THE POTENTIAL OF ACETYLCHOLINESTERASE FROM *Monopterus albus* BRAIN FOR THE DETECTION OF HEAVY METALS AND INSECTICIDES

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DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, excerpts, equations, summaries and references, which have been duly acknowledged.

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ABSTRACT

Environmental contamination of heavy metals and insecticides have been continuously increased in several areas in Malaysia because of industrial and human activity. Monitoring programmed was implemented to ensure the level of contamination can be controlled. In this study, Cholinesterase, (ChE) from the brain of eel, *Monopterus albus* was tested to determine the potential use as alternative biosensor, which is sensitive towards various insecticides and metal ions. Acetylcholinesterase (AChE) from the brain of *M. albus* was purified through ammonium sulphate precipitation and procainamide affinity chromatography. Enzyme recovery was obtained at 38.73% with the specific activity of 1847 U μg^{-1}. The Michaelis constant (K_m) value and maximal velocity (V_{max}) were determined at 8.910 mM and 29.44 μmol min^{-1} mg^{-1}, respectively, for acetylthiocholine iodide (ATC). Based on effective coefficient ratio, AChE from *M. albus* brain showed higher affinity to ATC compared to butyrylthiocholine iodide (BTC) and propionylthiocholine iodide (PTC) at the value of 3.304, 1.515, 2.965 $V_{max}.K_m^{-1}$, respectively. Optimum activity of AChE was obtained at the range of 25°C to 50°C and incubated in 0.1M Tris HCl buffer pH 9.0. Purified acetylcholinesterase was exposed with a different type of metal ions, and mercury shows the highest inhibition at the percentage of 62.9% followed by chromium at 59.22% while silver, arsenic, cadmium, cobalt, copper, nickel, zinc and lead not more than 50% inhibition (around 37 to 50%). Metal ions such mercury, zinc, chromium and copper show exponential decay type inhibition curves with half-maximal inhibitory concentration; IC_{50} was calculated in the ascending sensitivity order of 0.005, 0.595, 0.687 and 1.329 mgL^{-1}, respectively. Field trial works exhibited that the enzyme was applicable in sensing heavy metals pollution from the river which closes to the industrial and agricultural sites at near real-time and verified using ICP-OES. Inhibition study for insecticides resulted in this descending order of inhibition; Dimethoate> Parathion> Malathion> Diazinon> Chlorpyrifos> Bendiocarp> Methomyl> Acephate> Propoxur> Carbaryl> Trichlorfon> Carbofuran, with Dimethoate, Parathion, Malathion, Diazinon, Chlorpyrifos, Bendiocarp and Methomyl showing more than 50% inhibition at two ppm. Selected pesticides showed exponential decay type inhibition curves with calculated IC_{50} in the ascending sensitivity order of Bendiocarp, Dimethoate, Malathion and Parathion at the concentration of 1.639, 1.509, 0.874 and 0.162 mgL^{-1}, respectively. A 10-days field trial performed by testing purified ChE on *C. asiatica* treated with three commercial insecticides exhibited that the enzyme was applicable in sensing the presence of those compounds. This study proved that the potential use of acetylcholinesterase source from *M. albus* as a biomonitoring tool can be further utilized to develop a cheaper, easier and faster pesticides and heavy metals detection method as compared to conventional methods available.

ABSTRAK

POTENSI ASETILKOLINESTERASE DARI OTAK Monopterus albus SEBAGAI PENGESAN TERHADAP KEHADIRAN LOGAM BERAT DAN RACUN SERANGGA

Pencemaran alam sekitar oleh logam berat dan racun serangga terus meningkat di beberapa kawasan di Malaysia akibat daripada aktiviti perindustrian dan aktiviti manusia. Program pemantauan telah dilaksanakan untuk memastikan tahap pencemaran dapat dikawal. Dalam kajian ini, kolinesterase, (ChE) dari otak belut, *Monopterus albus* telah diuji untuk menentukan potensi penggunaan sebagai salah satu biopenderia alternatif yang sensitif terhadap pelbagai racun serangga dan logam berat. Asetilkolinesterase (AChE) dari otak *M. albus* ditulenkan melalui pemendakan amonium sulfat dan kromatografi keafinan procainamide. Perolehan enzim diperolehi pada 38.73% dengan aktiviti khusus 1847 U μg^{-1}. Nilai berterusan Michael (K_m) dan halaju maksima (V_{max}) ditentukan pada 8.910 mM dan 29.44 $\mu mol\ min^{-1}\ mg^{-1}$, masing-masing untuk asetiltiokolin iodida (ATC). Berdasarkan nisbah koefisien yang berkesan, AChE dari otak *M. albus* menunjukkan keafinan yang lebih tinggi kepada ATC berbanding dengan butiriltiokolin iodida (BTC) dan propioniltiokolin iodida (PTC) pada nilai masing-masing 3.304, 1.515, 2.965 $V_{max}.K_m^{-1}$. Aktiviti AChE yang optimum diperoleh dalam julat suhu diantara 25°C hingga 50°C dan diinkubasi didalam 0.1M penimbal Tris HCl pada pH 9.0. AChE yang ditulenkan telah didedah dengan pelbagai jenis logam berat yang berbeza dimana, merkuri menunjukkan perencatan tertinggi pada peratusan 62.9% diikuti oleh kromium pada 59.22% manakala perak, arsenik, kadmium, kobalt, kuprum, nikel, zink dan plumbum tidak melebihi 50% peratusan perencatan iaitu sekitar (37 hingga 50%). Ion-ion logam seperti merkuri, zink, kromium dan kuprum menunjukkan lengkung taburan pereputan jenis perencatan dengan kepekatan separa perencat; IC_{50} yang dikira dalam susunan kepekaan menaik sebanyak 0.005, 0.595, 0.687 dan 1.329 mgL^{-1}. Kerja-kerja percubaan lapangan menunjukkan bahawa enzim ini terpakai dalam mengesan pencemaran logam berat dari sungai yang menghampiri dengan tapak industri dan aktiviti pertanian dan telah disahkan dengan menggunakan bacaan ICP-OES. Kajian perencatan untuk racun serangga dalam susunan menurun; Dimethoate> Parathion> Malathion> Diazinon> Chlorpyrifos> Bendiocarp> Methomyl> Acephate> Propoxur> Carbaryl> Trichlorfon> Carbofuran, dengan Dimethoate, Parathion, Malathion, Diazinon, Chlorpyrifos, Bendiocarp dan Methomyl menunjukkan lebih daripada 50% peratusan perencatan pada dua ppm. Racun serangga yang terpilih menunjukkan lengkung taburan pereputan jenis perencatan dengan kepekatan separa perencat; IC_{50} yang dikira dalam susunan kepekaan yang menurun dari Bendiocarp, Dimethoate, Malathion dan Parathion pada kepekatan 1.639, 1.509, 0.874 dan 0.162 mgL^{-1}. Percubaan lapangan selama 10 hari dilakukan dengan menguji ketulenan ChE pada tumbuhan *C. asiatica* yang didedahkan dengan tiga racun serangga yang komersil iaitu yang ada dipasaran yang telah memperlihatkan bahawa enzim tersebut dapat digunakan untuk melihat kehadiran perencatan terhadap racun serangga tersebut. Kajian ini membuktikan bahawa sumber asetilkolinesterase dari *M. albus* berpotensi sebagai alat biokawalan dan boleh digunakan untuk membangunkan kaedah pengesanan racun serangga dan logam berat yang lebih murah, mudah dan cepat berbanding kaedah konvensional yang tersedia.

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%	-	Percentage
°C	-	Degree celcius
µg/g	-	Microgram per gram
µg/L	-	microgram perlitre
Abs	-	Absorbance
Ag	-	Silver
Al	-	Aluminium
ANOVA	-	Analysis of variance
APS	-	Ammonium persulfate
As	-	Arsenic
ATC	-	Acetylthiocholine iodide
BSA	-	Bovine serum albumin
BTC	-	Butyrylthiocholine iodide
CBB	-	Commasie brilliant blue
Cd	-	Cadmium
ChE	-	Cholinesterase
Cr	-	Chromium
Cu	-	Copper
$CuCl_2$	-	Copper (II) chloride
$CuSO_4$	-	Copper (II) sulfate
DOE	-	Department of Environment
DTNB	-	5, 5-dithio-bis-2-nitrobenzoate
et al.,	-	and all
FAO	-	Food and agricultural organization
Fe	-	Ferum
g	-	Gram
GF	-	Gel filtration
Hg	-	Mercury
HPLC	-	High performance liquid chromatography

IC_{50}	-	Initial concentration that cause 50% inhibition
ICP-OES	-	Inductively coupled plasma optical emission spectrometry
KCl	-	Potassium chloride
kDa	-	Kilo Dalton
K_m	-	Biomolecular constant
L	-	Liter
M	-	Molarity
mM	-	Milimolar
mg/L	-	Miligram perliter
min	-	Minute
MS	-	Mass spectrometry
MW	-	Molecular weight
NaN_3	-	Sodium nitrite
Ni	-	Nickel
PAGE	-	Polyacrylamide gel electrophoresis
Pb	-	Lead
PMSF	-	Phenylmethylsulfonyl fluoride
ppb	-	Part per billion
ppm	-	Part per million
PTC	-	Propionylthiocholine iodide
rf	-	Retention factor
ROS	-	Reactive oxygen species
RT	-	Retention time
SDS	-	Sodium dodecyl sulfate
TCA	-	Trichloroacetic acid
TEMED	-	Tetramethyl-ethylene diamine
U	-	Unit
UV	-	Ultra-violet
V_{max}	-	Maximum velocity
WHO	-	World health organization
Zn	-	Zinc

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CHAPTER 1

INTRODUCTION

Malaysia is a well known's country of rapid development in terms of economic, educational, agricultural and industrial resources. Today, the industrial is becoming increasingly important, which is a catalyst in the development of the Malaysian economy and contributes to the country's income and growth. Throughout the rapid development, there are some conflicts between economic benefits and environmental risks in use of considering from waste disposal of the industrial, illegal plantations and human activities. However, the excessive application of the waste has side effects by the decreasing of clean water sources, the increasing of health problems, the pollution of aquatic and marine life, increasing of contamination of heavy metal and affecting non-target organisms (Lu *et al.,* 2013). Due to Centrals for Disease Control and Prevention website, Water sources are essential to humans as it is estimated that 80% of the illness and 1/3 mortality in developing countries are due to the use of contaminated water (Young, Dooge & Rodda, 1994).

Exposure to toxicant such as industrial or agricultural waste containing metals, fertiliser and pesticides could develop a wide range of deleterious effects on exposed organisms. Those toxicants ions can induce harmful effects on living organisms at ecologically relevant concentrations and have been considered as an essential environmental contaminant. Moreover, most of the compound is highly persistence and resistance. Heavy metals are unable to be degraded or destroyed by the environmental stressor. Although they act as a trace element to regulate the metabolism of the human body such as copper, zinc and cobalt, continuous exposure of heavy metals at high concentration may lead to bioaccumulation. Bioaccumulation is an increase in the concentration of xenobiotics in a biological system over time. The food chain would also be affected if this affected the marine environment. This will influence and eventually affect the human entire food chain. For example, behavioural disorders and biochemical changes have been observed in the central nervous system (CNS) of heavy metals-exposed animals (Senger *et al.*, 2006). Utusan Online, (2016) reported the 39 victims of food poisoning at Batu Gajah, Perak caused by organophosphate toxin exposure caused diarrhea, vomiting and headache.

Due to the increase of environmental pollutions, the river rehabilitation technique will be used. The technique is physical, chemical and biological. Physical technique includes the construction of a filter system in the river for the processing of the ventilation system in the water to enhance water quality. The technique is found to be effective in the short-term period but less practical for a long-term period. Other than that, collecting all the solid waste in the river may also be one of the physical techniques. The chemical technique involves the treatment of water quality using certain chemicals, for example, chlorine. Meanwhile, the biological technique is based on the concept of green technology and bioremediation. The concept of this technique is using bacteria that have been cultured that able to treat contamination water. The bacteria would act as a substrate (bio-flocculant) of suspended substances in the polluted river and also using a microbial process that particular emphasis on one of the processes (degradation). This technique has proven effective and reliable due to its eco-friendly features, but they are costly, need professional worker to handle and a long time to apply.

1.1 Problem statement

Monitoring technique may also be used as controlling the contamination by daily checking of the BOD (Biochemical Oxygen Demand) and COD (Chemical Oxygen Demand). They are the main parameters analysed to indicate the degree of pollution in the river (Sawyer & McCarty, 1988). BOD is the measurement of the amount of oxygen required biologically to decompose organic matter under aerobic condition. In contrast, COD is the measurement of total oxygen required to oxidise all biologically available and inert organic matter into carbon dioxide and water. This technique is widely used parameters for organic pollution measurements, but it still needs to send to the laboratory for analysis. This process requires HPLC (High-performance liquid chromatography), ICP-MS (Inductively coupled plasma mass spectrometry) for heavy metal detection, and GCMS (Gas chromatography-mass spectrometry) for the volatile compound. This sophisticated instrument is very complicated to run and need the professional worker to handle, expensive and takes a long time to analyse.

Therefore, a new alternative source was introduced, which is biosensor. A biosensor is one of the biotechnology hubs, and it is an analytical tool that consists of biological elements capable of detecting changes or biological phenomena of life due to reactions to environmental factors. The biosensor is also applied to monitor the environment using protease enzymes and acetylcholinesterase to detect the presence of pollutants in a river or a water sample (Hayat *et al.,* 2016; Sabullah *et al.,* 2015). Since aquatic life becomes the final target of toxicant accumulation, the fish body was manipulated to determine the contamination level of the river other than a significant protein source for locals. Heavy metals and pesticides are capable of depositing in each of the fish organs. For this reason, environmental monitoring is crucial to be applied as an early warning indicator of pollution or related water quality problems. Other than that, it also enables us to take appropriate action to protect the public health and environment, the effect of heavy metals on fish would ultimately affect humans. An alternative aquaculture source such as Asian swamp eel (*Monopterus albus*) has potential value to be commercialized on a large scale not only to be consumed but contribution in the development of biosensor kit. Therefore, the objective of this study is to elucidate the response of acetylcholinesterase enzyme towards metal ion and insecticides. The use of ChE (Cholinesterase) extracted from the aquatic organism such as eel to act as a biomarker for the detection of anti-cholinesterase has been developed based on the study of biological responses of organisms to pollutants especially in aquatic system. This study can be possibly regarded as a basis for future application of this species, whether in environmental monitoring or aquaculture industry.

Freshwater fish, *M. albus* can be found in freshwater sources and river in Malaysia. They are economically relevant to villagers and the aquaculture industry. River contamination by heavy metals, mainly industrial and agricultural effluent, will give an adverse impact on the growth and sustainability of the fish since they thrive in the river. It is known that heavy metals and pesticides have shown an adverse effect on the freshwater life including fish biochemistry; resulting in swimming performance, slow growth, gross morphological changes and overall changes in protein expression (Mookerjee *et al.*, 2006; Javed, 2011; Mishra & Mohanty, 2009). Therefore, it becomes increasingly important to have baseline data on how these fishes adapt to a change in their environment with regards to their ability to reproduce and survive in an increasingly hostile environment.

1.2 Hypothesis of this study

This study will increase the potential of *Monopterus albus* to become an important species that permits the recognition of lower contamination level of heavy metals and insecticides. The use of sentinel species (bio-indicator) in bio-monitoring is important in assessing environmental risk.

1.3 Objectives of this study

This study will provide fundamental information on the interaction between acetylcholinesterase from *M. albus* with heavy metals and pesticides, carbamate and organophosphate. Moreover, the sensitivity of *M. albus* AChE was elucidated to become as an alternative source for biosensor development. Therefore, this study was conducted with the following objectives:

1. To purify Acetylcholinesterase (AChE) from the brain of *Monopterus albus*, through the process of ammonium sulfate precipitation and procainamide-based chromatography.
2. To optimized and characterize the purify enzyme using pH profile, temperature profile and substrate specificity.
3. To evaluate the inhibition effects of insecticides and metal ions on purified enzyme.

CHAPTER 2

LITERATURE REVIEW

2.1 Biosensor

Biosensors are research instruments that convert an electrical signal from a biological response. Biosensors are necessary to be highly specific and should be reusable, regardless of physical parameters, including pH and temperature. The founders Clark and Lyons started Biosensors in the 1960s. The proteins, tissue-based, immunosensors, DNA biosensors, and thermal and piezoelectric biosensors are various types used. In 1967, Updike and Hicks announced the first enzyme-based sensor. Installation techniques; adsorbing enzymes by van der Waals forces, ionic bonding or covalent connectivity, are the subject of enzymatic biosensors. Oxidoreductases, polyphenol oxidases, peroxidases and amine oxidases are the widely used enzymes (Muguruma, 2018).

The development of high-performance electrochemical biosensors requires enzyme immobilization. Biosecurity and the activation of enzymes were impaired. Several methods of achieving successful enzyme immobilization have been discussed, including the use of enzymes in separate matrices and covalent attachment on the substrate's surface. The advanced construction of nanomaterials offers the possibility to modify properties and thus to improve application and function in the immobilization of enzymes (Turner *et al.,* 1987)

Unlike biomarkers, biosensors used natural components that are extracted by purifying or extracting from the living organism or synthesized artificially to increase performance by raising environmental stressor responsiveness and stability (Bohunicky & Mousa, 2011). This detection method will produce reliable outcomes, whether in whole or in part, financial results, lower timescales on location, and poor handling compared with all biomarker testing (Thévenot *et al.,* 2001; Mascini & Tombelli, 2008). As the portable sensor instrument, the biosensor has been designed to handle biological elements such as enzymes and antibodies using a multi-analyte or physicochemical detection system (Mascini & Tombelli, 2008; Ngoepe *et al.,* 2013).

The development of the biosensor process from year to year is linked with the growing of companies in water supply, manufactures, farming and mining, as well as urbanizations, in which the demand for convincing water quality and non-toxicant water or product is fulfilled by this situation (Tothill, 2001). For example, the detection of infection and diabetic levels (Bohunicky & Musa, 2011; Mach *et al.,* 2011) have been widely requested to be used as a biosensor in biomedical diagnoses. In other cases, this approach was used to detect the presence of allergens and pathogens in its goods in food industries and agriculture (Narsaiah *et al.,* 2012; Rigi *et al.,* 2013). There was no question that the biosensor had been included in the environmental monitoring system for evaluating toxins, such as silage effluent (Stephens *et al.,* 1997), Bacterial pathogenes (Liao *et al.,* 2007), pesticides (Viswanathan *et al.,* 2009).

The valuable use of biosensors as a diagnostic device in various fields of research as well as the development of biosensor kits can be elaborated. The bacterium detection function has been exploited by the mobile biosensors package Microtox ® Bioassay, DeltaTox Bioassay, PolytoxTM Bioassay, Rhizobium melitos Biosaid and MTT check. A recombinant acetylcholinesterase that is sensitive to contagion is also used in an example of the biosensor using enzyme-basis kits such as demutox sensor (Shruthi *et al.,* 2014)