UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF AN INHIBITIVE ACETYLCHOLINESTERASE-BASED ASSAY FOR INSECTICIDES

THAM LIK GIN

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By

THAM LIK GIN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Dedicated to my parents and brother.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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January 2008

Chairman: Mohd. Yunus bin Shukor, PhD

Faculty : Biotechnology and Biomolecular Sciences

An inhibition study on *Pangasius pangasius* brain Acetylcholinesterase (AChE) was conducted. A custom-synthesized affinity column chromatography utilizing activated Sephacryl S-1000 was successfully coupled to procainamide. AChE loaded onto the column and run with a flow rate of 0.2 ml/min was shown to give a good binding capacity with a recovery yield of 51.97% and a purification fold of 7.52. Overall, total protein content (mg) decreases while specific activity increased, thus conforming to a typical successful purification process. Further analysis with effect o of substrate using Acetylthiocholine Iodide (ATC), Butyrylthiocholine Iodide (BTC) and Propionylthiocholine Iodide (PTC) shows that the apparent Michaelis-Menten ($K_{m\ app}$) of the partially purified enzyme was 0.1265 mM and the apparent maximal velocity ($V_{max\ app}$) was 0.6981 µmol/min/mg, with a substrate specificity in the order of acetylthiocholine iodide (ATC) > propionylthiocholine iodide (PTC) > butyrylthiocholine iodide (BTC). This, strongly suggest that AChE was the main bulk of the successfully bound and purified enzyme for this column. Sodium dodecyl sulphate - Polyacrylmide gel electrophoresis (SDS-PAGE) carried out shows a

greatly reduced number of band in the gel, again fortified the efficiency of the procainamide-based affinity chromatography column in obtaining a highly partial purified AChE. Temperature optimization studies show that the AChE has an optimum activity at 25°C. Primary and secondary screening of 16 xenobiotics comprising 11 pesticides and 5 solvents shows that 8 pesticides and no solvents show inhibition effect on AChE. Further studies with eight of the xenobiotics shows that, partially purified AChE exhibits more sensitivity to the xenobiotics than crude AChE. Half life (IC_{50}) studies carried out show that different sources of AChE have different sensitivities towards individual insecticides. Meanwhile, comparison between *P. pangasius* and *E. electricus* AChE shows that AChE from *P. pangasius* is more sensitive to carbofuran (0.0025 mg/l), carbaryl (0.0544 mg/l), methomyl (0.0124 mg/l), parathion (0.0401 mg/l), diazinon (0.1112 mg/l) and chlorpyrifos (0.0176 mg/l), while *E. electricus* is more sensitive to bendiocarb (0.0193 mg/l) and malathion (0.0278 mg/l). Comparison of the insecticides relative sensitivity of AChE from within each source revealed that both fish AChE (*P. pangasius* and *E. electricus*) have more similarity in insecticides sensitivity rank order compared to insect (*M. domestica*). Copper, silver and mercury show 58%, 68% and 86% inhibition of AChE respectively while lead, cadmium and arsenic did not inhibit AChE at 10 mg/l. Half life (IC_{50}) studies carried out show that *E. electricus* is a better indicator for all three heavy metal compared to partially purified AChE from *P. pangasius*. The result shows that AChE from *P. pangasius* have the potential to be used as an environmental bioindicator for the detection of selected insecticides and heavy metals.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PEMBANGUNAN SATU ASAI PERENCATAN BERDASARKAN ASETILKOLINESTERASE UNTUK RACUN SERANGGA

Oleh

THAM LIK GIN

Januari 2008

Pengerusi: Mohd. Yunus bin Shukor, PhD

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Satu kajian perencatan ke atas Asetilkolinesterase (AChE) dari otak *Pangasius pangasius* telah dijalankan. Satu kolum gel kromatografi keafinan telah berjaya disintesis dengan mengandingkan Sephacryl S-1000 teraktif yang epoksi kepada prosainamid. AChE yang dimasukkan dalam kolum pada kadar pengaliran 0.2 ml/min menunjukkan lekatan kapasiti yang baik dengan hasil perolehan 51.97% dan faktor penulenan 7.52. Secara umumnya, jumlah isikandungan protein (mg) menurun manakala aktiviti spesifik meningkat, secara langsung mencirikan satu kejayaan proses penulenan tipikal. Analisis selanjutnya dengan kesan penggunaan substrat mengunakan Asetiltiokolin Iodida (ATC), Butiriltiokoline Iodida (BTC) dan Propioniltiokolin Iodida (PTC) menunjukkan bahawa pekali Michaelis ($K_{m\ app}$) enzim separa tulen adalah 0.1265 mM dan kelajuan maksimum ($V_{max\ app}$) adalah 0.6981 µmol/min/mg, dengan substrat spesifikasi dalam turutan Asetiltiokolin Iodida (ATC) > Propioniltiokolin Iodida (PTC) > Butiriltiokolin Iodida (BTC). Ini menunjukkan bahawa AChE adalah enzim utama yang berjaya dilekat dan ditulenkan daripada kolum ini. Poliakrilamide gel elektroforesis-sodium dodesil sulfat (SDS-PAGE) yang

dijalankan menunjukkan pengurangan nombor jalur protein yang banyak pada gel, sekali lagi mengambarkan efisiensi kolum gel keafinan berdasarkan procainamide dalam menghasilkan AChE separa tulen yang berkualiti. Optimasi suhu menunjukkan bahawa AChE mempunyai aktiviti optimum pada 25°C. Hasil penyaringan primer dan sekunder untuk 16 xenobiotik yang menggabungkan 11 racun serangga dan 5 pelarut menunjukkan 8 racun serangga menunjukkan perencatan keatas AChE manakala kesemua pelarut tidak menunjukkan perencatan keatas AChE. Analisis lanjutan dengan lapan xenobiotik tersebut menunjukkan bahawa AChE separa tulen adalah lebih sensitif terhadap xenobiotik daripada AChE ekstrak kasar. Kajian terhadap kepekatan racun perosak yang menyebabkan 50% perencatan aktiviti enzim (IC_{50}) menunjukkan bahawa AChE dari sumber yang berlainan mempunyai sensitiviti berbeza terhadap individu racun serangga. Secara umumnya, dari jumlah racun serangga yang dikaji, AChE dari *M. domestica* adalah lebih sensitif kepada perencatan. Dalam pada itu, perbandingan AChE antara *P. pangasius* dan *E. electricus* menunjukkan AChE daripada *P. pangasius* adalah lebih sensitif kepada karbofuran (0.0025 mg/l), karbaril (0.0544 mg/l), metomil (0.0124 mg/l), paration (0.0401 mg/l), diazinon (0.1112 mg/l) dan klorpirifos (0.0176 mg/l), manakala *E. electricus* adalah lebih sensitif terhadap bendiokarb (0.0193 mg/l) dan malation (0.0278 mg/l). Perbandingan di antara sensitiviti relatif racun serangga dalam satu satu sumber AChE menunjukkan bahawa kedua-dua AChE ikan (*P. pangasius* and *E. electricus*) mempunyai lebih persamaan dalam turutan sensitiviti jika dibandingkan dengan serangga (*M. domestica*). Kuprum, perak dan merkuri masing-masing menunjukkan 58%, 68% dan 86% perencatan keatas AChE manakala plumbum, kadmium dan arsenik tidak merencat AChE pada 10 mg/l. Kajian separuh hayat (IC_{50}) yang dijalankan menunjukkan bahawa *E. electricus* adalah penunjuk

yang lebih baik untuk ketiga-tiga logam berat yang dikaji jika dibandingkan dengan AChE dari *P. pangasius* separa tulen. Keputusan ini menunjukkan bahawa AChE dari *P. pangasius* mempunyai potensi untuk digunakan sebagai penunjuk biologi alam sekitar dalam pengesanan sesetengah racun serangga dan logam berat.

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Last but not least, I would like to recognize my parents and brother for their unconditional sacrifices, love and undying support. Thank you for believing in me and for making me the person I am today.

I certify that an Examination Committee has met on 3rd January 2008 to conduct the final examination of Tham Lik Gin on his Master of Science thesis entitled "Development of an Inhibitive Acetylcholinesterase-Based Assay for Insecticides" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the degree of Master of Science.

Members of the Examination Committee were as follows:

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Date : 21 February 2008

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirements for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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Date : 21 February 2008

DECLARATION

I hereby declare that the thesis is based on my original work except for quotation and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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THAM LIK GIN

Date: 28 January 2008

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LIST OF ABBREVIATIONS

Å	Angstrom
≈	Almost equal to
Δ Abs	Changes of Absorbance
°C	Degree Celsius
%	Percent
3D	Three Dimensional
AChE	Acetylcholinesterase
ACh	Acetylcholine
ATC	Acetylthiocholine Iodide
BuChE	Butyrylcholinesterase
BTC	Butyrylthiocholine Iodide
ChE	Cholinesterase
CE	Carbofuran equivalence
DDVP	Dichlorvos
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DDT	Dichlorodiphenyltrichloroethane
g	Gravity (Relative Centrifugal force)
HPLC-MS	High Performance Liquid Chromatography Mass Spectrophotometer
HCL	Hydrochloric acid
IC_{50}	Fifty percent inhibition concentration
GC-MS	Gas Chromatography Mass Spectrophotometer
kDa	Kilo Dalton

$K_{m\ (app)}$	Apparent Michaelis-Menten Constant
M	Molar
mg	Miligram
mM	Milimolar
mg/l	Milligram per liter
µl	Microliter
nm	Nanometer
NaOH	Sodium hydroxide
OP	Organophosphate
pH	-log concentration of H^+ ion (*Puissance hydrogene)*
PTC	Propionylthiocholine Iodide
PMSF	Phenylmethylsulfonylfluoride
PAGE	Polyacrylamide gel electrophoresis
SEM	Standard Error Mean
SDS	Sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
$V_{max\ (app)}$	Apparent Maximum velocity
v/v	Volume/volume
w/v	Weight/volume

CHAPTER 1

INTRODUCTION

Study on pesticide toxicity has assumed importance because of the green revolution, which put emphasis on the need to increase yield, and reduce the cost of food production in the agricultural industry.

The study is focused in insecticides, which mostly are acetylcholinesterase inhibitors. Insectisides are classified into organophosphate, carbamate and organochlorine group. However, only the organophosphates and carbamates are used since most of the organochlorine is already banned.

Since carbamates and organophosphates not only inhibit insect AChE but also strongly interfere with neural transmission in other organisms, including humans, they represent a potential hazard for the environment and human health. Hence, continuous assessment and monitoring is required (Del Carlo *et al*., 2002; Council Directive 80/778, 1980). The advantage of these groups of pesticides naturally comes with widespread use in agricultural sector. Coupled with easy accessibility of these compounds, intoxication cases were high. For example, between 750,000 and 3,000,000 human organophosphates intoxication per year are estimated worldwide (Worek *et al.*, 2005; Kwong, 2002; Forget, 1991; Jeyaratnam, 1990), resulting in several hundreds thousands of fatalities annually (Gunnell and Eddleston, 2003; Eddleston *et al*., 2002).

Owing to their toxic effects on non-target organisms, most pesticides may produce serious detrimental effects on the ecosystems. Aquatic habitats are particularly vulnerable to contamination by pesticides, due to leaching and runoff water from treated areas (Bretaud *et al*., 2000). Taking into account the potential environmental impact of organophosphates and carbamates, many studies concerning detection and toxicity analysis of these pesticides have been carried out and demonstrated mainly for environmental application (Del Carlo *et al*., 2002; Rodriguez-Fuentes and Gold-Bouchot, 2000).

One of this type of studies is using biological source for environmental assessment. For example, brain AChE has been used for this purpose. This is mainly because of brain AChE sensitivity and specificity in detecting trace amounts of carbamates and OPs. Acetylcholinesterase inhibition studies were done by many using different sources of AChE such as from insect, mammalian, birds and crustaceans. Fish have been used traditionally as a biomarker for pesticides toxicity and generally not as a source of AChE for monitoring contamination of pesticides to marine and freshwater ecosystem (De La Torre *et al*., 2002; Karen *et al*., 2001; Rodriguez-Fuentes and Gold-Bouchot, 2000).

It is envisaged that AChE from local freshwater fish might be useful for monitoring pesticides contamination in water. Taking into account the quite substantial aquaculture industry in Malaysia and the existence of large amount of freshwater bodies, freshwater fish may be useful as biological indicators and that fish AChE may be a useful tool for monitoring contamination.

In this study, four major objectives will be accomplished. The objectives of this study are:

1. To develop an affinity gel chromatography to purify AChE.
2. To partially purify AChE from *P. pangasius* using the developed affinity gel.
3. To compare the efficiency of crude and partially purified AChE from *P. pangasius* to detect Carbamates and Organophosphates.
4. To determine the IC_{50} of selected insecticides using crude AChE from *P. pangasius*, partially purified AChE from *P. pangasius* and AChE from *M. domestica* and *E. electricus*.

CHAPTER 2

LITERATURE REVIEW

2.1 Acetylcholinesterase (AChE)

Acetylcholine acetylhydrolase (AChE, EC 3.1.1.7) is the enzyme that participates in chemical transmission of certain nerve impulses, with the specific role of breaking down the neurotransmitter acetylcholine released during the nerve signal relay in cholinergic synapses and neuromuscular junctions (Salles *et al.,* 2006). It is found primarily in the brain, nervous system, muscle tissues, blood and liver and is widely distributed among vertebrate and invertebrate animals. However, studies using specific substrates and inhibitors have indicated that AChE predominates in brain tissues (De La Torre *et al*., 2002; Habig *et al*., 1988).

Of all the pesticide-induced changes, inhibition of acetylcholinesterase (AChE) is most often studied (Singh and Kumar, 2000). Acetylcholine and noradrenaline are the two main transmitters in vertebrate nervous systems (Mitchell, 2004).

Acetycholine is an ammonium compound. It was the first transmitter to be isolated in 1920 (Mushigeri and David, 2005). The arrival of nerve impulses at the synaptic knob depolarizes the presynaptic membrane, causing calcium channels to open, increasing the permeability of the membrane to calcium (Ca^{2+}) ions (Poli *et al.,* 2001). As the calcium ions rush into the synaptic knob they cause synaptic vesicles to fuse with the presynaptic membrane, releasing their content into the synaptic cleft (exocytosis). The vesicles then return to the cytoplasm where they are refilled with

the transmitter substance, acetylcholine. Acetylcholine diffuses across the synaptic cleft, creating a delay of about 0.5 milliseconds, and attaches to a specific receptor site (a protein) on the postsynaptic membrane that recognizes the molecular structure of the acetylcholine molecules. The arrival of the acetylcholine causes a change in the shape of the receptor site, which results in ion channels opening up in the postsynaptic membrane (Lehninger, 1993).

Entry of sodium ions through the postsynaptic membrane causes depolarization of the membrane. This excites the cell, making it more likely to set up a nerve impulse (action potential). After the nerve impulse was transmitted across the synapse, acetylcholine is immediately removed from the synaptic cleft by the enzyme acetylcholinesterase (Figure 1). This enzyme is situated on the postsynaptic membrane and hydrolyses the acetylcholine to choline and acetic acid. The choline is then reabsorbed into the synaptic knob to be recycled into acetylcholine by synthetic pathways in the vesicles (Taylor *et al.,* 1998).

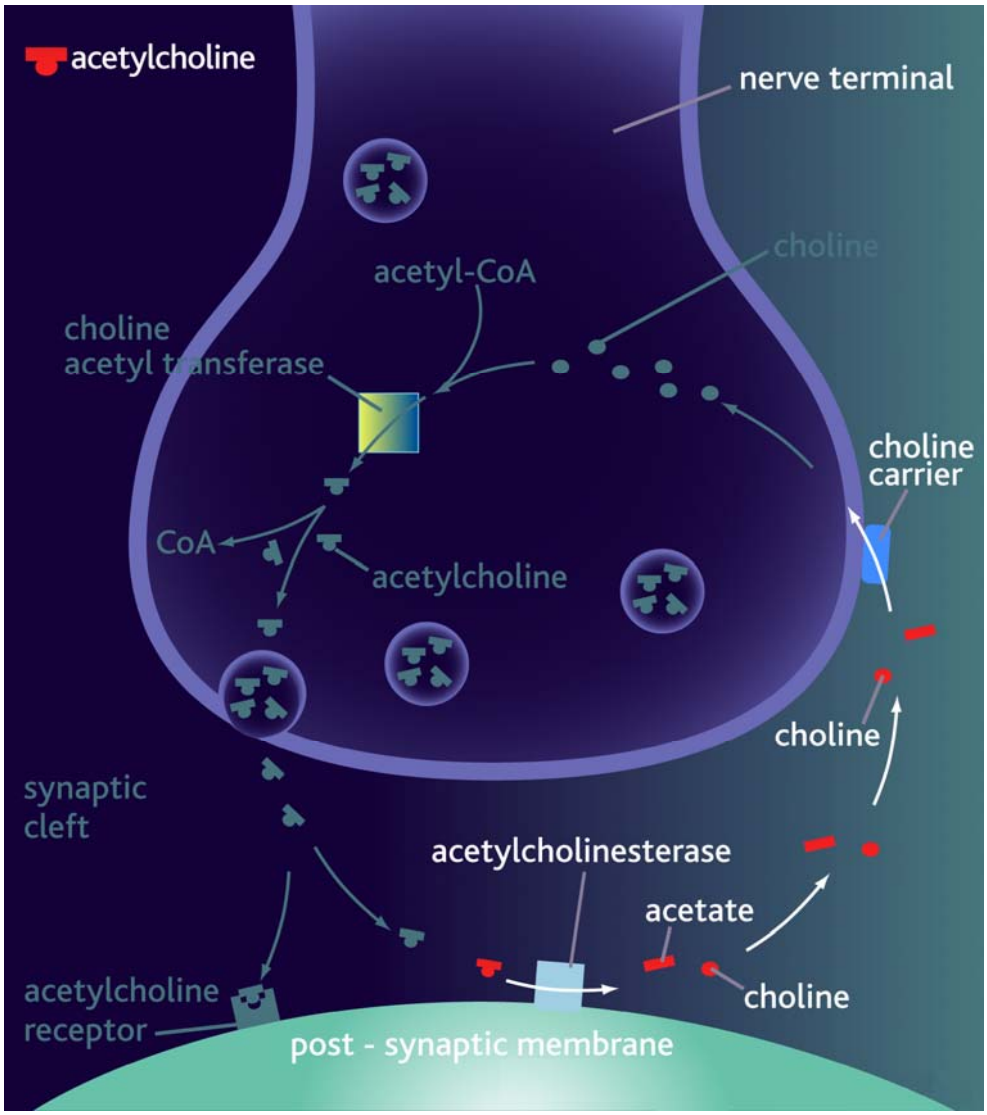

Figure 1: Acetylcholine transmission in the synapse (Katzung, 2001).