Acetylcholinesterase from the brain of *Monopterus albus* as detection of metal ions

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Abstract. Overwhelming amount of heavy metals discharged due to industrialization is a serious global concern. Thus, an investigation was done on acetylcholinesterase (AChE) from Asian swamp eel, *Monopterus albus*, as an alternative biosensor to detect various metal ions. AChE from the brain of *M. albus* was purified through ammonium sulphate precipitation and procainamide affinity chromatography. Enzyme recovery was obtained at 38.73% with the specific activity of 1847 U µg\textsuperscript{-1}. The Michaelis constant (K\textsubscript{m}) value and maximal velocity (V\textsubscript{max}) were determined at 8.910 mM and 29.44 µmol min\textsuperscript{-1} mg\textsuperscript{-1}, respectively for acetylthiocholine iodide (ATC). Based on effective coefficient ratio, AChE from *M. albus* brain showed higher affinity to ATC compared to butyrylthiocholine iodide (BTC) and propionylthiocholine iodide (PTC) at the value of 3.304, 1.515, 2.965 V\textsubscript{max}.K\textsubscript{m}\textsuperscript{-1} respectively. Optimum activity of AChE was obtained at 40°C and incubated in 0.1M Tris HCl buffer pH 9.0. Inhibition study performed on 10 heavy metals resulted in this descending order of inhibition mercury < chromium < zinc < copper < argentum < arsenic < silver < cobalt < cadmium < lead < nickel, with mercury and chromium showing more than 50% inhibition at 10 ppm. Data from this study can be further utilized to develop a cheaper, easier, and faster heavy metal detection method as compared to conventional methods available.

1. Introduction

Environmental contamination of substantial metals has been consistently expanded in a few areas in Malaysia due to civilization as well as the increasing number of industrial activities. Heavy metals particularly copper, cadmium, mercury, argentum, arsenic, zinc and silver are well known to cause aberration in living organism via bioaccumulation in each organ. Moreover, an excessive amount of these heavy metals within biological system could cause detrimental effects and inhibition of numerous metabolisms. This has been demonstrated by [1], where heavy metals prompt a modified physiology in fish including abnormal swimming velocity and behaviour, and nuclear damage. [2] additionally expressed that copper may cause the generation of responsive oxygen species which may disturb biochemical capacities and cellular morphology at high toxic level. Moreover, [3] mentioned that toxic metals can interfere with the normal functions of the animal central nervous system. In this way, this issue led to the advancement of biosensor by exploiting on the study of biological responses of
organisms exposed to heavy metal contaminants. In order to excess the implication of heavy metals towards living creatures, biomonitoring was introduced using aquatic organism and utilized for the recognition of heavy metals in the polluted area.

Fish have high sensitivity towards temperature changes, natural environment and water quality deterioration and additionally aquatic contamination that influences the fish health, which might bring mortalities and ecosystem degradation. This situation shows fish is the best candidates for biomarker development to monitor the present of heavy metal contamination because it could easily to accumulate toxins in each of fish organs (gills, liver, blood, muscle, and mind) which is high sensitivity to the occurrence of xenobiotics [4]. Fish biomarker which incorporates the evaluation of biomolecular, cellular and physiological modification were used for monitoring the biological impact of toxicant particularly metal introduction [5-7]. The investigation on the biochemical stage is another alternative way with rapid and low cost to evaluating the ecological contamination, which this method known as biosensor. Accordingly, the uses of extracted enzyme Cholinesterase (ChE) from aquatic organisms as a biosensor for the detection of anti-cholinesterase effect has been developed based on the alteration in biological response due to the exposure of contaminants in aquatic system. This is because the ChE is the crucial enzyme that presents abundantly in the brain, skeletal muscle and erythrocyte membrane that catalyses the hydrolysis of acetylcholine to choline and acetic acid [8].

ChE is an important compound from the serine hydrolases group, which catalyses the hydrolytic cleavage of acyl group in various esters of choline [9]. It is a group of a compound that comprises acetylcholinesterase (AChE; EC 3.1.1.7), butyrylcholinesterase (BChE; EC 3.1.1.8) and propionylcholinesterase (PChE; EC 3.1.1.8) classified dependent on their preference for particular substrates. Acetylcholinesterase (AChE) has a high preference for acetylcholine; Butyrylcholinesterase (BChE) is better in degrading butyrylcholine; Propionylcholinesterase favours propionylcholine [10]. Thus, in this study, ChE was isolated from the brain of Asian Swamp Eel, M. albus, in order to evaluate the inhibitory effect of metal ions toward the enzyme activity and discover its capability as a new local source of biosensor for environmental contaminant.

2. Materials and methods

2.1 Chemicals
Heavy metals such as silver (Ag²⁺), arsenic (As⁵⁺), cadmium (Cd²⁺), chromium (Cr⁶⁺), cobalt (Co²⁺), copper (Cu²⁺), mercury (Hg²⁺), nickel (Ni²⁺), zinc (Zn²⁺) and lead (Pb²⁺), acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC), propionylthiocholine iodide (PTC), 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB) were purchase from Sigma-Aldrich. These solutions were freshly prepared before used.

2.2 Preparation of brain AChE extractions
*Monopterus albus* were acquired from Meru, Selangor, Malaysia. The fish were brought alive to the laboratory and selected (according to apparent health condition) for the experiment. A day before starting the experiment, the fish was let to starved to reduce the potential dietary influences on organic metabolites status of the fish. The fish was killed in a box full of ice followed by the dissection of fish brain. The brain sample was then weighted followed by homogenization process using an Ultra-Turrax T25 Homogenizer in 0.1 M sodium phosphate buffer pH 7.5 containing 1 mM phenylmethyl sulfonyl fluoride with buffer ration 1: 4 (brain (w): buffer(v)). After centrifugation process at 10,000 xg for 30 minutes at 4 °C, the supernatant of the sample was collected and stored at -20 °C for purification process. Thawing process of the sample takes place after it was left at ambient temperature.

2.3 Ammonium Sulfate Precipitation
The extractant was then precipitate according to the method developed by [11] using different percentage concentration of ammonium sulfate; 0-20, 20-30, 30-40, 40-50, 50-60, 60-70% (w/v). The
mixture was centrifuged at 10,000 xg for 10 minutes at 4 °C. The supernatant from each centrifuge tube was decanted and continued for the next precipitation process while the pellet was resolubilized with a small volume of 25 mM sodium phosphate pH 7.5. The residual ammonium sulfate from the protein solution was removed by dialysis and ultrafiltration. The fraction with high AChE activity was selected for purification process.

2.4 Preparation of affinity purified AChE
Procamainamide affinity chromatography was used to partially purify the AChE from the selected fraction after precipitation. The matrix was packed in a column syringe to a bed height of 4 cm. Then, 1 mL of the supernatant was loaded into the affinity column containing procainamide-sepharose CL-6B and washed with 6 mL of buffer that contained 20 mM sodium phosphate buffer pH 7.0 with gravity flow rate. This stage is important to remove the unbounded protein to the matrix from the column. For eluting buffer, 20 mM sodium phosphate buffer pH 7.0 containing 1M NaCl was then loaded to elute the ChE from *M. albus* which is bounded to the affinity matrix. One mL of fractions was collected and assayed for enzyme activity and protein concentration determination. The fraction with highest activity was pulled and stored at -25°C.

2.5 ChE activity and protein content determination
A minor modification of [12] method was used to determine the enzyme activity of *M. albus* using 96-well microplate at the wavelength of 405 nm. A 200 µL of sodium phosphate buffer (0.1 M, pH 7.0), 20 µL of 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) (0.1 mM) and 10 µL sample ChE were loaded into the microplate wells and incubated for 15 min and obtained the initial reading. Next, 20 µL of the substrates (5.0 mM acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC) and propionylthiocholine iodide (PTC)) were then added to the mixture and incubated for 10 min and before obtained the final reading. ChE activity was specified as the amount of substrate (µM) broken down by ChE per minute (U) with the extinction coefficient of 13.6 mM-1 cm-1 while the specific activity is expressed as µmole/min/mg of protein or U mg-1 of protein. Protein content determination was calculated as designated by [13]. Bovine serum albumin (BSA) was used as standard quantitative value of the protein. The assays were run in the dark area and all the tests were carried out in triplicates.

2.6 Optimal substrate specificity
The substrate specificity for AChE of *M. albus* was determined with three different synthetic substrates, which are ATC, BTC and PTC, with concentrations ranging from 0, 0.5, 1.5, 1.5, 2, 2.5, 5 and 10 mM of each substrates in sodium phosphate buffer (0.1 M, pH 7) at ambient temperature. The substrate was added into reaction mixture and the reading of absorbance at 405 nm was recorded after 10 min. Michaelis-Menten curves was obtained from GraphPad Prism Software version 5.0 in order to determine the maximal velocity (*V* max) of ChE activity and biomolecular constant (*K* m).

2.7 Optimal pH and temperature profile
ChE from *M. albus* was incubated in three different buffers with an overlapping buffer system consisting of 0.1M acetate buffer (pH 3-6), 0.1M sodium phosphate buffer (pH 6–8), and 0.1M Tris–HCl buffer (pH 7–10) to determine the optimum pH for the enzyme. The optimal temperature of the assay was determined from the reaction mixture incubated in different temperatures ranging from 15 to 50°C. Beyond this range of temperature, ChE was considered fully denatured.

2.8 The effect of metal ion
Ten types of heavy metals, namely silver (Ag²⁺), arsenic (As⁵⁺), cadmium (Cd²⁺), chromium (Cr⁶⁺), cobalt (Co²⁺), copper (Cu²⁺), mercury (Hg²⁺), nickel (Ni²⁺), zinc (Zn²⁺) and lead (Pb²⁺) were tested on purified AChE of *M. albus* to study the effect of these metal ions on the enzyme activity. These metals were selected because of their capability to give an adverse impact to the environment. The reaction
mixture contains 150 μL of sodium phosphate buffer (0.1 M, pH 7.5), 50 μL of the metal ion with the final concentration of 10ppm, 20 μL of DTNB (0.1 mM), and 10 μL of AChE while for control, the reaction mixture contains 200 μL of sodium phosphate buffer (0.1 M, pH 7.5), 20 μL of DTNB (0.1 mM), and 10 μL of AChE. The reaction mixture was then incubated for 30 min followed by the addition of 20 μL of the substrate into the mixture. The mixture was left for another 10 min of incubation before the absorbance was recorded at the end of the incubation time at the wavelength of 405 nm.

2.9 Statistical Analysis
The means ± standard deviations (SE) were analysed using GraphPad Prism version 5.0. Comparison between two or more groups was calculated based on Student’s t-test or one-way analysis of variance (ANOVA) with post hoc analysis by Tukey’s test.

3. Results and Discussion

3.1 Sample extraction and purification
Figure 1 shows the ChE from *M. albus* brain extraction was precipitated and purified through ammonium sulphate 30% and procainamide-based affinity chromatography. The highest enzyme activity was observed at fraction number 40 while the highest protein content at fraction number 42. Table 1 shows the ChE was purified at 20.53-fold with 38.73% of yield. The yield was decreasing due to the loss of enzyme activity caused by external factor such temperature that was higher compared to the optimum temperature of the enzyme [14]. The amount of total protein decreased while, the specific enzyme activity increased throughout this purification process. This is because, the purification is necessary to minimise any inference from other protein to ensure that only maximum performance can be reached by the desired purified protein [15-18]. The purification process does not affect the enzyme activity through it seems to modify the molecular structure and enzyme activity only can be affected by temperature, pH and concentration of the enzyme and substrate [19]. In this study, the specific activity of the enzyme increases throughout the purification process at the end of the experiment.

![Figure 1](image_url)

**Figure 1.** Profile of procainamide-based affinity chromatography purification on ChE from *M. albus* brain. Error bars represent mean ± standard error (n=3).
Table 1. Purification table of AChE from *M. albus*. The specific activity from each step of purification is displayed in (U/μg), which means μmole/min/mg of protein.

| Procedure                                      | Total protein (μg) | Total ChE activity (U) | Specific activity (U/μg) | Purification folds | Yield (%) |
|------------------------------------------------|--------------------|------------------------|--------------------------|--------------------|-----------|
| Crude homogenate                               | 0.53               | 47.68                  | 89.96                    | 1                  | 100       |
| Ammonium sulphate precipitation (20-30%)       | 0.12               | 41.82                  | 398.5                    | 4.43               | 87.71     |
| Procainamide-Sepharose CL-6B                   | 0.01               | 18.47                  | 1847                     | 20.53              | 38.73     |

3.2 Kinetic study

An obeyed Michaelis-Menten kinetics that displays the hydrolysation of the three different synthetic substrates, ATC, BTC and PTC at various concentrations by the ChE is shown in Figure 2. As indicated by the outcomes appeared in each of the three reaction, the increase in hydrolytic activity influenced by the substrate concentration. On the contrary, at above 5 mM substrate concentration, the enzyme exhibited a plateau state. Plateau state demonstrates that the reaction becomes independent of substrate concentration. Table 2 shows that the lowest $K_m$ value recorded when PTC was used as the substrate. Conversely, maximum value (3.304) of catalytic efficiency ($V_{max}/K_m$) was detected with the usage of ATC as substrate. Substrate specificity is the ability of the enzyme to recognise and bind to its substrate typically measured using catalytic efficiency ($V_{max}/K_m$). This result is in agreement with that of [20]. Hence, it can be concluded that ATC was the preferable substrate for kinetic study of ChE from *M. albus*.

![Figure 2](image_url). Substrate specificity of purified ChE from *M. albus* brain in three synthetic substrates, namely, acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC) and propionylthiocholine iodide (PTC). Error bars represent mean ± standard error (n=3).
Table 2. Comparison table for maximum velocity (V_{max}) and biomolecular constant (K_m) for acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC) and propionylthiocholine iodide (PTC) of ChE from M. albus brain.

| Substrates | ATC  | BTC  | PTC  |
|------------|------|------|------|
| V_{max} (μmol/min/mg) | 29.44 | 14.09 | 5.126 |
| K_m (mM)   | 8.910 | 9.300 | 1.729 |

Catalytic efficiencies: \( \frac{V_{max}}{K_m} \)
- ATC: 3.304
- BTC: 1.515
- PTC: 2.965

3.3 Optimal pH and temperature
The optimal pH for ChE from M. albus was shown in Figure 3, where the maximum ChE falls at pH 9 of 0.1M Tris-HCl buffer. The enzyme-substrate complex formation is influenced by pH. According to [21], changes in pH caused protonation of imidazole group of histidine that exist at the catalytic triad thus effect the formation of enzyme-substrate complex. Therefore, high concentration of protons at low pH will disrupt the interaction of substrate towards ChE. ChE mechanism will be affected by the alteration of histidine conformation [22]. The same condition also may happen at high pH which the binding of the enzyme and substrate affected by the change of the substrate charge. The optimum pH for most forms of ChE is between pH 7.0 to 9.0 [23]. Figure 4 displayed the effects of different range of temperature on ChE activity. The optimum ChE activity was determined in the range of 30 to 50°C which statistically no significant different (p>0.05). At lower temperature, minimum activity of the ChE was observed. As the temperature increase, the activity rose and reached maximum value that given velocity was reached and a bell-shaped curve was later demonstrated. However, the ChE activity decreased sharply at higher temperatures because there will be enough thermal energy that can disrupt the enzyme conformation and inhibit the activity of the enzyme. As stated by [24], at higher temperature, the protein will undergo denaturation process and resulting loss of protein stability. Hence, this theory explained the sudden decrease in enzyme activity at temperature above 40°C as shown in Figure 4. The increase of ChE activity as a function of water temperature has previously been reported by [25] for the bluegill, Lepomis macrochirus. Inactivation of enzyme activity begins at 56°C and all cholinesterase activity lost at 70°C [26].

![Figure 3. pH profile of purified AChE activity from M. albus brain in three buffers, namely, acetate buffer, sodium phosphate buffer and tris-HCl buffer with mean point of triplicate assay and Y error bars denoted as standard deviation of mean.](image-url)
3.4 Metal ion inhibition study

In this *in vitro* test, all the optimal condition of assay parameters was combined followed by separately incubated with 10 metal ions with the concentration of 10 ppm. Figure 5 shows that ChE was inhibited by silver (Ag$^{2+}$), arsenic (As$^{5+}$), cadmium (Cd$^{2+}$), chromium (Cr$^{6+}$), cobalt (Co$^{2+}$), copper (Cu$^{2+}$), mercury (Hg$^{2+}$), nickel (Ni$^{2+}$), zinc (Zn$^{2+}$) and lead (Pb$^{2+}$) by lowering activity to 52.88, 52.22, 57.35, 40.78, 56.74, 51.73, 37.10, 62.59, 50.47 and 57.79% respectively when ATC was used as the substrate. Cr and Hg displayed the highest inhibition, lowering the activity of ChE to less than 50% with a significance value (P<0.005) between them. [27] reported that the inhibition of mercury is through binding at histidine, methionine, tryptophan, threonine and asparagine residue. Other than that Hg and Cu were categorized as the highest rank of heavy metal toxicity, and other previous studies was proved this statement such [28-33]. Cr is the seventh most abundant element on earth’s crust and the exposure with this metal ions in a short time consequence to irritation especially at ulcers on the skin and nasal mucosa [34-36]. Past investigations demonstrated that the heavy metal blocked the utilization of substrate either specifically binds at the active site of enzyme or binding at the allosteric site which caused the conformational change and substrate failure to shape compound substrate complex [37-39,6-7,40]. Normally, different heavy metals gave different inhibition or sensitivity on the activity of purified ChE [41-43]. Furthermore, it was proved that most of heavy metals showed the capability to inhibit *in vitro* or *in vivo* ChE activity, especially in fish [44-45].
Figure 5. Effect of different types of heavy metals on the enzymatic activity of purified ChE from *M. albus* brain after inhibition at 10ppm. Error bars represent mean ± standard error (n=3). Statistical significance of different from control: *P <0.001.

4. Conclusion

In this research, the optimum assay parameters for purified AChE from *M. albus*; pH and temperature, were successfully determined with ATC being preferred as the specific synthetic substrate throughout this study. Purified ChE from *M. albus* brain shows inhibition of activity after exposed with heavy metals which later can be expanded and developed into an alternative method for biosensing based on these results. Heavy metals especially mercury and chromium showed significant inhabitation toward brain ChE activity as they reduce half of its activity. Hence, this study suggests that *M. albus* could be a possible new source of ChE to replace the existing commercial ChE. In future, recommended to investigate the capability of the purified ChE to detect other types of contaminations such as pesticides, drug, detergents or dyes.
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