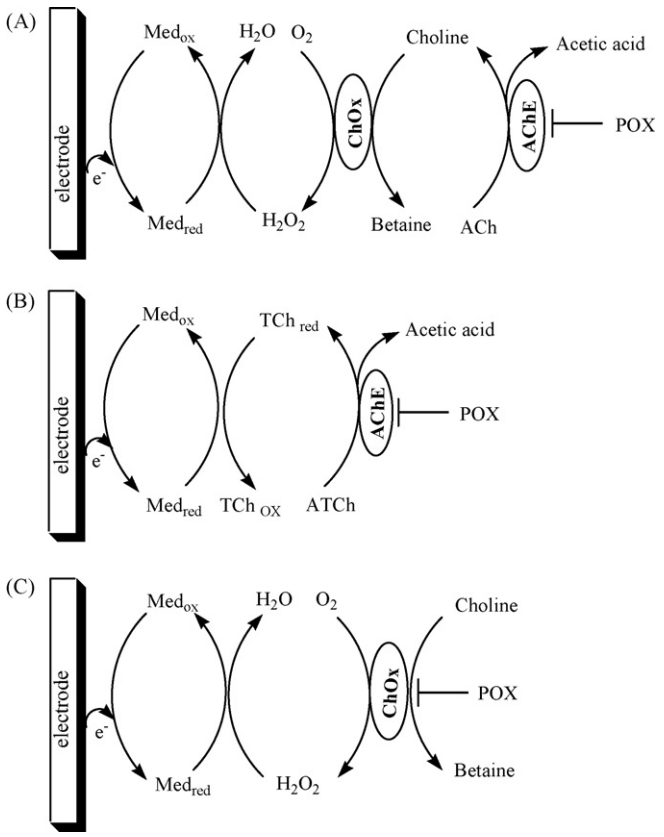

Scheme 1. Schematic description of amperometric POX biosensors for (A) bi-enzyme biosensors, (B) mono-enzyme cholinestrase-based biosensors and (C) the mono-enzyme ChOx-based biosensor. TCh, ATCh and Med represent thiocholine, acetylthiocholine and mediator respectively.

current is proportional to OP concentration.

$$\underset{\text{ATCh}}{(CH_3)_3N^+CH_2CH_2SOC(O)CH_3} \xrightarrow{H_2O,\ AChE} \underset{\text{TCh}}{(CH_3)_3N^+CH_2CH_2SH} + CH_3COOH \quad \text{(III)}$$

However, reaction (III) is often faced with difficulties such as formation of by-products deposited on the electrode or spontaneous hydrolysis of the substrate (Ivanov et al., 2003). The high working potential necessary for anodic oxidation of the TCh is an extra problem that limits the application of the biosensor especially for testing of samples containing electrochemically active species (Ivanov et al., 2003). In such biosensors the reducing of working potential is of critical importance. Because when the biosensor is used for the determination of pesticide residues in soil or plants, phenolic compounds may interfere with the detection of pesticides. However, by modification of the transducers using mediators such as co-phethalocyanine (Skladal, 1996) and TCNQ (Albareda-Sirvent et al., 2001) a decrease in working potential can be achieved.

A preferred avenue is to employ mono-enzyme biosensor capable of selective recognition of OP compounds. In our preceding studies, it was proven that OP pesticides such as paraoxon (POX) or methyl parathion could also inhibit ChOx (Tavakoli et al., 2005). Based on this experience, we made effort to design a mono-enzyme biosensor (Scheme 1C) in which the detection of POX can be accomplished through its inhibitory effect on ChOx. Following the inhibition of ChOx by POX, the biocatalytic production of H_2O_2 is reduced. This would subsequently decrease the cathodic current.

In order to level up the sensitivity of the biosensor, Prussian blue (PB), an artificial peroxidase, was used as a selective mediator for H_2O_2 reduction (Karyakin and Karyakina, 1999). PB-modified screen-printed electrodes have already been used in the bridge assembly of various enzyme sensors for the detection of oxidase substrates (Deng et al., 1998; Garjonyte and Malinauskas, 2000; O'Halloran et al., 2001). Characteristics such as high efficiency of electron transfer (E^o about 0.0 V vs. Ag/AgCl), stability of electrochemical characteristics and simplicity of method makes PB more favorable modifier than the other mediators, such as TCNQ for H_2O_2 detection (Suprun et al., 2005). Therefore, by immobilization of ChOx on PB modified screen-printed electrode, a highly selective ChOx/PB sensor for determination of POX has been developed.

2. Experimental

2.1. Reagents

Choline oxidase (E.C. 1.1.3.17) from *Alcaligenes* species (17 U mg^{-1}), glutaraldehyde 25% (v/v), Nafion 5% (v/v), bovine serum albumin (BSA), choline chloride, paraoxon and ethyl parathion (EPA) were purchased from Sigma (St. Louis, MO, USA). Potassium phosphate (K_2HPO_4 and KH_2PO_4), potassium ferricyanide ($K_3Fe(CN)_6$), ferric chloride ($FeCl_3$), potassium chloride (KCl), cadmium sulfate hydrate ($CdSO_4{\cdot}8H_2O$), copper (II) sulfate-pentahydrate ($CuSO_4{\cdot}5H_2O$) and lead nitrate [$Pb(NO_3)_2$] were obtained from Merk (Darmstate, Germany). Diazinon was purchased from Afrashimi Kumesh (Tehran, Iran). Decis, pyrimicarb, atrazine and chlorpyrifos were kindly gifted by Drs. B. Heidary Alizadeh and J. Khalghani of Plant Pests and Diseases Research Institute (Tehran, Iran). Unless otherwise stated, all samples were prepared in 50 mM phosphate buffer plus 0.1 M KCl, pH 7.0 and called as phosphate buffer solution (PBS).

2.2. Electrodes

Screen-printed electrodes (SPEs) were obtained from the Laboratory of Biosensors, the University of Florence, Italy. Each SPE (cell) was composed of three plane electrodes printed on a PVC sheet: a circular-graphite working electrode (diameter of 3 mm) on the center and a silver pseudo-reference electrode and a graphite counter electrode on both sides.

2.3. Preparation of Prussian blue-modified SPEs

Prior to electrode modification by PB, SPEs were pretreated by applying an anodic potential of 1.7 V for 3 min in PBS. Then the process for SPEs modification by PB was accomplished by placing 10 μl of a "precursor solution" exclusively on the surface of the working electrode. This solution was prepared by mixing 20 μl of 0.1 M potassium ferricyanide in 10 mM HCl with 20 μl of 0.1 M ferric chloride in 10 mM HCl. The solution was left on the electrode for 20 min at room temperature and then rinsed with few milliliters of 10 mM HCl. The electrodes were then left 60 min in the oven at 100 °C to obtain a more stable and active layer of PB (Ricci et al., 2003). The PB-modified electrodes were stored dry at room temperature in the dark.

2.4. Enzyme immobilization

According to García-Jareño et al. (1996) significant increase of PB stability can be achieved by covering electrode by Nafion. In addition, the Nafion film has high chemical stability, good biocompatibility and also ability to resist interferences from anions and biological macromolecules (Furbee et al., 1993; Andrieux et al., 1990; Liu and Deng, 1995). Therefore, the enzyme immobilization was carried out using Nafion. By adding 0.8 mg of BSA to 20 μl ChOx solution (0.47 mg/ml in PBS) followed by addition of 6 μl of

Nafion (5% v/v in ethanol) a mixture was prepared. Then 2 μl of the mixture solution was applied exclusively on the PB-modified working electrode (prepared in Section 2.3). Thereafter, the enzyme was cross-linked by adding 1 μl of a 0.05% (v/v) glutaraldehyde to the mixture solution and then left to evaporate at room temperature. Finally, the electrodes were washed with a solution of 0.1 M glycine to saturate all the free aldehyde groups (Ricci et al., 2003).

2.5. Amperometric measurement of enzyme activity

ChOx activity was assayed by chrono-amperometric determination of H_2O_2 produced under the catalytic activity of the enzyme on a PB-modified SPE. Electrochemical measurements were performed with a potentiostat/galvanostat (Model 263A, EG&G, USA).

The assay was carried out in PBS with an applied potential of −50 mV vs. Ag/AgCl (Ricci et al., 2003). A drop (50 μl) of PBS containing different amounts of choline (up to 1.5 mM) was placed onto the ChOx-PB-SPE cell in such a way that all three plane-electrodes were covered with the solution. By applying the potential, the current was monitored continuously and recorded after 10 s.

2.6. POX determination procedure

POX could be determined by controlling the inhibitory effect of POX on ChOx-PB-modified SPE. The decrease in electric current produced by H_2O_2 as a product of enzymatic reaction is proportional to POX concentration. In our previous study (Tavakoli et al., 2005), POX showed an uncompetitive inhibitory effect on ChOx; therefore, inhibitory analysis was carried out in the presence of a certain concentration of choline at which the enzymatic velocity was reached to its max. 50 μl of freshly prepared inhibitor solution containing a certain amount (enzyme-saturated concentration) of choline was dropped onto the working electrode surface of sensor. For determination of the optimum incubation time, different periods (5–30 min) were examined. The incubation was followed by three times rinsing the sensor with deionized water. Then, the response was measured towards choline as described in Section 2.5. Finally, the inhibition percent ($I\%$), as the relative decay of the biosensor response, was calculated based on Eq. (2):

$$I\% = 100\ \frac{(I_o - I)}{I_o} \quad (2)$$

where I_o and I represent the biosensor response before and after incubation, respectively.

2.7. Selectivity of biosensor

In order to control the selectivity of biosensor the same procedure for POX determination (Section 2.6) was applied for assay of some widely used pesticides such as atrazine, decis, pyrimicarb; some OP compounds such as chlorpyrifos, diazinon, ethyl parathion; and some heavy metal ions, as well. The final concentrations of the tested metal ions (Cu^{2+}, Pb^{2+} and Cd^{2+}) were 0.3 mg/l in de-ionized water. Exceptionally, in the case of heavy metal ion solutions, ChOx was incubated in the absence of substrate (choline).

3. Results and discussion

3.1. Optimum enzyme concentration

The biosensor response on choline or inhibitor may be affected by the enzyme loading on the electrode surface. In order to optimize the biosensor response to POX, a set of four electrodes with different ChOx loading was prepared. The experimental results depicted in Table 1 indicate that at the enzyme-saturated concentration of choline, the peak current was decreased by lowering the enzyme loading. At the enzyme loading below 0.47 mg/ml the signal/noise ratio was not satisfactory for studying the inhibition process (data not shown). So, this concentration was chosen as minimum enzyme loading for producing a significant basic current. In order to establish a relationship between ChOx loading and inhibition caused by POX, the inhibition percentage was measured against the enzyme loading change while POX and choline concentrations as well as the incubation time were kept constant. Table 1 shows the relative percent inhibition (RI%) for ChOx loading from 0.47 to 0.95 mg/ml. These experimental results were obtained after 5 min incubation of ChOx with 4 μM POX at the enzyme-saturated choline concentration. As expected, RI% reduced by increasing the enzyme loading. Indeed, when the total enzyme activity is low due to a small amount of immobilized enzyme, the applied POX concentration caused a considerable inhibition (RI% = 45). In contrast, by loading a large amount of immobilized enzyme, only a low percent of enzyme is inhibited by the applied POX. Consequently, the data depicted in Table 1 indicates that the biosensor B (prepared using 0.47 mg/ml of enzyme) could provide a sufficient analytical response. So, this concentration of ChOx was chosen as optimum enzyme loading.

3.2. Substrate concentration dependency of the biosensor for POX determination

In order to calibrate the ChOx/PB/SPE biosensor for OP compounds, at first it was necessary to find the optimum conditions for obtaining high response toward choline as substrate. Therefore, the analytical parameters such as response time, linear range, detection limit, sensitivity and reproducibility of the biosensor for determination of choline were optimized. All measurements were performed in PBS at pH 7.0 and applied potential of −50 mV. The ChOx/PB/SPE achieved 95% of steady state currents within 10 s. The calibration plot (Fig. 1A) gave the linear working range up to the value of 0.4 mM of choline concentration ($y = 0.3551x + 0.0132$, $R^2 = 0.9901$, where y and x denote the current in μA and concentration of choline in mM, respectively). The minimum detectable current response was observed at 12.7 μM choline concentration. The calibration curve shows a sensitivity of 5.03 μA mM^{-1} cm^{-2} with a R.S.D. of 5% ($N = 3$). The R.S.D. value found to be 3.3% at 0.9 mM choline concentration.

In the next step, the effect of substrate concentration on the biosensor response toward POX was investigated. To examine this parameter, choline concentrations were varied in the range of 0.09–2 mM and the relative inhibition percent was tested after 5 min incubation with 4 μM POX. As seen in Fig. 1B, by increasing substrate concentration, the enzyme inhibition percent increased

Table 1
Amperometric responses and relative percent inhibitions for different ChOx loadings on Prussian blue-modified SPEs.

Modified SPEs	[ChOx*] (mg/ml)	$[Choline]_{Vmax}$ (mM/l)	Amperometric current (nA)	Relative inhibition (RI%)
A	0.34	0.3	100 ± 15.0	100
B	0.47	0.9	215 ± 7.1	45 ± 2.2
C	0.68	1.4	340 ± 13.5	33 ± 1.5
D	0.95	2.0	450 ± 20.2	21 ± 1.0

Amperometric current responses were obtained at a concentration of choline at ChOx V_{max}. RI% was calculated based on the description of experimental section after 5 min incubation of enzyme electrode in a 4 μM POX solution containing a concentration of choline at ChOx V_{max}. For ChOx immobilization, 2 μl of enzyme solution was used according to the enzyme immobilization method explained in Section 2.

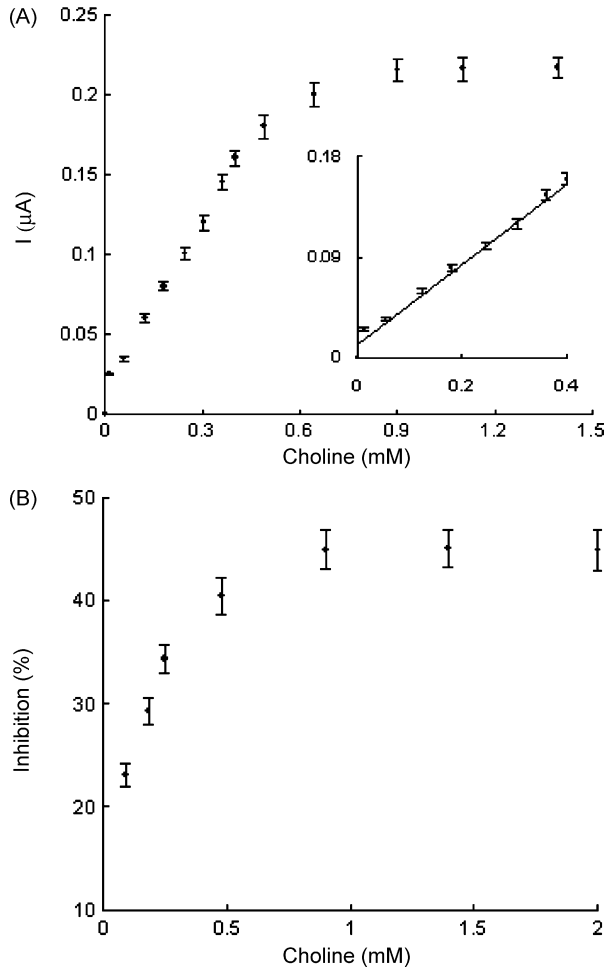

Fig. 1. (A) Calibration curve of ChOx-PB-SPE for choline. For the experimental condition detailes see text (Section 2.5). The inset shows the linear range and detection limit for choline. The points are mean values of three independent measurements. (B) Effect of substrate concentration on ChOx inhibition. Concentration of POX was 4 μ and incubation time was 5 min. Other conditions were the same as (A).

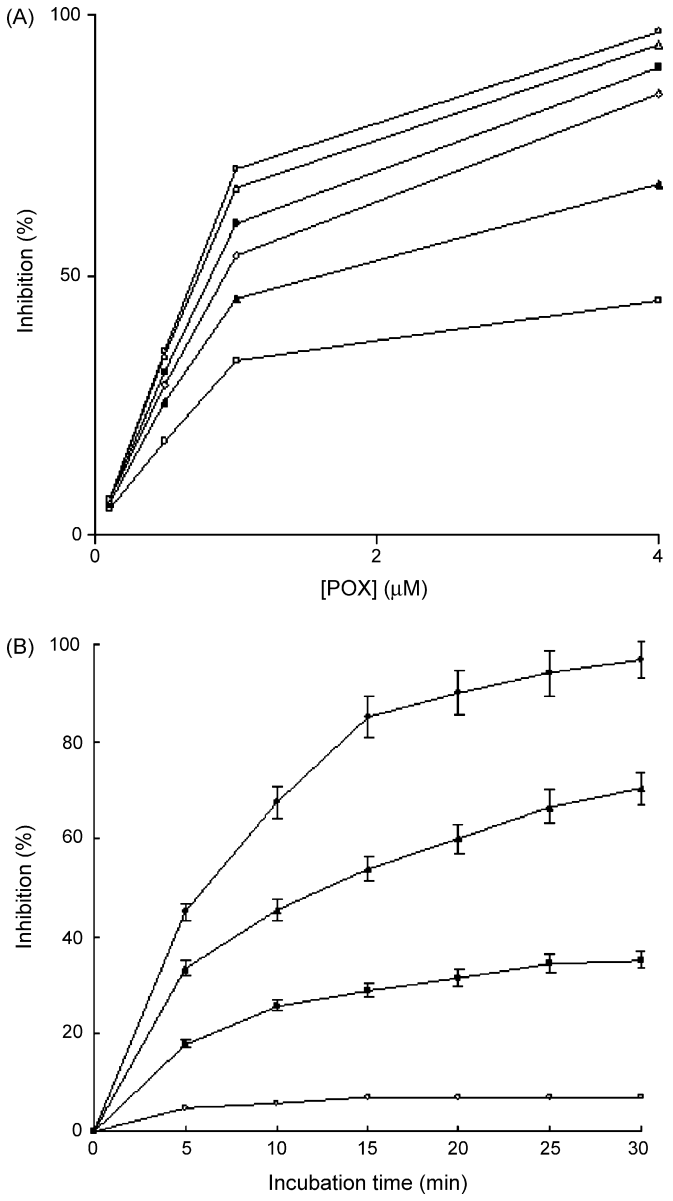

Fig. 2. Effect of incubation time and POX concentration on ChOx inhibition. (A) Represents the inhibition percent against POX concentration at different incubation times (from down to up 5, 10, 15, 20, 25 and 30 min, respectively). (B) Represents the inhibition percent at different incubation times in the presence of POX (from down to up 0.1, 0.5, 1 and 4 μM). The POX samples were prepared in 0.9 mM choline chloride solution. Other conditions were the same as Fig. 1. For the experimental condition details see text (Section 2.6).

until reaching a plateau. Therefore, low pesticide concentrations could only be detected at substrate concentration above 0.9 mM, where the enzyme was saturated with the substrate. By evaluation of the relative inhibition percentage obtained by both the active and inhibited forms of the enzyme, one may conclude that all the enzyme molecules must be involved in the reaction and this could only be possible if the enzyme is saturated with substrate. Moreover, the inhibition of ChOx with POX is shown to be uncompetitive (Tavakoli et al., 2005). Uncompetitive inhibitor can only bind to the enzyme–substrate complex. By increasing the substrate, the enzyme–substrate complex increases so that more POX molecules can bind. But after the saturation of enzyme with choline, further increase in choline amount would not yield more enzyme–substrate complex. Therefore to achieve maximum inhibition, it is rational that the enzyme be kept saturated with substrate.

3.3. Effect of incubation time on POX determination

The effect of incubation time (5, 10, 15, 20, 25 and 30 min) on ChOx inhibition was examined at four POX concentrations (0.1, 0.5, 1 and 4 μM) while the enzyme was saturated with substrate (0.9 mM). The results are shown in Fig. 2. These data can be considered from two points of view. At first, it is important to notice the inhibition profile against POX concentration at different incubation times. As shown in Fig. 2A, the plots were found to be linear only at the POX concentration from 0.1 to 1 μM. At concentrations higher than 1 μM it tends to reach a plateau. Consequently the POX concentration over 1 μM is out of the linear range of the biosensor response. Therefore, we should ignore it in the next step of optimization.

In the next step, the inhibition percentage plotted against incubation time at different POX concentrations (Fig. 2B). It is important to note that after 5 min incubation, the POX inhibition is almost stopped at low concentration (0.1 μM). But the highest increase in response was pronounced at 4 μM POX; however, as discussed, this concentration is out of the sensitivity determining range. At concentrations of 0.5 and 1 μM POX, the response still increased gradually. There is always a tradeoff between incubation time and sensitivity. However, many practical analytical applications require rapid analysis. In view of the fact that the slope of sensitivity is less than that of

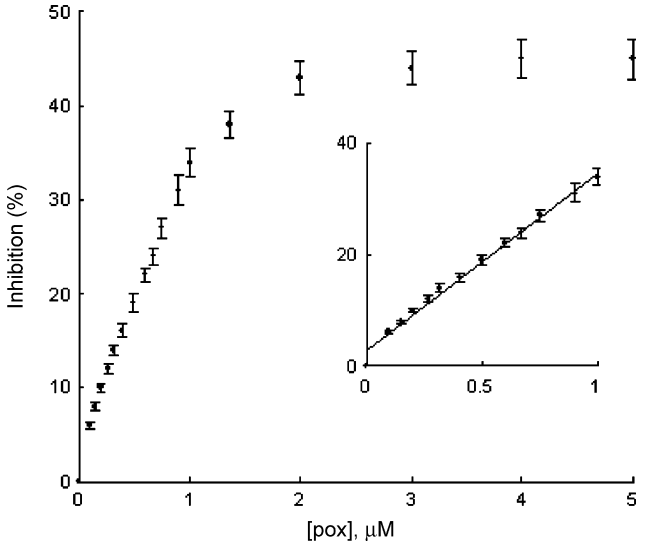

Fig. 3. Calibration curve for POX detection. For the experimental condition detailes see text (Section 2.6).

incubation time (Fig. 2B), it is useful to choose the lowest incubation time that leads to a reasonable sensitivity. As shown in Fig. 2, in any case, a significant enzyme inhibition with a reasonable sensitivity was pronounced after 5 min incubation. Therefore, 5 min incubation time was selected for further analysis. In such a condition, the ChOx/PB/SPE was able to detect POX with a relative standard deviation below 5%. However, longer incubation times could be used to obtain more sensitive responses where fast analysis is not crucial.

This value is suited favorably with most of the data reported in the literature for other enzyme electrodes. For example, the incubation times of 5, 3–5, 10 and even 60 min have been reported by Kok et al. (2002), La Rosa et al. (1994), Suprun et al. (2005), Skladal (1992) and Cho et al. (1999), respectively.

3.4. Analytical characteristics of the sensor for POX determination

3.4.1. POX calibration curve

Determination of POX was carried out by 5 min pre-incubation of immobilized enzyme with appropriate concentration of inhibitor in the presence of enzyme-saturated (0.9 mM) choline solution. The calibration curve was obtained by plotting the inhibition percentage versus POX concentration (Fig. 3.). The plot was found to be linear over the range 0.1–1 μM (6–34% inhibition) with the linear regression equation of $y = 32.002x + 2.7284$, $R^2 = 0.991$ and standard deviation 2%, $N = 3$. As shown in Fig. 3, 0.1 μM of POX is the minimum detection value. The sensitivity of the biosensor was 32% per μM.

3.4.2. Selectivity

Selectivity is an important criterion for any analytical tool. Table 2 shows the response of the biosensor to a range of OP compounds such as chlorpyrifos, diazinon and EPA and other interfering compounds that are commonly encountered in real samples. As can be seen, chlorpyrifos and diazinon did not interfere with the biosensor response; however, the biosensor showed a weak response to EPA yielding a response slope ratio (POX/EPA) of 16. This indicates high selectivity of ChOx/PB/SPE biosensor to POX relative to three other OP compounds. Besides, the other widely used pesticides and insecticides such as atrazine, decis and pyrimicarb showed no interference. Also by applying up to 0.3 mg/l of heavy metal cations such as Cu^{2+}, Pb^{2+} or Cd^{2}, no interference was observed (data not shown). This could be accounted as an advantage over the AChE-based biosensors, which are unable to differentiate between OP and other neurotoxic compounds (Trojanowicz, 2002).

Table 2
Selectivity of the ChOx-PB-SPE to OP compounds.

Compound	Concentration (μM)	Biosensor response (RI%)
Paraoxon	1	34
Chlorpyrifos	1	0
Diazinon	1	0
Ethyl parathion	1	11
Ethyl parathion	10	29
Decis	1	0
Pyrimicarb	1	0
Atrazine	1	0

3.4.3. Storage stability

To investigate the long-term storage stability, the biosensor response to 0.9 mM choline was controlled every 2 days and then it was stored at 4 °C in PBS, pH 7.0. As demonstrated in Fig. 4, only an average decrease of 10% in response was observed at the end of a 30 days period.

3.4.4. Reproducibility

The ChOx/PB/SPE biosensor demonstrated a low relative standard deviation of 2% for the reproducibility of responses in the same electrode ($N = 3$) and up to 5% for the reproducibility of the results from electrode to electrode ($N = 3$).

3.4.5. Biosensor evaluation

The results obtained by the mono-enzyme ChOx-based biosensor were compared with those previously reported bi-enzyme biosensors (Palleschi et al., 1992; Bernabei et al., 1993; Cremisini et al., 1995; Curulli et al., 2001; Ciucu et al., 2003). Although sensitive and useful for environmental monitoring, the two-enzyme biosensors had the disadvantages of long incubation time (20–120 min) and high working potential (350–700 mV vs. Ag/AgCl); while the present mono-enzyme biosensor, showed the advantage of omitting AChE and applying ChOx as both specific biosensing element and detecting system. Accordingly, the characteristics such as lower cost, simplicity, selectivity, and short incubation time (5 min) made the ChOx/PB/SPE biosensor more favorable with regard to bi-enzyme biosensors. On the other hand, compare to the previously developed mono-enzyme biosensors, it showed the advantage that the synthetic and unstable substrate of ATCh was replaced with choline as a native and stable substrate. A decrease in the working potential (−50 mV vs. Ag/AgCl) was also achieved by modification of the transducers by PB.

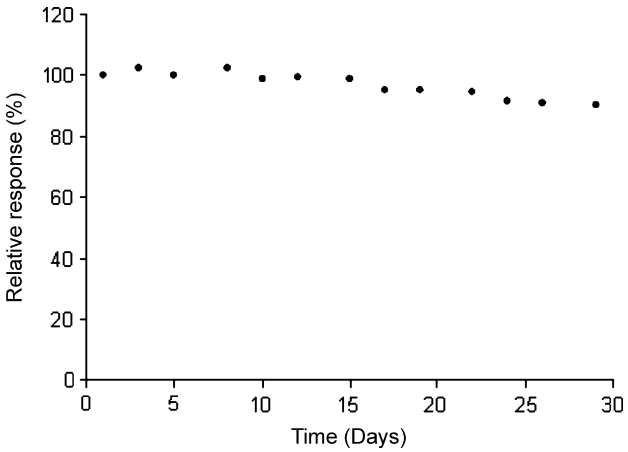

Fig. 4. The biosensor long-term stability when stored at 4 °C in PBS pH 7.0 between measurements. For the experimental condition details see text (Section 2.5).

4. Conclusion

The problem of the detection of OP compounds using enzymatic inhibition biosensors has already been tackled by numerous researchers using electrochemical multi-enzyme systems (Cremisini et al., 1995; Fennouh et al., 1997; Ciucu et al., 2003). However, in the multi-enzyme biosensors in which AChE and ChOx co-immobilized on the electrode surface, the inhibition of ChOx by POX at the incubation step has usually been neglected. While, in ChOx/PB/SPE biosensor the inhibition of ChOx was essential for POX detection. In addition to the lower cost and simplicity of this biosensor, introducing ChOx as a new bio-sensing element for POX compounds showed some advantages over the conventional systems; it represented a good selectivity to POX. Moreover, choline as a native and stable substrate makes this biosensor more favorable with regard to other previously reported mono-enzymatic AChE-based biosensors. These characteristics make the ChOx/PB/SPE biosensor attractive for further development as a potential analytical method and could bring it closer to commercial purposes. The efficiency of the biosensor response for real samples is under investigation in our laboratory.

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