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Flow Injection Biosensor for the Detection of Anti-Cholinesterases

Myung-Sun Chung, Yong-Tae Lee,† and Hye-Sung Lee*

Department of Food Science and Nutrition, College of Human Ecology, Kyungpook National University, Taegu 702-701, Korea

†Department of Biochemistry, Yeungnam University, Kyongsan 712-749, Korea

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A potentiometric flow injection biosensor for the analysis of anti-cholinesterases (anti-ChEs), based on inhibition of enzyme activity, was developed. The sensor system consists of a reactor with acetylcholinesterase (AChE) immobilized on controlled pore glass and a detector with an H^+-selective PVC-based membrane electrode. The principle of the analysis is based on the fact that the degree of inhibition of AChE by an anti-ChE is dependent on the concentration of the anti-ChE in contact with AChE. The sensor system was optimized by changing systematically the operating parameters of the sensor to evaluate the effect of the changes on sensor response to ACh. The optimized biosensor was applied to the analysis of paraoxon, an organophosphorus pesticide. Treatment of the inhibited enzyme with pyridine-2-aldoxime fully restored the enzyme activity allowing repeated use of the sensor.

Keywords: Acetylcholinesterase, Anti-cholinesterase, Biosensor, Flow injection analysis.

Introduction

There have been numerous reports on the development of biosensors using enzymes as bioreceptors (Blum and Coulet, 1991; Scheller and Schubert, 1992; Wagner and Guilbault, 1994). These devices are based on the conjugation of an enzyme and an electrochemical electrode; the enzyme recognizes the substrate to be determined and specifically converts it into a product that is measured by the adjacent electrode. Recently, some biosensors have also appeared based on the inhibition of immobilized enzymes; for example, cholinesterase (ChE)-based sensors for the detection of anti-ChEs such as organophosphorus nerve gases and pesticides which act as inhibitors of ChEs (Tran-Minh *et al.*, 1985; Tran-Minh *et al.*, 1990; Dumschat *et al.*, 1991; Skládal, 1991; Takruni *et al.*, 1993; Cho and Cha, 1994; Ivnitskii and Rishpon, 1994; Martorell *et al.*, 1994; Evtyugin *et al.*, 1996; Ghindilis *et al.*, 1996; Skládal, 1996; Trojanowitz and Hitchman, 1996). The principle of these biosensors is based on the fact that the degree of inhibition of ChE by an anti-ChE is dependent on the concentration of the anti-ChE in contact with the enzyme, and the degree of inhibition can be determined by monitoring the decrease of enzyme activity through the measurement of chemical changes in the enzyme reaction (Giang and Hall, 1951; Mendoza *et al.*, 1968; Guilbault *et al.*, 1970; Alfthan *et al.*, 1989; Zoun and Spierenburg, 1989; Argauer and Brown, 1994; Evtyugin *et al.*, 1997). Acetylcholinesterase (AChE) and butyrylcholinesterase have been widely used as bioreceptors for this type of biosensors. According to the method selected for measurement of enzyme activity, a variety of potentiometric (Tran-Minh *et al.*, 1985; Tran-Minh *et al.*, 1990; Dumschat *et al.*, 1991; Cho and Cha, 1994; Ivnitskii and Rishpon, 1994; Evtyugin *et al.*, 1996; Ghindilis *et al.*, 1996; Skládal, 1996; Trojanowitz and Hitchman, 1996), amperometric (Skládal, 1991; Martorell *et al.*, 1994; Trojanowitz and Hitchman, 1996), and spectrophotometric (Takruni *et al.*, 1993) biosensors were developed.

AChE catalyzes the hydrolysis of acetylcholine to yield choline and acetic acid, thus its activity can be determined by measuring the pH change potentiometrically with an H^+-selective electrode. Sensors based on this principle can be built either by immobilizing AChE directly on an H^+-selective electrode or in flow injection analysis (FIA) type that offers advantages of highly reproducible timing and high sample throughput (El Yamani *et al.*, 1988; Kumaran and Tran-Minh, 1992). In the potentiometric FIA method, the enzyme is normally immobilized on glass beads to be packed into a glass column and an electrochemical electrode is used as the detector. Since ion-selective glass electrodes are expensive and have a much shorter life-span

* To whom correspondence should be addressed.
Tel: 82-53-950-6231; Fax: 82-53-950-6229
E-mail: hslee@bh.kyungpook.ac.kr

when used for measuring enzyme inhibition, in which they are incubated in toxic sample solutions for long periods of time, it is desirable to use disposable detectors that can be prepared rapidly and inexpensively in the lab.

In this paper, we describe the development of an FIA enzyme sensor with such a disposable detector that consists of a tubular H^+-selective membrane electrode fabricated by incorporating a neutral carrier within a PVC matrix. The reactor consisted of AChE immobilized onto controlled pore glass (CPG). The sensor was optimized by changing systematically the operating variables of the sensor and examining the effect of these changes on sensor response to ACh. The usefulness of the sensor was evaluated by the application of the optimized sensor to the analysis of paraoxon, an anti-ChE. It is well-known that several oximes restore ChE activity after the enzyme has been irreversibly inhibited by anti-ChEs (El Yamani *et al.*, 1988; Tran-Minh *et al.*, 1990; Kumaran and Tran-Minh, 1992; Takruni *et al.*, 1993; Trojanowitz and Hitchman, 1996). In this work, reactivation of the inhibited AChE by pyridine-2-aldoxime (PAM) for the repeated use of the proposed sensor was also studied.

Materials and Methods

Materials Polyvinyl chloride (PVC, high molecular weight), tridodecylamine, and tetrahydrofuran (THF) were purchased from Fluka (Buchs, Switzerland) and dioctyl phthalate from Aldrich (Milwaukee, USA). Sodium tetraphenylborate, acetylcholinestrase (V-S type from electric eel, 1000–2000 units/mg protein), acetylcholine chloride, glutaraldehyde (25% aqueous solution), pyridine-2-aldoxime methiodide and N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES) were obtained from Sigma (St. Louis, USA). Three types of aminopropyl CPG obtained from Sigma were used as solid supports for enzyme immobilization: (a) 80–120 mesh (average pore size 700 Å, amine content 77 μmol/g), (b) 200–400 mesh (500 Å, 70 μmol/g), and (c) 200–400 mesh (75 Å, 152 μmol/g). Paraoxon-ethyl was from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

FIA manifold The configuration of the FIA manifold used is shown in Fig. 1. The carrier stream was pumped through the FIA manifold by a multi-channel peristaltic pump (Ismatec model IPC). Samples were injected into the carrier stream using a four-way rotary injection valve (Rheodyne). The decrease of pH in the sample plug was sensed with a homemade membrane electrode and a double junction reference electrode (Orion 90-02) in combination with a pH/mV meter (Mettler model 340), and was recorded on a flatbed recorder (Kipp & Zonen model BD 111). The carrier consisted of HEPES buffer containing 20 mM $MgCl_2$ and 100 mM NaCl which were added for enzyme stabilization (Dudai, 1974). This buffer solution will be referred to "HEPES working buffer" below. Reactors were assembled by packing AChE-immobilized CPG in a 2.06 mm i.d. Tygon tubing. The components of the system were connected with a 0.89 mm i.d. Tygon tubing.

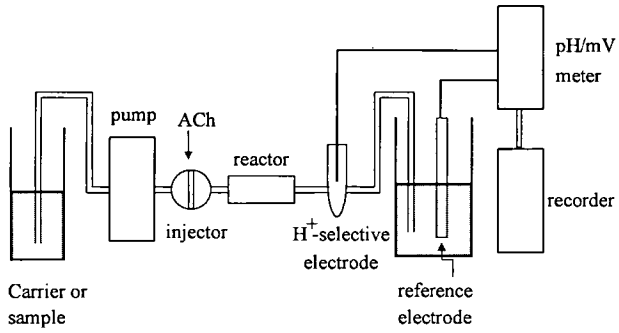

Fig. 1. Schematic diagram of the AChE-based flow injection biosensor.

Detector, tubular H^+-selective membrane electrode The casting solution for the H^+-selective PVC-based membrane electrode was prepared by dissolving 20 mg of tridodecylamine (neutral carrier), 132 mg of dioctyl phthalate (plasticizer), 1.4 mg of sodium tetraphenylborate (liphophilic additive), and 51 mg of PVC (polymeric matrix) in 1 ml of THF. For the construction of the H^+-selective electrode in tubular form (Meyerhoff and Kovach, 1983), an 18-gauge syringe needle was inserted in a ca. 45 mm Tygon tubing (i.d. 0.64 mm) and a 5 mm hole was cut out lengthwise with a razor blade. Onto the cut-out region was poured 6 drops of the casting solution with THF being evaporated after each addition. After pulling out the needle from the tubing, the membrane tubing was inserted through the two holes drilled on the wall of a plastic centrifuge tube of 1.5 ml capacity (outer jacket) and the holes were sealed with cyclohexanone-PVC paste (1 ml/1 mg) with the membrane facing down (Fig. 2). The detector was completed by pouring 0.5 M citrate buffer solution (pH 4.5) and two drops of 10^{-2} M KCl (internal filling solution) into the jacket and placing an electroplated silver wire in the internal solution.

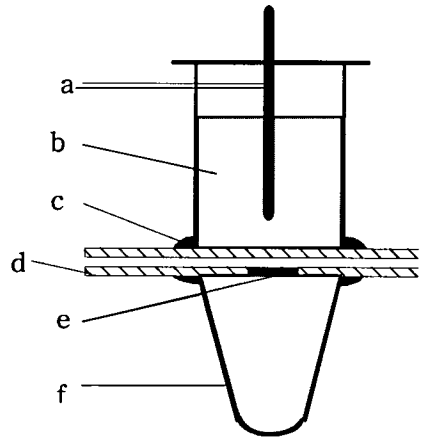

Fig. 2. Schematic diagram of the detector of the AChE-based biosensor, H^+-selective electrode. (a) Ag/AgCl wire, (b) internal solution, 0.5 M citrate buffer, pH 4.5 and 10^{-2} M KCl, (c) sealant, (d) Tygon tubing (i.d. 0.64 mm), (e) tridodecylamine-PVC membrane, and (f) 1.5 ml centrifuge tube (outer plastic jacket).

Immobilization of AChE AChE was immobilized according to the procedure of Gnanasekaran and Mottola (1985). Functionalization (aminosilane-treatment) of CPG was omitted by using aminopropyl CPG that is commercially available. To 1 g of aminopropyl CPG was added 3.3 ml of 2.5% (w/v) solution of glutaraldehyde in 0.05 M phosphate buffer, pH 7 (PB). The mixture was shaken in a shaker for 1 h and the solid then washed with distilled water to remove the unreacted glutaraldehyde. To activated glass was added AChE (330 units) in 2.4 ml of PB, and the mixture was stored in a refrigerator at 4°C overnight. The beads were washed successively with distilled water, 1 M KCl, distilled water and 2.5 mM HEPES working buffer (pH 7.5), and then stored in a refrigerator at 4°C until they were used.

Performance of the detector In order to evaluate the performance of the H^+-selective membrane electrode, while passing 2.5 mM HEPES working buffer (pH 7.5) through a reactor-free FIA system at 1 ml/min flow rate, 50 μl of the pH standard solutions over pH range 5–10 were injected into the carrier stream to measure the detector response.

Response of the detector to ACh The response of the sensor to ACh may result from nonspecific sensing of the electrode for unreacted ACh (a cation) rather than the pH change by the enzyme reaction. To examine this possibility, the response of a reactor-free sensor to ACh over the concentration range 10^{-3}–10^{0} M was measured. The carrier was 2.5 mM HEPES working buffer (pH 7.5) at 1 ml/min flow rate.

Optimization of the sensor system To optimize the sensor system, the various operating variables were changed as follows and the effect of the changes on sensor response was examined.

(i) Size of glass beads Three commercially available CPGs were tested for use as solid supports for enzyme immobilization. They were aminopropyl CPG of (a) 80–120 mesh (average pore size 700 Å), (b) 200–400 mesh (500 Å), and (c) 200–400 mesh (75 Å). The conditions for operating the sensor were: reactor length, 7 cm (i.d. 2.06 mm); carrier, 2.5 mM HEPES working buffer (pH 7.5); flow rate, 1 ml/min; substrate sample injected, 50 μl of 10^{-3}, 10^{-2}, and 10^{-1} M ACh.

(ii) Reactor length The effect of varying the reactor tube length on sensor response was examined using reactors of 4, 7, and 10 cm length (i.d. 2.06 mm). The CPGs used were of 80–120 mesh (700 Å) and 200–400 mesh (500 Å), and the rest of the operating conditions were the same as those used in (i).

(iii) Flow rate The effect of varying the flow rate on sensor response was examined. Flow rates employed were 0.4, 0.7, 1.0, and 1.3 ml/min. The CPG used was of 200–400 mesh (500 Å) and the rest of the operating conditions were the same as those used in (i).

(iv) Injection volume of ACh The difference between the sensor responses for using 50 and 100 μl of substrate sample was compared. The carrier flow rate was 0.7 ml/min and the rest of the operating conditions were the same as those used in (iii).

(v) Concentration of carrier buffer The variation of sensor response for different concentrations of carrier buffer was examined using 2.5 mM HEPES working buffer (pH 7.5) over the concentration range 0.5–5 mM. The substrate sample injected was 100 μl of 10^{-2} M ACh and the rest of the operating conditions were the same as those used in (iv).

(vi) pH of carrier The variation of sensor response for different pHs of carrier buffer was examined using 2.5 mM HEPES working buffer over the pH range 7.0–8.5. The operating conditions were the same as those used in (v).

Application of the sensor to analysis of paraoxon In order to evaluate the general utility of the sensor for the detection of anti-ChEs, the sensor was applied to the analysis of paraoxon, an anti-ChE. First, the variation of percent inhibition of the enzyme for different incubation times (5–35 min) was examined. Percent inhibition was determined by comparing the enzyme activity before and after passage of paraoxon through the reactor for given periods of time. Following this, a calibration curve for paraoxon over the concentration range 10^{-8}–10^{-5} M was obtained. The paraoxon working solutions were prepared by diluting the stock solution of paraoxon in ethanol with 2.5 mM HEPES working buffer, pH 7.8 (2% solution). The incubation time was 20 min. The operating conditions for these two experiments were: CPG, 200–400 mesh (500 Å); reactor length, 4 and 7 cm; carrier, 2.5 mM HEPES working buffer (pH 7.8); flow rate, 0.7 ml/min; substrate sample, 100 μl of 3.16×10^{-3} (4 cm reactor) or 10^{-2} M (7 cm reactor) ACh.

Reactivation of the inhibited AChE by 2-PAM was studied. The enzyme was inhibited by 10^{-5} M paraoxon as described above and the recovery of the enzyme activity after different time period of the passage of 20 μM 2-PAM in HEPES working buffer (pH 7.8) was examined. Reactivation of the enzyme inhibited by 10^{-6} M paraoxon was similarly examined.

Stability of the reactor enzyme The decline in activity of immobilized enzyme over the period of six months was examined. The operating conditions were the same as those used in (i) of the optimization experiment.

Results and Discussion

Performance of the detector The pH response of the H^+-selective membrane electrode is shown in Fig. 3. The electrode showed a near-Nernstian response (54 mV/decade) with a wide range of linear response (pH 5–10). Therefore, the fabricated tubular electrode was considered to be satisfactory as the detector for the proposed sensor.

Response of the detector to ACh The response of the detector to ACh in the concentration range up to 10^{-2} M was negligible (2 mV at 10^{-2} M). The responses to 10^{-1} and 1 M ACh were 7 and 131 mV, respectively. The results indicate that the concentration of ACh injected should be below 10^{-1} M.

Optimization of the sensor system Glass tubes initially used as the reactor columns often caused back pressure

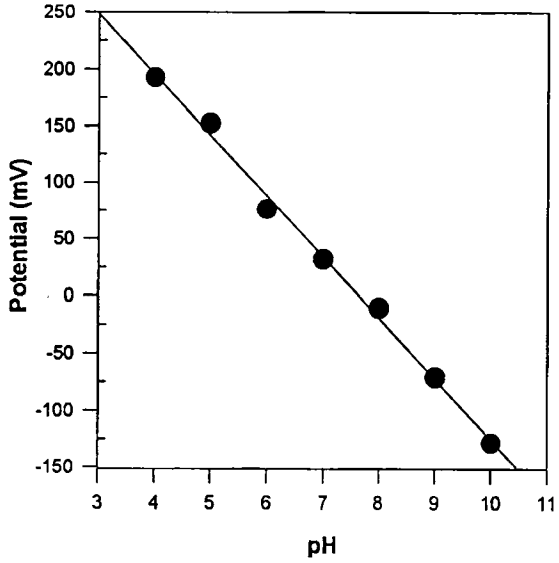

Fig. 3. Response of H^+-selective detector to pH standard solutions. The carrier was 2.5 mM HEPES buffer (pH 7.5) at a 1.0 ml/min flow rate and the injection volume was 50 μl.

problems. Thus, Tygon tubing was tested as an alternative to glass tube. The new columns produced neither resistance to carrier flow, nor any undesirable reactor characteristics. Accordingly, Tygon tubing was chosen as the reactor column for the construction of the AChE-based FIA sensor.

The variation of peak height for using different CPGs as solid supports for enzyme immobilization is shown in Fig. 4. CPGs of 80–120 mesh (average pore size 700 Å) and 200–400 mesh (500 Å) showed three-fold higher response than that of 200–400 mesh (75 Å). It appears that the pore size rather than the bead size is important for high sensor response. Greater pore size will allow faster mass transfer of the species giving a higher response.

The variation of sensor response for different reactor lengths is presented in Fig. 5. At ACh concentrations of 10^{-3}–10^{-2} M, where response due to unreacted ACh is negligible, the reactors of 4 and 7 cm length (i.d. 2.06 mm) gave greater responses than that of 10 cm length. This implies that the 4 cm reactor contains a limited but sufficient quantity of enzyme to effect an almost total conversion. A longer reactor, due to the greater amount of enzyme, will give greater peak height, but, due to the longer time for diffusion, will yield broader peaks. Reactors of 4–7 cm length were considered to offer the best compromise when peak height and sharpness are simultaneously considered.

The experiments carried out to explore the relation between the peak height and flow rate revealed that a faster flow rate, due to the shorter enzyme-ACh contact time, gives smaller but sharper peaks. The flow rate of 0.7 ml/min appeared to be a reasonable one.

Increasing the sample size of ACh from 50 to 100 μl brought about a nearly two-fold increase in sensor response to ACh in the concentration range of 10^{-4}–10^{-2} M, where the detector response to unreacted ACh is negligible. Also, there was no significant decrease in the ratio of peak height to peak width. Therefore, the sample volume of 100 μl was a better choice than that of 50 μl.

The effect of varying carrier buffer concentration on sensor response is shown in Fig. 6. Higher concentration of buffer having higher buffering capacity brought about a lower response but higher peak height/peak width. Here, again, a compromise is required and 2.5 mM seemed to be a convenient compromise.

The effect of varying the carrier pH on sensor response is shown in Fig. 7. The pH of 7.8 seemed to be an appropriate one where both peak height and peak height/peak width are relatively high.

In summary, the optimum parameters chosen for the

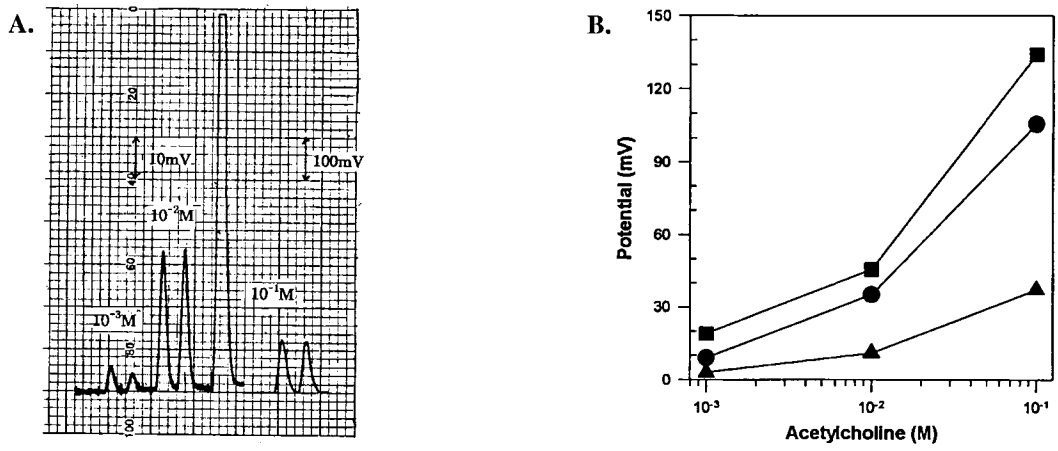

Fig. 4. Variation of sensor response for using different CPGs to immobilize the enzyme. A. Flow-injection peaks. B. Response curves. CPGs, 80–120 mesh (pore size 700 Å) (●), 200–400 mesh (500 Å) (■), 200–400 mesh (75 Å) (▲); reactor length, 7 cm; carrier, 2.5 mM HEPES buffer (pH 7.5); flow rate, 1 ml/min; injection volume of ACh, 50 μl.

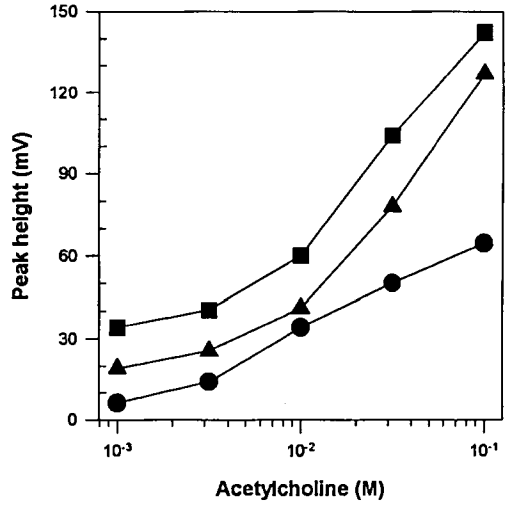

Fig. 5. Variation of sensor response for different reactor lengths. Reactor tube length, 4 cm (●), 7 cm (■), 10 cm (▲); CPG, 200–400 mesh (500 Å); carrier, 2.5 mM HEPES buffer, pH 7.5; flow rate, 1 ml/min; injection volume of ACh, 50 μl.

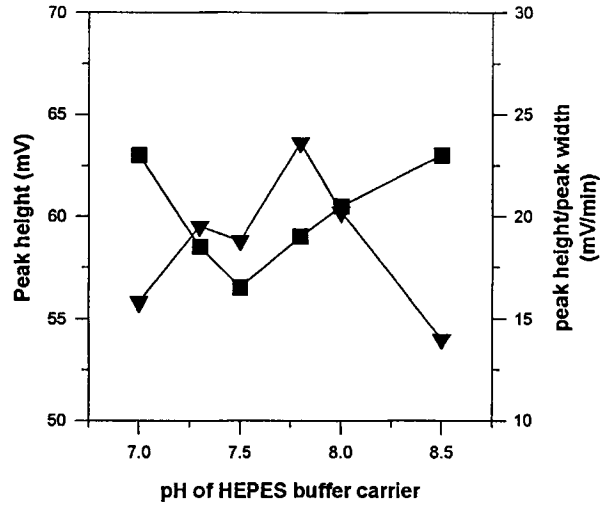

Fig. 7. Effect of carrier buffer pH on peak height (■) and peak height to peak width ratio (▲). Carrier, 2.5 mM HEPES buffer; flow rate, 0.7 mL/min; CPG, 200–400 mesh (500 Å); reactor length, 7 cm; injection volume of ACh (10^{-2} M), 100 μl.

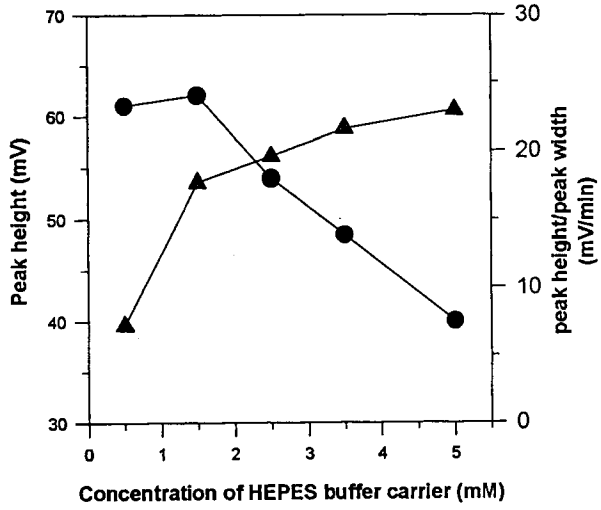

Fig. 6. Effect of carrier buffer concentration on peak height (●) and peak height to peak width ratio (▲). Carrier, HEPES buffer (pH 7.5); CPG, 200–400 mesh (500 Å); reactor length, 7 cm; flow rate, 0.7 ml/min; injection volume of ACh (10^{-2} M), 100 μl.

operation of the proposed sensor are: aminopropyl CPG of 200–400 mesh (500 Å pore size) as the support for enzyme immobilization, the reactor tube of 4–7 cm length Tygon tubing (2.06 mm i.d.), the carrier of 2.5 mM HEPES buffer (pH 7.8) at the flow rate of 0.7 ml/min, and the substrate sample of 3.16×10^{-3} and 10^{-2} M ACh for the 4 and 7 cm reactors, respectively.

Application of the sensor to the analysis of paraoxon Percent inhibition of enzyme progressively increased as the enzyme-paraoxon contact time was increased. The percent inhibition after passing 10^{-6} M paraoxon in HEPES working buffer for 10 min was 49%. After 30 min, it was 88%. Percent inhibition of enzyme by paraoxon over the concentration range 10^{-8}–10^{-5} M after a 20 min enzyme-paraoxon contact time is shown in Fig. 8. The sensitivity of the measurement for relatively low paraoxon concentrations was higher with the 4 cm reactor and 3.16×10^{-3} M ACh than that with the 7 cm reactor and 10^{-2} M ACh. The detection limit (the concentration where the sigmoidal curve originates) under the former and latter conditions were ca. 10^{-8} and 10^{-7} M, respectively. This level of sensitivity in the determination of paraoxon

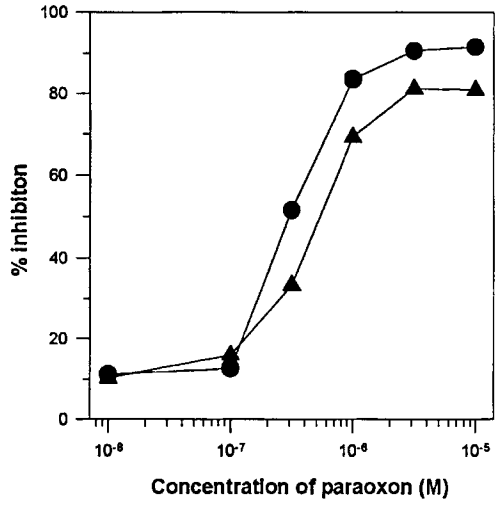

Fig. 8. Calibration curves for paraoxon. Incubation time, 20 min; reactor length, 4 cm (▲) and 7 cm (●); CPG, 200–400 mesh (500 Å); carrier, 2.5 mM HEPES buffer, pH 7.8; flow rate, 0.7 ml/min; ACh concentration, 3.16×10^{-3} M for 4 cm and 10^{-2} M for 7 cm reactor; injection volume of ACh, 100 μl.

was comparable to those observed previously in conventional enzymatic analysis (Giang and Hall, 1951) and in the analysis by AChE sensors (Tran-Minh *et al.*, 1990; Kumaran and Tran-Minh, 1992; Cho and Cha, 1994). Therefore, the proposed sensor is suitable for the sensitive determination of anti-ChEs.

It was found that 20 μM 2-PAM fully restored AChE that had been inhibited by 10^{-6} or 10^{-5} M paraoxon over a 20 min contact time. The incubation times needed were 5 and 30 min, respectively. Figure 9 shows the flow injection peaks after inhibition of the enzyme by 10^{-5} M paraoxon and reactivation of the enzyme by 2-PAM. The results show that 2-PAM treatment of the inhibited enzyme enables repeated use of the reactor.

Storage stability of the immobilized enzyme The storage stability of reactor enzyme was remarkable. The decrease of enzyme activity up to six weeks was negligible. After 6 months, the activity was decreased to 62% of the initial activity.

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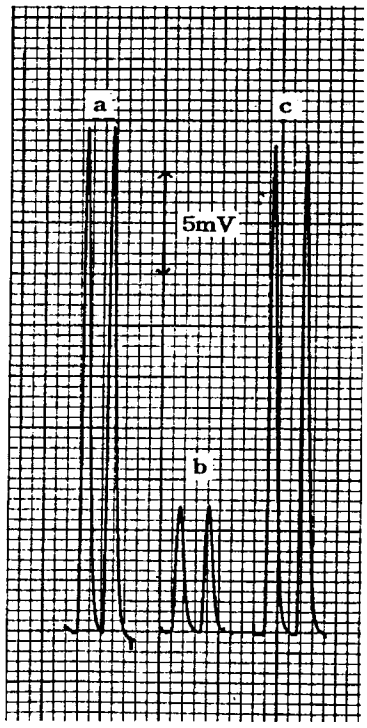

Fig. 9. Dynamic sensor responses (a) before enzyme inhibition, (b) after enzyme inhibition by 10^{-6} M paraoxon (20 min incubation time), and (c) after enzyme reactivation by 20 μM PAM (20 min incubation time). CPG, 200–400 mesh (500 Å); reactor length, 4 cm; carrier, 2.5 mM HEPES buffer (pH 7.8); flow rate, 0.7 ml/min; injection volume of ACh (3.16×10^{-3} M), 100 μl.

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