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An alternative bioassay using *Anabas testudineus* (Climbing perch) colinesterase for metal ions detection

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Abstract

Climbing Perch or its scientific name, *Anabas testudineus* is one of the freshwater fish belonging to the family of Anabantidae. It is widely distributed in ponds, swamps and estuaries in Asia. In this study, cholinesterase (ChE) was partially purified from the liver of *A. testudineus* through ion exchange chromatography. This purification method provided a recovery yield of 5.36% with a purification fold of 6.6. The optimum conditions for ChE assay were identified to be 2.5 mM of butyrylthiocholine iodide (BTC) with pH 8.0 in Tris-HCl buffer at 40°C. Substrate specificity profile also indicated that ChE favours BTC as substrate because it records the highest catalytic efficiency (V_{max}/K_m). Protein analysis through Native-PAGE showed that ion exchange chromatography is an effective method to partially purify ChE. Metal ion inhibition tests were conducted and mercury (Hg) was found to show the highest inhibition effect (87.30%) whereas lead (Pb) shows the lowest inhibition effect (28.01%). All these findings showed that partially purified ChE from the liver of A. testudineus is suitable to be used as a bioindicator to detect the presence of metal ions.

Introduction

Cholinesterases (ChE) are important enzymes that are present in both vertebrates and invertebrates. It is a family of enzyme that comprises of acetylcholinesterase (AChE; EC 3.1.1.7), butyrylcholinesterase (BChE; EC 3.1.1.8) and propionylcholinesterase (PChE; EC 3.1.1.8) which are closely related to BChE. The aspects that distinguishes AChE from BChE is related to substrate binding and catalytic mechanism. Apart from the natural substrates, ChE also hydrolyse esters of thiocholine such as butyrylthiocholine, acetylthiocholine, propionylthiocholine, acetyl-β-methylthiocholine as well as indophenylacetate, o-nitrophenylacetate, and α-napthyl acetate (Andreescu and Marty, 2006). In addition, tissue distribution, kinetic properties and sensitivity to inhibitors are also some of the ways to differentiate between AChE and BChE (Romani *et al*., 2011). The crystaline structure, sequence comparisons and site-specific mutagenesis could also be used to distinguish between AChE and BChE (Taylor *et al*., 1995).

In biochemistry, cholinesterases especially AChE in the brain tissue readily hydrolyses acetylcholine, a neurotransmitter in synaptic cleft into choline and acetic acid (Mayberry *et al*., 2015). BChE is found most abundantly in the liver and plasma followed by skin and leg muscle (Lockridge, 2014). According to Ashani *et al*. (1991), BChE could act as a useful prophylaxis against soman poisoning in mice. BChE can also degrade cocaine into inactive metabolite (Larrimore *et al*., 2013). This proves that the alternative function of BChE in the liver is to act as detoxifier other than co-regulator in neurotransmitter metabolism (Çokugras, 2003). The liver performs an important role in biosynthesis and the ChE activity is an indicator for liver function in patients with liver disease (Meng *et al*., 2013). All types of ChE are targets for inhibitors such as pesticides and heavy metals. Unfortunately, misapplication of pesticides which might also contain heavy metals may affect to the non target organism as well as contaminating the environment.

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Fish could be easily exposed to and take up heavy metals that are increasingly present in the aquatic environment. Fish are part of the food chain and as a protein source for human. Owing to bioaccumulation and biomagnification of heavy metals, fish are considered as good indicators of water resource quality as the fish health reflects the severity of water pollution (Rautenberg *et al*., 2015). Heavy metals especially mercury, silver, cadmium, lead, zinc, arsenic, chromium, and copper are known to cause abnormalities in fish. According to Fatima *et al.* (2014) and Sabullah *et al.* (2014a), heavy metals lead to an altered physiology in fish and hepatonuclear damage. Moreover, a study by Zheng *et al.* (2003) stated that toxic heavy metals does have the ability to interrupt normal functioning of the animal central nervous system (CNS). Therefore, to assess the effects of heavy metals toward living organisms, the ChE enzyme present in fish was used for the detection of heavy metals (Sabullah *et al*., 2014b).

In this study, crude ChE was extracted from the liver of climbing perch *(Anabas testudineus)* or locally known as "ikan puyu". Next, crude ChE was partially purified using ion-exchange chromatography with diethylaminoethyl-cellulose (DEAE-cellulose) as matrix. Native polyacrylamide gel electrophoresis (Native-PAGE) was used to analyse and separate the protein in sample for purity assessment. The partially purified ChE was assayed for various parameters. Lastly, the inhibitive test of heavy metals toward ChE was carried out to study the effect of heavy metals on ChE activity.

Materials and Methods

Chemicals

Acethylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC), propionylcholine iodide (PTC), phenylmethylsufonyl fluoride, 5,5'- dithiobis (2-nitrobenzoicacid) (DTNB), and diethylaminoethyl cellulose (DEAE-cellulose) were purchased from Sigma-Aldrich, Germany. Bovine serum albumin (BSA) and Bradford solution were purchased from Bio-Rad, USA.

Preparation of crude homogenate

A. testudineus was bought alive from the night market and acclimatised in laboratory for two days. The fish was freeze-killed by immersion in ice for 45 min. The fish liver was extracted and weighed. Next, the crude liver sample was homogenised together with 0.1 M sodium phosphate buffer, pH 7.5 containing 2 mM phenylmethylsufonyl fluoride (the liver and the buffer ratio was 1:4) using an Ultra-Turrax T25 homogeniser (IKA, Germany). The homogenate was then centrifuged at 10000 ×g with the temperature of 4°C for 30 min. The supernatant was collected and stored in a clean microcentrifuge tube at -20°C for further purification while the pellet was discarded (Hayat *et al.*, 2015).

Ammonium sulphate precipitation

The homogenised liver extract was subjected to protein fractionation using ammonium sulfate precipitation following the method developed by Green and Hughes (1955). Starting with 0 to 30% (w/v) of ammonium sulfate, the mixture was transferred into a screw-cap polycarbonate centrifuge tubes and centrifuged at 10000 × g for 15 min at 4°C. The supernatant was collected and ready for dialysis, while the pellet was kept for the next round of precipitation and repeated from 30-40, 40-50, 50-60, 60-70 and 70-80% (w/v) of ammonium sulphate.

Partial purification (ion exchange chromatography)

The column (GE Healthcare, USA) was packed with diethylaminoethyl cellulose (DEAE-cellulose) with the dimension of 5 cm diameter and 70 cm height. Then, 15 mL of crude supernatant was loaded into the matrix. Next, 120 mL of 20 mM sodium phosphate buffer with pH 7.0 was loaded onto the column with the flow rate calibrated at 1 mL/min. For elution buffer, 20 mM sodium phosphate buffer pH 7.0 containing 1 M NaCl was then loaded to elute the ChE of *A. testudineus*. The eluted fractions were collected in the volume of 1 mL and stored in 1 ml microcentrifuge tubes. The protein content and ChE activity for each eluted fraction were determined using the Bradford method and Ellman method respectively. The fraction that showed the highest activity was concentrated and dialysed using Vivaspin 20 centrifugal concentrators (Sartorious, Germany) at 5000 ×g for 10 min. Purified ChE was stored at -25 °C.

Native polyacrylamide gel electrophoresis (Native-PAGE)

Native-PAGE was carried out based on the method developed by Laemmli (1970) with slight modifications. The polyacrylamide gel was set up with resolving gel (12%) and stacking gel (4%). 5 µL of high range protein marker solution was added into the well along with the mixture consisting of 9 µL ChE and 1 µL sample buffer. Gel electrophoresis was conducted at 15 A for 30 min or until tracking gel were completely out from the gel.

ChE activity determination

The activity of *A. testudineus* ChE was measured using the method from Ellman *et al*. (1961) with slight adjustment such as the usage of 96-well microplate at the wavelength of 405 nm. 20 µL of DTNB with concentration of 0.1 mM, 200 µL of sodium phosphate buffer with concentration of 0.1 M at pH 7.0 and 10 µL of ChE were loaded into the microplate wells and incubated for 15 minutes. Next, 20 µL of 5.0 mM of BTC was added into the mixture and incubated for another 10 min to allow the reaction to take place. The ChE activity was expressed as the quantity of substrate (µM) hydrolysed by ChE per minute (U) with the extinction coefficient of 13.6 mM^{-1} cm^{-1} while the specific activity was given as µmole/min/mg of protein or U/mg of protein. All of these assays were carried out in the dark and a series of triplicates of tests were carried out.

Protein content determination

Protein content for each fraction was measured using the Bradford method (1976). Bovine serum albumin (BSA) was chosen as a standard for the quantitative determination of protein.

Substrate specificity

The substrate specificity of *A. testudineus* ChE was determined with three different substrates namely acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC) and propionylthilcholine iodide (PTC). The concentrations of each substrate ranging from 0.5 to 5.0 mM were used. The substrate was added into the assay reaction mixture and incubated for 10 min and the absorbance was read at 405 nm. To determine the biomolecular constant (K_m) and maximal velocity (V_{max}) of ChE activity, Michaelis-menten curves were plotted using GraphPad Prism Software version 5.

pH and temperature profile

The optimum pH of ChE from *A. testudineus* was determined using overlapping buffer systems. These buffers include acetate buffer (0.1 M with pH of 3.0, 4.0, 5.0 and 5.5), sodium phosphate buffer (0.1 M with pH of 5.5, 6.0, 7.0 and 8.0) and tris-HCl buffer (0.1 M and pH of 7.0, 8.0, 9.0 and 10.0).

To determine the optimal temperature of the ChE sample, the reaction mixture was incubated at 15, 20, 25, 30, 35, 40, 45 and 50°C. Beyond this temperature, the tertiary structure of ChE was fully denatured.

The effect of metal ions

The effect of metal ions on the enzyme activity of ChE from *A. testudineus* was determined by incubating it with 10 types of metal ions namely arsenic (As^{5+}), chromium (Cr^{6+}), copper (Cu^{2+}), cadmium (Cd^{2+}), zinc (Zn^{2+}), lead (Pb^{2+}), silver (Ag^{2+}), cobalt (Co^{2+}), nickel (Ni^{2+}) and mercury (Hg^{2+}) (Sigma-Aldrich, Germany). These metals were selected due to their adverse effects on the environment. The reaction mixture contains 150 µL of sodium phosphate buffer (0.1 M, pH 7.5), 50 µL of metal ion with final concentration of 10 mg/L, 20 µL of DTNB (0.1 mM) and 10 µL of the ChE. The reaction mixture was incubated for 15 min at room temperature and followed by the addition of 20 µL of the substrate. The mixture was then incubated for another 10 min. The absorbance reading at the wavelength of 405 nm was taken at the end of incubation time.

Half maximal inhibitory concentration (IC_{50})

The half maximal inhibitory concentration (IC_{50}) study was conducted by using the most inhibitive heavy metal on ChE activity in the previous study. ChE was incubated in different concentrations of the selected heavy metal and the inhibition profile was analysed and IC_{50} value of the selected heavy metal was determined using Graph Pad Prism 5.

Results and Discussion

Purification of cholinesterase (ChE)

The fraction with the highest ChE activity was at 0-30% (w/v) of ammonium sulphate with the value of 59.69 U for BTC (data not shown). This fraction was collected and used for subsequent purification. Table 1 shows that the total protein significantly decreased throughout subsequent purification steps. This phenomenon was due to the removal of unwanted proteins from the sample through ion exchange chromatography method. The total activity also decreased throughout the purification. This might due to the fluctuation of temperature during purification processes. This purification method produced 6.6 fold of purification with the enzyme activity retained at only 5.36%. The purification fold from this experiment was higher compared to a previous study conducted by Tham *et al.* (2009), who only obtained a 4.8 purification fold using edrophonium-Sephacryl S400 column. However, the use of Procainamide-Sepharose affinity chromatography is more suitable and effective compared to any DEAE-Sephadex column chromatography (Mehrani, 2004). Li *et al.* (2008) also demonstrated that one-step procainamide purification could eliminate a remarkable amount of proteins including albumin. Moreover, single-step procainamide affinity purification recovers 70% of BChE by elution with 1 M NaCl. The drawbacks of this affinity chromatography are that it is expensive

Table 1. Purification table of ChE from the liver of *A. testudineus*; the specific activity from each step of purification was expressed in (U/mg) which indicates µmol hydrolysed/min/mg of protein

Sample	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification fold	Yield (%)
Crude	1830.30	785.1	2.33	1.0	100.00
Ammonium Sulphate Precipitation	895.31	132.11	6.78	2.9	48.91
DEAE-Cellulose	98.09	6.34	15.47	6.6	5.36

and time consuming since it requires affinity preparation (Mehrani, 2004).

Native polyacrylamide gel electrophoresis (Native-PAGE)

The purification of ChE using DEAE-cellulose as matrix in ion exchange chromatography aided to exclude the undesired proteins and only retains the desired protein, which is ChE from the sample. There are three bands present and based on standard curve equation from Figure 1, the molecular weight were measured to be at 131.21 kDa (first band), 82.98 kDa (second band) and 63.86 kDa (third band). In another study, the molecular weight of BChE of Leporinus microcephalus, a neotropical fish was found to be 160 kDa (Salles *et al*., 2006). This molecular weight of *L. microcephalus* BChE is very close to the molecular weight obtained from the first band for the partially purified *A. testudineus* liver ChE where the color intensity was darker compared to other bands at Lane D. Although a single band was not obtained from this experiment, the presence of ChE was verified through enzymatic assay. Therefore, this protein analysis through Native-PAGE helps to elucidate the effectiveness of purification on the liver extract from *A. testudineus*.

Substrate specificity

Substrate specificity assay was carried out by using three different substrates, namely ATC, BTC and PTC at different concentration ranging from 0 to 5.0 mM. From Figure 2, three of the reactions showed increasing enzymatic activity with the increasing of substrate concentration. However, at concentration of 2.5 mM onwards, the increment of enzymatic activity was at steady or plateau state. The V_{max} value indicated the highest amount of product formed through the interaction of partially purified ChE with substrate. The K_m value indicates enzyme affinity towards a particular substrate. Hence, the lower the Km value, the higher the enzyme affinity towards the substrate. The highest V_{max} value exhibited at

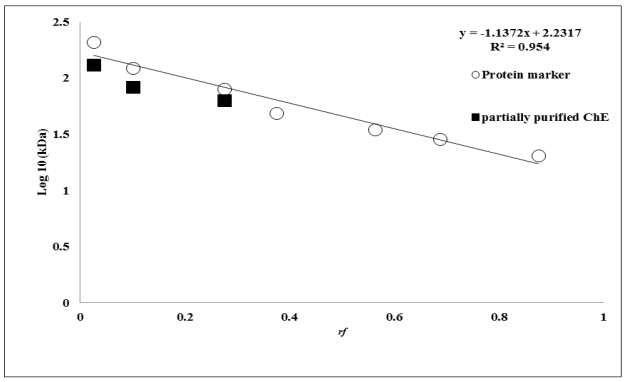

Figure 1. Determination of the molecular weight of partially purified ChE from *A. testudenius* liver by interpolating the retention factor *(rf)* of protein markers. Overlapping of partially purified ChE with other protein was indicated by the squares.

substrate ATC, followed by BTC and PTC with the value of 18.04, 15.63, and 13.28, respectively. As for K_m value, the partially purified ChE showed the highest affinity towards BTC followed by ATC and PTC with the value of 1.97, 4.50 and 6.4, respectively. However, partially purified ChE from the liver of *A. testudineus* showed the highest catalytic efficiencies (V_{max}/ K_m) for BTC followed by ATC and PTC with the value of 7.93, 4.00 and 2.08, respectively. This result proved that this partially purified ChE contains the most BChE. Hence, it coincides with the finding of Lockridge (2014) who stated that BChE are found most abundantly in liver. Therefore, BTC with a concentration of 2.5 mM was selected as the preferred substrate for further analysis even though ATC exhibited higher V_{max} value due to highest catalytic efficiencies of BTC.

Optimum pH profile

In this pH profile study, three types of buffers with different pH values were used to determine the optimum pH condition of partially purified BChE. The optimum pH for partially purified ChE was at pH 8.0 in 0.1 M Tris-HCl buffer (Table 2). The ChE was highly sensitive towards extreme pH conditions such at pH 3 and pH 10. According to Dziri *et al.* (1998),

Table 2. pH and Temperature profile of partially purified ChE from the liver of *A. testudineus*. Error bars represent mean ± standard error (n=3)

Effects	Enzyme activity (U)	Stdev
pH		
Acetate buffer (pH 3)	1.19	0.192
Acetate buffer (pH 4)	0.42	0.505
Acetate buffer (pH 5)	0.83	0.168
Acetate buffer (pH 5.5)	1.60	0.048
Phosphate beffer (pH 5.5)	0.78	1.683
Phosphate beffer (pH 6)	2.38	0.865
Phosphate beffer (pH 7)	10.27	0.529
Phosphate beffer (pH 8)	11.56	0.106
Tris-HCl buffer (pH 7)	10.40	0.202
Tris-HCl buffer (pH 8)	26.96	0.337
Tris-HCl buffer (pH 9)	17.02	1.755
Tris-HCl buffer (pH 10)	10.08	1.390
Temperature (°C)		
15	6.85	1.851
20	11.22	1.923
25	12.89	2.019
30	15.44	0.673
35	17.95	0.240
40	30.07	1.226
45	19.41	1.596
50	7.85	0.240

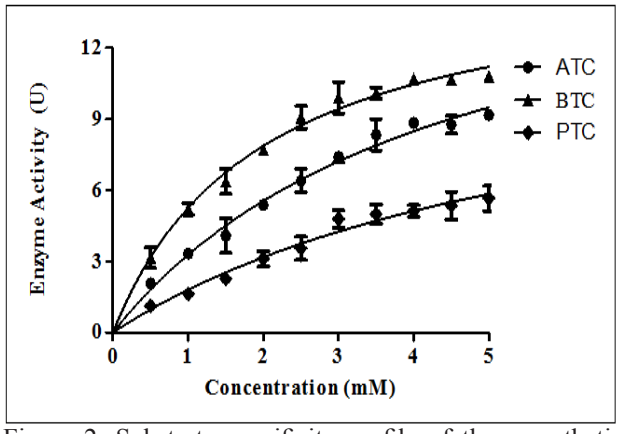

Figure 2. Substrate specificity profile of three synthetic substrates, namely, acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC), and propionylthilcholine iodide (PTC), with different concentration ranging from 0 to 5.0 mM incubated with partially purified ChE from liver extract of *A.testudineus* to study its substrate specificity. All values represent mean ± standard error (n=3).

pH has a substantial influence on the adsorption and stability of enzyme. Thus, this indicates that pH has a profound effect towards the formation of enzyme-substrate complex formation. Under extreme low or high pH, this condition will prevent the formation of enzyme-substrate complex formation and will eventually leads to the loss of enzymatic function.

At low pH such as pH 3, the catalytic activity of ChE was incapacitated due to the protonation of imidazole group of histidine in the catalytic triad (Masson *et al*., 2002). When the pH rises, deprotonation of imidazole group of histidine occurs and the enzyme regains its function, thus able to operate effectively. The optimum pH of ChE, which was pH 8.0 in 0.1 M Tris-HCl showed that the imidazole group of histidine in the catalytic triad was in the ideal natural state. Therefore, the enzyme-substrates complexes were stabilised and the enzyme exhibited the highest activity. Hence, Tris-HCl with pH 8.0 was selected as the optimum buffer for all further assays.

Optimum temperature profile

Temperature is one of the most important factors influencing both metabolism and behaviour of animals, as well as the toxicity of xenobiotics (Świergosz-Kowalewska *et al*., 2014). In general, the curve obtained from temperature profiling showed a bell shaped curve (Table 2). ChE activity was relatively slow at low temperatures and increased drastically as the temperature increased. At a higher temperature, the ChE activity decreased steeply. This result is similar to a previous study where *Mytilus* sp. showed the highest AChE activity during summer months and lowest AChE activity during winter months (Pfeifer *et al.*, 2005).

The logical explanation behind the low ChE activity at 15°C and 20°C were due to low kinetic energy for ChE. Therefore, the formation of enzyme-substrate complex was scarce. Thus, the ChE activity was low at these temperatures. As temperature rises, the ChE activity rises significantly. The ChE activity peaked at 40°C as shown in Table 2. The increase in kinetic energy of ChE will in turn lead to an increase in its movement frequency. As a result, there will be higher chances of enzyme-substrate collision and

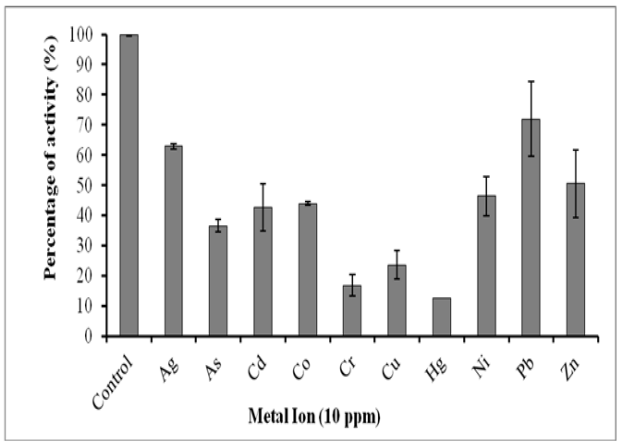

Figure 3. Percentage of enzyme activity after inhibition by heavy metal at 10 ppm. Error bars represent mean ± standard error (n=3)

formation that ultimately lead to higher ChE activity. However, beyond optimum temperature, the ChE activity plummeted. This is because the structural integrity of ChE has been compromised. High temperature causes the molecule in ChE to vibrate violently and this leads to molecular instability. In the end, the bonds that hold ChE molecules together break apart and the denaturation of enzyme started. Therefore, a suitable temperature is crucial due to the pervasive influence of temperature on biological systems (Somero, 2004).

Effect of metal ions on ChE activity

Figure 3 shows that all of the tested heavy metals ion have the ability to inhibit ChE activity. ChE activity was significantly inhibited *in vitro* by Ag, As, Cd, Co, Cr, Cu, Hg, Ni, Pb and Zn by lowering the activity to 62.87, 36.48, 42.67, 43.97, 16.78, 23.62, 12.70, 46.42, 71.99, and 50.49%, respectively. Mercury showed the highest inhibition effect (87.3%) whereas lead showed the lowest inhibition effect (28.01%). Mercury has the highest inhibition effect because it has high affinity to sulfhydryl groups that are presents in ChE (Cabecinhas *et al.*, 2015).

Both mercury and copper showed high inhibition effect towards ChE activity. This finding coincides with the previous study, which showed that copper and mercury have high inhibition effect towards ChE activity of *Pomatoschistus microps* and *Puntius javanicus* (Vieira *et al*., 2009; Sabullah *et al*., 2015). The toxicity of the tested metals can be summarised as follows: Hg ≤ Cr ≤ Cu ≤ As ≤ Cd ≤ Co ≤ Ni ≤ Zn ≤ Ag ≤ Pb. Since the inhibition potentiality of mercury was the highest, it was used for the next round of screening on its IC_{50} value on the partially purified ChE.

Conclusion

Cholinesterase (ChE) extracted from the liver of *A. testudineus* was partially purified with the use of DEAE-cellulose as a matrix in ion exchange. Native PAGE protein profile also proved that the capability of DEAE cellulose in removing large amounts of proteins and leaving only the desired protein. The substrate specificity study showed that partially purified ChE was more likely to catalyse BTC as a substrate due to its lowest Km value and highest catalytic efficiencies value. Optimum pH and temperature were combined to study the effect of heavy metals. Metal ion inhibition tests proved that all of the metals ion used were able to inhibit ChE. Mercury (Hg) showed the most potent inhibitor towards ChE whereas lead (Pb) showed the least inhibitions toward ChE. The outcome showed that ChE activity is suitable to be used as a biosensor for polluted water. To determine the efficacy of ChE as biomarker, further studies using different pesticides or toxic compounds shall be conducted.

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