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A new biosensing material based on sol-gel encapsulated housefly acetylcholinesterase: Characterisation and application

[Bahan baru biosensor berdasarkan sol-gel terperangkap asetilkolinesterase (lalat rumah): Pencirian dan penggunaan]

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Key words: biosensing material, sol-gel, acetylcholinesterase, organophosphate pesticides, encapsulation

Abstract

A new biosensing material based on sol-gel encapsulation with enzyme was developed for the rapid screening of organophosphate residues. The newly developed biosensor prototype can utilise this biosensing material as a bioreceptor. The encapsulation was carried out at room temperature and phosphate buffer was used to minimise the denaturation of enzymes. Three types of organophosphate pesticides with different toxicity i.e. dichlorvos (category I – highly toxic and hazardous compound), chlorpyrifos and fenitrothion (category II – slightly toxic compounds) were used in this study. The calibration plots for dichlorvos, chlorpyrifos and fenitrothion with dynamic range between 0.2–1.0 ppm, 20–100 ppm and 125–725 ppm respectively were obtained.

Competitive inhibition was observed when halves of maximum inhibition (I_{50}) of these organophosphate pesticides (OP) were reacted with various concentrations of substrate, acetylthiocholine iodide (ATCI). Enzymatic inhibition showed that the response of the biosensing material to these OP was highly reproducible. The lower percentage of enzyme leaching (20%) shows that the microencapsulation process is able to entrap enzyme in the sol-gel matrix. The enzyme-doped sol-gel monolith was found to be stable at least for 3 months under storage at 4 °C. In addition, the gel can be mass-produced thus making it disposable after a single use. Recovery data were collected for dichlorvos on two varieties of vegetables, amaranth or chinese spinach *(Amaranthus viridis)* and convolvulus *(Ipomoea aquatica*). The percentage recovery of approximately 80% for spiked samples with average RSD of 2.44% shows that this newly developed biosensing material can be used as a bioreceptor for the biosensor device.

Introduction

The development of new materials that can improve the analytical capabilities of sensor devices is an important aspect of biosensor technology. One of the most challenging aspects in the development of these

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biological support matrices is the immobilisation and integration of biological molecules in the host matrix and retaining the functionality of the biomolecules.

A number of techniques such as physical adsorption, covalent attachment, entrapment and encapsulation in polymer as well as in inorganic matrices have been explored over the years. This is to achieve a reproducible and robust immobilisation technique that preserves the activity of the biological molecule.

Silica host matrices, made by the sol-gel process have emerged as a promising platform for encapsulation of biomolecules such as enzymes, antibodies and cells. Enzymes find a more stable environment upon encapsulation in silica host because the polymeric framework grows around the biomolecule, creating cage and thus protecting the enzyme from aggregation and unfolding (Bhatia et al. 2000).

These sol-gel matrices are chemically inert, thermally stable, hydrophilic and transparent, enabling spectroscopic monitoring of the entrapped sample. Microencapsulation in the pores of the sol-gel matrix does not decrease the affinity and activity of biomolecules because the enzyme is not really bound to the matrix (Diaz and Peinado 1997).

The purpose of this work was to develop a sol-gel encapsulated housefly acetylcholinesterase as a biosensing material that will be used in the development of biosensor device for screening of pesticide residues in vegetables.

Materials and methods

Direct absorbance measurements were recorded using a double beam Varian (Model Cary 100) UV-Vis spectrophotometer. A Dell PC was used for on-line data acquisition. The wavelength was 412 nm and pathlength of 1 cm. The instrument parameters were controlled by the Cary-Win software (Varian). Data were collected and processed by this software as well.

Acetylcholinesterase (1.25 U/μg) from housefly (HFAChE) was purchased from Taiwan Agricultural Research Institute (TARI). Acetylthiocholine iodide (ATCI) and 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Co. The organophosphate pesticides, dichlorvos (99%) and chlorpyrifos (99%) were purchased from Riedel de Haen (Seelze, Germany).

Stock standards of 100 μg/mL concentrations were prepared in methanol and stored at 4 °C. Tetramethyl orthosilicate (TMOS) was purchased from Tokyo Kasei, TCI (grade 99%). Methanol, hydrochloric acid (HCl) and other chemicals used were from Merck. A phosphate buffer solution of pH 8 and 0.1 M was prepared. The disposable methacrylate cuvettes were obtained from Dispolab-Kartell, 1.5 mL.

Preparation of sol-gel

The sol-gels were prepared according to that described by Ellerby et al. (1992) employing a two-step method. The acid catalysed silica sol was first prepared by mixing 4.5 mL of TMOS, 1.4 mL of H_2O and 0.1 mL HCl in a glass-vial according to Kumar et al. (2000). The mixture was stirred until a clear solution was obtained. The vial was sealed and polymerisation took place for 24 h to obtain a suitable silicate polymer gel.

Enzyme entrapment in the sol-gel matrices

A mixture of 70 μL of buffer, 20 μL of HFAChE (0.5 U/mL) and 30 μL of sol were put into a disposable cuvette and the sol-gel solidified within seconds. The enzyme was encapsulated completely within the growing network. The cuvettes were sealed with parafilm to prevent cracking, through rapid evaporation of the gels during ageing. Complete gelation took approximately 15 min after which time, the gel was stored at 4 °C for ageing.

Leaching test

Two different cuvettes were used to add 20 μL of HFAChE, and 20 μL of sol

respectively. The third cuvette was added with 30 µL of sol, 70 µL of buffer and 20 µL of AChE and immobilisation was allowed for one min before it was measured. One µL of buffer was added to each cuvette and the optical density of protein was measured at wavelength 280 nm using spectrophotometer.

Recovery test

Vegetable sample (spinach) was chopped into small pieces and a subsample (50 g) was weighed and placed in a blending jar. It was homogenised with 50 µL of 1 000 ppm dichlorvos for one hour. After incubation, a volume of 50 mL methanol was added and the mixture was blended for 5 min. The extract was filtered through a filter paper and the filtrate was collected.

For the control, similar procedures were carried out except the homogenization with dichlorvos. The analytical procedure for the enzymatic inhibition was similar to work reported by Salmah et al. (2002a). A volume of 50 µL filtrate was incubated in a cuvette containing AChE-doped sol-gel for 10 min. The absorbance was determined spectrophotometrically after 5 min of substrate addition.

Results and discussion

Characterisation of enzyme-doped sol-gel monolith

TMOS-derived sol-gel monolith was optically transparent and porous and hence suitable for the encapsulation of AChE. Previous results of the optimised working conditions such as composition ratio of buffer + enzyme to sol was 3:1, enzyme quantity of 0.5 U and working substrate concentration of 5.0×10^{-3} M were used for the characterisation of the biosensing material (Salmah et al. 2002b).

Leaching test was performed successfully prior to the characterisation study where leaching percentage of the enzyme-doped sol-gel monolith compared to free enzyme are shown in *Table 1*. The optical density of protein (280 nm) measured at wavelength 280 nm showed that 80% of the encapsulated AChE was retained in the sol-gel matrices with a gelation period of 1 min. According to Bhatia et al. (2002), the pores of the enzyme-doped silica matrix should be small enough to prevent leaching of encapsulated macromolecules but large enough to allow unrestricted transport of buffer ions, molecules of substrate and analyte.

The monolith was characterised using enzymatic inhibition whereby spectrophotometric measurement of cholinesterase activity was performed using thiocholine ester (ATCI) as a substrate. Thiocholine reacts with the Ellman's reagent (Ellman et al. 1961), 5,5'-dithiobis (2-nitrobenzoic acid) to produce the yellow anion 5-thio-2-nitrobenzoate and measured at 412 nm.

Dichlorvos is a highly toxic compound category I and very corrosive. Chlorpyrifos and fenitrothion are in category II, slightly toxic compounds and moderately irritative to skin within 72 h (Ware 1978). Different toxicity of these compounds will reflect in the enzyme inhibition range.

Figure 1 shows the calibration plots for inhibition of enzyme by dichlorvos, chlorpyrifos and fenitrothion with dynamic ranges between 0.2–1.0 ppm, 20–100 ppm and 125–725 ppm respectively. The dynamic range of these OP is differed from each other showing different toxicity strength of each category of OP. The correlation coefficients are 0.991, 0.977 and 0.974 respectively for dichlorvos, chlorpyrifos and

Table 1. Leaching test

Sample	Average $OD_{280\ nm}$	Leaching (%)
Free enzyme, AChE	0.515	0
Sol solution	0.015	0
AchE-doped sol-gel monolith	0.102	19.8

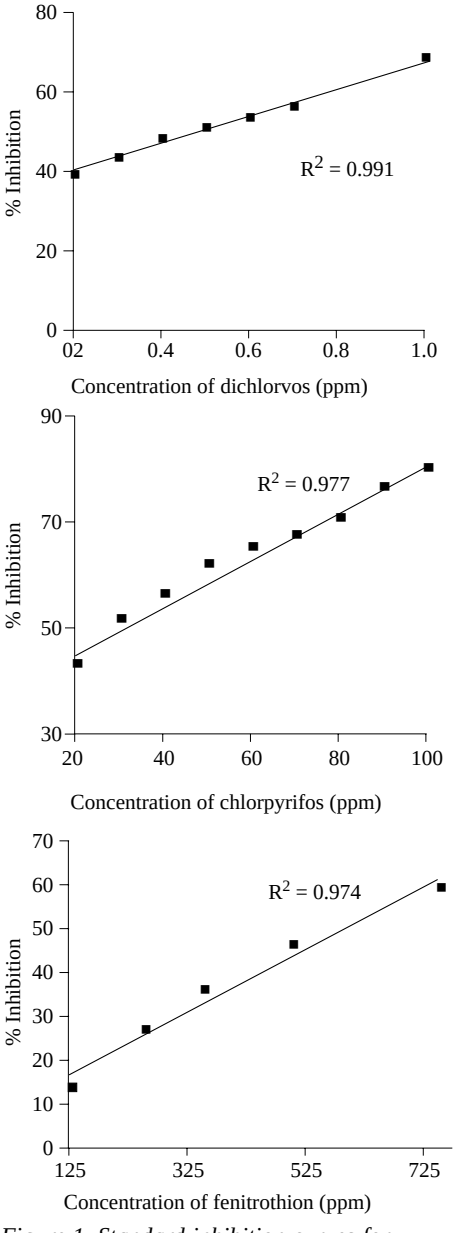

Figure 1. Standard inhibition curves for dichlorvos, chlorpyrifos and fenitrothion

fenitrothion. The difference in toxicity was confirmed when 50% of the maximum inhibition showed different I_{50} values i.e. 0.5, 55 and 350 ppm for dichlorvos, chlorpyrifos and fenitrothion respectively.

Since the inhibition was competitive between substrate and inhibitor for the esteritic site of enzyme, the inhibitor was

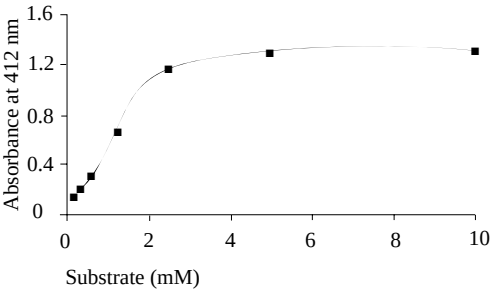

Figure 2. Calibration plot of substrate, ATCI

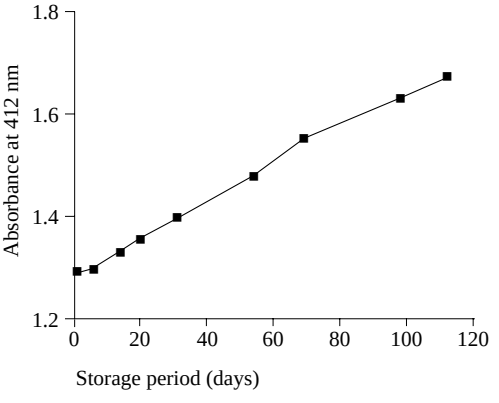

Figure 3. Stability of enzyme-doped sol-gel monolith under storage at 4 °C

incubated 10 min prior to the addition of the substrate. It was found at approximately substrate concentration of 5.0 x 10^{-3} M, the hydrolysis was already saturated with 50% inhibition as shown in *Figure 2*. It was found that the response of the developed biosensing material to dichlorvos, chlorpyrifos and fenitrothion were highly reproducible with average RSD of 4.18%.

Stability of AChE-doped sol-gel monolith

The monoliths were kept in cuvettes at 4 °C for stability test for a period of 2, 7, 15, 32, 55, 70, 99 and 113 days after entrapment. Three sol-gel monoliths were analysed by recording the absorbance at that period of time. *Figure 3* shows that the increase in absorbance is proportional to the storage time, which indicates that longer storage or ageing would increase the quantity of pores (but not increasing the size of pores) thus increasing the stability of entrapped

biomolecules. The enzyme was quite stable in the sol-gel matrix for 3-month storage. However, storage in room temperature was not advisable since the activity of the immobilised enzyme was rapidly decreased *(Figure 4)*.

Recovery test

Two vegetable samples were used in this experiment, i.e. chinese spinach and convolvulus *(Table 2)*. Three analyses were performed each time. The actual concentration of dichlorvos present in the spiked spinach was 1.0 ppm per 50 g sample (50 µL of 1 000 ppm dichlorvos was used to spike the vegetable). Average inhibition of 60.7% and 59.9% was obtained for chinese spinach and convolvulus with RSD values of 1.4% and 3.5% (n = 3), respectively. It was found that concentrations of dichlorvos in chinese spinach and convolvulus were 0.78 ppm and 0.76 ppm respectively when compared with standard inhibition curve of dichlorvos *(Figure 1)*.

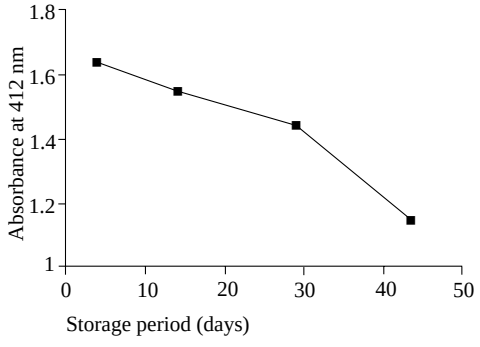

Figure 4. Stability of enzyme-doped sol-gel monolith under storage at room temperature

Conclusion

A new biosensing material used in the development of biosensor device for rapid screening of organophosphate residues has been developed. The encapsulation process was carried out at room temperature and phosphate buffer was used to minimise the denaturation of enzymes. The twenty percentage of enzyme leaching shows that the microencapsulation process is able to entrap enzyme in the sol-gel matrix.

The enzyme-doped sol-gel monolith can be mass-produced and possessed good storage stability for 3 months and therefore can be discarded after a single use. Furthermore, dichlorvos recoveries of 78.3% and 76.0% in respective chinese spinach and convolvulus were obtained. In conclusion the encapsulation of acetylcholinesterase was done successfully in the sol-gel matrix. This newly developed biosensing material can be used as a bioreceptor for a biosensor device.

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Table 2. Recovery of dichlorvos (absorbance measurement at 412 nm) from spiked samples (chinese spinach and convolvulus) at the 1.0 ppm level

Replicate	Chinese spinach		Convolvulus	
	Spiked	Unspiked (control)	Spiked	Unspiked (control)
1	0.6058 (60.1%)*	1.4947	0.5825 (61.2%)*	1.4886
2	0.5942 (60.9%)*	1.503	0.6239 (58.4%)*	1.5475
3	0.5890 (61.2%)*	1.6102	0.5996 (60.1%)*	1.4903
% RSD	1.44		3.45	
+ve control	1.5189		1.5012	

*Percentage of inhibition which was calculated based on positive control
+ve control = Enzyme assay without vegetable sample or pesticide

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Abstrak

Bahan biosensor berdasarkan pemerangkapan enzim ke dalam sol-gel telah dibangunkan untuk penyaringan pantas sisa baki organofosfat. Prototaip biosensor yang baru dibangunkan juga boleh menggunakan bahan biosensor ini sebagai bioreseptor. Pemerangkapan dilakukan pada suhu bilik dan penimbal fosfat digunakan untuk meminimumkan penyahaslian enzim. Tiga jenis pestisid organofosfat yang berlainan ketoksikan iaitu diklorvos (kategori I – ketoksikan tinggi dan sebatian berbahaya), klorpirifos dan fenitrotion (kategori II – sebatian yang sedikit toksik) digunakan dalam kajian ini. Plot kalibrasi untuk diklorvos, klorpirifos dan fenitrotion dengan julat dinamik antara 0.2–1.0 ppm, 20–100 ppm dan 125–725 ppm telah diperoleh.

Perencatan bersaing diperhatikan apabila separuh perencatan maksimum (I_{50}) oleh pestisid ini bertindak balas dengan pelbagai kepekatan substrat, asetiltiokolin iodida (ATCI). Perencatan enzim menunjukkan bahan biosensor memberikan gerak balas yang tinggi terhadap pestisid organofosfat. Peratus larut resap yang rendah (20%) menunjukkan proses pemerangkapan mikro berupaya memerangkap enzim ke dalam matrik sol-gel. Monolit sol-gel terdop enzim didapati stabil sekurang-kurangnya selama 3 bulan pada suhu 4 °C. Gel ini boleh dihasilkan dengan banyak dan dengan itu ia dipakai buang selepas sekali penggunaan. Data perolehan semula diklorvos untuk dua varieti sayur, bayam *(Amaranthus viridis)* dan kangkung *(Ipomoea aquatica)* dikumpulkan. Peratus perolehan semula, 80% diperoleh untuk sampel yang ditambah diklorvos dengan purata sisihan piawai (RSD) 2.44%. Ini menunjukkan bahan biosensor yang baru dibangunkan ini boleh digunakan sebagai bioreseptor untuk peralatan biosensor.

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