In vivo and in vitro effects on cholinesterase of blood of *Oreochromis mossambicus* by copper

Ain Aqilah Basirun¹ · Siti Aqlima Ahmad¹ · Mohd Khalizan Sabullah² · Nur Adeela Yasid¹ · Hassan Mohd Daud³ · Ariff Khalid⁴ · Mohd Yunus Shukor¹

Received: 8 December 2017 / Accepted: 23 January 2019 / Published online: 2 February 2019
© King Abdulaziz City for Science and Technology 2019

Abstract

The present study is aimed to evaluate the effects of sub-acute toxicity testing of copper sulphate (CuSO_4), on behavioural, histological and biochemical changes of the *Oreochromis mossambicus* (black tilapia) blood tissues. The effects were assessed according to the previous results on sub-acute toxicity test after exposing fish to several concentrations (0.0, 2.5, 5.0, and 10.0 mg/L). The observations of scanning electron microscope, and transmission electron microscope studies revealed severe histopathological changes on the surface and the cellular changes in blood tissues, respectively. The morphological alterations in blood involved irregular structure of red blood cell and blood clot formation. CuSO_4 affected the biochemical alteration of the blood cholinesterase also known as serum cholinesterase (ChE). Blood ChE inhibited up to 80% of activity when exposed to 10.0 mg/L CuSO_4 . The findings from this study can further improve the quality standards of aquaculture industry and the fundamental basis in selecting suitable strains among freshwater fish species to be used as bioindicator.

Keywords Acute toxicology · Blood · Copper contamination · Cholinesterase · *Oreochromis mossambicus*

Introduction

Over the past few decades, the contamination of heavy metals in the surroundings, specifically in aquatic system has drastically increased. Heavy metals are able to transform into persistent metallic composites which can be accumulated in organisms' body system, disrupting the food chain and eventually threaten the human life (Zhou et al. 2008). The occurrence of heavy metals spillage in the rivers and

lakes is due to the inconsiderate disposal of excess heavy metal usage from human activities such as urbanisation, agriculture, rapid industrial development, and deforestation (Ibemenuga 2016).

The accumulation of heavy metals in water system will affect all the aquatic organisms especially fish. Heavy accumulation of copper in Malaysia is alarming, especially in Mamut River, near Ranau, Sabah (van der Ent and Edraki 2016; Ali et al. 2014). Copper pollution occurs due to an open-pit copper mine located in the headwater of Mamut River, which has operated since 1975 and ceased its operation in 1999. Beyond this time range, this mine became a source of copper, causing several environmental problems beyond Ranau areas. The main source of heavy metals originated from the runoff of the mine site, in addition to the floatation process used in preparing copper concentrates (Ali et al. 2004). Copper plays crucial role in several integral parts of enzymes regarding respiration, collagen synthesis and to reduce free radicals (Acosta et al. 2016). Organisms require a small amount of copper to regulate body metabolism. According to Mashifane and Moyo (2014), several copper compounds were effectively utilised in water treatment as they preserve the discolouration of water and monitor or eliminate algae development

✉ Mohd Yunus Shukor
mohdyunus@upm.edu.my; yunus.upm@gmail.com

¹ Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, UPM 43400 Serdang, Selangor, Malaysia

² Faculty of Science and Natural Resources, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia

³ Department of Veterinary Laboratory Diagnostic, Faculty of Veterinary Medicine, Universiti Putra Malaysia, UPM 43400 Serdang, Selangor, Malaysia

⁴ Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia

and fish parasites in freshwater and marine systems. However, massive usage of copper can lead to copper pollution that would give several negative influences on the aquatic living systems. Copper is tremendously toxic to aquatic life (Ezeonyejiaku et al. 2011; Sabullah et al. 2014a). Copper exposure can generally disrupt the neural processes, protein function and chemosensory abilities (Dew et al. 2012). Consequences of copper poisoning include several organ defects to organisms. As such, copper can encourage larval mortality that will endanger the productivity of aquatic living systems, movement limitation of organisms in their habitat and cell degeneration (Gandhewar et al. 2014; Sabullah et al. 2015a, b, c, d).

Accumulation of heavy metal in aquatic system will affect the sustainability and productivity of aquatic organisms (Ismail and Mat Saleh 2012). Heavy metals such as copper, cadmium, and zinc are the most abundant metal ions that were excessively accumulated in the water. Freshwater fish, which is the most stable subsistence income provider and a major contributor to aquaculture, will be the most to be affected (Sabullah et al. 2014b). Thus, a new alternative source of aquaculture to be used in this project known as *Oreochromis mossambicus* (black tilapia), which has been detected to be marketed of commercialised on a large scale along with other freshwater fishes such as *Clarius batracus* (catfish) and *Puntius javanicus* (lampam Jawa) (Padrilah et al. 2017). Toxicity testing, gross morphology change, conventional enzyme assays and protein chemistry will be analysed by studying the change in fish gross morphology, sub-cellular and biochemical alterations in fish tissues and organs as a result of acute selected heavy metals in the form of metal ion-contamination in the water. Histological effect of the fish organs can also be analysed under microscopy, transmission electron microscope, TEM to observe the changes caused by heavy metal exposure. Despite of the morphology of fish, biochemical alterations can also present as pollution biomarker. Cholinesterase (ChE), a ubiquitous enzyme was selected as the best biomarker for heavy-metal detection as the response of inhibition towards vast range of inhibitors was closely accompanied by a rise in mortality and survival of aquatic organism, impaired due to inhibition (Nunes 2011). ChE is one of versatile enzyme in which it is capable to be a component on enzyme-based biomarker in vivo and in vitro methods (Basirun et al. 2018; Sabullah et al. 2014c). ChE is an enzyme from the serine hydrolases group, which catalyses the hydrolytic cleavage of acyl group of various esters of choline. The most common ChEs are acetylcholinesterase (AChE; E.C. 3.1.1.7), butyrylcholinesterase (BChE; E.C. 3.1.1.8) and propionylcholinesterase (PrChE; E.C. 3.1.1.8). Vertebrates basically have acetylcholine neurotransmitter in their neuromuscular junction and synapses (Lopes et al. 2014). There are two genes present in vertebrates, which are AChE and BChE (Gomes et al. 2014).

Therefore, this study can be referred as the basic procedure for environmental monitoring especially in aquatic system and for species reproducibility and conservation. The outcomes of this study suggest the suitability of *O. mossambicus* (black tilapia) as a bioindicator of copper pollution as the species is well established in Malaysia and copper pollution is an immediate problem especially in the Ranau area in the state of Sabah.

Materials and methods

Specimen treatment

The treatment of acute, non-renewal toxicity testing was done using *O. mossambicus* weighed within 200–300 g obtained from Department of Fisheries, Semeniyih. The fish were brought to Bioremediation laboratory, Universiti Putra Malaysia to be acclimatised about 10 days prior to treatment. The water supply used is 60 L of chlorine-free tap water filtered with top filter pump (Ki-Ki). Anti-chlorine was also added to reduce the chlorine exposure towards fish. The temperature and pH of water were replicated in the laboratory condition between 27 and 30 °C and pH 6–7, respectively. The fish were left in the dark to resemble their natural habitat. Water was renewed once a week to maintain the hygiene and cleanliness of the water. The treatment was done in control condition within 96 h to generate LC₅₀ data. The fish were grouped into six animals in each aquarium. The treatment includes the exposure of copper (CuSO₄) into the aquarium with several ascending concentrations of 0.0, 0.2, 1.0, 2.5, 5.0, 7.5, and 10.0 mg/L with triplicate. The behavioural changes were observed during the treatment period. Semi-quantitative analysis was carried out by observing several changes to the fish upon prolonged exposure period.

Chemicals

Pentahydrate copper sulphate (CuSO₄·5H₂O) (HmbG® Chemicals), phenylmethylsulfonyl fluoride, acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC), propionylcholine iodide (PTC), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), glutaraldehyde, sodium cacodylate, and acetone from Sigma-Aldrich.

Sample preparation

Scanning electron microscope (SEM)

The treated fish were then freeze-killed and decapitated. The blood from femoral vein of the cut-off fish was collected and fixed into 4% glutaraldehyde at 4 °C within 24 h. The blood

was centrifuged at 3000×g for 5 min. The blood was then washed with 0.1 M sodium cacodylate buffer three times for 10 min each. The blood was centrifuged with every change of washing buffer. Post fixation was done with 1% osmium tetroxide for 2 h at 4 °C. Washing step was repeated and continued with dehydration process using series of acetone concentration (35%, 50%, 75%, 90%) for 10 min each. The blood sample was then dehydrated by 100% of acetone for 15 min with triplicate dehydration. Dehydrated blood sample was then dried under critical point drying for 30 min using critical dryer. The mounting process was performed and the sample was coated using gold coating prior to viewing under SEM. The alteration of the treated sample was observed and photographed.

Transmission electron microscope (TEM)

Blood sample was fixed with 4% glutaraldehyde for 24 h. The sample was centrifuged at 3000×g for min. The serum was mixed into the blood sample and the sample was settled down to clot. The clotted blood was then cut into 1 mm³ prior to washing with 0.1 M sodium cacodylate buffer three times for 10 min each. The blood was then post-fixed with 1% osmium tetroxide for 2 h at 4 °C. The sample was then washed again. The dehydration was done using a series of acetone concentrations of 35%, 50%, 75%, and 90% for 10 min for each concentration. The sample was immersed in absolute acetone concentration three times for 10 min each. The dehydrated sample was infiltrated with the mixture of acetone and resin with the ratios of 1:1 for 1h and 1:3 for 2 h. 100% resin was used to infiltrate the sample overnight. The sample was then embedded onto the beam capsule along with resin. Polymerisation took place for 24–48 h at 60 °C. The sample was sectioned by following the ultrathin sectioning. The selected area of sectioned sample was cut and dry. The sample was placed on glass slide and stained with uranyl acetate for 15 min. The slide was washed with distilled water two times prior to staining with lead stain for 10 min. The ready sample of treated blood and untreated blood was viewed under TEM and the morphological was observed.

Cholinesterase activity determination

Cholinesterase (ChE) extraction was done by homogenising the sample in 0.1 M sodium phosphate buffer pH 7 and phenylmethylsulfonylfluoride (PMSF) with the ratio of sample-to-buffer of 1:4. The crude ChE extracts were obtained from the centrifugation at 10,000×g, 4 °C for 25 min. ChE activity of crude was tested using the Ellman assay method (Ellman et al. 1961). The assay has used the synthetic substrates of acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC), and propionylthiocholine iodide (PTC). The mixture of 200 µl of 0.1 M sodium phosphate buffer

(pH 7.0), 20 µl of 0.1 mM 5,5-dithio-bis-2-nitrobenzoate (DTNB) and 10 µl ChE were pipetted into the microplate. The mixture was incubated for 15 min prior to the addition of 20 µl of substrates. After adding the substrates, the mixtures were incubated for 10 min then the absorbance was read. The experiment was done in triplicates. ChE activity of treated sample was compared with the purified untreated ChE blood sample.

The activity of ChE is expressed as the amount of µmol substrate that reacts with ChE per min. The enzymatic reaction rate was quantified against a blank for each activity measurement. ChE-specific activity was determined by a calculation using Eq. 1 and Eq. 2 as follows :

ChE activity = µmole/min/mL = Unit (U)

$$U = \frac{\Delta_{405}}{\epsilon} \times \frac{TV}{SV}, \quad (1)$$

where Δ_{405} is the wavelength at 405 nm after 10 min of incubation (final–initial), ϵ is the extinction coefficient (0.0136 µM/cm⁻¹) of DTNB at the wavelength of 405 nm, TV is the total assay volume (L), SV is the sample volume (mL).

Specific activity = µmol/min/mg = U/mg

$$U/mg = \frac{\text{Total U}}{\text{Total mg}}, \quad (2)$$

mg is the milligram.

ChE inhibition study

ChE inhibition study was done in vitro using copper in the form of metal ion, Cu²⁺ with increasing concentration from 0.1 mg/L up to 10 mg/L. ChE inhibition was assayed by following the Ellman's method (Ellman et al. 1961) with optimal condition of pH buffer, temperature, and specific substrate. The inhibition profile was analysed and IC₅₀ value of copper was generated using GraphPad Prism (Sabullah et al. 2015a, b, c, d).

Purification of ChE by affinity chromatography

ChE blood from the untreated sample was purified through affinity chromatography using Procainamide–sepharyl 6B as matrix. The samples were loaded into the column and washed using 20 mM sodium phosphate buffer. The samples were purified with the flow rate of 1.0 mL/min. The samples were eluted out using 1 M sodium chloride (NaCl). The purification profile was analysed and the enzyme activity of the purified ChE was determined using Ellman assay method. The peaks with the highest enzyme activity were collected for ChE optimisation study. Optimisation process, that includes substrate specificity, optimal pH, and optimal

temperature, was done. For substrate specificity study, ChE activity was determined using three synthetic substrates, ATC, BTC, and PTC. The kinetic study of ChE including maximal velocity, $V_{\max}$ and Michaelis–Menten constant, K_m was analysed using Graphpad Prism software. The equation of K_m and $V_{\max}$ determination was used as shown in Eq. (3).

$$v_i = \frac{V_{\max(\text{app})} \times [S]}{K_{m(\text{app})} + [S]}, \quad (3)$$

where v_i is the initial reaction velocity, $V_{\max(\text{app})}$ is the apparent maximal reaction velocity; attained when all enzyme active sites are filled with substrate molecules ($\mu\text{mol}/\text{min}/\text{mg}$), $[S]$ is the substrate concentration (mM), $K_{m(\text{app})}$ is the apparent Michaelis–Menten constant (mM).

For optimal pH determination, ChE was assayed in the overlapping buffering system of 0.1 M acetate buffer (pH 3–5), 0.1 M sodium phosphate buffer (pH 5.5–8), and 0.1 M tris–HCl buffer (pH 7–10). For optimal temperature study, ChE was incubated in different temperatures ranging from 15, 20, 25, 30, 35, 40, 50 and 60 °C.

ChE inhibition study

Purified ChE inhibition study was done in vitro using copper in the form of metal ion, Cu^{2+} with increasing concentrations from 0.1 to 10 mg/L. ChE inhibition was assayed, following the Ellman's method (Ellman et al. 1961) with optimal condition of pH buffer, temperature, and specific substrate. The inhibition profile was analysed and IC_{50} value of copper was generated using GraphPad Prism software.

Result and discussion

Behavioural changes of *O. mossambicus* upon the CuSO_4 exposure

The study on fish behaviours can contribute vast knowledge and information as it is correlated with the physiological biomarker in aquatic species. Based on Table 1, upon adding CuSO_4 , *O. mossambicus* were also recorded to change

their swimming pattern as they tend to swim heavily at the water surface. In addition, the least-observed effects of concentration (LOEC) value for CuSO_4 was 5.0 mg/L (grey-coloured column) as the fish tend to swim at the surface of water beginning at 5.0 mg/L of CuSO_4 exposure. This change is due to oxygen suffocation experienced by the fish to survive in the contaminated water. At 20.0 mg/L of exposure, fish movement has become inverted which is related to impaired nerve brain that will be explained under histology analysis using three different features of microscopes namely LM, SEM, and TEM. Previous study by Bose et al. (2013) reported that exposure of copper to fish can induce several related stress responses such as ion's regulations, olfaction impairment, and distorted swimming performance.

Histological alteration of *O. mossambicus* blood tissue affected by CuSO_4

Histological alterations' observation under scanning electron microscope (SEM)

Blood sample of *O. mossambicus* was collected from the end of the lateral line of the body that locates the main blood vessel of the fish. Blood morphology was assessed under two different resolutions of SEM and TEM. The present study did not assess the histology on blood smear or apply any anaesthetic agent in collecting the blood sample while the fish is alive. Hence, the blood smear was not involved in this study. The use of both SEM and TEM is to observe the structure of blood cell and cellular alterations after being exposed to sub-acute CuSO_4 toxicity testing, respectively. Figure 1 illustrates the structure of blood and blood content in the control fish (A) and treated fish with series of CuSO_4 concentrations of 2.5 mg/L (B), 5.0 mg/L (C), 10.0 mg/L (D), and 20.0 mg/L (E). Normal fish showed normal structure with normal red blood cell (RBC) also known as erythrocytes and low amount of leucocytes. The alterations in blood tissue suggest that the organism has been attacked by xenobiotic; in this case, copper. Besides, blood alterations in fish or damage of hemopoietic tissue organs may be associated with either changes in environmental conditions or water-borne

Table 1 Behavioural changes and morphological deformities of *O. mossambicus* upon exposure of different concentrations of CuSO_4

	CuSO ₄ concentration (mg/L)							
	Control	2.5	5.0	10.0	20.0	30.0	40.0	50.0
Swimming pattern	R	R	S	S	S. Iv	S. Iv	S. Iv	S. Iv
Fin and tail movement	N	N	In	In	In	In	In	In
Mucus secretion	—	+	++	+++	+++	+++	+++	+++
Gills operculum condition	N	N	W	W	W	W	W	W

R relax, N normal, In inactive, W wide, Iv inverted, W.GC wide and gills come out, S surface and inverted
—: none, +: Little, ++: Moderate, +++: A lot

Fig. 1 The representative section images of the normal *O. mossambicus* blood (a) and exposed by CuSO_4 with different concentration [2.5 mg/L (b), 5.0 mg/L (c), 10.0 mg/L (d), 20.0 mg/L (e)]. Normal red blood cell (RBC) (R) is shown in a. Formation of erythrocytes

(1), irregular structure of RBC (2), and blood clot formation (3) were found in the exposed blood tissue under visualisation of SEM. (Magnification: $\times 1000$)

pollutants (Kumar et al. 2017). Meanwhile, the formation of leucocytes (1) in Fig. 1 acts as a defence mechanism for the fish upon exposure. Formation of monocytes was visualised at the exposure of 5.0 mg/L CuSO_4 . This largest leucocyte has a similar function as neutrophil involved in phagocytosis. The mechanism of action proceeds when the monocytes leave the bloodstream and are revolutionised as tissue microphage where they discharge all the cell

waste like dead cell debris and attacked microorganism. Defence mechanism by leucocyte is related to CuSO_4 exposure after post-exposure effect of the fish, which causes the fish to have physical and internal injuries upon exposure. Abnormal red blood cells (2) were also spotted in SEM micrograph of exposed blood cells as shown in Fig. 1. Increase in exposure of up to 10.0 mg/L of CuSO_4 resulted in several changes in the morphology of the RBC

that includes losing the ellipsoidal shape acquiring different shapes. Among the abnormal cells are the tear drop-like cells, acanthocytes, crenated cells and sickle cells. These abnormalities have been similarly found in previous studies on the effects of copper on other teleost species (Kumar et al. 2017; Privitera and Meli 2016; Sabullah et al. 2014d; Nussey et al. 1995). 20.0 mg/L of CuSO_4 exposure resulted in the enlargement of RBC size, sticking of cells together and caused the presence of spherocytes, which have all been reported by Kumar et al. (2017) who studied the effect of copper on *Clarias batrachus* blood. Blood coagulation or clotting formation (3) is a vital mechanism for fish to counteract the effects of CuSO_4 exposure as it forms fibrin from the disintegrated thrombin, which acts like a mesh to reduce bleeding; in this case,

the physical wound was faced by the fish upon exposure (Ivanov et al. 2017).

Histological alterations' observation under transmission electron microscope (TEM)

TEM ultrastructure magnified the blood cells up to cellular visualisation. All fish species have nucleated RBC (Wong et al. 2012). Hence, Fig. 2 displays the normal nucleated RBC (A) and exposed nucleated RBC by 2.5 mg/L (B), 5.0 mg/L (C), 10.0 mg/L (D), 20.0 mg/L (E). Several anomalies include abnormal RBC structure (1), protrusion and vesicle formation on RBC membrane (2), disintegration of RBC nucleus (3), and excess formation of leucocytes as defence mechanism to the CuSO_4 attack. Abnormal RBC was spotted when it lost its ellipsoidal shape. Changes in RBC structural

Fig. 2 Ultrastructure of normal *O. mossambicus* blood cell (a) and exposed to CuSO_4 with different concentrations [2.5 mg/L (b), 5.0 mg/L (c), 10.0 mg/L (d), 20.0 mg/L (e)]. Normal red blood cell (RBC) was shown in a. Several anomalies include abnormal RBC (1), protrusion and vesiculation of RBC (2), disintegration of RBC nucleus (3), and formation of leucocytes include monocytes (M), lymphocytes (L), and basophils (B), visualised under TEM. (Magnification: $\times 4000$)

formation have caused the fish to receive insufficient oxygen supply as the gaseous exchange in fish respiratory system involves the blood capillaries and the pillar cells in the blood vessel located in gills. Blood vessel system connects the whole fish body system as blood is known as transport mechanism for nutrient, gaseous, and waste throughout the organism's body. Formation of protrusions on the plasma membrane (membrane vesiculation) as shown in Fig. 2c has been well documented in human red blood cells but is less known in fish erythrocytes. Human red cells exposed to high intracellular Ca^{2+} concentration (Allan et al. 1976) or dimyristoyl phosphatidylcholine (Ott et al. 1981) were first transformed from discocytic to echinocytic morphology displaying numerous spicula on the membrane surface with subsequent release of microparticles. In the present study the sites of membrane protrusions clearly corresponded to the sites for releasing vesicles. This anomaly has been also reported in the study by Wong et al. (2012) on silver sea bream erythrocytes. The protrusion was followed by post-apoptotic necrosis, which led to the leakage in cytoplasmic content due to integration of nucleus (3). New experimental cell system has been developed by Bratosin et al. (2011), which assesses the aquatic pollution degree through apoptosis of nucleated RBC from the teleost.

ChE inhibition study on *O. mossambicus* blood exposed by CuSO_4

Cholinesterase (ChE) from untreated and healthy *O. mossambicus* blood was extracted to study its specificity towards three selective artificial substrates; acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC), and propionylthiocholine iodide (PTC) to determine the cholinesterase-type abundance in *O. mossambicus* blood plasma. Figure 3a illustrated that PTC substrate was hydrolysed the highest by ChE with 15.45 U in blood. The result showed that crude ChE

extracted from blood of *O. mossambicus* is dominant with propionylcholinesterase (PrChE), which contradicts the finding by Ahmad et al. (2016a, b, c) stating that blood serum of *Anabas testudineus* is dominant with butyrylcholinesterase (BChE) even though early findings published that blood samples of aquatic organism contain 80% of ChE compared to other tissues (Augustinsson 1961). Blood extracted from several aquatic species showed similar result with present study, which hydrolysed PTC higher compared to ATC and BTC (Talesa et al. 1994; Bonacci et al. 2004). ChE from exposed fish was assessed using PTC with inhibition profile shown as in Fig. 3b. The graph indicates the inhibition of ChE at the lowest CuSO_4 concentration tested, which is 2.5 mg/L. It was seen that the inhibition has declined at about 80% of the control ChE. ChE was reported to be totally inhibited at concentration of 10.0 mg/L CuSO_4 . These results indicate that copper acts as an inducer of toxic stress on the *O. mossambicus*, thus suggesting that ChE from *O. mossambicus* blood may serve as a significant biomarker for copper-induced toxicity.

In vitro analysis of *O. mossambicus* blood cholinesterase exposed by CuSO_4

Based on crude profile in Fig. 3, ChE extracted from *O. mossambicus* blood was seen dominant with PrChE. Hence, the activity determination was proceeded using PTC as substrate. Purification data, enzyme activity and protein content of partially purified PrChE from *O. mossambicus* brain through affinity chromatography were recorded in Fig. 4. Based on the profile, low amount of PrChE and protein was detected starting from the fraction of 1–20. However, the highest peak of PrChE amount was protruded at fraction 23 showing the bonded ChE in the Procainamide–Sephacryl 6B matrix, which was eluted at that time by high ionic strength of elution buffer. Table 2 tabulates the purification fold and

Fig. 3 Enzyme activity of *O. mossambicus* blood cholinesterase hydrolysed three substrates, ATC, BTC, and PTC (a). ChE inhibition study of *O. mossambicus* blood treated by CuSO_4 with concentration of 2.5 mg/L, 5.0 mg/L, 10.0 mg/L, and 20.0 mg/L (b). Error bars represent mean $\pm$ standard error ($n=3$)

Fig. 4 Profile of purified ChE from blood extract of *O. mossambicus* on Procainamide–Sephacryl 6B affinity column. Values are mean $\pm$ SD ($n=3$)

recovery of PrChE, which are 4.89 and 80.20%, respectively. Similar with the enzyme recovered from purification of brain, this result showed that less ChE activity was lost during the purification process since the matrix uses Procainamide–Sephacryl 6B ligand affinity column known as the specific ligand binding for ChE. In general, decrease of protein recovery is associated with the loss in ChE activity due to enzyme denaturing caused by excessive temperature exposure or the ChE enzyme that remained in the crude cells to sediment down during centrifugal process (Sabullah et al. 2014e; Hayat et al. 2016, 2017). Hence, affinity chromatography by Procainamide–Sephacryl 6B was suitably applied to partially purify PrChE from the blood extract of *O. mossambicus*. After that, the partially purified PrChE fractions (21–24) with higher enzyme activity were pulled and then used for optimisation and inhibition studies.

SDS-PAGE of partially purified ChE from the blood of *O. mossambicus* in a 12% polyacrylamide gel was illustrated in Fig. 5. Broad range of prestained protein marker marked as Lane A was used as the indicator for estimating PrChE MW extracted from *O. mossambicus* blood. Lane C showed three visible bands from partially purified PrChE blood. Based on the MW protein standard curve (Fig. 6), those three bands are with the MW of 147.25, 101.39, and 62.46 kDa. In SDS-PAGE, the mobility of the protein separation was observed

based on the MW. Hence, the lowest MW appeared first throughout the gel. The lowest MW recorded is in accordance with that obtained by Sabullah et al. (2015d) and Ahmad et al. (2016a, b, c); the extracted ChE from two different freshwater fish species known as *A. testudineus* and *P. javanicus*. In addition, whole blood including blood plasma and serum is usually predominant with acetylcholinesterase and pseudocholinesterase. ChE in blood is vital for signal transduction in central nervous system since blood is one of the important transport mechanisms in organism's body (Ghazala et al. 2014). The different molecular weight in blood was expressed from the same monomer, but in different aggregation. Frequently, the studies on ChE in blood are gaining much attention; however, there are few studies examining MW determination of ChE extracted from blood. Different MW of PrChE in *O. mossambicus* blood did not affect enzyme quantification as the presence of ChE was verified through enzymatic assay (Hayat et al. 2016).

Optimisation studies of *O. mossambicus* blood cholinesterase

Substrate specificity

The enzyme activity in Fig. 7 indicates PrChE in the brain of *O. mossambicus* hydrolysed PTC at higher rate compared to ATC and BTC, indicating a perceptibly high enzyme activity for its regular substrate. ATC recorded the lowest K_m value compared to BTC and PTC. PrChE kinetic parameter test was done to validate the substrate specificity profile. This present study shows the kinetic data; the highest maximal velocity (V_{max}) of PrChE towards PTC, suggesting that PrChE has hydrolysed PTC at a higher rate compared to ATC and BTC. Catalytic efficiency was calculated in Table 3 with BTC displaying the highest ratio. This is because both PTC and BTC are the non-specific substrates for PrChE as PrChE was categorised as pseudocholinesterase (Sandrini et al. 2013; Ahmad et al. 2016c). Based on statistical analysis using method of Tukey's test, the study also recorded that the optimum substrate concentration of PTC for maximum PrChE activity is 3.0 mM, which is significantly different than other substrates ($p < 0.05$). Thus, this concentration of PTC was applied for further analysis of AChE activity.

Table 2 Comparison between extraction and purification method of *O. mossambicus* ChE blood

Blood	Volume (mL)	Protein	Activity	Total protein (mg)	Total ChE (U)	Specific activity	Fold	Yield
Crude	20	1.03	15.46	20.60	309.19	15.02	1	100
Affinity chromatography	10	0.34	24.80	3.37	247.98	73.54	4.89	80.20

ChE activity was expressed in U (μ mole/min) from each purification step

Fig. 5 SDS-PAGE of partially purified ChE from the blood of *O. mossambicus* in a 12% polyacrylamide gel. Lane A is the well load with broad range molecular weight proteins marker, Lane B with Supernatant and Lane C with purified protein was recovered from procainamide affinity column. Each protein from the gel was detected by modified CBB G-250

Fig. 6 Purified ChE from *O. mossambicus* blood was detected based on broad protein range standard curve at 147.44, 114.14, and 62.46 kDa

pH profile

Figure 8 shows the optimised pH profile of blood PrChE with the optimum pH at tris-HCl, pH 9. The bell-shaped graph at increasing pH of sodium acetate buffer demonstrated that the ChE activity has gradually increased until reaching pH 7 of sodium phosphate buffer. At pH 8 of

Fig. 7 Michaelis–Menten plot of *O. mossambicus* ChE blood incubated with different synthetic substrates; acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC), and propionylthiocholine iodide (PTC), at various concentrations ranging from 0 to 4.0 mM. Error bars represent mean $\pm$ standard error ($n=3$)

Table 3 Maximal velocity, $V_{\max}$ and Michaelis–Menten constant, K_m values of three synthetic substrates on partially purified ChE from blood extract of *O. mossambicus* to study its substrate specificity properties

Substrates	$V_{\max}$, specific activity (U/mg protein)	K_m (mM)	Catalytic efficiencies ($V_{\max}/K_m$)
ATC	9.33	8.42	1.11
BTC	5.64	1.17	4.82
PTC	9.46	3.06	3.10

The results were obtained using GraphPad Prism

Fig. 8 Optimisation studies of pH for the ChE from *O. mossambicus* blood using three different buffers. Values are mean $\pm$ SD ($n=3$)

tris-HCl buffer, the activity was increased until pH 9. The activity kept declining at pH 10 and pH 11. This fluctuation was due to the suitability of pH for optimum enzyme activity. ChE activity at pH 9 was significantly difference with pH 8 ($p < 0.01$) but not significantly difference with pH 10 ($p > 0.05$). However, pH 9 was selected for blood PrChE optimisation as it possesses the highest enzyme activity among buffer systems used. In addition, previous study by (Ahmad et al. 2016c) extracted ChE from *Anabas testudineus* blood reported a similar result on tris-HCl as an optimum buffer system.

Temperature profile

Figure 9 shows the temperature profile of ChE extracted from *O. mossambicus* blood. At low temperature, PrChE activity was truncated due to low molecular motion between enzyme and substrate collision in low temperature. The activity kept arising until it reaches the prime temperature of 40 °C. The optimum temperature of PrChE in blood was the highest compared to other organs. This result was in agreement with the statement by Ahmad et al. (2016a, b, c) that the range of ChE temperature in blood is between 30 and 40 °C. At 50 °C, the activity has significantly declined, indicating the denaturation of ChE active site by extreme temperature condition. The optimum temperature for blood ChE in this study was quite high since the blood is from one of the fish organisms with the poikilothermic ability (Lu et al. 2016). Hence, the optimum temperature of 40 °C was selected as the incubation temperature for the analysis on the AChE activity.

Fig. 9 Optimisation of temperature for ChE from *O. mossambicus* blood. Values are mean $\pm$ SD ($n = 3$)

Half maximal inhibitory concentration (IC₅₀) study of ChE extracted from *O. mossambicus* blood

Half maximal inhibitory concentration (IC₅₀) studies of copper on five selected organs have depicted different patterns of inhibition curves. Generally, IC₅₀ is determined for checking antagonist potency. Antagonist can be toxicants, drugs, pesticides, and in this case, Cu. IC₅₀ can be calculated by determining the concentration of Cu required, eliciting half of the percentage activity of tested ChE. Lower IC₅₀ value will give a greater potency of the antagonist and the lower the concentration of toxicants required to inhibit the maximum biological response.

Figure 10 depicts the IC₅₀ study on Cu of blood PrChE generated from Graphpad Prism software. Based on the inhibition profile, PrChE extracted from *O. mossambicus* blood was found to be very sensitive towards Cu as the IC₅₀ recorded was 0.2972 mg/L with R^2 value of 0.9978. It was astonishing as the result proved that blood PrChE was very sensitive as it requires less than 1 mg/L of Cu to be inhibited. Furthermore, the result concluded the sensitivity of blood PrChE on Cu in both methods of in vivo (Fig. 3) and in vitro analysis. According to Ahmad et al. (2016a, b, c), IC₅₀ of heavy metals on fish blood PrChE recorded from this finding was 1.048 mg/L, which is not much different with the present study. This finding can prove that blood PrChE is very sensitive and preferable as a biosensor for detecting heavy metals in polluted area due to the blood properties as a transport mechanism in an organism's body.

Conclusion

This study demonstrated that copper at the concentration of as low as 5 mg/L elicits behavioural changes in fish that can be a visible biomarker for copper toxicity. Histopathological

Fig. 10 Percentage inhibition of blood ChE by Cu with series of concentrations (1–10 mg/L). IC₅₀ was analysed using GraphPad Prism. Error bars represent mean $\pm$ standard error ($n = 3$)

changes in the present study are in agreement with the study recorded in the *O. mossambicus* exposed by CuSO₄ within 96-h of exposure period. It was astonishing as the result proved that blood PrChE was very sensitive as it requires less than 1 mg/L of Cu to be inhibited. Furthermore, the result concluded the sensitivity of blood PrChE on Cu in both methods of in vivo and in vitro analysis. In water pollution control, aquatic bioassays through biochemical changes such cholinesterase inhibition in blood are essential as to determine whether a potential toxicant is unsafe to aquatic life and if so, to discover the affiliation between the toxicant concentration and its effect on marine animals.

Acknowledgements This research is supported by the funding of the Grants from Putra-IPS Grant (9481400), Putra-IPS Grant (934700), and Graduate Research Fellowship Universiti Putra Malaysia.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interests regarding the publication of this article.

References

- Acosta DS, Danielle NM, Altenhofen S, Luzardo MD, Costa PG, Bianchini A, Bonan CD, da Silva RS, Dafre AL (2016) Copper at low levels impairs memory of adult zebrafish (*Danio rerio*) and affects swimming performance of larvae. *Comp Biochem Physiol C Pharmacol* 185–186:122–130
- Ahmad S, Sabullah M, Basirun A, Khalid A, Iqbal I, Shamaan N, Syed M, Shukor M (2016a) Evaluation of cholinesterase from the muscle and blood of *Anabas testudineus* as detection of metal ions. *Fresenius Environ Bull* 25:4253–4260
- Ahmad SA, Sabullah MK, Shamaan NA, Shukor MYA, Jirangon H, Khalid A, Syed MA (2016b) Evaluation of acetylcholinesterase source from fish, *Tor tambroides* for detection of carbamate. *J Environ Biol* 37:479–484
- Ahmad SA, Wong YF, Shukor MY, Sabullah MK, Yasid NA, Hayat NM, Shamaan NA, Khalid A, Syed MA (2016c) An alternative bioassay using *Anabas testudineus* (Climbing perch) cholinesterase for metal ions detection. *Int Food Res J* 23:1446–1452
- Ali MF, Heng LY, Ratnam W, Nais J, Ripin R (2004) Metal distribution and contamination of the Mamut River, Malaysia, caused by copper mine discharge. *Bull Environ Contam Toxicol* 73:535–542. <https://doi.org/10.1007/s00128-004-0462-5>
- Ali BNM, Lin CY, Cleophas F, Abdullah MH, Musta B (2014) Assessment of heavy metals contamination in Mamut river sediments using sediment quality guidelines and geochemical indices. *Environ Monit Assess* 187(1):4190–4201
- Allan D, Billah MM, Finean JB, Michell RH (1976) Release of diacylglycerol-enriched vesicles from erythrocytes with increased intracellular (Ca²⁺). *Nature* 261:58–60
- Augustinsson K-B (1961) Multiple forms of esterase in vertebrate blood plasma. *Ann N Y Acad Sci* 94:844–860
- Basirun AA, Ahmad SA, Yasid NA, Sabullah MK, Daud HM, Sha'arani S, Khalid A, Shukor MY (2018) Toxicological effects, behavioural and biochemical responses of *Oreochromis mossambicus* gills and its cholinesterase by copper. *Int J Environ Sci Technol*. <https://doi.org/10.1007/s13762-018-1711-1>
- Bonacci S, Browne MA, Dissanayake A, Hagger JA, Corsi I, Focardi S, Galloway TS (2004) Esterase activities in the bivalve mollusc *Adamussium colbecki* as a biomarker for pollution monitoring in the Antarctic marine environment. *Mar Pollut Bull* 49:445–455. <https://doi.org/10.1016/j.marpolbul.2004.02.033>
- Bose MTJ, Ilavazhahan M, Tamilselvi R, Viswanathan M (2013) Effect of heavy metals on the histopathology of gills and brain of fresh water fish *Catla catla*. *Biomed Pharmacol J* 6:99–105
- Bratosin D, Fagadar-Cosma E, Gheorghe A-M et al (2011) In vitro toxic—and ecotoxicological assessment of porphyrine nanomaterials by flow cytometry using nucleated erythrocytes. *Carpath J Earth Environ Sci* 6:225–234
- Dew WA, Wood CM, Pyle GG (2012) Effects of continuous copper exposure and calcium on the olfactory response of fathead minnows. *Environ Sci Technol* 46:9019–9026. <https://doi.org/10.1021/es300670p>
- Ellman GL, Courtney KD, Andres V, Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 7:88–95. [https://doi.org/10.1016/0006-2952\(61\)90145-9](https://doi.org/10.1016/0006-2952(61)90145-9)
- Ezeonyejiaku CD, Obiakor MO, Ezenwelu CO (2011) Toxicity of copper sulphate and behavioral locomotor response of tilapia (*Oreochromis niloticus*) and catfish (*Clarias gariepinus*) species. *Online J Anim Feed Res (OJAfr)* 1:130–134
- Gandhewar SS, Zade SB, Sitre SR (2014) Assessment of toxic potential of three different heavy metals to *Clarias batrachus* (Linn.) utilizing static acute bioassay. *J Appl Nat Sci* 6(1):117–120. <https://doi.org/10.31018/jans.v6i1.385>
- Ghazala MS, Ahmad L, Sultana S, Alghanim K, Al-Misned F, Ahmad Z (2014) Fish cholinesterases as biomarkers of sublethal effects of organophosphorus and carbamates in tissues of *Labeo rohita*. *J Biochem Mol Toxicol* 28:137–142. <https://doi.org/10.1002/jbt.21545>
- Gomes IDL, Lemos MFL, Soares AMVM, Barata C, Faria M (2014) The use of cholinesterase as potential biomarker: in vitro characterization in the polychaete *Capitella teleta*. *Mar Pollut Bull* 85:179–185. <https://doi.org/10.1016/j.marpolbul.2014.05.053>
- Hayat NM, Shamaan NA, Sabullah MK, Shukor MY, Syed MA, Khalid A, Dahalan FA, Ahmad SA (2016) The use of *Lates calcarifer* as a biomarker for heavy metals detection. *Rendiconti Lincei* 27:463–472. <https://doi.org/10.1007/s12210-015-0501-7>
- Hayat N, Ahmad S, Shamaan N, Sabullah M, Syed M, Khalid A, Khalil K, Dahalan F (2017) Characterisation of cholinesterase from kidney tissue of Asian seabass *Lates calcarifer* and its inhibition in presence of metal ions. *J Environ Biol* 38:381–388
- Ibemenuga KN (2016) Bioaccumulation and toxic effects of some heavy metals in freshwater fishes. *Anim Res Int* 10:1792–1798
- Ismail I, Mat Saleh I (2012) Analysis of heavy metals in water and fish (*Tilapia* sp.) samples from Tasik Mutiara, Puchong. *Malays J Anal Sci* 16:346–352
- Ivanov I, Matafonov A, Sun M, Cheng Q, Dickeson SK, Verhamme IM, Emsley J, Gailani D (2017) Proteolytic properties of single-chain factor XII: a mechanism for triggering contact activation. *Blood*. <https://doi.org/10.1182/blood-2016-10-744110>
- Kumar M, Kumar D, Kumar R (2017) Sublethal effects of cadmium and copper on the blood characteristics of catfish *Clarias batrachus* (Linn.). *Int J Adv Res Biol Sci* 4:123–128
- Lopes RM, Filho MVS, de Salles JB, Bastos VLFC, Bastos JC (2014) Cholinesterase activity of muscle tissue from freshwater fishes: characterization and sensitivity analysis to the organophosphate methyl-paraoxon: ChE activity of muscle tissue from freshwater fishes. *Environ Toxicol Chem* 33:1331–1336. <https://doi.org/10.1002/etc.2556>
- Lu Y, Wu Z, Song Z, Xiao P, Liu Y, Zhang P, You F (2016) Insight into the heat resistance of fish via blood: effects of heat stress on metabolism, oxidative stress and antioxidant response of

- olive flounder *Paralichthys olivaceus* and turbot *Scophthalmus maximus*. Fish Shellfish Immunol 58:125–135. <https://doi.org/10.1016/j.fsi.2016.09.008>
- Mashifane TB, Moyo NAG (2014) Acute toxicity of selected heavy metals to *Oreochromis mossambicus* fry and fingerlings. Afr J Aquat Sci 39:279–285. <https://doi.org/10.2989/16085914.2014.960358>
- Nunes B (2011) The use of cholinesterases in ecotoxicology. Rev Environ Contam Toxicol 212:29–59. https://doi.org/10.1007/978-1-4419-8453-1_2
- Nussey G, Van Vuren JHJ, Preez HH, Du (1995) Effect of copper on blood coagulation of *Oreochromis mossambicus* (Cichlidae). Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 111:359–367. [https://doi.org/10.1016/0742-8413\(95\)00062-3](https://doi.org/10.1016/0742-8413(95)00062-3)
- Ott P, Hope MJ, Verkleij AJ, Roelofs B, Brodbeck U, Van Deenen LL (1981) Effect of dimyristyl phosphatidyl choline on intact erythrocyte; release of spectrin-free vesicle without ATP depletion. Biochem Biophys Acta 641:79–87
- Padrilah SN, Ahmad SA, Yasid NA, Sabullah MK, Daud HM, Khalid A, Shukor MY (2017) Toxic effects of copper on liver and cholinesterase of *Clarias gariepinus*. Environ Sci Pollut Res Int. <https://doi.org/10.1007/s11356-017-9923-3>
- Privitera G, Meli G (2016) An unusual cause of anemia in cirrhosis: spur cell anemia, a case report with review of literature. Gastroenterol Hepatol Bed Bench 9:335–339
- Sabullah M, Ahmad S, Hussain J, Gansau A, Sulaiman M (2014a) Acute effect of copper on *Puntius javanicus* survival and a current opinion for future biomarker development. J Environ Bioremediat Toxicol 2:28–32
- Sabullah M, Ahmad S, Shukor M, Syed M, Shamaan N (2014b) The evaluation of *Periophtalmodon schlosseri* as a source of acetylcholinesterase for the detection of insecticides. Bull Environ Sci Manag 1:20–24
- Sabullah M, Ahmad S, Sulaiman M, Shukor M, Syed M, Shamaan N (2014c) The development of an inhibitive assay for heavy metals using the acetylcholinesterase from *Periophtalmodon schlosseri*. J Environ Bioremediat Toxicol 1:20–24
- Sabullah MK, Shukor MY, Sulaiman MR, Shamaan NA, Syed MA, Khalid A, Ahmad SA (2014d) The effect of copper on the ultrastructure of *Puntius javanicus* hepatocyte. Aust J Basic Appl Sci 8:245–251
- Sabullah MK, Sulaiman MR, Shukor MYA, Syed MA, Shamaan NA, Khalid A, Ahmad SA (2014e) The assessment of cholinesterase from the liver of *Puntius javanicus* as detection of metal ions. Sci World J. <https://doi.org/10.1155/2014/571094>
- Sabullah MK, Ahmad SA, Shukor MY, Shamaan NA, Khalid A, Gansau AJ, Dahalan FA, Sulaiman MR (2015a) Acetylcholinesterase from *Puntius javanicus* for the detection of carbamates and organophosphates. J Chem Pharm Sci 8:348–353
- Sabullah MK, Shukor MYA, Shamaan NA, Khalid A, Gansau AJ, Sulaiman MR, Jirangon H, Ahmad SA (2015b) Purification and anticholinesterase sensitivity of cholinesterase extracted from liver tissue of *Puntius Javanicus*. ResearchGate. <https://doi.org/10.17957/IJAB/15.0012>
- Sabullah MK, Sulaiman MR, Shukor MYA, Shamaan NA, Khalid A, Ahmad SA (2015c) In vitro and in vivo effects of *Puntius javanicus* cholinesterase by copper. Fresenius Environ Bull 24:4615–4621
- Sabullah MK, Abd. Shukor MY, Shamaan NA, Khalid A, Gansau AJ, Sulaiman MR, Jirangon H, Ahmad SA (2015d) Purification and anticholinesterase sensitivity of cholinesterase extracted from liver tissue of *Puntius javanicus*. Int J Agric Biol 17:1025–1030. <https://doi.org/10.17957/IJAB/15.0012>
- Sandrini JZ, Rola RC, Lopes FM et al (2013) Effects of glyphosate on cholinesterase activity of the mussel *Perna perna* and the fish *Danio rerio* and *Jenynsia multidentata*: in vitro studies. Aquat Toxicol 130–131:171–173. <https://doi.org/10.1016/j.aquatox.2013.01.006>
- Talesa V, Grauso M, Principato GB, Giovannini E, Norton SJ, Rosi G (1994) Presence of soluble tetrameric (blood) and membrane-bound dimeric forms of cholinesterase in the mollusk *Murex brandaris* (Gastropoda: Neogastropoda). J Exp Zool 270:233–244. <https://doi.org/10.1002/jez.1402700302>
- Van Der Ent A, Edraki M (2016) Environmental geochemistry of the abandoned Mamut Copper Mine (Sabah) Malaysia. Environ Geochem Health. <https://doi.org/10.1007/s10653-016-9892-3>
- Wong SK, Zhang X-H, Woo NYS (2012) *Vibrio alginolyticus* thermolabile hemolysin (TLH) induces apoptosis, membrane vesiculation and necrosis in sea bream erythrocytes. Aquaculture 330–333:29–36. <https://doi.org/10.1016/j.aquaculture.2011.12.012>
- Zhou Q, Zhang J, Fu J, Shi J, Jiang G (2008) Biomonitoring: an appealing tool for assessment of metal pollution in the aquatic ecosystem. Anal Chim Acta 606:135–150. <https://doi.org/10.1016/j.aca.2007.11.018>