Assessment of *Clarias batrachus* as a source of acetylcholinesterase (AChE) for the detection of insecticides

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**Abstract:** An inhibitory assay of insecticides using Acetylcholinesterase (AChE) from the local fish *Clarias batrachus* is reported. AChE was assayed according to the modified method of Ellman. Screening of insecticide and heavy metals showed that carbofuran and carbaryl strongly inhibited *C. batrachus* AChE. The inhibition concentration (IC) values (and the 95% confidence interval) for both carbofuran and carbaryl inhibition on *C. batrachus* AChE at 6.66 (5.97-7.52) and 130.00 (119.3-142.5) µg l⁻¹, respectively, was within the IC₅₀ range of Electrophorus electricus at 6.20 (6.03-6.39) and 133.01 (122.40-145.50) µg l⁻¹, respectively, and were much lower than bovine AChE at 20.94 (19.53-22.58) and 418.80 (390.60-451.60) µg l⁻¹, respectively. The results showed that *C. batrachus* have the potential to be used as a cheaper and more readily available source of AChE than other more commercially available sources.

**Key words:** C. batrachus, AChE, Carbamate

PDF of full length paper is available with author (*yunus@biotech.upm.edu.my*)

**Materials and Methods**

**Chemicals and enzymes:** Bendiocarb, carbaryl, carbofuran, methomyl, acephate, chlorpyrifos, diazinon, dimethoate, malathion, parathion, silver (ii), aluminum (iii), arsenic (vi), cadmium (ii), chromium (vi), copper (ii), ferrous iron, mercury (ii), magnesium (ii), manganese (ii), nickel (ii), lead (ii), zinc (ii), acetilthiocholine iodide (ATC), β mercaptoethanol and procainamide hydrochloride were purchased from Sigma-Aldrich. 5.5-dithio-bis (2-nitrobenzoic acid) (DTNB) was purchased from Fluka Chemie GmbH. Phenylmethylsulfonyl fluoride (PMSF) was sourced from AMRESCO®. Two commercial AChEs from eel (*Electrophorus electricus*, Lot no 044K7655, 349 units/mg solid) and bovine (Bos Taurus, Lot no 91H9324, 0.35 units/mg solid) were from Sigma (St. Louis, USA). All other chemicals used in this study were of analytical grade.

**Preparation of brain AChE extracts from *C. batrachus***: *Clarias batrachus* weighing 800-1000 g and approximately 20 cm in length was obtained from the hatchery at University Putra Malaysia, Serdang, Selangor Darul Ehsan. The fish were checked for external signs of injury or disease. Only healthy and disease-free fish were used for experimentation. They were acclimatized to laboratory conditions for two days in aquarium filled with 20 liter of chlorine-free tap water at 25°C and fully aerated. The fish were fed with commercial fish pellet prior to the experiment. To reduce possible dietary influences on the metabolite status, fish were starved a day before the start of the experiment. Water was changed daily to maintain the cleanliness and to eliminate the interfering products of respiration and excretion. The fish were killed by decapitation and the whole brain was dissected out immediately. Approximately one gram of brain was homogenized in 10 ml of 20% (w/v) of 0.1 M sodium phosphate buffer pH 8.0 containing 1 mM phenylmethylsulfonyl fluoride (PMSF) as an

**Introduction**

The use of enzymes in the biological assessment of the environmental impact of chemical substances has increased in the past few years. Acetylcholinesterase (AChE), which is the enzyme that inactivates the neurotransmitter acetylcholine presents at the synapse is one of the widely used enzymes for this purpose. The inhibition of cholinesterase activity has been successfully used to detect insecticides in various samples (Souza *et al.*, 2003).

However, the use of fish AChE as a source of enzyme is still not widely pursued. The current sources of AChE for pesticide bioassay and biosensor technology are *Drosophila melanogaster* and *Electrophorus electricus*. AChE from *D. melanogaster* is more sensitive than *E. electricus* but the latter is less expensive, could detect insecticides within the permissible limit allowed by many countries and is commercially readily available (Villatte *et al.*, 1998). Fish has been traditionally used as one of the organisms of choice for the bioassay of a variety of toxicants such as detergents (Kumar *et al.*, 2007), pesticides (Mondal *et al.*, 2007), textile dyes (Soni *et al.*, 2006) and heavy metals (Oyewo and Don Pedro, 2008). This reflects the sensitivity of fish to toxicants. With this in mind, this study is aimed at evaluating the possibility of using AChE from the local catfish *C. batrachus* as a cheaper and more available source of AChE for the assay of insecticide. The use of *C. batrachus* takes into account of the quite substantial aquaculture industry use of this species in Malaysia. This species has also been reported to be a sensitive biomarker for carbamate such as carbaryl (Parveen *et al.*, 2004). In this work we demonstrate that the AChE from this species has similar sensitivity to commercial AChEs and is an excellent candidate for the detection of insecticide.
antiprotease using an Ultra-Turrax T25 homogenizer fitted with a Teflon pestle. The brain suspension was homogenized and the crude extract was subjected to centrifugation at 15 000×g for 10 minutes at 4°C to remove debris. The resulting homogenate was then subjected to ultracentrifugation at 100,000×g in a Sorval® Ultra Pro 80-TH-641 for one hr at 4°C to separate the cytosol and membrane components. The pellet was discarded and the supernatant was used in the purification procedures. All procedures were performed at 4°C unless otherwise stated.

Isolation and partial purification of cholinesterase: Partial purification was performed using procarboxamid affinity 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 1ml were then collected 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) was then applied directly to the matrix. Collection of 1 ml fractions into each 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 was collected during the elution process were then pooled.

Concentration and dialysis: The partially purified sample was concentrated and dialyzed with 3 batch volumes of washing buffer using Viva Spin tubes at 2500 rpm at 4°C. The dialyzed partially purified AChE was stored at -20°C until subsequent use. At this temperature, the enzyme was stable for more than six months.

Enzyme activity determination: AChE activity was measured using the method developed by Ellman et al. (1961) with modification for a 96 well microplate assay. This method employs acetylthiocholine iodide (ATC) as a synthetic substrate for AChE. Acetylthiocholine iodide is broken down to thiococline and acetate by AChE and thiococline 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 measure of the activity of the enzyme AChE and can be measured using a microplate reader. AChE activity is expressed as the amount of Acetylthiocholine iodide (µmol) which is broken down by AChE per minute. The specific activity is defined as µmole hydrolyzed min⁻¹ mg⁻¹ 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 Ellman et al. (1961).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U mg⁻¹)</th>
<th>Purification fold</th>
<th>Yield (%)</th>
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<tbody>
<tr>
<td>Crude</td>
<td>563.3</td>
<td>43,047,419.6</td>
<td>76,424.2</td>
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<td>100.0</td>
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<tr>
<td>Affinity-edrophonium-Sephaeryl S4000</td>
<td>55.3</td>
<td>20,125,879.1</td>
<td>363,654.5</td>
<td>4.8</td>
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<tr>
<th>AChE</th>
<th>IC₅₀ (95% Confidence interval) µg⁻¹</th>
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<tr>
<td>Carbofuran</td>
<td>6.66 (5.97-7.52) 130.00 (119.3-142.5)</td>
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<td>Clarias batrachus</td>
<td>20.94 (19.53-22.58) 418.80 (390.60-451.60)</td>
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Assay for insecticide and effects of heavy metals as interference: The insecticide and heavy metals to be incorporated in the screening assay were dissolved in the respective solvents and were diluted with distilled water to a final concentration of 1 mg⁻¹. The reaction mixtures contained 150 µl of 0.1 M potassium phosphate buffer pH 8.0, 20 µl DTNB (0.067 mM) followed by 50 µl of 1 mg⁻¹ of the xenobiotics and subsequently 10 µl of enzymes. The mixtures of enzyme preparation were incubated in the dark for 30 min at room temperature. Finally, 20 µl of ATC (0.5 mM) was then added. Again, the mixture was left to stand for 10 minutes at room temperature before the absorbance was read at 405 nm wavelength. The control was run through the same procedure except substituting samples with potassium phosphate buffer pH 8.0.

Statistical analysis: Values are means ± SE of at least three replicates. All data were analyzed using Graphpad Prism version 3.0 and Graphpad InStat version 3.05. Comparison between groups was performed using a Student’s t-test or a one-way analysis of variance with post hoc analysis by Tukey’s test (Miller and Miller, 2000). p < 0.05 was considered statistically significant.

Results and Discussion

Partial purification of the enzyme: Preliminary studies showed that partial purification of the enzyme caused a significant (p<0.05) increase in the sensitivity of the enzyme towards carbamate and other organophosphates (OPs). Thus partial purification of the enzyme was carried out. Table 1 showed that partial purification of the enzyme was successful with approximately 5-fold purification and a good yield of nearly 50%.

Insecticides inhibition studies: Only two insecticide, carbaryl and carbofuran, showed an almost complete inhibition of C. batrachus AChE activity at 1.0 mg l⁻¹ (Fig. 1). Bendiocarb, methomyl and malathion showed significant inhibition to C. batrachus activity compared to control (p<0.05) while diazinon and parathion showed significant activation but the differences were small (<5%) and were considered as no effect. The entire OP tested in the experiment did not show any significant inhibition. This is because the OPs involved...
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in this experiment require bioactivation in order to be effective anticholinesterases (Fukuto, 1990). Table 2 shows the IC\textsubscript{50} (concentration causing 50% inhibition) values and their 95% confidence intervals for the two carbamates that inhibit C. batrachus AChE activity in comparison with commercial AChEs. In all cases the carbamates showed non linear inhibition curves and the regression model best suited this curve with high correlation coefficient value (0.99) was radioactive decay. The IC\textsubscript{50} values (and the 95% confidence interval) for both carbofuran and carbaryl inhibition on C. batrachus AChE at 6.66 (5.97-7.52) and 130.00 (119.3-142.5) µg l\textsuperscript{-1}, respectively, were within the IC\textsubscript{50} range of E. electricus at 6.21 and 133.01 µg l\textsuperscript{-1}, respectively, and were much lower than bovine AChE at 20.94 and 418.80 µg l\textsuperscript{-1}, respectively. This suggests that AChE from C. batrachus is suitable as a replacement for AChE from E. electricus.

From the results of the heavy metals screening, heavy metals such as copper and mercury showed significant lowering of the AChE activity (p<0.05) while silver, cadmium and magnesium showed a significant increase (p<0.05) but the effects were small (<5%) and were considered as no effect. No heavy metals were found to inhibit C. batrachus AChE activity (Fig. 2) suggesting specific detection of only for insecticides. As with most or all enzymes, metals at some concentrations have been shown to inhibit AChE in vitro, including in

![Fig. 1: The effect of various insecticides on the enzymatic inhibition of partially purified AChE from C. batrachus. The error bars represent mean ± standard deviation of three replicates](image1)

![Fig. 2: The effect of various heavy metals on the enzymatic inhibition of partially purified AChE from C. batrachus. The error bars represent mean ± standard deviation of three replicates](image2)
fish (Olson and Christensen, 1980; Bocquene et al., 1990). For example, Cd shows inhibition of Daphnia magnaAChE with an IC$_{50}$ of 3367 $\mu$g l$^{-1}$ (Guilhermino et al., 1996).

In this work we have found that a local fish, C. batrachus could be a newer source of AChE to replace the commercial AChE from E. electricus. The IC$_{50}$ values for two carbamates tested were found to be within the range of E. electricus indicating similar sensitivity. We also found that the AChE from C. batrachus was not inhibited by heavy metals suggesting that the enzyme could be used to detect insecticides from environmental samples without interference from heavy metals. Works are currently being carried out to purify the AChE from C. batrachus to characterize the enzyme and to study the inhibition of insecticides using a purified fraction instead of a partially purified fraction used in this study.

References


