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Characterisation of Purified Acetylcholinesterase (EC 3.1.1.7) from *Oreochromis mossambica* Brain Tissues

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ABSTRACT

This study reports on the characterization of a purified AChE from *Oreochromis mossambica* brain extract. The purification protocol involved the application of custom-synthesized affinity chromatography gel (Edrophonium–Sephacryl S-400) and the use of high performance liquid chromatography system (HPLC). Soluble AChE was partially purified 27.9 fold with a highest specific activity around 73.1×10^3 U/mg proteins. The partially purified AChE higher capability to hydrolyse acetylthiocholine (ATC) and shows less degradation against propionylthiocholine (PTC) and also butyrylthiocholine (BTC). Based on enzyme kinetic analysis, the partially purified AChE exhibits the apparent Michaelis constants K_m, for ATC, PTC and BTC in the range of 125, 260 and 600 µM and the maximum velocities V_{max} were 276, 59 and 36 µmol/min/mg protein, respectively. The apparent inhibition constant (k_i) values of eserine, propidium and carbofuran were 0.24 $\mu M^{-1}min^{-1}$, 65 $\mu M^{-1}min^{-1}$ and 0.41 $\mu M^{-1}min^{-1}$ $\mu M^{-1}min^{-1}$, respectively. The purified enzyme is apparently an AChE since it capable to hydrolyzes ATC at a higher rate compared to other synthetic substrates, at pH 7.0 and 25ºC, and is inhibited by it specific inhibitor which is eserine but not by iso-OMPA.

INTRODUCTION

Acetylcholinesterase (EC 3.1.1.7; AChE) is in the group of hydrolase enzyme that play role to rapidly degrade acetylcholine in the synaptic gap to terminate the signal transmission at cholinergic synapses. In agreement with its biological function, AChE considered as a very rapid- acting enzyme, operating at nearly diffusion-limited rates. AChE shows genetic and biomolecular polymorphism with their distributions, functions and physiological roles differ among organism [1,2]. Therefore, the characteristic of the enzyme is associated with biochemical and physiological properties is also highly variable.

The study of AChE in fish was started in 1943, when it was shown that abundance of AChE in common carp; *Cyprinus carpio* brain and further researches was conducted on the other teleost where the results are similar [3]. Previous studies also show the differences of AChE kinetic studies, and sensitivity to inhibitor varied among different fish species [4,5]. Even though there are several studies of the fish AChE properties, but most of researchers have been conducted for a small or limited number of fish species. Moreover, their studies have been mostly concerned with their local source. Currently, the properties of AChE among fish species either marine or freshwater habitat from local source are not well studied.

Commonly, the studies on AChE have been conducted with crude preparations which contain other esterases or known as pseudacholinesterase with likely overlapping substrate specificities. However, purified AChE has obciously advantages over crude extract in the term of kinetic studies of each substrate either ATC, BTC and PTC, and specific inhibitor interactions. Even though several different procedures have been developed to purify AChE from any samples, affinity chromatography has been proved to be an alternative and the most effective method for purification of AChE with provides a high recovery or yield which require in characteristic and inhibitory studies [6–9].

Most studies of AChE enzyme use non-local source. In this work the main aim is to provide fundamental knowledge on AChE from local fish species of *O. mossambica*. This research has been carried out using *O. mossambica* because of their availability, commercial importance and can be locally produced. The main objectives of this study are to purify and

characterize AChE extract from the brain tissues of *O. mossambica* as well to evaluate the effectiveness of AChE as an *in vitro* inhibition assay system for pesticides.

MATERIALS AND METHODS

Chemicals

Synthetic substrate; Acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC), and Propionylthiocholine iodide (PTC), and chromogen namely 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Fluka Chemika. 1, 4-butanediol diglycidyl ether, bovine serum albumin, edrophonium chloride, eserine, iso-OMPA, propidium iodide, Triflumuron, Atrazine, Carbaryl, Carbofuran, Caumaphos, Dicamba, Endosulfan, Flucytrinat, Glyphosate, Diazinon, Diurone, Imidacloprid, Metolachlor, Paraquat, Parathion and Simazine were obtained from Sigma Aldrich. Broad range SDS molecular weight marker was purchased from BioLabs and High Range Native-PAGE marker from Amersham biosciences. Most all the chemicals used in the experiments were of analytical grade with acceptable purity.

Test organism

Oreochromis mossambica was used as fresh water test organism. The fish weighing 150-180 g and length 14-15 cm were bought from the hatchery at Universiti Putra Malaysia (UPM), Serdang, Selangor, Malaysia. Each fish was observed to select only normal, healthy and disease-free fish were used in this study.

Enzyme assay

Each activity of AChE from crude extract, supernatant and purified was determined using Ellman Assay [10] with slight modification for microassay system using 96 well micro plate readers [11,12]. Each yellow color which develops over time show the activity of AChE is present then measured at 405 nm on a microplate reader. AChE activity was calculated from the extinction coefficient; EC of DTNB at 0.136 mM-1cm-1 which the activity is expressed as the amount of thiocholine produced from ATC which is hydrolyzed by AChE per minutes. Each reagent was dissolved in 0.1 M potassium phosphate buffer pH 8, except DTNB which was prepared in sodium phosphate buffer (20 mM, pH 7.5).

Each of the well microplate contain with the mixture of 0.5 mM ATC (20 µl), 0.067 mM DTNB (20 µl), 0.1 mM potassium phosphate buffer pH 8.0 (200 µl) and 10 µl of AChE sample. All the reagents were put into the same well and carried out in triplicate using a multichannel pipettor. The AChE activity was introduced by loading the 10 µl AChE sample solution to the wells. The protein content of AChE in each well were determined based on Bradford method [13], while bovine serum albumin was selected as a standard of protein concentration.

Synthesis of Edrophonium–Sephacryl S-400 Affinity gel

The Edrophonium–Sephacryl S-400 affinity gel was synthesized referring to the procedure of Hodgson and Chubb [6].

Preparation of brain extract

AChE was extracted from *O. mossambica* brain tissues. Each fish was killed by kept in a box of ice and the fish brain were dissected out immediately to weight. The brain tissues were homogenized in cold solution of 0.1 M sodium phosphate buffer pH 8 at the ratio of 1:1 (w/v) using an Ultra-Turrax T25 homogenizer at medium speed. The crude extract was subjected to centrifuged at 15 000×g for 10 minutes at 4ºC. The resulting supernatant was continued to ultracentrifugation at 100, 000×g for an hour at 4ºC. The pellet was removed, and only the supernatant was used in the purification methods. All procedures were performed at 4°C.

Purification of AChE by affinity chromatography

Edrophonium–Sephacryl S-400 column (1.6 × 5.5cm) with bed volume of 10 ml was equilibrated with washing buffer; 20 mM sodium phosphate buffer pH 7.5 at chilled condition. The flow rate was calibrated at 0.3 ml/min. The supernatant was filtered with a syringed filter (0.45 µm) then loaded into the column and then washed with washing buffer until the all the unbounded protein was removed; below the detection limit by using Bradford method. The adsorbed enzyme in the matrix was then eluted with an eluting buffer; 20 mM sodium phosphate buffer pH 7.5 containing NaCl gradient from 0 to 1.0 M at a flow rate of 0.3 ml/min using gradient mixer.

Each fractions contained with eluted samples were collected and tested to determined enzyme activity based on Ellman *et al*. [10] and Kato *et al*., [11,12] method. Only the fractions that containing high activities were pooled and subsequently ultrafiltration using a cellulose triacetate filter with a molecular mass cut-off point of 10 kDa in a 5 ml Amicon™ ultrafiltration cell to concentrating and desalting the enzyme. Activity and protein concentration of the partially purified enzyme were determined. This concentrated enzyme was stored at -20 ºC until subsequent use for enzyme purification studies.

Purification of AChE by Superose™ Gel Filtration using HPLC

The Amersham Superose™ 12 HR 10/30 pre-packed gel filtration column with a composite cross-linked agarose matrix was used to further purify AChE extract. The bed dimensions are 30 cm x 1 cm, and the bed volume is approximately 24 ml. An Agilent™ 1100 Series HPLC unit was connected to Superose™ 12 HR column. The affinity column was equilibrated with washing buffer containing 0.2 M KCl with added 1 mM NaN_3 as a bacteriostatic agent and the flow rate was set at 0.5 ml per minute. The concentrated AChE sample from affinity chromatography was introduced into the column by loading it into a Rheodyne™ sample injector. The AChE sample was then flushed into the affinity column at flow rate of 0.5 ml/min and then the protein was eluted with the same buffer.

The eluents were collected using a fraction collector at 0.5 ml/min per tube and assayed for AChE activity and protein content. The protein content was also observed using the HPLC combined wth UV detector at a fixed wavelength of 280 nm. Fractions containing high activities were pooled and subsequently concentrated. The purity profile of partially purified AChE was performed on non-denaturing polyacrylamide electrophoresis gel (Native-PAGE) with high molecular weight calibration kit as marker; porcine thyroid thyroglobulin (669 kDa), equine spleen ferritin (440 kDa), bovine liver catalase (232 kDa), lactate dehydrogenase (140 kDa) and bovine serum albumin (66 kDa). Proteins in the gels were detected with Coomassie Brilliant Blue R-250 staining.

Effect of pH and temperature on AChE activity

The purified AChE was tested to determine the optimum assay condition by mix the enzyme separately at the different pH of buffer [Acetate buffer (pH 4 to 6), Potasium phosphate buffer (pH 6 to 8) and Tris-HCl (7 to 9), and different temperatures ranging from 15 - 80°C for 10 min incubation time using ATC.

Kinetic study and substrate specificity determination

The effect of substrate concentrations was carried out using three different synthetic substrates; Acetylthiocholine iodide (ATC), Propionylthiocholine iodide (PTC) and Butyrylthiocholine iodide (BTC) at different concentrations ranging from 0.05 to 40 mM and the basic protocol for enzyme activity was followed as described above. This experiment was carried out to determine the occurrence of substrate inhibition at high concentrations of substrates. The Michelis constant K_m and V_{max} were determined by analysis of Lineweaver and Burk transformation using Excel (Microsoft) software and Graphpad PRISM 4 software available from www.graphpad.com.

RESULTS

Purification of soluble AChE

Soluble AChE was partially purified from *O. mossambica*. A detailed summary of the purification scheme, employing various steps are compiled in **Table** 1. The concentrated eluent of AChE from Edrophonium–Sephacryl S-400 affinity column (**Fig. 1**) had a specific activity of 31.3 µmol min^{-1} mg^{-1} protein and a purification fold of 11.9. The Superose™ gel filtration column eluent resulted in a purification fold of 27.9 and yielded 16.8%. However, two shoulder peaks were obtained with the first peak shows very high AChE activity (231 nmol/min) compared to the second peak (99 nmol/min) (**Fig. 2**). Therefore, molecular mass of partially purified AChE from *O. mossambica* could not be determined.

Approximately 5.2 mg of partially purified AChE with a specific activity of 73.1 µmol min^{-1} mg^{-1} protein was obtained from one gram of *O. mossambica* brain tissues. Consistent results were obtained when the purification procedures were repeated several times. The purity of the enzyme was characterized in 5% Native-PAGE which produced three protein bands (band 1, 2 and 3) (**Fig. 3**) after the gel was stained with Coomassie Brilliant Blue R-250 solution.

Table 1. Partial purification scheme of soluble AChE enzyme from *O. mossambica* brain.

Procedure	Volume (ml)	Total activity (U)/10^3	Total protein (mg)	Specific activity (U/mg)/10^3	Purification fold (fold)	Yield (%)
Supernatant	7.0	2255	860.1	10.5	1.0	100
Affinity - Edrophonium-Sephacryl S400	3.5	1203	38.5	31.3	11.9	53.3
Superose™ gel filtration	1.0	379	5.2	73.1	27.9	16.8

Note: Soluble AChE was partially purified from *O. mossambica* brain by custom synthesized Edrophonium Sephacryl S-400 and Superose™ gel filtration column. The specific activity of AChE, expressed as µmol hydrolyzed/min/mg of protein or U/mg of protein, was calculated on the basis of an extinction coefficient of 13.6 mM^{-1} m^{-1}. The yield (%) was based on the specific activity of the enzyme. Data presented are typical of three independent experiments.

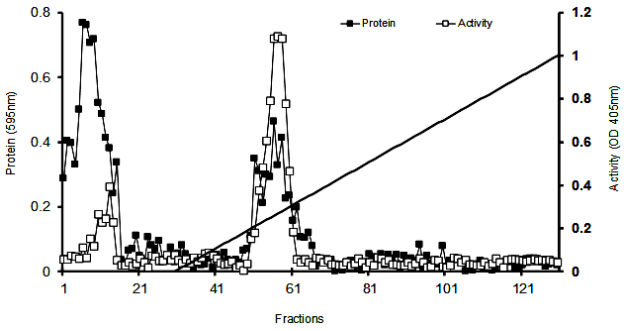

Fig. 1. Elution profile of partially purified AChE from *O. mossambica* on custom synthesized Edrophonium Sephacryl S-400 chromatography column.

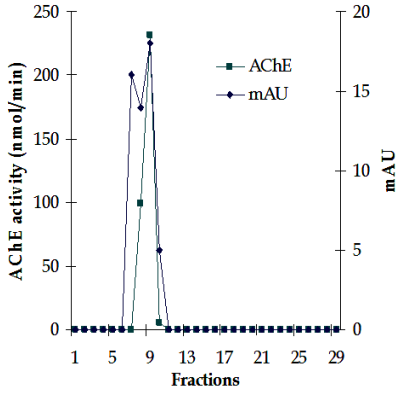

Fig. 2. The elution profile of pooled fractions (*O. mossambica*) from affinity chromatography on SuperoseTM gel filtration column. Protein amounts are given in milli absorbance units (mAu) measured at 280 nm.

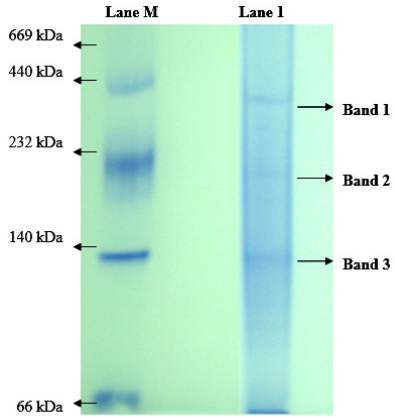

Fig. 3. Non-denaturing polyacrylamide gel electrophoresis (Native-PAGE) of partially purified AChE from *O. mossambica* in a 5 % polyacrylamide gel. Lane M, Native high molecular weight proteins and Lane 1, 5.19 mg/ml protein recovered from SuperoseTM gel filtration column were stained with Coomassie Brilliant Blue R-250.

Effect of pH and temperature

The results from **Fig. 4** show the pH profile for partially purified AChE. With rising pH values, activity increases to optimal pH 8 in potassium phosphate buffer (0.1 M) and drops significantly in the alkaline region, which is expressed as a bell-shaped optimum curve. The acetic acid buffer appears to have an inhibitory effect on AChE as the gap in activity between the

acetate buffer and the phosphate buffer is quite large. **Fig. 5** presents the effects on the temperature profile of partially purified AChE. This figure also shows a common bell-shaped profile of optimum temperature as with most enzymes [5,14]. The maximum temperature was observed at 30°C. At low temperature, the enzyme is retarded but not denatured, resulting in low activity.

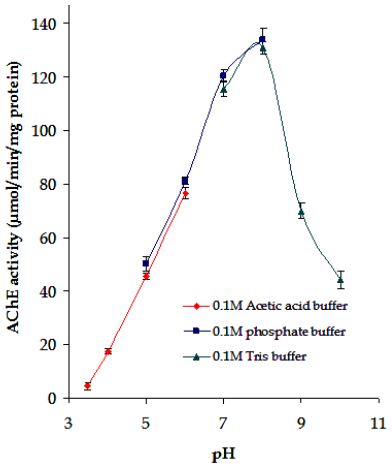

Fig. 4. Effect of pH on partially purified AChE from *O. mossambica* at a range of pH from pH 3.5 to 10. The error bars represent mean ± standard deviation for three replicates.

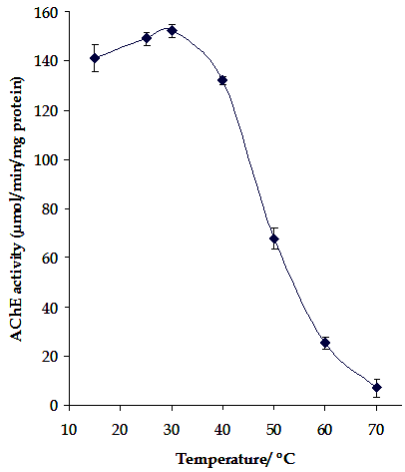

Fig. 5. Effects of temperatures on AChE activity with ATC as the substrate. The error bars represent mean ± standard deviation for three replicates.

Substrate Specificities and Kinetic Analysis of partially purified **AChE.**

The partially purified AChE was most active against ATC and shows less activity against PTC and BTC as shown in **Fig. 6**. No substrate inhibition was evident for ATC, PTC and BTC to a concentration of 2.5 mM, 5.0 mM and 10 mM, respectively (**Fig. 6**).

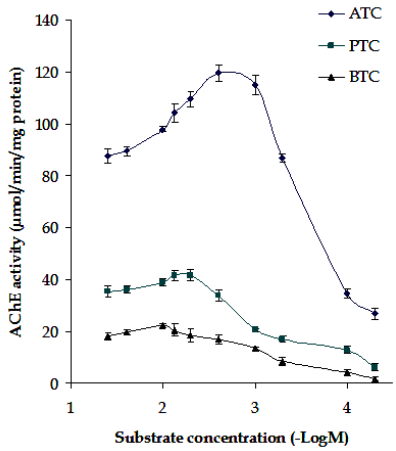

Fig. 6. Substrates specificity and effect of substrates concentration on the hydrolysis of ATC, PTC and BTC by partially purified AChE from *O. mossambica* at 25°C, and pH 7.5. The error bars represent mean ± standard deviation for three replicates.

The reaction rate reduced as the concentration for ATC, PTC and BTC were further increased, which indicated substrate inhibition. From a kinetic point of view, the partially purified AChE exhibited apparent K_m for ATC in the range of 125 ± 10 µM and V_{max} of 275.9 ± 6.7 µmol min^{-1} mg^{-1} proteins. The K_m ratio for ATC and PTC with BTC were 4.8 and 2.3 fold higher, respectively and in order of the hydrolyzing efficiencies V_{max} were 7.7 and 1.7 fold also higher, respectively (**Table 2**).

Table 2. Kinetics parameters of partially purified soluble AChE from *O. mossambica* hydrolyzing various substrates [acetylthiocholine (ATC), propionylthiocholine (PTC) and butyrylthiocholine (BTC)] at pH 8 and 25 ºC.

Substrates	$K_{m(app)}$ (mM ± SD)	$V_{max(app)}$ (µM/min/mg protein ± SD)	Relative rate hydrolysis (%)	Substrate inhibition K_{si} (mM)
ATC	0.125 ± 0.01 [a] 0.176 [b]	275.9 ± 6.7 [a] 217.4 [b]	100	2.5
PTC	0.26 ± 0.10 [a] 0.202 [b]	59.1 ± 5.8 [a] 56.3 [b]	21.4	5
BTC	0.60 ± 0.07 [a] 0.714 [b]	35.8 ± 1.5 [a] 44.6 [b]	13	10

Note: [a]: calculated using GraphPad Prism software. [b]: estimated using Lineweaver-Burke plots. For each substrate concentration there were at least three determinations. The straight lines and curve obtained by linear regression and analysis always showed correlation coefficient (R^2) values higher than 0.95.. For each substrate concentration there were at least three determinations.

Effect of inhibitors

The apparent IC_{50} values of eserine, propidium and carbofuran were 0.44 (0.36-0.58) µM, 7.92 (6.35-9.36) µM and 0.53 (0.43-0.64) µM for partially purified AChE (**Table 3**). Eserine was the most potent followed by carbofuran and propidium. Propidium, a purely non-competitive AChE inhibitor binding to the peripheral site [16], shows a very low IC_{50} value, which was found to decrease slightly. There was no inhibition by Iso-OMPA (specific BuChE inhibitor). Differences in each IC_{50} values can be explained by a dependence on inhibition kinetic parameters k_i. Indeed, they can be related to the more potent inhibitor effect related with its lower k_i values [15]. The apparent inhibition constant (k_i) values of eserine, propidium and carbofuran were0.24 ± 0.03 $\mu M^{-1}min^{-1}$, 64.9 ± 25.8 $\mu M^{-1}min^{-1}$ and 0.41 ± 0.04 $\mu M^{-1}min^{-1}$ (**Table 3**). Generally, except for propidium which showed a noncompetitive inhibition, the

remaining two compounds eserine and carbofuran inhibited purifed AChE competitively.

Table 3. Biomolecular rate constants (k_i) and IC_{50} values for *in vitro* inhibition by eserine, Iso-OMPA, propidium and carbofuran from partially purified AChE (*O. mossambica*).

Inhibitor	k_i ($\mu M^{-1}min^{-1}$)	IC_{50} (10^{-6}) (95% confidence limits)	ppb (µg/L) (95% confidence limits)	Inhibition type
Eserine	0.243 ± 0.026 [a] 0.590 [b]	0.441 (0.356-0.581) [a]	121.4 (98.0-160.0)	Competitive
Propidium	64.9 ± 25.8 [a] 50.99 [b]	7.91 (6.35-9.36) [a]	2390 (1865-3328)	Non-competitive
Iso-OMPA	N.M	N.M	117.8 (100.4-142.3) [a]	Competitive
Carbofuran	0.410 ± 0.035 [a] 0.544 [b]	0.532 (0.454-0.643)	3.714 [b]	

Note: [a]: calculated using GraphPad Prism software. [b]: estimated using Lineweaver-Burke plots. N.M: Not measurable. The concentrated eluants from Superose™ gel filtration chromatography were used to examine the effects of inhibitors and the enzyme activity was measured in the presence and absence of inhibitors

Pesticides screening

All the pesticides tested display no significant inhibition except carbaryl and carbofuran, which have a 26.6% and 30.8% inhibition (**Fig. 7**). Although organophosphate pesticides were also tested in these experiments, they do not show any significant inhibition. Partial purified AChE from affinity chromatography using Edrophonium-Sephacryl S-400 gel for both fish was sensitive to carbofuran and the sensitivity increase significantly with partially purified AChE derived from Superose™ 12 HR chromatography column. Partially purified AChE from *O. mossambica* shows an IC_{50} of 117.8 (100-142) ppb (**Table 3**) for carbofuran in 15 minutes of incubation time.

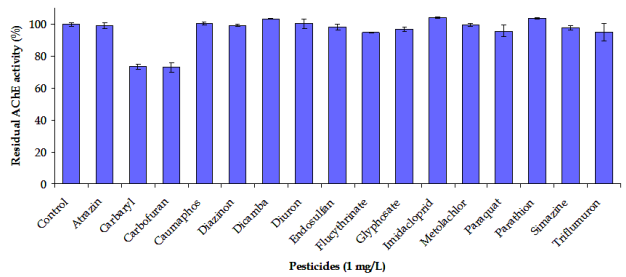

Fig. 7. Effect of various pesticides on the enzymatic inhibition of partially purified AChE from *O. mossambica.* Data were expressed as % AChE activity; where 100% represents the enzyme activity in the absence of the pesticides. Other parameters were held constant. The error bars represent mean ± standard deviation of three replicates. Carbaryl and carbofuran were considered inhibiting the crude enzyme AChE activity as seen by the apparent reduction in enzyme activity

DISCUSSIONS

The affinity ligand edrophonium prove the capability to purify AChE from various vertebrate and invertebrate since its first application to *Electrical electricus* [6,8]. Using the procedure described, the soluble AChE from *O. mossambica* can be partially purified within a week. The purification efficiency of AChE by affinity chromatography and modified gel filtration using HPLC unit is almost similar to other chromatographic procedures using procainamide [9,17,18], tacrine [19] and edrophonium [6–8]. The degree of purification is not readily comparable between organism from previous studies as it is apparently dependent on the amount of extraneous tissue present to begin with. In conclusion; the combined use of affinity chromatography (Edrophonium Sephacryl S-400) and gel filtration (Superose™) using HPLC were very efficient and selective in partially purifying soluble AChE from *O. mossambica*. This procedure can be readily applied in the purification of AChE from other types of tissue or organism.

The apparent K_{si} values of the partially purified AChE from *O. mossambica* are comparable with the purified AChE from *Schizaphis graminum* [20], *Leptinotarsa decemlineata* [21] and *Nephotettix cincticeps* [11]. The rank order from the highest K_m and $V_{max(}$ to the lowest were ATC > PTC > BTC. Similar K_m values had been also reported for the same substrate (ATC) for purified AChE from *Maia verrucosa* [22], *Murex brandaris* [23], *Nephotettix cincticeps* [11], *Schizaphis graminum* [20] and *O. cuniculus* [24].

The high inhibitory potency of eserine and carbofuran were probably due to the effect of its high affinity to the enzyme and the high carbamylation process during interactions between AChE and inhibitors. The apparent k_i values for carbofuran and eserine from *O. mossambica* (**Table 3**) fit with the inhibition constants values from purified AChE of *Tigriopus brevicornis* [1], *Electrophorus electricus* [25] and *Drosophila melanogaster* [26]. Propidium has been well established as a noncompetitive inhibitor that selectively binds to the peripheral anionic site of AChE [27]. AChE inhibition by propidium previously has been attributed totally to a conformational alteration at the acylation site induced by the binding of propidium to the peripheral site [16]. The partially purified AChE from *O. mossambica* was efficiently inhibited by eserine (an inhibitor for both AChE and BuChE or perhaps PChE) at a concentration of 5 µM but not by iso-OMPA (specific BuChE inhibitor) at the same concentration. Here can be comfirm that the enzyme was 'true' cholinesterase (AChE) based on their specific inhibitor. Referring to the studies of kinetics, substrates specificities and inhibitors sensitivities we conclude that AChE partially purified from *O. mossambica* possess typical characteristics of other AChE [3,14].

Generally, the levels of detection using partially purified AChE from *O. mossambica* are adequate to monitor carbamate pesticides levels in water samples and food as the permissible levels in vegetables is often higher than 1 mg/L (1000 ppb) for certain leafy vegetables. The results also show that most of the inhibition was more than 50% at 15 minutes incubation time. According to Silver, [3], the aquatic animal death was noticed when there were 40% to 70% of AChE was inhibited either *in vivo* and *in vitro* respectively. The results suggest that the AChE *in vitro* programs, using the described methodology provides a fast, low cost, easy to handle and capable of providing reliable analytical information for assessing carbofuran toxicity. The outcome has the potential to be used for futher studies on development of bioassay system for detection of carbamates in food and actual water samples, avoiding complicated chromatographic extraction, separation and detection.

Inhibition kinetics of purified AChEs with inhibitors

The aim of this study was to estimate the apparent inhibition rate of partially purified AChE by selected inhibitors on the basis of median inhibition concentration (IC_{50}) and biomolecular rate constants (k_i). Eserine, carbofuran and propidium were used because susceptibility of its inhibitions was one of the distinctive characteristic of AChE [1]. Iso-OMPA (specific BuChE inhibitor) was also employed as non selective inhibitor of AChE from BuChE [14]. Susceptibility of the enzymes to inhibition by eserine, carbofuran and propidium were determined over a range of 0-3.0 × 10^{-6} M, 0-2.0 × 10^{-6} M

and 0-10.0 × 10^{-6} M, respectively. Stock solutions of inhibitors were prepared in ethanol and diluted to the desired concentrations with dH_2O.

A series of reaction mixtures containing fixed concentration of inhibitor, DTNB and partially purified AChE in 0.1 mM potassium phosphate buffer (pH 8) were prepared in a 96 well microtiter plate and incubated for 15 minutes at 25ºC. Reactions were initiated by adding ATC to exacting mixtures and hydrolytic activity was determined after 10 minutes incubation at 25 ºC at 405 nm with microtiter plate reader. A parallel control was included for each experimental run and the rate of spontaneous hydrolysis calculated as described above. To characterize the inhibition type, the turnover rates of several measuring series with varying substrate concentrations [S] are performed, each series containing a constant amount of inhibitor [I]. One series without inhibitor is tested for control and for the determination of initial K_m and V_{max}. Substrate and inhibitor concentration were varied around their respective Michaelis and inhibition constant. The IC_{50} and k_i values were determined by using Graphpad PRISM 4 software available from www.graphpad.com and compared with data estimated from double-reciprocal method of Lineweaver Burk transformation using Excel (Microsoft) software [15]

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