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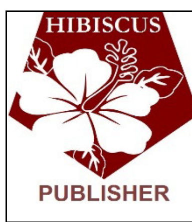

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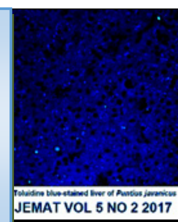

Partial Purification of Cholinesterase from *Pangasius pangasius* using Affinity Chromatography

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ABSTRACT

An affinity chromatography column to purify AChE was developed using procainamide as the ligand coupled with the epoxy activated Sephacryl-S1000 as the modified matrix. From this study, the procainamide matrix was found to be capable of binding acetylcholinesterase (AChE) from the brain of *Pangasius pangasius* efficiently, with percentage of yield of approximately 51.97% and a purification fold of 7.52. The value for Michaelis-Menten ($K_{m\ app}$) was 0.1265 mM and maximal velocity ($V_{max\ app}$) of the partially purified enzyme was 0.6981 µmol/min/mg, with a substrate specificity in the sequence of— acetylthiocholine iodide (ATC) > propinylcholine iodide (PTC) > butyrylcholine iodide (BTC). This strongly suggest that using this column, AChE can be purified successfully and efficiently. SDS-PAGE indicated a reduced band intensity, indicating that the procainamide-based affinity chromatography column produces acceptable partially purified enzyme adequate for the application in the rapid detection of carbamate and organophosphate (OP) at the large scale.

INTRODUCTION

As the global environmental health deteriorate, enforcement agency around the world are looking for ways that can rapidly detect pollutants, ranging from heavy metals, pesticides and other xenobiotics predominantly carbamates and organophosphate (OP) which are primarily associated with heavy usage in agriculture practices. Cholinesterase, especially AChE have been deployed as an environmental pollution biosensor. However, various problems exist which include low levels of AChE sensitivity and a high cost.

This paper describes the attempt to provide a cheap and sensitive source of AChE that can satisfy the above conditions. A fish (*Pangasius pangasius*) brain cholinesterase was chosen. This is a native fish in Malaysia and found scattered all over South East Asia. Recently, fish cholinesterase has been widely used as bioindicators in the detection of organophosphate and carbamate pesticides [1–11]. Cholinesterase is divided into the major groups; acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and propionylcholinesterase (PChE). All have different sensitivities towards carbamate and OP. These enzymes can be distinguished by their substrate specificity [12]. Acetylcholine hydrolysis rate by AChE is much faster compared to other choline esters, but AChE activity can be inactivated in the presence of butyrylcholine. BChE on the other hand can hydrolyzed both acetylcholine and butyrylcholine in a much appreciable rate.

Among the various chromatography techniques, affinity chromatography is the most preferred method. This is because, a typical affinity chromatography contains features such as high resolution, capacity and speed enabling it to trap the intended enzyme through a simultaneous processes of isolation,

concentration and stabilization achieving the final high level of enzyme purity. Affinity is more effective than other column chromatography methods because it uses the specific binding properties of protein and the sample volume is not a limiting factor provided the maintenance of correct binding conditions is observed during sample application. Furthermore, the total amount of loaded protein onto the column can be very high.

MATERIALS AND METHODS

Chemicals

Bovine serum albumin (BSA), Acetylthiocholine iodide (ATC), Propionylthiocholine chloride (PTC), β-mercaptoethanol and Procainamide hydrochloride ($C_{13}H_{21}N_{3}O$.HCl) were purchased from Sigma-Aldrich. 5, 5'-dithio-bis (2-nitrobenzoic acid) (DTNB) and Butyrylthiocholine iodide (BTC) was purchased from Fluka Chemie GmbH. N,N,N',N'–tetramethylethylethylenediamine (TEMED) and Acrylamide were the sources of AMRESCO®. Ammonium persulfate and Biorad Protein Assay from Bio-Rad Laboratories Inc. PageRuler™ Protein Ladder from FERMENTAS. Sodium dodecyl sulfate (SDS) from Applichem GmbH and Vivaspin4 from Vivascience. All other chemicals used in this study were of analytical or special grade.

Specimen

Pangasius pangasius was used as the fresh water test organisms. The fish weigh between 800 to 1000 g and length ≈ 30 cm were harvested from the hatchery at Universiti Putra Malaysia, Serdang, Selangor Darul Ehsan. This organism was chosen because it is abundantly available, and studies related to AChE originated from fish is still less especially related to pesticides compared to another organism. Fish in healthy and disease-free condition were chosen for the experiment. *P. pangasius* was breed under laboratory condition for two days for adaptation purposes. *P. pangasius* was acclimatized in aerated aquaria filled with chlorinated-free tap water at 25ºC. Commercial fish pelleted food was used as food to the fish prior to the experimentation. Fish were let to starve a day before the experiment begins to reduce metabolite changes due to dietary influences. To maintain the cleanliness and eliminate any possible interfering product from respiration and excretion, aquaria water was changed daily.

Preparation of brain AChE extracts from *Pangasius pangasius.*

P. pangasius brain was used to extract Acetylcholinesterase. The fish were decapitated, and the brain part was taken out immediately. Approximately, one gram of this part was homogenized in 20% (w/v) of 0.1 M sodium phosphate buffer pH 8.0 using an Ultra-Turrax T25 homogenizer fitted with a Teflon pestle. Phenylmethylsulfonyl fluoride was used to inactivate and remove unwanted serine proteases. The crude extract was obtained from homogenized brain suspension. The crude extract was centrifuged at 15 000×g for 10 minutes at 4ºC to removed debris. The precipitated pellet was then undergone ultracentrifugation at 100,000×g in a Sorval® Ultra Pro 80-TH-641 for one hour at 4ºC to separate the cytosol and membrane components. The supernatant was collected and used in the purification procedures. All procedures were performed at 4°C unless otherwise stated.

Customization of Column

Epoxy (Bisoxirane) activation

Sephacryl S-1000 (100ml settled gel) was washed in a sintered glass tunnel with a 1 L water, sucked dry to a wet cake, and was transferred to a 500-ml beaker. The gel was suspended in 75 ml 0.6M NaOH containing 150mg sodium borohydride and stirred with a paddle. 75 ml 1, 4-butanediol diglycidyl ether was added slowly and stirred constantly. The reaction mixture was stirred overnight at room temperature. Excess reagent was removed by washing the activated gel thoroughly with water. This washing step was continued until there was no longer oily film on the surface of the gel. Acetone was used to aid in the complete removal of bisoxirane groups. The gel was resuspended in water for ligand coupling [1,6].

Ligand coupling of procaine mide–Sephacryl S-1000 gel

The epoxy-activated Sephacryl S-1000 was washed with deionized distilled water on a sintered glass filter. The slurred gel was then transferred onto a coupling solution of 12 mM borate buffer, pH 11 containing 0.2 M procainamide. The pH of the slurry gel was adjusted to 12 by adding 1.0 M NaOH. The mixture was incubated at 25°C for 96 hours on a shaking incubator.

The gel was washed in sequence manner with 10 volumes each of 0.1 M sodium acetate, pH 4.5, 12 mM sodium borate, pH 10 and deionized water. The excess active groups on the gel were blocked by suspending the gel in 100 ml 1.0 M ethanolamine, pH 9.0 and were stirred at room temperature for 6 h. This step was meant to block all the remaining active groups on the amine-reactive supports. Finally, the gel was washed thoroughly with 1.0 M NaCl followed by deionized water [3].

Isolation and partial purification of Cholinesterase

Affinity chromatography was performed using procainamide, a ligand specific for the choline-binding site. The matrix was packed in the column with a bed height of 3 cm. The flow rate was maintained at 0.2 $mLmin^{-1}$. First, the matrix was washed with 5 batch volumes of 20 mM sodium phosphate buffer, pH 7.5 (washing buffer) to clean and equilibrate the column.

Secondly, the crude extract was loaded onto the affinity matrix. Thirdly, three batch volumes of washing buffer were used to wash the column matrix. One mL fraction was collected in separate Eppendorf tubes and kept on ice. Washing step continued until all of the non-absorbed proteins were washed out. Collected protein fractions were assayed for enzyme activity and protein content. Fractions with AChE activity were pooled.

Concentration and dialysis

The extracted AChE was concentrated and dialyzed with 3 batch volumes of washing buffer using Viva Spin tubes at 2500 rpm at 4°C. The dialyzed AChE was stored at -20°C until subsequent use.

Protein content

The Bradford's method was used to quantify the proteins amount with bovine serum albumin (BSA) as standard. The assay was performed using a microplate reader at 600 nm [13].

Activity and effect of substrates

AChE activity was determined using the method described by Ellman [14] with slightly modification for a 96 well microplate assay. This method uses acetylthiocholine iodide (ATC) as a synthetic substrate for AChE. ATC is broken down by AChE to produce thiocholine and acetate. Thiocholine is reacted with 5, 5'-dithio-bis-2-nitrobenzoate (DTNB) to produce a yellow colour. The formation of a yellow colour over time used to quantify the activity of AChE. Absorbance was measured using

a microplate reader. AChE activity is expressed as the amount of Acetylthiocholine iodide (μmol) which is broken down by AChE per minute. The specific activity is given as μmole hydrolyzed min^{-1} mg^{-1} of protein or U mg^{-1} of protein and was calculated on the basis of an extinction coefficient of 13.6 mM^{-1} m^{-1}. This experiment was carried out to determine the substrate specificity of the AChE. Enzyme kinetics analysis was used to determine substrate specificity. Through the Michaelis-Menten equation, the efficiency of the enzyme catalysis was analyzed.

Three substrates named as ATC, butyrylthiocholine iodide (BTC) and propionyl thiocholine iodide (PTC) were used. Concentrated Procainamide-Sephacryl S1000 affinity eluents were used to determine the AChE $K_{m(app)}$ and $V_{max(app)}$ values for *P. pangasius* at six different concentrations ranging from 0 to 2.50 mM for each substrate. For the control, AChE was substituted with the buffer to correct for nonenzymatic hydrolysis of each substrate. The experiment was conducted in triplicates. The $K_{m(app)}$ was determined by analysis of GraphPad PRISM 4 non-linear regression analysis for four-parameter logistic equation software available from www.graphpad.com.

Denaturing electrophoresis (SDS-PAGE) analysis of crude and partial purified AChE

SDS-PAGE was carried out in a 3-10% acrylamide gradient gel. Samples were prepared with the following steps: the protein, the solution was added deionized water, 0.06 M Tris-Cl, pH 6.8, glycerol and 0.0025% (w/v) bromophenol blue. SDS and β-mercaptoethanol were incorporated in the reducing sample buffer. The mixture was mixed and heated in a hot water bath at 90°C for 5 minutes and then cooled. The sample and stock sample buffer were in a ratio of 2:1. Seven μL of protein marker and 50 μL of this mixture were then loaded onto the polyacrylamide gradient gel. Both gels were run at 10 mA for the first 15 minutes, then at 20 mA for the next 90 minutes at room temperature. The gels were stained by using Coomassie Brilliant Blue R-250 Dye overnight. Destaining was carried out the next day for 30 minutes before it was stored and photographed.

RESULTS AND DISCUSSION

Fig. 1 shows the enzyme activity profile and protein content from the procainamide-based affinity chromatography. In this elution profile, fractions 1 to 6 were collected during the washing phase while fraction 7 to 12 was collected during the elution phase. A protein peak with no enzyme activity was detected during the washing phase. In the elution phase, both enzyme activity and protein were detected as represented by the two-overlapping peak. Soluble AChE was partially purified from *P. pangasius*.

A summary of the partial purification of AChE is shown in **Table 1**. The crude AChE from the brain of *P. pangasius* had a specific activity of 1.01 μmol $min^{-1}mg$ $protein^{-1}$. The supernatant obtained after ultracentrifugation had about 80.23% of the total AChE activity. When the supernatant was applied into the Procainamide–Sephacryl S-1000 affinity column, AChE was adsorbed by this gel. The bound protein was eluted from the column with high ionic strength phosphate buffer (20 mM sodium phosphate buffer pH 7.4, containing 1.0 M NaCl) at a flow rate of 0.2 $mLmin^{-1}$.

Sodium chloride (1 M) was used to change the ionic strength and lowered the energy bond between AChE and the procainamide ligand, leading to the desorption of AChE from the matrix. Most of the non-specifically bound proteins were removed in 20 mM sodium phosphate pH 7.4 washing buffer as can be seen from a typical purification profile of high enzyme activity and low protein content (**Fig. 1**). Later on, two 1.0 ml fractions (fraction 8 and 9) were collected and pooled (**Fig. 1**). Pooled fraction was concentrated and desalted by ultrafiltration using a polyethersulfone membrane filter with a molecular weight cut-off (MWCO) point of 5 kDa in a 4 mL ultrafiltration cell (Vivaspin 4). Ultrafiltration spin column was used to concentrate and dialyse the sample after partial purification to concentrate the sample to the desired level and to remove the initial salt content used to elute the AChE. Three wash cycles employed to remove the salt content.

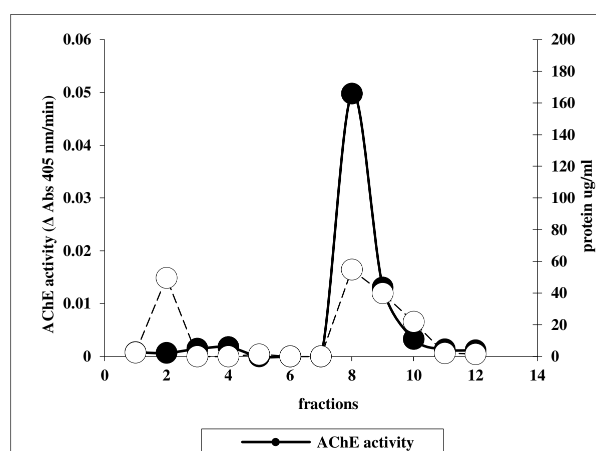

Fig. 1. Elution profile of partially purified AChE from P. pangasius on custom synthesized Procainamide–Sephacryl S-1000 chromatography column. Sample with a specific activity of 2.05 Umg^{-1} was loaded into a 5 mL column (1.6 x 2.5 cm) and was washed with 20 mM sodium phosphate buffer, pH 7.5 at 4°C. The elution was commenced with the same buffer containing 1 M NaCl. The elution was run and collected using a fraction collector at a flow rate of ≈ 0.2 $mLmin^{-1}$ per tube under the influence of gravity and assayed for AChE activity and protein as described in the text.

We observed that the AChE was successfully partially purified and concentrated. After this purification procedure, the partially purified enzymes contained 57.64 mg protein, with specific AChE activity of 7.6 μmol min^{-1} mg $protein^{-1}$. The purification fold is in tandem with the custom-made procainamide-based affinity gel used to bind BuChE (a sister enzyme) in [15] that harvested a 10-fold purification. These results were reproducible over several purifications similar to what was observed in other reports [1–11,16,17].

Most of the AChE activity was retained with a recovery of 80.23%, after centrifugation at 100,000× g for 1 hour at 4°C. After going through the procainamide-Sephacryl S1000 affinity column, 51.97% of enzyme activity was recovered with a 7.52 purification fold obtained.

Table 1. Purification table of partially purified AChE from *P. pangasius.*

Procedure	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (Umg^{-1})	Purification fold (X)	Yield (%)
Homogenate	4.8	843.53	834.07	1.01	1.00	100.00
Ultracentrifugation at 30,000 rpm for 1 hour	3.6	676.76	330.02	2.05	2.03	80.23
Affinity – Procainamide Sephacryl S1000	3.5	438.36	57.64	7.60	7.52	51.97

The specific activity of AChE expressed as µmole hydrolyzed min^{-1} mg^{-1} of protein or U mg^{-1} of protein and calculated based on the extinction coefficient of 13.6 mM^{-1} m^{-1}.

The temperature range from 15ºC to 70ºC was chosen to test the AChE activity. **Fig. 2** shows the temperature profile of AChE activity, where AChE achieved its maximum activity at 25°C. Based on the profile, AChE shows high stability at room temperature. The enzyme is retarded but not denatured in low temperature, resulting in low activity.

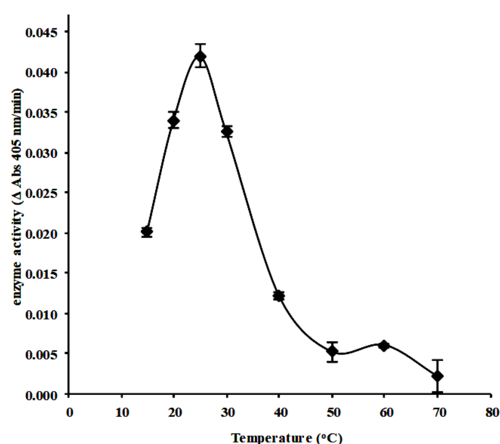

Fig. 2. The effect of temperature on the AChE activity from *P. pangasius.* Error bars are mean ± standard deviation of triplicates.

The optimum temperature for this AChE skews from the general optimal rates of mammalian AChE which is reported to be around 37 to 40°C. It was reported, however, that the optimal temperature for insects or any other organisms might be lower [18]. Since fish are poikilotherms, it is possible that warmer temperatures will denature and inactivate AChE. Acetylcholinesterase will undergo conformational changes caused by the temperature increment. This is due to the energy that can disrupt the non-covalent bond in AChE. At 70°C and above, the enzyme activity is negligible. Since the temperature for the reaction mixture has a dramatic effect on the kinetic parameters of an enzyme-catalyzed reaction, it is critical to carefully control the temperature during measurements of enzyme activity [19].

Table 2 shows the Michaelis-Menten constant ($K_{m\ app}$) and maximal velocity ($V_{max\ app}$) of the enzyme in hydrolyzing three different substrates, which were ATC, BTC and PTC. Comparison between these three substrates gave K_m value at 0.1265, 0.1325 and 0.4979 mM for ATC, PTC and BTC, respectively. ATC substrate recorded the lowest K_m values among those three substrates, indicating that the affinity of partially purified AChE is higher for ATC compared to BTC and PTC substrate.

The enzyme also displayed higher rates of hydrolysis when incubated with ATC but showed far less activity in the presence of BTC and PTC, based on the V_{max} values. Based on the table of $K_{m\ app}$ and $V_{max\ app}$, ATC gave the highest $V_{max\ app}$ and the lowest $K_{m\ app}$. Hence, the partially purified enzyme has the highest affinity towards ATC. Again, this result has supported the earlier assumption that the enzyme that had been partially purified was AChE.

Table 2. Comparison of $K_{m\ app}$ and $V_{max\ app}$ for ATC, BTC and PTC.

Substrates	ATC	BTC	PTC
$V_{max\ app}$ (µmol min^{-1} mg^{-1})	0.6981	0.1139	0.2746
$K_{m\ app}$ (mM)	0.1265	0.4979	0.1325
$V_{max\ app}/K_{m\ app}$ ($mg^{-1}m^{-1}$)	5.52 x 10^{-3}	0.23 x 10^{-3}	2.07 x 10^{-3}

The AChE enzyme was successfully partially purified from the brain of *P. pangasius* using Procainamide-Sephacryl S-1000 based affinity chromatography. The enzyme purified was an AChE based on substrate specificity kinetic studies, which shows that the enzyme has the highest $V_{max\ app}$ values compared to PTC and BTC as shown in **Table 2**. The rate of hydrolysis corresponds with the several previous reports [20,21]. The $V_{max\ app}$ value of ATC obtained is comparable with that obtained from different parts of the brain, which have a $V_{max\ app}$ value ranging from 0.196-0.923 µmol min^{-1} mg^{-1} [20].

The low Michaelis-Menten constant ($K_{m\ app}$) values obtained indicate ATC has high affinity compared with PTC and BTC towards extracted enzyme, indicating that the extracted enzyme is a true AChE. This $K_{m\ app}$ value of ATC is similar to that obtained from freshwater fish such as *Pseudorasbora parva*, *Carassius auratus auratus*, *Oncorrhychus mykiss* as reported by Shaonan et al. [22] and *Cnesterdon decemmaculatus* [23] with values of 0.111 mM, 0.112 mM, 0.114 mM and 0.17 mM, respectively. Catalytic efficiency calculated from the $V_{max\ app}/K_{m\ app}$ ratio gave significant differences between ATC, PTC and BTC. The catalytic efficiency value is even higher for ATC reported for *Cyprinus carpio* and *Cnesterodon decammaculatus* [23]. This underlines the efficiency of *P. pangasius* AChE.

The result of the SDS-PAGE shows large amounts of foreign proteins had been removed through affinity chromatography using Procainamide-Sephacryl S-1000 affinity column. This can be observed in lane 1 and lane 2 where there was an obvious difference between the numbers of bands (**Fig. 3**). Several minor bands were detected on the gel for the partially purified AChE samples. Band A indicates a probability for the location of the AChE based on previous studies [4-11]. The presence of these faint bands might indicate the presence of trace amounts of non-AChE proteins that were co-purified by the procainamide-based affinity column. The molecular weight of AChE obtained from this study cannot be determined from this electrophoresis result because the enzyme was only partially purified and further purification should be performed to determine the molecular weight of this AChE.

Several minor faint bands were detected on the gel for the partially purified AChE samples that were treated with reducing agents. The presence of these faint bands might indicate the presence of trace amounts of non-AChE proteins that were co-purified by the procainamide-based affinity column. The molecular weight of AChE obtained from this study cannot be determined from this electrophoresis result because the enzyme was only partially purified and further purification should be performed to determine the molecular weight of this AChE. The results support the effectiveness of the procainamide-Sephacryl S-1000 affinity column to remove a large amount of contaminating protein.

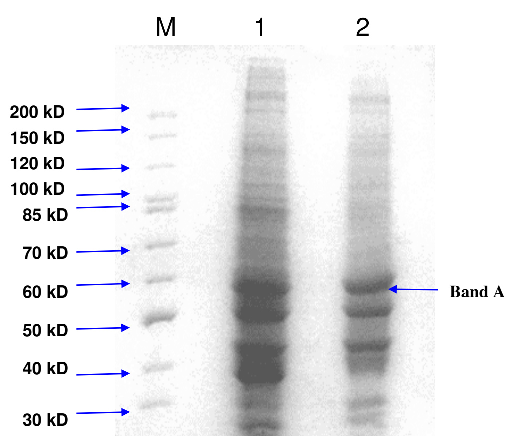

Fig. 3. Electrophoresis analysis of the crude and partially purified AChE from the brains of *P. pangasius* in a 3-10% polyacrylamide gel. Protein bands were stained using Coomassie Brilliant Blue R-250 dye. Lane M, molecular weight markers; Lane 1, crude AChE treated with reducing agents SDS and mercaptoethanol; Lane 2, partially purified AChE treated with reducing agents SDS and mercaptoethanol.

CONCLUSION

The procainamide-Sephacryl S-1000 affinity column employed in this study was able to partially purify the AChE from *P. pangasius* brain sample with high activity and fold purification. The SDS-PAGE analysis also showed the reduction of foreign protein after partial purification step.

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