Assay for Heavy Metals Using an Inhibitive Assay Based on the Acetylcholinesterase from *Clarias batrachus*

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INTRODUCTION

Heavy metals level in developing countries like Malaysia with an agricultural agenda is becoming a problem. The health problems caused by the chronic effect of heavy metals is costing the country millions of Ringgits in healthcare and monitoring of rivers and other aquatic habitats. There are about 180 river basins in Malaysia and between 5 to 10% of these basins has been reported to be polluted with inorganic and organic pollutants [1]. Hence, their monitoring is important. Currently, the yearly levels of heavy metals in these polluted rivers are not monitored due to high costs. One way to reduce the cost of monitoring is to use biomonitoring. The USEPA has recognized microbial and enzyme-based methods to biomonitors heavy metals [2]. Microbial-based methods such as Microtox and Polytox are not amenable to field trial works, as they require bulky incubators. An enzyme such as urease [3] and proteases [4–6] could be used, but they are time-consuming as each measurement takes more than one hour to complete.

Acetylcholinesterase (AChE) is usually used as an inhibitive assay for insecticides. A lesser-known property of AChE is its inhibition by heavy metals. In this work, we evaluate an AChE from brains of *Clarias batrachus* (catfish) exposed to wastes from aquaculture industry as an inhibitive assay for heavy metals. We discovered that the AChE was inhibited completely by Hg^{2+}, Ag^{2+}, Pb^{2+}, Cu^{2+}, Cd^{2+}, Cr^{6+} and Zn^{2+} during initial screening. When tested at various concentrations, the heavy metals exhibited exponential decay type inhibition curves. The calculated IC_{50} (mg/L) for the heavy metals Ag^{2+}, Cu^{2+}, Hg^{2+}, Cr^{6+} and Cd^{2+} were 0.088, 0.078, 0.071, 0.87 and 0.913, respectively. The IC_{50} for these heavy metals are comparable, and some are lower than the IC_{50} values from the cholinesterases from previously studied fish. The assay can be carried out in less than 30 minutes at ambient temperature.

KEYWORDS

*Clarias batrachus*  
acetylcholinesterase  
heavy metals  
inhibitive assay
Due to this and the fact that the fish cholinesterase assay is simple to perform and is also rapid makes fish cholinesterase-based assays an excellent candidate for the development of in situ bioassay methods for detecting toxicants especially heavy metals in soil and water bodies exposed to industrial effluents such as the Juru Industrial Estate where heavy metals pollution is often reported. The search for a more sensitive cholinesterase source for the detection of heavy metals meant that more fish needs to be screened and compare to existing results.

*Clarias batrachus* is reared at a large scale in Malaysia for its flesh palatability [20]. In this work, we discovered that the acetylcholinesterase from *Clarias batrachus* is sensitive to the heavy metals and it is anticipated that the cholinesterase from this fish can be part of the current battery of fish cholinesterase assay.

**MATERIALS AND METHODS**

**Chemicals**

Heavy metals such as silver (ii), copper (ii), mercury (ii), and cadmium (ii) were prepared by Atomic Absorption Spectrometry standard solutions from Merck. Working solutions at the concentrations of 10 mg l⁻¹, 5 mg l⁻¹, 2.5 mg l⁻¹, 1.0 mg l⁻¹ and 0.5 mg l⁻¹ were prepared by diluting them in deionized water, and all of them were stored in acid-washed polypropylene containers.

These solutions were prepared fresh daily. Acetylthiocholine iodide (ATC), β-mercaptoethanol and 5'-dithio-bis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich, Absorption Spectrometry standard solutions from MERCK (Merck, Darmstadt, Germany).

**Preparation of affinity purified AChE**

*Clarias batrachus* with an average weight of 750 g and about 25 cm in length were sourced from a local fish aquaculture farmer in Dengkil, Selangor. The whole brain was dissected out, and 10 grams of brain was homogenized (Ultra-Turrax T25 homogenizer) in 20% (w/v) of 100 mM sodium phosphate buffer at pH 8.0. Unbroken tissues were then centrifuged at 15 000g for 10 minutes at 4 °C. The supernatant was subjected to affinity purification.

A procainamide affinity chromatography was used to partially purified the AChE [21]. The matrix, packed in a glass column (1.6 cm x 20 cm) (Amershams) to a bed height of 10 cm matrix washed with 400 ml of buffer A (20 mM sodium phosphate buffer, pH 8.0). This procedure was to clean and equilibrate the column. About 10 mg of crude extract was loaded onto the affinity matrix and then washed with 500 ml of buffer A with a flow rate of 1 ml/min.

A linear gradient of 1 M NaCl in buffer A was used to elute AChE using a total volume of 100 ml. Fractions of 1 ml were then collected and assayed for activity and protein. Fractions exhibiting high AChE activity were then pooled and dialyzed in 2 L of buffer A at 4 °C overnight. The dialyzed fraction was then concentrated (Viva Spin) and stored at -20 °C until subsequent use.

**Determination of AChE Activity**

AChE activity was calculated on the basis of an extinction coefficient of 13.6 mM⁻¹ cm⁻¹ using the method developed by Ellman *et al.* [22]. One unit of activity is defined as one μmole ATC hydrolyzed/min. The reaction mixture was composed of 150 μl of potassium phosphate buffer (0.1 M, pH 8.0), DTNB (20 μl, 0.067 mM), carbamate (50 μl) and enzyme (10 μl). The mixture was incubated in the dark for 10 minutes at room temperature. Then, 20 μl of acetylthiocholine iodide (0.5 mM stock) was added. Again, the mixture was left to stand but for 10 minutes at room temperature before the absorbance was read at 405 nm. Experiment was conducted in triplicates. IC₅₀ of heavy metals was determined using a one-phase exponential decay model on GraphPad PRISM 4 for non-linear regression analysis software available from www.graphpad.com.

**Data and statistical analysis**

The percent inhibition was computed according to following formula:

\[
\% \text{ Inhibition} = \frac{\text{Test activity of control - test activity of sample}}{\text{Test activity of control}} \times 100\%
\]

Values are means ± SE. All data were analyzed using GraphPad Prism version 3.0. Comparison between groups was performed using a Student's t-test or one-way analysis of variance (ANOVA) with post hoc analysis by Tukey’s test. P < 0.05 was considered statistically significant.

**RESULTS AND DISCUSSIONS**

Fig. 1 showed that Hg²⁺, Cu²⁺, and Ag²⁺ inhibited almost 100% of AChE activity whereas cadmium and chromium show less inhibition as compared to the previous heavy metals (Fig. 1). When tested at various concentrations, the heavy metals exhibited exponential decay type inhibition curves (Figs. 2 to 6). The IC₅₀ for the heavy metals Hg²⁺, Cu²⁺, Ag²⁺, Cd²⁺, and Cr⁶⁺ including their 95% confidence interval were then calculated (Table 1). The percentage inhibition of mercury (Fig. 2), copper (Fig. 3), silver (Fig. 4), chromium (Fig. 5) and cadmium (Fig. 6), in all the heavy metals, tested the percentage inhibition is correlated to the concentration of the corresponding heavy metal.

The comparative LC₅₀ (lethal concentration that causes 50% toxicity), LD₅₀ (lethal dose that causes 50% toxicity), EC₅₀ (effective concentration that causes 50% response) and IC₅₀ (concentration that causes 50% inhibition) data for the metals; presented as 95% Confidence Intervals (CI) (where available) for different toxicity tests based on fish cholinesterases (Table 2) and other assays (Table 3) was then made. In general, the IC₅₀ value or EC₅₀ or LC₅₀ values are usually used to benchmark bioassays [4].

Schenker and Gentleman [23] suggested that in the occasion that non-overlap of confidence interval occurs, this usually signifies significant difference at the p<0.05 level, while overlapped interval does not necessarily means there is a difference or no significant differences at the p>0.05 level. An overlapped confidence interval provides a general view that more data and experimentation are needed to assess non-significance. Based on this, the result of the present assay indicated that mercury, copper and silver were significantly more sensitive (p<0.05) with no significant difference among them than the cholinesterases inhibitive assays for chromium and cadmium.

In comparing the result of this study with the previous study, copper is more sensitive as compared to the
cholinesterases inhibitive assays from *Pheriophtalmodon schlosseri* [9], *Osteochilus basselti* [13], and expected to show no difference to the cholinesterases inhibitive assays from *Electrophorus electricus* [10] and *Pangasius hypothalamus* [14]. Among the heavy metals, mercury is more sensitive to *C. batrachus* followed by copper and silver, but chromium and cadmium are less sensitive as compared to mercury, copper and silver (Fig 1).

Based on other inhibitive assays, the present assay for copper was more sensitive than rainbow trout, bromelain, Microtox™ and immobilized urease assays, equivalent in sensitivity to the Mo-reducing enzyme assay, and less sensitive than the papain and *Daphnia magna* assays, although it needs to be mentioned that the papain assay is measured as LOQ not IC₅₀. The present assay for mercury was equivalent in sensitivity to the papain and immobilized urease assays and less sensitive than the rest of the assays. The present assay for chromium was equivalent in sensitivity to the *Daphnia magna* assay and more sensitive than all of the other assays.
The results showed that fish AChE can be used for in vitro detection of heavy metals. Fish and aquatic organisms due to their aquatic environment are sensitive to toxicants such as heavy metals. For instance the heavy metals chromium, copper, cadmium and mercury has been shown to be very toxic to *Gambasia affinis* [25], *Mytilus galloprovincialis* [26], and *Pomatoschistus microps* [27], respectively. Metal ions inhibit enzymes because they could form ligands with amino and carboxyl groups, tryptophan (ring nitrogen), cysteine (thiol), methionine (thioether), serine, threonine, tyrosine (hydroxyl groups), asparagine and glutamine groups of protein [28]. In addition, the metal ion mercury could disrupt cysteine bridges leading to protein denaturation [7].

**CONCLUSION**

In conclusion, AChE from *Clarias batrachus* has shown to be sensitive to heavy metals with limit of detection (LOD) values that can be used for biomonitoring works. Due to the limited information regarding the use of AChE as a biomonitoring assay for heavy metals, this work adds new data and information that is useful for future biomonitoring studies using fish enzymes.

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**REFERENCES**


**Table 1.** I_{50} values for heavy metals that inhibit the fish cholinesterases enzyme activity.

<table>
<thead>
<tr>
<th>Heavy Metals</th>
<th>R²</th>
<th>I_{50} (mg/L) (95% CL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hg</td>
<td>0.99</td>
<td>0.071 (0.059-0.088)</td>
</tr>
<tr>
<td>Cu</td>
<td>0.99</td>
<td>0.078 (0.065-0.096)</td>
</tr>
<tr>
<td>Cr</td>
<td>0.98</td>
<td>0.088 (0.082-0.095)</td>
</tr>
<tr>
<td>Cd</td>
<td>0.99</td>
<td>0.87 (0.80-0.97)</td>
</tr>
</tbody>
</table>

**Table 2.** Comparison of this assay to various other fish cholinesterases assays.

<table>
<thead>
<tr>
<th>Fish species</th>
<th>I_{50} (mg/L) (95% Confidence Interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrophorus</td>
<td>0.074-0.084</td>
</tr>
<tr>
<td>electricus</td>
<td>0.29-1.690</td>
</tr>
<tr>
<td>schlosseri</td>
<td>0.056-0.123</td>
</tr>
<tr>
<td>Osteochilus hasselti</td>
<td>0.104-0.267</td>
</tr>
<tr>
<td>Pangasius hypophthalmus</td>
<td>0.093-0.267</td>
</tr>
</tbody>
</table>

**Table 3.** Comparison of this assay to immobilized urease, Microtox™ *Daphnia Magna*, fish bioassays (Rainbow trout), papain and bromelain assays.

<table>
<thead>
<tr>
<th>Met Immobilized urease</th>
<th>15-min Microtox™ <em>Daphnia Magna</em></th>
<th>48 hours Microtox™ <em>Daphnia Magna</em></th>
<th>96 hours Rainbow trout</th>
<th>Papain</th>
<th>Bromelain</th>
<th>This Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu</td>
<td>0.41-0.14</td>
<td>0.076-0.38</td>
<td>0.020-0.093</td>
<td>0.25</td>
<td>0.004</td>
<td>0.004</td>
</tr>
<tr>
<td>Hg</td>
<td>0.33-0.14</td>
<td>0.029-0.05</td>
<td>0.005-0.421</td>
<td>0.33</td>
<td>0.24-0.62</td>
<td>0.13-0.16</td>
</tr>
<tr>
<td>Ag</td>
<td>0.11-0.4</td>
<td>0.01-1.8</td>
<td>0.05-1.15</td>
<td>0.1</td>
<td>0.24-0.62</td>
<td>0.09-0.088</td>
</tr>
<tr>
<td>Cd</td>
<td>1.59-0.26</td>
<td>0.19-0.22</td>
<td>0.04-1.9</td>
<td>0.17</td>
<td>0.24-0.62</td>
<td>0.85-0.99</td>
</tr>
</tbody>
</table>

* Not detected

Fig. 6. Percentage inhibition of cadmium on the activity of the partially purified AChE from *Clarias batrachus*.


