The evaluation of *Periophtalmodon schlosseri* as a source of acetylcholinesterase for the detection of insecticides

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**INTRODUCTION**

Pesticides are used in agriculture to control the activity of the pests by an external contact on the surface reaction or by internal contact which is by consuming\(^1\). The crisis of this compound is improper applications which contaminate rivers and other water source endangered the aquatic organism, birds and human\(^2, 3\). The impact of pesticides toxicity are major public health concerns globally\(^3\).

Acetylcholinesterase (AChE) is a functional protein in the brain that is widely used as a traditionally analysis as to biomonitor the environmental pollution\(^4\). The alteration of the AChE activity in the tissues of fish and other is caused by inhibition of organophosphorous and carbamates exposure determine of this enzyme have potent as biomarker for such compounds\(^5-8\). Organophosphorous and carbamates insecticides are able to interact with the cholinergic system by binding at the activie site of AChE\(^9\). Heavy metals also affected to the inhibition of cholinesterase activity such chromium, arsenic, mercury and others\(^10, 11\). The persistence of heavy metals may accumulate in the ecosystem food chain and cannot be detoxified through degradation\(^12\).

Inhibition of AChE is not compulsory lethal, but the purpose of a highly sensitive AChE source from fish as a viewpoint species can detect the lowest detection level of contaminant. In this work, AChE was obtained source from the brain of mudskipper, *Periophtalmodon schlosseri* and will be purified by using procainamide-base affinity chromatography and level of inhibition will be determined. This study was carried out to add on data for further comparative studies on the local sea fish in Malaysia.

**MATERIALS AND METHODOLOGY**

**Chemicals**

Carbofuran, methomyl, carbaryl, parathion, malathion, diazinon, bendiocarb, chlorpyrifos, acephate, dimethoate and trichlorfon were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Bromine, acetylthiocholine iodide (ATC), propionylthiocholine chloride (PTC), β-mercaptoethanol and procainamide hydrochloride were purchased from Sigma-Aldrich. 5'-dithio-bis (2-nitrobenzoic acid) (DTNB) and butyrylthiocholine iodide (BTC) were purchased from Fluka Chemie GmbH. Biorad Protein Assay were purchased from from Bio-Rad Laboratories Inc. Vivasin4 was sourced from Vivascience. All other chemicals used in this study were of analytical or special grade.

**Specimens**

AChE from mudskippers, *Periophtalmodon Schlorsseri* was obtained from Pulau Melaka, Malacca, Malaysia (N 2°10’47.82 E 102°15’19.62”). Only healthy and disease-free fishes were used for the experiment. The specimens were brought alive to the laboratory and were frozen immediately. They were decapitated and their brains were dissected out immediately and weight.

**Preparation of Affinity Chromatography Columns; Epoxy (Bisoxirane) Activation**
Affinity procainamide chromatography was prepared according to the modified method of Ralston et al. (1983) [13]. Briefly, Sephacryl S-1000 (100ml settled gel) (Sigma, St. Louis, USA) was washed with 1 L of deionized water in a sintered glass funnel, sucked dry to a wet cake, and then transferred to a 500 ml beaker. The gel was suspended in 75 ml of 0.6 M NaOH containing 150 mg sodium borohydride (Sigma, St. Louis, USA) and stirred. About 75 ml of 1,4-butanediol diglycidyl ether (Sigma, St. Louis, USA) was slowly added with constant stirring. The reaction mixture was stirred at room temperature overnight. The activated gel was thoroughly washed with water to remove excess reagent. The washing was continued until there was no longer evidence of an oily film on the surface of the gel representing the remaining epoxy compound. Acetone was used to aid in the complete removal of 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 (Sigma, St. Louis, USA). The pH of the gel slurry was then adjusted to 12 by the addition of 1.0 M NaOH. The mixture was incubated at 25°C for 96 hours 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 excess active groups on the gel were blocked by suspending the gel in 100 ml of 1.0 M ethanolamine (pH 9.0) which acted to block all remaining active groups on the amine-reactive supports. The mixture was stirred at room temperature for 6 hours. Finally, the gel was 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 matrix was packed in the column and allowed to settle to obtain a bed height of 3 cm. Flow rate was maintained at 0.2 ml min-1. The matrix was first washed with 5 batch volumes of washing buffer (20 mM sodium phosphate buffer, pH 7.5) to clean and equilibrate the column. The crude extract was then loaded onto the affinity matrix. At least 3 batch volumes of washing buffer were then applied directly to the matrix. Fractions of 1 ml were then collected in Eppendorf tubes and kept on ice. Washing was continued until all non-absorbed proteins were washed out. At least 3 batch volumes of elution buffer (20 mM sodium phosphate buffer containing 1.0 M sodium chloride, pH 7.5) were then applied directly to the matrix. Collection of 1ml fractions into Eppendorf tubes continued until the elution process was completed. Enzyme activity and protein content determination was carried out for all the fractions collected. Fractions exhibiting high AChE activity collected during the elution process were then pooled. The partially purified sample was concentrated and dialyzed with 3 batch volumes of washing buffer using VivaSpin® tubes at 2500 rpm at 4°C. The dialyzed partially purified AChE was stored at -20°C until subsequent use.

Determination of protein content
Bradford’s method (1976) was used for quantitative determination of proteins with bovine serum albumin (BSA) as a standard [14].

Activity and Effect of Substrates
AChE activity was measured using the method developed by Ellman et al. (1961) with modification for a 96 well microplate assay [15]. This method employs acetylthiocholine iodide (ATC) as a synthetic substrate for AChE. ATC is broken down to thiocholine and acetate by AChE and thiocholine is reacted with 5, 5'-dithio-bis-2-nitrobenzoate (DTNB) to produce a yellow color. The quantity of yellow color which develops over time is a measured of the activity of AChE and can be measure using microplate reader. AChE activity is expressed as the amount of butyrylthiocholine iodide (μmol) which is broken down by AChE per minute. The specific activity is given as μmole hydrolyzed/min/mg of protein or U mg-1 of protein and was calculated on the basis of an extinction coefficient of 13.6 mM-1.cm-1. Experiment was carried out in triplicates. The Km(app) was determined by analysis of Graphpad PRISM 4 for non-linear regression analysis software available from www.graphpad.com. OPs were subjected to activation according to the modified method of Villate et al. (1998) [16]. The pesticide (25 μl) was incubated in 5 μl of 0.01 M pure bromine solution at room temperature for 20 minutes. The activation process was stopped with 20 μl of 5% ethanol, which acted as a reducing agent. Preliminary experiments showed that bromine and ethanol at the given concentration in this bioassay system did not inhibit AChE activities. The IC50 was determined using at least five different concentrations of carbamate and OPs. The assay mixture in a well contained 150 μl of potassium phosphate buffer (0.1 M, pH 8.0), DTNB (20 μl, 0.067 mM), carbamate (50 μl) and enzymes (10 μl). The mixture was incubated in the dark for 10 minutes at room temperature. Acetylthiocholine iodide (20 μl, 0.5 mM) was then added. Again, the mixture was left to stand but for 10 minutes at room temperature before the absorbance was read at 405 nm [17].

Standard analysis
To compare the value between other group, statistically analyze by a student’s t-test or a one-way analysis of variance with post hoc analysis by Tukey’ test with the 95% confidence interval was performed [18].

RESULTS AND DISCUSSION

Purification processes
Purification stage using affinity chromatography exhibited higher specific activity compared to the other stage after the sample was homogenized and centrifuged. AChE was successfully partial purified with the purification fold approximately 5.9 fold and yield 31.62% (Table 1).

Insecticides inhibition studies
The inhibition studies showed that carbamate group mostly inhibited the acetylcholinesterase activity while bendiocarb, cabaryl, carbofuran and propoxur inhibited more than 80% after incubated in 1 ppm (Figure 1). AChE cannot be inhibited by malathion and chlorpyrifos even though they exhibited significant activation which was a small difference compared to 3% of the control. The organophosphate test, AChE did not show extreme inhibition or can consider there were no inhibitions. Generally, organophosphate group poor inhibit acetylcholinesterase as proven by previous investigators [17, 19]because these compounds need biotransformation of non toxic OP to be active metabolites [20]. Further study to detect half life
(IC50) of carbamate group will be done in this work (Table 2). Result showed the IC50 values with the 95% confidence interval are 0.1124 (0.1025 to 0.1245), 0.0567 (0.0504 to 0.0648), 0.0633 (0.0537 to 0.0773), 0.0450 (0.0399 to 0.0517) and 0.0892 (0.0761 to 1.0777) for carbaryl, methomyl, bendiocarp, carbofuran and parathion, respectively. Among the various pesticides commonly applied in agriculture, carbofuran is more persistent than other carbamate. Carbofuran has been detected in ground, surface, and rain water due to its widespread use [21, 22]. Carbofuran is widely used for the foliar feeding insects and control of soil dwelling. The use is broadly applied in paddy field, sugar cane, vegetables and fruits in Malaysia making their detection important [23]. It is a broad spectrum of systemic acaricide and insecticide which is typical examples exhibit fairly high toxicity and more extensively [24, 25]. AChE inhibition is a sensitive indicator relative to clinical effects of exposure to carbofuran in both animals and humans [24, 26].

Table 1: The purification table of various procedure of AChE from Periophthalmodon schlosseri. (U) is equal to nmole/min/mg.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total activity (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Purification factor (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>1229.76</td>
<td>5.6</td>
<td>219.74</td>
<td>1.0</td>
<td>100.00</td>
</tr>
<tr>
<td>Crude supernatant (Centrifugal 30,000g, 4°C, 1 hour)</td>
<td>829.03</td>
<td>1.4</td>
<td>594.67</td>
<td>2.7</td>
<td>67.41</td>
</tr>
<tr>
<td>Affinity Chromatography (Procainamide Sepharl S-1000)</td>
<td>389.70</td>
<td>0.3</td>
<td>1292.07</td>
<td>5.9</td>
<td>31.62</td>
</tr>
</tbody>
</table>

Table 2: Half life (IC50) of partial purified AChE activity expose with various carbamate

<table>
<thead>
<tr>
<th>Carbamate Compound</th>
<th>IC50 (95% Confidence Interval) mg/L (ppm)</th>
<th>Limit of Detection (LOD) mg/L (ppm)</th>
<th>Limit of Quantitation (LOQ) mg/L (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbaryl</td>
<td>0.1124 (0.1025 to 0.1245)</td>
<td>0.00824</td>
<td>0.01531</td>
</tr>
<tr>
<td>Methomyl</td>
<td>0.0567 (0.0504 to 0.0648)</td>
<td>0.02722</td>
<td>0.03490</td>
</tr>
<tr>
<td>Bendiocarp</td>
<td>0.0633 (0.0537 to 0.0773)</td>
<td>0.02544</td>
<td>0.03576</td>
</tr>
<tr>
<td>Carbofuran</td>
<td>0.0450 (0.0399 to 0.0517)</td>
<td>0.02997</td>
<td>0.03384</td>
</tr>
<tr>
<td>Propoxur</td>
<td>0.0892 (0.0761 to 0.1077)</td>
<td>0.01430</td>
<td>0.03247</td>
</tr>
</tbody>
</table>

Each of these insecticides is toxic to fish when exposed in vivo and the mechanism of inhibition is probably through inhibiting cholinesterases function [27]. For example, carbaryl is toxic to Gobiocypris rarus (Chinese Rare Minnow) [26], Oncorhynchus mykiss (rainbow trout) [28] and Cirrhina mirigala (Cyprinidae) [29]; Tetradotoxin is toxic to Lagocephalus scleratus (Pufferfish, Fugu) [30] and Mytus Vittatus (‘Baung’) [31]; and propoxur is toxic Carassius auratus [32] and Mytus Vittatus (‘Baung’) [31].

**Figure 1**: The inhibition of the activity of partially purified AChE from Periophthalmodon schlosseri by insecticides with the mean point of triplicate assay and Y error bars which indicate ± Standard deviation of the mean.

The organophosphates are not toxic in their own and need activation by oxonation. This is accomplished using bromine water in vitro and enzymes in vivo. Parathion is converted to O,O-
CONCLUSION

This work can be concluded that the acetylcholinesterase source from Periophthalmodon schlosseri is the best bioindicator for various carbamate xenobiotic insecticides such as carbayl, metalomyl, bendiocarp, carbofuran and propoxur. For further studies, this enzyme will be tested with water polluted sample and compare with other AChE sources.

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Department of Wildlife and National Park (PERHILITAN), Cheras, Malaysia

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