Synthetisation of an affinity matrix (Procainamide Sepharose Cl-6b) for brain cholinesterase purification and separation source from *Monopterus albus*

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**Abstract.** Affinity chromatography for acetylcholinesterase; AChE namely Procainamide Sepharose CL-6B was synthesised through the coupling method between soluble procainamide hydrochloride immobilised by a cross-linked agarose size exclusion, sepharose CL-6B. 1,4-butanediol diglycidyl ether plays a role in building up a productive and rigid connecting of a biospecific ligand (Procainamide hydrochloride) to an insoluble matrix (Sephacryl CL-6B). Local freshwater eel brain was extracted and centrifuge at high speed. The supernatant was collected, and seven different volumes were separately loaded on to the column for isocratic purification where 12 fractions were collected at the end of elution stage. This study shows 1000 μL of extractant; considered as the maximum volume to load onto the column. Lastly, a stepwise elution was performed with five different concentrations of NaCl, and each of 1 mL fraction was collected then assay for determination of enzyme activity and protein content. The data shows AChE was successfully purified with percentage recovery of 38 % after 21 purification fold. Kinetic study strengthens the data where the efficient coefficient ratio of ATCi was much higher compared to PTCi and BTCi at 3.03, 2.67 and 1.52 $V_{max} \cdot K_m^{-1}$, respectively, prove that the collected fraction predominantly contained with AChE, which is a targeted enzyme to be used as a sensitive biosensor to detect the presence of carbamate and organophosphate contamination in the environment.

1. Introduction

Cholinesterase-based biosensors, either acetylcholinesterase; AChE or butyrylcholinesterase; BChE, is widely used as a rapid with low-cost preliminary screening tools of inhibitors, such as heavy metals and insecticides especially organophosphates and carbamates [1]. The sources of ChE depend on the organ for the example AChE, most abundant in brain tissue with a small amount of BChE, while the liver contains an abundant of BChE followed by AChE. Both ChE from the same family but different characteristics [2]. AChE plays a role to hydrolyse neurotransmitter acetylcholine at the synaptic cleft between neurons. At the same time, the small amount BChE in the brain is speculated as a backup role for the insufficient amount or absent of AChE [3,4]. Conversely, BChE activity in the liver is related to xenobiotic detoxification but slightly efficient to metabolise acetylcholine [5]. However, the sensitivity
of cholinesterase depends on the level of purity and the target of the substrate. The elimination of non-
targeted protein, protease, and other types of the contaminant is crucial to determine the success of ChE
puration. Moreover, ChE, such as AChE and BChE, are from the same group of esterase but different
in substrate specificity and inhibitor selectivity \[6\]. Sabullah et al., \[1\] and Hayat et al., \[2\] has proved
the application of procainamide-sepharyl 6B affinity chromatography coupled and DEAE-cellulose ion
exchange chromatography, respectively, to purify ChE from different types of organ. Thus, a
comparison purification efficiencies method is needed to be implemented to ensure the quality of
puration and separation. This study was performed to produce a purification matrix by synthesising
procainamide-based affinity chromatography and AChE from the brain extract of Monopterus albus
was purified using the affinity matrix; isocratic and stepwise purification, followed by a kinetic study using
three different synthetic substrates.

2. Methodology

2.1. Synthesisation of procainamide – sepharose CL-6b

An affinity matrix; Procainamide-Sepharose CL-6B, was synthesised through coupling method between
soluble biospecific ligand known as procainamide hydrochloride; Sigma-Aldrich brand, immobilised by
a cross-linked agarose size exclusion, sepharose CL-6B; Sigma-Aldrich brand. Epoxy activation was
performed using 1,4-butanediol diglycidyl ether; Sigma-Aldrich brand, to change the matrix chemically
between the products of a procedure and a ligand of preference by the response of covalent bond
formation. Next, a purification column with 0.9 cm in diameter and 10 cm in height syringe was packed
with the matrix and enable settling to acquire a bed height of 5 cm.

2.2. Purification and separation process

The brain tissue of local swamp eel, Monopterus albus (obtained from the wet market) was dissected
out, weight and homogenize using ultraturax T25 homogenizer in 0.1 M phosphate buffer, pH 7.5 at the
ratio of 1:4 [w/v]. After 10 minutes of high-speed centrifugation; 10,000 x g, the supernatant was
collected, and 100 μL was loaded on to the column. Affinity purification was carried out where three
batch volume of washing buffer; 25 mM, pH 7.5 phosphate buffer, was load followed by the collection
of 1 mL of fraction until the end of elution stage (Three batch volume of an eluting buffer; 25 mM, pH
7.5 phosphate buffer containing 1 M of NaCl). The flow rate was calibrated at 0.3 ml/ min. The
purification processes were repeated by changing the loading volume to 200, 400, 600, 800, 1000 and
2000 μL of supernatant. Next, a stepwise elution was performed using five batch volume of an eluting
buffer with different concentrations of NaCl; 0.2, 0.4, 0.6, 0.8 and 1.0 M of NaCl. Each collected
fractions were tested for ChE activity and protein content determination using a modified method of
Ellman \[7\] and Bradford \[8\], respectively.

2.3 Cholinesterase activity and protein assay

A 96 well microplate was used, and the Ellman assay was conducted by each well contained with the
mixture of 0.1 M phosphate buffer pH 7.5 (200 μL), purified AChE (10 μL), and 0.1 mM 5,5' dithiobis
(2-nitrobenzoic acid) or DTNB; Sigma-Aldrich brand (20 μL). After 15 minutes' incubation, 20 μL of
1.0 mM of acetylthiocholine iodide (ATCi; Sigma-Aldrich brand) was added followed by 10 minutes
incubation. The production of yellowish colour was read at a wavelength of 405 nm. The assay was
repeated by replacing the synthetic substrate ATCi with the same concentration of butyrylthiocholine
iodide (BTCi; Sigma-Aldrich brand), and propionylthiocholine iodide (PTCi; Sigma-Aldrich brand).
Molar absorption coefficients for the reduced DTNB at 13,600 M⁻¹.cm⁻¹. Bradford assay was performed
by determining the absorbance of each 20 μL of bovine serum albumin; Merck brand, protein standard
and fractions at the wavelength of 595 nm after 10 minutes incubation in 200 μL of Bradford reagent;
Bio-rad brand.

2.4. Synthetic substrate study
A kinetic study was conducted by incubation of purified AChE with three different synthetics; ATC, BTC, and PTC, with concentration ranging from 0.5 to 2.5 mM. Maximal velocity; Vmax and biomolecular constant; Km, were calculated using Lineweaver-Burk plots. Efficiency coefficient of each synthetic substrate was calculated based on the ratio of Vmax: Km.

3. Result and Discussion

AChE is an enzyme that could be used as a sensitive biosensor to detect the presence of contaminants such as carbamate insecticides and organophosphate in very low concentration [1,6]. Thus, the fish brain was selected as the enzyme is abundantly concentrated in this organ. However, there is no doubt the existence of pseudocholinesterase such as BChE and propionylcholinesterase that were probably contaminating the extraction. In this situation, purification is not enough to obtaining AChE from the brain extract. Combination with separation technique is crucial in order to enhance the purification process by isolating the enzyme that shares a similar characteristic with other types of cholinesterase.

In this study, the different volume of fish brain extract was loaded on to the column containing Procainamide-Sepharose CL-6B matrix. Then, by applying gravity force, the flow rate was controlled at 0.3 mL/min. The result shows two peaks were obtained from different stages where the first six fractions most of the protein was washed out using 25 mM phosphate buffer, pH 7.5 which known as washing stage, while targeted protein was eluted during the elution stage; fraction 7 to 12. Higher volume of sample associated with the higher protein concentration loaded onto column may cause overloading and increase multiple protein contamination in each collected fraction. Conversely, small volume loading significantly lowering the protein content as well as the targeted protein became too diluted after being purified. Figure 1 shows the most preferred loading capacity was at the volume of 1000 μL (~20 mg) of crude cholinesterase extract which this volume will maximize the elution of ChE and minimized the overloading of the sample.

![Figure 1. Isocratic purification profile of Monopterus albus brain ChE using Procainamide-Sepharose CL-6B as an affinity matrix of the column.](image_url)

Cholinesterase has a high affinity to covalently bind with procainamide. During the elution stage, fraction 9, 10 and 11 contained with all types of cholinesterase but at different concentrations. Thus, the separation technique is needed to maximize the isolation of the target enzyme, which is AChE instead of BChE. Usually, the method for purifying a protein requires a combination of methods. For the example, an extractant was priory purified using ion-exchange chromatography followed by size
exclusion chromatography, or vice versa [9]. Unfortunately, a combination of these expensive methods that take too long to the process may result in errors due to environmental factors such as temperature, unintended contaminant exposure or human error. Besides, the purified sample becomes too diluted, which may be affecting the activity of the enzyme.

Procainamide-based chromatography is the best method which efficiently isolating ChE from thousand proteins in an extract. However, the determination of the best-coupled system between biospecific ligand to an insoluble matrix to ensure the efficiency of purification and separation of two or three similar families of protein with distinct characteristic [10]. Combination of insoluble sepharose CL-6B with soluble procainamide aid by a spacer or arm that hold both compounds namely 1,4-butaneol diglycidyl ether. Other than that, sepharose CL-6B is not only working as a procainamide immobilizer. The resin is made for size exclusion chromatography with a broad fractionation range. Absolutely, this coupling strategy able to isolate cholinesterase by removing the massive amount of unwanted protein, followed by separation of any cholinesterase isoform based on biomolecular size. Thus, Procainamide-Sepharose CL-6B meets the required criteria compared to other types of chromatography. Three distinctive peaks related with cholinesterase isoform were obtained while the targeted fraction containing high activity of AChE; fraction 40 to 44; the third peak, was purified at a high yield around 38% after 21 purification fold (figure 2).

Figure 2. Brain extract was purified using Procainamide-Sepharose CL-6B as an affinity matrix. During the elution stage, stepwise elution (salt concentration from 0.2, 0.4, 0.6, 0.8, and 1.0 M) was performed, and fraction number 40 to 44, which were proved as acetylcholinesterase based on synthetic substrate test (blue box), were collected.

Purified ChE was incubated in different concentrations of three synthetic substrates. Based on catalytic efficiencies ratio (Vmax/Km), ATC shows the highest value followed by PTC and BTC at 3.03, 2.67 and 1.52, respectively (Figure 3). As preliminary screening, this study proves the collected fraction predominantly containing AChE, which this enzyme highly efficient to hydrolyse ATC. Alternatively, the types of cholinesterase could be determined by incubation with their specific inhibitor such as tetra(monoisopropyl)pyrophosphoramide (Iso-OMPA) specifically inhibit AChE, while ethopropazine are selective inhibitor for BChE. Usually, AChE was selected as a biosensor candidate as this enzyme are very sensitive to sense the presence of a nerve agent such as carbamates and organophosphate in very low concentration [11]. Conversely, BChE is sensitive towards metal ions as mentioned by Sabullah et al., [12] using BChE purified from the liver of Puntius javanicus. This study also could be applied to purify AChE or BChE from any tissue such gill [13], liver [12], kidney [14], muscle [15] and blood [16].
4. Conclusion

Loading capacity of M. albus brain extract was optimized at the volume of 1000 mL to enhance the purification of AChE with minimal overloading. Stepwise purification method successfully separates three distinctive peaks proved the present of cholinesterase isoform. Based on the kinetic study, the third peak proved AChE is the most abundant enzyme in the brain extract and could be used as a source of biosensor tool for environmental biomonitoring programme.

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