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# Acetylcholinesterase from *Puntius javanicus* for the detection of carbamates and organophosphates

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## ABSTRACT

A new alternative local source of acetylcholinesterase (AChE) has been found to be sensitive towards several insecticides. AChE was purified from the brain tissue of *Puntius javanicus*using affinity chromatography (procainamide hydrochloride coupled with sephacryl 6B) with the total recovery of 40% at 8.48 purification fold. All carbamate (CB) insecticides tested at the concentration of 1 mg/L were capable of lowering AChE activity to less than 30%; Bendiocarp (18.80%), carbaryl (5.96%,), carbofuran (6.12%), methomyl (13.91%) and propoxur (15.851%). The *P. javanicus* AChE was found to be unaffected by several activated organophosphates (OP) such as acephate and dimethoate, while trichlorfon slightly inhibited the enzyme activity ($p<0.05$). Chlorpyrifos, diazinon, malathion and parathion lowered AChE activity to 43.02, 40.97, 37.11 and 46.72%, respectively. Pesticides that inhibit AChE activities more than 50% were further tested in different concentrations to determine the half maximal inhibitory concentration ($IC_{50}$). Carbofuran and carbaryl showed the lowest $IC_{50}$ value at 0.035 and 0.031 mg/L, respectively, as both showed no significant differences ($p<0.05$), followed by bendiocarp, propoxur, methomyl, malathion, diazinon, parathion and chlorpyrifos at 0.045, 0.076, 0.090, 0.063, 0.103, 0.151 and 0.202 mg/L, respectively. Based on these results, the sensitivity of AChE from brain *P. javanicus* brain tissue shows promise as an alternative biosensor for the detection of insecticides contamination.

**Keywords:** *Puntius javanicus*, Acetylcholinesterase, Carbamate, Organophosphate.

## INTRODUCTION

Chemical applications are widely used to control pest activity in agriculture. Insecticides such as carbamates and organophosphates are used to control insect development by binding at the active site of AChE through carbamylation (carbamates) or phosphorylation (organophosphates) and inhibiting the metabolism of neurotransmitter in the nervous system that leads to lethality (Rosenberry et al.,2005; Sabullah et al., 2014a). Unfortunately, overuse and misapplication of these compounds may drift and affect non-target organisms, contaminating the food web and transfer to the final consumer through bioaccumulation, especially in humans (Van Geest et al., 2014). Soil leaching and groundwater runoff from the treated areas to waterways may affect aquatic life (Bonmatin et al., 2015). However, this problem can be through the use of aquatic organism as testing materials to expand the use of a biological by sensing the presence of anti-AChE. Fish is commonly used as a bioindicator for the presence of pesticides (Singh et al., 2007; Young et al., 2014). In previous studies, the relationship between the presence of xenobiotics in aquatic environments and ChE activity has been widely studied and employed as a biomarker in aquatic invertebrate and also vertebrate species (Brown et al., 2004; Sabullah et al., 2014b). Measurement of fish cholinesterase activity is a classical tool used to monitor pollution in both marine and continental waters. AChE isolated from local Malaysian fish such catfish; *Clarias batrachus* that is capable to be inhibited by carbaryl and carbofuran had proved the ability to discern the presence of anti-AChE (Tham et al., 2009). In this study, we reveal an alternative source by using AChE isolated from the brain tissue of *Puntius javanicus,* which was broadly sensitive towards several selected insecticides and as a candidate for future biosensor kit development.

## MATERIALS AND METHODS

**Brain Extraction:**P. javanicus weighing 300-400 g and approximately 15-20 cm in length were obtained from Kenyir Lake, Terengganu, Malaysia. The fish was killed by immersing it in a box of ice then the brain was immediately dissected and weighed. The brain was homogenised with the ratio of 1:5 (w/v) volume of 0.1 M sodium phosphate buffer, pH 7.5 containing 2 mM phenylmethylsulfonyl fluoride (PMSF; AMRESCO® brand) using an Ultra-Turrax T25 homogeniser until completely pulverised. The crude extract was subjected to centrifugation at 100,000xg for an hour at 4ºC. The supernatant was collected for purification procedures. All procedures were performed at 4°C unless stated otherwise.

**Purification of Acetylcholinesterase:** A ligand specific for the choline-binding site, procainamide hydrochloride (Sigma-Aldrich) coupled with Sephacryl 6B (Sigma-Aldrich) was used as an affinity matrix to purify the brain extract. The matrix was loaded in the column with a diameter of 1.5 cm and then allowed to sediment to obtain a bed height around 3 cm. The column was first washed with five batch volumes of washing buffer (20 mM sodium phosphate buffer, pH7.5) to clean and calibrate the matrix at the flow rate of 0.2 ml per min. 400µl of crude extract was then loaded into the column followed by three batch volumes of washing buffer. Three batch volumes of elution buffer (20 mM sodium phosphate buffer containing 1.0 M sodium chloride, pH 7.5) were applied directly into the column and1 ml fractions were collected until at the end of the elution stage. Enzyme activity (Ellman et al., 1961) and protein content determination (Bradford et al., 1976) were carried out for all the fractions collected and fractions that displayed high AChE activity were pooled. The purified AChE was concentrated and desalted using VivaSpin® tubes at 5000xg at 4°C. The dialysed purified AChE was stored at -25°C until subsequent use.

**Activity and Inhibition Studies:** Ellman et al. (1961) method was used to determine the activity of AChE with modification for a 96 well microplate assay. The synthetic substrate acetylthiocholine iodide (ATC) was used in this study. The AChE will hydrolyse ATC to acetate and thiocholine, followed by the production of yellow colour by the reaction of 5, 5'-dithio-bis-2-nitrobenzoate (DTNB) reagent with thiocholine. This yellow colour production was quantified by measuring it at the wavelength of 405nm using a microplate reader. AChE activity was calculated based on the amount of ATC (µmol) that was hydrolysed by *P. javanicus* AChE per minute per total protein, which was given as µmole hydrolysed/min/mg (U) with the extinction coefficient of 13.6 $mM^{-1}.cm^{-1}$. Purified AChE was incubated separately in 1 mg/L of carbamate (bendiocarb, carbofuran, methomyl, carbaryl and parathion: Sigma-Aldrich brand) and activated OP (malathion, diazinon, chlorpyrifos, acephate, dimethote and trichlorfon: Sigma-Aldrich brand). Activation of OP insecticides was performed by incubating each OP (25µl) in 5 µl of 0.01 M pure bromine for 15 minutes then 20 µl of 5% ethanol was loaded to stop the activation process, which acts as a reducing agent. The assay mixture contained 150 µl of potassium phosphate buffer (0.1 M, pH 7.5), 20 µl of 0.067 mM DTNB (Fluka), 50 µl of insecticides solution (1 mg/L) and 10 µl of enzymes followed by incubating it in the dark for 15 minutes at room temperature. 20 µl of 0.5 mM ATC (Sigma-Aldrich) was then added and left to stand for 10 minutes at room temperature before the absorbance was read at 405 nm.

$IC_{50}$ determination was carried out by incubating each of the insecticides at six different concentrations with *P. javanicus* AChE and the $IC_{50}$ value was analysed using Graphpad PRISM 5 software with non-linear regression analysis using one phase exponential decay model.

**Statistical Analysis:** All experiments were run in triplicates and each value are means of standard deviations, ± SD. Analysis of the mean AChE activity was evaluated using Graphpad Prism version 5.0. Comparison between each group was carried out using a Student's t-test for two groups or a one-way analysis of variance (ANOVA) with post hoc analysis by Tukey's test (Miller and Miller, 2000) for more than two groups. $p< 0.05$ was considered statistically significant**.**

## RESULTS AND DISCUSSION

**AChE Purification:** The purification process was carried out in this study because the increasing purity of the sample enhances its sensitivity towards the contaminant (Masdor and Said, 2011). AChE was purified from the brain extract of *P.javanicus* using Procainamide-based affinity column. The total recovery activity of AChE after purification was 40% with 8.48 fold purification (Table 1).

**Table.1.Purification table of *P. javanicus* AChE**

| Procedure | Total Activity (U) | Total Protein (mg) | Specific Activity (U/mg) | Purification fold | Recovery (100%) |
|---|---|---|---|---|---|
| Crude extract | 0.85 | 3.77 | 0.23 | 1 | 100.00 |
| Supernatant | 0.73 | 2.6 | 0.28 | 1.23 | 85.28 |
| Affinity Chromatography | 0.34 | 0.18 | 1.92 | 8.48 | 40.00 |

**Inhibition Studies:** Carbamate insecticides such as bendiocarp, carbaryl, carbofuran,,methomyl and propoxur exhibited inhibition by lowering AChE activity to 18.80, 6.12, 5.96, 13.91 and 15.851%, respectively (Figure 1). Gupta et al., (2011) reviewed insecticides such as methomyl and carbofuran that are extremely dengerous to living system. Anova analysis revealed several OP that showed significant inhibition compared to control ($p<0.05$) such as chlorpyrifos, diazinon, malathion andparathion with the percentage inhibition of 21.36, 16.41, 21.10 and 24.36%, respectively. Trichlorfon showed small inhibition approximately <5% while acephate and dimethoate were considered to show no effect as they exhibited no significant difference compared to controls ($p<0.05$). Yen et al., (2011), reported chlorpyrifos and diazinon at the concentration of 300 nM and 10 µM were capable of lowering the activity of zebra fish AChE to 81 and 55%, respectively. Malathion at the concentration of 6 mg/kg has significantly inhibited AChE activity of *Seriola dumerilli* from 141.22 to 69.16 U (Jebali et al., 2006). All of the insecticides that showed significant inhibition were subjected for $IC_{50}$ determination.

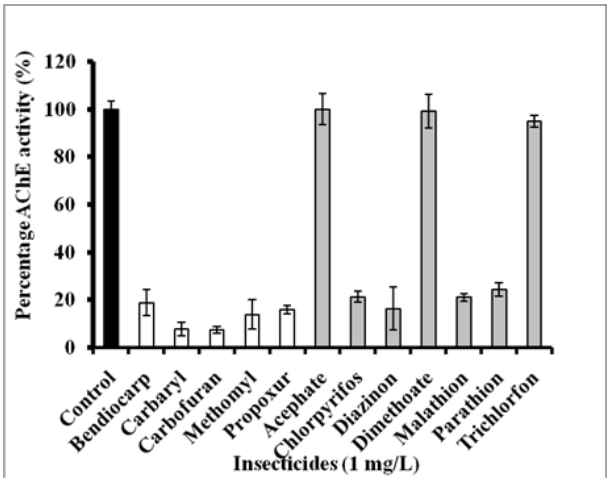


**Figure.1. Purified AChE was incubated in different of insecticide compound with the final concentration of 1 mg/L. White and grey bars represent as CB and OP, respectively.**

Table 2 shows $IC_{50}$ values and their 95% confidential intervals for selected insecticides in comparison with another source of AChE such *Electrophorus electricus* and and *Clarias batrachus*. Each value was analysed with nonlinear regression with the use of one phase decay. Although *C. batrachus* and *P. javanicus* both are economically important in Malaysia, in the terms of sensitivity, *P. javanicus* AChE was highly comparable with the commercial AChE extracted from *E. electricus*. Carbaryl and malathion from CB and OP group displayed the lowest $IC_{50}$ value at 0.031 mg/L and 0.063 mg/L, respectively (Figure 2). Farahat et al., (2003) mentioned that organophosphates gave a wide impact to the environment as this compound is capable to form aging or irreversible inhibition of several enzymes especially AChE. Prior of the reaction, biotransformation is needed to activate the sensitivity of organophosphates to their active metabolite, where the process of oxonation was biologically carried out by microsomal cytochrome oxidase P450-dependent in the liver (Fukuto, 1990). In this study, OP was chemically activated using bromine water as mentioned by (Barber, 1999).

**Table.2. The $IC_{50}$ values of selected insecticides with 95% confidential interval**

| | **$IC_{50}$ (95% Confidential interval) mg/L** | | |
|---|---|---|---|
| **Compound** | ***P. javanicus*** **(Present study)** | ***E. electricus*** **(Sharif *et al.* 2014)** | ***C. batrachus*** **(Tham *et al.* 2009)** |
| Bendiocarp | 0.045 (0.039 -0.054) | 0.015 (0.015-0.016) | N.D. |
| Carbaryl | 0.031 (0.026 -0.040) | 0.133 (0.122-0.145) | 0.130 (0.119-0.142) |
| Carbofuran | 0.035 (0.030 -0.045) | 0.006 (0.0063-0.0065) | 0.006 (0.005-0.007) |
| Methomyl | 0.090 (0.077 -0.108) | 0.026 (0.024-0.028) | N.D. |
| Propoxur | 0.076 (0.061 -0.099) | N.D. | N.D. |
| Chlorpyrifos | 0.202 (0.178 -0.232) | 0.060 (0.055-0.065) | N.D. |
| Diazinon | 0.103 (0.084 -0.132) | 0.177 (0.169-0.186) | N.D. |
| Malathion | 0.063 (0.053 -0.078) | 0.014 (0.013-0.014) | N.D. |
| Parathion | 0.151 (0.122 -0.198) | 0.068 (0.066-0.069) | N.D. |

N.D.=Not detected

Compared to organophosphates, carbamates can easily bind to the esteric site of AChE directly without any prior need of bioconversion. Common carp, *Cyprinus carpio* showed 50% inhibition after being exposed to carbofuran (Dembélé, 2000) and 40% to 60% inhibiton in Mosquito fish *Gambusia yucatana* (Rendón-von *et al.* 2005). *Oncorhynchus mykiss* showed significant inhibition toward carbaryl at the lower $IC_{50}$ value of 0.019 mg/l (Ferrari, 2004). Methomyl had proved to be adverse to freshwater fish such as *Pseudorasbora parva* with the

ability to inhibit several enzymes such as AChE, Gluthathione S-Transferase and glutamic oxaloacetic transaminase (Li, 2008). Carbaryl exhibited 13 to 20 times higher toxicity compared to malathion on squawfish (Beyers and Sikoski, 2009).

Most of the insecticides can be broadly applied for many types of crops. For the example, carbofuran is currently applied to oil palm plantations and also to protect the production of fruits, vegetables and paddy fields in Malaysia (Farahani et al., 2008). Other carbamate applications such as bendiocarp and propoxur are more for controlling the development of household pests (Goose, 1987). These kinds of insecticides showed low toxicity towards mammals and vertebrates since the activity of cholinesterase were back to normal within 24 hours after acute exposure (Dorko, 2011). This study shows that purified AChE was inhibited by all tested compounds in the CB group and by several compounds in the OP group. Because organophosphates such as trichlorfon, acephate and dimethoate were unable to inhibit *P. javanicus* AChE activity in this study, other local sources of AChE need to be screened for the ability to detect many kinds of nerve agents, especially these compounds. Moreover, the use of enzymes for the biomonitoring of toxicants has becoming more widespread in an effort to speed up the detection process, cost reduction and handling.

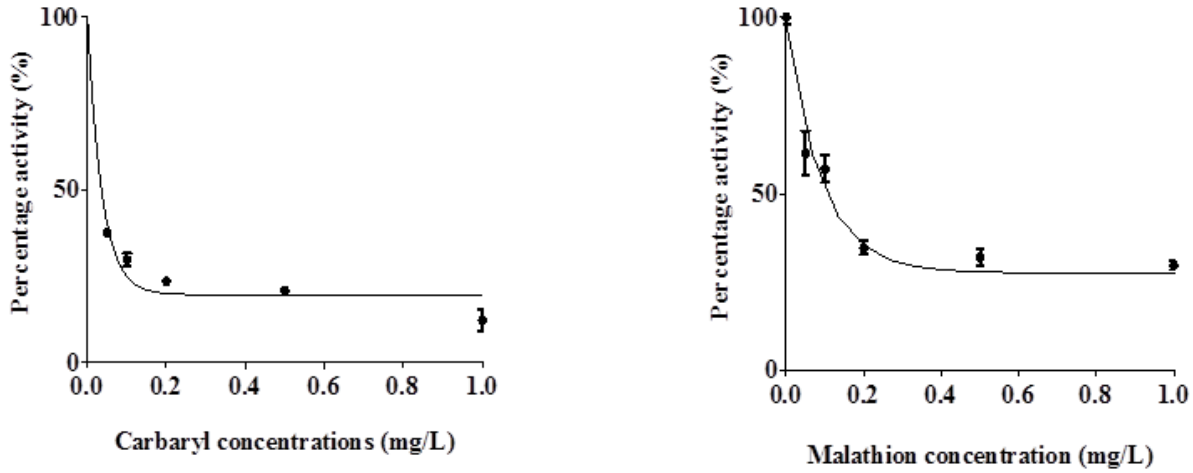


**Figure.2. Remaining activity of purified AChE after incubated in different concentration of carbaryl and malathion. Each point represented as mean of triplicate with standard deviation (SD).**

## CONCLUSION

These results show that AChE from *Puntius javanicus*is a potentially new local source to replace current commercial AChE for use in biomonitoring programmes. Further studies are needed for the development of this assay that is capable of detecting other pollutants such as detergents (Kucherenko, 2012), neurotoxin (Zakir Hossain, 2009), drug (Elumalai, 2015) and heavy metals (Frasco, 2007), which are threatening our environment and ecosystems stability. Their early detection is important as a prevention step to overcome the contamination of those compounds.

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