Profiling of Cholinesterase Extracted from The Brain Tissue of *Diodon hystrix* and Its Inhibition Reaction Towards Carbamates

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ABSTRACT

Diodon hystrix, commonly known as spot-fin porcupine fish is a salt-water fish belonging to the *Diodontidae* family. It is widely distributed in Sabah wet market due to its commercial value. This study exploits the effectiveness of Cholinesterase (ChE) obtained from the brain tissue of *D. hystrix* in detecting carbamates inhibitory activities. Carbamate pesticides known to inhibit ChE and toxic towards living organisms can contaminate the water bodies. By using diethylaminoethanol (DEAE) Sepharose ion exchange chromatography, a total of 40% recovery yield of ChE was obtained with a 165.77 purification fold. Furthermore, the ChE showed a high affinity towards acetylthiocholine iodide (ATC) with an optimum activity at pH 7.45 and temperature ranging from 20 to 40°C. Among five different types of carbamates, methomyl was found to have the highest percentage of inhibition analyzed using ChE inhibitory assay, followed by carbofuran, bendiocarb, carbaryl and propoxur with >85% inhibition rate. The results concluded that ChEs extracted from the *brain* tissue of *D. hystrix* are applicable to be used as a bioindicator in detecting the presence of carbamates.

INTRODUCTION

Almost all of today's applications require water such as in agriculture, industrial, animal husbandry, tourism, fishery and even simple individual tasks. However, unethical use of water resources is becoming worse

and this threatens the quality of water which will slowly and surely get more polluted with time (Gonzalez et al., 2009). The pollution of water bodies from direct and indirect use of natural or artificial compounds, such as carbamates can be toxic to the human body due to its ability to inhibit cholinesterase enzyme (ChE) (Hayat et al., 2015). In order to curb this issue, water monitoring and rehabilitation are needed. A fast, cost-effective and reliable way of contamination screening before using high-performance technology such as High Pressure Liquid Chromatography (HPLC) can help ease this process.

ChE is an enzyme responsible for lysing choline base esters which serve as neurotransmitters found in animals. This enzyme can be subdivided into acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and propionyl cholinesterase (PChE) with PChE being closely related to BChE (Ahmad et al., 2016). The most apparent difference between AChE and BChE is their ligand-binding specificity due to the difference in the number of aromatic residues in their gorge (Rosenberry et al., 2017). Nonetheless, all three sub-types of ChE are susceptible towards carbamate inhibitory activities which disable them from hydrolyzing choline-based esters.

Carbamate pesticides are widely used to control the development of pests through inhibition of cholinesterase activities, with high acute toxicity similar to the organophosphates (OP). Carbamates and OP differ in their mode of action during the formation of the ChE-inhibitor complex. OP are irreversible inhibitors where after being phosphorylated in ChE, cannot be reversed. Carbamates, on the other hand, are temporary or reversible inhibitors where decarbamylation via spontaneous hydrolysis would shorten the inhibition time (Darvesh et al., 2008). When AChE is inhibited in the body, acetylcholine will accumulate in synapses which can cause severe central nervous system disorder (Berman et al., 2017).

The bioaccumulation of toxicants in marine organisms causes severe effects to the fish, handlers and also humankind (Almroth, 2008). Multiple past studies had proven the sensitivity of fish towards toxicants (Monserrat et al., 2002; Sturm et al., 2000), making them a suitable biomarker. This study used *D. hystrix* mostly due to its availability and increasing commercial value in Sabah. It can be found at prices as low as RM 5 per kilogram but their commercial value in Sabah has been increasing in recent years, mostly as a delicacy for both tourists and locals. Other than that, *D. hystrix* interesting characteristics such its ability to host multiple endo and ectoparasites (Quilichini et al., 2010) with high survival endurance were also considered during the selection process. Observing the inhibition of ChE by pesticide *in vitro* helps to bypass detoxification defenses and bioactivation of compounds in non-target tissues (Assis et al., 2012). ChE based early-warning biomarker that was thoroughly researched and recently applied for pollution assessment showed tissue-specific variations in AChE responses towards inhibitors (Hayat et al., 2015). This can also be true for different species of fish. Hence, this study focused on the profiling of

ChE extracted from the brain tissue of *D. hystrix* where AChE was concentrated to test its ability to detect carbamate inhibitory activities.

MATERIALS AND METHODS

Chemicals

The materials used in the study include Acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC), propionyl thiocholine iodide (PTC), 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), diethylaminoethanol-Sepharose (DEAE-Sepharose), Bradford solution and bovine serum albumin (BSA) were obtained from Sigma-Aldrich, Germany.

Preparation of Crude Homogenate

A total of three *D. hystrix* was obtained from the Kota Kinabalu Wet Market. Each fish was approximately 500-800g in weight with 30-40 cm in length. After being killed, the brain was extracted and weighed. The crude brain sample was homogenized in 0.1 M sodium phosphate buffer pH 7.5 along with 2 mM phenylmethylsulfonyl fluoride (PMSF) with the ratio of brain and buffer; 1:4 (w/v). The homogenate was centrifuged at 10, 000 rpm (11200 $\times g$) for 13 minutes at 4°C. The supernatant was stored in a clean microcentrifuge tube at -20°C after separating it from the pallet prior to further analysis (Ahmad et al., 2016).

Partial Purification (Ion Exchange Chromatography)

DEAE-Sepharose was loaded into a disposable desalting column with a 0.9 cm diameter and was allowed to settle giving a 3 cm bed height. The column was then washed with a five-batch volume of washing buffer (20 mM sodium phosphate buffer, pH 7.5), cleaning the matrix and calibrating the flow rate. A total of 300 µL of crude supernatant was loaded into the column before running another three-batch volumes of washing buffer into the column. Next, five batch volumes of elution buffer (20 mM sodium phosphate buffer containing increasing NaCl concentration from 0.2 M to 1 M) was loaded into the column while simultaneously collecting 1 mL fractions until the end of the elution stage (Acikara, 2013). According to Ellman et al. (1961) and Bradford et al. (1976), enzyme activity and protein content determination are applied on all of the fractions collected with fractions showing high ChE activity combined (Sabullah et al., 2015).

Enzyme Activity Determination

The activity of ChE was determined using a 96 microplate well assay according to the method of Ellman et al. (1961) with a slight modification. This study used acetylthiocholine iodide (ATC) as a substrate for both pH and temperature profiling. A total of 20 µL DTNB (0.067 mM), 200 µL of 0.1 M sodium phosphate buffer at pH 7.45 and 10 µL extracted ChE

were loaded into the microplate wells and incubated at room temperature for 15 minutes. After that, 20 µL of 0.5 mM ATC was added, followed with another 10 minutes incubation at room temperature. The changes in colour of solution from colourless to yellow indicates a reaction. The enzyme activity was determined by calculating the quantity of substrate (µmol) hydrolyzed by ChE per minute and per protein (U or $\mu mol min^{-1} mg^{-1}$) with 13.6 $mM^{-1} cm^{-1}$ as extinction coefficient. Specific enzyme activity was indicated as $\mu mole\ min^{-1} mg^{-1}$ of protein or Umg^{-1} of protein. The assays were performed in the dark with each test done in triplicates.

Substrate Specificity

Acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC) and propionyl thiocholine iodide (PTC) were the three synthetic substrates used in determining ChE specificity. The concentration of each substrate was set from 0.5 to 2.5 mM. The assay reaction mixture was incubated for 10 minutes at room temperature before the absorbance reading at 405nm was taken (Sabullah et al., 2014). Biomolecular constant (K_m) and maximum velocity (V_m) was determined using Michaelis-Menten curves; plotted using GraphPad Prism Software version 5.

pH and Temperature Profiling

The overlapping buffering system was applied to test *D. hystrix* optimum pH. The buffers were comprised of acetate buffer (0.1 M; pH 3.0, 4.0 and 5.0), sodium phosphate buffer (0.1 M; pH 5.0, 6.0, 7.5 and 8.0) and Tris-HCl buffer (0.1 M; pH 7.5, 8.0 and 9.0). With reference to Ahmad et al. (2016), optimum temperature was deduced by incubating the reaction mixture at a selected temperature of 20, 25, 30, 40, 50 and 60 °C.

ChE Inhibitory Assay

The inhibitory effect of five types of carbamates, namely methomyl, carbofuran, bendiocarb, carbaryl and propoxur was tested by incubating them with D. hystrix ChE. Carbamates were selected due to its widespread use in agriculture to control pests and it could be toxic when they were discharged into the water bodies without proper treatment. The test mixture comprised 150 µL sodium phosphate buffer (0.1 M, pH 7.5), 50 µL of carbamates with 10 ppm final concentration, 20 µL DTNB (0.067 mM) and 10 µL of extracted ChE. For control, carbamates were replaced with sodium phosphate buffer. The mixture was incubated for 15 minutes at room temperature before the addition of 20 µL of substrate. It was then incubated again for 10 minutes at room temperature. Reading of absorbance was taken at 405 nm following 10 minutes incubation (Sabullah et al., 2015). ChE inhibition rates for each pesticide were then calculated using Equation 1.

$$Inhibition(\%) = \frac{Uc - Ui}{Uc}(100)$$

Equation 1: Carbamates inhibition rate towards ChE (%)

Uc = Control enzyme activity

Ui = Enzyme activity with carbamates inhibition

RESULTS AND DISCUSSIONS

Cholinesterase (ChE) Purification Profile

Figure 1 shows the purification profile of ChE by comparing the enzyme activity and the protein fractions obtained from ion exchange chromatography. A total of 37 fractions were obtained, each with a total volume of 1 mL. Fraction 14 displayed the highest ChE activity followed by fraction 12 and 13, while the highest protein concentration was observed in fraction 13. The low protein concentration with high enzyme activity on fraction 14 suggests that it has the highest amount of ChE. Fractions 12 to 16 were selected and combined for enzyme profiling due to their high enzyme activity and protein concentration. Table 1 shows the ChE specific activity and final yield of protein for each stage in the sample purification process from crude homogenate until the sample had gone through ion exchange chromatography.

Following the principle of anion-exchange gel chromatography of DEAE-Sepharose, it is understood that the positive charge matrix will exhibit ionic interaction towards a negative charge sample that passes through the column. Due to proteins having different
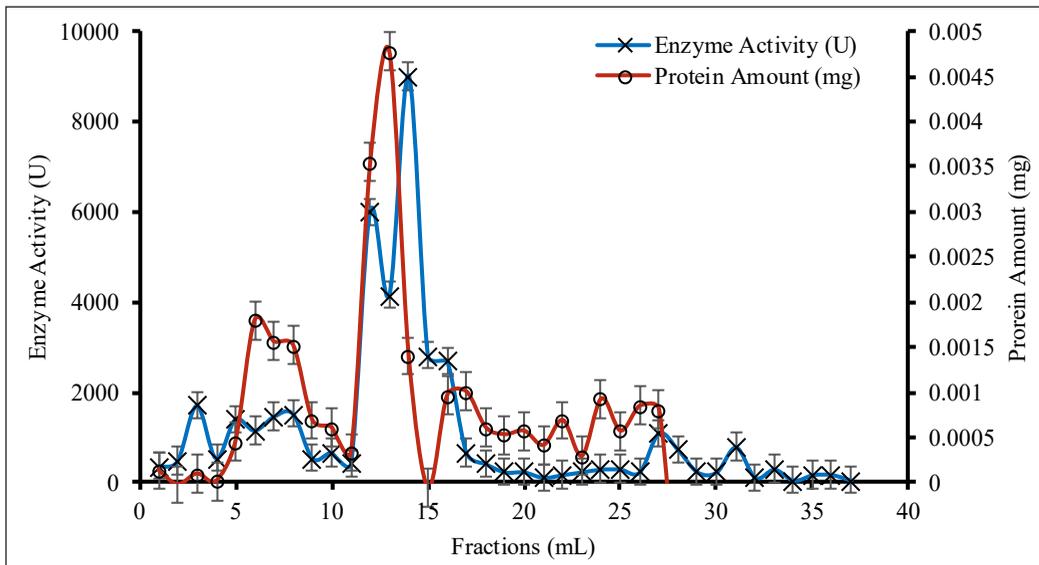
Figure 1. DEAE-Sepharose ion exchange chromatography profile on ChE purification from brain tissue of *D. hystrix* with error bars represented by mean ± standard error (n=3)

Table 1

ChE purification table of D. hystrix. *Specific activity displayed in(U mg^{-1}) indicates μmole/min/mg protein*

Sample	Total protein (mg)	Total ChE activity (U)	Specific activity (Umg^{-1})	Purification fold	Yield (%)
Crude homogenate	7.54	32720.59	4339.60	1.00	100
Supernatant (centrifuged;11200g, 13 minutes, 4°C)	3.66	32169.12	8789.38	2.03	98.31
DEAE Sepharose sample extract	0.009	13112.75	1456972.68	165.77	40.07

total charge and ionic strength, separation of other proteins from the targeted ChE can be achieved. It is important to know the isoelectric point of the targeted enzyme in order to make sure the ChE is negatively charged. Thus, the sample solution needs to have pH higher than the isoelectric point of ChE, which is approximately ±5.35 (Bon and Rieger, 1975). Different concentrations of NaCl helps in eluting ChE by displacement of bound proteins through ions in the buffer competing for the binding site where further increase in ionic strength displaces proteins based on their charge strength (Acikara, 2013). Figure 1 shows the abundance of protein amounts in fraction 13, although low enzyme activity was observed opposite to that of fraction 14. This displayed the principle of ion-exchange chromatography very clearly. This study can be further proven in Table 1, which shows the drastic decrease in protein amount due to foreign proteins being filtered from ChE at different NaCl concentrations. Purification is important in enzyme profiling which helps in increasing the ChE reactivity towards targeted toxicants. Further tests in the future using Native polyacrylamide gel electrophoresis (Native-PAGE) would better support the results obtained from ion-exchange chromatography (Hayat et al., 2015).

Substrate Specificity Profiling

Figure 2 shows hydrolyzation of three synthetic substrates (ATC, BTC, PTC) each with increasing concentration from 1 mM to 5 mM obeying Michaelis-Menten plot. All three substrates displayed similar increases in hydrolytic activity along with the increase in concentration. However, it can be observed that ChE activity starts to reach plateau nearing the 5 mM concentration. There is a clear significant difference to which substrate ChE from the D. hystrix brain reacts to. The curve indicated that ATC had the highest ChE activity, followed by PTC and lastly BTC. Following further analysis in Table 2, ATC displayed the lowest K_m value, thus having the highest affinity towards ChE from *D. hystrix* brain compared to BTC and PTC. Although PTC indicated a high V_{max} value, its catalytic efficiencies (V_{max}/K_m) still fall short in comparison to ATC. Both data indicated that ATC is the most favored substrate of ChE from *D. hystrix* brain, followed by PTC and BTC. This also suggests that the ChE obtained in the brain was mostly AChE.
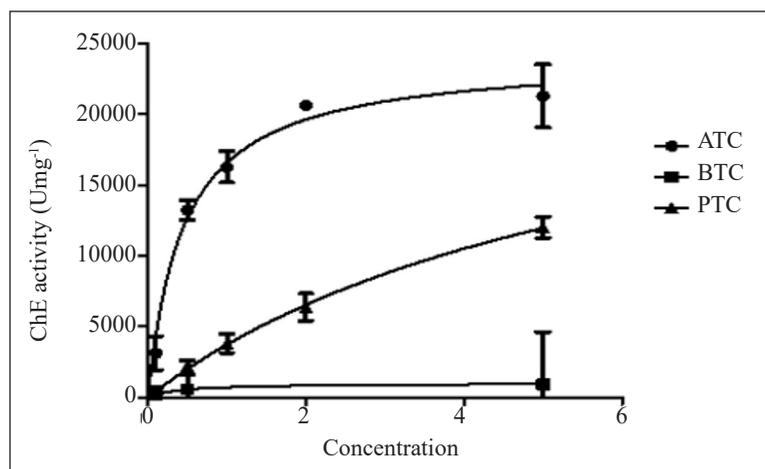
Figure 2. ChE profile on substrate specificity using three synthetic substrates (ATC, BTC, PTC). Error bar represented by mean ± standard error (n=3).

Table 2
Table of comparison of ATC, BTC and PTC on their maximum velocity (V_{max}), biomolecular constant (K_m) and catalytic efficiencies (V_{max}/K_m) with ChE from the brain tissue of D. hystrix

	Mean point (95% confidence intervals)		
Substrate	ATC	BTC	PTC
V_{max} (µmol/min/mg)	981.10	1547.00	3633.00
K_m (mM)	0.07	2.33	1.33
Catalytic efficiencies (V_{max}/K_m)	14015.71	663.95	2731.58

To further discuss, in the study done by Sabullah et al., (2014) on *P. javanicus* liver tissue, ChE was found to be specific towards BTC with (V_{max}/K_m) higher than ATC and PTC. However, a study on *M. albus* brain tissue by Khalidi et al. (2019) found that ATC had the highest (V_{max}/K_m), similar to the result obtained in this study. These results proved that different parts of organs and different species of fish incorporated ChE with different substrate specificity of either ATC, BTC or PTC.

Profiling of Optimum pH and Temperature

Figure 3 shows the pH profile of ChE in overlapping buffer system of acetate buffer, sodium phosphate buffer and Tris-HCl buffer. The highest ChE activity was indicated at pH ±(7.45-7.50) for both sodium phosphate buffer and Tris-HCl buffer with no significant difference between the two values. Similarly, both buffers showed a decline in enzyme activity, starting from pH 8.0 to 9.0. The pattern in Figure 3 indicates that ChE from *D. hystrix* is optimum and can be regulated in both sodium phosphate buffer and Tris-HCl buffer at pH 7.45 to 7.50. However, due to Tris-HCl high sensitivity towards temperature (AppliChem, 2008), sodium phosphate buffer is more suitable to be used for enzyme profiling. These

attributes are also dependent on the pK_a value of the buffer, which determines the range in which the buffer is considered adequate. Sodium phosphate buffer thus fits perfectly to optimize ChE from *D.hystrix* brain tissue with its pK_a value falls in the range of 5.8 to 8.0.

In Figure 4, ChE enzyme activity with different temperatures was displayed. Optimum temperature was at 40°C where a steady increase in enzyme activity could be observed from 20 to 40°C but no significant difference was observed (p>0.05). Similar to other enzymes, kinetic energy increases as temperature increases, thus resulting in higher enzyme activity. However, a sharp decline of enzyme activity was shown at higher temperature presumably due to denaturation of the biological shape of ChE resulting in a bell-shaped curve. The ChE starts to denature when the temperature is higher than its optimum temperature (Ahmad et al., 2016) which in this study was at temperature >40°C.
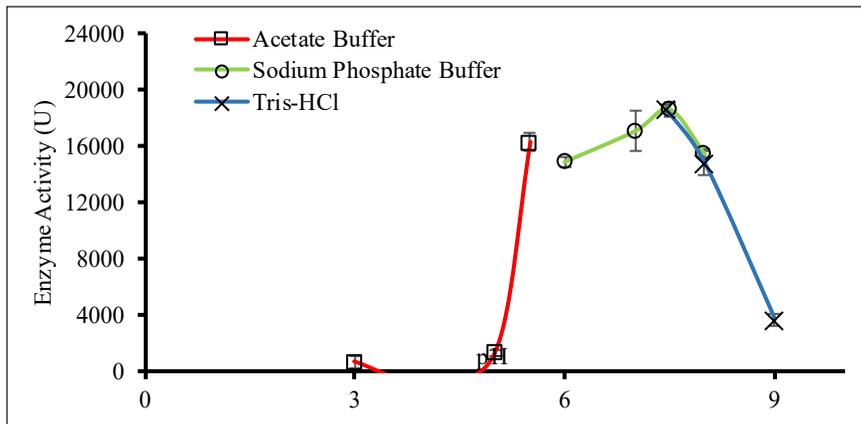
Figure 3. pH profile of ChE from *D. hystrix* brain tissues through overlapping buffer system of three different buffers (acetate buffer, sodium phosphate buffer and Tris-HCl buffer) with error bar represented by mean ± standard deviation (n=3)
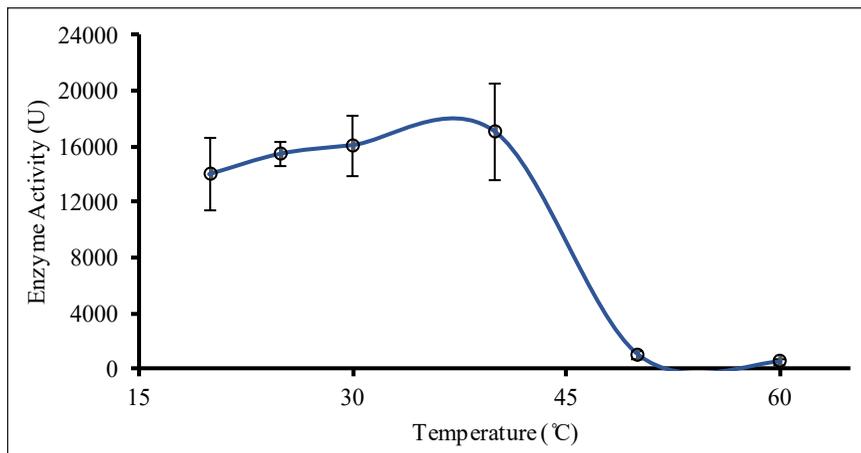
Figure 4. Temperature profile of ChE from *D. hystrix* brain tissues with error bar represented by mean ± standard deviation (n=3)

Similar to its substrate specificity, ChE extracted from different sources may or may not exhibit similar optimum pH and temperature. In this case, two studies by Ahmad et al. (2016) that evaluated the brain tissue of *Tor tambroides*[b] and liver tissue of *Anabas testudineus*[a] showcased that AChE optimum temperature was in the range of 25 to 35℃ for *T. tambroides*, falling into the same temperature range in this study. *A. testudineus* AChE showed an optimum temperature of 40℃, similar to the result obtained in this study despite using different organs and species, thus further solidifying the point made.

ChE Inhibitory Assay

Figure 5 shows the reactivity of ChE enzyme from *D. hystrix* brain tissue towards the inhibitory effect of bendiocarb, propoxur, methomyl, carbofuran and carbaryl at 10 ppm concentration. All of the carbamates elicit inhibitory activities higher than 85% with highest in methomyl at 98%. The strength of carbamate inhibitory activities are as follows; methomyl>carbofuran>bendiocarb>carbaryl>propoxur. According to Sabullah et al. (2015), in comparison to organophosphate, which can also inhibit ChE, carbamates bind to AChE esoteric sites without the need of any bioconversion. This study implied that carbamates-based pesticide could bind to AChE more easily than organophosphate. All of the tested carbamates are applied as a broad-spectrum insecticide that could kill more than one species of insects. In concurrent to their applications, methomyl and carbofuran are mainly used in fields and crops whereas bendiocarb, carbaryl and propoxur were also found in household pest control. It was stated by Gupta et al. (2011) that methomyl and carbofuran were highly dangerous towards any living system and this statement was further supported by Dorko et al. (2011) indicating that insecticides applied as household pest control had lower toxicity towards vertebrates. A study carried out by Mahboob et al. (2014) on *Labeo rohita* found
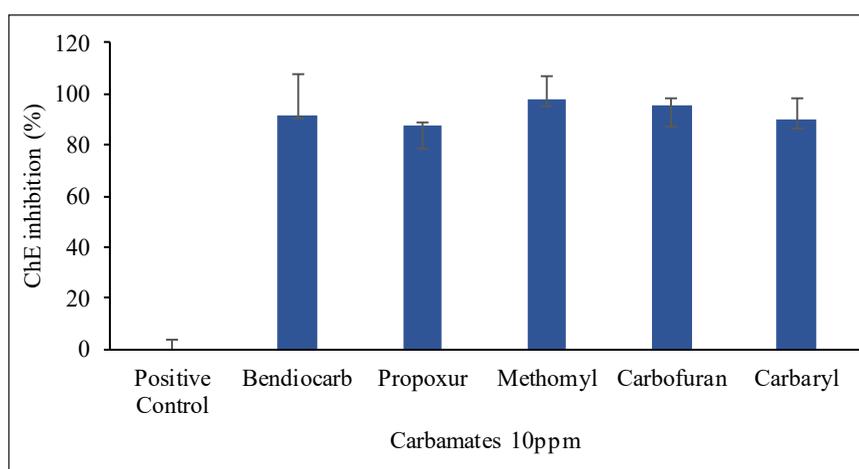
Figure 5. Inhibition test profile of five different types of carbamates at 10ppm concentration on ChE from *D. hystrix* brain tissues with error bar represented by mean ± standard deviation (n=3)

that carbofuran exposure disrupted the functions of organs that included the brain, gills, muscle, kidney, liver and even non-organs such as blood. These multiple findings thus solidified the result and present a logical explanation to why methomyl and carbofuran have the highest inhibitory activities in comparison to the other carbamates.

CONCLUSION

The extraction of cholinesterase (ChE) enzyme from the brain tissues of *D. hystrix* was achieved using DEAE-Sepharose ion-exchange chromatography. Through substrate specificity test, it was observed that the partially purified ChE enzyme was proned towards ATC with lowest K_m value (0.07011 mM) and highest catalytic efficiency value of 13993.72 ($V_{max}K_m^{-1}$). The optimum pH was in the range of pH ±7.45 and 7.5 with an optimum temperature at 20 to 40°C. ChE inhibitory assay showed all pesticides were able to inhibit ChE of *D. hystrix* with methomyl having the highest inhibiting ability at 98%, while propoxur with the lowest inhibition rate. This study proved that the extraction and partial purification of ChE from the brain tissues of *D. hystrix* were efficient and were able to show high reactivity towards carbamates inhibitory activities without applying higher cost consuming methods such as the HPLC. This also implies that ChE from *D. hystrix* brain tissues can be applied as a biomarker tool due to its ability in detecting carbamates inhibitory activities with the change in color following the Ellman assay principles. Further analysis regarding this enzyme on the wider variety of toxicants can help determine the efficacy of it as a biomarker.

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