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SEM of *Theobroma cacao*

Partial Purification and Characterization of the Acetylcholinesterase from *Poecilia reticulata* (Peters, 1859)

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ABSTRACT

Poecilia reticulata (P. reticulata) is a species of tropical fish in the family Poeciliidae that is found all over the world and is a favorite in freshwater aquariums. The Malay name for this fish is "Ikan Gapi," although the English term "Guppy" has become more common. The Million Fish, or Rainbow Fish, as it's known in other parts of the world. The goals of this research were to characterize the acetylcholinesterase that will be partially purified from *P. reticulata* brain extract using ammonium sulphate fractionation followed by an affinity chromatography method. The result shows that ammonium sulphate fractionation gave better purification fold over the procainamide affinity chromatographic method with fold of purification of 3.38 compared to 0.64 for the latter. Polyacrylamide gel electrophoresis (SDS-PAGE) shows a dramatic reduction of protein bands compared to crude extract. A pH of 9 in a Tris-HCl buffer system and a temperature of 15 °C were found to be ideal for the activity of the enzyme. Based on its high V_{max} and low K_m with acetylthiocholine iodide (ATC) as a substrate compared to butyryl thiocholine iodide (BTC) and propionyl thiocholine iodide (PTC), the substrate specificity profile identified acetylcholinesterase (AChE) as the major enzyme present in the purified enzyme. The molecular weights of the enzyme following affinity chromatographic purification are about 92 kDa.

INTRODUCTION

Native to northeastern South America, guppies have become widely distributed due to introductions to new environments. The caudal and dorsal fins of male guppies are for show, and they are smaller than those of females. Guppies in the wild feed on a wide range of foods, from benthic algae to aquatic insect larvae. Because of their adaptability and ability to thrive in a variety of environmental and ecological conditions, this species has been chosen as a model organism in the fields of ecology, evolution, and behavioural studies. For many people, the Guppy is one of the most well-known and popular aquarium fish. They add a lot of colour to tanks, and are peaceful, inexpensive, and simple to care for. It comes in a variety of colours. They can live for up to 2 years and range in size from 0.6 to 2.4 inches [1]. Guppies are also omnivores, meaning they eat a variety of plant and animal foods. Guppies are *Poeciliidae* family freshwater tropical fish native to South America. *Poecilia reticulata* is the scientific name for the common guppy. Guppies come in nearly 300 different varieties. They come in a variety of colours, sizes, and tail shapes. The name comes from Robert John Lechmere Guppy, who discovered them in Trinidad in 1866. An Ichthyologist took it back to the British Museum and named it *Girardinus guppii*. The fish's name has changed several times since then, including *Lebistes reticulatus,* and it is now known as *Poecilia reticulata*. They are also known as the Millions Fish because of their incredible breeding rate and The Rainbow Fish because of the wide range of colours they come in [2].

Inhibition of ChE-based assays have been utilized for different detection markers in both live organisms and the surrounding environment for quite some time now. Pesticides, industrial chemical waste, viruses, and heavy metals that are released on land may get concentrated in the bodies of marine species, making fish harmful to handlers and farmers, and ultimately to humans as the principal consumer [3]. Fish are a type of marine creature that has historically been examined and selected for use as biomarkers for toxicants and biosensors, chiefly for insecticides through the suppression of cholinesterase activity. *Drosophila melanogaster* and the electric eel *Electophorus electricus*

are two examples of fish AChE sources used in pesticide bioassay and biosensor technologies. Possible potential future fish sources that may be utilized as biomarker and biosensor agents for insecticides or any other toxicants include the guppy *Poecilia reticulata*, tiger grouper; *Epinephelus fuscoguttatus*, Javanese carp; *Puntius gonionotu*, and the grass carp; *Ctenopharyngodon Idella* all of which are native to Malaysian waters. Current studies use *Oreochromis mossambicus* [5], *Channa micropeltes* or Toman [4], and *Hemibagrus nemurus* or Baung [6]. This research reveals a new potential source of AChE by isolating it from *Poecilia reticulata* brain tissue. The purpose of this research is to partially purify and characterize AChE.

MATERIALS AND METHODS

Specimen

One hundred *P. reticulata* fish, of both sexes, were purchased in 2018 from a commercial source in Subang, Selangor, that carries the live and colorful variety of wild guppy fish. Each fish has a body weight of approximately 0.10 to 0.17 g and a length of roughly 2-3 cm. Fish supplied are in good shape and are untainted.

Preparation of crude homogenate

The first step was to kill the *P. reticulata* fish by freezing them for around 30 min. The brain of a fish is so little that it was collected by cutting off the fish's head rather than dissecting the entire animal. The heads were weighed and broken using mortar and pestle. Using a 1:4 buffer-to-sample ratio, we continually crushed the brain sample until homogeneous while adding 0.1 M sodium phosphate buffer, pH 7 and 0.01 mM phenylmethylsulfonyl fluoride (PMSF) as an antiprotease. Higher concentrations of PMSF were found to be inhibitory. The whole process was carried out at 4 ºC. After that, the homogenate was centrifuged for 20 min at 4 ºC and 10,000 ×g in a Sorvall Ultra Pro-TH-641. The supernatant was placed in a 15 ml Falcon tube and frozen at -25 C. Using the Bradford protein assay, total protein content in a sample was carried out with bovine serum albumin (BSA) as the reference protein [7].

Enzyme assay of ChE

P. reticulata ChE activity was determined using a modified version of the technique used by Ellman et al. [8]. This can be done using a 96-well microplate at the wavelength of 405 nm. The microplate wells were initially filled with 200 µL of sodium phosphate buffer (0.1 M, pH 7.0), 20 µL of DTNB (0.1 mM), and 10 µL of crude ChE, and then incubated for 15 min. After an incubation period of 10 min, 20 µL of ATC (2.5 mM) was added to the mixture. Specific activity is reported in terms of mmoles of substrate hydrolyzed per minute per milligram of protein, or units per milligram of protein per hour, with an extinction coefficient of 13.6 $mM^{-1}.cm^{-1}$. Below is a formula for determining ChE:

$$\text{Enzyme activity (U)} = \frac{\text{Absorbance /10 min}}{\varepsilon} \times (TV/TS)\ \mu l \text{ of well}$$

Where,

Δ Absorbance= Change in absorbance reading at 405 nm after 10 minutes of incubation (Final-Initial)
Ɛ = Specific extinction coefficient = 13.6 mM^{-1} cm^{-1}
TV = Total volume = 250 µL
TS = Total sample = 10 µL

Precipitation of protein by ammonium sulphate

Precipitation with ammonium sulfate is a typical technique for isolating proteins by changing their solubility in a salty environment. Before adding the specified amount of ammonium sulphate, the volume of the protein sample was determined using the nomogram or a standard table [9]. The amount of ammonium sulphate added will be different according to the volume of the sample. Solid ammonium sulphate was gradually added to the sample and stirred slowly for 15-20 minutes using a magnetic bar in a beaker of ice to preserve enzyme quality from heat produced by this reaction.

Then, the salt was removed by dialysis using a dialysis tube. The standard dialysis tubing has a molecular weight cut-off of 10 kDa. The resultant sample remains in the dialysis tube and will then further centrifuge solution at 15,000 ×g for 10 minutes at 4°C to pellet out protein. The pellet was dissolved in 0.1 M sodium phosphate buffer while supernatant will be collected and continued added with another amount of ammonium sulphate to prepare the next concentration, stirring and centrifugation also be repeated. Pellet protein from each % of saturation will then undergo protein assay and enzyme assay to determine the % of ammonium sulphate saturation with the highest enzyme activity [9].

Purification of ChE through affinity chromatography

Before loading the *P. reticulata* brain extract into the affinity column, which is a Procainamide-Sephacryl 6B column (16 mm in diameter and 50 mm in height), the column was filled with 20 mM sodium phosphate buffer at pH 7.0. Three times the volume of the matrix column's volume of crude brain extract (approximately 240 mL) was put into the matrix. The washing stage took place by loading 5 mL of wash buffer that consisted of 20 mM sodium phosphate buffer at pH 7.0 onto the column with a flow rate of 1 mL/min two times. This step was then followed by eluting buffer that comprised of 20 mM sodium phosphate buffer, pH 7.0 containing 1 M sodium chloride (NaCl) loaded into the column to elute the cholinesterase (ChE) of *P. reticulata* which was still bounded to the affinity matrix [10,11].

Enzyme parameter determination

Determination of optimum conditions for ChE activity was carried out on three parameters which are substrate specificity, pH and temperature. The reaction mixtures were prepared and tested according to the Ellman method.

Substrate specificity

P. reticulate ChE was tested for substrate specificity by incubating the enzyme with acetylthiocholine iodide (ATC), butyryl thiocholine iodide (BTC), and propionyl thiocholine iodide (PTCI) in sodium phosphate buffer (0.1 M, pH 7.0) at room temperature (PTC). We used substrate concentrations between 0.5 and 2.5 mM. After 10 minutes, a 405 nm reading was taken after the substrate was introduced to the assay reaction mixture. The maximal velocity (V_{max}) of ChE activity and biomolecular constant (K_m) were determined nonlinearly using the Michaelis-Menten curves plotted using the GraphPad Prism Software version 5.

Optimum pH

Following a previously established procedure [5] the ChE was incubated in buffers of several pH values to identify the ideal pH for its activity with an overlapping buffer system consisting of 0.1 M acetate buffer (pH 3 to 5.0), 0.1 M sodium phosphate buffer (pH 5.0 to 8), and 0.1 M Tris-HCl buffer (pH 7 to 10).

The mixture in a final volume of 250 µL contained 200 µL of tested buffer, 20 µL of 0.1 mM DTNB and 10 µL of partial purified ChE was incubated for 15 minutes at room temperature then 20 µL of 0.5 mM ATC was loaded and incubated for another 10 minutes before reading the absorbance at 405 nm. This assay was carried out at room temperature with minimal light exposure.

Optimum temperature

The effects of temperature will be carried out using the method of Bocquené *et al.,* [12]. The mixture in a final volume of 250 µL contained 200 µL of tested buffer, 20 µL of 0.1 mM DTNB and 10 µL of partially purified ChE. Then incubate in the water bath for 20 to 30 minutes at different temperatures ranging from 15, 20, 25, 30, 40 to 50 °C to cover the variety of temperatures optimal for ChE from fish. Then 20 µL of 0.5 mM ATC was loaded and incubated for another 10 minutes at room temperature before reading the absorbance. The optimal temperature for *P. reticulata* ChE was determined after that by enzyme assay. Beyond this temperature, ChE was assessed to be completely denatured.

SDS-PAGE gel preparation

SDS-PAGE was prepared according to the method of [13]. 10x Tris-glycine electrophoresis buffer (run buffer) stock solution was diluted with deionized water to 1x concentration and poured into the electrophoresis tank until it reached the required volume mark, depending on the number of gels prepared. The sample well was also filled with a run buffer so that the bubbles in the sample wells could be ruled out through the electrophoresis buffer. Before loading the sample into the well, the samples were first mixed with Coomassie Brilliant Blue dye in order to observe the mobility of the protein samples. The first well was loaded with 10 µL of low-range protein leader, followed by different types of protein samples in each well. To mix the sample with dye, 4 µL of blue dye was first pipetted on a parafilm and mixed with another 6 µL of a sample.

Statistical analysis

The following formula was used to determine the percentage of inhibition:

$$\% \text{ Inhibition} = \frac{\text{Test activity of control - test activity of sample}}{\text{Test activity of control}} \text{ x } 100\%$$

Means are shown with a standard error of the mean. Graphpad Prism version 3.0 was used for all statistical analysis. Student's t-tests or one-way ANOVAs with Tukey's post hoc test comparisons were used to determine statistical significance across groups. Statistical significance was assumed at the $P<0.05$ level.

ChE activities of crude based on specific substrates

Using acetylthiocholine (ATC), butyrylthiocholine (BTC), and propionylthiocholine (PTC) as substrates, total ChE activity was calculated to investigate activities of the enzyme in *P. reticulata* brain extract (**Fig. 1**). Among the AChEs tested, those from *P. reticulata* showed the least activity toward BTC and PTC and the greatest toward ATC. When compared to BTC and PTC, ATC showed the highest enzyme activity value, with a total ChE activity of 454.1605 U. Since acetylcholinesterase (AChE) catalyzes the hydrolysis of acetylcholine at the neuromuscular junction and central synapses, it plays an important function in controlling cholinergic neurotransmission in the fish brain [14].

In most teleost, AChE makes up to than 97% of the total brain cholinesterases [15]. Nunes [16] recorded that the major cholinesterase form in the brain tissues of *Dicentrarchus labrax* is AChE. Ding et al. also recorded that brain ChE extracted from *Oreochromis aurea* shows a specific affinity towards ATC compared to other substrates. The reason for high ChE activity in ATC may be due to its biochemical role in the brain. The next purification steps will focus more on AChE since it possesses the highest enzyme activity. Ding et al. [17] have noted that *Oreochromis aureus* brain ChE extract displays substrate specificity for ATC. The biological function of ChE in the brain may explain why it is particularly active in ATC. Because of its high enzyme activity, purifying efforts will be directed primarily against AChE in the next stage.

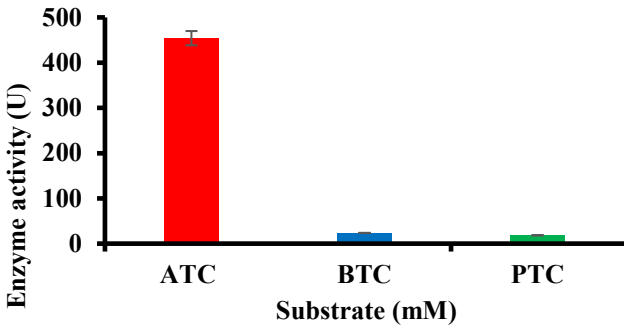

Fig. 1. *P. reticulata* brain extract total ChE activity broken down by substrates (ATC, BTC, and PTC). Mean value (with Y error bars) of triplicate assays of total ChE activities in *P. reticulata* brain extract.

Ammonium sulphate precipitation

For salting out proteins, ammonium sulphate $(NH_4)_2SO_4$ is often used salt because of its high solubility, low price and availability of pure material. Ammonium sulphate $(NH_4)_2SO_4$ is commonly used for salting out proteins due to its high solubility, inexpensive price, and readily available pure material. Only a limited number of proteins are highly soluble in water without the presence of salt, and the vast majority of them need at least a trace amount of salt to maintain their folded and stable state.

Proteins having both positively and negatively charged regions tend to aggregate in the presence of relatively small amounts of salt. Salt, on the other hand, prevents protein aggregation by neutralizing surface charges through its anions and cations. Adding additional salt to a solution raises the overall salt concentration, which in turn charges the protein surface and re-agglomerates the molecules. Proteins having both positively and negatively charged regions tend to aggregate in the presence of relatively small amounts of salt. However, the anions and cations in salt begin neutralizing charges on the protein surface, blocking the aggregation process. Adding more salt causes a higher concentration of salt, which in turn charges the protein's surface, causing the molecules to clump together further more [9]. Based on **Fig. 2**, the enzyme assay shows the highest AChE activity at 30-40% of ammonium sulphate precipitates with 207.384 U compared to other concentrations. This indicates that desired enzyme which is AChE was successfully purified with the highest value at 30-40% of ammonium sulphate.

The % with the highest AChE activity will then be used for further purification process through affinity chromatography.

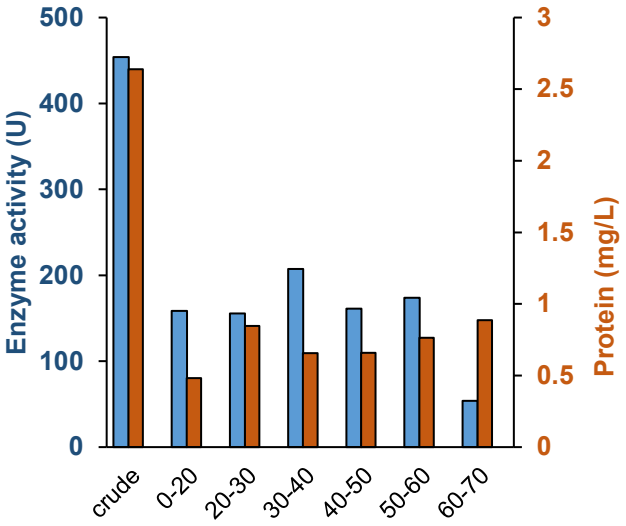

Fig. 2. Protein and enzyme activity for each ammonium sulphate fraction.

Purification of AChE by affinity chromatography

Purification of AChE process was done by affinity chromatography to isolate targeted protein from a pool of protein. The most used technique is affinity chromatography because of the unique specific binding qualities it employs. It has a specific ligand for ChE to bind, which is procainamide. The elution profile in **Fig. 3** shows AChE activity within the range of fraction 0 to 38 (1 mL per min) where the sample was incubated in ATC.

AChE was eluted after eluting buffer containing NaCl-phosphate buffer was loaded into the column. 1 M NaCl in sodium phosphate buffer was used to remove the AChE bound to the column. NaCl act to loosen the covalent interaction between AChE and the immobilised substrate in the column. From 38 fractions, AChE showed the highest enzyme activity at fraction 26. A decreased amount of protein was detected during the elution stage due to the removal of unbound or non-targeted protein during the washing stage because these non-targeted protein does not have the binding site for the covalent interaction to the column. The decreasing value in enzyme activity might be due to the effect of surrounding temperature that disrupts the stability of ChE and unavoidable loss of ChE during purification. Only fraction 26 was used for enzyme characterisation and inhibition study.

Ammonium sulphate fractionation gave the best purification method in terms of fold purification and yield while the affinity step after it, which serves as a polishing step was detrimental to the yield of the enzyme and fold purification (**Table 1**). Further studies are needed to assess the diminishing reason for this step since numerous purification experiments with this method reported good results [4,10,18–22]. The decreased purification fold during affinity chromatography may be due to improper ChE storage and handling ChE samples at low temperatures or the presence of numerous proteases.

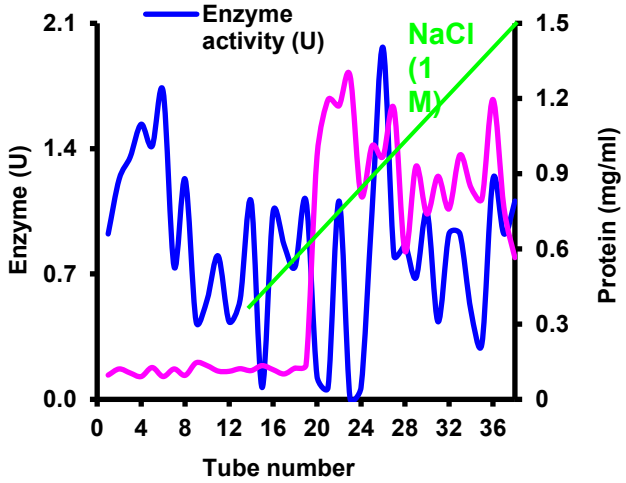

Fig. 3. Procainamide-Sephacryl 6B affinity chromatography elution profile of partly purified acetylcholinesterase (AChE) from *P. reticulata* brain extract.

Table 1. *P. reticulata* brain extract AChE purification table. Each purification process yielded a particular activity value in (U/mg), represented as mmol hydrolyzed/min/mg of protein.

Procedure	Vol. (mL)	Activity (U)	Total activity (U)	Protein content (mg/mL)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude	20	22.71	454.16	2.65	52.98	8.57	1	100
Ammonium sulphate	9.3	22.29	207.38	0.77	7.17	28.94	3.3765	45.66
Affinity	6	3.48	20.91	0.64	3.82	5.47	0.6383	4.60

Sodium Dodecyl Sulphate-Polyacrylamide gel (SDS-PAGE)

Lane P in **Fig. 4** illustrates the affinity chromatographic (Procainamide-Sephacryl 6B) purification of ChE. The level of purity of the partly purified ChE was established using SDS-PAGE. The molecular weights of the proteins may be calculated and separated using this method. Polyacrylamide gels separate smaller molecules like a protein in the same way as agarose does, but the matrix contains fewer holes. Coomassie blue was used to stain the electrophoresis.

Depending on their sequence, proteins can take on any charge at any pH. This means that the charge-to-mass ratio of proteins is very variable. The issue was fixed by adding a negative charge to the proteins through the detergent SDS. The SDS shattered the protein's secondary, tertiary, and quaternary structure, causing the subunits to become separated. Molecular weight was used as a cutoff point during SDS-PAGE separation. The molecular weights of the uncharacterized protein bands following affinity chromatographic purification are within 92 kDa, as seen in the gel profile. Three bands were observed each in Lane P, A and C. Lane P was loaded with pure brain crude of *P. reticulata* while A and C were loaded with purified ammonium sulphate precipitation and affinity chromatography sample respectively. Each protein band with an unknown molecular weight was assigned a value using the equation created from the graph of the protein marker standard curve where the log molecular weights are plotted against the retention factor (cm) in **Fig. 5**.

The protein band from partly purified AChE is shown in red circle. This size is considered within the range of numerous enzyme purified from fish. *Colossoma macropomum* chymotrypsin esterase, for instance, is 62 kDa in size [23]. However, several studies have indicated a molecular weight of 220 kDa for AChE from the fish species Piracucu (*Arapaima gigas*) [24]). For AChE from *P. reticulata* the protein band resides on the 92 kDa of low range leader. Lane A band is very unclear (**Fig. 6**) may be due to the very low % of AChE which has been successfully purified through affinity chromatography. Most fish AChE is multimeric and ranges from dimer to tetramers. More studies are needed to clearly assess the true form of the AChE whether truly monomer or tetramer.

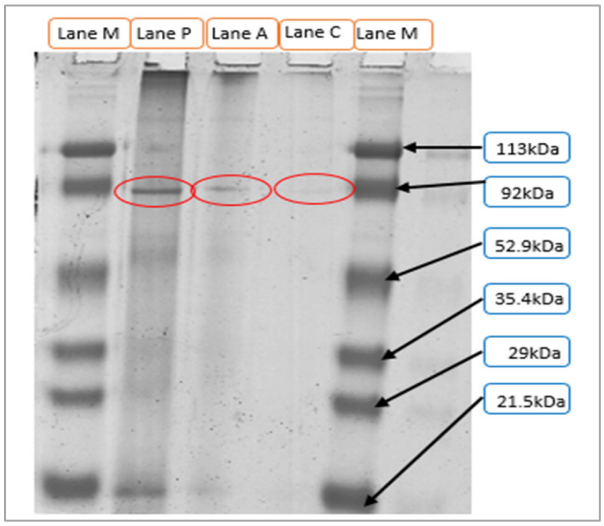

Fig. 4. SDS-PAGE protein profile using brain extract from *P. reticulata.*

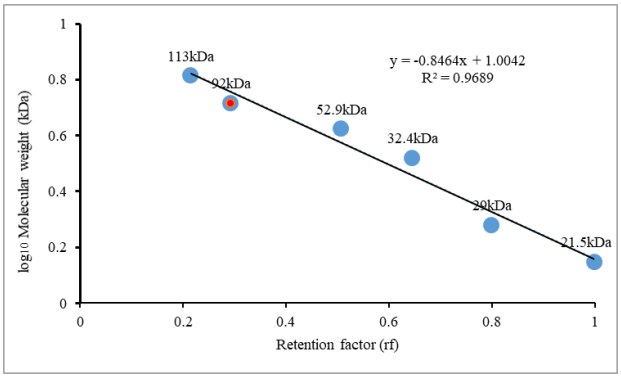

Fig. 5. Protein marker retention factor (rf) interpolation yields the molecular weight of isolated *P. reticulata* ChE. Dotted red represents purified ChE.

Optimisation of assay conditions for AChE

Substrate specificity profile

The substrate specificity assay was conducted using three different synthetic substrates: ATC, BTC and PTC. The concentrations of substrate vary starting from 0 mM to 2.5 mM. **Fig. 6** shows that all three synthetic substrates were hydrolysed by ChE. Generally, the increase in substrate concentration will increase with enzyme activity. When compared to BTC and PTC, ATC's K_m value is the lowest. These results actually indicated that ChE has a higher affinity towards ATC. In addition, ChE exhibited the capability of hydrolysing ATC at a higher rate compared to BTC and PTC. This can be clearly seen by analysing its V_{max} value (**Table 2**). ATC was shown to have the greatest ratio of catalytic efficiency in a series of calculations, thus proving that a partial purified enzyme sample collected from brain extract of *P. reticulata* predominantly contains AChE. This observation is in agreement with the data obtained in several studies [4,22,25–32]. The result also showed that the ChE in brain extract prefers to hydrolyse ATC, followed by PTC and BTC. AChE from the muscle of *P. reticulata* shows strong substrate inhibition at concentrations of substrates higher than 5 mM [33].

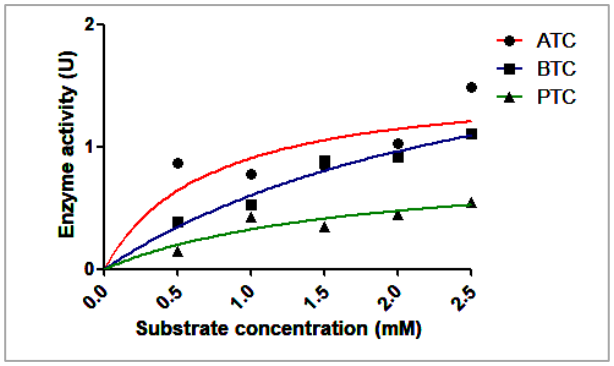

Fig. 6. The selectivity of the AChE from *P. reticulata* brain extract was investigated by incubating the enzyme with three synthetic substrates at doses ranging from 0 mM to 2.5 mM. Means and standard deviations (n=3) are displayed for all data.

Table 2. The differences between the substrates ATC, BTC, and PTC of ChE from the brains of *P. reticulata* in terms of maximum velocity (V_{max}) and the Michaelis constant (K_m).

Substrates	V_{max}, Specific activity (U/mg)	K_m (mM)	Catalytic efficiencies (V_{max}/K_m)
ATC	1.546	0.7035	2.197584
BTC	2.358	2.898	0.813665
PTC	0.892	1.753	0.508842

Effect of pH on AChE activity

The optimal pH for AChE activity was estimated using three buffers of varying pH values. Tris-HCl buffer (pH 8.0), phosphate buffer (pH 5.5), and acetate buffer (pH 3.5) (7 to 10). The pH profile shows a very low enzyme activity at pH range from 3 to 6, a slight increase at pH 7 and a drop again at pH 8 and an increase in enzyme activity from pH 9 to optimum pH 10 (**Fig. 7**). However, according to Ellman, at pH above 9 ATC starts to hydrolyse resulting the increased in enzyme activity. In addition, Tris does not have a buffering power at pH >9.1.

The ionization state of acidic and basic amino acids in the protein, for instance, can be modified by the pH of the protein. The side chains of acidic amino acids include carboxyl groups, while the side chains of basic amino acids have amine groups. Amino acids' ionization states determine the ionic bonding that ultimately form proteins in three dimensions. This can change the enzyme's ability to recognize proteins, rendering it inactive. Alterations to the pH can also alter the charge characteristics of the substrate, rendering it inactive or preventing it from binding to the active site.

In general, the optimum pH for each enzyme varies. From observation, it can be seen that the AChE possessed low enzyme activity at low pH ranging from 3 to 5.5. The explanation behind this is that a high concentration of protons will naturally interrupt the interaction between substrate towards AChE due to the protonation of an imidazole group of histidine at the catalytic triad of the enzyme. The change in histidine conformation may affect

AChE mechanism. Inversely, the phenomenon of imidazole group deprotonation will occur at catalytic sites with high pH such as from 6 to 8. This process could lead to the effective binding between substrate and enzyme hence increasing enzyme activity [20]. It was determined that pH 7.0 is the most optimum for AChE activity. The enzyme-substrate complexes became more stable once the imidazole group in AChE was neutralized.

AChE may hydrolyze its substrate very well in this environment. After pH 7, there was a noticeable drop in activity. Probably, the ionization constant of the ring nitrogen in the imidazole group of the catalytic triad provided the basis for this observation, as this is the pH-dependent intermediate in the hydrolytic process [24]. The findings were consistent with those of a research by Zhukovskii et al. [34] where the freshwater fish *Abramis ballerus*' ChE activity was highest at a pH of 9. To better characterize AChE, Tris-HCl buffer was used as the buffering system. Toman (*Channa micropeltes*) AChE was shown to have the maximum activity on ATC at 0.1 M, and this finding may be compared to that study's findings [4]. Therefore, AChE activity was studied further at the optimal pH of 9 in Tris-HCl buffer.

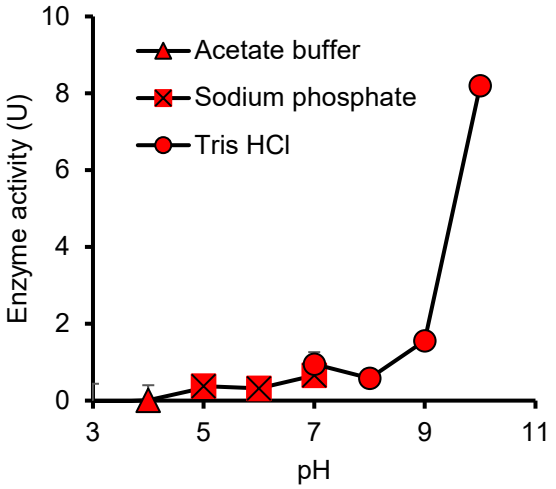

Fig. 7. pH profile on the activity of the partially purified AChE from brain extract of *P. reticulata*. Error bars represent mean ± standard deviation (n=3).

Effect of temperature on AChE activity

AChE optimum temperature was determined to be in the range of 15 to 50 °C. **Fig. 8** shows a fluctuating pattern in the temperature profile. Normally the higher the temperature, the kinetic energy will increase thus increasing the rate of collision between AChE molecules and ATC substrates. A high rate of collision results in the high formation of enzyme-substrate complexes and thus increase the enzyme activity and the number of products. However, the optimum temperature of AChE from the brain extract of *P. reticulata* is at 15 °C. AChE activity decreases as the temperature increases. This shows that AChE from *P. reticulata* is very sensitive to high temperatures and needs proper handling and storage to maintain its quality. Although the optimal temperature is lower compared to AChE of other fish species like *Rachycentron canadum* [24] AChE from different species possess different characteristics thus giving different optimal temperatures.

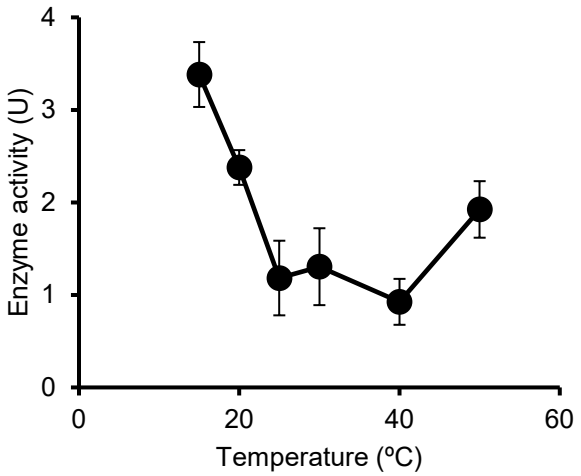

Fig. 8. Temperature profile on the activity of partially purified AChE from brain extract of *P. reticulata*. Error bars represent mean ± standard deviation (n=3).

CONCLUSION

Crude extract of ChE from the brain of *P. reticulata* was partially purified using procainamide-Sephacryl 6B. Acetylthiocholine iodide (ATC) was selected as the specific synthetic substrate for this assay with the maximal velocity and lowest biomolecular constant at 1.546 U/mg and 0.7035 mM respectively, with the highest catalytic efficiency ratio of 2.1976. The optimum substrate concentration was at 2.5 mM ATC, pH at 9.0 and the optimum temperature was at 15 ºC. Based on substrate specificity, the brain tissues of *P. reticulata* predominantly contain AChE with the highest catalytic efficiency when tested with ATC as the substrate according to the kinetic study. Further characterization of the enzyme can also be improved by further purification to obtain a homogenous enzyme suitable to be used in sequencing studies.

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