Defence Science Journal, Vol. 59, No. 3, May 2009, pp. 300-304

Sarin Assay using Acetylcholinesterases and Electrochemical Sensor Strip

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ABSTRACT

An electrochemical sensor strip was used for sarin assay. Three different acetylcholinesterases (AChEs) were chosen as promising recognition elements. viz., human recombinant, electric eel, and bovine erythrocytes origin. Human recombinant AChE seems to be the most sensitive to inhibition by sarin when the achieved limit of detection (0.45×10^{-8} mol/l) and IC50 [$(9.77\pm 8.08)\times10^{-6}$ mol/l] are considered. On the contrary, AChE from bovine erythrocytes proved to reach highest IC50 ($5.37\pm 4.52)\times10^{-7}$ mol/l and the one from electric eel reached the highest limit of detection 0.93×10^{-8} mol/l. From the AChEs tested as biorecognition element, human recombinant seems to be the best for construction of new ChE detectors.

Keywords: sarin, biosensor, detection, organophosphate, AChE, amperometric, nerve agent

1. INTRODUCTION

O-isopropylmethylfluorophosphonate is well known nerve agent 'sarin' that is an acronyme of scientist who first synthesised it: Schrader, Ambros, Rudiger, and van der Linde. The North Atlantic Treaty Organisation (NATO) encoded sarin as "GB". In laboratory temperature, it is a colourless and odourless liquid. Though sarin is able to chemically react with multiple structures, the most important toxic effect, common for organophosphorus insecticides and nerve agents, is targeted towards serine in active site of acetylcholinesterase (AChE). The reaction has several steps; however, the first nucleophilic addition is the rate-determining step followed by slower elimination of the fluoride ion[1]. Structure of AChE inhibited by sarin is given in Fig. 1; the structure was completed according to Hornberg[2], *et al.*

Another enzyme being inhibited in a similar way as AChE is butyrylchoinesterase (BuChE)[3]. Even some studies propose BuChE as a scavenger when one is intoxicated with sarin [4]. There is also an effort to develop therapeutics for sarin intoxication treatment. The most promising are compounds with oxime functional groups[5]. Currently available are pralidoxime, obidoxime, HI-6, HLO-7, and methoxime[6,7]. Some novel pyridinium oximes seem to prove equal or better efficacy than the currently available were referred in this way[8,9].

Several analytical methods were taken for approachable as sarin assay. Estimation of intoxication could be based on measuring BuChE activity in collected serum[10]. This way, only appoint at misusing of organophosphate. Some more elaborative techniques could be used for identification of misused organophosphate. Holland[11], *et al*. proposed refluoridation of nerve agent, partial purification and its following assay by gas chromatography with MS detection[11]. The conventional detection systems are more extensive in amount of available techniques; however, simple and inexpensive method allowing to detect toxicologically subliminal amount of sarin and others organophosphates is still missing. We could introduce some commercialised devices. Armies of the Warsaw Treaty of Friendship, Cooperation and Assistance were equipped by Soviet CHP-71, GSP, and GSA detectors. Nowadays, more instrumental devices such as mobile mass spectrometer Raid (Bruker Daltonics, MA, USA) are available. There were also attempts to construct functional biosensors for several organophosphates using AChE in a way as biorecognition component, i.e. element responsible for analyte recognising[12].

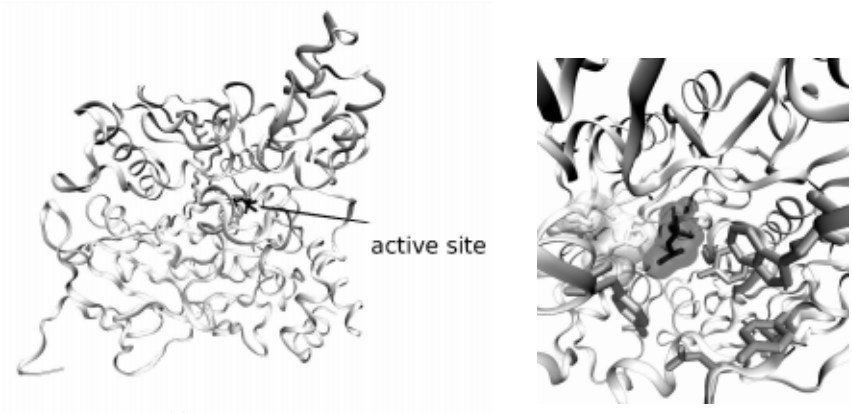
Though AChE-based biosensors could be used for multiple organophosphates, including nerve agents, detection, the current research is focused on pesticides assay as well. The AChE biosensors use AChE as biorecognition component tightly connected with proper device enabling to follow enzyme activity and evaluate concentration of organophosphate as range of found inhibition. This study aims to develop electrochemical biosensor based on AChE being able to detect sarin at low level. For this reason, some available AChEs from different organisms, i.e. species differences are being tested, for independent assay because of different affinity towards sarin and approachability of developed biosensors is being considered.

2. EXPERIMENTAL

2.1 Chemicals and Enzymes

Acetylthiocholine chloride (ATChCl) as well as three different origin AChEs: human recombinant (3,200 IU/mg), from bovine erythrocytes (0.3 IU/mg), and from electric eel (1052 IU/mg) were purchased from Sigma-Aldrich (Czech

Received 8 August 2008



(a) (b)

Figure 1. (a) Crystal structure of acetylcholinesterase from Mus musculus inhibited by sarin (PDB code 2JGG); (b) Detail of active site of mouse acetylcholinesterase inhibited by sarin. The enzyme is represented by grey ribbon and selected residues by tubes: catalytic triad residues are colourated white (Glu334, His447) and black (Ser203 with bound sarin). Peripheral site (Tyr72, Tyr124, Trp286, Tyr341, Asp74) is colourated grey. VMD software (NIH Center for Research Resources, University of Illinois) was used for structure drawing.

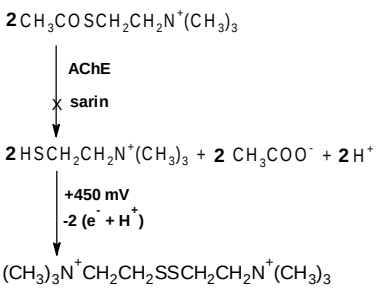
Republic branch). Sarin was kindly provided by Military Technical Institute (Brno, Czech Republic) in 95% purity. The purity was evaluated by acidimetric titration. Isopropanol was used for sarin dilution for the final concentration applied onto sensor. All other chemicals were of the analytical standard quality. Deionized water prepared by Millipore system was used throughout the experiments.

2.2 Device Used

Electrochemical device Bioanalyzer BA1 with adjusted data collecting frequency 0.1 s^{-1} and instrument limit of detection 0.1 nA was obtained from BVT (Brno, Czech Republic). Device was operated through PC (serial port connection) using software Bioanalyzer-BTA (BVT). Screen printed sensors (25.4 × 7.3 mm, thickness 0.6 mm) were used for experiments realisation. Sensor included platinum working (circle shaped, dia 1.0 mm), *Ag*/*AgCl* reference, and platinum auxiliary electrodes (thermally-composited powder). Electrodes were burned on corundum ceramic base. Conductive parts except of electrodes and outputting contacts were isolated by varnish. Sensor was connected with bioanalyzer through elastic cable (0.4 m) allowing separating of bioanalyzer from digester. The completed device is presented in Fig. 2.

2.3 Measuring setup

Principle of electrochemical sensor is obvious from the following scheme:



The first reaction was catalysed by AChE. This reaction could not proceed when sarin or another inhibitor (nerve agent) was present. Concentration of thiocholine accumulated within first reaction was depleted by applied voltage (here +450 mV). Electrochemical oxidation of thiocholine is the visible step when proper electrochemical analysator is used. Dithiocholine is the final product of cascade.

AChE was suspended into phosphate buffer (50 mM, pH 7.4) with albumin 1mg/ml and activity was adjusted at 0.05 IU/µl using Ellman´s method according the previous optimisation[13, 14]. Electrochemical strip was fixed horizontally and voltage +450 mV was applied between working and reference electrodes. The voltage +450 mV is slightly higher than the voltage needed for oxidation of thiocholine; decrease of potential could be realised in the future using some electrochemical modificants[15]. However, the modificants were not considered for the present experiments in order to avoid pertinent influencing of experiments. One measuring cycle could be represented by the following steps:

Step 1. 20 µl of 1 mM ATChCl was applied over electrode
Step 2. 10 µl of sample (sarin solution) was given into ATChCl solution
Step 3. Finally, 10 µl of AChE was injected into mixture
Step 4. Output current was measured after two minutes stabilisation

Experiment was realised at standard conditions for temperature and pressure (SATP). Nonlinear fitting was realised in proper software, Origin 6.1 (Northampton, MA, USA). The Boltzmann equation was chosen for data processing.

3. RESULTS AND DISCUSSION

Sensor was performed in the described manner. In the first round, these were followed by interference of organic solvent in the proposed assay. The achieved data indicates at strong inhibition of used solvents. Monovalent alcohols, methanol and ethanol, inhibited about 30% of AChE activity when presented in 5% concentration in the substrate. Propanol

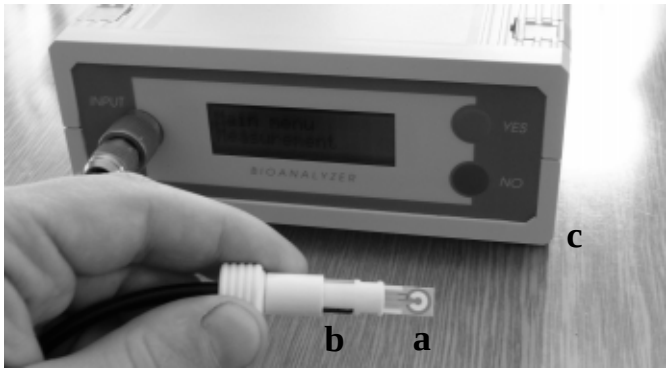


Figure 2. Photograph of used device. Screen printed sensor *a* with three electrodes is partially inserted into holder *b*. Output signal is measured using an amperometric potentiostat Bioanalyzer *c*.

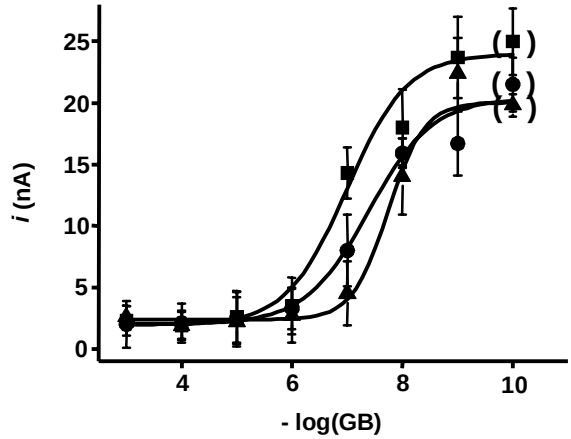


Figure 3. Inhibition of different origin AChEs by sarin. Used AChEs were following: human recombinant (■), electric eel (●), and the one from bovine erythrocytes (▲). Error bars indicate standard deviation. Value in the brackets represents substrate without sarin.

was found interfering in assay due to redox reaction by given applied voltage. Dimethyl sulfoxide and acetonitrile were found to be worse than ethanol, and methanol: the inhibition was about 40% for acetonitrile and even more than 60% for dimethyl sulfoxide. The best for assay seems to be isopropanol that proved inhibition less than 5% when compared to initial activity. In order to avoid inhibition, isopropanol was used throughout experiments.

Three calibration curves: for human recombinant, electric eel and bovine erythrocytes AChE as biorecognition component were constructed. The curves are shown in Figure 3. Curves start at relatively high concentration of sarin: 1 mM. Calibration was carried out up concentration of sarin that was not statistically different (*t*-test, *P*= 0.05) to signal achieved by blank sample processing.

Though the activities of given AChE were adjusted to the same value, the curves were of different shapes. Probably, the most common cholinesterase for biosensor construction is the one from electric eel[16] and some electric eel mutants[17]. The presented data appoint at lower steepness of curve obtained by electric eel AChE performance when compared with the other two AChE species. Due to lower steepness, the limit of detection is highest from the tested AChEs just for the electric eel one. On the other side, IC50 had the middle value. The steepness of curves obtained by human recombinant and bovine erythrocytes AChEs proved similar shape. However, limit of detection as well as IC50 was lower for human recombinant AChE when compared with the one from bovine erythrocytes. The IC50 as well as limit of detection values are summarised in Table 1.

As the results obtained indicate, there are species differences in the inhibition of AChE. Such results were confirmed earlier by Gray and Dawson[18] and Wiesner[19], *et al*. are concerned. As far as experimental data, AChE from bovine erythrocytes is typical for construction of neither biosensor nor enzymatic assay for organophosphates detection. Some works proposed following of bovine AChE activity as a marker of cattle intoxication[20]. We should appoint at better analytical parameters when AChE from bovine erythrocytes is used for sarin assay in comparison with that one from the electric eel. The best limit of detection was obtained for performance of human recombinant AChE. Reached limit of detection was 0.45×10^{-8} mol/l for sarin solution. Though this enzyme has been marketed recently, some studies appoint at feasibility of its performance[21, 22].

Presented assay seems to be approachable for detection of nerve agent sarin. Simple performance of electrochemical strip is very intriguing for diagnostic and pharmaceutical studies as well[23-28]. The assay of sarin represents two main advantages: (i) assay is quite cheap as the total cost per assay are approximately slightly above one eurocent when only reagents are considered; and (ii) assay allows detecting noxious agent represented by sarin within a very short time. One measuring cycle would be completed within four minutes, consisting of two minutes of signal stabilisation and another two minutes of sample and reagents manipulation. This time interval could be considered as relatively long.

On the other hand, optimisation of time interval was not the primary aim of the present study. The time interval could be improved in a multiple way. The manipulation with reagent and sample was not realised with intention

Table 1. Summary of IC50 and limits of detections (LOD) when different origin AChE species are used for sarin assay based on electrochemical sensor

	Human recombinant	Electric eel	Bovine erythrocytes
IC50 (mol/l)	$(9.77\pm 8.08)\times10^{-6}$	$(2.40\pm 2.27)\times10^{-7}$	$(5.37\pm 4.52)\times10^{-7}$
LOD (mol/l)	0.45×10^{-8}	0.93×10^{-8}	0.88×10^{-8}

to minimise it as possible. Two minute of signal stabilisation was chosen to improve experiment's reproducibility. This time interval could be shortened when assay is performed in field conditions. If a washing step is employed after measuring cycle, the strip could be reused. This is an advantage if typical performance of biosensor with intercepted AChE[26]is considered. The most promising way to detect low amount of nerve agent sarin is through sensor based on recognition capability of human recombinant AChE rather than the more common electric eel one.

The total costs of assay per one measuring cycle seem to be low enough to be widely used. Employed sensor could be reused after simple washing when no organophosphate is positively detected. AChE is the most expensive reagent in pertinent assay. The estimated costs of AChE needed per one measuring cycle are about one eurocent that would be taken for assay budget. It should be emphasised that sarin was chosen as a model nerve agent. The other nerve agents, e.g. soman, tabun, and VX could be assayed as well.

CONCLUSIONS

Assay based on electrochemical strip was used for sarin assay consequently employing three different AChEs – human recombinant, from electric eel, and the one from bovine erythrocytes. Assay based on all three origin AChEs was approachable for sarin detection. However, the performance of human-recombinant AChE seems to be the most promising. On the other hand, AChE from bovine erythrocytes was less sensitive to inhibition by sarin when IC50 is considered.

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Contributors

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