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Assay for Heavy Metals Using an Inhibitive Assay Based on the Acetylcholinesterase from *Channa striatus*

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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 striped snakehead (*Channa striatus*) wastes from aquaculture industry as an inhibitive assay for heavy metals. We discovered that the AChE was inhibited almost completely by Hg^{2+}, Ag^{2+} and Cu^{2+} during an initial screening. When tested at various concentrations, the heavy metals exhibited exponential decay type inhibition curves. The calculated IC_{50} for the heavy metals Hg^{2+}, Ag^{2+}, Pb^{2+}, Cu^{2+} and Cr^{6+} were 0.08432, 0.1008, 0.1255, 0.0871, and 0.1771, 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.

INTRODUCTION

The increasing in heavy metals contamination has becoming a major problem due to its major usage in agricultural sector including in Malaysia [1]. Huge amount of money has been spent in order to observe the water sources quality and healthcare concern caused by the long-time effects of heavy metals [2]. It has been reported that about 10% of Malaysia's river basin has been contaminated with pollutants. Monitoring of the contaminated areas is important as the contamination level keep rising, yet the highly cost needed leads to lacking in heavy metal monitoring [3]. Thus, biomonitoring technique has been initiated in order to reduce the monitoring cost [4]. Some mechanism including both enzyme and microbe-based techniques has been recognized by the USEPA, yet there are few limitations to be considered. Protease is an example of the enzyme used for monitoring the toxicant, but the quantification process may take longer time to be completed [5].

Acetylcholinesterase (AChE) from the family of Cholinesterases (ChE) is a crucial enzyme which mainly resides in both invertebrates and vertebrates [6]. Despite commonly being used in pesticides monitoring; it can also be used in heavy metals detection [7]. Currently enzyme sources for pesticide bioassay and biosensor technology were taken from *Drosophila melanogaster* and *Electrophorus electricus*. However, these sources are considered as inefficient as the enzyme costs more than hundreds of Malaysia ringgit per milligram. The sensitivity of fish towards toxicants are widely known and the extracted cholinesterase from fish is used for the bioassay of heavy metals [8]. This method has been done as reported in *Pangasius hypophthalmus*, *Electrophorus electricus* and *Lates calcarifer* with few results appeared to have better sensitivity. As fish cholinesterase assay is simpler to perform compare to other sources, this make fish based cholinesterase assay as excellent candidate for in situ bioassay. This bioassay method is suitable to be used for detecting any toxicants especially heavy metals toxicant in soil and water, as soil and water are more prone to heavy metals contaminations from industrial effluents. One of the most current reported heavy metals pollution in Malaysia was in Juru Industrial Estate [5]. Therefore, screening of a number of types of fish need to be done in order to identify the source for better sensitive cholinesterase for heavy metals detection.

Channa striatus which locally known as striped snakehead is reared at a huge scale for its flesh in Malaysia [9]. In this work we discovered that the acetylcholinesterase from *C. striatus* is sensitive to the heavy metals Ag, Cu, Hg, Cr and Pb. It is expected that the cholinesterase from *C. striatus* can be part of the present switch of fish cholinesterase assays.

MATERIALS AND METHODS

Chemicals

Heavy metals such as mercury (ii), molybdenum (vi), arsenic (v), cadmium (ii), lead (ii), copper (ii), zinc (ii), chromium (vi) and silver (ii) were prepared from 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

C. striatus, with an average weight of 750 g and about 25 cm in length were sourced from a local fish aquaculture farmer in Dengkil, Selangor in 2012. The whole brain was dissected out and 10 g 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 ºC. This is followed by ultracentrifugation of the supernatant at 100,000 ×g (Sorval) for one hr at 4 ºC. The supernatant was subjected to affinity purification. A procainamide affinity chromatography was used to partially purify the AChE [10]. 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 ºC overnight. The dialyzed fraction was then concentrated (Viva Spin) and stored at -20 ºC 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*. [11]. 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 then 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. IC_{50} 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:

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

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 a 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 Hg^{2+}, Ag^{2+} and Cu^{2+} inhibited almost 100% of AChE activity while Cr^{6+} and Pb^{2+} showed inhibition of more than 30% of AChE activity **(Fig. 1).**

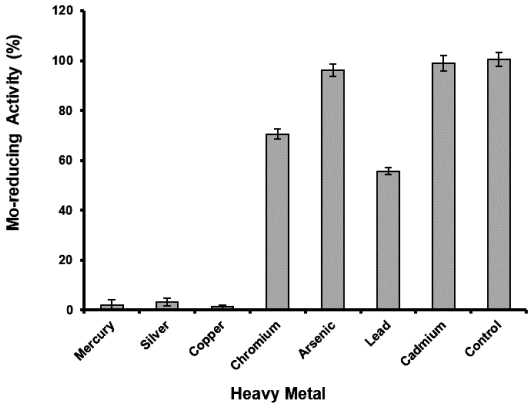

Fig. 1. The effect of metal ions on the activity of the partially purified AChE from *C. striatus*. Data represents mean± SEM, n=3.

When tested at various concentrations, the heavy metals exhibited exponential decay type inhibition curves (**Figs. 2 to 6**).

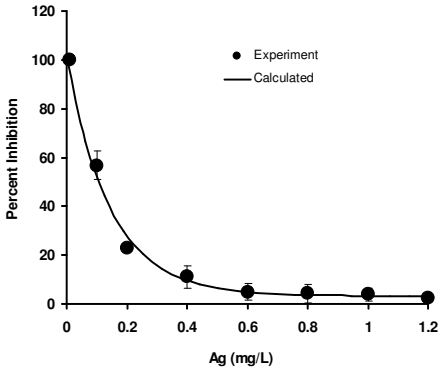

Fig. 2. Ag exponential decay type inhibition curves at different concentrations.

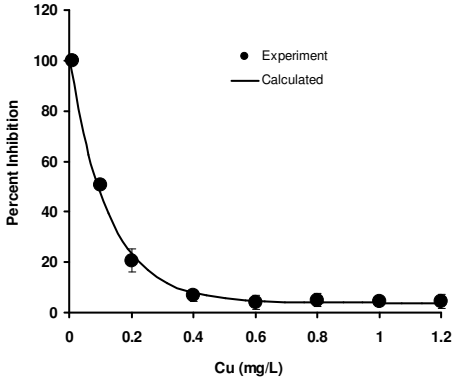

Fig. 3. Cu exponential decay type inhibition curves at different concentrations.

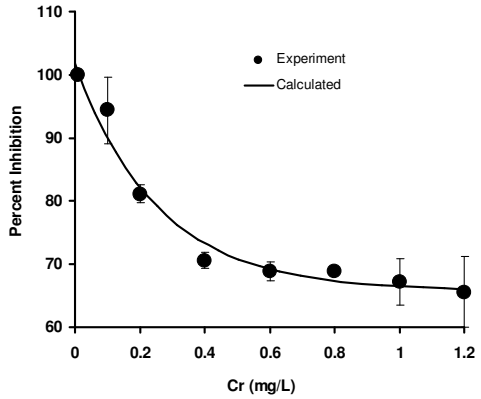

Fig. 4. Cr exponential decay type inhibition curves at different concentrations.

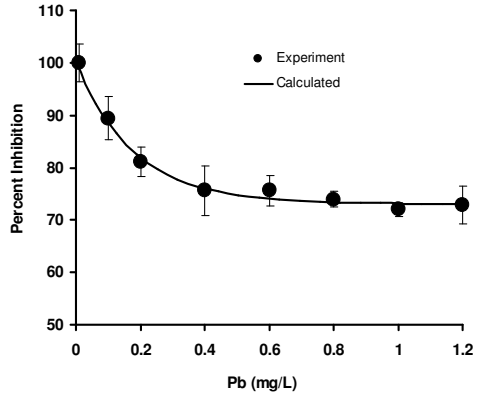

Fig. 5. Pb exponential decay type inhibition curves at different concentrations.

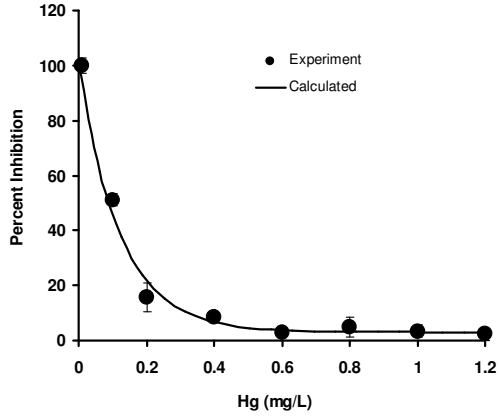

Fig. 6. Hg exponential decay type inhibition curves at different concentrations.

The calculated IC_{50} values for the heavy metals are 0.08432, 0.0871, 0.1008, 0.1255, 0.1771 mg/L, in the order of Hg^{2+} < Cu^{2+} < Ag^{2+} < Pb^{2+} < Cr^{6+}, respectively, including their 95% confidence interval were then calculated **(Table 1).**

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

Heavy metals	R^2	IC_{50} (mg /l) (95% CI)
Hg^{2+}	0.99	0.08432 (0.07472 to 0.09673)
Ag^{+}	0.99	0.1008 (0.08963 to 0.1152)
Pb^{2+}	0.98	0.1255 (0.09367 to 0.1899)
Cu^{2+}	0.99	0.0871 (0.07994 to 0.09567)
Cr^{6+}	0.97	0.1771 (0.1334 to 0.2634)

Greenland et al. [12] has stated that the comparison between two values may have significant difference if two confidence intervals does not overlap, but the test may still have $P < 0.05$. If the two values produce overlapping of 95% of confidence interval, then the significant difference may or may not be available [13, 14]. Based on this, the present assay for copper was significantly more sensitive ($p<0.05$) than the cholinesterases inhibitive assays from the immobilized urease, and bromelain, is equivalent in sensitivity to the cholinesterases inhibitive assays from rainbow trout and papain, and less sensitive than the cholinesterases inhibitive assays from Microtox™ and *Daphnia magna*.

Based on other inhibitive assays, the present assay for copper was significantly more sensitive ($p<0.05$) 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. The present assay for mercury was equivalent in sensitivity to the papain and immobilized urease assays and significantly less sensitive ($p<0.05$) than the rest of the assays. The high tendency of mercury to be attracted towards sulfhydryl groups of the enzyme may be the cause of the enzyme activity inhibiton [15]. The present assay for chromium was equivalent in sensitivity to the *Daphnia magna* assay and significantly more sensitive ($p<0.05$) than all of the other assays.

This discovery is corresponding to the previous research, where both Mercury and Copper exhibited high inhibition effect towards AChE activity in *Electrophorus electricus and Pangasius hypophthalmus* [7, 25]. Identification of heavy metals can be done using in vitro method as proven from the results. The aquatic organisms are highly susceptible towards toxicant for which they live in an aquatic habitat [26]. For instance the heavy metals chromium, copper, cadmium and mercury has been shown to be very toxic to *Gambusia affinis* [27], *Mytilus galloprovincialis* [28], and *Pomatoschistus microps* [29], 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 [30]. In addition, the metal ion mercury could disrupt cysteine bridges leading to protein denaturation [31].

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

	IC_{50} (mg/L) (95% Confidence Interval)								
Fish species	Ag	Cu	Hg	Cr	As	Pb	Cd	Zn	Author
Anabas testudineus									[16]
Electrophorus electricus	0.074-0.29	0.64-1.690	0.084-0.115						[7]
Periophtalmodon schlosseri		0.064-0.104	0.328-0.427	0.099-0.128	0.122-0.167				[17]
Lates calcarifer						0.1			[18]
Cholinesterase from kidney *Osteochilus hasselti*		0.186-0.213	0.377-0.556	0.067-0.086					[19]
Pangasius hypophthalmus	0.082-0.095	0.065-0.096	0.059-0.088	0.80-0.97		1.042-1.164	0.85-0.99	1.443-1.727	[20]

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

	LC_{50} or IC_{50} (mg/l)						
Metals	Immobilized urease[a]	15-min. Microtox™ [a, b]	48 hours *Daphnia Magna*[a]	96 hours Rainbow trout[a, c]	Papain[b]	Bromelain[d]	This Assay
Cu	0.41±0.14	0.076-3.8	0.020-0.093	0.25	0.004 (LOQ)	0.163-0.305	0.087
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	0.08432
Cr	36.1±2.5	13	0.1-1.8	11	n.d.	n.d.	0.1771
Pb	>250	1.7-3.0	3.6	8.0	2.16±0.53	n.d.	0.1255
Ag	n.d.	n.d.	1.930	0.05	0.33-0.49	n.d.	0.1008

[a] [21]
[b] [22]
[c] [23]
[d] [24]
n.d. Not detected

CONCLUSION

In conclusion, AChE from *C. striatus*. has been shown to be sensitive to heavy metals with 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 enzymes.

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