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Research article

The development of an inhibitive assay for heavy metals using the acetylcholinesterase from *Periophtalmodon schlosseri*

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ABSTRACT

Acetylcholinesterase (AChE) assay is among the most rapid, simplest and economic assay in existence. AChE is known to be inhibited strongly by heavy metals aside from insecticides. Its use as an inhibitive assay for heavy metals is less well known. We report on the AChE from *Periophtalmodon schlosseri* as a sensitive assay for heavy metal ions. Heavy metals exhibited exponential decay type inhibition curves with calculated IC_{50} for copper, mercury, chromium and arsenic at 0.088, 0.371, 0.112 and 0.141 mg l^{-1}, respectively. The LOD for copper, mercury, chromium and arsenic were 0.093, 0.126, 0.099 and 0.127 mg l^{-1}, respectively. The LOQ for copper, mercury, chromium and arsenic were 0.001, 0.209, 0.103 and 0.219 mg l^{-1}, respectively. The IC_{50}s for these heavy metals are lower than the values for immobilised urease, *Daphnia magna*, Microtox™, rainbow trout, papain and bromelain assays.

INTRODUCTION

Industrialization has introduced much pollution into the environment. Heavy metals are among the most toxic pollutant that has emerged from industrial activities and they have reached toxic level over the years [1, 2]. Their detection is urgently required but the cost of instrumental analysis has impeded widespread monitoring. Thus, a rapid, simple and field trial friendly method must be developed to monitor the levels of heavy metals in the environment. Inhibitive determination of heavy metals using enzymes is a relatively emerging new technology because enzyme assays are usually rapid, able to detect bioavailable metal ions, does not require skilled technician and is amenable to field trial works [3]. Enzymes that have been used for inhibitive determination of heavy metal traces include peroxidase, xanthine oxidase, invertase, glucose oxidase and the proteases bromelain, trypsin and papain [4, 5, 6]. However, newer sources of more sensitive enzymes are needed. It has been known that fish is especially sensitive to toxicants and the use of fish for the bioassay of a variety of toxicants have been reported [7, 8]. The use of acetylcholinesterase (AChE) from fish as an inhibitive enzyme assay for insecticides is well documented [9, 10], but their

use for the detection of heavy metals are rarely ventured. Metals at some concentrations have been shown to inhibit AChE in vitro, including in fish [11, 12, 13]. However the sensitivity level is not adequate for biomonitoring works. In this work we discovered that the acetylcholinesterase from *Periophtalmodon schlosseri* is sensitive to the heavy metals copper, chromium and mercury and this sensitivity could be used for the detection of these heavy metals in the environment.

MATERIALS AND METHODOLOGY

Chemicals

Acetylthiocholine iodide (ATC), β-mercaptoethanol and procainamide hydrochloride were purchased from Sigma-Aldrich. 5'-dithio-bis (2-nitrobenzoic acid) (DTNB) were purchased from Fluka Chemie GmbH. Vivaspin4 was from Vivascience. All other chemicals used in this study were of analytical or special grade. Silver (ii), arsenic (v), cadmium (ii), chromium (vi), copper (ii), mercury (ii), lead (ii), and zinc (ii) Atomic Absorption Spectrometry standard solutions from MERCK (Merck, Darmstadt, Germany). From these stock solutions, 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.

Preparation of Brain AChE Extracts

Periophtalmodon schlosseri was obtained from Malaysia National Park, Kuala Atok, Pahang Malaysia at GPS N 4º20'7.98" E 102º23'41.1" and brought alive to the laboratory. Only healthy and disease-free fishes were used for the experiment. They were decapitated and the brains were dissected out immediately and weighed. Homogenization of the brain was carried out using an Ultra-Turrax T25 homogenizer fitted with a Teflon pestle. Briefly, one gram of brain was homogenized in 20% (w/v) of 0.1 M sodium phosphate buffer pH 8.0. The crude extract was subjected to centrifugation at 15 000×g for 10 minutes at 4 ºC to remove debris and the resulting homogenate was then subjected to ultracentrifugation at 100,000×g in a Sorval® Ultra Pro 80-TH-641 ultracentrifuge for one hr at 4 ºC. The pellet was discarded and the supernatant was used in the purification procedures.

Isolation and Partial Purification of Cholinesterase

Affinity chromatography was performed using procainamide, a ligand specific for the choline-binding site [14]. 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 each Eppendorf tube 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 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 collected during the elution process were then pooled. The partially purified sample was concentrated and dialyzed with 3 batch volumes of washing buffer using Viva Spin tubes at 2500 rpm at 4 ºC. The dialyzed partially purified AChE was stored at -20 ºC until subsequent use.

Activity and substrate specificity

AChE activity was measured in a 96 well microplate assay format using according to Ellman et al. (1961) with modification [15]. Acetylthiocholine iodide (ATC) was used as a synthetic substrate for AChE. Acetylthiocholine iodide 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. AChE activity is expressed as the amount of acethylthiocholine iodide (μmol) which is broken down by AChE per minute. The specific activity is given as μmole ATC hydrolyzed/min/mg of protein or U/mg of protein and was calculated on the basis of an extinction coefficient of 13.6 $mM^{-1}.cm^{-1}$ [13]. 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. The IC_{50} value was statistically analyzed using Graphpad PRISM 4 non-linear regression analysis and the model used was radioactive decay [16].

Statistical Analysis

Values are means ± SE. 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 (ANOVA) with post hoc analysis by Tukey's test with the 95% confidential interval was performed [18].

RESULTS AND DISCUSSION

Heavy metals are toxic elements that cause toxicity to organism [17]. Figure 1 showed that chromium, copper and mercury inhibited 80%, 61.3% and 35% of AChE activity, respectively. When tested at various concentrations, the heavy metals exhibited exponential decay type inhibition curves. Heavy metals exhibited exponential decay type inhibition curves with calculated IC_{50} for copper, mercury, chromium and arsenic at 0.104, 0.371, 0.112 and 0.141 mg l^{-1}, respectively. The comparative LC_{50}, EC_{50} and IC_{50} data for the metals; presented as 95% Confidence Intervals (where available) for different toxicity tests is shown in Table 1. Comparison was made with fish (rainbow trout), daphnid (*Daphnia magna*), immobilized urease, *R. meliloti*, papain, bromelain and Microtox™ toxicity data in the same table. Scehenker and Gentleman (2001) [5] demonstrated that non-overlap of confidence interval usually signifies significant difference at the $p<0.05$ level while overlapped interval does not necessary means difference or no significant differences at the $p<0.05$ level. Overlapped confidence interval provides a general view that more data and experimentation are needed to assess non-significance. Based on this, 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 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. The limit of detection (LOD) is the lowest concentration that can be detected with confidence (99% confidence interval) and is usually assigned as three times the

standard deviation of the blank for the y-intercept [18]. The LOD for copper, mercury, chromium and arsenic were 0.093, 0.126, 0.099 and 0.127 mg l^{-1}, respectively. The limit of quantitation (LOQ) is the concentration level above which the concentration can be determined with acceptable precision (usually RSD < 10 to 25%) and accuracy (usually 80-120% recovery) and is usually assigned as ten times the standard deviation of the blank for the y-intercept [18]. The LOQ for copper, mercury, chromium and arsenic were 0.001, 0.209, 0.103 and 0.219 mg l^{-1}, respectively. Samples that gave 10% inhibition to AChE activity in theory would contain these metal ions well above the maximum permissible limit (MPL) level as outlined by the Malaysian Department of Environment [19]. Repeated measurements of the assay suggested that the assay was reproducible with CV (Coefficient of Variation) of the replicated data ranging from 3.5 to 4.4%.

The mechanism of heavy metals inactivation of enzyme is probably through binding towards amino acids in the active site of enzymes. Metal ions form specific binding with amino and carbonyl groups, tryptophan (ring nitrogen), cysteine (thiol), methionine (thioether), serine, threonine, tyrosine (hydroxyl groups), asparagine and glutamine groups [20]. In addition, according to Frasco *et. al*., (2008), the general mechanism of mercury inactivation of AChE from fish, fruit fly, human and eel is through reacting with the sulfhydryl groups [12]. This group is the most reactive nucleophilic sites of protein amino acid side chains. Mercury, especially mercuric chloride, inhibits AChE activity by binding to a single residue to form R-S-Hg-Cl, inactivating the enzyme in the process. In addition, mercury could also react with the S–S bond in general. Binding leads to the cleavage of the disulfide bond forming R-S-Cl and Cl-Hg-S-R groups. Since S–S bridges provides stability to protein tertiary structure, their destruction lower the stability of proteins.

It has been known that fish is especially sensitive to toxicants and the use of fish for the bioassay and bioindicator of a variety of toxic xenobiotics such as detergents [21], pesticides [22], textile dyes [23] and heavy metals [8, 16] have been reported. The toxicity of chromium to fish has been demonstrated in *Gambusia affinis* [24] and *Daphnia magna* [25]. According to Najimi (1997), copper and cadmium show inhibition to *Mytilus galloprovincialis* and *Perna perna* [26]. Mercury is a well known toxic element and showed toxicity to the estuarine fish *Pomatoschistus microps* and many others [27].

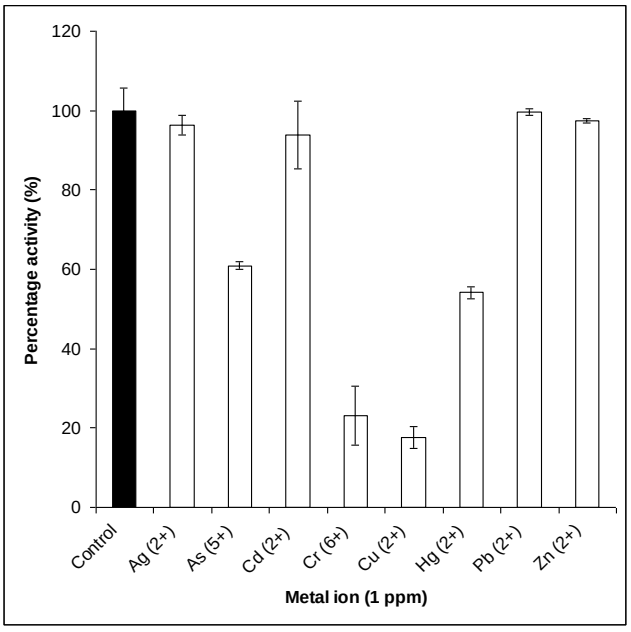

Figure 1. The effect of metal ions on the activity of the partially purified AChE from *Periophthalmodon schlosseri*. Data represents mean± SEM, n=3.

CONCLUSION

In conclusion, AChE from *Periophtalmodon schlosseri* 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 add new data and information that is useful for future biomonitoring studies using enzymes. Currently, we are carrying out field trial works to assess the efficacy of the assay to detect heavy metals in comparison with instrumental techniques.

Table 1. Comparison of the proposed assay to fish (rainbow trout), Daphnid (*Daphnia magna*), immobilized urease, *R. meliloti*, papain, bromelain and Microtox™ toxicity assays.

	EC_{50}, LC_{50} or IC_{50} (mg l^{-1})							
Metal	Immobilized urease[a]	15-min. Microtox™ [a]	48hr *Daphnia magna* [a]	96hr Rainbow trout [a]	Papain [b]	Bromelain [c]	Mo-reducing enzyme[d]	This Study (95% C.I.*)
Cu	0.41±0.14	0.8	0.020-0.093	0.25	0.004 (LOQ‡)	0.163-0.305	0.099±0.01	0.088 (0.064 to 0.104)
Hg	0.33±0.021	0.05	0.005-0.21	0.033-0.21	0.24-0.62	0.130 -0.160	n.i.	0.371 (0.328 to 0.427)
Cr	36.1±2.5	18.0	0.10-1.8	11.0	n.i	n.i	n.i	0.112 (0.099 to 0.128)
As	n.i.	43.60	5.4	43.00	n.i.	n.i	n.i.	0.141 (0.122 to 0.167)

[a] Jung *et al*., 1995.
[b] Shukor *et al*., 2006.
[c] Shukor *et al*., 2008.
[d] Shukor *et al*., 2009.
n.i. No inhibition.
‡ Limit of Quantitation
*Confidence Interval

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