INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
13–379/2014/16–2–389–394
http://www.fspublishers.org

Full Length Article

Assessment of Acetylcholinesterase from *Channa micropeltes* as a Source of Enzyme for Insecticides Detection

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Abstract

In this work we assess the potential of acetylcholinesterase (AChE) from *Channa micropeltes* (Toman) as a sensitive test for the presence of insecticides. The partial purification and characterization of a soluble AChE from *C. micropeltes* brain tissues using affinity chromatography gel (procainamide–Sephacryl S-1000) showed that the partially purified AChE was most active on acetylthiocholine (ATC) but had low activities on propionylthiocholine (PTC) and butyrylthiocholine (BTC), indicating that the partially purified fraction was predominantly AChE. Soluble AChE was partially purified 9.27-fold with a 91.12% yield. The partially purified AChE displayed the highest activity on ATC at pH 7 and at 30^{o}C using 0.1 M Tris buffer. The enzyme exhibited Michaelis-Menten kinetic constants, K_m, for ATC, BTC and PTC at 36, 77 and 250 µM, respectively, and the maximum velocities, V_{max}, were 18.75, 0.12 and 0.05 µmol/min/mg protein, respectively. Moreover, the AChE from *C. micropeltes* presented comparable sensitivity to carbamates and organophosphates insecticides than that from *Electrophorus electricus* by comparing half maximal inhibitory concentration values, therefore the enzyme is a valuable source for insecticides detection in Malaysian waters at lower cost. © 2014 Friends Science Publishers

Keywords: AChE; *Channa micropeltes*; Affinity Chromatography; Biomarker

Introduction

The extensive use of organophosphate (OP) and carbamate pesticides is a concern due to the neurotoxicity properties of the compounds (Gill *et al.*, 2011). In Malaysia, the presence of pesticides were reported in the Selangor River, which is one of the major rivers used as a source of drinking water, due to intense agricultural activities (Leong *et al.*, 2007). Another study conducted along Malaysian shore also indicate the detection of pesticides in fishes although the levels were below the maximum residue limits most of the time (Santhi *et al.*, 2012).

Aquatic organisms have been widely used as biomarkers to detect various pesticides and toxicants, which can inhibit activities of cholinesterases (de la Torre *et al*., 2002; Shaonan *et al*., 2004; Tham *et al*., 2009; Assis *et al*., 2010). An example of the effect of pesticides on fish has been tested on a snakehead fish, *Channa striata* (Van Cong *et al.*, 2006). Similar types of fish that are natives to Malaysian waters, such as *C. striatus, C. micropeltes and C. lucius* (Mohsin and Ambak, 1983), are therefore potentially useful as biomarker agents for pesticides or insecticides. Moreover, mussels (Perić and Petrović, 2011) and snails (Chitmanat *et al.*, 2008) have been employed to detect the presence of pollutants by linking it to the inhibition of cholinesterase activities.

Two classes of cholinesterase, i.e. acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), can be specifically distinguished based on the substrate (Radić *et al.*, 1991). AChE is found in nervous system and its functions are not yet fully studied (Carlock *et al*., 1999). The hydrolysis rate of acetylcholine by AChE is faster than BChE. Only BChE can hydrolyze both butyrylcholine and acetylcholine while AChE is inactive on butyrylcholine. Nevertheless, AChE is easily inhibited by OP and carbamate insecticides, providing a convenient and rapid means of monitoring the presence of pollutants in the environment.

A previous *in vivo* work on a snakehead fish has shown that the brain cholinesterase activity from the organism is very sensitive to insecticides (Van Cong *et al.*, 2006) and hence can be a replacement for the expensive *Electrophorus electricus*, which is commonly used as biosensor for the detection of insecticide (Tham *et al.*, 2009). The main aim of this work is to assess the sensitivity of AChE from *C. micropeltes* on carbamates and OPs assay

To cite this paper: Sharif**,** M.S.A., M.I.E. Halmi, A. Syahir, W.L.W. Johari and M.Y. Shukor, 2014. Assessment of acetylcholinesterase from *Channa micropeltes* as a source of enzyme for insecticides detection. *Int. J. Agric. Biol*., 16: 389–394

in vitro. The sensitivity of AChE from *C. micropeltes* in comparison of AChE from *Electrophorus electricus* will be then evaluated. This work proves that AChE from *C. micropeltes* is the potential source for a cheap and local indicator of OP and carbamate insecticides in the tropics.

Materials and Methods

Chemicals

Carbofuran, methomyl, carbaryl, parathion, malathion, diazinon, bendiocarb, chlorpyrifos, acephate, dimethote and trichlorfon were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Bromine, acetylthiocholine iodide (ATC), propionylthiocholine chloride (PTC), β-mercaptoethanol, procainamide hydrochloride, 1,4-butanediol diglycidyl ether and sodium borohydride were purchased from Sigma-Aldrich. 5'-dithio-bis (2-nitrobenzoic acid) (DTNB) and butyrylthiocholine iodide (BTC) were purchased from Fluka Chemie GmbH. Commercial AChE preparation from eel (*E. electricus*, 349 units/mg solid) was purchased from Sigma (St. Louis, USA). Biorad Protein Assay was purchased from Bio-Rad Laboratories Inc. Vivaspin4 was from Vivascience. All other chemicals used in this study were of analytical or special grade.

Specimen

C. micropeltes was used as the fresh water test organisms in this study. The fishes (weight: 900-1200 g; average length: 36 cm) were obtained from Snoc International Sdn Bhd, Selangor, Malaysia. The selected fish was decapitated and the whole brain was promptly removed. The process of obtaining crude extract of the brain cells were laid out by Tham *et al.* (2009). The supernatant of the crude extract was further purified using the following procedures.

Preparation of Affinity Chromatography Columns

Epoxy (bisoxirane) activation: Affinity procainamide chromatography was prepared according to the modified method of Ralston *et al.* (1983). Briefly, 100 mL of Sephacryl S-1000 (settled gel, Sigma, St. Louis, USA) was washed with 1 L of deionized water in a sintered glass tunnel, dried, and then transferred to a 500-mL beaker. 75 mL of 0.6 M sodium hydroxide containing 150 mg sodium borohydride was subsequently added and continuously stirred before the addition of approximately 75 mL of 1,4-butanediol diglycidylether. The mixture was left stirred at room temperature overnight. The activated gel was then thoroughly washed with water to remove excess reagent until there was no oily film (representing epoxy compound) on the surface of the gel. Acetone was used to completely remove bisoxirane groups. The gel was resuspended in water for ligand coupling.

Ligand coupling of procainamide–sephacryl S-1000 gel: The epoxy-activated Sephacryl S-1000 was washed with deionized water on a sintered glass filter. The gel slurry was transferred onto a coupling solution of 12 mM of borate buffer (pH 11.0) containing 0.2 M of procainamide. The pH was then adjusted to 12 by the addition of sodium hydroxide. The mixture was incubated at 25°C for 96 h on a shaking incubator. The gel was washed in sequence with 10 volumes each of 0.1 M sodium acetate (pH 4.5), 12 mM sodium borate (pH 10) and deionized water. The gel was then suspended in 100 ml of 1.0 M ethanolamine (pH 9.0) to block excess active groups and the mixture was stirred for 6 hours. The gel was finally washed thoroughly with 1 L of 1.0 M NaCl followed by 5 L of deionized water.

Partial Purification of AChE using Affinity Procainamide Chromatography

The procedure for partial purification of AChE using affinity procainamide chromatography has been previously described in details (Tham *et al.*, 2009). For quantitative measurement of proteins, Bradford protein assay (1976) was employed using bovine serum albumin as a standard.

Activity and Effect of Substrates

The measurement of AChE activities and the effect of substrates have been previously described (Ellman *et al.*, 1961; Tham *et al.*, 2009). Briefly, AChE activity was measured from the development of yellow color over time through reaction of thiocholine and 5, 5'-dithio-bis-2-nitrobenzoate (DTNB). AChE catalyzes the hydrolysis of acetylthiocholine iodide (ATC) producing thiocholine and acetate. The activity is defined as the hydrolysis of ATC (μmol) per minute.

In order to analyze the efficiency of the enzyme catalysis, the substrate specificity was determined through enzyme kinetics utilizing Michaelis-Menten equation. Three different substrates i.e. ATC, butyrylthiocholine iodide (BTC) and propionylthiocholine iodide (PTC) were tested. Experiment was performed in triplicates. The apparent K_m value ($K_{m,app}$), was determined by non-linear regression analysis using Graphpad PRISM 4.

OPs were activated prior to assaying according to the modified method of Villatte *et al.* (1998). The pesticide (25 μL) was incubated in 5 μL of 0.01 M pure bromine solution at room temperature for 20 min. 20 μL of 5% ethanol was added to stop the activation process. Preliminary experiments showed that bromine and ethanol at given concentration did not inhibit AChE activities. The half maximal inhibitory concentration (IC_{50}) was determined using at least five different concentrations of carbamate and OPs. The assay mixture contained 0.1 M potassium phosphate buffer (pH 8.0), 0.067 mM DTNB, pesticides and enzymes, and was incubated in the dark (10 min) following methods by Tham *et al.* (2009). A 20 μL of 0.5 mM ATC

was subsequently added into the mixture and was left for 10 min. The absorbance of the reaction mixture was read at 405 nm. The experiment was conducted at room temperature (Tham *et al.*, 2009).

Effect of pH and Temperature

The effect of pH and temperature for purified AChE was determined by measuring the enzyme activities at the different pH using an overlapping buffer system consisting of acetate buffer (pH 4 to 6), potassium phosphate buffer (pH 6 to 8) and Tris-Cl (pH 7 to 9) and temperatures (15 to 80°C) for 10 min and enzyme activity was assayed with ATC as previously described.

Results

Partial Purification

Fig. 1 shows the elution profile of enzyme activity and protein content from the procainamide-based affinity chromatography. AChE activity was detected in the washed fraction and this was due to column overloading. The bound protein was eluted from the column at a flow rate of 0.2 mL/min with high ionic strength phosphate buffer (1.0 M NaCl in 20 mM sodium phosphate buffer pH 7.4). Tubes exhibiting high AChE activities were pooled. Changing of the ionic strength of the solution using 1 M NaCl lowered the energy of the bond between AChE and the procainamide ligand, leading to the desorption of AChE from the matrix. It is probable that the explanation for protein peaks that were not coinciding with enzyme activity peaks was due to non-specific hydrophobic binding of protein via other interactions (Scopes, 1994). The purification yielded 55 mg protein, with specific AChE activity of 793,674 µmol/min/mg. 91.12% of enzyme activity was recovered and a 9.27-fold purification was obtained (Table 1).

Kinetic Studies

Table 2 shows the Michaelis-Menten constant ($K_{m,app}$) and maximal velocity ($V_{max,app}$) of the enzyme in hydrolyzing three different substrates; ATC, BTC and PTC. Both BTC and PTC recorded higher K_m values than that of ATC, indicating that the affinity of the partially purified enzyme towards BTC and PTC were lower compared to ATC. The enzyme also displayed higher rates of hydrolysis when incubated with ATC but showed far less activity in the presence of BTC and PTC, based on the V_{max} values. Based on the table of $K_{m,app}$ and $V_{max,app}$, ATC gave the highest $V_{max,app}$ and the lowest $K_{m,app}$. The catalytic efficiency calculated from the $V_{max,app}/K_{m,app}$ shows that ATC exhibited the highest efficiency followed by BTC and PTC. This supported the evidence that the enzyme that was partially purified was indeed AChE.

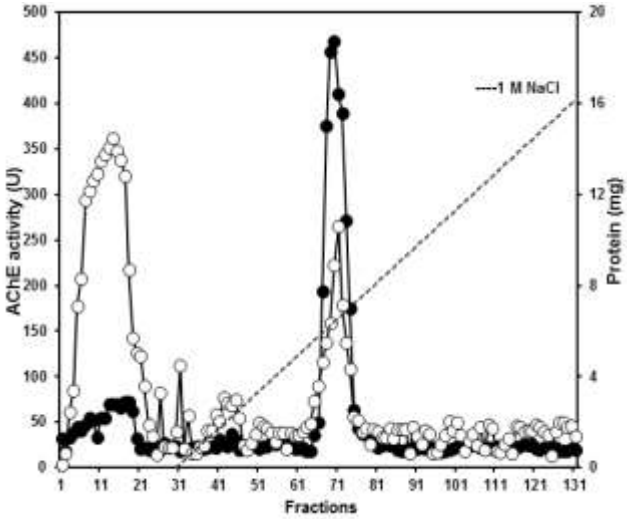

Fig. 1: Elution profile of partially purified AChE from *Channa micropeltes* on custom synthesized Procainamide–Sephacryl S-1000 chromatography column showing protein (○) and enzyme activity (●) peaks

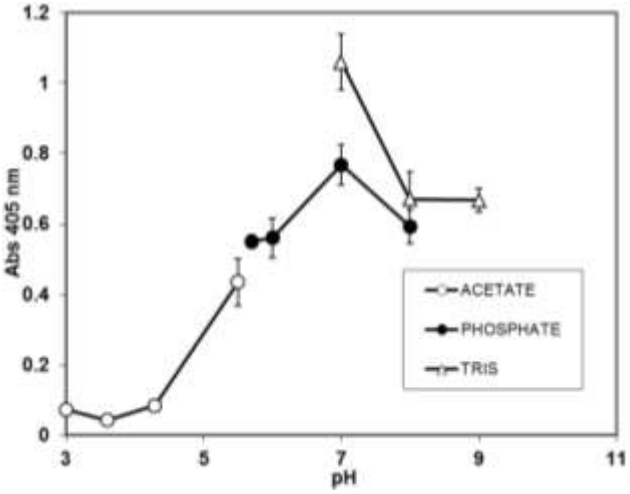

Fig. 2: Effect of pH on the activity of partially purified AChE from *Channa micropeltes* at room temperature using an overlapping buffer system consisting of 50 mM acetate (○), phosphate (●) or Tris-Cl (△) buffers. Each point represents the mean of triplicate assays. Data are mean± standard error (n=3)

The Effect of pH on AChE Activity

The assays were carried out using an overlapping buffer system consisting of acetic acid buffer (pH 3.0 to 5.5), sodium phosphate buffer (pH 5.0 to 8.0) and Tris-Cl buffer (pH 7.0 to 10.0). The partially purified AChE displayed highest activity towards ATC at pH 7, using 0.1 M Tris buffer, with a specific enzyme activity of 34.12 µmole/min/mg protein (Fig. 2).

Table 1: Purification table of partially purified AChE from *Channa micropeltes*

Procedure	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)[a]	Purification fold (X)	Yield (%)
Homogenate	12.5	48,205,875	563	85,582	1.00	100.00
Ultracentrifugation at 100,000×g for 1 h	7.5	35,033,818	293	119,708	1.40	72.68
Affinity-Procainamide Sephacryl S1000	2	43,924,625	55	793,674	9.27	91.12

[a]µmole hydrolyzed/min/mg of protein. The extinction coefficient used is 13.6 $mM^{-1}cm^{-1}$

The Effect of Temperature on AChE Activity

Fig. 3 shows a bell-shaped graph from the temperature profile, where AChE achieved its maximum activity at 30°C. At lower temperature, the enzyme is retarded but not denatured, resulting in low activity.

The Effect of Insecticides on AChE Activity

Screening of insecticides showed that all of the carbamates and the OPs malathion, parathion and diazinon gave strong inhibition while other OPs such as trichlorfon, acephate and dimethoate showed less than 50% inhibition to AChE activity (Fig. 4). IC_{50}s for various insecticides chosen for further studies are shown in Table 3 in comparison with another source of AChE. Other comparisons on IC_{50}s of fish cholinesterases on pesticides can be found elsewhere (Assis *et al.*, 2011). From the experimental data, if there is no overlap between two associated confidence intervals, it usually indicates significant difference at the $p<0.05$ level. An overlapped confidence interval indicates that more experimentation are required to evaluate non-significance (Schenker and Gentleman, 2001). Based on this premise, the AChE from *C. micropeltes* showed comparable sensitivity to carbamates and organophosphates than that from *E. electricus*. AChE from *C. micropeltes* was more sensitive to the carbamates carbaryl, methomyl and bendiocarb than that from *E. electricus.* The latter was more sensitive to the carbamate carbofuran while propoxur showed similar sensitivity towards both sources with an overlapped confidence interval. As for organophosphate insecticides, *C. micropeltes* was more sensitive to parathion and diazinon than *E. electricus* while the latter was more sensitive to malathion while chlorpyrifos showed similar sensitivity towards both sources with an overlapped confidence interval (Table 3).

Discussion

C. micropeltes is highly prized for its medicinal properties in healing wounds of internal organs and is widely found in Malaysian fresh water aquatic bodies (Muhamad and Mohamad, 2012). The results from this study will be useful for comparison purposes on other local fish species and as a precursor for the development of an assay for insecticide pollutants. The purification fold and yield obtained are within the reported range for other AChEs using the custom made procainamide based affinity gel (Talesa *et al.*, 1992; Forget and Bocquené, 1999; Gao and Zhu, 2001).

Table 2: Comparison of $K_{m\ app}$ and $V_{max\ app}$ of AChE from *Channa micropeltes* for ATC, BTC and PTC

Parameter	unit	ATC	BTC	PTC
$V_{max\ app}$	µmol/min/mg	18.75	0.12	0.05
$K_{m\ app}$	µM	36	77	250
$V_{max\ app}/K_{m\ app}$	L/min/mg	0.52	0.001	0.002

Table 3: Comparisons of the sensitivity of *C. micropeltes* AChE to various insecticides with AChEs from *E. electricus*

Insecticides	*C. micropeltes* IC_{50} (Confidence Interval) mg/L	*E. electricus* IC_{50} (Confidence Interval) mg/L
Carbofuran	0.0081 (0.0074-0.0089)	0.006 (0.0063-0.0065)
Carbaryl	0.07922 (0.0697-0.0917)	0.133 (0.122-0.145)
Methomyl	0.0192 (0.0178-0.0208)	0.026 (0.024-0.028)
Propoxur	0.0679 (0.0616-0.0757)	0.060(0.055-0.067)
Bendiocarb	0.0379 (0.0341-0.0427)	0.015 (0.015-0.016)
Parathion-oxon	0.0316 (0.0279-0.0363)	0.068 (0.066-0.069)
Malathion-oxon	0.0242 (0.0192-0.0327)	0.014 (0.013-0.014)
Diazinon-oxon	0.0599 (0.0554-0.0652)	0.177 (0.169-0.186)
Chlorpyrifos-oxon	0.0522 (0.0418-0.0693)	0.060 (0.055-0.065)

Note: All values have a correlation coefficient of 0.99

The enzyme has high affinity for ATC followed by PTC and BTC as apparent from the low Michaelis-Menten constant ($K_{m,app}$) values indicating that the sample was a true AChE. The $K_{m,app}$ value obtained for ATC is within the range obtained from other freshwater fish such as *Pseudorasbora parva, Carassius auratus auratus, Oncorrhychus mykiss* (Shaonan *et al.*, 2004) and *Cnesterdon decemmaculatus* (de la Torre *et al.*, 2002). Catalytic efficiency calculated from the ratio indicated significant differences between ATC and the other substrates tested. The catalytic efficiency value was within the range reported for ATC from *Cyprinus carpio* and *Cnesterodon decammaculatus* (de la Torre *et al.*, 2002).

The optimum pH value obtained in this study coincided with the near neutral pH values reported for AChE from mammalian, insects and fish tissues (Villatte *et al.*, 1998; Forget and Bocquené, 1999; Anitha *et al.*, 2004; Şahin *et al.*, 2005). The pH profile clearly shows that at the extreme ends of the pH range, the enzyme activity declines significantly. This is due to the change of charge distribution at the substrate-binding site thereby directly influencing the enzyme-substrate complex interaction. The active site of AChE contains side chains with charge groups, which stabilize enzyme-substrate complex via hydrogen and ionic bonds. The ionic states of these charged groups are sensitive to pH. Thus, a change in pH values would dramatically change the ionic states of the active sites and molecular

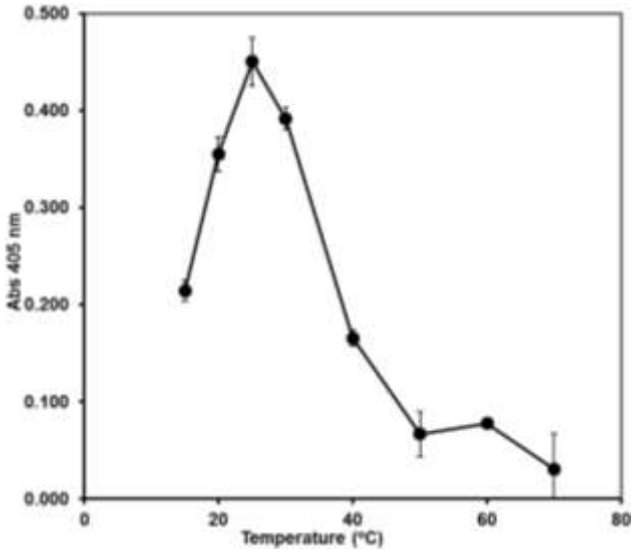

Fig. 3: The effect of temperature on AChE activity from *C. micropeltes*. Data are mean ± standard error (n=3)

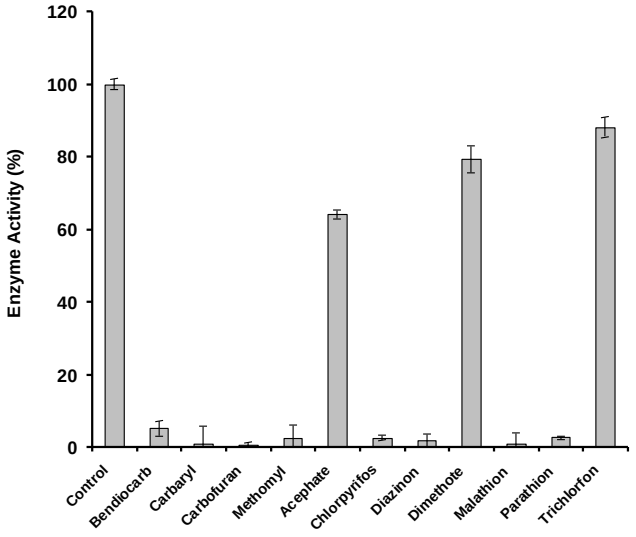

Fig. 4: Effect of various pesticides (0.1 mg/L) on partially purified AChE from *Channa micropeltes.* Data are mean± standard error (n=3)

areas, leading to a loss in the enzyme activity (Dziri *et al.*, 1997).

With an optimal temperature recorded at 30°C, this temperature skews from the general optimal rates of mammalian cholinesterase which is reported to be around 37°C to 40°C. It was reported that the optimal temperature for insects or any other organisms might be lower (Fairbrother *et al.*, 1991). Since fish are poikilotherms, it is possible that warmer temperatures will denature and inactivate AChE. Beyond the optimum temperature, the energy of the vibration within the molecule is great enough to disrupt the non-covalent bonds that maintain the three-dimensional structure of the enzyme, resulting in conformational changes. At 70°C and above, the enzyme activity is negligible. It is important to control the temperature during the catalysis due to the effects that it will bring to the kinetic parameters of the enzymatic reaction (Copeland, 2000). The insecticides screening results showed that the AChE from this organism could be further developed into a sensitive inhibitive assay for insecticides. The bromine oxidation technique in this work was adequate to fully oxidize the organophosphates. However, oxonation using bromine is limited to OP compounds that require oxidative desulfuration for activiation. OP compounds that are oxygen analog in the active form are activated by other procedures (Villatte *et al.*, 1998).

Pollutions such as azo dyes (Pathak and Madamwar, 2010; Ruiz-Arias *et al.*, 2010), detergents (Malaviya and Sharma, 2011; Carvalho-Neta *et al.*, 2012), hydrocarbons (Chavan and Mukherji, 2010; Kang *et al.*, 2010; Hung *et al.*, 2011; Tripathi *et al.*, 2011), heavy metals (Sani *et al.*, 2010; Kavita *et al.*, 2011) and insecticides (Deshpande *et al.*, 2011; Rama Krishna and Philip, 2011) are serious health threats and the development of an assay for insecticides is hoped to increase the biomonitoring efficiency of toxic xenobiotics. A previous work has shown that *C. punctatus* could be used as a biomarker for monitoring the presence of insecticide (Agrahari *et al.*, 2006) and there is a possibility that AChE from this organism could be used as a new source of AChE for the detection of insecticides.

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(Received 25 March 2013; Accepted 30 July 2013)