Assessment of *Monopterus albus* liver as a source of Cholinesterase for the detection of heavy metals

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**Abstract.** Heavy metals pollution has now become a serious environmental problem worldwide especially in Malaysia River. As a precaution, continuous environmental monitoring is needed to minimize heavy metal to the ecosystem. Inhibitive enzyme assay based on enzyme including cholinesterase has been introduced as a rapid, cheap and reliable method to assess the level of contamination in the river. In this study, the Asian swamp eel, *Monopterus albus*, was selected and determined of the sensitivity level towards heavy metals. The liver of *M. albus* was extracted and purified using ion exchange chromatography of which DEAE sepharose as the matrix of the column. Based on the Ellman assay, cholinesterase was obtained at 4.86 purification fold with the percentage recovery of 30.32 %. The enzyme works optimally at pH 9 (0.1 M Tris-HCl buffer) and 25 oC, while BTC; 369x103 U.mg-1 was selected as a the preferable substrate which shows highest catalytic efficiencies compared to ATC and BTC at 1457 x 103, 1220 x 103 and 488 x 103 Vmax.Km-1, respectively, Cholinesterase was tested with eight metal ions at the concentration of 5 ppm and the ascending order of inhibition is as followed; arsenic = chromium ≤ plumbum ≤ copper ≤ argentum = nickel < mercury. From this study, the ability of cholinesterase partially purified from the liver tissue of *M. albus* has the potential to be an assay for heavy metals.

**Keywords:** *Monopterus albus, Cholinesterase, Ion exchange chromatography, Heavy metal*

1. **Introduction**

The increasing, rapid and vast industrial activities has resulted in the continuous deposition of heavy metal in the environment, which raised implied concerns. This problem occurs in the most modern and developing country due to due to urbanisation as well as high demand among users. Industrial waste containing heavy metal is either accidentally or uncontrolled released into the soil or finally transferred via leaching or transported by groundwater from industrial areas to the nearby water source [1]–[4]. Exposure to as low as 1 ppm level of toxic metal ion such mercury, cadmium, chromium and lead can cause a severe effect to the biological system such neurotoxicity, hepatotoxicity, nephrotoxicity, malfunction to the vital metabolism, inhibition of enzymes activity and overproduction of reactive oxygen species [5]–[8]. Due to its non-degradable properties, heavy metal is very hard to detoxify biologically and can concentrate in the animal body through the food chain. Moreover, several heavy metals able to be oxidized and caused more harmful effect to living organism. As an example, the less toxic form of elemental mercury, Hg0, comparatively speaking are easily oxidized to the divalent mercury, Hg2+ which is highly toxic and mobilized in the form of free particles into the air or can be adsorbed into the water, sediment and living organism.
The ingestion of contaminated food and water is the main reason for human exposure to heavy metal compared to inhalation and external dermal contact. Due to its bioconcentration and biomagnification factor, heavy metal in the aquatic organism is the main cause of food poisoning. In 1956, the first outbreak of Minamata disease was discovered at Minamata city, Japan. This disease is related to the neurological disorder, numbness of limb, loss of peripheral vision and ultimately death caused by severe poisoning after consuming the contaminated fish product or shellfish containing organic mercury [9], [10].

So far, numerous technique has been established for heavy metal examination including X-ray fluorescence analyzers, inductively coupled plasma, anodic stripping voltammetry, and atomic absorption spectrometry. These systems show sensitivity and accuracy in quantifying the concentration and the level of heavy metals in nature. Notwithstanding, these system are for the most part costly, time-consuming, require repetitive test and need a highly skilled technician to operate. Hence, there is a need to identified and developed sensitive heavy metals detection with rapid determination especially for in-situ analysis through the development of biosensor.

Nowadays, the biosensor has gotten a good attention and consideration among researcher for detection of toxic metal in the environment [11]–[15]. Biorecognition component normally depends on biomolecule such enzyme, antibodies, or nucleic acids, or perhaps the whole cells that could specifically respond with the targeted elements. At the same time, the biological respond ably to quantify or semi-quantify based on colourimetric approach. Cholinesterase; ChE has been used to develop a colourimetric enzyme assay for pesticides such as carbamate and organophosphate. However, more species need to study to obtain the most sensitive ChE which capable to detect other than pesticides such heavy metals, detergent, and drug. In this study, ChE from the liver tissue of Asian swamp eel to elucidate the sensitivity towards selected metal ion and prove to be an alternative environmental biosensor.

2. Materials and Methods

2.1 General preparation
Asian swamp eel; Monopterus albus was bought alive at the wet market, Tuaran, Sabah. The fish was transferred in an aquarium containing 40 L of free chlorinated tap water with full aeration. A day before experimental, the water was replaced and the fish has stopped to feed to avoid contamination of organic material. The fish was sacrificed by immersed in a box of ice for 20 minutes followed by liver dissection and immediately weight. The liver was crushed by using mortar and pestle, then transferred into a beaker containing 0.1 M sodium phosphate buffer at the ratio of 1:4 (weight of liver (g): volume of buffer (v)). The extraction was performed using a digital homogenizer; UltraTurax model T25, then transferred into a tube for centrifugation at 10,000 x g for 15 minutes in the refrigerated condition. The supernatant was collected and used for purification purpose.

2.2 Ion Exchange Chromatography (IEX) purification
Purification using IEX was performed by using DEAE sepharose as the matrixes which was loaded into column until it reaches 3 cm height. The matrix (1.9 cm x 3 cm) was equilibrated with 25 mM sodium phosphate buffer, pH 7.5 and flow rate was calibrated to 0.2 ml/min prior to load 200 µl of supernatant onto the matrix. The column was wash with three batches volume of 20 mM sodium phosphate buffer (washing buffer, pH 7.5) and followed by elution with 20 mM sodium phosphate buffer containing 1 M NaCl. Fractions of 1 mL was collected starting from the washing stage until end of eluting stage. Finally, the column washed with five batches of 20 mM sodium phosphate buffer, pH 7.5 to ensure the cleanness of the matrix.
2.3 Cholinesterase (ChE) enzyme and protein assay

The activity of the enzyme was assayed based on the method of Ellman et al., [16] with slight modification using 96 microplates well. Each well was load with 0.2 mL of sodium phosphate buffer (0.1 M, pH 7.5), 20 μL chromogenic 5,5'-dithiobis-2-nitrobenzoic acid; DTNB (0.067mM) and 10 µL purified enzyme. After 30 minutes preincubation, 20 µL acetylthiocholine iodide; ATC was added and the absorbance was initially read at 405 nm using a multimode detector, while final reading was recorded after 10 minutes incubation in ambient temperature. The yellow product indicates the presence of ChE activity was calculated using an extinction coefficient of 13.6 mM⁻¹cm⁻¹.

The quantitative determination amount of protein was carried out based on the method developed by Bradford, [17] with bovine serum albumin (BSA) as a standard protein graph. 20 μl of ChE was loaded into the well of microplate following by 200 μl of Bradford reagent. The absorbance at 595nm was recorded after 10 minutes incubation at ambient temperature.

2.4 Enzymatic parameter and substrate specificity determination

Optimum pH determination was carried out by replacing the buffer followed by separately ChE incubation in three different buffer; acetate buffer (ranging from pH 3 to 5.5), phosphate buffer (ranging from pH 6 to 8) and tris-HCl buffer (ranging from pH 7 to 10). For temperature profile, preincubation was performed in a separate temperature ranging from 15 to 60°C before addition of substrate.

ChE was separately tested with three substrates namely acetylthiocholine iodide; ATCi, butyrylthiocholine iodide; BTCi, and propionylthiocholine iodide; PTCi, with concentrations ranging from 0.5 to 10 mM. The most preferred substrate was selected based on the measurement of Michaelis–Menten kinetics; maximal velocity (Vmax) and biomolecular constant (Km), using GraphPad PRISM ver 5 software, followed by the selection of the highest ratio of effective coefficient (Vmax.Km⁻¹).

2.5 Inhibition study

Each of metal ions; Argentum (Ag 2+), arsenic (As 5+), cadmium (Cd 2+), chromium (Cr 6+), copper (Cu 2+), lead (Pb 2+), mercury (Hg 2+), and nickel (Ni 2+), was prepared at the final concentration of 10 mg/L. The test was performed by 30 minute incubation of the mixture containing 150 μL of 0.1 M sodium phosphate buffer, 50 μL of single metal ion, 20 μL 0.067 mM DTNB and 10 μL of ChE. Then, initial and final reading was record after addition of substrate at 405 nm. Percentage activity was determined by comparing each of metal ions with the control; replace metal ion with tap water.

3. Results and discussion

3.1 Purification profile

Purification was performed using IEX chromatography with a total of 25 fractions. ChE enzymatic activity was determined based on the colourimetric response in which the chromogens DTNB reacted with ATCi substrate to produce an intensely yellow colour product known as 5-thio-2-nitrobenzoate [18]. Figure 1 shows ChE from M. albus liver extract was purified (fraction 10 to 12) at 4.86 fold with the percentage recovery of 30.32 %; by comparing the specific and total activity of crude homogenate, respectively. Low percentage recovery due to non-specific protein adsorption, ligand leakage and excessive temperature exposure [19]. However the yield was acceptable for the next test since other studies obtained lower than 50% [20], [13], [21], [7].
Figure 1. Purification profile of cholinesterase from the liver tissue of *Monopterus albus*. (U) denoted as mmol⁻¹.min⁻¹.mg⁻¹.

3.2 Enzymatic parameter

3.2.1 Temperature profile

The bell shape shows the best incubation temperature for *M. albus* ChE to be at 25°C (Figure 2) which the similar result also was obtained from *Clarias gariepinus* liver ChE [22]. However, at higher temperatures the ChE activity decreased drastically considering the protein was fully denatured due to loss of stability and structure [23]. Low temperature explained that the ChE activity was retarded or only minimum due to the lack of effective energy to form ES complex. Thus, this study exhibited the optimum temperature for *M. albus* ChE assay at 25°C.

Figure 2. Purified ChE was incubated separately in different temperature ranging from 15 to 45°C. ChE activity was read at the wavelength of 405nm (final – initial reading).
3.2.2 pH profile
Due to the limited capacity of each buffer; acetate, phosphate and tris-HCl buffer, an overlapping system was introduce to study the optimum pH of *M. albus* liver ChE. Figure 3 display the activity of ChE was affected within a specific range of pH. Optimum pH was determined at 9 in 0.1 M tris-HCl buffer. As the optimal pH achieved with respect to the enzyme active site, the efficiency of the ChE to react with specific substrates will increase. pH strongly implies buffering system which resists changes in the activity of the ChE during experimental protocol. Thus, it is fundamental to maintain a good buffering system to reduce error such as rapid denaturation of the protein content and enzyme activity. This data in contrast with ChE from the liver tissue of *Puntius javanicus* where the optimum pH at 7.5 in sodium phosphate buffer [13]. However, several studies show an optimal condition of ChE activity around pH 7 to 9 in tris-HCl buffer such ChE from the liver and gill tissue of *Anabas testudineus* and *Lates calcarifer*, respectively [7], [12], [21].

![Figure 3](image_url)

**Figure 3.** Purified ChE was incubated separately with different buffering condition at different range from pH 3 to 10. ChE activity was read at the wavelength of 405nm (final – initial reading).

3.2.3 Substrate specificity
An obeyed Michaelis-Menten kinetics is displayed in Figure 4 that shows the hydrolysation of the three substrates, namely ATC, BTC, and PTC, at the concentrations ranging from 0.5 to 2.5 mM by the purified ChE. Based on all three reactions, the drastic increase in enzymatic activity associated by the increase in substrate concentrations. Inversely, at above 1 mM substrate concentration, *M. albus* demonstrated a plateau state or reach saturation point. BTC and PTC shows no significant in maximal velocity; $V_{max}$ (P>0.05), however the catalytic efficiency of the enzyme when incubated with BTC showed the highest value followed by PTC and ATC at $1457 \times 10^3$, $1220 \times 10^3$ and $488 \times 10^3 \text{V}_m\text{K}_m^{-1}$ (Table 1). Thus, it can be concluded that BTC was selected as the ideal substrate for this assay and high possibilities the major type of ChE in the liver sample is butyrylcholinesterase; BChE. This study was in line with Hayat et al., [7], Aqlima et al., [21], Padrilah et al., [22] and Sabullah et al., [13] using BTC as the specific substrate for ChE from the liver of *Lates calcarifer*, *Anabas testudineus*, *Clarias gariepinus* and *Puntius javanicus*, respectively.
Figure 4. Incubation of *M. albus* liver ChE in three synthetic substrates with different concentrations. Error bars represent mean ± standard error (n=3).

Table 1. Comparison $K_m$ dan $V_{max}$ between ATC, BTC dan PTC.

|       | ATC          | BTC          | PTC          |
|-------|--------------|--------------|--------------|
| $V_{max}$ (U/mg) | 271$x10^3$   | 369$x10^3$   | 378$x10^3$   |
| $K_m$ (mM)    | 0.56         | 0.25         | 0.31         |
| Catalytic efficiencies | $V_{max}/K_m$ | 488$x10^3$   | 1457$x10^3$  | 1220$x10^3$ |

3.3 Inhibition test

In this study, the detection for metal ions was developed using the reaction of ChE inhibitive assay. It is normal that ChE is inhibited by the existence of metals ions since this element deteriorate enzyme or other types of protein via several mechanisms. Hence, colourless (or yellow paler) product was produced associated with the inhibition of ChE activity. For Hg$^{2+}$, a noticeable colourless solution was formed and considering almost inhibit 100% of ChE activity compared to other metal ions only show paler yellow colour product; different intensities, when reacted with 10 ppm concentration. The ascending order of inhibition is as followed; As$^{5+}$ = Cr$^{6+}$ ≤ Pb$^{2+}$ ≤ Cu$^{2+}$ ≤ Ag$^{+}$ = Ni$^{2+}$ < Hg$^{2+}$ (Figure 5). Metal ion capable to inhibit ChE activity through the formation of ES complex or interaction with the functional group of amino acids such hydroxyl, aromatic, charged and sulphhydryl group [24]. Zhang et al., [25] prove about the alteration of amino acids structure affected by metal ion. Moreover, different monovalent metal ion complex shows differences in the form of skeletal structure associated with the toxic effect, its character and biological function when interact with heavy metals. Vrček and Šinko, [26] compared the composition of amino acids between albumin and AChE which the result shows cysteine is high in albumin while AChE...
contained more methionine and tryptophan, while both proteins have almost similar number of histidine. All of these amino acid strongly sensitive and easily to interact with silver, gold, mercury, copper and zinc [15], [25], [26].

Figure 5. Effect of different types of 10 mg.L⁻¹ metal ions on the ChE activity of M. albus. Error bars represent mean ± standard error (n=3).

4. Conclusion
In this study, the enzymatic parameters such pH and temperature for purified ChE from M. albus liver were successfully determined and it can be concluded that BTC was preferred as the specific synthetic substrate. The sensitivity of ChE inhibition by selected heavy metals was proved and the results suggested a promising biosensor kit for the detection of heavy metals pollution in aquatic environment. Further work is recommended to investigate the ability of the M. albus ChE to detect other contaminants such as detergents, dyes, pesticides, and drugs.

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