VERSITA

Cent. Eur. J. Chem. • 10(6) • 2012 • 1760-1765
DOI: 10.2478/s11532-012-0101-7

Central European Journal of **Chemistry**

Enantioselective inhibition of immobilized acetylcholinesterase in biosensor determination of pesticides

Short Communication

Marzena Kaniewska[1*], Justyna Jońca[1], Iwona Połeć[2], Tomasz Sikora[1], Jean-Louis Marty[3], Marek Trojanowicz[1]

[1]*Department of Chemistry, University of Warsaw, 02-093 Warsaw, Poland*

[2]*Institute of Industrial Organic Chemistry, 03-236 Warsaw, Poland*

[3]*BioMem - IMAGES, University of Perpignan, 66860 Perpignan Cedex, France*

Received 12 January 2012; Accepted 7 July 2012

Abstract: Chiral effects for the inhibition of acetylcholinesterase by organophosphorus pesticides were investigated for insecticide malathion and malaoxon, which is a metabolic product of malathion in living organisms. Studies were carried out using a bienzymatic biosensor with immobilized acetylcholinesterase, choline oxidase, and with Prussian Blue used as a mediator. In both cases the R enantiomers accelerate acetylocholinesterase inhibition. The chiral effect in inhibition was much more pronounced in fast flow measurements than in batch measurements.

Keywords: *Biosensor • Enantioselectivity • Enantioselective inhibition • Acetylcholinesterase • Malaoxon*

1. Introduction

Determinations based on inhibition of enzymes are important analytical tools especially for environmental and food analysis. Although they can provide mostly information on the presence of a certain level of a given group of compounds, such screening is important for the determination of particular analytes using more complex analytical methods. Much effort in the last two decades was focused mainly on the development of integrated biosensors with immobilized enzymes [1], and especially using cholinesterases for inhibitive determination of pesticide residues in food and environmental samples [2]. Besides using different natural enzymes of different origin, also site-directed mutagenesis of cholinesterases were employed in order to obtain enzymes with improved inhibitive response to particular pesticides [3,4]. Differences in limit of detection for different pesticides observed for amperometric biosensors with engineered cholinesterases may reach even 3 orders of magnitude [5,6]. Amperometric biosensors with immobilized acetylcholinesterase (AChE) can be also successfully employed for the continuous monitoring in flow-injection systems [7,8]. The flow measurements in microfluidic format were also reported with fluorimetric detection [9]. Recently reported method for screening of pesticides with AChE and fluorimetric detection was based on the use of emissive core-shell silica particles containing fluorophore [10]. In the development of new methods based on inhibition of cholinesterases an increase in application of different nanomaterials is observed, which are mainly used for effective immobilization of enzymes, such as carbon nanotubes [11,12], or TiO_2-decorated graphene nanohybrids [13]. Also highly sensitive fluorimetric optosensors were reported with nanostructured films of AChE and CdTe quantum dots [14].

There is a large representation of chiral compounds among pesticides. About 25% of the active pesticide ingredients appear in the form of enantiomers [15]. A vast majority of these compounds is produced however and distributed as a racemate. In order to reduce environmental danger of harmful pesticide residues it is important to investigate the behaviour of individual enantiomers and use the chosen form according to

* *E-mail: mkaniewska@chem.uw.edu.pl*

Table 1. The purity of the enantiomers and the optical purity confirmed by gas chromatography and optical rotation.

	Purity GC [%]	Optical rotation $[\alpha]_D^t$	Optical rotation $[\alpha]_D^t$ literature
R-malathion	96.1	+79.44° (c=2.14, t=23.9 C) e.e. 94.3%	+81.2° (t=22 C)
S-malathion	94.8	-71.43° (c=2.80, t=23.8 C) e.e. 88.4%	-80° (t=22 C)
R-malaoxon	96.7	+49.21° (c=2.54, t=23.8 C) e.e. 98.4%	+50° (t=18 C)
S-malaoxon	94.3	-46.32° (c=2.31, t=24.2 C) e.e. 89.4%	-46.6° (t=18 C)

the observed differences. Numerous examples have been described where the enantiomers of the same compound vary in biological activity, the response rate or decomposition time. It is a very common that only one of the enantiomers has the desired effect in relation to the target organism; in other cases the two enantiomers work differently in different organisms, which may have inadvertently come into contact with them [16-19]. A comprehensive overview of the differences in the activity and toxicity of pesticide enantiomers has been published [20]. The toxicity of pesticides in relation to non-target organisms may be a result of more active compounds formed in the process of metabolism. There are also cases in which only one of the enantiomers is a source of toxic metabolic products [21].

The operating principle for a large number of organophosphorus pesticides is precisely acetylcholinesterase inhibition. The inhibition studies using non-immobilized enzymes and organophosphorus pesticides in solution and *in vivo* in water microorganisms, indicate differences in the inhibition rate and toxicity against selected organisms for the enantiomers of the compounds used for tests. These differences may depend significantly on the origin of the enzyme [22-24]. The pesticide malathion is used against organisms harmful to crops, insects, as well as mites. In living organisms malathion is metabolized and a more toxic compound – malaoxon – is produced. The differences in the acetylcholinesterase inhibition rate, which are dependent on the enantiomer applied, as well as on the origin of the enzyme, have been observed. R malaoxon inhibited RB AChE (rat brain) 8.6 times faster than S malaoxon [25]. In turn, BE AChE (bovine erythrocytes) and EE AChE (electric eel) difference in the rate of inhibition of R / S are 22.5 and 16 respectively [26]. All of the above studies of the degree of enzyme activity inhibition have been conducted with the use of spectroscopic methods.

The purpose of this work was to examine if the inhibitive response of a biosensor with immobilized acetylcholinesterase can be different in various experimental conditions to enantiomers of malaoxon and malathion. To our best knowledge, it is the first attempt presented in literature to examine the differences in the inhibition of an immobilized enzyme for biosensing purposes where significant differences in the responses for both enantiomers have been observed.

2. Experimental procedure

Choline oxidase EC 1.1.3.17, (247.5 U mg^{-1}), acetylcholinesterase EC 3.1.1.7 (65.8 U mg^{-1}), acetylthiocholine chloride, tiocholine chloride, BSA, glutaraldehyde 50%, Nafion 5%, potassium hexacyanoferrate, PVA were purchased from Sigma, while concentrated phosphoric acid, potassium chloride, potassium nitrate, ferric chloride from POCH, Gliwice, Poland. B 394-strain acetylcholinesterase, a mutant strain of the enzyme isolated from the fruit fly was obtained from GTB Technology (Toulouse, France). R-malaoxon, S-malaoxon, S-malathion, R-malathion were synthesized in the Institute of Organic Industry in Warsaw [30,31]. The purity of the enantiomers was confirmed by gas chromatography, and optical purity was determined, as well. The results can be seen in the Table 1.

For the preparation of biosensors different possibilities of the immobilization of acetylcholinesterase (AChE) on the surface of the screen-printed electrode were examined. Amperometric measurements were carried out using Potentiostat CH Instruments 830 (CH Instruments Inc, USA). Screen-printed electrodes (SPE) “Florence graphite sensors” were purchased from Palm Instruments BV, Holland (working electrode in the form of a circle with a 2.5 mm diameter made of graphite

paste surrounded by a graphite auxiliary electrode and a silver reference electrode).

Different methods of enzyme immobilization for the preparation of amperometric AChE biosensor, reported earlier in the literature, were examined in this work [27-29]. The best results were obtained for the method reported in our earlier work with D-amino acid oxidase biosensor [29]. For the preparation of biosensor 8 µL of enzyme solution was placed on the surface of the graphite working electrode of SPE biosensor, which was modified with PB and covered with a Nafion layer. The membrane was obtained by mixing 5 µL acetylcholinesterase ((AChE) EC 3.1.1.7 65.8 U mL^{-1}), 40 µL choline oxidase ((AChE) EC 1.1.3.17, 247.5 U mL^{-1}) and 5 µL of BSA solution (50 g L^{-1}) in 0.05 mol L^{-1} phosphate buffer with the addition of 0.1 mol L^{-1} KCl, and then with 10 µL of 2.5% glutaraldehyde solution in water. The ready biosensor was left for 5 hours in a refrigerator for drying. Measurements were also carried out for B 394-strain acetylcholinesterase. The enzyme was mixed with photo-curing polymer PVA (polyvinyl acetate) in the ratio 1:1. Then 3 µL of this homogenous mixture was placed on the surface of the working electrode. The amount of the immobilized enzyme was calculated as 4 mU. Electrodes prepared this way were placed for 3 h under a neon lamp at 4°C. Amperometric measurements were carried out with electrode immersed in a solution of 0.1 mol L^{-1} phosphate buffer of pH 7.0 containing 0.1 mol L^{-1} KCl. The applied potential was 410 mV against pseudo-reference Ag/AgCl electrode.

For the flow measurements a pump (Minipuls 2, Gilson (France) combined with an injection valve with a loop-volume 100 µL, model Rheodyne 5020 from IDEX (USA) supplied phosphate buffer solution as a carrier to the biosensor surface at a constant flow-rate. The substrate and pesticide solutions were dispensed with an injection valve. The biosensor was mounted on a tripod at an angle of 45°, so that the solution could freely rinse the surface.

All inhibition measurements were performed in series, on the same day for both enantiomers.

3. Results and discussion

In the introductory part of this work different possibilities of the immobilization of acetylcholinesterase (AChE) on the surface of the screen-printed electrodes were examined. AChE-based biosensors require the use of a mediator, *e.g.* cobaltophtalocyanine usually imprinted during the preparation of the screen-printed electrode. As it was impossible to obtain screen-printed electrodes with an imprinted mediator – and a previous comparison with biosensors using cobaltophtalocyanine and Prussian Blue pointed out a very similar results [27], it was decided that the more accessible mediator PB might be used.

The biosensors based on two steps cross-linking of enzyme on the working electrode modified with deposited Prussian Blue (PB) [27] did not exhibit a satisfactory enough large response to substrates acetylcholine chloride or acetylthiocholine chloride. Another method of enzyme immobilization with acetylcholinesterase and choline oxidase was subsequently examined [28]. In this case the employed procedure consisted of enzyme immobilization on a layer of glutaraldehyde and Nafion mixture. A mixture composed of acetylcholinesterase, choline oxidase, BSA, glutaraldehyde and Nafion was then deposited on the surface of the working electrode coated with a mediator. The electrodes with the biocatalytic layer were dried at room temperature. The ready biosensor exhibited a high degree of sensitivity; however, the enzyme layer became unstuck after a few hours of measurements in static conditions. Therefore, the sensor would not have been suitable for flow measurements. For further tests, the biocatalytic layer was protected by an extra layer of 1% Nafion (3 µL per electrode). However, this treatment did not result in any significant improvement either.

Finally, it was decided to employ the procedure developed earlier for amperometric D-amino acid sensor [29]. The obtained biosensor showed response to acetylcholine chloride, both in batch and flow conditions. The static measurements were conducted in 0.05 mol L^{-1} phosphate buffer with an addition of 0.1 mol L^{-1} KCl, pH 7. AChE catalyses the reaction of deacylation of acetylcholine. The resulting choline is then oxidized to betaine due to the presence of choline oxidase, which catalyzes this reaction. The remaining product is the hydrogen peroxide, which the mediator is sensitive to.

Calibration curves were prepared for the biosensors in the range of acetylcholine chloride concentrations from 1 to 600 µmol L^{-1}. The range of linear response was 10-500 µmol L^{-1}, however, a decline of the baseline was observed at higher concentrations of the substrate. Therefore, substrate concentration of 100 µmol L^{-1} was selected for measurements of inhibition.

The detection of pesticides proceeded in several stages. Initially, the electrode response times were measured for the substrate in phosphate buffer. The average value of the current before inhibition (I_0) corresponds to the activity of the enzyme immobilized at the electrode. Then the electrode was incubated for 20 minutes in the solution of a given concentration of

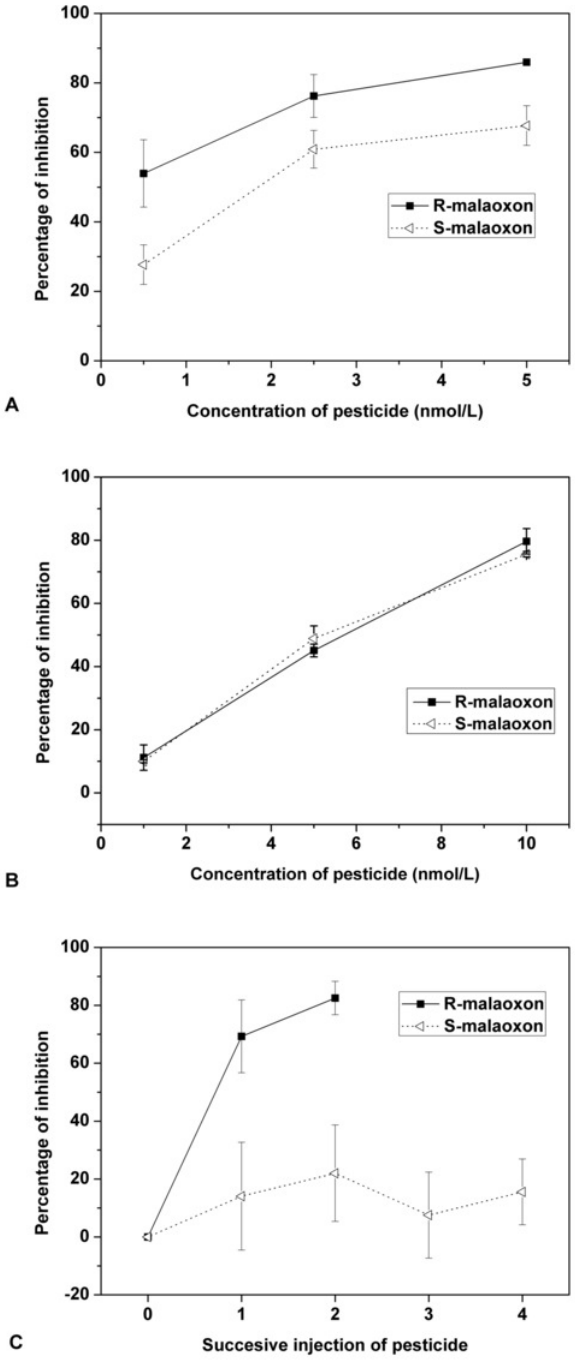

Figure 1. The percentage of inhibition of AChE by malaoxon enantiomers. (A) Inhibition of EE AChE in batch measurement. (B) Inhibition of B394 strain AChE in batch measurements. (C) Inhibition of EE AChE in the flow system. 100 μL of solution of the substrate at a concentration of 400 μmol L^{-1} and 100 μL of inhibitor solution were injected.

the pesticide. The final stage was a triple measurement of current after inhibition. This average value (I_1) corresponds to the remaining activity of the inhibited enzyme.

The differences in the inhibition of the immobilized electric eel AChE (EE AChE) by the malaoxon enantiomers are presented in Fig. 1A. For the inhibition measurements, only freshly prepared biosensors were always used, as the lifetime of the biosensor was usually 5-7 days, and a gradual loss of activity of the immobilized enzyme was observed. Batch measurements indicated that the enzyme immobilized by the R-enantiomer is inhibited stronger than the one immobilized with the S-enantiomer by approximately 1.25 times. For malathion the inhibition ratio for the enantiomers R/S was 1.3. A different situation was observed in the case of biosensors with B394 strain acetylcholinesterase. The results showing the inhibition of the B394 enzyme immobilized on the electrode by malaoxon can be seen in Fig. 1B. The biosensor with immobilized B394 strain acetylcholinesterase showed practically no difference in inhibition by two examined pesticides (malaoxon and malathion).

The obtained results show, that biosensors with commercially available EE AChE show a much higher degree of inhibition than B394 strain AChE in the same concentration of the pesticide.

The flow measurements of the inhibition by the examined pesticides were carried out for the biosensor with immobilized EE AChE. At the flow rate of 625 µL min^{-1}, the biosensor showed a linear response to the analyte (acetylcholine) in the range of concentration from 50 to 800 µmol L^{-1}. The biosensor showed a stable response and had a good level of repeatability. For 30 measurements no significant reduction in the signal was observed. Flow measurements consisted of triple recording of the biosensor response to the substrate. First a 100 µL of a 400 µmol L^{-1} substrate solution was injected to carrier solution flow in over the sensing surface. Then the biosensor was rinsed with an equal volume (100 µL) of 40 nmol L^{-1} solution of the pesticide in buffer. The pesticide was injected into the buffer rinsing the surface of biosensor with the same speed and in the same volume as the substrate. After each injection, the measurements were carried out after 300 seconds, which was necessary for the signal to return to the baseline values in phosphate buffer. Fig. 1C shows the changes in enzyme activity after subsequent injections of malaoxon enantiomers at a concentration of 40 nmol L^{-1} in the flow system. The same correlation was also noted for the enantiomers of malathion.

4. Conclusions

It is known from biological literature that enantiomers of organophosphorus pesticides

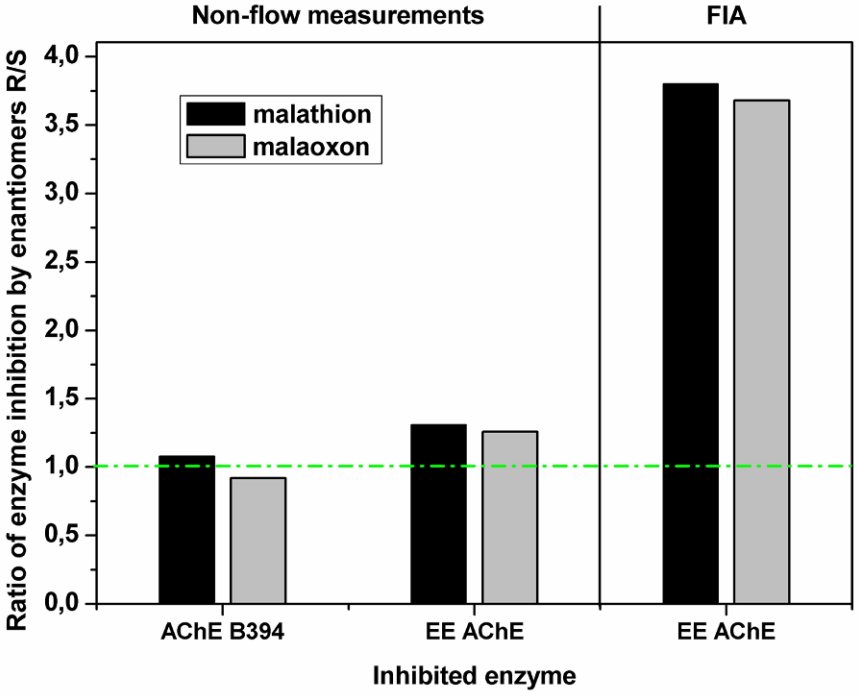

Figure 2. The value of the enzyme inhibition for biosensors inhibited by enantiomers of malathion and malaoxon. The concentration of the inhibitor at batch measurements for malaoxon was 5 nmol L^{-1} for both enzymes, while for malathion it was 5 nmol L^{-1} for EE AChE and 1 mmol L^{-1} for AChE B394. In flow conditions, the concentration of both pesticides was 40 nmol L^{-1}.

inhibit acetylcholineserases to different degrees. Although analytical literature on AChE biosensors is very wide, there was not, to our best knowledge, any attempts to study how much this phenomenon can be visible with enzymes immobilized for biosensing, partly due to the lack of commercially available enantiomers of organophosphorus pesticides on the market.

The screen-printed biosensor developed in this work gives a fast and stable response. The chiral effect in inhibition was much more pronounced in fast flow measurements than in steady-state measurements. The results of this study confirm also that the differences in inhibition depend on the origin of the enzyme. The overall results obtained in this study are shown in Fig. 2, as a ratio of inhibition by R and S enantiomer for both pesticides. The greater differentiation obtained for the enzyme from electric eel and much higher difference in flow conditions was observed, although the R/S ratio values reported earlier for dissolved AChEs of various origins were much higher, and depending on the origin of enzyme they ranged from 3.4 to 22.5 [26]. The obtained differences in biosensor response for enantiomers of examined particular pesticides are not sufficient for selective determination of particular enantiomers, but they may in some cases affect accuracy of determination of total content of analyes, which seems to be especially important conclusion from this work.

References

[1] A. Amine, H. Mohammadi, I. Bourais, G. Palleschi, Biosensors Bioelectron. 21, 1405 (2006)
[2] S. Andresscu, J.-L. Marty, Biomol. Eng. 23, 1 (2006)
[3] Y. Boublik, P. Saint-Aguet, A. Loguarre, M. Arnaud, F. Villatte, S. Estrada-Mondaca, D. Fournier, Protein. Eng. 15, 43 (2002)
[4] H. Schulze, S.B. Muench, F. Villatte, R.D. Schmid, T.T. Bachmann, Anal. Chem. 77, 5823 (2005)
[5] A. Gałęzowska, T. Sikora, G. Istamboulie, M. Trojanowicz, I. Połeć, G.S. Nunes, T. Noguer, J.-L. Marty, Sens. Mater. 20, 299 (2008)
[6] G. Valdes-Ramirez, M. Cortina, M.T. Ramirez-Silva, J.-L. Marty, Anal. Bioanal. Chem. 392, 699 (2008)
[7] G. Jeanty, A. Wojciechowska, J.-L. Marty, M. Trojanowicz, Anal. Bioanal. Chem. 373, 691 (2002)
[8] D. Du, J. Wang, J.N. Smith, C. Timchalk, Y. Lin, Anal. Chem. 81, 9314 (2009)
[9] A.G. Hadd, S.C. Jacobson, J.M. Ramsey, Anal. Chem. 71, 5206 (1999)
[10] X. Shen, F. Liang, G. Zhang, D. Zhang, Analyst 137,

2119 (2012)
[11] S. Viswanathan, H. Radecka, J. Radecki, Biosensors Bioelectron. 24, 2772 (2009)
[12] I. Ion, A.C. Ion, Mater. Sci. Eng. C, 32, 1001 (2012)
[13] K. Wang, H.-N. Li, J. Wu, C. Ju, J.-J. Yan, Q. Liu, B. Qiu, Analyst 136, 3349 (2011)
[14] Z. Zheng, Y. Zhou, X. Li, S. Liu, Z. Tang, Biosensors Bioelectron. 26, 3081 (2011)
[15] A.W. Garrison, Environ Sci Technol. 40, 16 (2006)
[16] I. J. Buerge, T. Poiger, M. D. Müller, H-R. Buser. Environ. Sci. Technol. 37, 2668 (2003)
[17] A. W. Garrison, P. Schmitt, D. Martens, A. Kettrup, Environ. Sci. Technol. 30, 2449 (1996)
[18] A. Miyazaki, T. Nakamura, M. Kawaradani, S. Marumo, J. Agric. Food. Chem. 36, 835 (1988)
[19] Q. Zhou, C. Xu, Y. Zhang, W. Liu, J. Agric. Food Chem. 57, 1624 (2009)
[20] N. Kurihara, J. Miyamoto, G.D. Paulson, B. Zeeh, M.W. Skidmore, R.M. Hollingworth, H.A. Kuiper, Pure Appl Chem. 69, 2007 (1997)
[21] J. Miyamoto, H. Kaneko, Y. Takamatsu, J. Biochem Toxicol. 1, 79 (1986)
[22] M.G. Nillos, G. Rodriguez-Fuentes, J. Gan, D. Schlenk, Environm. Toxicol. Chem. 26, 1949 (2007)
[23] K.D. Lin, F. Zang, S.S. Zhou, W. Lui, J. Gan, Z. Pan, Environm. Toxicol. Chem. 26, 2339 (2007)
[24] S. Zhou, K. Lin, H. Yang, L. Li, W. Liu, J. Li, Chem. Res. Toxicol. 20, 400 (2007)
[25] C.E. Berkman, D.A. Quinn, C.M. Thompson, Chem. Res. Toxicol. 6, 724 (1993)
[26] O.P. Rodriguez, G.W. Muth, C.E. Berkman, K. Kim, C.M. Thompson, Bull. Environ. Contam. Toxicol. 58, 171 (1997)
[27] F. Arduini, F. Ricci, C.S. Tuta, D. Moscone, A. Amine, G. Palleschi, Anal. Chim. Acta 580, 155 (2006)
[28] A. Ivanov, G. Evtugyn, H. Budnikov, F. Ricci, D. Moscone, G. Palleschi, Anal. Bioanal. Chem. 377, 624 (2003)
[29] M. Wcislo, D. Compagnone, M. Trojanowicz, Bioelectrochemistry 71, 91 (2007)
[30] I. Połeć , L. Cieślak , B. Śledziński , H. Ksycińska, Pestic. Sci. 53, 165 (1998)
[31] C.E. Berkman, C.M. Thompson, S.R. Perrin, Chem. Res. Toxicol. 6, 718 (1993