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

Abubakar M. Umar^{1,2}, Tham Lik Gin¹, Natarajan Perumal¹, Hassan Mohd Daud³, Nur Adeela Yasid¹, and Mohd Yunus Shukor^{1*}

¹Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

²Department of Biological Sciences, Faculty of Science, Gombe State University, P.M.B. 027, Gombe, Nigeria.

³Department of Veterinary Clinical Studies, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

*Corresponding author:

Associate Prof. Dr. Mohd Yunus Shukor
Department of Biochemistry,
Faculty of Biotechnology and Biomolecular Sciences,
Universiti Putra Malaysia,
UPM 43400 Serdang, Selangor, Malaysia
Tel: +60389478292

Email: mohdyunus@upm.edu.my / yunus.upm@gmail.com

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ABSTRACT

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 Hg²⁺, Ag²⁺, Pb²⁺, Cu²⁺, Cd²⁺, Cr⁶⁺ and Zn²⁺ during initial screening. When tested at various concentrations, the heavy metals exhibited exponential decay type inhibition curves. The calculated IC₅₀ (mg/L) for the heavy metals Ag²⁺, Cu²⁺, Hg²⁺, Cr⁶⁺ and Cd²⁺ were 0.088, 0.078, 0.071, 0.87 and 0.913, respectively. The IC₅₀ for these heavy metals are comparable, and some are lower than the IC₅₀ values from the cholinesterases from previously studied fish. The assay can be carried out in less than 30 minutes at ambient temperature.

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 biomonitor toxicants [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 is normally used for biomonitoring of insecticides, but it could also be used to detect heavy metals [7]. The current sources of the enzyme for pesticide bioassay and biosensor technology are from the fruit fly *Drosophila melanogaster* and the electric eel *Electrophorus electricus* [8]. Currently, these sources are very expensive reaching several hundred Malaysian Ringgit per milligram.

It has been known that fish is especially sensitive to toxicants and the *in vitro* use of fish cholinesterases for the bioassay of a variety of toxicants including heavy metals have been reported such as in *Anabas testudineus* [9] *Electrophorus electricus* [10], *Periophthalmodon schlosseri*, [11], *Lates calcarifer* (cholinesterase from kidney) [12], *Osteochilus hasselti* [13] *Pangasius hypophthalmus* [14], where some results show better sensitivity than established inhibitive assays mentioned previously. Changes in cholinesterase activity are generally accepted as a tool for assessing pollution due to organophosphorus and carbamates compounds [15][16]. Other studies confirmed that ChEs are inhibited by PAHs, many heavy metals and surfactant [17]; [18] [19].

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), chromium (vi) 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 removed by centrifugation at 15 000×g for 10 minutes at 4 °C. This is followed by ultracentrifugation of the supernatant at 100,000-×g (Sorval) for one hr at 4 °C. The supernatant was subjected to affinity purification.

A procainamide affinity chromatography was used to partially purify the AChE [21]. The matrix, packed in a glass column (1.6 cm x 20 cm) (Amersham) 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:

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

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 *Pheriophthalmodon 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.

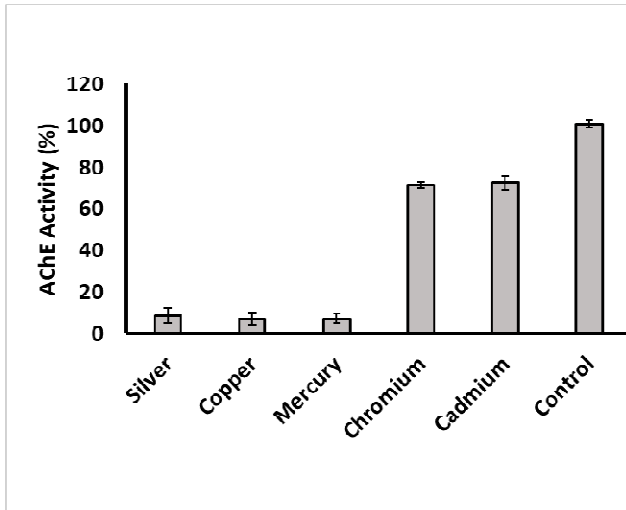


Fig. 1: The effect of metal ions on the activity of the partially purified AChE from *Clarias batrachus*. Data represents mean± SEM, n=3.

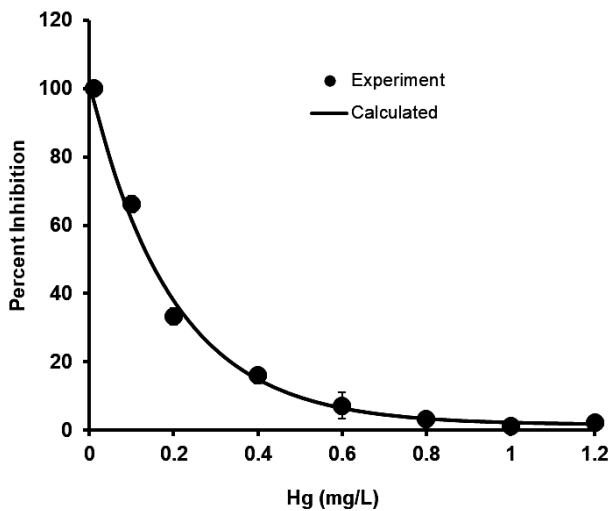


Fig. 2. Percentage inhibition of mercury on the activity of the partially purified AChE from *Clarias batrachus*.

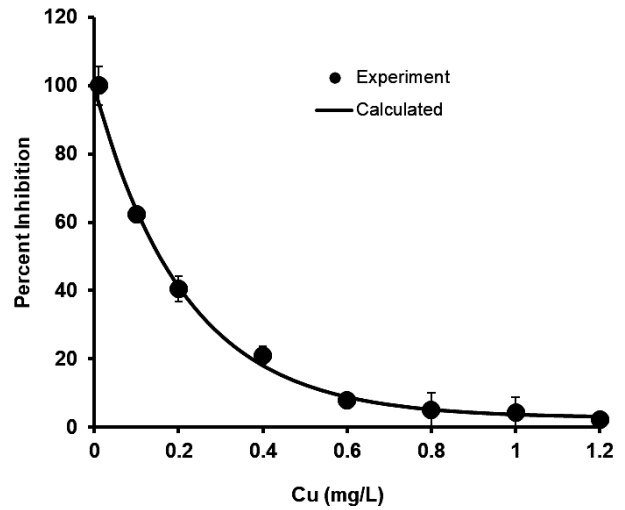


Fig. 3. Percentage inhibition of copper on the activity of the partially purified AChE from *Clarias batrachus*.

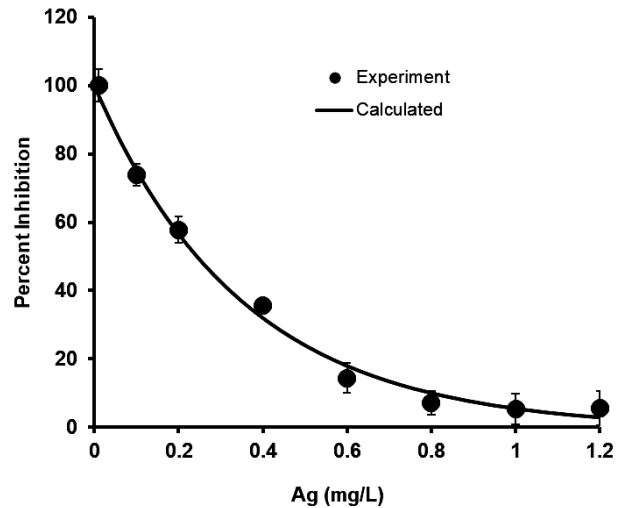


Fig. 4. Percentage inhibition of silver on the activity of the partially purified AChE from *Clarias batrachus*.

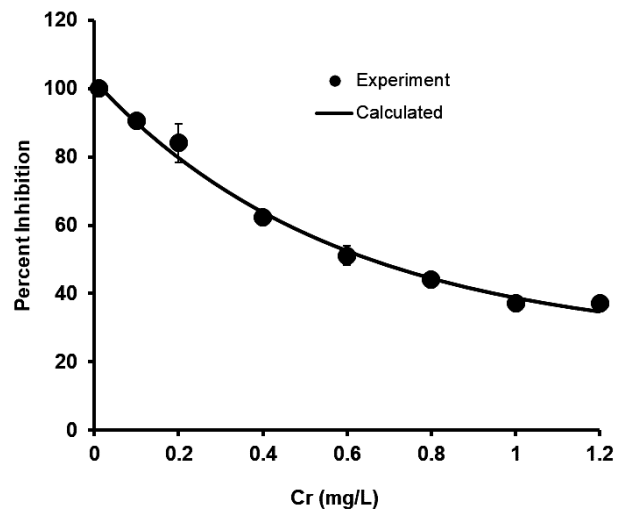


Fig. 5. Percentage inhibition of chromium on the activity of the partially purified AChE from *Clarias batrachus*.

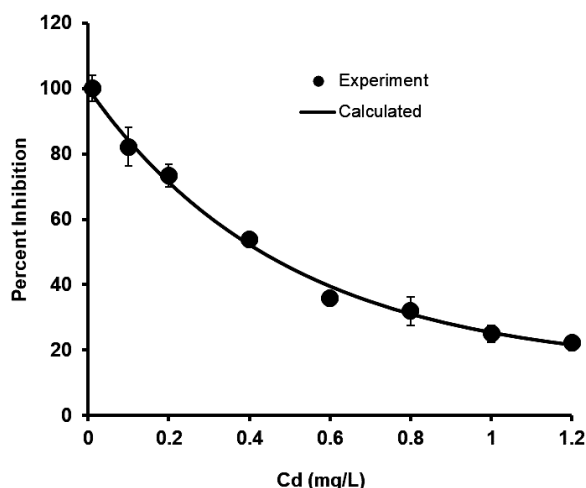


Fig. 6. Percentage inhibition of cadmium on the activity of the partially purified AChE from *Clarias batrachus*.

Table 1. IC₅₀ values for heavy metals that inhibit the fish cholinesterases enzyme activity.

Heavy Metals	R ²	IC ₅₀ (mg/L) (95% CL)
Hg ²⁺	0.99	0.071 (0.059-0.088)
Cu ²⁺	0.99	0.078 (0.065-0.096)
Ag ²⁺	0.98	0.088 (0.082-0.095)
Cr ⁶⁺	0.99	0.87 (0.80-0.97)
Cd ²⁺	0.99	0.913 (0.85-0.99)

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

Fish species	IC ₅₀ (mg/L) (95% Confidence Interval)					Author
	Ag	Cu	Hg	Cr	Cd	
<i>Electrophorus electricus</i>	0.074-0.29	0.64-1.690	0.084-0.115			[10]
<i>Periophthalmodon schlosseri</i>		0.064-0.104	0.328-0.427	0.099-0.128		[11]
<i>Osteochilus hasselti</i>		0.186-0.213	0.377-0.556	0.067-0.086		[13]
<i>Pangasius hypophthalmus</i>	0.082-0.095	0.065-0.096	0.059-0.088	0.80-0.97	0.85-0.99	[14]

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

Met	Immobilized urease ^a	LC ₅₀ or IC ₅₀ (mg/l)					This Assay
		15-min. Microtox TM ^{a,b}	48 hours <i>Daphnia Magna</i> ^a	96 hours Rainbow trout ^c	Papain ^b	Bromelain ^d	
Cu	0.41±0.14	0.076-3.8	0.020-0.093	0.25	0.004 (LOQ)	0.163-0.305	0.065-0.096
Hg	0.33±0.021	0.029-0.05	0.0052-0.21	0.033-0.21	0.24-0.62	0.13-0.16	0.059-0.088
Cr	36.1±2.5	13	0.1-1.8	11	n.d.	n.d.	0.85-0.97
Cd	1.59±0.26	19-220	0.041-1.9	0.15-2.5	0.1 (LOQ)	n.d.	0.85-0.99
Ag	n.d.	n.d.	1.930	0.05	0.33-0.49	n.d.	0.082-0.095

^a [3]

^b [24]

^c [5]

^d [4]

n.d. Not detected

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 toxicant such as heavy metals. For instance the heavy metals chromium, copper, cadmium and mercury has been shown to be very toxic to *Gambusia affinis* [25], *Mytilus galloprovincialis* [26], and

Pomatoschistus microps [27], respectively. Metal ions inhibit enzymes because they could form ligands with amino and carbonyl 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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