UNIVERSITI PUTRA MALAYSIA

PURIFICATION AND CHARACTERIZATION OF ACETYLCHOLINESTERASE FROM CLARIAS BATRACHUS AND OREOCHROMIS MOSSAMBICA BRAIN TISSUES

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PURIFICATION AND CHARACTERIZATION OF ACETYLCHOLINESTERASE FROM *CLARIAS BATRACHUS* AND *OREOCHROMIS MOSSAMBICA* BRAIN TISSUES

By

NATARAJAN PERUMAL

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

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"Dedicated to my father, Perumal Sakaravathy and

mother, Thanam Perumal- the unconventional scholars,

to my siblings, and to the teachers and lecturers who

have taught me everything…

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

PURIFICATION AND CHARACTERIZATION OF ACETYLCHOLINESTERASE FROM *CLARIAS BATRACHUS* AND *OREOCHROMIS MOSSAMBICA* BRAIN TISSUES

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November 2006

Chairman : Professor Mohd. Arif Syed, PhD

Faculty : Biotechnology and Biomolecular Sciences

This study reports on the purification and characterization of a soluble AChE (EC 3.1.1.7) from *Clarias batrachus* and *Oreochromis mossambica* brain tissues. The purification protocol involved homogenization, centrifugation, ultrafiltration, application of custom-synthesized affinity chromatography gel (Edrophonium–Sephacryl S-400) and the use of high performance liquid chromatography system (HPLC). The affinity matrix was synthesized by coupling an AChE-specific inhibitor, edrophonium chloride to epoxy-activated Sephacryl S-400 matrix. Soluble AChE from *C. batrachus* and *O. mossambica* were purified 26.4 and 27.9 fold with a specific activity of 59.7×10^3 and 73.1×10^3 U/mg proteins, respectively. The molecular weight of AChE for *C. batrachus* estimated on Superose^{TM} gel filtration column under nondenaturing conditions is 311 kDa. Native polyacrylamide gel electrophoresis (Native-PAGE) under non-denaturing conditions showed only one major molecular form of protein for *C. batrachus* with a molecular weight of about 309 kDa, while AChE from *O. mossambica*

could not be purified. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate and beta-mercaptoethanol (SDS-PAGE) gave only one band for *C. batrachus* with an estimated molecular weight of 74 kDa. Based on the molecular weights obtained for *C. batrachus* from both SDS-PAGE and Native-PAGE, the purified AChE can be postulated as being a tetramer form linked with disulfide bonds. Acetylcholinesterases purified from brain tissues samples of *C. batrachus* and partially purified from *O. mossambica* have been analyzed further on substrate and sensitivity to inhibitors to distinguish from butrylcholinesterase (BuChE). The AChE from *C. batrachus* and *O. mossambica* were most active against acetylthiocholine (ATC) and shows less activity against propionylthiocholine (PTC) and butyrylthiocholine (BTC). From a kinetic point of view, the purified AChE from *C. batrachus* exhibit the Michaelis constants K_m, for ATC, PTC and BTC in the range of 97, 138 and 238 µM and the maximum velocities V_{max} were 347, 64 and 25 µmol/min/mg protein, respectively. Meanwhile, partially purified AChE from *O. mossambica* exhibit $K_{m(app)}$ for ATC, PTC and BTC in the range of 125, 260 and 600 µM and $V_{max(app)}$ were 276, 59 and 36 µmol/min/mg protein, respectively. The turnover number (k_{cat}) for purified AChE from *C. batrachus* with ATC as a substrate was 0.19×10^5 min^{-1}. The inhibition constant (k_i) values of eserine, propidium and carbofuran were 0.34, 81 and 0.51 $\mu M^{-1}min^{-1}$ for *C. batrachus* and 0.24, 65 and 0.41 $\mu M^{-1}min^{-1}$ for *O. mossambica*, respectively. This enzyme is apparently an AChE since it hydrolyzes ATC at a higher rate than other substrates, such as BTC and PTC, at pH 7.0 and 25ºC, and is inhibited by eserine but not by iso-OMPA.

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

PENULENAN DAN PENCIRIAN ASETILKOLINESTERASE DARIPADA TISU OTAK *CLARIAS BATRACHUS* DAN *OREOCHROMIS MOSSAMBICA*

Oleh

NATARAJAN PERUMAL

November 2006

Pengerusi : Profesor Mohd. Arif Syed. PhD

Fakulti : Bioteknologi dan Sains Biomolekul

Kajian ini melaporkan mengenai penulenan dan pencirian asetilkolinesterase (AChE) larut yang diekstrak daripada tisu otak *Clarias batrachus* dan *Oreochromis mossambica*. Asetilkolinesterase telah ditulenkan separa menggunakan cara homogenisasi, pengemparan, penurasan ultra, gel kromatografi keafinan (Edrofonium–Sephacril S-400) yang telah disintesis di dalam makmal, diikuti dengan kromatografi cecair berprestasi tinggi (HPLC). Matrik afiniti disintesis dengan menggandingkan perencat spesifik AChE "edrofonium" klorida kepada matrik Sephacril S-400 teraktif yang epoksi. Asetilkolinesterase terlarut dari *C. batrachus* dan *O. mossambica* telah ditulenkan dengan faktor penulenan masing-masing sebanyak 26.4 dan 27.9 kali ganda dan aktiviti spesifik masing-masing sebanyak 60×10^3 and 73×10^3 U/mg protein. Berat molekul asetilkolinesterase dari *C. batrachus* dianggarkan seberat 310 kDa di dalam keadaan tidak ternyahasli dengan menggunakan kolum Superose^{TM}. Elektroforesis gel poliakrilamida (Native-PAGE) di bawah keadaan tidak

ternyahasli telah menunjukkan hanya satu bentuk utama molekul protein bagi *C. batrachus* dengan berat molekul kira-kira 310 kDa, manakala AChE daripada *O. mossambica* tidak berjaya ditulenkan. Elektroforesis gel poliakrilamida dengan kehadiran sodium dodesil sulfat dan beta-merkaptoetanol (SDS-PAGE) memberikan hanya satu jalur protein untuk *C. batrachus* dengan anggaran berat molekul 74 kDa. Berdasarkan berat molekul-berat molekul yang diperolehi daripada *C. batrachus* bagi kedua-dua SDS-PAGE dan Native-PAGE, AChE yang telah ditulenkan bolehlah dipostulatkan sebagai bentuk tetramer yang dihubungkan oleh ikatan-ikatan disulfida. Asetilkolinesterase yang telah ditulenkan daripada sampel otak *C. batrachus* dan yang telah ditulenkan separa daripada *O. mossambica* telah dianalisis selanjutnya menggunakan substrat dan kesensitifan kepada perencat-perencat bagi membezakannya daripada butirilkolinesterase (BuChE). Asetilkolinesterase daripada *C. batrachus* dan *O. mossambica* didapati paling aktif terhadap Asetiltiokolin (ATC) dan menunjukkan aktiviti yang rendah terhadap propioniltiokolin (PTC) dan butiriltiokolin (BTC). Secara kinetiknya AChE daripada *C. batrachus* menunjukkan pekali Michaelis K_m bagi ATC, PTC dan BTC masing-masing dalam julat 97, 138 dan 238 µM dan kelajuan awal maksimum V_{max}, masing-masing 347, 64 dan 25 µmol/min/mg protein. Manakala AChE daripada *O. mossambica* menunjukkan pekali Michaelis $K_{m(app)}$ bagi ATC, PTC dan BTC masing-masing sebanyak 125, 260 dan 600 µM dan kelajuan maksimum awal $V_{max(app)}$ masing-masing 276, 59 dan 36 µmol/min/mg protein. Nombor pusingan (k_{cat}) bagi AChE yang telah ditulenkan dari *C. batrachus* dengan ATC sebagai substrat ialah 0.19×10^5 min^{-1}. Nilai pekali perencatan bagi eserine, propidium dan karbofuran ialah masing-masing 0.34, 81 and 0.51 $\mu M^{-1}min^{-1}$ untuk *C.*

batrachus manakala 0.24, 65 and 0.41 $\mu M^{-1}min^{-1}$ bagi *O. mossambica*. Maka jelaslah enzim ini adalah AChE kerana ia telah menghidrolisis ATC pada kadar yang lebih tinggi berbanding dengan lain-lain substrat seperti BTC dan PTC pada pH 7.0 dan suhu 25ºC, dan juga ianya direncatkan oleh eserine tetapi bukan oleh iso-OMPA.

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Finally, I am forever indebted to my parents and to my siblings who gently offer counsel and unconditional support at each turn of the road.

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I certify that an Examination Committee met on 1st November 2006 to conduct the final examination of Natarajan Perumal on his Master Of Science thesis entitled "Purification and Characterisation of Acetylcholinesterase from *Clarias batrachus* and *Oreochromis mossambica* Brain Tissues" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows;

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

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Date: 16 JANUARY 2007

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations 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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NATARAJAN PERUMAL

Date:

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LIST OF ABBREVIATIONS

Å	Angstrom
APS	Ammonium persulphate
≤	Lesser then or equal
≥	Greater then or equal
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChR	Acetylcholine receptor
ATC	Acetylthiocholine iodine
BTC	Butyrylthiocholine
BuCh	Butyrycholine
BuChE	Butyrylcholinesterase
ChAT	Cholineacetyltransferase
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
g	gravity (Relative centrifugal force)
HPLC	High performance liquid chromatography
IC_{50}	50% inhibition concentration
Iso-OMPA	Tetramonoisopropylpyrophosphotetramide
IU	International unit
k_{cat}	Turnover number
kDa	kiloDalton
K_i	Inhibition constant
K_m	Michaelis constant
K_{si}	Substrate inhibition constant

L	Liter
M	Muscarinic receptors
M	Molar
m	Meter
mAU	milliabsorbance unit
mol	Mole
NaOH	Sodium hydroxide
PAGE	Polyacrylamide gel electrophoresis
PAS	Peripheral anionic site
ppb	Parts per billion
ppm	Parts per million
psi	Pounds per square inch
PTC	Propionylcholine
rpm	Revolutions per minute
S.D.	Standard deviation
SDS	Sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
UV	Ultraviolet
V	Volt
vAChT	Vesicular-ACh transporter
V_{max}	Maximum initial velocity

CHAPTER I

INTRODUCTION

"ACETYLCHOLINESTERASE NEVER CEASED TO AMAZED, EXCITE OR CHARM US, with its wide ramifications, unexpected roles, strange forms and complex inhibition," according to Brzin *et al*. (1984). These words described perfectly how important the charm and complexity of Acetylcholinesterase is becoming more powerful as we learn more about it.

Acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7; AChE) is a serine hydrolase that serves principally to terminate signal transmission at cholinergic synapses by rapid hydrolysis of the excitatory neurotransmitter acetylcholine in the synaptic gap. In accordance with its biological role, AChE is a very rapid-acting enzyme, operating at nearly diffusion-limited rates. Acetylcholinesterase exhibit genetic and molecular polymorphism and their distributions and physiological roles differ among species (Forget and Bocquene, 1999). As a consequence, the characteristic associated with biochemical and physiological properties is also highly variable.

Investigation of AChE in fish was initiated in 1943, when it was demonstrated that common carp *Cyprinus carpio* brain tissues contains AChE and further investigations have found similar results for other teleost (Silver, 1974). Previous studies also show that the AChE kinetic studies and sensitivity to inhibitor varied among different fish species (Chuiko, 2000). Although there are numerous studies of the properties of fish AChE, they have been mainly conducted for a

limited number of fish species and have been mostly concerned with non local source. Currently, characteristic differences of AChE among fresh water fish species from local source are not well studied.

Most of the studies on AChE have been carried out with relatively crude preparations which contain other esterases with possible overlapping substrate specificities. The use of purified AChE has obvious advantages over crude homogenates in kinetic studies of substrate and inhibitor interactions, especially when other esterases are incapable of hydrolyzing compounds under investigation. Although many different methods have been used for the purification of AChE, affinity chromatography has been demonstrated to be the most effective technique for purification. It usually provides a high yield with an adequate purity of AChE which is particularly desirable in many characteristic and inhibitory studies.

Most studies of AChE enzyme use non-local source. In this work the main aim is to provide fundamental knowledge on AChE from local fish species of *Clarias batrachus* and *Oreochromis mossambica*. This research has been carried out using *C. batrachus* and *O. mossambica* because of their availability, commercial importance and can be locally produced. The objectives of this study are;

1. To purify and characterize AChE from the brain tissues of *C. batrachus* and *O. mossambica.*

2. To evaluate the effectiveness of AChE as an *in vitro* inhibition assay system for pesticides.