Acetylcholinesterase (AChE) of *Diodon hystrix* brain as an alternative biomolecule in heavy metals biosensing

Noreen Nordin[1], Rahmath Abdulla[1], Siti Aqlima Ahmad[2], and Mohd Khalizan Sabullah[1*]

[1] *Faculty Science and Natural Resources, Universiti Malaysia Sabah, 88400, Kota Kinabalu, Sabah, Malaysia*
[2] *Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[*] *Corresponding author. E-mail: khalizan@ums.edu.my*

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The continuous discharge of toxic materials into the environment has been an alarming issue faces around the globe. Hence, matching effort of monitoring activity is vital to coping with the overwhelming amount of metal ions. Along with the significant current research being conducted, this study aims to investigate the sensitivity of acetylcholinesterase (AChE) of Sabah porcupine fish, *Diodon hystrix* as an alternative biosensor in the detection of heavy metals. The enzyme was precipitated followed by the purification using ammonium sulfate precipitation and procainamide-affinity chromatography, respectively, with a total recovery of 66.67% with the specific activity of 2297.50 U/mg. The enzyme works optimally at pH 9 with the best incubation temperature of 30°C. The Michaelis constant (K_m) and maximal velocity (V_{max}) of 1.171 mM and 879257 mol/min/mg denotes the highest catalytic efficiency (V_{max}/K_m) of acetylthiocholine iodide (ATC) as its preferable substrate. Inhibition study tested on 10 metal ions resulted in increasing toxicity order of $Cr^{6+} < Co^{2+} < Ag^{2+} < Cu^{2+} < Pb^{2+} < As^{5+} < Cd^{2+} < Zn^{2+} < Ni^{2+} < Hg^{2+}$, with only Hg^{2+} exhibited the half-maximal inhibitory concentration (IC50) of 0.48 mg/L. From the study, it suggests that the D. hystrix AChE as the potential conventional biosensor for heavy metals detection.

Keywords: Acetylcholinesterase; *Diodon hystrix*; Heavy metals; Pollution

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1. Introduction

Internationally, estimated around two million tons of anthropogenic waste was being deposited into the water bodies daily [1, 2]. Human-made waste releasing multiple extraneous by-products, particularly heavy metals that were tarnishing the water quality and negatively impact to the aquatic life [3, 4]. With this massive unloading scale, derived from various social activities such as manufacture industries, crop cultivation, minerals quarry and construction development were exclaiming the significant loss we may face within the near future [5–7]. These goings-on were resulting in the depletion of hygienic water supply and bio-network commotion, predominantly in aquatic ecosystem.

The improper discharge of anthropogenic waste containing heavy metals enters the soil, likely to leach into the groundwater and finally reach the neighboring water source [8, 9]. Although some heavy metals like copper, chromium and nickel play an essential role in a biological system, metal ions can produce high toxicity at the minute concentration [10]. The exposure of toxic heavy metals may trigger some lethal effects such as neurotoxicity, nephrotoxicity, enzyme inhibition and excess production of reactive oxygen species as metal ions may accumulate in the body [11, 12]. The aquatic organism, for instance fish had appeared with physiological disorders like irregular swimming velocity, aggressive behavior, low feed intake and

cellular injury when exposed to metal ions [13].

With the increasing pollution rate, profound control and monitoring endeavors were vital in a way to cope with this problem. Numerous monitoring techniques have been introduced using inductively coupled plasma, atomic absorption spectrometry and flow injection mercury system in the detection of metal ions concentration [14, 15]. Though all systems display high sensitivity and accuracy, opt for rapid in-situ analysis was looked-for in quantification. By using enzyme-like cholinesterase from an aquatic organism such as fish, this allows for biochemical study regarding the altered biological responses due to the toxicant's exposure, in which leads to the development of biosensor. The Sabah porcupine fish, *Diodon hystrix* was covered with tiny fins in revolutionized scales and venomous skin. The specialized features it used mainly for defense mechanism against predators [15]. This study aims to extract and purify acetylcholinesterase from the brain tissue of *Diodon hystrix*, followed by elucidation the sensitivity towards metal ions which an alternative option for development of biosensor to detect the presence of heavy metals contamination in the environment.

2. Materials and methods

2.1. Materials

The ten number of *D. hystrix* with weight between 0.8 and 1.3 kilograms and body diameter of 28 - 34 centimeters were obtained from local market at Kota Kinabalu, Sabah.

2.2. Extraction of brain AChE

The brain of *D. hystrix* was extracted, weighed and homogenized in 0.1 M sodium phosphate buffer pH 8 with ratio of 1:5 (w/v) using Ultra-Turrax T-25 homogenizer at 4°C. The crude homogenate was centrifuged at 10,000 xg for 10 minutes at 4°C to remove the intact part. The 500 μL of crude and supernatant were taken and stored at -20°C, respectively, for enzyme assay and protein content determination.

2.3. Ammonium sulfate precipitation

The precipitation of supernatant was carried out with Green and Hughes [16] method using varying ammonium sulfate percentage concentration; 0-20, 20-30, 30-40, 40-50, 50-60, 60-70% (w/v). The centrifugation taken place at 10,000 xg for 10 minutes at 4°C. The extractant from each tube was decanted and proceeded for next precipitation percentage. The pellet was resolubilized with 0.1 M sodium phosphate buffer pH 8. The protein solution then dialyzed to eliminate the salt residue. The fraction show high AChE activity was used for affinity purification stage.

2.4. Affinity-purification AChE

The selected fraction was purified using Procainamide-based affinity chromatography. The column syringe was packed with procainamide-sepharose CL-6B matrix to a 5 cm bed height. 1 mL of supernatant was loaded and washed with 5 batch volume of 20 mM sodium phosphate pH 7.5 with gravity flow rate. The 5 batch volume of 20 mM sodium phosphate buffer pH 7.5 containing 1 M NaCl was loaded for elution of bounded-AChE from affinity matrix. 1 mL fractions were collected for assay of enzyme activity and protein content. The fraction shows the highest enzyme activity was stored at -20°C.

2.5. AChE activity and protein content assay

The AChE assay was carried out in modified Ellman [17] method using 96-well microplate. Mixture of 200 μL buffer (0.1 M sodium phosphate, pH 8), 20 μL,5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) (0.5 mM) and 10 μL AChE sample were loaded into microplate wells and incubated for 15 minutes. The first reading was obtained at 405 nm wavelength. Next, a 20 μL acetylthiocholine iodide (ATC) (2 mM) was added into the reaction mixture, incubated for 10 minutes and the final reading was obtained. The AChE activity was defined as the number of substrates (mM) broken down by AChE per minute (U) with 13.6 m/Mcm extinction coefficient. The specific activity was specified as μmole/min/mg or U/mg of protein. The protein content assay was conducted with Bradford [18] method. The bovine serum albumin (BSA) was utilized to construct standard protein plot.

2.6. Enzyme parameters

The optimal substrate specificity test was carried out using the three different synthetic substrates; acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC) and propionylthiochioline iodide (PTC). The various concentration (0, 0.5, 1.0, 1.5, 2.0, and 2.5 mM) of substrates were added to the reaction mixture and reading was obtained after 10 minutes. The maximal velocity (V_{max}) and biomolecular constant (K_m) values were obtained after construction of the Michaelis-Menten curve via Graphpad Prism software version 5.0.

The optimum pH was determined by an overlapping buffer system comprising of acetate buffer (0.1 M, pH 3-5), sodium phosphate buffer (0.1 M, pH, pH 5-8) and Tris-HCl (0.1 M, pH 7-9). The optimum assay temperature was examined by assaying the reaction mixture at varying temperature ranging of 15-60 °C.

2.7. Heavy metals assay and IC_{50}

Ten heavy metals; which cobalt (Co^{2+}), chromium (Cr^{6+}), copper (Cu^{2+}), cadmium (Cd^{2+}), lead (Pb^{2+}), zinc (Zn^{2+}), nickel (Ni^{2+}), arsenic (As^{2+}), silver (Ag^{2+}) and mercury (Hg^{2+}) at 10 mg/L concentration were tested on AChE activity. The inhibition reaction including 150 μL buffer (0.1 M Tris-HCl, pH 9), 50 μL metal ions (10 mg/L), 20 μL 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) (0.5 mM) and 10 μL AChE sample were loaded into 96-microplate wells and incubated for 15 minutes before first reading was taken. A 20 μL acetylthiocholine iodide (ATC) (2 mM) was loaded into the reaction mixture, incubated for 10 minutes and the final reading was obtained at wavelength of 405 nm.

The determination of half maximal inhibitory concentration (IC_{50}) was carried out by means of the highest inhibitive metal ions on AChE activity. The AChE was exposed to multiple concentrations (0.5–10 mg/L) and obtained linear regression analysis on Graphpad Prism software version 5.0.

2.8. Statistical analysis

The data presented in mean ± standard deviations (SD) were analyzed using Graphpad Prism software version 5.0. The comparison of two or more groups of data were analyzed via post hoc analysis of Tukey's test and one-way analysis of variance (ANOVA).

3. Results and discussions

3.1. Purification profile

The AChE from *D. hystrix* brain was highly precipitated at 40% of ammonium sulfate concentration and continued with purification process using procainamide-affinity column. Fig. 1 shows 50 fractions were collected, while the highest AChE activity exhibited at the fraction number 30 and 31. The AChE was recovered at 66.67% after 20.42 purification fold . The decrement in total protein recovery (mg) and AChE activity (U) applied with removal of unwanted protein such pseudocholinesterase and proteases throughout the process [19]. This condition was supported as purification works by maximizing the performance of the purified protein-interest with the increasing specific activity [20–22]. The AChE lost its catalytic properties as external factors such as inapt temperature and pH condition to denature the protein. The surface interactions, ligand leakage and non-specific protein absorption inside the matrix column influencing the low percentage yield of recovery as in Table 1 [23].

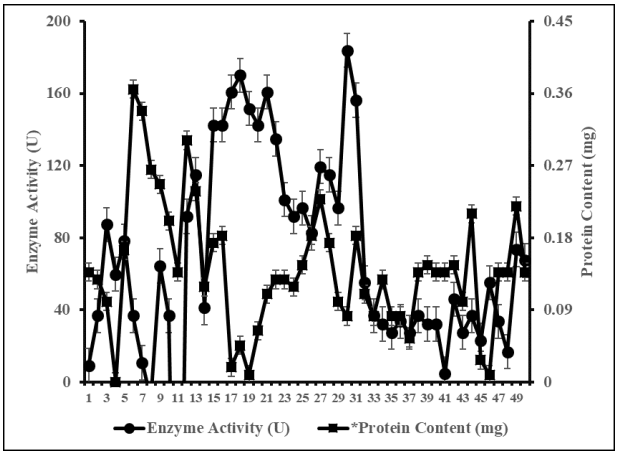

Fig. 1. The purification profile of AChE from *Diodon hystrix* brain with mean of triplicates ± standard deviations (SD).

3.2. Kinetic study

The hydrolyzation of three synthetic substrates; ATC, BTC, and PTC by the purified AChE were demonstrated an obeyed Michaelis-Menten kinetics as in Fig. 2. A steady increase in hydrolytic activity with increasing substrate concentration. Contrarily, the AChE activity reached a plateau state at a concentration above 1 mM, which explained the independency of AChE on the concentration of substrates. The lowest Km shows the high affinity of AChE towards BTC whiles ATC denotes the high Vmax value compared to other substrates. The catalytic efficiencies (V_{max}/K_m) were calculated in Table 2 and showed the preferences of purified AChE towards ATC with displayed the highest ratio. With the abundancy of AChE in the brain, the hydrolyzation of ATC works efficiently in comparison to BTC and PTC [23, 24]. Several notable studies from [12, 25–29] agreed with the finding of the present study in stating preferable ATC substrates for AChE activity.

3.3. PH and temperature profile

The pH optimization involving an overlapping buffer system, and the result shows the maximal activity of AChE achieved in 0.1 M Tris-HCl buffer at pH 9. The enzyme-substrate binding reached an optimum level with achieved maximal pH condition. Theoretically, alterations in pH affecting the enzyme-substrate complex. The AChE catalytic triad histidine-containing imidazole group undergo protonation and interrupt the structural of the active site for conformation of enzyme-substrate [30]. At low pH, high proton concentration disrupts the ionic bonding and consequently impair catalytic efficiency. Conversely, high pH instigating the proton losing at imidazole group to produce

Table 1. The purification table from various AChE procedure from *Diodon hystrix* brain.

Procedure	Total Recovery		Specific activity (U/mg)	Purification fold (fold)	Yield (%)
	Total activity (U)	Total protein (mg)			
Crude extract	275.70	2.45	112.53	1	100
Ammonium sulfate precipitation (40%)	205.88	0.83	248.50	2.20	74.68
Affinity-Procainamide - Sepharose CL-6B	183.80	0.08	2297.50	20.42	66.67

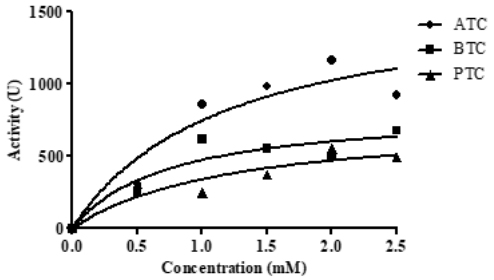

Fig. 2. The substrate specificity of *D. hystrix* AChE brain in 0 to 2.5 mM acetylthiocholine iodide, butyrythiocholine iodide and propionylthiocholine iodide, respectively. Data represented in mean ± standard deviation (n=3).

Table 2. Kinetic table of biomolecular constant (K_m) and maximal velocity (V_{max}) for ATC, BTC and PTC of *Diodon hystrix* AChE.

Mean point (95% confidence intervals)			
Substrates	ATC	BTC	PTC
V_{max} (μmol/min/mg)	$405x10^3$	$204x10^3$	$188x10^3$
K_m (mM)	1.171	0.7381	1.226
Catalytic efficiencies V_{max}/K_m	$879x10^3$	$277x10^3$	$153x10^3$

imidazolate ions. The pH finding of the present study was supported by the previous study by [29]. Fig. 4 displays the bell-shaped curve of AChE activity in response to six different temperature (10, 20, 25, 30, 40 and 50°C). The curve demonstrated as AChE reached optimum activity at 30°C and slowly degraded after incubated at high temperature. Critically, the temperature was influencing reaction rate. Low temperature shows enzyme retardation at the absence of enough kinetic energy to facilitate enzyme-substrate interaction. The same situation works at high temperature in which heat cause instability of protein structure and induce more enzyme denaturation [31, 32]. Therefore, this clarified the dropped activity at 50°C, considering that enzyme to lost catalytic properties at 70°C [33].

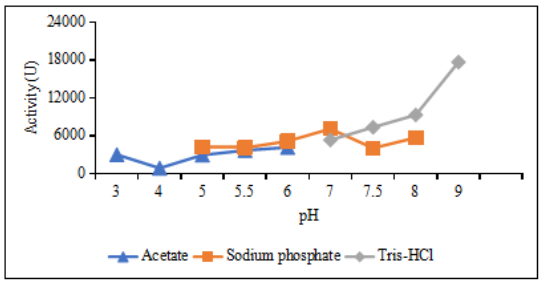

Fig. 3. The pH optimization of AChE activity in expression of mean of triplicates ± standard deviation.

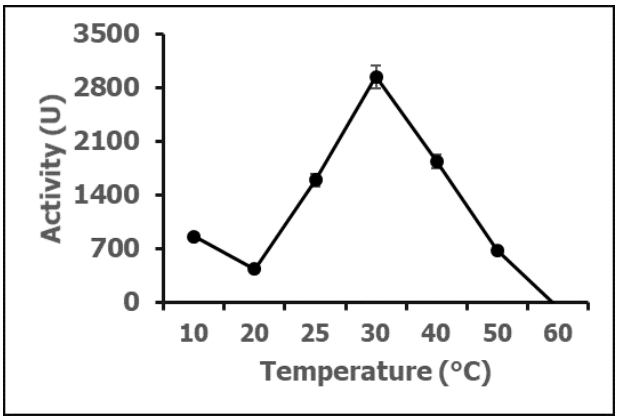

Fig. 4. The temperature optimization of AChE activity in expression of mean of triplicates ± standard deviation.

3.4. Heavy metals inhibition assay

The *in vitro* bioassay of ten metal ions was tested on the purified AChE from *D. hystrix* brain. As in Fig. 5, AChE was inhibited by mercury (Hg^{2+}), nickel (Ni^{2+}), zinc (Zn^{2+}), cadmium (Cd^{2+}), arsenic (As^{5+}), lead (Pb^{2+}) and copper (Cu^{2+}) by lowering AChE activity to 5.88%, 52.94%, 54.41%, 67.65%, 70.59%, 88.24% and 91.18%, respectively. Silver (Ag^{2+}), cobalt (Co^{2+}) and chromium (Cr^{6+}) discreetly exhibited zero inhibition with remaining indifferent activity. Overall, the inhibition assay resulted in ascending toxicity order of $Cr^{6+} < Co^{2+} < Ag^{2+} < Cu^{2+} < Pb^{2+} < As^{5+} < Cd^{2+} < Zn^{2+} < Ni^{2+} < Hg^{2+}$.

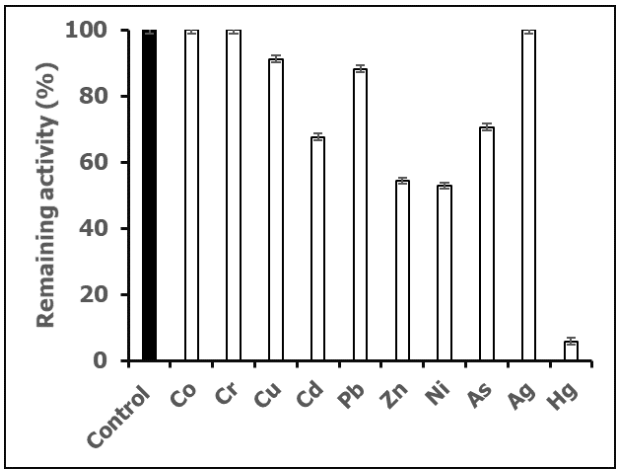

Fig. 5. Heavy metals bioassay of 10 mg/L concentration on AChE activity from *Diodon hystrix* brain. Data represented in mean of triplicates ± standard deviation.

In theory, heavy metals inhibit enzyme through the binding on the amino acid residues such as histidine, tryptophan, cysteine and methionine at either active or allosteric site [34]. The inhibitive mechanism explained when positively-charged metal ions bind to puller negatively-charged protein residues and ultimately altering the regular form of binding sites to form poor catalytic efficiency. The imidazole group in histidine creates strong cation-π pull to draws free metal ions and the ensuing failure of substrate binding [35, 36]. Mercury (Hg^{2+}) produce irreversible neurological, cognitive, cardiovascular and immunological injuries in visualizing noxious result [37, 38]. The commercialized fish samples from Persian Gulf and Palk Bay, Southeastern India were reportedly containing hazardous metal traces such as Cd^{2+}, Pb^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+} [39, 40].

3.5. IC_{50} determination

Among ten tested heavy metals, only mercury (Hg^{2+}) was expressed the strong inhibition with more than 50% AChE activity from Diodon hystrix brain. The IC_{50} value was calculated by the mean of the one-phase exponential decay model for Hg^{2+}, as shown in Fig. 6. The calculated value was 0.48 mg/L with a 95% confidence interval of 0.29 – 1.26 mg/L. Hg2+ specifically binds to the cysteine bridge and irreversibly inhibit enzyme activity [41]. Numerous reports have been specified on the significant inhibition of Hg^{2+} on cholinesterase activity as in [19, 42–44]. Besides, the developed assay of the present study was more sensitive compared to [13].

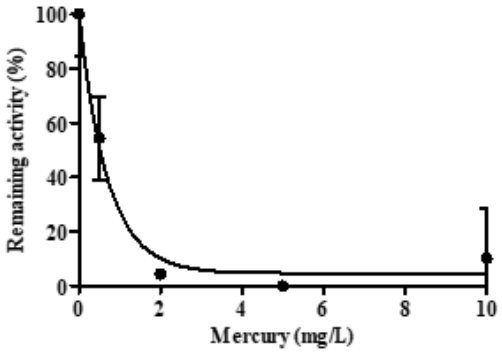

Fig. 6. The inhibitory effect of Hg^{2+} on AChE activity from *Diodon hystrix* brain.

4. Conclusion

The present study was successful in determining the optimal AChE parameters including pH, temperature and substrate specificity with highlighting the high preferences of purified AChE towards ATC. The sensitivity of purified AChE from *Diodon hystrix* brain with respect to metal ions exposure provides a promising opt for development of biosensor in sensing heavy metals contamination. Continuous research should provide more ideas and innovations in creating the sophisticated biomonitoring kit; hence it is suggested to investigate the potential of AChE *D. hystrix* in detecting other types of pollutants such as insecticides and many more.

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