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Evaluation of acetylcholinesterase source from fish, *Tor tambroides* for detection of carbamate

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Abstract

Acetylcholinesterase (AChE) from the brain tissue of local freshwater fish, *Tor tambroides* was isolated through affinity purification. Acetylthiocholine iodide (ATCi) was preferable synthetic substrate to purified AChE with highest maximal velocity (V_{max}) and lowest biomolecular constant (K_m) at 113.60 Umg^{-1} and 0.0689 mM, respectively, with highest catalytic efficiency ratio (V_{max}/K_m) of 1648.77. The optimum pH was 7.5 with sodium phosphate buffer as medium, while optimal temperature was in the range of 25 to 35 °C. Bendiocarp, carbofuran, carbaryl, methomyl and propoxur significantly lowered the AChE activity greater than 50%, and the IC_{50} value was estimated at inhibitor concentration of 0.0758, 0.0643, 0.0555, 0.0817 and 0.0538 ppm, respectively.

Key words

Acetylcholinesterase, Brain, Carbamate, Organophosphate, *Tor tambroides*

Introduction

At present, chemical pesticides are widely used in the Malaysian agriculture sector. Pesticides are well known as an effective way to control pest activity inexpensively (Abhilash and Singh, 2009). However, there are several conflicts between economic benefits and environmental risks in use of pesticides in agriculture. Farmers use pesticides to enhance crop productivity and reduce loss and at the same time to ensure high quality of products. However, excessive application of pesticides has side effects by polluting environment and affecting non-target organisms (Lu *et al*., 2013). Pollution can also occur through leaching of pesticides into large bodies of water sources (Neumann *et al.,* 2002; Sabullah *et al.,* 2014a).

Pesticides such as carbamates and organophosphates are widely used to control insect pests. However, these compounds also have adverse effects on other organisms such as aquatic organisms and humans (Barata *et al.,* 2004). Carbamates and organophosphates are nerve agents that inhibit cholinesterase activity at the active site of enzyme through carbamylation and phosphorylation processes respectively (Bartolini *et al.,* 2009). This inhibition causes accumulation of acetylcholine at muscarinic and nicotinic receptors at the synaptic cleft (Zhou *et al.,* 2003; Peňa-Llopis *et al.,* 2003). Researchers manipulate use of acetylcholinesterase as an alternative tool for detection of pesticide through inhibitive assay (Tham *et al.,* 2009; Sabullah *et al.,* 2015). This alternative tool work as preliminary screening which result is rapidly generated to

determine the existence of toxicants in sample as compared to high instrument such as high performance liquid chromatography and gas chromatography which is expensive and time consuming. Monteiro *et al.* (2005) and Rendón-von Osten *et al.* (2005) mentioned that fish species vary in cholinesterase biochemistry, especially their sensitivity towards nerve agent. Therefore, additional bioassay data are needed to study interaction or inhibition degree of acetylcholinesterase from various fish species towards an anti-cholinesterase.

In the present study, AChE from local fresh water fish, *Tor tambroides* was selected to evaluate its capability in detecting the lowest concentration of bendiocarp, carbaryl, carbofuran, methomyl and propoxur. Prior to evaluation, optimal condition for assay of enzyme is required inmodel organism. The objective of the present study was to develop a template for biosensor through inhibition study and half inhibitory (IC_{50}) determination on the selected carbamate.

Materials and Methods

Extraction and specimen preparation : The specimen, *Tor tambroides* was isolated from Endau-Rompin, Johor State Park, which is a protected and uncontaminated area. The specimen was transported to laboratory and acclimatised in an aquarium with 60 l water. The aquarium was fully aerated for three days. The specimen was fed two times per day with commercial fish food. Two days before sacrifice, water was changed and the specimen was not fed to reduce possible dietary effects on metabolite status. The specimen was sacrificed in a box of ice for 30 min. The brain tissue was dissected and immediately weighed. Homogenisation of tissue was performed in 0.1 M sodium phosphate buffer, pH 8 containing 1 M phenyl methyl sulfonyl fluoride (the tissue and buffer ratio was 1:4) using an Ultra-Turrax T25 homogeniser. Crude extracts were then centrifuged at 100, 000 x g for 1 hr at 4 °C. The supernatant collected was used for purification procedure. The entire experiment was carried out in cold room (Tham *et al.,* 2009). Purification was performed using packed affinity column (diameter 1.5 cm, height 5.5 cm) containing Procainamide Sephacryl S-1000 as specific ligand to isolate acetylcholinesterase from the supernatant. The column was washed with 10 ml of degassed 20 mM sodium phosphate buffer and flow rate was adjusted at 0.2 ml min^{-1}. The supernatant (400 µl) was loaded into the column and allowed to absorb into the column. Without letting column to dry, washing stage was carried out by loading 6 ml of 20 mM sodium phosphate buffer into the column. The adsorbed enzyme was then eluted with 20 mM sodium phosphate buffer containing 1 M NaCl. Fractions of 1 ml were collected starting from washing stage until the end of elution stage. Each fraction was tested for enzyme activity and protein content following the method of Ellman *et al.* (1961) and Bradford (1976), respectively. The most enzymatically active fractions were subsequently concentrated and desalted by VivaSpin® tubes at 5000 xg for 10 min at 4 °C. Purified sample was stored at -20 °C until subsequent use.

Assay of enzyme activity : The present experiment was carried out to determine maximum activity that could be reached in this bioassay. In a typical assay, 200 µl of 0.1 sodium phosphate buffer pH 7.5, 20 µl of 0.067 mM dithiobisnitrobenzoate (DTNB), 10 µl of purified AChE, and 20 µl of 0.5 mM acetylthiocholine iodide were loaded in a microplate well and incubated for 10 min. The activity was quantified spectrophotometrically at wavelength of 405 nm against blank The enzyme activity was expressed as U (nmol $^{-1}$ $min\ mg^{-1}$).

Effect of temperature, pH and substrate on enzyme activity : Purified ChE activity was tested by incubating enzyme with three synthetic substrates at 0.1 M each of acetylthiocholine iodide (ATCi), butyrylthiocholineiodide (BTCi) and propionylthiocholineiodide (PTCi) and different concentrations of substrate from 0.1, 0.5, 1, 2 and 2.5 mM for each substrate. Maximum velocity (V_{max}) and Michaelis-Menten constant (K_m) values were determined using Graphpad Prism 5.0. Effect of pH was elucidated by equilibrating purified AChE for 15 min with different buffers and different pH conditions namely 0.1 M each of acetate buffer (pH 3.0, 4.0, 5.0, and 5.5), sodiumphosphate buffer (pH 5.0, 6.0, 7.0, 7.5, 8.0, 8.5, and 9.0) and Tris-HCl buffer (pH 7.0, 7.5, 8.0, 8.5, 9.0, and 10). Effect of temperature was determined by incubating the assay mixture at 15, 20, 25, 30, 35, 40 and 50 °C for 15 min.

Carbamate screening : *In vitro* sensitivity of *Tor tambroides* was investigated to carbamates as anticholinesterase compounds using bendiocarp, carbaryl, carbofuran, methomyl and propoxur (Sigma). Purified AChE was incubated for 30 min at room temperature with all the carbamate test solutions at following concentrations: 0, 0.02, 0.05, 0.1, 0.2, 0.5 and 1.0 mg l^{-1}. Substrate was then added and AChE activity was determined after 10 min of incubation.

Statistical analysis : Each data point was represented as mean ± SD. Student *t*-test was used to compare two mean points, while differences among more than two groups were analysed using ANOVA followed by Tukey's multiple comparison test at 5% of significant level using Graph Pad Prism 5.0 software (Miller and Miller, 2000). $P< 0.05$ was considered statistically significant.

Results and Discussion

Soluble AChE was purified from brain tissue of freshwater fish, *Tor tambroides*. A summary of partial

purification of AChE is shown in Table 1. In Procainamide Sephacryl S-1000 affinity column step, 53 purification fold was obtained and ChE activity remained bound to column with recovery of 38.41% of original activity. Partially purified enzyme contained 2.47 mg protein, with specific AChE activities of 138.05 µmol min^{-1} mg $protein^{-1}$.

The present study proved that affinity chromatography using Procainamide Sephacryl S-1000 successfully purified *Tor tambroides* AChE even in the presence of high level of proteins mixture. Previous studies ultilizing the affinity ligand procainamide method also successfully purified AChE from various other species such *Lates calcarifer* brain (Hayat *et al.,* 2016) and *Puntius javanicus* liver (Sabullah *et al.,* 2014b).

As shown in Fig.1, the rate of enzymatic hydrolysis of ATCi, BTCi, and PTCi increased from 0 to 2.5 mM, thus proving the capability of enzyme to hydrolyse substrates with different frequencies. All the substrates showed significant difference in term of catalytic reaction ($p < 0.05$). Purified AChE also showed capability to hydrolyse ATCi with highest rate as compared to BTCi and PTCi with maximal velocity (V_{max}) reaching to 113.60 µmol min^{-1} mg^{-1} (Table 2). AChE substrate affinity was also determined. BTCi and PTCi displayed higher K_m values as compared to ATCi, which proved that affinity of *T. tambroides* AChE towards BTC and PTC was lower than ATCi. In other words, based on the apparent biomolecular constant (K_m) value, the affinity of *T. tambroides* AChE was more preferabed to ATCi. The V_{max}/K_m ratio showed catalytic efficiency towards purified sample. In the present study, ATCi showed highest V_{max}/K_m ratio among the substrates. Above 1.0 mM of ATCi, BTCi and PTCi concentration, AChE activity exhibited saturation.

Brain tissue contain abundance of AChE (Tsim and Soreq, 2012) and AChE hydrolyses ATCi more efficiently than other substrates (Hayat *et al.,* 2015). Most studies utilise acetylthiocholine iodide as specific substrate for brain acetylcholinesterase from various species such birds (Santos *et al.,* 2012), fish (Tham *et al.,* 2009; Aidil *et al.,* 2013) and insects (Temeyer and Chen, 2012).

Fig. 2 shows the influence of pH on enzyme activity. The bell-shaped curve in Fig. 2 indicates that the enzyme activity was relatively low at pH 3 and slowly increased to pH 5.5 in acetate buffer. The enzyme activity sharply increased in sodium phosphate buffer from pH 5.5 to 7.5 and the activity levelled out at pH 8.0. However, the activity decreased beyond pH 8.5 to 10 in Tris-HCl buffer. The result showed that *T. tambroides* AChE activity has a broad pH

Table 1 : Purification of solubeAChE from brain tissue of *Tor tambroides*

Procedure	Total protein (mg)	Total activity (U)	Specific activity (Umg^{-1})	Purification fold	Yield (%)
Crude extract	340.70	887.87	2.61	1	100
Supernatant (100,000g, 1 hour, 4°C)	115.59	743.38	6.43	2.50	83.73
Affinity column (Procainamide Sephacryl S-1000)	2.47	340.99	138.05	53.00	38.41

Table 2 : Kinetics properties of AChE from *Tor tambroides* hydrolyzing the synthetic substrates

Substrate	ATC	BTC	PTC
V_{max} (µmole/min/mg)	113.60	95.64	61.20
K_m (mM)	0.0689	0.1268	0.1830
V_{max}/K_m	1648.77	754.26	334.43

Table 3 : Sensitivity of AChE from *Tor tambroides* to pesticide

Compound	Concentration ($mg l^{-1}$)		
	IC_{50} 95% confidence intervals	LOD	LOQ
Bendiocarp	0.0758 (0.05815 to 0.1090)	0.00578	0.033185
Carbofuran	0.0643 (0.0482 to 0.0966)	0.007539	0.030741
Carbaryl	0.0555 (0.0439 to 0.0754)	0.007102	0.02631
Methomyl	0.0817 (0.0571 to 0.1438)	0.010371	0.05058
Propoxur	0.0538 (0.0420 to 0.0746)	0.003615	0.018777

*LOD=Limit of Detection, LOQ=Limit of quantification

range as the activity of each mean point from pH 7.0 to 8.5 showed no significant difference ($p< 0.05$) either in sodium phosphate buffer or Tris-HCl buffer. pH 7.5 was selected as optimum pH as mean point was highest among the others. Optimum condition near to neutral pH from other sources of AChE has been reported (Ding *et al.,* 2011; Sharif *et al.,* 2014).

The optimum temperature of *T. tambroides* AChE for degradation of acetylthhiocholine iodide was determined. Fig. 3 shows that the activity of purified AChE increased until it reached optimal temperature between 25 to 35 °C with no significant difference (p>0.05) in activity at 25, 30 and 35 °C. Beyond 50 °C AChE activity was completely lost.

Temperature plays a major role in promoting enzyme-substrate affinity. Aliriz and Turkoglu (2003) reported optimum activity of AChE in *Chalcalburnustarichii pallasis* at 25 °C, and highest temperature of optimum AChE activity was reported by Assis *et al.* (2010) in *Colossomamacro pomum* at 45 °C. In contrast, Kreimer *et al.* (1995) showed that AChE isolated from *Torpedo californica*is totally denatured after incubation at 45 °C for 3 min and this thermal denaturation was an irreversible process.

The variants of carbamate pesticides with concentration of 1 ppm were tested in the present study and significantly inhibited (p<0.05) *T. tambroides* AChE by more than 50% as compared to control (Fig. 4). Carbaryl, carbofuran and propoxur showed no significant difference by lowering the AChE activity below 20%. IC_{50} was determined by incubating *T. tambroides* AChE in different carbamate. concentrations Data analysis using Graph Pad Prism software, displayed non-linear curves (exponential decay)

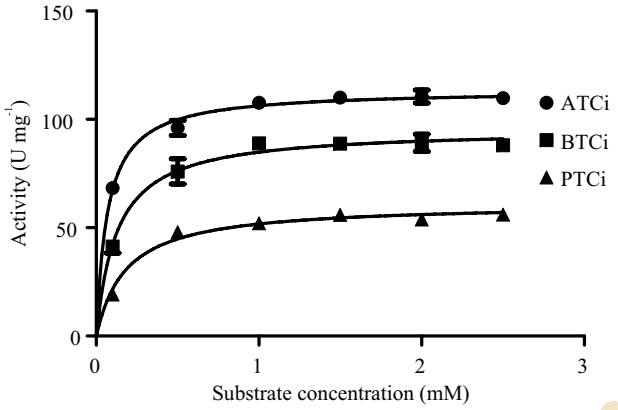

Fig. 1 : Substrates specificity and effect of substrates concentration on the hydrolysis of ATCi, PTC and BTC by partially purified AChE from *Tor tambroides*. Each point represents mean of three determinations with standard deviation of means

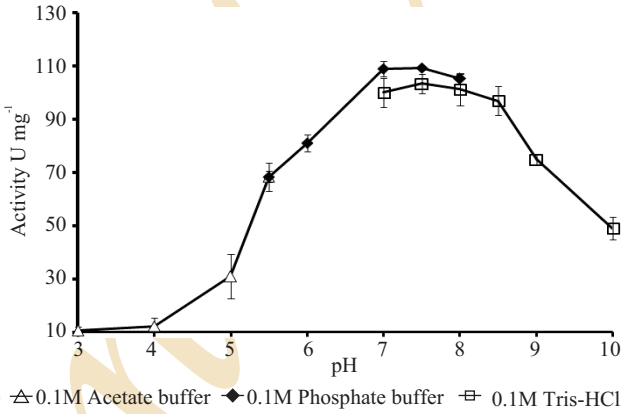

Fig. 2 : Activity of AChE enzyme from *Tor tambroides* at pH ranging from 3.0 to 10. Error bars represent mean ±SD for triplicate

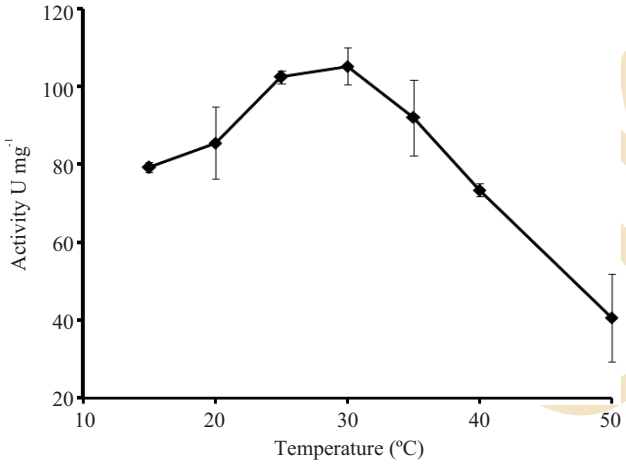

Fig. 3 : Effect of temperature on *Tor tambroides* AChE activity with ATCi as substrate. Error bars represent mean ±SD for triplicate

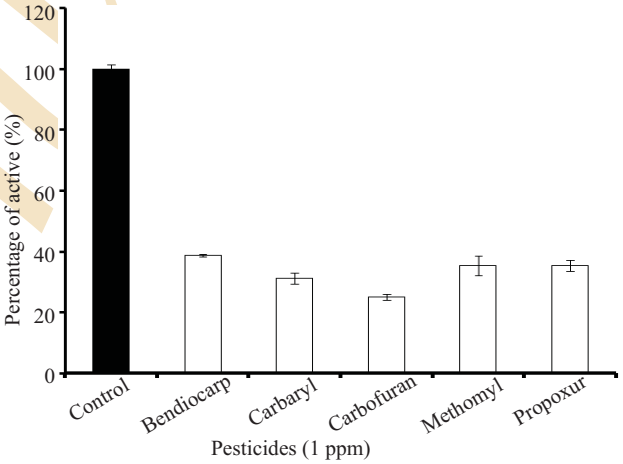

Fig. 4 : : Effect of various pesticides on enzymatic activity of AChE from *Tor tambroides*. Data are expressed as percentage of AChE activity. All values represent mean ±SD

based on the inhibition of AChE from lowest concentration to highest inhibition at highest carbamate concentration, with high correlation coefficient value of 0.95. Carbofuran and carbaryl showed the lowest IC_{50} value of 0.0363 and 0.0389 ppm, respectively, but with no significant difference ($p>$ 0.05) followed by propoxur, bendiocarp and methomyl (Table 3).

Enzymes have been used as rapid and sensitive biosensors for the detection of various toxicants. Enzymatic biosensors also help in distinguish toxic levels of compound in various organisms or species (Guven *et al.,* 2003). AChE is an enzyme, widely used in detecting the presence of contaminants such as carbamate and organophosphate in the medium (Arduini *et al.,* 2006; No *et al.,* 2007). In the present study, several tests were conducted to find a new source of AChE that were sensitive towards selected carbamates based on the inhibition of enzyme activity.

As shown in Fig. 4, it was found that carbamate significantly inhibited *Tor tambroides* AChE activity. Inhibition occur due to carbamylation of serine residue from the esteristic site of AChE by carbamate consequently causing the blocking of substrate metabolisation (Weinbroum, 2005). Long term effects of carbamates exposure is of great concern due to its toxicity. Tham *et al.,* (2009) showed that AChE from local Malaysian fish, *Clariasbatrachus* was sensitive towards carbofuran and carbaryl with IC_{50} of 6.66 and 130.00 $\mu g\ l^{-1}$, respectively,and data was almost similar to the test on the commercial AChE sourced from *Electrophorus electricus* with IC_{50} value of 6.2 and 133.01 $\mu g\ l^{-1}$ for carbofuran and carbaryl, respectively. The present study also showed that AChE isolated from *T. tambroides* was able to detect more types of carbamates with acceptable sensitivity. All the variants of carbamates significantly inhibited AChE activity as compared to control.

In conclusion, due to the sensitivity of *T. tambroides* AChE towards carbamate can considered as an alternative source of environmental biosensor. Further studies are recommended to identify the ability of *T. tambroides* AChE to detect other toxic compounds such heavy metals and organophosphate, prior to proceeding with commercial kit development.

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