Covalent immobilization of *Drosophila* acetylcholinesterase for biosensor applications

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The synthesized cDNA coding for AChE (acetylcholinesterase) was subcloned in pENTR™/D-TOPO® plasmid and expressed using baculovirus expression vector and Sf9 insect cells as host. Purified enzyme (specific activity 36 374 μmol · min^{-1} · mg^{-1}) was immobilized on pre-activated perlite (a porous silica matrix) by silanization and glutaraldehyde treatment. The total enzyme immobilized was then measured, and total and specific activity of immobilized AChE was compared with that of soluble enzyme. Using this perlite support not only resulted in a great amount of maintained immobilized enzyme activity (more than 70%, specific activity 26 238 μmol · min^{-1} · mg^{-1}), but also significantly improved stability against temperature (8.7- and 17.7-fold at 50 and 60 °C respectively), urea (2.7-fold) and acetonitrile (1.7-fold). Kinetic studies showed that the K_m value for immobilized enzyme is very similar to the soluble one (0.088 and 0.081 mM respectively). In addition, immobilized enzymes retained 80% of their initial activity after 16 consecutive reactor batch cycles. A comparison of the inhibitory effect of paraoxon on soluble and immobilized AChE showed that immobilization increased the linearity of the inhibition plot particularly in the range 0.1 nM–0.1 μM.

Introduction

AChE (acetylcholinesterase) (EC 3.1.1.7) is a serine hydrolase which catalyses the hydrolysis of the neurotransmitter acetylcholine [1]. Carbamates and organophosphates are potent inhibitors of AChE which carbamylate or phosphorylate the serine residue of the active-site triad which causes the blocking of the nerve signal transference into the postsynaptic membrane [2]. Because of the uniform mechanism of signal transduction in animals, these inhibitors have been used as agricultural pesticides, as well as chemical warfare agents (nerve agents) [3]. Among all methods, biosensors based on AChE appear to be the most simple, rapid and cheap technique for detection of such agents [4].

Development of efficient biosensors requires a suitable immobilization of enzyme in a stable and active condition. The use of immobilized enzyme, especially in a flow-injection system will decrease the price per analysis considerably plus increasing the sample throughput in analytical experiments. In such systems, the immobilized enzyme should be packed in a suitable reactor in such a way that allows even liquid flow throughout [5].

Various immobilization methods, such as simple adsorption, covalent attachment, sol–gel encapsulation and enzyme cross-linking have been described for the immobilization of AChE for biosensor applications [6–9]. Nevertheless, cross-linking with glutaraldehyde is the most widely used method for AChE immobilization [10]. For instance, Singh et al. [3] have immobilized AChE on amine-silanized silica beads using the homobifunctional linker glutaraldehyde. Also, Li et al. [11] have covalently immobilized AChE with glutaraldehyde vapour on the surface of screen-printed electrodes.

Covalent immobilization of enzyme has some advantages such as good stability, low protein loss and high surface covering. However, it may cause loss of protein activity due to the chemical reactions involved. Therefore, for achievement of a functional immobilized enzyme, we should have a detailed understanding of the protein immobilization process and its effect on enzyme conformation and function [12].

Since the 1970s, inorganic materials, such as silica beads, alumina, controlled pore glass and zeolite, have been used as enzyme supports, particularly in FET (field-effect

Key words: acetylcholinesterase, biosensor, *Drosophila melanogaster*, immobilization, paraoxon, perlite.

Abbreviations used: AChE, acetylcholinesterase; APTS, 3-aminopropyltriethoxysilane; ATCh, acetylthiocholine; SEM, scanning electron microscopy.

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transistor)-based biosensors [13]. These inorganic supports have some advantages over organic supports in enzyme immobilization, such as their mechanical, thermal and chemical stability. They are non-toxic, non-swelling and resistant toward pH changes and microbial attack [14–16].

Perlite is an amorphous porous aluminium silicate with a high content of silica of more than 70%. Commercially, the term perlite is used to describe either natural or expanded perlite which is formed by heating quickly [17]. In addition, compared with the other inorganic supports, perlite is inexpensive [18] and, because of its hydrophilic character, provides a desirable microenvironment to decrease resistance to mass transfer [13].

In the present study, expressed and purified *Drosophila melanogaster* AChE was immobilized on perlite. Primary amino groups were made available on the surface of the support by chemical treatment which provided an extra event for cross-linking with enzymes using the bifunctional reagent glutaraldehyde. The kinetic parameters of both soluble and immobilized enzymes were determined. Also storage stability, operational stability, thermal stability and stability of the immobilized enzyme in the presence of organic solvent (acetonitrile) and urea were compared with soluble enzyme. Finally, the response of the immobilized system to an AChE inhibitor (paraoxon) was compared with that of the soluble enzyme.

Experimental

All experiments were performed at least in triplicate.

Materials

A BaculoDirect™ baculovirus expression kit was purchased from Invitrogen. Glutaraldehyde, Triton X-100, DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)] and ATCh (acetylthiocholine) were purchased from Sigma–Aldrich. Procainamide–Sepharose affinity gel was kindly donated by Professor Oksana Lockridge (Eppley Institute, University of Nebraska Medical Center, Omaha, NE, U.S.A.). Perlite was kindly given by Kan Azar Co. Paraoxon was purchased from Supelco. All other chemicals were of analytical grade and were purchased from Merck. Aqueous solutions were prepared using doubly distilled deionized water.

AChE production and purification

The cDNA coding for AChE was synthesized by Entelechon GmbH using the sequence reported by Hall and Spierer [19] and subcloned into pENTR™/D-TOPO® (Invitrogen). BaculoDirect™ C-Term Linear DNA (Invitrogen) derived from AcMNPV (*Autographa californica* multiple nuclear polyhedrosis virus) DNA, was used to build the recombinant baculovirus. It allows the transferral of the gene of interest from the entry plasmid to the baculovirus DNA directly *in vitro*, without the need of recombination in bacterial cells, using specific recombination sites from bacteriophage lambda. The presence of HSV1tk (herpes simplex virus thymidine kinase) gene located between the two recombination sites allows inhibition of replication of non-recombinant baculovirus in the presence of ganciclovir [20]. Propagation of recombinant baculovirus and expression of AChE were made in a *Spodoptera frugiperda*-derived Sf9 cell line. Sf9 cells were maintained in suspension or adherent cultures in Grace's insect cell culture medium (Gibco) supplemented with 10% fetal bovine serum [21].

Secreted proteins were recovered in the medium 4 days post-infection. Cells were pelleted by centrifugation at 800 ***g*** for 10 min, and supernatant was then filtered through a 0.22-μm-pore-size cellulose acetate syringe filter. Proteins were precipitated in 4 M ammonium sulfate and, after centrifugation at 10000 ***g*** for 15 min, were resuspended in 25 mM phosphate buffer (pH 7.4). Enzyme solution was purified on procainamide–Sepharose 4B affinity gel using 10 mM procainamide and 1 M NaCl as eluting buffer [22,23].

Support preparation and AChE immobilization

Perlite support was prepared as described previously [18] with some modifications. Briefly, perlite powder of special mesh size was incubated in methanol for 12 h to remove any organic contaminant. After rinsing and oven-drying at 80 °C for 1 h, 5 M NaOH was added, and the solution was refluxed for 30 min at 120 °C. Precipitated perlite was then washed with excess water and was incubated in 15% APTS solution in acetate buffer (pH 3.3) at 75 °C for 4 h. Alkylamine-derivatized perlite powder was washed thoroughly with a large flow of water in order to remove APTS molecules which did not attach to the surface. It was then soaked in 10% (v/v) glutaraldehyde in deionized water for 1 h at room temperature (24–25 °C). The activated support was washed extensively after glutaraldehyde treatment to remove any adsorbed cross-linker and dried thoroughly at room temperature.

For immobilization, 2 g of dried active perlite was soaked in 3 ml of AChE solution (440 μg/ml, specific activity 36374 $\mu mol \cdot min^{-1} \cdot mg^{-1}$) in PBS, and the suspension was incubated at 4 °C overnight with gentle stirring. The mixture then was washed with PBS and incubated in 100 mM glycine for 30 min in order to block any unreacted aldehyde groups. Finally, it was suspended in 0.05% Tween 20 and 0.5 M NaCl in PBS to remove non-specifically adsorbed enzymes (all steps were carried out on ice). The resulting immobilized enzymes were stored at 4 °C in 25 mM PBS (pH 7.4) before usage. The amount of immobilized enzymes on perlite was determined by measuring the residual enzyme present in all the supernatants.

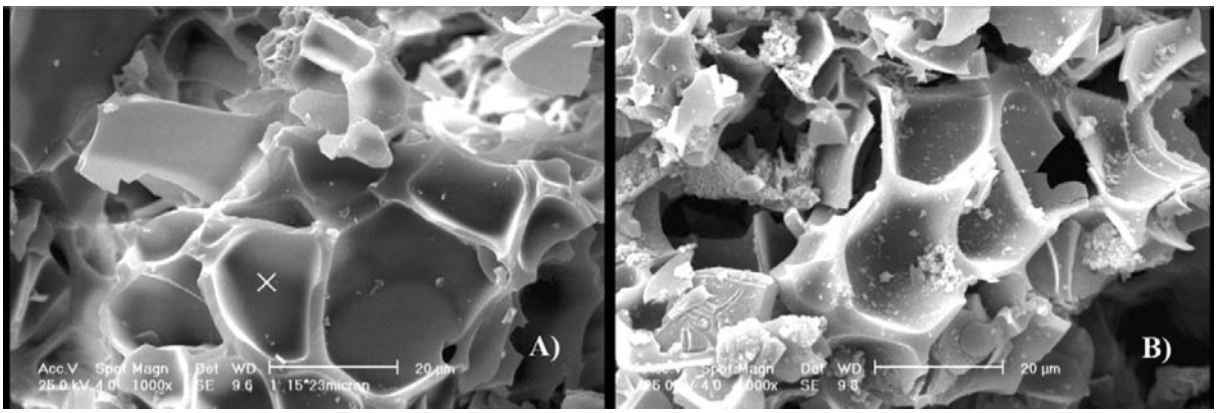

Figure 1 Scanning electron micrographs of perlite particles (A) before chemical treatment and (B) after AChE immobilization

Protein determination

The amount of protein in the different samples was determined using the Bradford method using BSA as standard [24].

Biochemical assay

AChE activity was assayed using ATCh as substrate using the Ellman method [25,26].

The activity of immobilized enzyme was measured as following: 30 μl of immobilized enzyme suspension was added to each reaction mixture in vials, which were sealed and then vortex-mixed for 1 min before being centrifuged at 16000 ***g*** for 30 s. The colour intensity of supernatant was immediately measured at 412 nm to determine product concentration. The concentration-dependent activity and reproducible signals obtained in the present study illustrate the reliability of the measurement approach.

In order to determine kinetic parameters of soluble and immobilized enzyme, a serial dilution of ATCh ranging from 0.01 to 6 mM was used. Values of V_0 were determined over a short reaction time during the initial linear phase of the reaction.

Electron microscopy

The morphology of perlite particles were studied with a Philips scanning electron microscope (XL-30). Before imaging, samples were sputtered with approx. 5–10 nm of gold/palladium mixture (18% palladium) with a BAL-TEC SCD 005 Cool Sputter Coater.

Stability measurement

The thermal stability was determined by studying irreversible thermal inactivation of enzyme incubated in 25 mM PBS (pH 7.4) at 50 and 60 °C. Periodically, samples of the incubated enzymes were removed and incubated on ice for at least 30 min and, subsequently, the remaining activity was determined. The activity of the same enzyme solution, kept on ice, was considered as the control (100%).

Moreover, the stability of soluble and immobilized enzyme in the presence of 4 M urea or 20% (v/v) acetonitrile was measured. Periodically, a 30 μl aliquot of each incubated enzyme was removed and assayed. In both cases, stability was considered by comparing the remaining activity of the soluble and immobilized AChE.

To consider operational stability, a 30 μl aliquot of the immobilized enzyme was packed into a flow-through cell containing a filter with 0.2 μm pores to make an enzyme reactor. Flow system comprised a four-channel Heidolph peristaltic pump equipped with Tygon tubing (1.8 mm internal diameter). All of the experiments were carried out at room temperature using a flow rate of 2 ml/min. To determine the number of times that the enzyme reactor can be reused, the enzyme reactor was exposed repeatedly to substrate solution and phosphate buffer subsequently for 2 min. Each time, the waste (coloured product solution) was sampled and measured spectrophotometrically at 412 nm, and the percentage of the residual activity was compared with original activity.

To determine the shelf life of the immobilized enzyme at 4 °C, enzyme reactors were prepared and stored at 4 °C, and their activities were measured once each day.

Results and discussion

Enzyme immobilization

AChE was immobilized on a modified porous silica support, perlite, using glutaraldehyde as a cross-linker. Porosity and surface property of perlite were analysed by SEM (scanning electron microscopy) before and after immobilization (Figure 1). It is obvious that perlite is a porous support with a pore size of approx. 15–25 μm and chemical modifications of the immobilization process did not affect porosity

Table 1 Enzyme loading and kinetic parameters of soluble and immobilized AChE

Enzyme	K_m (app) (mM)	Yield of immobilization (%)	Protein content (μg/g support)	Remaining activity of the immobilized enzyme (%)
Soluble AChE	0.081 ± 0.003	–	–	–
Immobilized AChE	0.088 ± 0.015	100	132	70

and surface properties of the support. After the immobilization process, the perlite suspension was centrifuged. The activity and protein concentration of supernatant were measured, and no enzymatic activity and protein concentration were detected. Furthermore, blank activated perlite had no detectable activity. It was therefore assumed that all enzyme molecules were bonded to the support surface. The amount of total immobilized enzyme per unit of perlite particles was 132 μg/mg, and immobilized AChE maintained 70% of its activity compared with soluble enzyme during the immobilization procedure (specific activity 26238 μmol $\cdot$ $min^{-1} \cdot mg^{-1}$) (Table 1). The activity loss of immobilized AChE might be attributed to the unsuitable orientation of some immobilized molecules on the support surface. Also, it is accepted that external and internal mass transfer limitations, decreased protein flexibility resulting from multipoint attachment and/or enzyme overcrowding on the surface of the support might affect the performances of the immobilized system [26].

According to previous literature searches and regarding the concentration of glutaraldehyde (10%, v/v, in the present study) and SEM studies, it seems that glutaraldehyde polymerized on the surface of the perlite acts as an enzyme linker [18,27]. According to the *Drosophila melanogaster* AChE crystal structure [28] there are more than 20 lysine residues available on the AChE surface which could react with glutaraldehyde linkers. The presence of such a great quantity of lysine residue on the surface of AChE may cause multipoint attachment of the enzyme and simultaneously decrease molecular mobility [29], leading to decreasing immobilized AChE activity.

Kinetic characterization

The kinetic parameter values of the immobilized and soluble AChE were determined from the Michaelis–Menten equation (Table 1). For both immobilized and soluble AChE, initial estimates of V_{max} and K_m were obtained using the Hanes equation, a linear transformation of the Michaelis–Menten equation (without considering substrate inhibition range) [30]. Considerably, comparing the K_m values of immobilized and soluble AChE did not show significant difference. The amount of K_m for immobilized enzyme strongly depends on mass transfer or diffusion resistance of support to substrate molecules, which is one of the most important factors in using immobilized enzymes in designing enzyme-based biosensors. As shown in Figure 1(B), the side view of particles clearly demonstrates the porous structure of the perlite support. Porous immobilization supports involve two kinds of mass transfer resistance: (i) diffusion limit or resistance to mass transfer from bulk solution to the diffusion layer at the support surface, which is similar to non-porous particles, and (ii) mass transfer resistance as a result of diffusion from bulk solution through the porous matrix. Such effects could increase the K_m < 30-fold [3]. However, in this case, it seems that these resistances did not affect the mass transfer of ATCh.

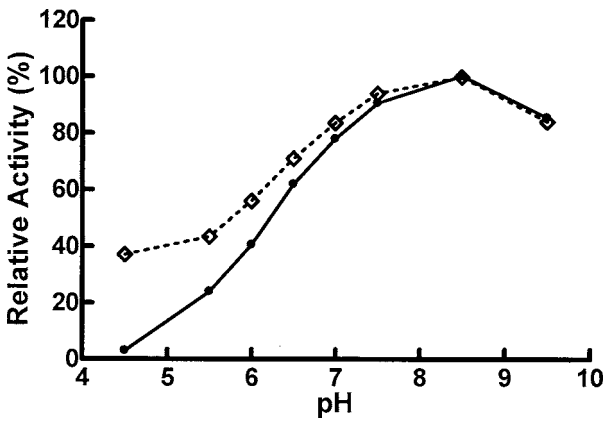

Figure 2 Effect of pH on the activity of (●) soluble and (◇) immobilized AChE

All reactions were carried out at room temperature at various pHs (0.2 M phosphate buffer), and enzymes were added to the reaction mixtures before relative activity determination.

The kinetic profile of the immobilized enzyme appears similar to that of soluble enzyme in solution, even though the concentration of substrate inhibition for both soluble and immobilized enzymes was similar (research not shown).

Effect of pH

The influence of pH on the activity of soluble and immobilized AChE was observed (Figure 2). The results show that the optimum pH for the activity of both kind of AChE is approx. 8.5 and was not affected by immobilization procedure. The activity of both soluble and immobilized enzyme decreased sharply in basic and acidic media. However, as shown in Figure 2, the decrease in the activity of soluble AChE in acidic pH is greater than that of immobilized enzyme. According to previous studies, enzymes immobilized on a perlite support showed better stability at different pH values and have wider pH range than soluble

Table 2 Stability of soluble and immobilized AChE

For each condition, the $t_{1/2}$ (min) was calculated for each denaturing agent.

	$t_{1/2}$ (min)			
Enzyme	50 °C	60 °C	20% acetonitrile	4 M urea
Soluble AChE	1.2 ± 0.1	0.3 ± 0.1	0.9 ± 0.1	1.3 ± 0.1
Immobilized AChE	10.4 ± 1.0	5.3 ± 0.5	1.5 ± 0.1	3.5 ± 0. 2
Immobilized/soluble ratio	8.7	17.7	1.7	2.7

enzymes, but this effect was more significant at lower pH [18]. As mentioned above, while enzymes were immobilized on siliceous activated support, the presence of protonated unchanged amino groups and also Si-OH groups on the surface of the support might repel protons from the region in the vicinity of the surface and create a higher pH at the boundary layer between the support and the bulk solution. This microenvironment with a pH slightly higher than that of the bulk solution led to the lowering of apparent optimal pH which occurs in the case of immobilized enzymes. This phenomenon may be one of the reasons for better stability of immobilized enzymes at lower pH.

Thermal, organic solvent and urea denaturation

Stability was assayed with three denaturing agents and was characterized by the half-life ($t_{1/2}$), the time it takes for the activity to reduce to half of the original activity (Table 2). In all cases, denaturation was irreversible and $t_{1/2}$ values were calculated by using first-order kinetics.

Figure 3(A) compares thermal stability of free and immobilized AChE at 50 and 60 °C in terms of residual activity. It appears that immobilization decreased the irreversible thermal denaturation of AChE. The $t_{1/2}$ of immobilized AChE at 50 and 60 °C were 8.7- and 17.7-fold higher respectively than the values of the native enzyme.

Detection of some environmental pollutant such as insecticides or heavy metals in soil or food using immobilized AChE as the biological compartment of a biosensor requires their extraction with organic solvent. Although the solvent should be eliminated before the assay, low amounts may remain in the solution and inactivate the enzyme.

In the present study, acetonitrile is used as a model because it is soluble in water (log P −0.34 and dielectric constant 37.5 at 20 °C [31,32]). Acetonitrile possess a relatively high denaturation capacity (64.3 for α-chymotrypsin [32]). Addition of acetonitrile in protein solution could induce denaturation through two effects: the weakening of tertiary structure by lowering the hydrophobic bonding capacity of non-polar residue side chains, and the enhancement of secondary structure such as α-helix or β-structure. The enhanced helix structure decreases the chemical potential of the denatured state through the diminished protein–acetonitrile contact [31,33]. The $t_{1/2}$ of immobilized

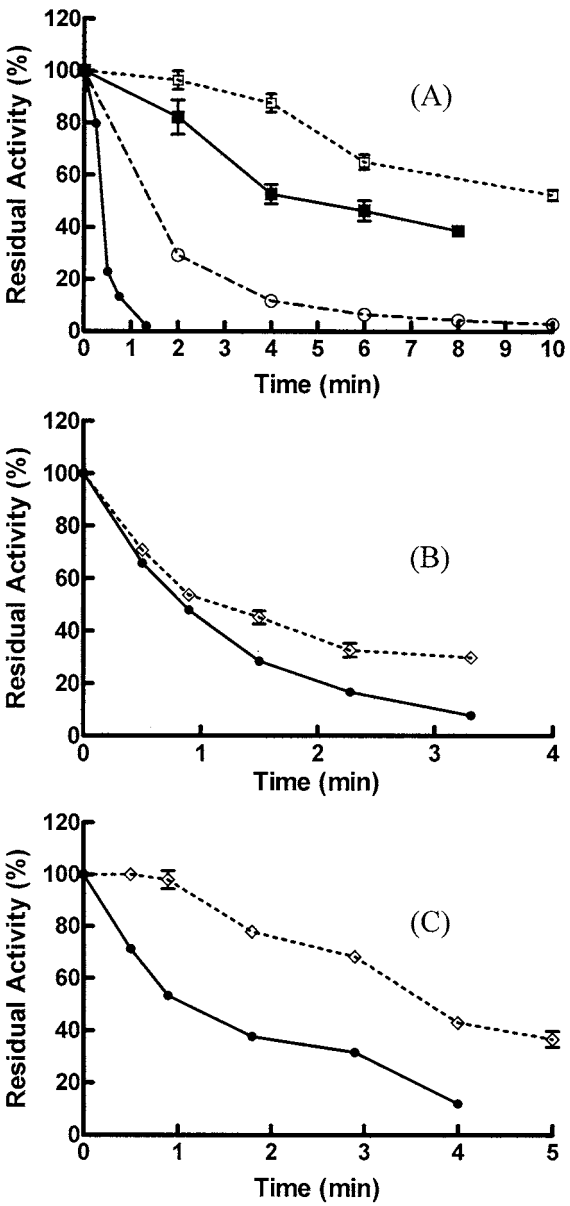

Figure 3 Effect of denaturation agents on AChE stability

Inactivation was performed in phosphate buffer (pH 7.4) with 6 mg of perlite particles. (**A**) Thermal stability of soluble and immobilized AChE: soluble AChE at 60 °C (●), immobilized AChE at 60 °C (■), soluble AChE at 50 °C (○), immobilized AChE at 50 °C (□). (**B** and **C**) Denaturation of soluble and immobilized AChE by 20% acetonitrile and 4 M urea respectively: soluble AChE (●), immobilized AChE (◊). Results are means ± S.D. for at least three experiments.

AChE in 20% (v/v) acetonitrile was 1.7-fold higher than that of soluble enzyme (Table 2 and Figure 3B).

Typical denaturants such as urea are known to bind preferentially to proteins in a large number and denature their native structures [34]. Immobilization also improved the stability of AChE in the presence of 4 M urea, and

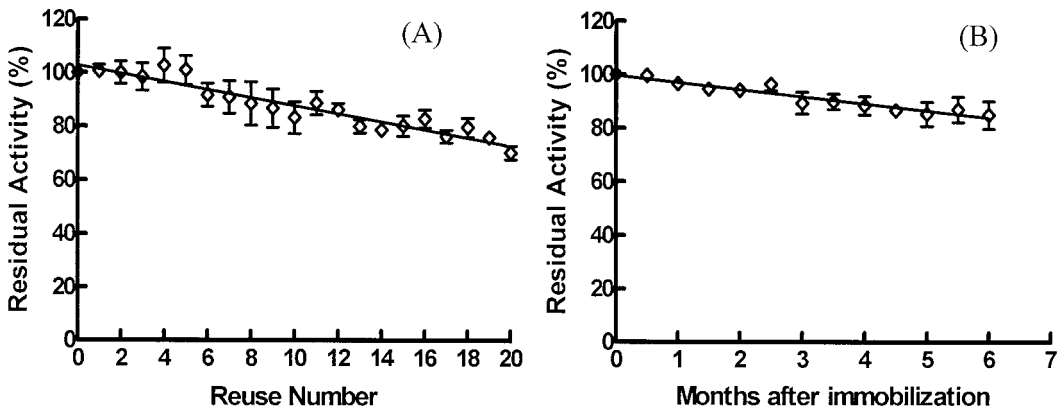

Figure 4 Operational (A) and storage (B) stability of immobilized AChE

(**A**) Operational stability of immobilized AChE on perlite particles. All experiments were carried out at room temperature, pH 7.4, and the substrate flow rate was 2 ml/min. (**B**) Storage stability of immobilized AChE on perlite particles in 4 °C. Results are means ± S.D. for at least three experiments.

the $t_{1/2}$ of the enzyme increased 2.7-fold (Table 2 and Figure 3C). The enhanced stability in acetonitrile and urea particularly reflects that covalent binding provides effective protection against enzyme inactivation caused by structural denaturation.

The native protein molecule is suggested to be characterized by a specific structural region where the unfolding process starts. Accordingly, enzyme stabilization and decrease of the effects of denaturing agents on inactivation of enzymes by immobilization not only is the result of blocking this unfolding nucleus [35], but also could be due to the protection of immobilized enzymes from the structural rearrangement and lower flexibility of immobilized form, owing to the likely multipoint attachments to the support [36–38].

Operational stability

Operational stability or repeatability refers to the measurement-to-measurement precision of a single immobilized module, over a period of time under continuous or intermittent monitoring, which is a function of a variety of factors, the most important of which is the ability of the immobilized enzyme to retain activity on the surface of immobilization support [39]. Increased operational stability of immobilized enzymes is essential in order to achieve the cost benefits [40]. Thus the ability for long-term stability of immobilized AChE on a perlite support was tested by assaying for activity after repeated washing in a flow system. As shown in Figure 4(A), the immobilized enzyme maintained more than 70% of its original activity after 20 reuses. In some studies of surface treatment, the amount of activity decreased in the initial few washes and then remained constant [40]. It is believed that the dominant loss mechanism is continuous desorption of non-covalently bound protein after the immobilization procedure. In the present study, enzyme bleed from the perlite was found to be below detection limits. The activity decrement followed first-order kinetics.

Storage stability

Storage stability or shelf life refers to the stability of the immobilized enzymes when not in use (stored) [39]. As shown in Figure 4(B), the immobilized enzyme maintained more than 85% of its original activity after 6 months of storage at 4 °C.

Some reports have showed that porous matrices are significantly superior in maintaining enzyme activity. A porous structure might provide a microenvironment that protects enzyme from deactivation. Pores can protect protein molecules from shear forces that protein molecules on the outside surface are subjected to during mixing or contact with other particles [3]. Another factor could be multipoint attachments of enzyme molecules to the support.

Inhibition measurement

The inhibition effect of paraoxon on soluble and immobilized AChE activity was measured, and the percentage of inhibition was plotted against the paraoxon concentration to give an inhibition curve (Figure 5). As can be seen, the inhibition curves exhibited a sigmoidal shape. For soluble and immobilized enzyme, an inhibition effect was observed for concentrations greater than 10 and 1 pM respectively. The highest inhibition value was measured for paraoxon concentrations of 0.1 and 1 μM for soluble and immobilized enzyme respectively. For both free and immobilized enzyme, a linear decrease of the activity was found in the range 0.1 nM–0.1 μM. Interestingly, as shown in Figure 5, immobilized AChE exhibited a more linear appearance in various concentrations of paraoxon particularly in the above-mentioned range [$y = 30.158 \cdot \log(x) + 290.68$, $r^2 = 0.843$ for free AChE and $y = 24.634 \cdot \log(x) + 256.87$, $r^2 = 0.969$ for immobilized

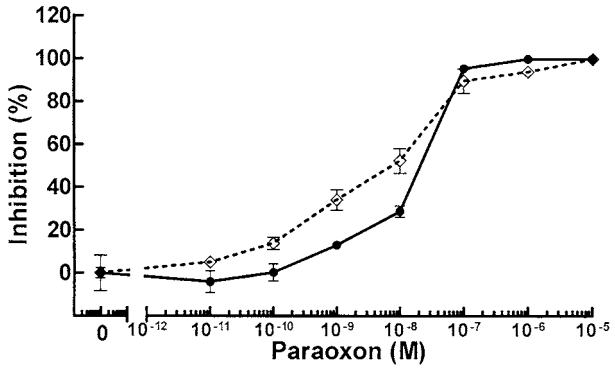

Figure 5 Inhibition plot of organophosphate paraoxon on to (●) soluble and (◇) immobilized AChE on perlite particles

Results are means ± S.D. for at least three experiments.

AChE]. This could be due to the porous property of perlite support which controlled the flow of paraoxon to the immobilized enzymes.

Conclusions

Over the last decade, several strategies have been formulated to construct immobilized AChE for pesticide and insecticide biosensor applications. As the demand for such products increases over the coming years, the efficiency of the immobilization methods and stability of immobilized system will become even more important.

The immobilization method used in the present study resulted in a very good efficiency of enzyme attachment, specific activity and kinetic parameters. It means that the immobilization on perlite does not affect AChE structural condition or behaviour. Thermal inactivation and other denaturation studies showed that these multipoint enzyme–support attachments promoted an increase in the stability of the immobilized enzymes. A more linear behaviour of immobilized enzyme in the presence of various concentrations of paraoxon puts this system forward as a good candidate for pesticide biosensor investigations.

Taking all of the above-mentioned elements and regarding the availability of perlite at a very low cost, it appears that the perlite–enzyme complex developed in the present study can be used as a high-performance cheap bioreactor for various biochemical processing applications, particularly in high-temperature media.

Acknowledgements

We acknowledge Professor Oksana Lockridge for her gift and recommendations. We also thank Shahram Barzegar for his helpful comments.

Funding

This work was supported by New Ideas Research Institute [grant number NIRI-071187].

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Received 10 January 2008/7 April 2008; accepted 20 June 2008
Published as Immediate Publication 20 August 2008, doi:10.1042/BA20080005