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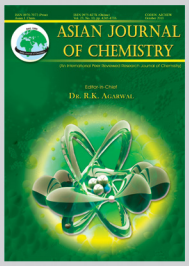

Reconstitution of Acetylcholinesterase into Liposomes and Electrochemical Assay of Its Activity and Inhibition by Methyl Parathion

YUHUI WANG, JIN CHEN, QIAOQIAO REN and JIGEN MIAO*

College of Chemistry and Life Sciences, Zhejiang Normal University, Jinhua 321004, P.R. China

*Corresponding author: Fax/Tel: +86 579 82283173; E-mail: mjg5354@zjnu.cn

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A biomimetic structure was constructed by reconstituting acetylcholinesterase (AChE) into liposomes for the electrochemical determination of methyl parathion using square wave voltammetry. Compared with free AChE, the *michaelis* constant (K_m) drops and the maximum reaction rate (V_{max}) increases for the reconstituted AChE. Several parameters for the experiments were optimized including the concentration of liposome and acetone, the time of enzyme reaction and the time of AChE inhibition by methyl parathion. The logarithmic plot of inhibition rate *versus* methyl parathion shows a close linearity ranging from 0.1 ng/mL to 1 μg/mL. The detection limit is calculated to be 0.49 ng/mL at 10 % inhibition. The reconstituted AChE exhibits improved performance of enzymatic activity and inhibition sensitivity since the biomimetic structure offers a microenvironment close to the situation of natural enzyme *in vivo*. Bio-inspired AChE-liposome system shows the potential to provide a promising and sensitive medium in organophosphates assay with AChE.

Key Words: Biomimetic, Enzyme inhibition, Organophosphate, Electroanalysis.

INTRODUCTION

Organophosphates (OPs) are widely used as pesticides in agriculture. They are potential toxic residues in food and environment[1,2]. Organophosphates poison insects and mammals primarily by the inhibition of acetylcholinesterase (AChE)[3]. Acetylcholinesterase inhibition has been utilized to develop various bioassays or biosensors for faster, simpler and potentially *in situ* alternatives to conventional analytical methods such as gas chromatography and high performance liquid chromatography for detection of organophosphates[4-6]. However, the detection sensitivity is unsatisfactory.

Acetylcholinesterase is distributed in excitable membrane of nerve tissue and muscle. Acetylcholinesterase from electric eel, frequently used for the development of bioassays or biosensors, is an integral membrane protein[7]. The natural environment of cell membrane is of importance to the properties of the enzyme[8-10]. However, most of reported studies on AChE-based bioassays or biosensors were carried out using a purified AChE separated from membrane system[11,12].

As we know, the activity of the enzyme in bioassays depends largely on whether the experimental conditions are close to the situation of natural enzymes *in vivo*. We therefore assume that the sensitivity of AChE-based bioassays toward organophosphates inhibition can be improved if a sensing experiment is performed on a biomimetic structure by integrating AChE with liposome. In this work, AChE was reconstituted into a liposome and used for the determination of methyl parathion. The results show that the reconstituted AChE improved the enzymatic activity and inhibition performance obviously.

EXPERIMENTAL

Acetylcholinesterase (from electric eel, type VI-S, EC 3.1.1.7), acetylthioch-oline (ATCh) chloride and 2,6-dichloroindophenol (2,6-DCIP) sodium salt were purchased from Sigma (St. Louis, USA). Cholesterin and egg phospholipid were obtained from Sinopharm Chemical Reagent Co. Ltd., (Shanghai, China) and methyl parathion from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Stock solutions of methyl parathion were prepared in acetone. All other chemicals from commercial sources were of analytical grade. Ultra-pure water was prepared from Milli-Q.

Electrochemical experiments of cyclic voltammetry (CV) and square wave voltammetry (SWV) were conducted on an Autolab PGSTAT 30 electrochemical workstation (Ecochemie, Netherlands). A conventional three-electrode system was employed with a glassy carbon (GC) electrode as working electrode, an Ag/AgCl electrode as reference electrode and a platinum wire as counter electrode. The glassy carbon electrode was polished according to literature to achieve mirror surface[13]. The determination of AChE enzymatic activity was carried out at 37 ºC. The supporting electrolyte is 50 mM pH 7.5 phosphate buffer solution (PBS) containing 0.1 M KCl.

Preparation of the liposomes and reconstituted AChE: Liposomes were prepared by dissolving 6 mg egg phospholipid and 1.2 mg cholesterin in 2 mL chloroform, which whereafter are dried under nitrogen to allow the solvent to be vaporized off. Then 6 mL water was added and sonicated to obtain a homogeneous solution of liposomes.

Acetylcholinesterase was reconstituted into the structure of liposomes by mixing 1 U/mL AChE and the liposomes in equal volumes, before which the liposome solution was diluted 50 fold with water if unmentioned.

Electrochemical study of reconstituted AChE and determination of methyl parathion: The inhibition of methyl parathion on the reconstituted AChE was determined by incubating 50 μL reconstituted AChE with 200 μL methyl parathion of different concentration in PBS containing 1.5 % acetone for a designed time before enzyme reaction. Then, the aforementioned 250 μL solution was added into and mixed with a reaction mixture consisting of 1.55 mL PBS, 100 μL 8 mM ATCh and 100 μL 1 mM 2,6-dichloroindophenol in a final volume of 2 mL. The enzymatic reaction was allowed to take place for 15 min.

Cyclic voltammetry and square wave voltammetry were employed to measure the redox current of 2,6-dichloroindophenol. Here, the activity of AChE is related with the decrease of 2,6-dichloroindophenol and thus the change (ΔI) of square wave voltammetry signal within the first 15 min was defined as the activity of AChE. The degree of inhibition was calculated as the relative decrease of enzyme activity using the formula[13]:

$$\text{Inhibition } \% = [(\Delta I_0 - \Delta I_i)/\Delta I_0] \times 100\ \% = (1 - \Delta I_i/\Delta I_0) \times 100\ \%$$

where ΔI_0 is initial AChE activity in the absence of methyl parathion and the ΔI_i is the residual AChE activity after inhibited. Both enzymatic reaction and inhibition experiments were carried out at 37 ºC.

RESULTS AND DISCUSSION

Cyclic voltammetrys were obtained on a glassy carbon electrode after different time of enzyme reaction with a solution of mixed AChE, 2,6-DCIP and ATCh (not shown). 2,6-Dichloroindophenol gives a pair of well-behaved redox peak with the E_{pa} of 130 mV, the E_{pc} of 76 mV and the peak separation of 54 mV. The result suggests that 2,6-DCIP is a good candidate as an electrochemical indicator. In the presence of AChE and its substrate ATCh, ATCh is hydrolyzed by the AChE to produce thiocholine. The resulting thiocholine subsequently reacts with the 2,6-DCIP. As a result, the concentration of 2,6-DCIP drops, which accordingly decreases the redox signal of 2,6-DCIP [13].

Square wave voltammetry is a pulse technique with the advantages of great speed, excellent sensitivity and good discrimination against background currents with high signal-to-noise ratio. Well-defined square wave voltammetry spectra in the inset of Fig. 1 were obtained for free AChE. The signal of 2,6-DCIP decreases as a result of enzymatic reaction, demonstrating that the square wave voltammetry is a reliable and reproducible technique for assessing AChE activity. Acetylcholinesterase from electric eel (type VI-S, EC 3.1.1.7) is a membrane-bound enzyme found in excitable tissues. This type of AChE can be reconstituted with the liposomes. Fig. 1 shows the effect of enzymatic reaction time on the change of square wave voltammetry peak values of 2,6-DCIP. The result shows that the reaction speed of reconstituted AChE is higher than that of free AChE. Obviously, the reconstituted AChE has higher catalytic activity than the free AChE. At early time points, the 2,6-DCIP concentration decreases linearly with time due to the increasing thiocholine from enzymatic reaction. After 15 min, the substrate ATCh is being depleted, so the curve starts to level off. Therefore, the following experiments were performed on the systems undergoing enzymatic reaction of 15 min.

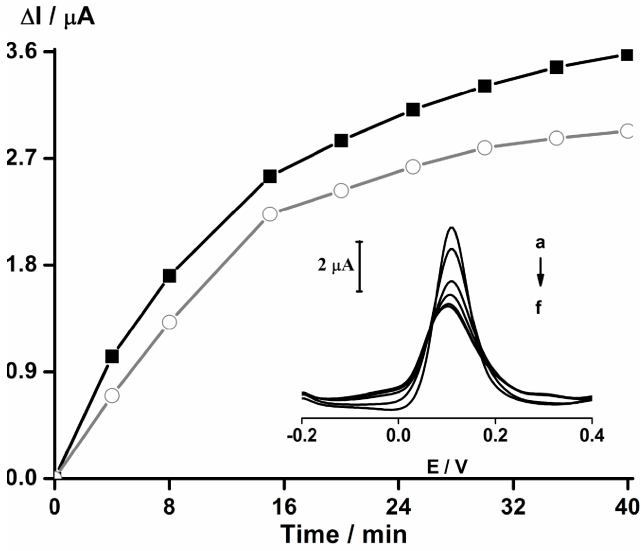

Fig. 1. The effects of enzymatic reaction time on the change of SWV peak values of 2,6-DCIP with (O): free AChE and (■): reconstituted AChE. The inset: SWV spectra of 2, 6-DCIP with different enzyme reaction time (a) 0; (b) 4; (c) 8; (d) 15; (e) 20; (f) 25 min for free AChE. Potential step: 10 mV, amplitude: 25 mV and frequency: 10 Hz.

As indicated from Fig. 2, the reaction velocities are dependent on the concentration of substrate ATCh in the both systems of free and reconstituted AChE. Both of them are fast at lower concentration of substrate and approach to be a maximum (V_{max}) at high concentration where the saturation occurs with all of enzyme present as enzyme-substrate (ES) complex. The V_{max} value of reconstituted AChE is bigger than that of free AChE. Using the c-intercept of the Lineweaver-Burk plots (inset of Fig. 2), *Michaelis* constant (K_m) and the maximum reaction rate (V_{max}) give 0.033 mM and 0.36 A/min respectively for reconstituted AChE, while K_m and V_{max} give 0.047 mM and 0.34 A/min for free AChE. It means that the reconstituted AChE exhibited better catalytical performance probably because its structure is closer to natural enzyme than free enzyme.

In order to achieve the satisfactory inhibitory effect within a short time, the influence of incubation time on the inhibition percentage was investigated. As shown in Fig. 3, the inhibition percentages of both free AChE and reconstituted AChE level off after 10 min of incubation due to inhibition saturation. It suggests that 10 min is sufficient to obtain the satisfactory inhibitory results. The reconstituted AChE exhibits higher inhibition percentage (*i.e.*, *ca.* 37.50 %) than the free AChE (*i.e.*, *ca.* 29.03 %). It means that the reconstituted AChE has

higher sensitivity towards the determination of the methyl parathion than free AChE.

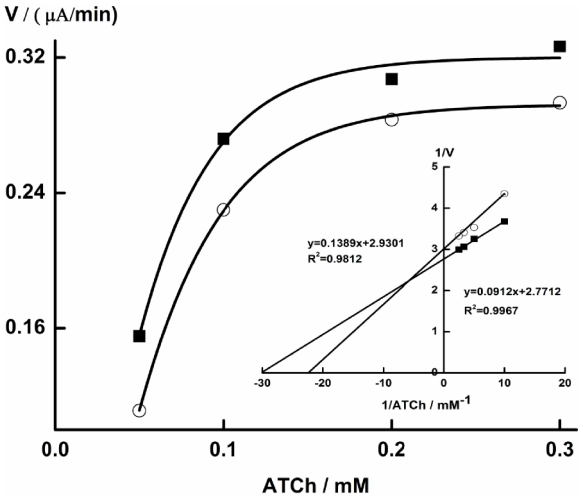

Fig. 2. Michaelis-Menten plots and Lineweaver-Burk curves (inset) of free AChE (O) and reconstituted AChE (■)

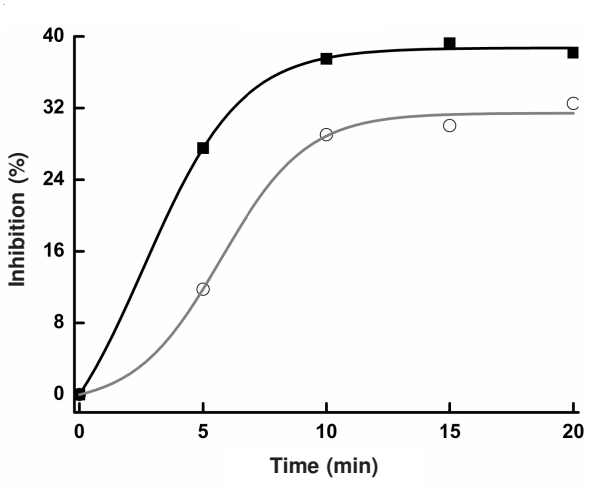

Fig. 3. Effect of incubation time on inhibition percentage of free AChE (O) and reconstituted (■) AChE by 0.6 mg/mL methyl parathion in PBS containing 1.5 % acetone

The effect of liposome concentration on the activity and inhibition rate of free AChE and reconstituted AChE was studied. As shown in Fig. 4, with the increase of liposome concentration, the activity of AChE increases due to the formation of biomimetic microenvironment for AChE. When the liposome was diluted by 50 fold, maximum activity is reached. Higher concentration liposome makes constituted AChE activity decrease. Classical Ellman's method using UV spectrometry also gives similar results (not shown). Interestingly, the inhibition rate increases also with the decrease of dilution fold of liposome from 100 to 50. Both activity and inhibition rate are the highest for the reconstituted AChE with 50-fold diluted liposome. It means that both of them can be improved by the formed biomimetic microenvironment of liposome for AChE. As a compromise, 50-fold diluted liposome was chosen as the optimal concentration for the subsequent experiments.

Methyl parathion has an extremely low solubility in water and a high solubility in organic solvents. The use of AChE capable of also functionalizing in organic solvent-containing

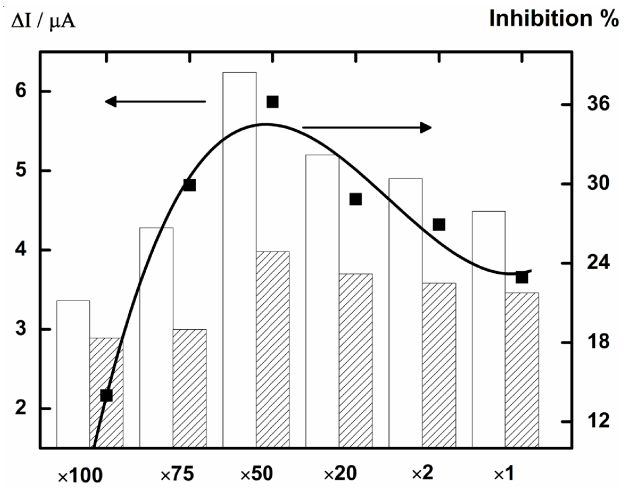

Fig. 4. Effect of liposome concentration on AChE activity and inhibition percentage before (■) and after (▨) inhibited by 0.6 mg/mL methyl parathion in PBS containing 1.5 % acetone for 10 min incubation, where AChE was reconstituted into liposome by mixing 1 U/mL AChE and the liposome in equal volumes, before which 1 mg/mL liposome solution was used without dilution (×1) or with dilution of 2, 20, 50, 75 and 100 fold with water

solution represents wider applications. Therefore, the effect of the acetone concentration on the activity of reconstituted AChE and its inhibition by methyl parathion was studied. To optimize the results, several concentrations of acetone from 0 to 2.5 % were tested (Fig. 5). On the one hand, with the increase of acetone concentration, the activity of reconstituted AChE decreases due to the competition between acetone and water molecules at the hydration layer of enzymes[14]. On the other hand, methyl parathion is dissolved and dispersed better at higher concentration of acetone. As a compromise, 1.5 % acetone gives the highest inhibition rate about 53.27%, far higher than that without acetone.

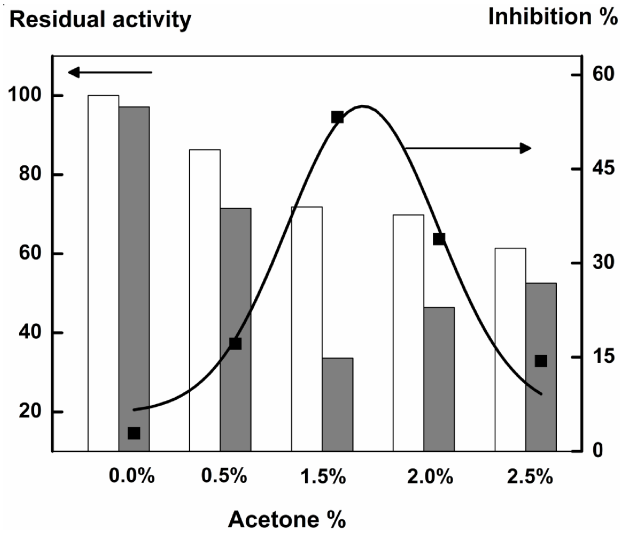

Fig. 5. Effect of acetone concentration on residual activity of reconstituted AChE and its inhibition efficiency by 1 μg/mL methyl parathion in PBS containing acetone of different concentration for 10 min incubation

Using the optimized conditions established in the above studies, calibration curve for assaying methyl parathion was generated in Fig. 6. The data of inhibition percentage are

recorded as a function of methyl parathion concentration and standard deviation is represented as an error bar. One can see from the figure that the inhibition curve exhibits a sigmoid shape. The logarithmic plot of inhibition rate *versus* methyl parathion ranging from 0.1 ng/mL to 1 µg/mL shows a close linearity. The detection limit is calculated to be 0.49 ng/mL^{-1} at 10 % inhibition.

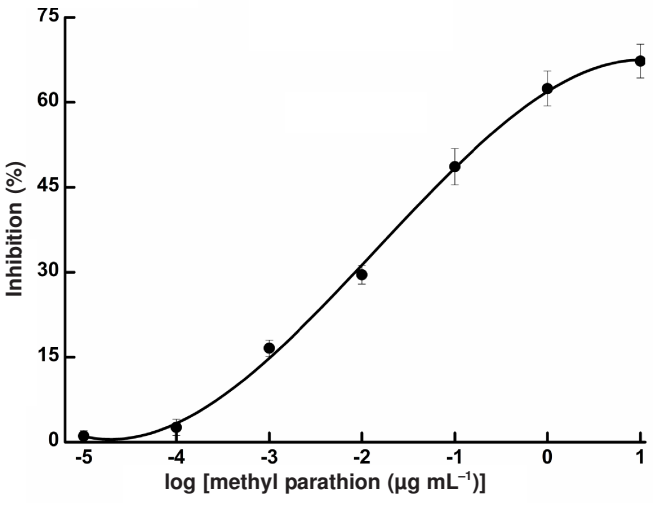

Fig. 6. Calibration curve for assaying methyl parathion using reconstituted AChE

Conclusion

Acetylcholinesterase was reconstituted into liposomes to construct a biomimetic structure for assaying parathion methyl using square wave voltammetry method. The reconstituted AChE exhibits improved catalytical activity and inhibition sensitivity. The microenvironment from liposome is significantly beneficial to give a natural state of AChE and allow detection of methyl parathion with an improved sensitivity.

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REFERENCES

1. H.M. Stapleton, S. Klosterhaus, S. Eagle, J. Fuh, J.D. Meeker, A. Blum and T.F. Webster, *Environ. Sci. Technol.*, **43**, 7490 (2009).
2. D. Wang, D.P. Weston and M.J. Lydy, *Talanta*, **78**, 1345 (2009).
3. H. Schulze, S. Vorlová, F. Villatte, T.T. Bachmann and R.D. Schmid, *Biosens Bioelectron*, **18**, 201 (2003).
4. S. Sotiropoulou, D. Fournier and N.A. Chaniotakis, *Biosens. Bioelectron*, **20**, 2347 (2005).
5. B. Kuswandi, C.I. Fikriyah and A.A. Gani, *Talanta*, **74**, 613 (2008).
6. M. Stoytcheva, R. Zlatev, Z. Velkova, B. Valdez, M. Ovalle and L. Petkov, *Electrochim. Acta*, **54**,1721 (2009).
7. I. Silman and J.L. Sussman, *Chem. Biol. Interact.*, **175**, 3 (2008).
8. Y. Cho, T.S. Ko, S.H. Cha and D.E. Sokm, *Neurochem. Res.*, **20**, 1995681-1995687 (1995).
9. T.F. Schmidt, L. Caseli, T. Viitala and O.N. Oliveira Jr., *BBA-Biomembranes,* **1778**, 2291 (2008).
10. H. Umakoshi, L.Q. Tuan, T. Shimanocuhi and R. Kuboi, *Biochem. Eng. J.*, **46**, 313 (2009).
11. M. Pohanka, D. Jun and K. Kamil, *Talanta,* **77**, 451 (2008).
12. S. Viswanathan, H. Radecka and J. Radecki, *Biosen. Bioelectron.*, **24**, 2772 (2009).
13. J. Qiu, J. Chen, Q. Ma and Y. Miao, *Chemosphere*, **77**, 129 (2009).
14. V.S. Somerset, M.J. Klink, P.G. Baker and E.L. Iwuoha, *J. Environ. Sci. Health B*, **42**, 297 (2007).