EXPRESSION, CHARACTERIZATION, AND LOCALIZATION OF ACETYLCHOLINESTERASE-1 FROM THE AFRICAN MALARIA MOSQUITO, *ANOPHELES GAMBIAE*

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DEDICATION

I dedicate this humble piece of work to my wife and our daughter, who strongly support me to pursue my dreams. I dedicate this humble piece of work in honorable memory of my father and my mother.

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%	Percentage
>	More than
±	Plus or minus
°C	Temperature degree(s) at Celsius
3D	Three dimensional
µg	Microgram
µl	Microliter
AA	Amino acid
ACh	Acetylcholine
AChE	Acetylcholinesterase
ace	Acetylcholinesterase gene
A β MTC	Acetyl-(β-methyl) thiocholine iodide
*Ag*AChE1	*Anopheles gambiae* AChE1
*Ag*AChE2	*Anopheles gambiae* AChE2
*Agace*1	*Anopheles gambiae ace*1
*Agace*2	*Anopheles gambiae ace*2
*Ag*Actin	*Anopheles gambiae* actin
AP	Alkaline phosphatase
ATC	Acetylthiocholine iodide
Bac-to-Bac	A bacterial artificial chromosome to baculovirus
BCA	Bicinchoninic acid
BChE	Butyrylcholinesterase
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	Base pair
BSA	Bovine serum albumin
BTC	S-butyrylthiocholine iodide

BW284C51	1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-onedibromide
CA	Carbamate
cDNA	Complementary deoxyribonucleic acid
ChE	Cholinesterase
cm	Centimeter(s)
CNS	Central nervous system
Con A	Concanavalin A
Da	Dalton
ddH_2O	Deionized distilled water
DEPC	Diethylpyrocarbonate
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
dsDNA	Double strand DNA
DS	Dextran Sulfate
DTNB	5,5-dithiobis(2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
Fig.	Figure
g	Gram(s); Relative centrifuge force: gravity
GLM	General Linear Models
h	Hour(s)
HPLC	High performance Liquid chromatography
[I]	Inhibitor
IC_{50}	Half maximal inhibitory concentration
IEDB	Immune Epitope Database
IgG	Immunoglobulin G
Inc	Incorporation
k_2	Unimolecular bonding rate constant, phosphorylation or carbamylation constant
K_d	Dissociation constant
k_i	Bimolecular bonding rate constant

K_M	Michaelis constant
Krpm	Kilo rotations per minute
LSD	Least Significant Difference
M	Molar solution; mean
MAW	Methanol/acetone/water
mg	Milligram
min	Minutes
ml	Milliliter
mM	Millimole
MOI	Multiplicity of Infection
mol	Mole: measures an amount of substance
MR4/ATCC	The Malaria Reference and Research Reagent Resource Center /American Type Culture Collection
mRNA	Messenger ribonucleic acid
MW	Molecular Weight
N	Sample size (or n); normality
NBT	Nitro blue tetrazolium
NCBI	National Center for Biotechnology Information
ng	Nanogram
Ni-NTA	Nickel-nitrilotriacetic acid
nl	Nanoliter
nm	nanometer
nt	Nucleotide
OP	Organophosphorus pesticide(s)
ORF	Open reading frame
P	P - value: a probability statement
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PTC	Propionylthiocholine iodide
PTW	0.1% Tween-20 in 1 × PBS

r^2	Average correlation coefficient
RNA	Ribonucleic acid
rpm	Revolution per minute
S	Second(s) or s
[S]	Substrate
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SE	Standard error
Sf	*Spodoptera frugiperda*
SFM	Serum-free medium
ssDNA	Single strand DNA
TCIS	Transcription initiation site
TESPA	3-aminopropyl-triethoxysilane
TLIS	Translation initiation site
TLTS	Translation termination site
U	Enzyme or antibiotic units
UTR	Untranslated region
v	Velocity
V_{max}	Maximal velocity
X	Times

CHAPTER I

GENERAL INTRODUCTION

Background

Of more than 10 million animal species on the earth, insects are the largest and most diverse group with at least one million species. Insects appeared 550 million years ago, 250 million years earlier than the ancestors of humans (Dimarcq, 2002; Ware and Whitacre, 2004). During the evolutionary course, some insects have had a tremendous impact on mankind. About one thousand insect species became pests (Tvedten, 2007), which either compete for food with people and domestic animals, or transmit diseases to them. Humans have struggled to control insect pests for centuries (Chaddick and Leek, 1972; Benner, 2006).

To manage insect pests, several methods for prevention, suppression, and control have been used, including cultural, physical, biological, and chemical control. Compared with other control measures today, chemical control is highly effective and easily applicable. Since the 1930s, methyl bromide, dichloro-diphenyl-trichloroethane (DDT), and organophosphorus (OP) insecticides have been developed and used (Thompson et al., 2005). These insecticides are inexpensive but effective against a broad spectrum of pests. However, there have been significant problems associated with the application of these insecticides. They are hazardous to humans, domestic animals, and natural environments. Another challenge is that lots of insect pests are resistant to these insecticides (Tvedten, 2007).

As first recognized by Mercurialis (an Italian physician) in 1577, some insects are vectors

of human diseases (Harwood and James, 1979). They transmit a variety of pathogens, such as viruses, bacteria, fungi, protozoa, and metazoan parasites, which lead to millions of human deaths every year. Mosquitoes are the most important insect vectors of human diseases. Over 70 *Anopheles* species transmit the protozoa *Plasmodium* that causes malaria (Lane, 1997). Malaria is one of the most significant and serious infectious diseases (Klowden, 1995). It infects 300 to 500 million people and kills 1.5 - 2.7 million humans annually -- mainly African children (Von Seidlein et al., 1998). *Anopheles gambiae* (Diptera: Culicidae) is a principal vector of malaria in Africa.

In all insect species, acetylcholinesterase (AChE) plays a crucial role in *A. gambiae* cholinergic synaptic transmission. AChE, therefore, is the target site of inhibition by OP and carbamate (CA) insecticides (Ware and Whitacre, 2004). Both OP and CA are very effective in controlling of *A. gambiae*. Unfortunately, *A. gambiae* has developed high resistance by gene mutation or expression increase (Djouaka, et al., 2008).

Objectives

The discovery of two different AChE genes (*ace*1 *and ace*2) from *A. gambiae* (Weill et al., 2002) has raised some fundamentally important questions: First, what are the differences between two AChE genes in the genome on characteristics, structures, and main functions including the OP and CA resistance? Second, how sensitive are these AChEs to substrates and inhibitors? Third, where and when are AChEs expressed in *A. gambiae*, and what are their expression patterns in different organs and tissue locations at different stages? To control *A. gambiae*, basic information is required. In this project, I

studied *A. gambiae* AChE1. My two objectives were as follows:

1. Characterize *Ag*AChE1 by sequencing, compare *Ag*AChE1 with *Ag*AChE2 and AChEs from other insects, express *Ag*AChE1 in *Spodoptera frugiperda* (Lepidoptera: Noctuidae) cells (*Sf*21). Optimize this expression system for high protein production. Develop an efficient purification method to get highly purified protein. Characterize the biochemical and molecular properties of *Ag*AChE1. Refine information on the kinetics of substrate hydrolysis and inhibitor specificity.

2. Characterize spatial and temporal expression patterns of *A. gambiae* AChE1 by using immunohistochemistry and *in situ* hybridization to suggest *in vivo* functions. This includes both mRNA localization and protein localization on tissue sections.

Studies of molecular structure, heterogeneity, function, and evolution of AChEs from *A. gambiae* will: (1) Generate useful information on the molecular complexity and heterogeneity for further study of biological functions of multiple AChEs. (2) Lead to the development of more selective anti-cholinesterase agents. Provide a basis for both insecticide design and understanding the structure for mutagenic resistance to OP and CA insecticides. Improve the selectivity of new insecticides for the African malaria mosquito control while reducing their toxicity toward humans and other vertebrates.

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CHAPTER II

LITERATURE REVIEW

1. The life cycle and behavior of *Anopheles gambiae*

There are over 3,000 species of mosquito worldwide (Yuval, 2006), about 380 of which belong to the genus of *Anopheles* (Gosling, 2005). Among them, 70 species are vectors of malaria (Lane, 1997), and about 40 are important (Service, 2000). *A. gambiae*, as an important malaria vector transmits the serious parasite, *Plasmodium falciparum*. It also carries and spreads three other malaria parasites: *P. vivax*, *P. malariae*, and *P. ovale* (Johnson, 2006). Malaria is a dangerous and sometimes fatal disease (Klowden, 1995). The parasites grow in red blood cells. At maturation, they destroy the host cells. Symptoms include high fever, chills, and flu-like illness. Malaria is epidemic mainly in tropical Africa, Central America, the Amazon basin, South and Southeast Asia. Malaria was eradicated or greatly reduced in many countries including the USA between the 1940s and 1960s (Lengeler et al., 2004).

A. gambiae has four stages in its life cycle: egg, larva, pupa, and adult. The adults of *A. gambiae* have a slender body, black and white scales on the wings, and long, needle-shaped, piercing mouthparts. The palps are as long as the proboscis. Their abdomens stick up when they rest. The females usually mate once. The females suck blood from people and, thus, transmit malaria. A blood meal is necessary for female *A. gambiae* to lay eggs. They often suck blood indoors and may rest indoors for a few hours after a blood meal. They lay their eggs singly directly on the water surface with floats on either side, not in rafts. They lay eggs at intervals throughout their life. One female usually lays 50-200 brown boat-shaped eggs per oviposition (Service, 2000). Most eggs

hatch into larvae within 2-3 days. There are four larval instars. Larvae do not have a siphon to take air. So the body is parallel to the surface of the water. They develop into pupae in 4-7 days. A pupa has a comma-shape, and does not feed. But they have to come to the water surface frequently to breathe. A pupa has a pair of respiratory trumpets on the cephalothorax. Usually, the pupal period lasts 1-3 days. The larvae and pupae are called wigglers and tumblers, respectively. Under good conditions, the whole life cycle from eggs to adults takes about 7-13 days (Fradin, 1998; Triplehorn and Johnson, 2005; Chen et al., 2006; Koutsos et al., 2007).

2. Basic background knowledge about acetylcholinesterase

The characteristics of acetylcholinesterase

Cholinesterases can be divided into two subfamilies based on their catalytic and inhibitory specificities: acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8). AChE is a serine hydrolase, which is found at the cholinergic nerve terminals. AChE breaks down the neurotransmitter acetylcholine (ACh) at the synaptic cleft so that the next nerve impulse can be transmitted across the synaptic gap. BChE can also hydrolyze acetylcholine. The difference between AChE and BChE is that the former hydrolyses ACh more quickly than the latter does, and that they have different inhibitory specificity (Bon and Greenfield, 2003; Greig et al., 2005).

The mechanism of AChE

Signals from nerve cells to muscle cells are carried by neurotransmitters in both

vertebrates and invertebrates. Of course, neurotransmitters must be cleaned up immediately after the message is passed; otherwise, the next signal is mixed up. ACh is an important neurotransmitter. AChE exists in the basement membrane around cholinergic nerve terminals. It plays an important role by removing synaptic old ACh through rapid and efficient hydrolysis into choline and acetate (Schumacher et al, 1986; Pope et al., 2005), and choline will be recycled for generating a new neurotransmitter for subsequent signaling. AChE breaks up each molecule of ACh in about 80 microseconds (Guillebeau, 2005). There are three amino acid residues implicated in the catalytic triad of the active site of the enzyme: serine, histidine, and glutamate (Taylor, 1991). AChE is a key enzyme in the insect nervous system (Fournier *et al*., 1992).

The history of AChE research

In 1914, Dale observed a phenomenon of ACh inactivated in a cat. He proposed the existence of an enzyme to remove ACh (Dale, 1914). A dozen years later, Loewi and Navratil demonstrated a prolonged action of ACh by using inhibition of the proposed enzyme with physostigmine (Burn and Rand, 1965). Stedman, et al. extracted the enzyme from horses in 1932, which they called choline-esterase (Stedman et al., 1932). Nachmansonhn purified AChE from electric eels (Silver, 1974). Leuzinger et al. successfully crystallized the enzyme from electric eels in 1968. The three dimensional (3D) structure of *Torpedo californica* AChE was first determined in 1991 by Sussman et al. Harel et al. (2000) reported the recombinant expression and three-dimensional structure determination of AChE and its complexes with inhibitors had been determined from the fruit fly, *Drosophila melanogaster* (Diptera: Drosophilidae).

Insect AChE and insecticide resistance

Insect AChE can be terminated by insecticides, which leads to insect paralysis and death. However, some insects show obvious resistance, such as mosquitoes and aphids (Zhu and Zhang, 2005).

In general, there are three mechanisms of resistance to insecticides: (1) reduction in penetration, (2) increased metabolism by esterases, mixed-function oxidases, or glutathione transferases, and (3) modification of the insecticide target by gene amplification, over transcription, or point mutations (Mutero, et al. 1994). Up to now, numerous studies have focused on insect AChEs because they are the targets of pesticides of the organophosphorus (OP) and carbamate (CA) classes. OPs and CAs are widely used to control various pests. OP and CA compounds can phosphorylate or carbamylate the active site serine of AChEs to block the hydrolysis of neurotransmitter ACh (Eldefrawi, 1985). Once AChE is carbamylated or phosphorylated, it no longer hydrolyzes the neurotransmitter **(**Boublik, et al. 2002). That leads to paralysis and death of susceptible strains. So far, more than 30 agricultural pest species have been reported to have AChEs that are insensitive to OPs or CAs, including *A. gambiae* (Zhu and Gao, 1999; N'Guessan et al. 2003; Casimiro et al., 2006). On the other hand, resistance also provides a model for studying adaptation of eukaryotes to a toxic environment.

3. Progress and prospect of insect AChE research

For years AChE has been one of the well-studied insect enzymes due to its physiological and toxicological significance. To date, insect AChEs have been studied biochemically and molecular biologically in at least 20 insect species (Zhu and Zhang, 2005).

Biochemical studies on insect AChE

The biochemical studies have been carried out in several insect species, including isolation and characterization of AChE from the fruit fly (*D. melanogaster*) (Gnagey et al., 1987), distribution and purification of AChE from the horn fly (*Haematobia irritans*) (Zhu and Zhang, 2005), and purification and characterization of AChE from the lesser grain borer (*Rhyzopertha dominica*) (Guedes et al., 1998), the Colorado potato beetle (*Leptinotarsa decemlineata*) (Zhu and Clark, 1994; 1995), the western corn rootworm (*Diabrotica virgifera virgifera*) (Gao et al., 1998), and the western tarnished plant bug (*Lygus hesperus*) (Zhu et al., 1991). As summarized by Zhu and his colleagues (1994; 2005), insect AChEs have the following features:

1. Substrate inhibition occurs at high concentrations of ACh in some insects. That is, the inhibition of AChE activity by ACh in the reaction catalyzed by AChE.

2. AChEs appear to be highly sensitive to eserine and 1, 5-bis (4-allyldimethylammoniumphenyl)-pentan-3- onedibromide (BW284C51) but less so to ethopropazine. BW284C51 and ethopropazine are specific inhibitors for AChE and BChE, respectively.

3. There is relatively low activity from insect AChE as compared with AChE activity from vertebrates.

Molecular biology on insect AChEs

The molecular studies have targeted some insect species. For example, mosquitoes include the malaria mosquito (*A. stephensi*) (Hall and Malcolm, 1991), the yellow fever mosquito (*Aedes aegypti*) (Liu et al., 1998), the African malaria mosquito (*A. gambiae*) (Weill et al., 2002), and the northern house mosquito (*Culex pipiens*) (Weill et al., 2003). Aphids include cotton aphid (*Aphis gossypii* Glover) (Li and Han, 2002; Javed et al., 2003), the greenbug (*Schizaphis graminum*) (Gao et al., 2002), and the potato peach aphid, which includes 3 species: *Myzus persicae* Sulzer (Javed et al., 2003), *Bemisia tabaci* Gennadius, and *Trialeurodes vaporariorum* Westwood (Javed et al., 2003; Zhu and Zhang, 2005). Flies include the fruit fly (*D. melanogaster*) (Hall and Spierer, 1986), the housefly (*Musca domestica*) (Huang et al, 1997), and the Australian sheep blowfly (*Lucilia cuprina*) (Chen et al., 2001). Other insects include the western tarnished plant bug (*L. Hesperus*) (Zhu et al., 1991), the cotton bollworm (*Helicoverpa armigera*) (Hübner) (Ren et al., 2002), the Colorado potato beetle (*L. decemlineata*) (Zhu and Clark, 1995), and the green rice leafhopper (*Nephotettix cincticeps*) (Tomita et al., 2000).

Most molecular studies on insect AChE have focused on understanding the molecular basis of altered AChE in conferring insecticide resistance. Point mutations, such as serine to glycine at certain positions of AChE, can cause decreased sensitivity of the enzyme to various insecticides in some insect species. Examples include the fruit fly (*D. melanogaster*) (Mutero et al, 1994), the Colorado potato beetle (*L. decemlineata*) (Zhu et al., 1996; Zhu and Clark, 1997), the housefly (*M. domestrica*) (Huang et al, 1997), the

yellow fever mosquito (*Aedes aegypti*) (Vaughan et al., 1997), the northern house mosquito (*C. pipiens*) (Weill et al., 2003; Weill et al 2004), and the African malaria mosquito (*A. gambiae*) (Etang et al., 2003; Weill et al 2004). These studies have implied that the tertiary structure of AChEs is very important for not only nervous system functioning but also insecticide efficacy. However, studies in the green rice leafhopper (*N. cincticeps*) could not identify any sequence changes in the AChE even though the sensitivities of AChE to inhibition were decreased when the insects were confirmed to be insecticide-resistant (Nomura et al., 2000).

Vertebrates have AChE and BChE. BChE has not been found in insects (Toutant, 1989). *Drosophila* was confirmed to have only one single AChE gene *ace*2 (Hall and Spierer, 1986; Myers *et al*., 2000). In 2000, the AChE gene of the fruit fly (*D. melanogaster*) was expressed successfully, and a three dimensional structure of the complex was determined by X-ray crystallography using *in vitro* expressed protein (Harel at el., 2000). Some insect species have two AChE genes to encode the enzymes with different substrate and inhibitor specificity. For instance, the northern house mosquito *(C. pipiens)* was first to be identified as having two AChE genes (Bourguet *et al.*, 1996). Todate, others with two AChE genes include the African malaria mosquito (*A. gambiae*) (Weill et al., 2002), the cotton aphid (*A. gossypii*) (Li & Han, 2002), the greenbug (*S. graminum*) (Gao *et al*., 2002), the diamondback moth (*Plutella xylostella*) (Baek *et al*., 2005), and the German cockroach (*Blattella germanica*) (Kim et al., 2006). There is some evidence suggesting that only one of the two gene products acts as the primary AChE to hydrolyze ACh (Weill *et al*., 2003; Nabeshima *et al*., 2004).

Unresolved questions in insect AChE research

There are many unresolved issues concerning insect AChE even though it is one of the most studied insect enzymes.

First of all, what are the differences in AChEs between vertebrates and insects? Up to now, only AChEs are reported in insects and there are no reports about BChE from any invertebrates. Several studies suggest that there are both AChE and BChE in some insects, such as the greenbug (*S. graminum*) (Zhu and Gao, 1999) and the western flower thrips (*Frankliniella occidentalis* Pergande) (Novozhilov et al., 1989; Liu et al., 1994). This hypothesis was based on two properties of insect AChE. The AChEs of aphids and thrips lack substrate inhibition at high concentrations and have low levels of substrate specificity. These two properties are consistent with the characteristics of a vertebrate BChE. On the other hand, this enzyme can be inhibited by sulfhydryl reagents such as 5, 5-dithio-bis (2-nitrobenzoic acid) (DTNB) (Toutant, 1989; Liu et al., 1994). Neither AChE of vertebrates nor AChE of other insect species has this typical characteristic of BChE (Toutant, 1989).

Secondly, why do some insects have two AChE genes? In vertebrates, there are multiple isoforms of AChE encoded by a single gene (Legay et al., 1993). In insects, after two AChE genes were identified in the northern house mosquito *(C. pipiens)* (Bourguet *et al.*, 1996), at least six insect species are known to have two AChE genes. *Drosophila* has only one AChE gene (Myers *et al*., 2000), why do other insects need more than one

AChE gene? Kim et al. (2006) analyzed 33 animal species and suggested that two *ace* genes were derived from a duplication event long before insects were differentiated. It is possible that *ace*1 was lost in some insects, such as the Cyclorrhapha dipterans (Higher fly), during the course of evolution (Weill *et al*., 2002). While most insects possess two *ace*s, only *ace*1 correlates with AChE insensitivity against the OP and CA pesticides (Weill *et al*., 2003; Nabeshima *et al*., 2004; Kim et al., 2006). This indicates that *ace*1 may play an important role in ACh hydrolysis. The functions of the second AChE in insects are still not clear. Little is known about the differences between two AChE genes in the genomes on characteristics, structures, or functions. Some studies have biochemically identified reduced sensitivities of AChEs associated with OP and/or CA resistance. But their corresponding AChE sequences are not available for functional assignment. For instance, the green rice leafhopper (*N. cincticeps*) contains a mixture of sensitive and insensitive AChE loci in a CA-resistant strain. No difference was found in the amino acid sequences between the resistant and susceptible strains (Nomura et al., 2000). It is likely that uncloned gene is responsible for the insecticide-resistance. If it is true, then the question is which one plays a role in neurotransmission? It might be hypothesized that if only one AChE is involved in neurotransmission, its protein must be expressed at cholinergic synapses.

Thirdly, of insect AChEs, substrate inhibition seems to be a common property (Toutant, 1989). AChEs from some insects do not exhibit excessive substrate inhibition, such as the greenbug (*S. graminum*) (Zhu and Gao, 1999), the western flower thrips (*Frankliniella occidentalis* Pergande) (Novozhilov et al., 1989; Liu et al., 1994), and some OP-resistant

strains of other insects (Zhu and Clark, 1995). What is the molecular mechanism of substrate inhibition in AChE? Is substrate inhibition caused by the binding of substrate molecules to peripheral anionic sites leading to an inactive enzyme-substrate-substrate complex (Radic et al., 1991)? Perhaps these insects have a different AChE that is sensitive to substrate concentration.

4. Research on *A. gambiae* AChEs

A. gambiae has two AChE genes, *ace*1 *and ace*2 (Weill et al., 2002). *Ace*2 is homologous to *D. melanogaster* AChE, whereas *ace*1 has no homologue in the fly (Weill et al., 2003). The gene *ace*1 was found in 15 mosquito species. In *A. gambiae, ace*1 is made up of 9 exons encoding polypeptides of 534 amino acids, while *ace*2 contains 4 - 9 exons encoding polypeptides of 569 amino acids. *A. gambiae* AChE1 (*Ag*AChE1) and AChE2 (*Ag*AChE2) (respectively encoded by *ace*1 and *ace*2) display 53% similarity at the amino acid level. A major difference between them is a 31-residue insertion in the AChE2 sequence (Weill et al., 2002).

Weill et al (2002) suggested that target AChEs are encoded by different types of AChE genes in various insect groups. In Dipteran, for example, only *ace*2 exists in Drosophilidae and Muscidae. *Ace*1 and *ace*2 exist in the family Culicidae. *Ace*1 is linked with insecticide resistance and probably encodes the OP-targeted AChE, but *ace2* is not associated with insecticide resistance (Malcolm et al., 1998; Mori et al., 2001). In the *ace*1 gene of *An. gambiae*, a mutation of glycine to serine at the 119th residue (GGT to

AGT in cDNA sequence) leads to high insecticide resistance. The substitution is exactly located in an oxyanion hole, indicating the pesticide insensitivity is related to its interference with the AChE catalytic functions. This resistance or high insensitivity is displayed in northern house mosquito (*C. pipiens*) at the same position (G119S) (Weill et al., 2004). Interestingly, a mutation of glycine to histidine at the 117^{th} position of human BChE is known to alter substrate specificity and confer insecticide resistance (Lockridge *et al*., 1997). In the blowfly carboxylesterase, a mutation of glycine to aspartic acid at the 137^{th} residue also alters substrate specificity (Newcomb *et al*., 1997). In both cases, OP hydrolysis is enhanced. There is a high degree of AChE1 insensitivity in *A. gambiae* (N'Guessan *et al.*, 2003). Five distinct mutations exist in *D. melanogaster* resistant strains, each providing a low resistance ratio (Mutéro *et al*., 1994). The resistance mechanism in *A. gambiae* could be detoxification (Vulule et al., 1999) or other mutations, such as leucine-to-serine substitution (Etang et al., 2003).

A sequence analysis of AChE shows that C^{286} and R^{339} of *A. gambiae* AChE are conserved at the opening of the active site of the model (Pang, 2006). The study revealed that these conserved residues are found in four insects and seventeen other invertebrate species. Neither residue is found in the active site of mammalian AChEs. Consequently, insecticides safe to vertebrates can be developed to interact with C^{286} or R^{339} and specifically block AChEs of mosquitoes and agricultural pests, if both residues are also not found in the active site of important invertebrates, such as, pollinators, beneficial predators, parasitic insects, and nematodes.

5. Localization of specific protein and nucleic acids

The background of localization

In situ hybridization is a technique that detects where a specific nucleic acid is located by visualizing a reporter molecule or probe that hybridizes with the target. The target can be DNA or RNA in the tissue. The probes can be labeled DNA or RNA molecules. In recent years, RNA probes have become more popular than DNA probes due to high sensitivity, even though RNA probes are harder to make and less stable.

In situ hybridization was first performed in the late 1960s (Gall and Pardue, 1969). Now, it is used successfully in a wide range of experiments (Brown, 1998). Both radioactively and non-radioactively labeled probes can be used for *in situ* hybridization on tissue sections or whole body mount. Non-radioactive probes result in better morphological resolution and are less hazardous. If probes derived from different regions of a gene yield an identical hybridization pattern, the hybridization specificity is verified.

Immunohistochemistry is used to detect protein targets in tissues by the use of specific antibodies. The antigen-antibody complexes are visualized by an enzyme, a fluorescent compound, or a radioactive element linked to the antibody molecules. This technique, developed in the early 1970s, has been widely used in various biomedical applications (Shi et al., 2001). The use of different fluorochromes coupled with secondary antibodies has been optimized for co-localization studies at the cellular level. In the 1990s, indirect immunohistochemistry has become a universal method, which uses a specific antibody

and an antispecies secondary antibody coupled with a fluorochrome, enzyme, or biotin to detect target proteins.

Localization studies on insect AChE

Localization studies on insect AChEs have been done in a few species such as the German cockroach (*Blattella germanica*) (Kim et al., 2006) and the fruit fly (*D. melanogaster*) (Zador and Budai, 1994; Zador, 1989). The German cockroach has two AChE genes. Based on the transcription patterns from *in situ* hybridization, both *ace*1 and *ace*2 encode active AChEs mainly expressed in the central nerve system (CNS). *Ace*1 is the predominant gene to encode AChE1 for synaptic transmission. The minor AChE2 coexists with AChE1 in the neuron network. However, the physiological function of AChE2 remains unknown (Kim et al., 2006). The transcripts of the AChE gene in *D. melanogaster* embryos were detected in CNS and the lateral chordotonal neurons as well (Zador and Budai, 1994). The expression of *D. melanogaster* AChE is also found in CNS of larvae and in the head and thorax of adults (Zador, 1989).

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CHAPTER III

EXPRESSION AND PURIFICATION OF ACETYLCHOLINESTERASE-1 FROM THE AFRICAN MALARIA MOSQUITO, *ANOPHELES GAMBIAE*

Abstract

Insect acetylcholinesterase (AChE, EC 3.1.1.7) plays an important role to break down neurotransmitter acetylcholine at synaptic clefts. Therefore, it is one of the most important target enzymes for insecticides. While two AChE genes (*ace*1 and *ace*2) have been reported from *Anopheles gambiae,* little is known about their biochemical properties. Recombinant expression and characterization of highly purified wild-type and mutant AChEs have served as a reliable platform for studying structure-function relationships. In this study, a cDNA fragment of *ace*1 from *A. gambiae* EST was subcloned, *Ag*AChE1 was expressed, and the protein purification scheme was optimized. After baculovirus amplification and expression, the final concentration of *Ag*AChE1 was up to 56 μg/ml and purified 2.5×10^3 fold. The three-step purification procedure took approximately eight hours and yield 51% of the protein near homogeneity. This system could be useful for recombinant AChE purification from other insect species.

Key words: Neurotransmission, Mosquito, Recombinant bacmid DNA, Heterologous expression, Optimized purification, Chromatography.

Introduction

Acetylcholinesterase is a serine hydrolase and well known for its important function at cholinergic synapses (Keller et al., 2001). It is one of the most efficient enzymes in nature and one of the best studied insect enzymes (Taylor and Radic 1994). To characterize a protein of interest (such as functional studies, protein-protein interactions, enzyme kinetics), determine its structure, prepare its antibody, and develop reagents and drugs, both high quality and a large quantity of the protein is needed. Very often, the original source of the protein is scarce. A recombinant protein is usually easier to prepare and purify than a natural protein, as the protein of interest can be fused with short tag peptides or partner proteins to improve expression level, solubility, detection, and purification of protein (Rosenberg, 2005; Müller, 2005).

There are two systems for protein expression, prokaryotic (bacterial) and eukaryotic (usually yeast, insect cell, or mammalian cell). Prokaryotic systems provide rapid growth, high expression, and minimum media, but no glycosylation. Extra steps are often needed for refolding and endotoxin removal. Eukaryotic systems overcome the problems of prokaryotic ones, but suffer from the slow growth, costly production, and variable yield (Lu et al., 2004). The advantages of using the insect cell baculovirus-expression system include lower cost for cell culturing than in mammalian cells and higher protein quality than in yeast (Tamás and Shewry, 2006).

AChEs have been purified to various degrees from at least 20 insect species for biochemical and toxicological analysis (Gnagey et al., 1987; Gao et al., 1998; Guedes et al., 1998; Zhu et al., 1991; Zhu and Clark, 1994; 1995; Zhu and Zhang, 2005). Molecular cloning of AChEs is achieved in more than 30 insect species, including flies, mosquitoes, wasps, aphids, moths, beetles, and cockroaches (Hall and Spierer, 1986; Hall and Malcolm, 1991; Anthony et al., 1995; Huang et al, 1997; Liu et al., 1998; Tomita et al., 2000; Chen et al., 2001; Gao et al., 2002; Li and Han, 2002; Ren & Han, 2002; Vontas et al., 2002; Weill et al., 2002, 2003; Javed et al., 2003; Ni et al., 2003; Zhu and Zhang, 2005; Lee et al., 2006). Sequence comparison and phylogenetic analysis indicate that most insects contain two AChE genes (*ace*1 and *ace*2) which arose from ancient gene duplication before the radiation of arthropod species (Kim et al. 2006). It is unclear if both AChEs function to break down ACh at cholinergic synapses.

Protein expression in insect cells infected with a baculovius vector was developed in the early 1980s (Smith et al., 1983). *Drosophila melanogaster* (Diptera, Drosophilidae) AChE has been expressed in *Drosophila* Schneider Line 2 cells using Lipofectin reagent (Life Technology, MD, USA) for X-ray structural determination (Incardona & Rosenberry, 1996; Harel at el., 2000). Shang et al. (2007) reported that expression of two AChEs from *Bombyx mori* (Lepidoptera: Bombycidae) in baculovirus infected *Trichoplusia ni* (Lepidoptera: Noctuidae) cells. Baculoviruses compose the most diverse

groups of arthropod viruses. Recent studies have shown that Noctuidae, such as *Autographa californica* (Lepidoptera: Noctuidae) and *Spodoptera frugiperda* (Lepidoptera: Noctuidae), is the best family of hosts for these viruses (Dong et al., 2005; Tamás and Shewry, 2006). The baculovirus expression system accepts large inserts of DNA and produces recombinant protein at a high level (Ikonomou et al., 2003; Philipps et al., 2005).

Anopheles gambiae (Diptera: Culicidae) is a principal vector of malaria parasites which cause nearly two million human deaths each year (Von Seidlein et al., 1998). The genomic sequence of *A. gambiae* has been completely determined (Holt et al., 2002). *A. gambiae* has two AChE genes that are 53% similar (Weill et al., 2002; 2003). However, none of them is expressed and purified for characterization. *A. gambiae* carrying AChE1 (G119S) exhibits high insecticide resistance, and G119 resides in the oxyanion hole (Weill et al., 2002) (Figure 1). To prepare *Ag*AChE1 enzyme for characterization of its properties and structure-function relationships, a cDNA clone encoding *Ag*AChE1 was incorporated into baculovirus, and expressed in *Spodoptera frugiperda* (*Sf*21) cells, and the enzyme was purified for future studies.

Materials and methods

Chemicals and materials

Wizard Minipreps DNA Purification kit (Promiga Corporation, WI, USA), alkaline phosphatase (Sigma Corporation, MO, USA), nitro-blue tetrazolium (NBT) and bromo-chloro-indoryl phosphate (BCIP) (Bio-Rad Laboratories, MO, USA), QIAprep Spin Miniprep kit (Qiagen Incorporation, CA, USA), Cellfectin (Invitrogen Life Technologies, CA, USA), Sf-900 II SFM (Invitrogen Life Technologies, CA, USA), pGEM T vector (Promega Corporation, WI, USA), nitrocellulose membrane (Osmonics Incorporation, Gloucester, MA, USA), Concanavalin A Sepharose (Sigma Corporation, MO, USA), and Ni-NTA agarose (Qiagen Incorporation, CA, USA) were purchased from the companies. Cell line *Spodoptera frugiperda* 21 (Invitrogen Life Technologies, CA, USA) was maintained in our laboratory for six years. *Ag*AChE1 EST clone (BM629847) was kindly provided by MR4/ATCC (the Malaria Reference and Research Reagent Resource Center /American Type Culture Collection).

Construction of *Ag*AChE1/pMFH6 and AgAChE1 expression

Subcloning of *Ag*AChE1 fragment: The EST clone (BM629847) (MR4/ATCC) was completely sequenced using vector- and gene-specific primers. The assembled cDNA was aligned with its gene to detect sequence variations. After the gene was analyzed, the region corresponding to the catalytic domain was amplified in a polymerase chain

reaction using primers j910 (GGAATTCACGACAACGATCCGCTG, nucleotides 702-725) and j911 (ACTCGAGGCTGCTTTCGCACG, reverse complement of nucleotides 2353- 2373). The thermal cycling conditions were 35 cycles of 94 ºC for 10 s, 45 ºC for 5 s and 60 ºC for 4 min. The PCR product (1.67 kb) was subcloned into pGEM T vector according to the manufacturer's directions. The ligation mixture was transformed into *E. coli* competent cells (DH5α). Transformations were grown in liquid media for plasmid isolation using QIAprep Spin Miniprep kit. Sequencing was performed at the Recombinant DNA/Protein Resource Facility at Oklahoma State University to confirm the correct insertion of the cDNA fragment.

Preparation of recombinant bacmid DNA: The 1.67 kb cDNA fragment was subcloned into pMFH6, a modified pFastBac1 plasmid, to generate *Ag*AChE1/pMFH6 (Ji et al., 2003; Lu and Jiang, 2007), which allows efficient secretion of *Ag*AChE1 containing a C-terminal hexa histidine tag. The recombinant plasmid was transformed into competent cells (*E. coli* DH10Bac), which contains a helper plasmid for transposition into the bacmid. White colonies, after streaking on the selection plate, were picked for culturing and bacmid isolation using Wizard Plus Minipreps DNA Purification System. Transposition of *Ag*AChE1 fragment was verified by PCR analysis.

Transfection of *Spodoptera frugiperda* (*Sf*21) cells: The insect cell line was maintained and propagated in *Sf*-900 II SFM (serum-free medium) with added antibiotics (2 ml of

Sf-900 II SFM containing 50 U/ml penicillin and 50 µg/ml streptomycin final concentration). *Sf*21 cells were transfected with recombinant bacmid DNA using Cellfectin (Invitrogen). The initial viral stock (V_0) was harvested at 48-72 hours (h) post-transfection. The virus titer was maximized through serial infection of Sf21 cells. The protein level in the medium was examined by immunoblot analysis and enzyme activity assay (Rivkin et al. 2006). The final viral stock, containing the highest level of baculovirus, was stored at -80 °C for further experiments.

Expression of *A. gambiae* AChE1: *Sf*21 cells (at 2.4×10^6 cells/ml) in 300 ml of Ultimate insect serum-free medium (Invitrogen) were infected with the viral stock at a multiplicity of infection (MOI) ranging from 0.05 to 0.1 and grown at 27 °C for 72 h with agitation at 100 rpm. After the cells were removed by centrifugation at 5,000 g for 10 min, the cell culture supernatant was diluted with equal volume of deionized distilled water (ddH_2O) and the solution pH was adjusted to 6.2.

Immunoblot analysis: After removing the cells by centrifugation at 5,000 g for 5 min, the cultural supernatant (80 µl) was mixed with 20 µl 5 × sodium dodecyl sulfate (SDS) sample buffer and incubated at 100 °C for 5 min. The cells were mixed with cell lysis buffer and incubated on ice for 30 min. After centrifugation at 10,000 g for 15 minutes (min) at 4 °C, 80 µl cellular supernatant was transferred to a new tube, and the cellular pellet was suspended in 80 µl ddH_2O. Both 80 µl samples, cellular supernatant and cellular pellet suspension, were treated with 20 µl 5 × SDS sample buffer and incubated

at 100 °C for 5 min. Following SDS-PAGE separation, the proteins were transferred onto a nitrocellulose membrane. Immunodetection was carried out using rabbit anti-$(His)_5$-tag antibody and goat anti-rabbit IgG (Immunoglobulin G) conjugated to alkaline phosphatase. The membrane was developed using NBT and BCIP.

Purification of *Ag*AChE1 from the cell culture supernatant

Preparing protein supernatant: After expression, the insect cell culture was centrifuged at 5,000 g for 10 min at 4 °C to remove the cells. The supernatant was aliquoted and stored in -80 °C.

Dextran sulfate (DS) chromatography: The thawed cell culture supernatant (600 ml) and 600 ml ddH_2O was mixed and the pH of the mixture was adjusted to 6.2 using 1N sodium hydroxide. DS coupled to Sepharose CL-6B (Nakamura et al., 1985) (75 ml) was mixed with the solution for one hour (h) at 4 °C, and loaded onto an empty column (100 ml – column volume). The column was washed with 200 ml buffer A (10 mM potassium phosphate, 0.01% Tween 20, pH 6.2). The proteins were eluted with 300 ml 1M NaCl in buffer A.

Concanavalin A (Con A) chromatography: $MgCl_2$ was added to the eluted proteins to a final concentration of 2 mM, and the pH of the solution was adjusted to 7.5 using 1N

sodium hydroxide. Con A Sepharose (10 ml) was mixed with the protein for one h at 4 ^{o}C, and loaded onto an empty column (30 ml). The column was washed with 50 ml buffer B (20 mM sodium phosphate, 0.5 M NaCl, 0.01% Tween 20, pH 7.5). The proteins were eluted with 210 ml 0.4 M methyl α-D-manno-pyranoside in buffer B.

Nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography: The eluted proteins (200 ml), after pH adjustment to 8.0 using 1N sodium hydroxide, were mixed with 3 ml Ni-NTA agarose for one h at 4 °C, and loaded onto an empty column (10 ml). The column was washed with 15 ml buffer C (20 mM Tris-HCl, 0.3M NaCl, 10 mM imidazole, 0.01% Tween-20, pH 8.0). The protein was eluted stepwise with 80 mM (6 ml), 100 mM (2 ml), and 250 mM (8.5 ml) imidazole in buffer C, respectively, the purified enzyme was stored at -80 ºC in the presence of 5% glycerol.

Protein concentration measurement: Protein concentration was determined by a modified Bradford method using a commercial kit (Pierce) (Smith et al., 1985) and bovine serum albumin (BSA) as a standard. The assay was done at room temperature using a VERSAmax microplate reader at 560 nm.

Determination of *Ag*AChE1 activity: Purified *Ag*AChE1 activity was determined based on the modified Ellman method (Zhu and Clark, 1994) using ATC and DTNB in a total volume of 100 µl. The assay was done at room temperature on a 96-well microplate using

a VERSAmax microplate reader (Molecular Devices). One unit of AChE activity is defined as the amount of enzyme hydrolyzing one µmol of ATC in one min. The activity (µmol/min/ml = U (enzyme unit) /ml) was calculated as [detection number (mOD/min) × dilution factor × 1,000 (M to mM or µmol/ml) × assay dilution factor] / [13600 (ε: $M^{-1}cm^{-1}$) × 0.3 cm (light path) × 1,000 (mOD to OD)]. The specific activity (U/mg protein) was calculated as activity (U/ml) / protein concentration (mg/ml).

Results

Features of *A. gambiae ace*1

In order to decide the *Ag*AChE1 cDNA fragment for recombinant DNA construction, *Agace*1 sequence was analyzed by searching GenBank at National Center for Biotechnology Information (NCBI). The full-length *Ag*AChE1 cDNA, 3,574 bp long, contains an open reading frame (ORF) ranging from nucleotides 276-2441 (Figure 2). The 5' untranslated region (UTR) corresponds to exon 1, exon 2, and 5' end of exon 3 of the gene. The sizes of introns 1-3 (954, 3925 and 1938 bp) are significantly longer than those of introns 4-8 (86, 79, 86, 66, 107 bp). The rest of exon 3 encodes a 24-residue signal peptide for secretion and, along with exon 4, encodes a Ser/Ala-rich pro-region. Since its counterpart was absent in the purified greenbug (*Schizaphis graminum*) AChE1 (Gao and Zhu, 2001), it was suspected that proteolytic processing also occurs in the

maturation of *A. gambiae* AChE1. Exons 5-8 encode the entire catalytic domain, followed by a carboxyl-terminal tail critical for self-association and membrane anchorage. Exon 9 encodes the tail and 3'UTR (1,118 bp). The AATAAA motif near the 3' end may act as the signal for polyadenylation. A comparison of the cDNA and gene sequences revealed eight synonymous substitutions. Based on the deduced *Ag*AChE1 protein sequence, a region of 1, 671 bp from *Agace*1 cDNA (nucleotide 702 to 2372) was selected for recombinant DNA constrction. This insert covered all parts of exons 5, 6, 7, 8, and 54 nucleotides of exon 9 (Figure 2 and 3).

Construction of *Ag*AChE1/pMFH6 and *Ag*AChE1 expression

To express the enzyme for functional analysis, the region coding for the catalytic domain was amplified by PCR and inserted into the *Eco*RI and *Xho*I sites of pMFH6. The insertion was confirmed by sequencing. The recombinant bacmid DNA 139 kb, which contains a bacmid vector, donor segments, and an *Agace*1 insert of 1.67 kb, was constructed. Successful transposition of the insert was verified to the bacmid by PCR analysis. The DNA insert was confirmed using two vector-specific primers and three gene-specific primers.

The resulting plasmid (*Ag*AChE1/pMFH6) was used to generate a viral stock through transposition, transfection and serial amplification. A suitable viral stock was obtained for large-scale expression. The protein was examined by SDS-PAGE analysis from

Coomassie blue staining (not shown), silver staining, and Western blot (Figure 4). Then expression was performed after amplification. Under the optimal conditions, the recombinant AChE was secreted by the baculovirus-infected *Sf21* cells at a final concentration of 3.8 mg/L.

Purification from the cell culture supernatant

A single protein band was detected on SDS-PAGE by Coomassie staining, silver staining and immunoblot. The overall purification factor and yield were 2491-fold and 51.8% (Table 1). *Ag*AChE1 from cell culture supernatant was purified by ion exchange and affinity chromatography. Following an ion exchange step, the captured protein was eluted from the polycationic resin in an enriched form free of medium components that interfere with affinity chromatography. A fifteen-fold increase in specific activity was achieved using Con A-Sepharose. Similar to the ion exchange step, *A. gambiae* AChE1 strongly associated with the resin and came off the column in a large volume. The recombinant protein in the pooled fractions bound to the Ni^{2+}-NTA agarose so tightly that, while 80-100 mM imidazole efficiently removed loosely associated proteins, most *A. gambiae* AChE1 remained attached to the column, until 250 mM imidazole was applied. The eluted enzyme was essentially pure and recognized by the monoclonal antibodies against the hexahistidine tag (Figure 5). The overall purification factor was 2.5×10^3. 476 µg of

purified *Ag*AChE1 with a specific activity of 523.10 U/mg was obtained from 600 ml of cell culture supernatant (Table 1).

Discussion

Acetylcholinesterase plays an important role in insect central nervous system. Although molecular cloning of AChEs has been studied in at least 30 insect species, only a few insect AChEs have been expressed as recombinant protein. In this study, I subcloned a fragment of AChE1 cDNA from *A. gambiae* EST (BM629847), heterologously express the protein, and purify it by ion exchange and affinity chromatography. *Ag*AChE1 was cloned into pMFH6 which contains a secretion peptide of *Apis mellifera* (Hymenoptera: Apidae) and a C-terminal hexahistidine tag. The former allows efficient secretion of *Ag*AChE1, and the latter helps the purification of *Ag*AChE1. A recombinant baculovirus was constructed to express *Ag*AChE1 catalytic domain in a soluble active form. After optimizing the expression conditions and purification procedures, a high yield of recombinant proteins was obtained.

Multiple forms of AChE exist in some insect species (Li & Han, 2002). However, in this study, only one form was found in AChE1 of *A. gambiae* by SDS-PAGE analysis. Furthermore, the procainamide-based affinity ligand had excellent affinity for the AChE

of the greenbug (*Schizaphis graminum*, Hymenoptera: Aphidiidae) (Gao & Zhu, 2001), but it did not bind the AChE1 of *A. gambiae* efficiently (data not shown). This suggests that *A. gambiae* AChE1 is different from the greenbug AChEs.

To increase the yield of *Ag*AChE1, expression and purification procedures were optimized. In this study, selection of ion exchange and affinity chromatography was based on the charge, glycosylation, and hexahistidine tag of *Ag*AChE1. A different pH in each step and the salt or ligand concentration in elution buffers were also vital factors. For instance, the pH was adjusted to 6.2 for DS column, 7.5 for Con A column, and 8.0 for Ni-NTA column. This optimized three-step scheme enhanced the purity and enriched the enzyme. To improve yield, fractions containing low levels of *Ag*AChE1 can be diluted 10 times for a second pass through a nickel affinity column. On the other hand, shortening the purification time is also important for maintaining the activity of *Ag*AChE1. In addition, I found that using proper stepwise elution instead of gradient elution helped to improve the product concentration and purity. The entire procedure took approximately 8 hours and gave about 51% final yield. *Ag*AChE1 protein has been expressed and purified, making characterization of *Ag*AChE1 properties more practicable. After the antibody preparation, localization of spatial and temporal expression patterns of *Ag*AChE1 will be possible. The success of *Ag*AChE1 expression and purification also facilitates the *Ag*AChE1 crystallization in future work.

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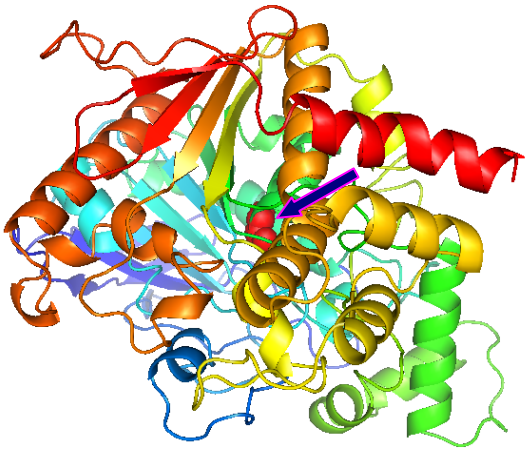

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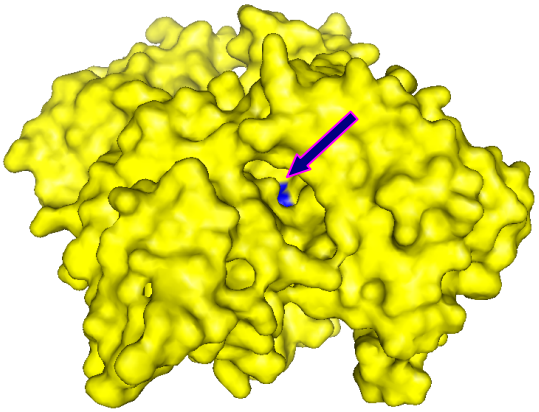
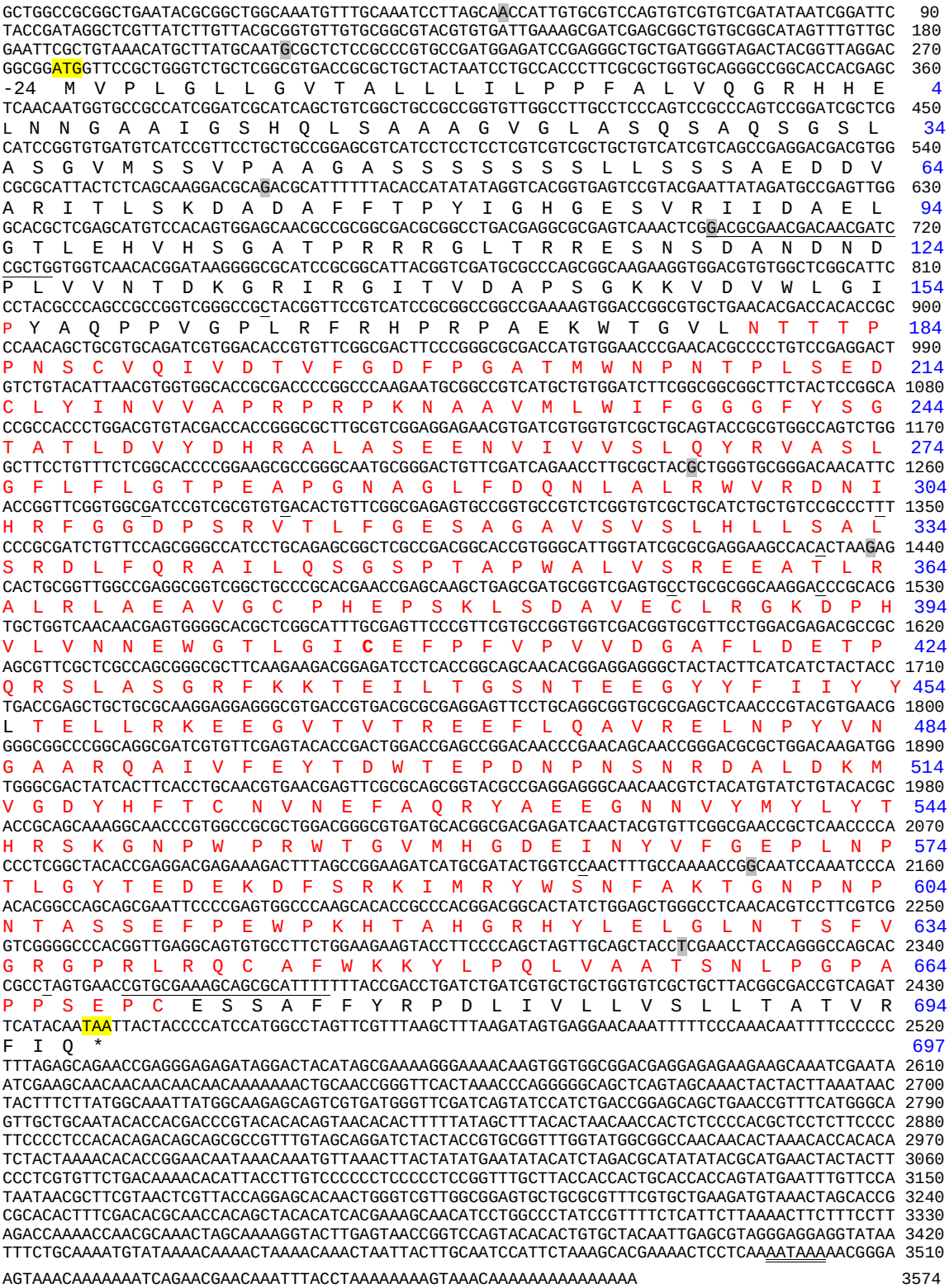


B

Figure 1. A ribbon (A) and space-filling (B) model for *Ag*AChE1. The substitution (G119S) is localized in an oxyanion hole marked by the arrow. The model was built using SWISS-MODEL (Torsten Schwede's Structural Bioinformatics Group at the Biozentrum, University of Basel, Switzerland) and presented by PyMOL (created by PyMOL Warren Lyford DeLano and commercialized by DeLano Scientific LLC, San Carlos, CA, USA).



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GTCTGTACATTAACGTGGTGGCACCGCGACCCCGGCCCAAGAATGCGGCCGTCATGCTGTGGATCTTCGGCGGCGGCTTCTACTCCGGCA 1080
C L Y I N V V A P R P R P K N A A V M L W I F G G G F Y S G 244
CCGCCACCCTGGACGTGTACGACCACCGGGCGCTTGCGTCGGAGGAGAACGTGATCGTGGTGTCGCTGCAGTACCGCGTGGCCAGTCTGG 1170
T A T L D V Y D H R A L A S E E N V I V V S L Q Y R V A S L 274
GCTTCCTGTTTCTCGGCACCCCGGAAGCGCCGGGCAATGCGGGACTGTTCGATCAGAACCTTGCGCTACGCTGGGTGCGGGACAACATTC 1260
G F L F L G T P E A P G N A G L F D Q N L A L R W V R D N I 304
ACCGGTTCGGTGGCGATCCGTCGCGTGTGACACTGTTCGGCGAGAGTGCCGGTGCCGTCTCGGTGTCGCTGCATCTGCTGTCCGCCCTTT 1350
H R F G G D P S R V T L F G E S A G A V S V S L H L L S A L 334
CCCGCGATCTGTTCCAGCGGGCCATCCTGCAGAGCGGCTCGCCGACGGCACCGTGGGCATTGGTATCGCGCGAGGAAGCCACACTAAGAG 1440
S R D L F Q R A I L Q S G S P T A P W A L V S R E E A T L R 364
CACTGCGGTTGGCCGAGGCGGTCGGCTGCCCGCACGAACCGAGCAAGCTGAGCGATGCGGTCGAGTGCCTGCGCGGCAAGGACCCGCACG 1530
A L R L A E A V G C P H E P S K L S D A V E C L R G K D P H 394
TGCTGGTCAACAACGAGTGGGGCACGCTCGGCATTTGCGAGTTCCCGTTCGTGCCGGTGGTCGACGGTGCGTTCCTGGACGAGACGCCGC 1620
V L V N N E W G T L G I C E F P F V P V V D G A F L D E T P 424
AGCGTTCGCTCGCCAGCGGGCGCTTCAAGAAGACGGAGATCCTCACCGGCAGCAACACGGAGGAGGGCTACTACTTCATCATCTACTACC 1710
Q R S L A S G R F K K T E I L T G S N T E E G Y Y F I I Y Y 454
TGACCGAGCTGCTGCGCAAGGAGGAGGGCGTGACCGTGACGCGCGAGGAGTTCCTGCAGGCGGTGCGCGAGCTCAACCCGTACGTGAACG 1800
L T E L L R K E E G V T V T R E E F L Q A V R E L N P Y V N 484
GGGCGGCCCGGCAGGCGATCGTGTTCGAGTACACCGACTGGACCGAGCCGGACAACCCGAACAGCAACCGGGACGCGCTGGACAAGATGG 1890
G A A R Q A I V F E Y T D W T E P D N P N S N R D A L D K M 514
TGGGCGACTATCACTTCACCTGCAACGTGAACGAGTTCGCGCAGCGGTACGCCGAGGAGGGCAACAACGTCTACATGTATCTGTACACGC 1980
V G D Y H F T C N V N E F A Q R Y A E E G N N V Y M Y L Y T 544
ACCGCAGCAAAGGCAACCCGTGGCCGCGCTGGACGGGCGTGATGCACGGCGACGAGATCAACTACGTGTTCGGCGAACCGCTCAACCCCA 2070
H R S K G N P W P R W T G V M H G D E I N Y V F G E P L N P 574
CCCTCGGCTACACCGAGGACGAGAAAGACTTTAGCCGGAAGATCATGCGATACTGGTCCAACTTTGCCAAAACCGGCAATCCAAATCCCA 2160
T L G Y T E D E K D F S R K I M R Y W S N F A K T G N P N P 604
ACACGGCCAGCAGCGAATTCCCCGAGTGGCCCAAGCACACCGCCCACGGACGGCACTATCTGGAGCTGGGCCTCAACACGTCCTTCGTCG 2250
N T A S S E F P E W P K H T A H G R H Y L E L G L N T S F V 634
GTCGGGGCCCACGGTTGAGGCAGTGTGCCTTCTGGAAGAAGTACCTTCCCCAGCTAGTTGCAGCTACCTCGAACCTACCAGGGCCAGCAC 2340
G R G P R L R Q C A F W K K Y L P Q L V A A T S N L P G P A 664
CGCCTAGTGAACCGTGCGAAAGCAGCGCATTTTTTTACCGACCTGATCTGATCGTGCTGCTGGTGTCGCTGCTTACGGCGACCGTCAGAT 2430
P P S E P C E S S A F F Y R P D L I V L L V S L L T A T V R 694
TCATACAATAATTACTACCCCATCCATGGCCTAGTTCGTTTAAGCTTTAAGATAGTGAGGAACAAATTTTTCCCAAACAATTTTCCCCCC 2520
F I Q * 697
TTTAGAGCAGAACCGAGGGAGAGATAGGACTACATAGCGAAAAGGGAAAACAAGTGGTGGCGGACGAGGAGAGAAGAAGCAAATCGAATA 2610
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TACTTTCTTATGGCAAATTATGGCAAGAGCAGTCGTGATGGGTTCGATCAGTATCCATCTGACCGGAGCAGCTGAACCGTTTCATGGGCA 2790
GTTGCTGCAATACACCACGACCCGTACACACAGTAACACACTTTTTATAGCTTTACACTAACAACCACTCTCCCCACGCTCCTCTTCCCC 2880
TTCCCCTCCACACAGACAGCAGCGCCGTTTGTAGCAGGATCTACTACCGTGCGGTTTGGTATGGCGGCCAACAACACTAAACACCACACA 2970
TCTACTAAAACACACCGGAACAATAAACAAATGTTAAACTTACTATATGAATATACATCTAGACGCATATATACGCATGAACTACTACTT 3060
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TAATAACGCTTCGTAACTCGTTACCAGGAGCACAACTGGGTCGTTGGCGGAGTGCTGCGCGTTTCGTGCTGAAGATGTAAACTAGCACCG 3240
CGCACACTTTCGACACGCAACCACAGCTACACATCACGAAAGCAACATCCTGGCCCTATCCGTTTTCTCATTCTTAAAACTTCTTTCCTT 3330
AGACCAAAACCAACGCAAACTAGCAAAAGGTACTTGAGTAACCGGTCCAGTACACACTGTGCTACAATTGAGCGTAGGGAGGAGGTATAA 3420
TTTCTGCAAAATGTATAAAACAAAACTAAAACAAACTAATTACTTGCAATCCATTCTAAAGCACGAAAACTCCTCAAAATAAAAACGGGA 3510
AGTAAACAAAAAAATCAGAACGAACAAATTTACCTAAAAAAAAGTAAACAAAAAAAAAAAAAAA 3574

Figure 2. Nucleotide sequences of *A. gambiae* AChE1. The last nucleotide of each exon is *shaded* to indicate the splicing junctions. Single nucleotide differences between the cDNA and the gene are *underlined*. The polyadenylation signal (AATAAA) is *double underlined*. Amino acid residues, shown in one-letter abbreviations, are aligned with the second nucleotide of each codon. The predicted catalytic domain is in *red*. The start and stop codons are *shaded* with *yellow*. The primer binding sites for recombinant expression in the baculovirus-insect cell system are *underlined*.

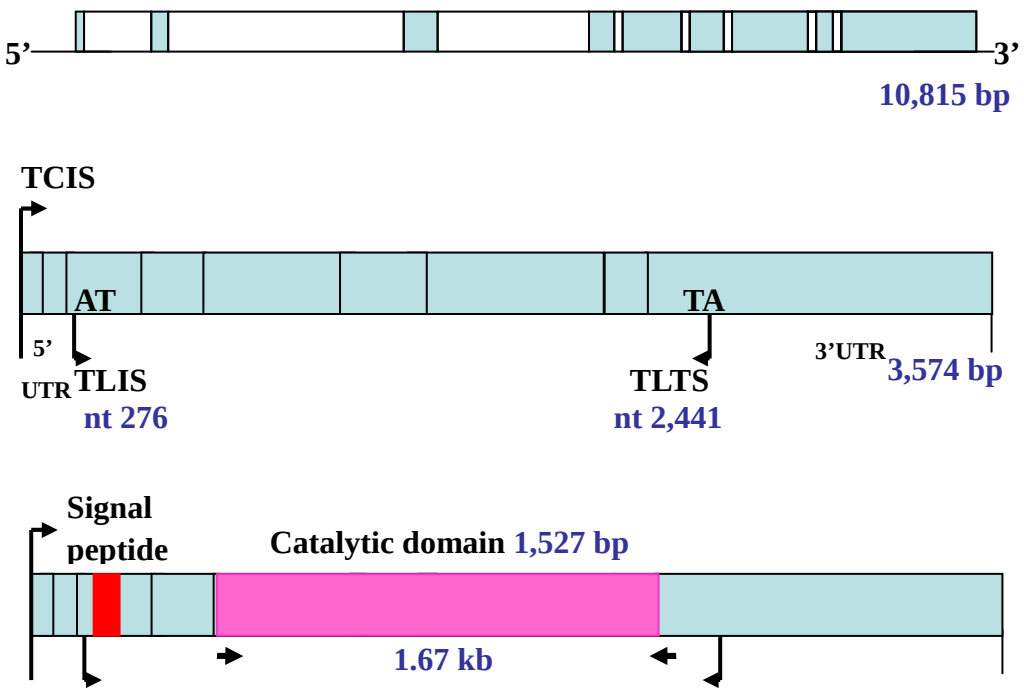

Figure 3. Schematic diagram of nucleotide sequences of *A. gambiae ace*1. *Agace*1 gene, 10,815 bp, contains 9 exons and 8 introns (upper). The full-length cDNA, 3,574 bp long, contains an open reading frame (ORF) ranging from nucleotides 276-2,441 (middle). Exons 5-8 encode the entire catalytic domain, 1,527 bp. A region of 1, 671 bp from *Agace*1 cDNA (nucleotide 702 to 2372) was selected for recombinant DNA (lower). TCIS: Transcription initiation site. TLIS: Translation initiation site. TLTS: Translation termination site.

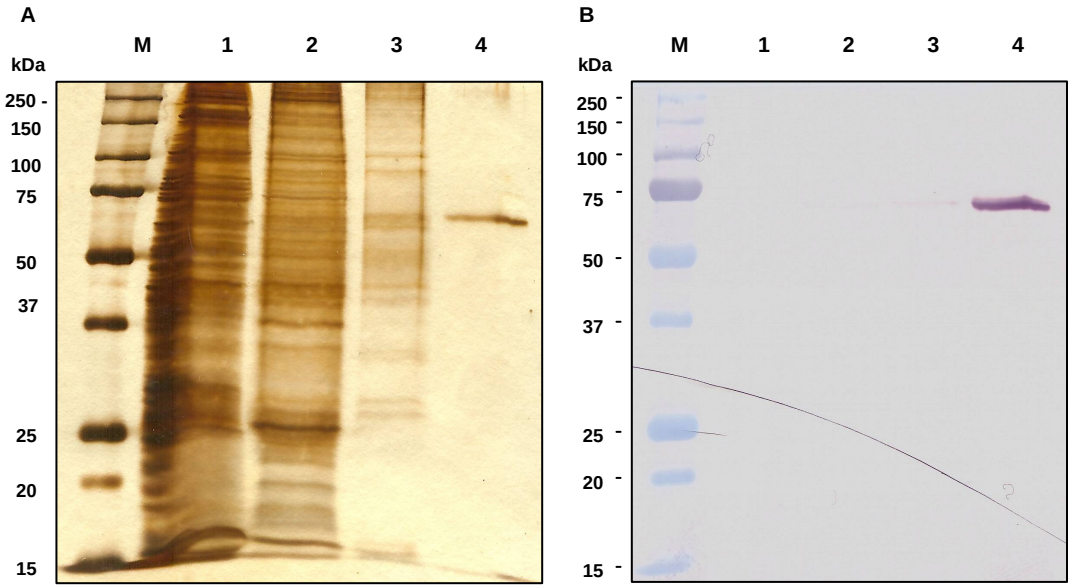

Figure 4. SDS-PAGE analysis of *Ag*AChE1. *Ag*AChE1 was loaded to each lane of 12 µl on 10% gels. The gels were run for 35 minutes. M: Marker, lane 1: Cell supernatant, lane 2: After DS column, lane 3: After Concanavalin A column, lane 4: After Nickel column. A: Silver staining. B: Immunoblot.

Table 1. Purification of AChE1 from *Anopheles gambiae*

Sample	Volume (ml)	Protein		Activity		Yield (%)	Specific activity (U/mg)	Purification (fold)
		(μg/ml)	(mg)	(U/ml)	(U)			
Medium	600	3820	2292	0.81	4.8×10^2	100	0.21	1
Dextran sulfate	300	978	293	1.18	3.5×10^2	73.1	1.21	6
Con A	210	81	17.0	1.47	3.1×10^2	64.0	18.16	86
Ni-NTA	8.5	56	0.48	29.54	2.5×10^2	51.8	523.10	2491

U =μM/min

CHARPTER IV

CHARACTERIZATION OF ACETYLCHOLINESTERASE-1 PROPERTIES FROM THE AFRICAN MALARIA MOSQUITO, *ANOPHELES GAMBIAE*

Abstract

Being the target enzymes of organophosphorus and carbamate pesticides, acetylcholinesterases (AChEs) and their genes have been isolated from susceptible and resistant insects to study the molecular basis of target site sensitivity and resistance. However, due to the existence of other resistance mechanisms, it can be problematic to correlate directly a mutation with resistance phenotype. In this work, biochemical and molecular properties of *Anopheles gambiae* AChE1 (*Ag*AChE1) were characterized. *Ag*AChE1 sequence deduced from cDNA was predicted and analyzed. The best range for *Ag*AChE1 reaction with acetylcholine is pH 7.0-8.5. The enzyme migrated as a single band at 65 and 130 kDa positions on SDS-polyacrylamide gels under reducing and nonreducing conditions, respectively. While K_M's of the *Ag*AChE1 for ATC (68 μM), AβMTC (79 μM), PTC (63 μM) and BTC (60 μM) were comparable, the V_{max}'s were substantially different: 209, 122, 84 and 15 μM/min/mg, in the order given. The IC_{50}'s showed that *Ag*AChE1 was highly sensitive to inhibition by eserine and BW284C51, but was less so by ethopropazine. The bimolecular association rate constant k_i and the dissociation constant K_d of six inhibitors for *Ag*AChE1 were $4.00 \times 10^3 \sim 2.19 \times 10^6$ M^{-1} min^{-1} and $1.19 \times 10^{-3} \sim 9.90 \times 10^{-8}$ M. Unimolecular bonding rate constant k_2 ranged from $0.06 \sim 53.62$ min^{-1}. *Ag*AChE1 is most sensitive to malaoxon and BW284C51, least so to carbaryl and ethopropazine. The affinity of BW284C51 was about 1.21×10^4 -fold greater than the affinity of carbaryl. *A. gambiae* AChE1 is a physiologically relevant enzyme for ACh hydrolysis at cholinergic synapses. The data may help to better understand the development of insecticide resistance in the African malaria mosquito.

Key words: Neurotransmission, Insecticide resistance, Mosquito, Malaria, Acetylcholine.

Introduction

Acetylcholinesterases play an essential role in neurotransmission at cholinergic synapses by rapidly hydrolyzing acetylcholine in insects and other animals including humans (Toutant, 1989). Classes of pesticides, such as organophosphates (OP) and carbamates (CA), have been developed to inhibit AChEs, which competitively inhibit AChE and lead to accumulation of the neurotransmitter and continuous stimulation of their nervous system (Fournier and Mutéro. 1994; Bourguet et al., 1997; Kozaki et al., 2002; Pope et al., 2005). Because vertebrate AChEs are similar in structure and function to the insect enzymes, application of chemical pesticides such as OPs is strictly controlled to prevent accidental exposure of people and livestock to these toxic compounds. On the other hand, severe resistance has developed in many insect pests (Ware and Whitacre, 2004; Tvedten, 2007), rendering the existing insecticides ineffective against insect pests and human disease vectors. This situation calls for the development of a new generation of compounds that are highly selective against the target enzymes in insects. In-depth understandings of their structure function and molecular properties are prerequisites for the potential success of this approach.

Insect AChEs, due to their physiological and toxicological significance, have been

intensively studied (Zhu and Zhang, 2005). So far, over 70 arthropod AChE cDNA sequences are available at GenBank. Only *Drosophila* AChE has been expressed in insect cells for X-ray structural determination (Harel at el., 2000). Many insects contain two AChE genes, which originated from ancient gene duplication (Kim et al., 2006). While higher flies lost one of the two genes later on (Weill *et al*., 2002). It is not clear why some insects have two AChEs and what their functions are. Recently, people reported that *ace*1 mutations were responsible for resistance to the OP, for instance, mosquito (*Culex pipens* and *Anopheles gambiae*), aphid (*Myzus persicae* and *Aphis gossypii*), and moth (*Plutella xylostella*) (Weill *et al*., 2002, 2003; Nabeshima *et al*., 2003; Toda *et al*., 2004; Andrew *et al*., 2004; Baek *et al*., 2005). In fact, most biochemical and molecular studies on insect AChEs fail to pinpoint the exact cause for insecticide resistance due to the existence of two genes and other resistant mechanisms.

The African malaria mosquito, *Anopheles gambiae* (Diptera: Culicidae), is a principal vector of malaria parasites which cause nearly two million human deaths each year and infects many more in the world (Von Seidlein et al., 1998). The mosquito carries two AChE genes but none of them has been characterized biochemically. The two genes are 53% similar in amino acid sequence. *A. gambiae* AChE2 contains a 31-residue insert and is more similar to *Drosophila* AChE (Weill et al., 2002). Intrigued by the possibility to develop highly specific and environmentally safe pesticides against this disease vector (Pang, 2007), the properties of *A. gambiae* AChE1 were characterized.

Materials and methods

Chemicals

Acetyl-(β-methyl)thiocholine iodide (AβMTC), acetylthiocholine iodide (ATC), 1,5-bis(4-allyldimethylammonium phenyl)-pentan-3-one dibromide (BW284C51), bicinchoninic acid (BCA), *S*-butyrylthiocholine iodide (BTC), 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB), eserine, ethopropazine, propionylthiocholine iodide (PTC), and carbaryl were purchased from Sigma-Aldrich Inc or generously provided by Dr. Kun-yan Zhu at Kansas State University. Paraoxon and malaoxon were kindly provided by Dr. Carey N. Pope at Oklahoma State University. PNGase and O-Glycosidase were purchased from New England BioLabs Inc (Ipswich, MA. USA) and Sigma-Aldrich Inc (St. Louis, MO. USA) to detect *N*-linked and O-linked glycosylation.

Feature prediction of *Ag*AChE1

The deduced amino acid sequence of *A. gambiae* AChE1 was analyzed by using Biology WorkBench. The sequence was aligned with those of AgAChE2 and AChEs from other animals using a ClustalW (version 2.0). The molecular weight (MW), isoelectric points (pI), and amino acid composition of recombinant *Ag*AChE1 were calculated using MacVector (version 7.2).

Characterization of *Ag*AChE1 properties

Determination of protein concentration and activity: *Ag*AChE1 concentration was measured by a modified Bradford method (Smith et al., 1985). The activity was determined based on the modified Ellman method (Zhu and Clark, 1994) (see chapter III).

Optimal pH: To determine the optimal pH for *Ag*AChE1, 17 µl amphoteric buffer (1:5 diluted polybuffer 96, Amersham Biosciences) at ten different pH from 5.5 to 10.0, diluted protein sample (3 µl) and substrate solution (80 µl) were mixed for kinetic measurement at 405 nm. In the control, *Ag*AChE1 was replaced by buffer C (3 µl, see chapter III). After activity measurement, pH of each mixture was determined using a microelectrode (Sentron pH-System) at room temperature at the Recombinant DNA/Protein Resource Facility at Oklahoma State University.

Association status: Molecular weight (MW) of *Ag*AChE1 was determined by gel filtration chromatography on a HPLC column. *Ag*AChE1 was eluted in 50 mM sodium phosphate, pH of 7.5, containing 300 mM NaCl. The HPLC gel filtration column was calibrated with molecular weight standards (670 kDa thyroglobulin, 158 kDa IgG, 44 kDa chicken ovalbumin, 17 kDa equine myoglobin, and 1.35 kDa vitamin B12). The activity in the fractions (fraction / ten sec) was measured and shown along with the absorbance at 214 nm.

Electrophoretic analysis of *Ag*AChE1 on SDS-polyacrylamide gels: The samples from

cell supernatant, DS column, Con A column, and Ni-NTA column were treated with SDS sample buffer with or without β-mercaptoethanol and separated by 10% and 7.5% SDS-polyacrylamide gel electrophoresis (PAGE) followed by Coomassie blue staining, silver staining, or anti-$(His)_5$ immunoblotting using monoclonal antibodies (Qiagen Incorporation, CA).

Native polyacrylamide gel electrophoresis: Nondenaturing gel (4% stacking, 7.5% separating, no SDS) was used for 12.5 µl protein sample mixed with 2.5 µl 6 × native loading dye. Electrophoresis was followed by immunobloting.

***N*-linked and *O*-linked deglycosylation:** For *N*-linked glycosylation study, two tubes were prepared for the tests on a reducing gel and a nonreducing gel. The reduced sample contained SDS and β-mercaptoethanol, while the non-reduced sample contained SDS only. Each 12 µl protein sample with 3 µl loading dye (with or without β -mercaptoethanol) was denatured in 1 × glycoprotein denaturing buffer (Sigma Incorporation, MO) in each tube at 100 °C for 10 min. Then 2 µl 10 × G7 buffer (0.5 M sodium phosphate, pH 7.5) and 2 µl 10% NP-40 (Nonidet P-40) were added into each tube. Finally, 2 µl PNGase F was added, and the reactions were incubated at 37 °C for 1 h. For *O*-linked glycosylation study, two tubes were prepared for the tests on a reducing gel and a nonreducing gel as well. Each 12 µl protein sample with 3 µl loading dye (with or without β -mercaptoethanol) was boiled for 5 min. The samples were incubated in 4 µl of 5 × reaction buffer (Sigma) at 37 °C for 1 h. Then 2 µl of *O*-glycosidase was added into each tube, and the reactions were incubated at 37 °C for 3 h. The reaction samples from both

N-linked and *O*-linked glycosylation were separated by 10% or 7.5% SDS-PAGE followed by Coomassie blue staining, silver staining, or immunoblotting

Kinetics of substrate hydrolysis and inhibitor specificity

Substrate specificity: Kinetics of *Ag*AChE1 was determined using four synthetic substrates at eleven different concentrations from 10 µM to 1,000 µM. The substrates were acetylthiocholine iodide (ATC), acetyl-(β-methyl) thiocholine iodide (A β MTC), propionylthiocholine iodide (PTC), and S-butyrylthiocholine iodide (BTC) (Appendix 1). The reactions were monitored at 405 nm for 2 min at room temperature and a pH of 7.5 using a Microplater reader (Molecular Devices, CA) (Gao and Zhu, 2001). Substrates only were included to correct nonenzymatic hydrolysis of the substrates. The maximum velocities (V_{max}) and Michaelis constants (K_M) for each substrate were calculated by fitting the substrate-velocity curve using Prism 3.0 (GraphPad Software Inc., CA). The data were analyzed by using General Linear Models (GLM) procedure (Der, 2002). Data are expressed as the mean ± SD.

Inhibitor specificity – IC_{50} : Inhibition assays of *Ag*AChE1 were performed as described by Gao and Zhu (2001) using six inhibitors including eserine, 5-bis(4-allyldimethylammonium-phenyl) -pentan-3-onedibromide (BW284C51), ethopropazine, carbaryl, paraoxon, and malaoxon (Appendix 2). Carbaryl and eserine are carbamate inhibitors, while paraoxon and malaoxon are organophosphorus inhibitors. *Ag*AChE1 was pre-incubated with each inhibitor at 6 -12 different concentrations at room

temperature for 10 min. The residual activity of *Ag*AChE1 was determined as described above after the ATC and DTNB solutions are added to the reaction mixture. The final concentrations of ATC and DTNB were 600 µM and 48 µM, separately. IC_{50} for each inhibitor was determined by nonlinear regression using Sigmoidal dose response equation in Prism 3.0.

Determination of K_d, k_2, and k_i for inhibition of *Ag*AChE1: *Ag*AChE1 inhibition assays of unimolecular bonding rate constant (k_2. phosphorylation or carbamylation rate constant), dissociation constant (K_d) and bimolecular association rate constant (k_i) were performed as described by Hart and O'Brien (1973) using the same six inhibitors as above. 10 µl inhibitor at 7 different concentrations was mixed with 80 µlsolution of ATC (600 µM) and DTNB (48 µM) mixture, then mixed with 10 µl*Ag*AChE1, and the reaction was inspected immediately for 5 min. The value was estimated from four replicates with controls. The concentrations falling into about 20-80% residual activity of each inhibitor were used for kinetic constants (k_2, K_d, and k_i) calculation:

$$K_d=K_M\ [I]\ /\{(K_M+[S])\ (v_c/v_0\ -1)\}$$

where S is ATC concentration, v_c/v_0 is the velocity ratio of a control and inhibition reactions at the same substrate concentration $[S]$. k_2 values were obtained from

$$k_2=(\Delta \ln v\ /\Delta t)\ \{\ (\ K_d\ /[I])\ /\ [1-\ K_M\ /(\ K_M\ +[S])]+1\}$$

k_2 was direct calculated by the zero-time method. k_i values were evaluated by a formula

$$k_i = k_2 / K_d$$

Before K_d and k_2 measurement, K_M for ATC and IC_{50} for each inhibitor was determined so that the inhibitor concentration range can be chosen around IC_{50} for the kinetic constant studies. Constants for each inhibitor were analized by using Prism 3.0.

Substrate or product inhibition: The substrate or product inhibition was determined in two ways. First, at four different concentrations (15, 30, 60, and 120 mM) of substrate ATC, absorbance change at 405 nm was monitored for 2 min on a 96-well Microplater reader at room temperature and a pH of 7.5. Controls without *Ag*AChE1 were used to correct nonenzymatic hydrolysis of the substrate. At 30 min, when the enzyme activity was near zero, 20 µl reaction mixture solution was removed into a new well from each reaction. Then, 200 µl fresh substrate solution at the same concentration or buffer (200 µl) were added into treatment and control wells. If activity recovery is from former, the inhibition is partly from product inhibition, because the substrate concentration does not change. Activity and OD were determined again for 2 min. Secondly, acetate, choline, or both at 1, 10, 100, and 1,000 mM was mixed with the same amount of *Ag*AChE1 to detect for possible product inhibitions.

Results

Feature prediction and analysis of *Ag*AChE1

To study the properties of *Ag*AChE1, cDNA and protein sequence were analyzed. The

open reading frame encodes 557 amino acid (AA) residues: 241 non-polar, 186 polar, 64 acidic, and 66 basic AAs (Table 1). There are three predicted *N*-linked glycosylation sites (N^{180}, N^{573}, and N^{630}). The enzyme contains the catalytic triad comprising S^{320}, E^{446} and H^{560}, as well as the ten hydrophobic residues (F, W, and Y) lining the active site gorge (Figure 1). Six absolutely conserved cysteine residues may form three disulfide bonds. While C^{407} is located at the entry point of the active site in insect AChE1s, C^{670} of the adjacent subunits may form an interchain disulfide linkage (Figures 1 and 2). Antibody epitope prediction showed that deduced *Ag*AChE1 has 11 binding sites (antigenic determinants) (Figure 3).

The deduced protein sequences from cDNAs of *A. gambiae* AChE1 and AChE2 are 39% identitical. The Expect (E) value is 4e-107 with a score of 392 bits. Figure 4 shows the conservative regions, conservative substitutions, and deletions. The less conserved regions are needed to design specific probes for *in situ* hybridization.

The primary structure of AChE1 was compared with those from other animal species. Seventeen invertebrate sequences *A. gambiae* AChE1 and 2, *C. pipiens* AChE1 and 2, *A. aegypti* AChE, and *A. stephensi* AChE, *A. gossypii* AChE1 and 2, *P. xylostella* AChE1 and 2, *B. germanica* AChE1 and 2, *D. melanogaster* AChE, *Rhipicephalus microplus* AChE1 and 2, and *Caenorhabditis elegans* AChE1 and 4. Two vertebrate sequences are *Homo sapiens* and *Mus musculus* AChEs. A multiple sequence analysis of AChEs revealed that in *A. gambiae* AChE1 from residues 415 ~ 457, Y, W, G, H, E, F, and G are

conserved (Figure 5). Deletions and insertions are also observed (not shown). D^{444}, Y^{448} and N^{455} are conserved in AChEs arthropod species but not the vertebrates.

The properties of *Ag*AChE1

Reaction PH: Enzyme activity of *Ag*AChE1 increased rapidly from pH 5.5 to 7.0, and then increased slowly from pH 7.0 to 8.5. The activity reached its peak at pH 8.5. The activity decreased quickly from pH 8.5 (Figure 6). The study indicates that 7.0-8.5 of pH is the best range for *Ag*AChE1 reaction with acetylcholine.

Molecular weight and specific activity of *Ag*AChE1: The calculated MW and pI are 62.92 kDa and 5.95, respectively. The calculated MW is consistent with the mobility on the reducing SDS-PAGE gel (Figure 7A). The gel filtration experiment showed that *Ag*AChE1 had an apparent molecular mass of 60.41 kDa, suggesting the enzyme interacted with the column matrix (Figure 8). The specific activity of *Ag*AChE1 is 523.10 U/mg.

Association of *Ag*AChE1 subunits: Under nonreducing condition, *Ag*AChE1 migrated to a position of 126 kDa (Figure 7B). This suggests that each molecule of *Ag*AChE1 may consist of two identical subunits connected with an interchain disulfide bond.

Deglycosylation: In order to examine whether or not *Ag*AChE1 is glycosylated, *Ag*AChE1 was incubated with *N*- and *O*-glycosidases and subjected to SDS-PAGE

analysis (Figure 9). The results indicated that *Ag*AChE1 is glycosylated at Asn position(s), but not modified by *O*-linked glycosylation (data not shown).

Kinetics of substrate hydrolysis and inhibitor specificity

Substrate specificity of *Ag*AChE1 was determined by kinetic studies using ATC, AβMTC, PTC, and BTC (Figure 10). The concentrations of substrates for *Ag*AChE1 were from 8 µM to 96 µM. The V_{max} /K_M were from 3.10 to 1.33 for ATC, AβMTC, and PTC. The efficiency of *Ag*AChE1 in hydrolyzing ATC, AβMTC, and PTC were statistically significant higher than BTC (0.25) as indicated by their V_{max} /K_M ratio (Table 2). In contrast, the V_{max} of *Ag*AChE1 for ATC, AβMTC, and PTC were significantly higher than that for BTC as indicated by their V_{max} values, 12.4, 6.2, and 5.3-fold, separately. These results demonstrate that ATC is a better substrate for this enzyme whereas BTC is a poor substrate for it.

Sensitivities of *Ag*AChE1 to six inhibitors, eserine, BW284C51, ethopropazine, carbaryl, paraoxon, and malaoxon were studied (Figure 11). *Ag*AChE1 was more strongly inhibited by eserine, followed by BW284C51, and least inhibited by ethopropazine. The half inhibition concentration (IC_{50}) showed that *Ag*AChE1 was 5×10^2 and 1.2×10^4-fold less sensitive to inhibition by ethopropazine than by BW284C51 and by eserine, respectively (Table 3). Based on IC_{50}, the order from the most potent CA and OP inhibitors to the least ones was malaoxon >carbaryl > paraoxon >eserine. Eserine was 40.17, 1.2×10^2, and 2.4×10^2-fold less sensitive to inhibition by paraoxon, carbaryl, and malaoxon, respectively

(Table 3).

Kinetic constants K_d, k_2, and k_i for inhibition of *Ag*AChE1 suggested that *Ag*AChE1 is more sensitive to malaoxon and BW284C51, and less sensitive to ethopropazine and carbaryl. The bimolecular rate constant k_i showed that *Ag*AChE1 sensitivity to malaoxon is 2.64×10^2 and 5.46×10^2 -fold higher than that to ethopropazine and carbaryl, respectively. The order from the most potent OP and CA inhibitors to the least ones was malaoxon > eserine > paraoxon > carbaryl. Carbaryl was 5.46×10^2, 15, and 14 -fold less sensitive to inhibition by paraoxon, eserine, and malaoxon, in the order given (Table 4).

A comparison between the compounds with lowest and highest K_d indicates that the affinity of BW284C51 was about 1.21×10^4 -times greater than that of carbaryl. The order from higher affinity of *Ag*AChE1 by OP and CA inhibitors to lower ones was malaoxon > paraoxon > eserine > carbaryl. For instance, the binding affinity of *Ag*AChE1 by malaoxon was 6.16×10^2 -fold greater than by carbaryl. In unimolecular bonding rate constant k_2, the bonding rate of eserine was about 8.38×10^2 -times greater than that of ethopropazine

Clearly, inhibition exists at high concentrations of *Ag*AChE1 substrates (Figure 12). The inhibition determination study indicated that the inhibition was partly from product inhibition. The evidence is from both activity and OD numbers (Figure 13). The activities recovered a lot after the substrate solution was added (Figure 13A). In these reactions, the substrate concentration almost did not change, but the products concentration reduced

eleven-fold. OD numbers confirmed this conclusion (Figure 13 B). At the same time, acetate and choline as products showed product inhibition as well (Figure 14).

Discussion

AChEs are serine hydrolases commonly found in vertebrates and invertebrates. This study aimed at characterization of *Ag*AChE1 properties. First of all, *Ag*AChE1 sequence was predicted and analyzed. In doing so, a lot of information was obtained on *Ag*AChE1 properties. Especially, some predictions, such as conserved cysteine residues, putative *N*-linked glycosylation sites, catalytic triad, and the hydrophobic residues lining the active site gorge, helped the determination of *Ag*AChE1 status. In the future characterization of spatial and temporal expression patterns of *Ag*AChE1, the antibody can be either produced from *Ag*AChE1 or synthesized based on the antibody epitope prediction (Lindskog et al., 2005), by which the probe design can avoid the conserved region showed in the sequence alignment of *Ag*AChE1 and *Ag*AChE2. The primary structure of AChE comparison would be useful for the design of selective insecticides.

In this study, *A. gambiae* AChE1 catalytic domain was determined and examined. It has a molecular mass of 63 kDa and forms a dimer. The optimal reaction pH is 7.0-8.0. *Ag*AChE1 hydrolyzes acetylthiocholine iodide much faster than butyrylthiocholine iodide, and is more sensitive to eserine than to ethopropazine. The V_{max} ratio of BTC and ATC was 0.07, lower than that of *Drosophila* (0.6) (Gnagey et al., 1987) or western corn

rootworm (*Diabrotica virgifera*, 0.1) (Gao at el., 1998) but higher than that of *Schizaphis graminum* (0.05) (Gao and Zhu, 2001) and lesser grain borer, *Rhyzopertha dominica* (0.03)(Guedes et al., 1998). AChEs from different insect species differ in their substrate specificity.

While V_{max}/K_M measures AChE catalytic efficiency, K_M and V_{max} are related to the affinity of a substrate to AChE and catalytic activity, respectively. V_{max}/K_M of *A. gambiae* AChE1 in hydrolyzing ATC was 3.1, which was lower than that from Colorado potato beetle, *Leptinotarsa decemlineata* (11.9) (Zhu and Clark, 1994), or western corn rootworm, *Diabrotica virgifera* (9.4) (Gao at el., 1998). But it was higher than that from lesser grain borer, *Rhyzopertha dominica* (0.8 for OP susceptible and 1.3 for OP resistant strain) (Guedes et al., 1998). These results indicated that the AChE catalytic efficiency is different among various insect species.

Some inhibitors suppress acetylcholinesterase activity by covalently binding to a serine residue in the active site in the base of the gorge of acetylcholinesterase. The inhibitory power is usually expressed in two ways: the 50% inhibition of an inhibitor concentration (IC_{50}) under defined conditions, and the bimolecular rate constant (k_i) for the reaction (Forsberg and Puu, 1984). To determine k_i, two parameters are needed to be characterized. The dissociation constant K_d representing the affinity of enzyme-inhibitor binding and the unimolecular bonding rate constant k_2 (i.e. the rate constant for the carbamylation or phosphorylation). The ratio k_2 / K_d gives an overall rate of inhibition, that is the bimolecular reaction constant k_i (Main, 1964; Fukuto, 1990).

In general, BW284C51 and ethopropazine are powerful inhibitors of ChE. Eserine is a general inhibitor of ChE, BW284C51 is a specific inhibitor of AChE, and ethopropazine is a specific inhibitor of BChE. *A. gambiae* AChE1 is a true AChE to fulfill the physiological function of ACh hydrolysis at cholinergic synapses. This conclusion is based on two pieces of evidence. First, IC_{50} results showed that *Ag*AChE1 was highly sensitive to inhibition by eserine and BW284C51, but was less so by ethopropazine. Secondly, the constants of k_i and K_d demonstrated that the sensitivities of six inhibitors for *Ag*AChE1 have a 5.41×10^2 -fold difference. *Ag*AChE1 sensitivity to BW284C51 is 1.36×10^2 -fold higher than that to ethopropazine. The affinity of BW284C51 was about 78.13 times greater than that of ethopropzine.

It should be noted that the rate constant for phosphorylation and carbamylation (k_2), sensitivity (k_i), and affinity (K_d) are influenced by many factors, such as three-dimensional structure and size of an inhibitor (Forsberg and Puu, 1984). Some OP inhibitors suppressed AChEs do not interaction always by the same mechanism. They sometimes even act in noncholinergic processes (Pope, 1999; Sultatos, 2007).

So far, a decrease in AChE activity at a high concentration of substrate has been found in some insect AChEs (Zhu & Zhang, 2005). The findings of this study provided evidence that the *Ag*AChE1 activity reduction is partly from product inhibition.

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Table 1. Amino acid composition of of *Anopheles gambiae* AChE1

Property	Number	Percentage	AA number and number			
Non-polar	241	43.27	A(41)	V(42)	L(51)	I(17)
			P(45)	M(6)	F(26)	W(13)
Polar	186	33.39	G(45)	S(32)	T(35)	C(8)
			Y(21)	N(33)	Q(12)	
Acidic	64	11.49	D(26)	E(38)		
Basic	66	11.85	K(18)	R(35)	H(13)	

```
-24  M V P L G L L G V T A L L L I L P P F A L V Q G R H H E    4
L N N G A A I G S H Q L S A A A G V G L A S Q S A Q S G S L   34
A S G V M S S V P A A G A S S S S S S S L L S S S A E D D V   64
A R I T L S K D A D A F F T P Y I G H G E S V R I I D A E L   94
G T L E H V H S G A T P R R R G L T R R E S N S D A N D N D  124
P L V V N T D K G R I R G I T V D A P S G K K V D V W L G I  154
P Y A Q P P V G P L R F R H P R P A E K W T G V L N× T T T P  184
P N S C& V Q I V D T V F G D F P G A T M W+ N P N T P L S E D  214
C& L Y I N V V A P R P R P K N A A V M L W+ I F G G G F Y+ S G  244
T A T L D V Y+ D H R A L A S E E N V I V V S L Q Y R V A S L  274
G F L F L G T P E A P G N A G L F D Q N L A L R W V R D N I  304
H R F G G D P S R V T L F G E S A G A V S V S L H L L S A L  334
S R D L F Q R A I L Q S G S P T A P W+ A L V S R E E A T L R  364
A L R L A E A V G C$ P H E P S K L S D A V E C$ L R G K D P H  394
V L V N N E W G T L G I C E F+ P F+ V P V V D G A F L D E T P  424
Q R S L A S G R F K K T E I L T G S N T E E G Y Y F+ I I Y+ Y  454
L T E L L R K E E G V T V T R E E F L Q A V R E L N P Y V N  484
G A A R Q A I V F E Y T D W T E P D N P N S N R D A L D K M  514
V G D Y H F T C• N V N E F A Q R Y A E E G N N V Y M Y L Y T  544
H R S K G N P W+ P R W T G V M H G D E I N Y V F G E P L N× P  574
T L G Y T E D E K D F S R K I M R Y W S N F A K T G N P N P  604
N T A S S E F P E W P K H T A H G R H Y L E L G L N× T S F V  634
G R G P R L R Q C• A F W K K Y L P Q L V A A T S N L P G P A  664
P P S E P C# E S S A F F Y R P D L I V L L V S L L T A T V R  694
F I Q *                                                    697
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Figure 1. Deduced amino acid sequences of *A. gambiae* AChE1. Amino acid residues are shown in one-letter abbreviations. The predicted signal peptide (-24 ~ -1) is *double underlined*, putative *N*-linked glycosylation sites are marked "×", and the stop codon is indicated by "*". Positions of the cysteine residues conserved in all AChEs are marked "& – &, $ – $, and ● – ●" to show disulfide bond connectivity. The unique Cys residue present in insect AChE1s is *double underlined*, whereas the one possibly involved in interchain disulfide linkage is labeled "#". The catalytic triad (Ser, His and Glu) is in *bold*

and *shaded*, and the hydrophobic residues (F, W, and Y) lining the active site gorge are labeled with "+". The primer binding sites for recombinant expression in the baculovirus-insect cell system are *underlined*.

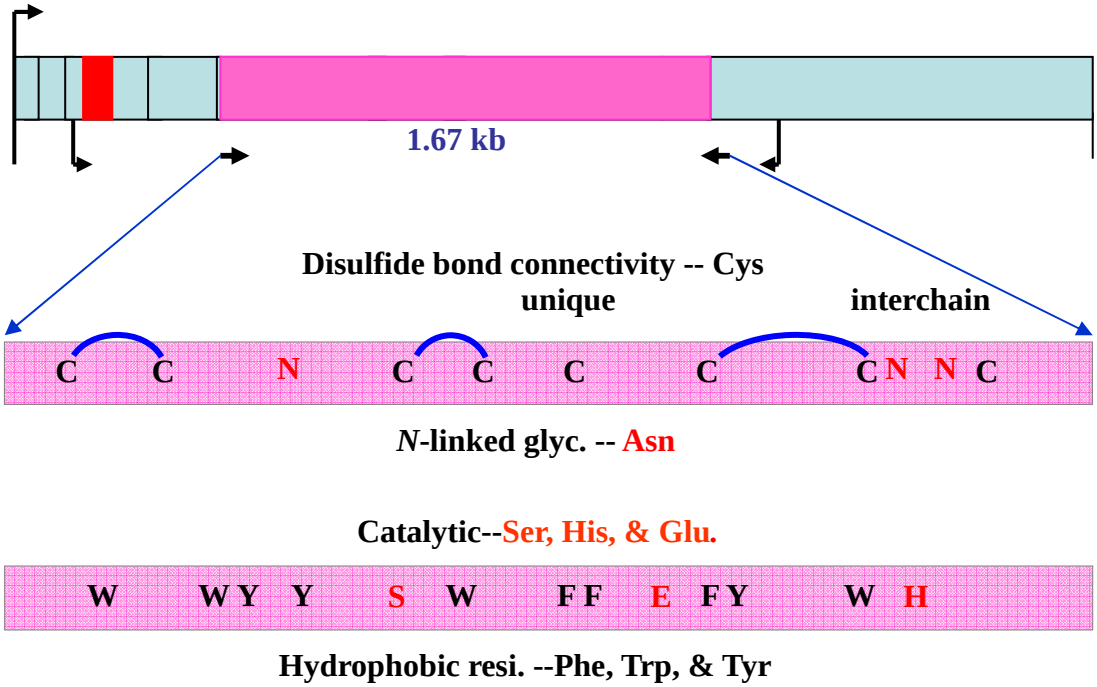


Figure 2. Schematic diagram of deduced amino acid sequences of *A. gambiae* AChE1. *Ag*AChE1 catalytic domain – coding region (1, 671 bp) was selected for recombinant expression (upper). The disulfide bond connectivity, unique Cys residue, interchain disulfide linkage, and putative *N*-linked glycosylation (N) are shown in the middle diagram. The catalytic triad and the hydrophobic residues lining the active site gorge are labeled in the lower diagram.

Number	Start AA	End AA	Peptide	Length of Peptide
1	3	8	NPNTPL	6
2	20	25	PRPRPK	6
3	219	224	ETPQRS	6
4	239	244	SNTEEG	6
5	255	260	LLRKEE	6
6	292	307	YTDWTEPDNPNSNRDA	16
7	324	333	FAQRYAEEGN	10
8	340	351	YTHRSKGNPWPR	12
9	375	383	YTEDEKDFS	9
10	396	404	TGNPNPNTA	9
11	407	418	EFPEWPKHTAHG	12

Figure 3. Antibody epitope prediction from recombinant *Ag*AChE1. *Ag*AChE1 was analyzed, and 11 peptides were predicted by using Emini Surface Accessibility Prediction method from IEDB Analysis Resource program.

```
Score =  392 bits (1008),  Expect = 4e-107
Identities = 232/591 (39%), Positives = 333/591 (56%), Gaps = 55/591 (9%)

AChE-1    6   DPLVVNTDKGRIRGITVDAPSGKKVDVWLGIPYAQPPVGPLRFRHPRPAEKWTGVLNTTT    65
              D LVV T  G IRG +     G++V V+ G+P+A+PPV  LRF+ P PAE W GVL+ T
AChE-2   34   DRLVVQTSSGPIRGRSTMV-QGREVHVFNGVPFAKPPVDSLRFKKPVPAEPWHGVLDATR    92

AChE-1   66   PPNSCVQIVDTVFGDFPGATMWNPNTPLSEDCLYINVVAP-RPRPKNA------------    112
              P SC+Q     F  F G   MWNPNT +SEDCLY+N+   P + R ++
AChE-2   93   LPPSCIQERYEYFPGFAGEEMWNPNTNVSEDCLYLNIWVPTKTRLRHGRGLNFGSNDYFQ    152

AChE-1  113   ---------------AVMLWIFGGGFYSGTATLDVYDHRALASEENVIVVSLQYRVASLG    157
                            A+++WI+GGGF SGT+TLD+Y+    LA+  NVIV S+QYRV + G
AChE-2  153   DDDDFQRQHQSKGGLAMLVWIYGGGFMSGTSTLDIYNAEILAAVGNVIVASMQYRVGAFG    212

AChE-1  158   FLFLGT------PEAPGNAGLFDQNLALRWVRDNIHRFGGDPSRVTLFGESAGAVSVSLH    211
              FL+L          +APGN G++DQ LA+RW+++N    FGGDP  +TLFGESAG   SVSLH
AChE-2  213   FLYLAPYINGYEEDAPGNMGMWDQALAIRWLKENAKAFGGDPDLITLFGESAGGSSVSLH    272

AChE-1  212   LLSALSRDLFQRAILQSGSPTAPWALVSREEATLRALRLAEAVGCPHEPSKLSDA--VEC    269
              LLS ++R L +R ILQSG+  APW+ ++ E+A    A   L +   C     K S +   ++C
AChE-2  273   LLSPVTRGLSKRGILQSGTLNAPWSHMTAEKALQIAEGLIDDCNCNLTMLKESPSTVMQC    332

AChE-1  270   LRGKDPHVLVNNEWGTL-GICEFPFVPVVDGAFLDETPQRSLASGRFKKTEILTGSNTEE    328
              +R  D   +    +W +  GI  FP   P +DG F+    P   L    +   +IL  GSN +E
AChE-2  333   MRNVDAKTISVQQWNSYSGILGFPSAPTIDGVFMTADPMTMLREANLEGIDILVGSNRDE    392

AChE-1  329   GYYFIIYYLTELLRKEEGVTVTREEFLQAVRELNPYVNGAARQAIVFEYTDWTEPDNPNS    388
              G YF++Y   +    K+    ++ R++FL+ +  +    +    R+AI+F+YT W   ++
AChE-2  393   GTYFLLYDFIDYFEKDAATSLPRDKFLEIMNTIFNKASEPEREAIIFQYTGWESGNDGYQ    452

AChE-1  389   NRDALDKMVGDYHFTCNVNEFAQRYAEEGNNVYMYLYTHRSKGNPWPRWTGVMHGDEINY    448
              N+  + + VGD+ F C   NEFA    E G +V+ Y +THR+  + W  W GV+HGDE+ Y
AChE-2  453   NQHQVGRAVGDHFFICPTNEFALGLTERGASVHYYYFTHRTSTSLWGEWMGVLHGDEVEY    512

AChE-1  449   VFGEPLNPTLGYTEDEKDFSRKIMRYWSNFAKTGNPNPNTASSEFPEWPKHTAHGRHYL-    507
              +FG+P+N +L Y + E+D SR+++   S FA+TGNP     + E    WP +T     Y
AChE-2  513   IFGQPMNASLQYRQRERDLSRRMVLSVSEFARTGNP-----ALEGEHWPLYTRENPIYFI    567

AChE-1  508   -----ELGLNTSFVGRGPRLRQCAFWKKYLPQLVAATSNLPGPAPPSEPCE     553
                   E  L     GRGP    CAFW  +LP+L A +        P  +PC+
AChE-2  568   FNAEGEDDLRGEKYGRGPMATSCAFWNDFLPRLRAWS------VPLKDPCK     612
```

Figure 4. The sequence comparison of AChE1 and AChE2 from *An. gambiae.* The *Ag*AChE1 and *Ag*AChE2 protein sequences deduced from cDNAs show the conservative regions. Conservative substitutions are marked “+”, and gaps are shown as “-”.

Figure 5. Alignment of the partial amino acid sequences of *A. gambiae* AChE1 and 2 with other AChEs sequences. A total of nineteen amino acid sequences are aligned using ClustalW of MEGA 4. Seventeen belong to invertebrates, including mosquitoes (*A. gambiae* AChE1 and 2, *C. pipiens* AChE1 and 2, *A. aegypti* AChE, *A. stephensi* AChE), aphid (*A. gossypii* AChE1 and 2), moth (*P. xylostella* AChE1 and 2), cockroach (*B. germanica* AChE1 and 2), fly (*D. melanogaster* AChE), tick (*Rhipicephalus microplus* AChE1 and 2), and nematode (*Caenorhabditis elegans* AChE1 and 4). Two AChEs belong to vertebrates, including human (*Homo sapiens* AChE) and mouse (*Mus musculus* AChE). Conserved AAs in AChEs from the vertebrate and invertebrate species are shaded in yellow. Conserved AAs in AChEs from the invertebrate species but not the vertebrates are shaded in red.

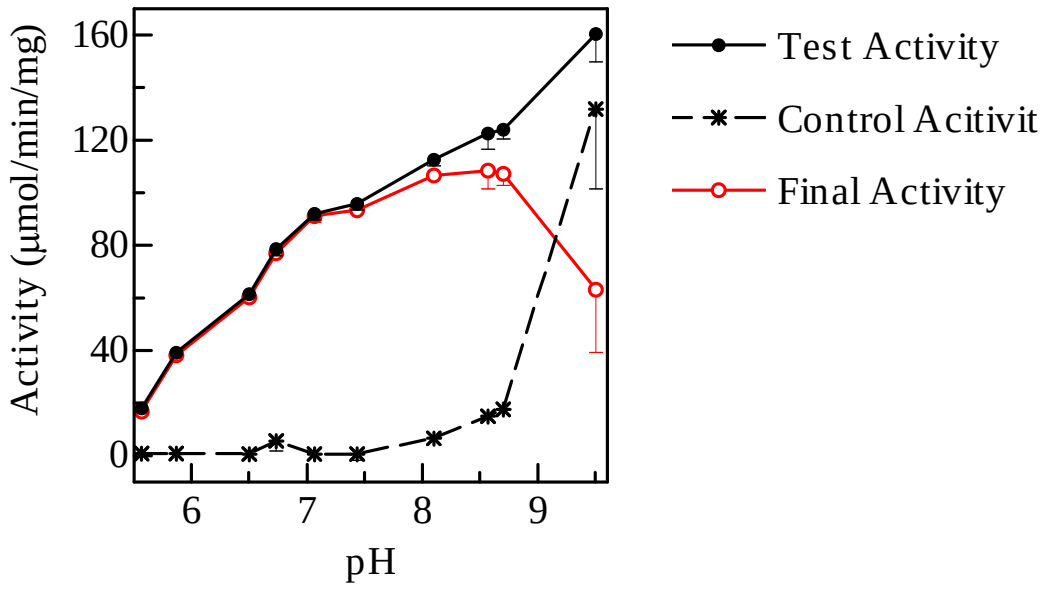


Figure 6. Effects of pH on *Ag*AChE1 activity. Activity was calculated based on the mean of three replications (n=3). Final activity is equal to test activity minus control (blank) activity. Vertical bars indicate standard deviations (SD) of the mean.

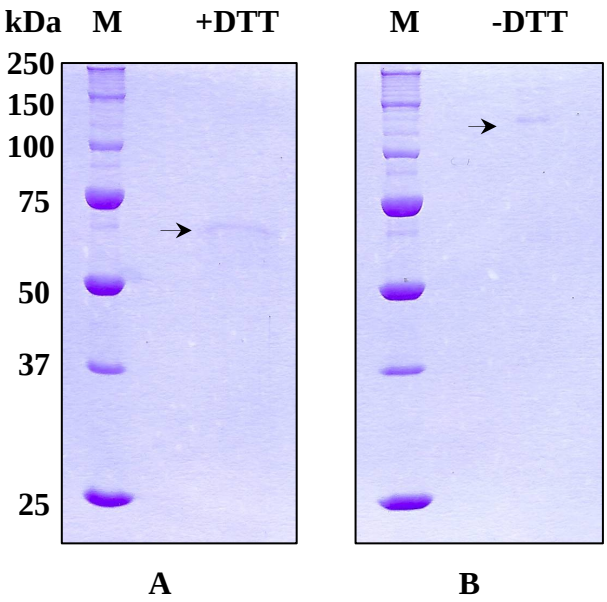


Figure 7. SDS-PAGE analysis of *Ag*AChE1 under reducing (A) and non-reducing (B) conditions. M: Marker, +DTT: with dithiothreitol, –DTT: without dithiothreitol. Arrow indicates the protein band.

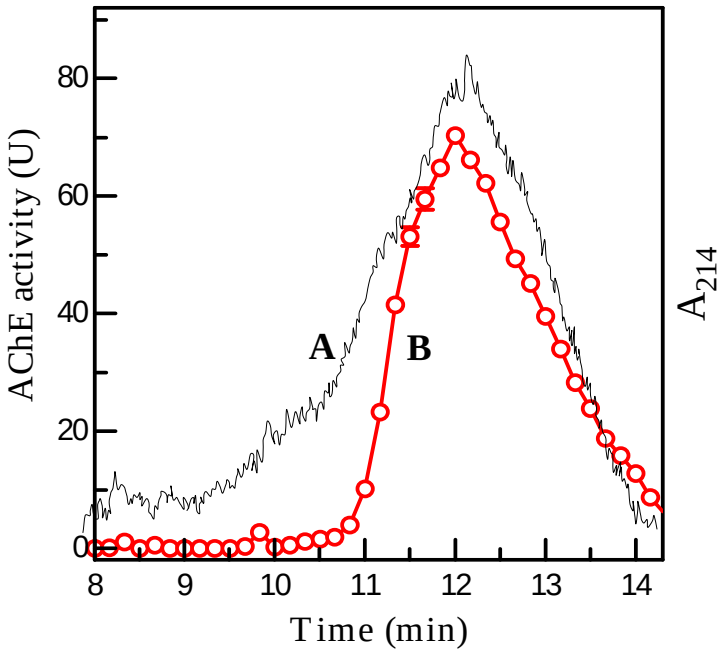


Figure 8. Gel filtration analysis of *Ag*AChE1. A: absorbance at 214 nm (——). B: enzyme activity in different fractions (○——○).

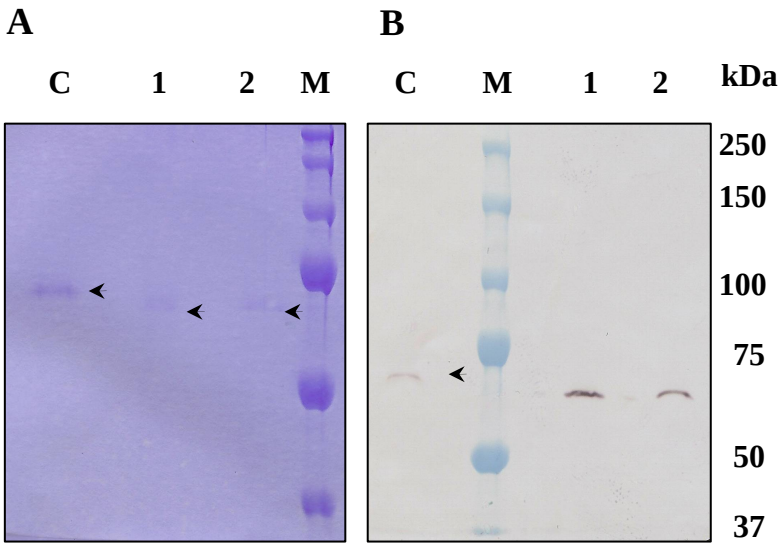


Figure 9. Deglycosylation of *Ag*AChE1 by *N*-glycosidase. The purified enzyme was treated with a buffer (lane "C") or PNGase F (lanes 1 and 2), separated by 10% (A) or 7.5% (B) SDS-PAGE under reducing condition, and visualized by Coomassie Blue staining (A) and monoclonal antibodies against the hexahistidine tag (B). Sizes and positions of the molecular weight markers are indicated on the *right*. Arrows indicate the protein and immunoreactive bands.

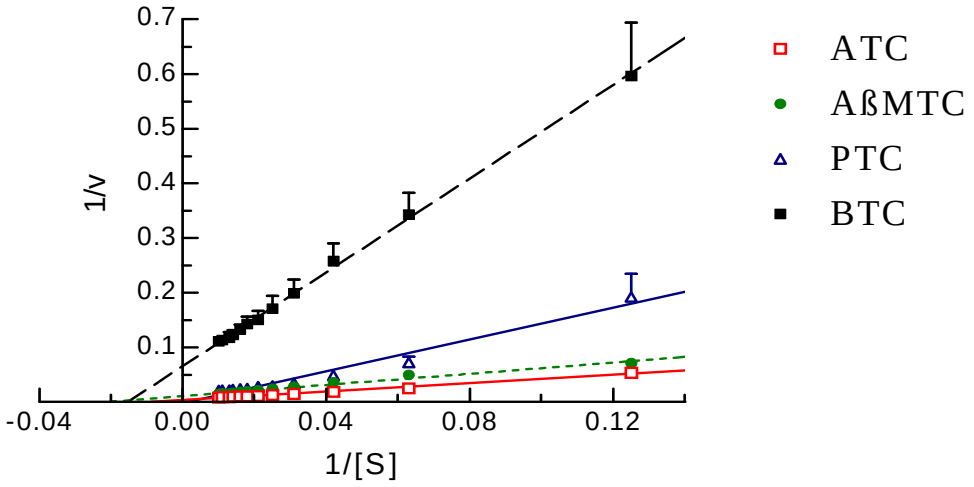


Figure 10. Determination of the enzymatic properties of *Ag*AChE1 using different substrates. Hydrolysis of ATC (□——□), AβMTC (●- -●), PTC (Δ——Δ), and BTC (■- -■) by the purified enzyme was measured as described in Materials and Methods. Each point on the double reciprocal plot represents mean ± SD (n = 4). K_M and V_{max} values for each substrate were derived from the nonlinear regression analysis on the *v* versus [S] plot.

Table 2. Kinetic parameters of AChE1 purified from *Anopheles gambiae* in hydrolyzing four substrates*

Substrate	K_M (μM)	V_{max} (μmol/min/mg protein)	V_{max}/K_M
ATC	67.51 ± 12.68 a	209.20 ± 20.49 a	3.10 a
AβMTC	78.56 ± 6.51 a	122.10 ± 5.61 b	1.55 b
PTC	63.26 ± 15.53 a	84.12 ± 10.49 c	1.33 b
BTC	59.78 ± 14.34 a	15.04 ± 1.79 d	0.25 c

* Results are presented as the mean ± SE (n=4). Same letters indicate the values not significantly different (LSD test. P>0.05) (Ramsey, 1993).

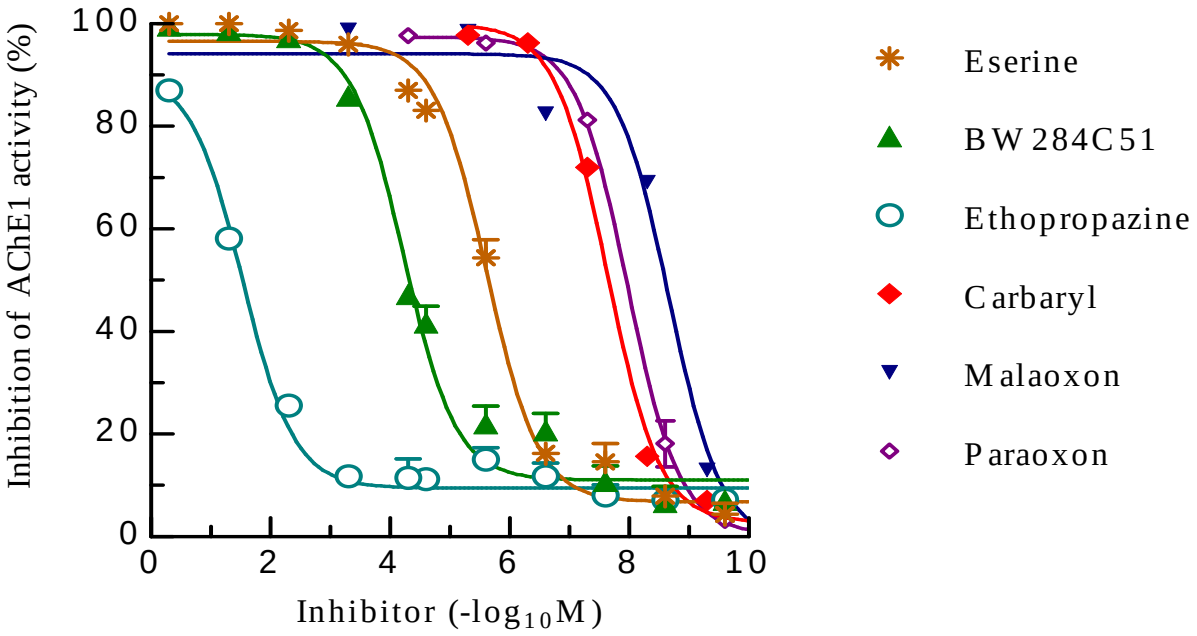


Figure 11. Inhibition of *Ag*AChE1 by six inhibitors at various concentrations. After incubation with its inhibitors for 10 min at 25 °C, the purified enzyme was reacted with ATC-DTNB and monitored by a microplate reader at 405 nm. The inhibition of activity, shown as mean ± SD (n = 3), is plotted against the inhibitor concentrations. (A) carbaryl (♦—♦), eserine (□—□), BW284C51 (▲—▲), and ethopropazine (○—○). (B) malaoxon (▼—▼),and paraoxon (◊—◊).

Table 3. IC_{50} of inhibitors towards *Anopheles gambiae* AChE1*

Inhibitor	IC_{50} (μM)	r^2
Carbaryl	0.02 ±0.001	1.00
Eserine	2.41 ±1.78	0.99
BW284C51	57.8 ±0.09	0.94
Ethopropazine	$(2.9\pm1.5)\times10^{4}$	0.99
Paraoxon	0.06 ±0.007	0.99
Malaoxon	0.01 ±0.002	0.98

* Results are presented as the mean ± SD (n=3). r^2: average correlation coefficient.

Table 4. Kinetic constants (K_d, k_2, and k_i) for inhibition of *Ag*AChE1

Inhibitor	K_d ($\times 10^{-6}$ M)	k_2 (min^{-1})	k_i ($\times 10^{6}$ $M^{-1}min^{-1}$)
Carbaryl	1194.009±46.200	0.694±0.112	0.004±0.0003
Eserine	890.390±2.500	53.619±2.243	0.060±0.005
BW284C51	0.099±0.788	0.111±0.026	1.130±0.656
Ethopropazine	7.735±0.400	0.064±0.012	0.008±0.003
Paraoxon	3.799±2.164	0.215±0.061	0.056±0.004
Malaoxon	1.938±0.100	4.237±0.230	2.186±0.033

* Results are presented as the mean ± SD (n=4).

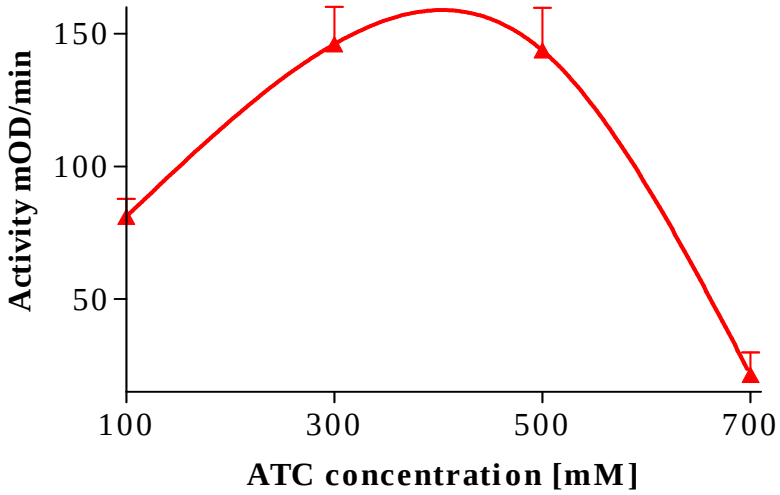
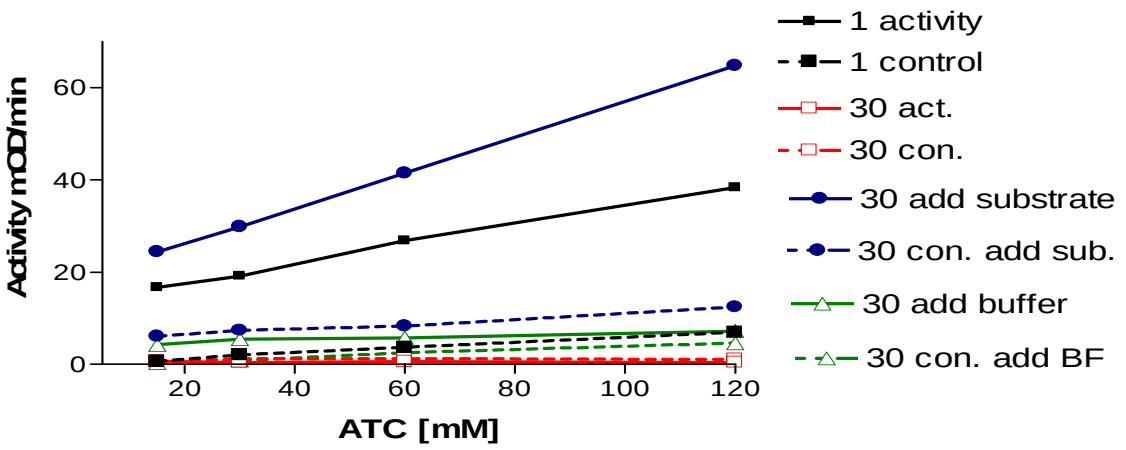
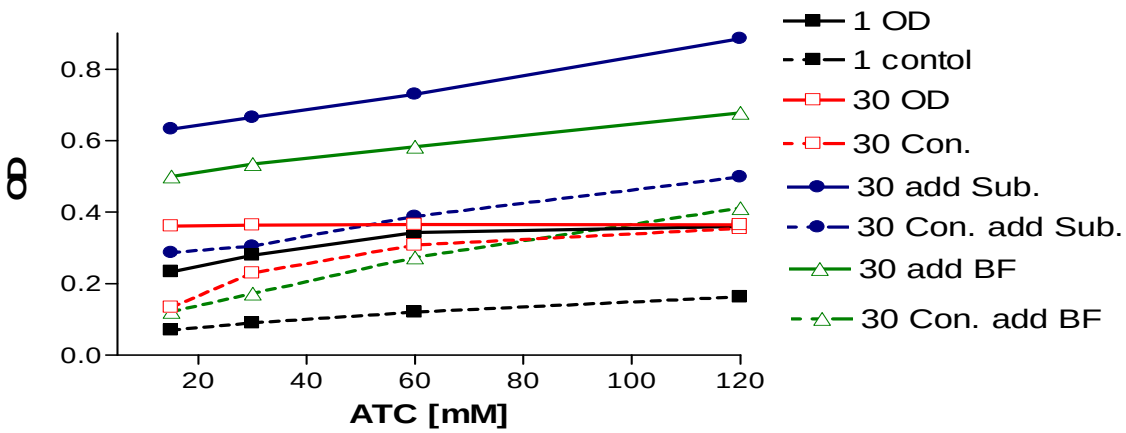


Figure 12. Inhibition of *Ag*AChE1 activity from high substrate concentration. Vertical bars indicate standard deviation (SD) of the mean (n=3).



A



B

Figure 13. Product inhibition determined from activity recovery and OD accumulation of *Ag*AChE1 at different concentrations. The reaction was monitored at 25 °C and a pH 7.5 for 2 min on a 96-well Microplater reader at 405 nm. Activity and OD were determined again for 2 min. A: Activity, B: OD. Solid line: the reaction with diluted *Ag*AChE1, Dot line: Without *Ag*AChE1 (instead buffer). Con: control. Sub: substrate (ATC). BF: buffer.

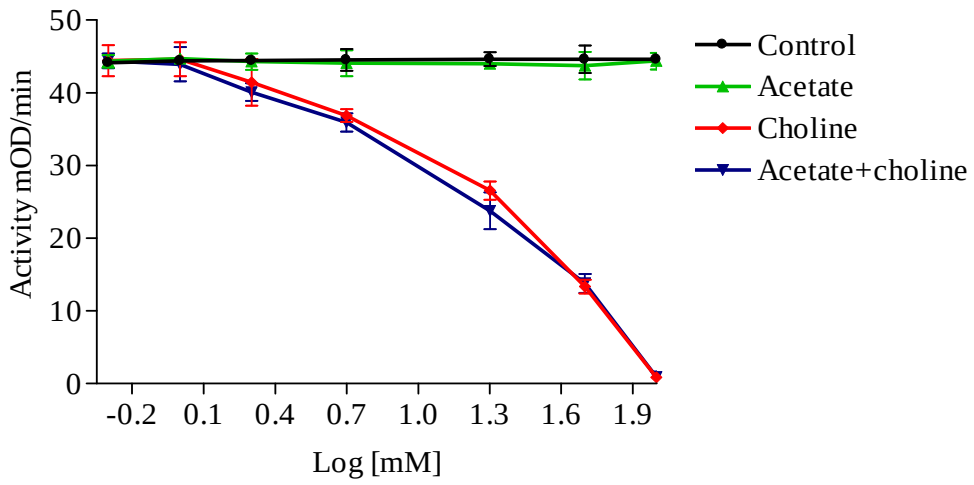
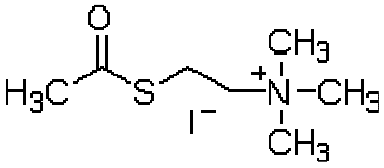
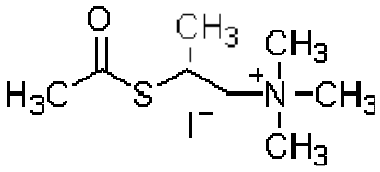


Figure 14. Inhibitory effect of acetate and choline on *Ag*AChE1. Acetate, choline, or both (10 µl at different concentrations) was incubated with 10 µl diluted *Ag*AChE1 and 80 µl substrate mixture (1% of 6 mM DTNB, 1% of 75 mM ATC, and 1% of acetone in 0.1 M phosphate buffer, pH 7.5). The reaction was monitored at room temperature for 2 min using a microplater reader at 405 nm. Con, control; Ace, acetate; Cho, choline; Ace + Cho, acetate and choline.

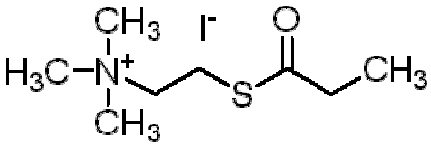
Appendix 1. The structures of substrates used in this study



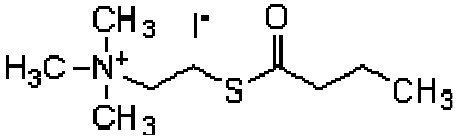
Acetylthiocholine iodide



Acetyl-(β-methyl) thiocholine iodide

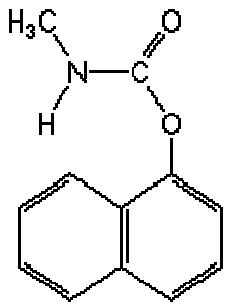


Propionylthiocholine iodide

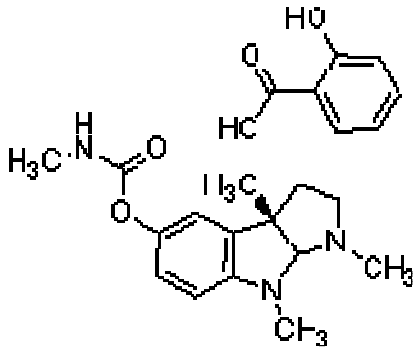


S-butyrylthiocholine iodide

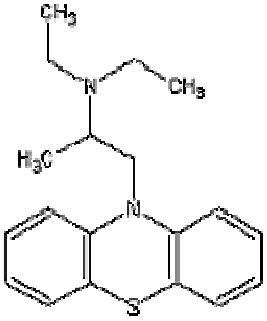
Appendix 2. The structures of inhibitors used in this study



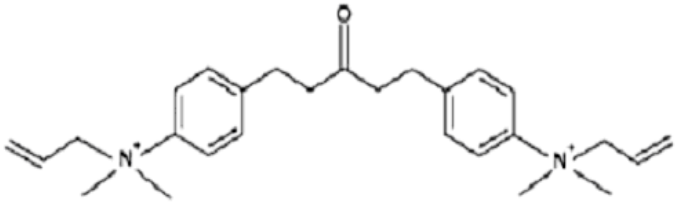
Carbaryl



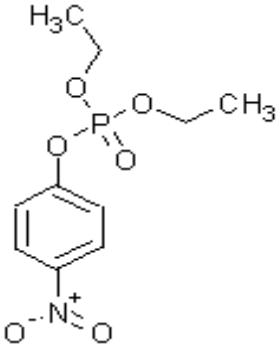
Eserine



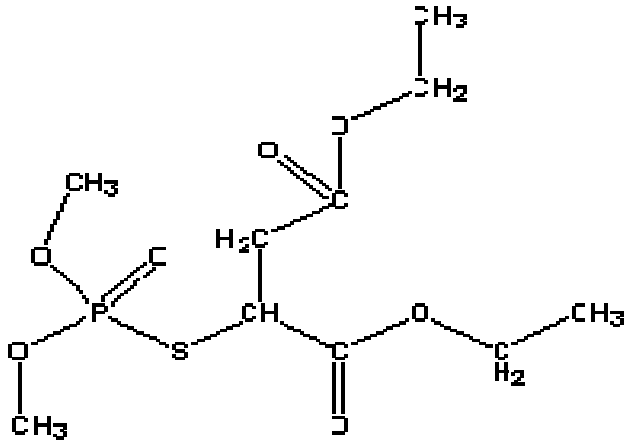
Ethopropazine



BW284C51



Paraoxon



Malaoxon

CHAPTER V

CHARACTERIZATION OF SPATIAL AND TEMPORAL EXPRESSION PATTERNS OF *AG*ACHE1 BY *IN SITU* HYBRIDIZATION AND IMMUNOHISTOCHEMISTRY

Abstract

In situ hybridization (ISH) and immunohistochemistry (IHC) are commonly used to characterize spatial and temporal expression patterns of a gene and to detect its protein product, respectively. In this study, acetylcholinesterase-1 (AChE1) and its mRNA were localized in the African malaria mosquito, *Anopheles gambiae*. IHC showed that *Ag*AChE1 protein exists mainly in the central nervous system (CNS) (brain and ganglia) of *A. gambiae* larvae, pupae, and adults. The protein is also found in the peripheral nervous system (PNS), such as the base of antennal and mid-gut nerves of *A. gambiae* adults. ISH indicated that *Agace*1 mRNA is predominantly in the CNS. These results suggested that the main function of *Ag*AChE1 is in cholinergic synapses of the CNS to hydrolyze acetylcholine. The hybridization signals were clearly detected on the neuropile or three brain lobes and the cell bodies of the CNS using the antisense probe. Signals were much weaker when sense probe was used. The sense and antisense DNA probes were prepared by asymmetric PCR.

Key words: Neurotransmission, Mosquito, Immunohistochemistry, Single strand DNA probes, Asymmetric PCR, *In situ* hybridization.

Introduction

Acetylcholinesterases are commonly found in vertebrates and invertebrates, which exists at cholinergic nerve terminals (Vigny et al., 1983). Insect AChE is one of the most studied insect enzymes due to its physiological and toxicological significance (Zhu and Zhang, 2005). Immunohistochemistry (IHC) and/or *in situ* hybridization (ISH) are used to characterize spatial and temporal expression patterns of a protein and/or a gene.

To detect protein expression, there are some methods, such as staining and measurement of protein activity, western blotting, and immunohistochemistry. Staining and measurement of extracted protein activity is common. Western blotting can be used to estimate the protein quantity, while protein extraction and antibody preparation are practical. They are labor consuming and neither can detect protein expression patterns in tissues. IHC detects the protein *in situ* (Zador and Maroy, 1987; Zador, 1989).

There are several ways to analyze gene expression, such as northern blotting, polymerase chain reaction (PCR), and ISH. Northern blotting measures the quantity of mRNA and the level of mRNA is critical (Miyazaki et al., 1994). PCR, a powerful tool of DNA amplification, detect mRNA at low abundance (Eisenstein, 1990). Both northern blotting and PCR detect gene expression in vitro. ISH, however, can detect mRNA transcripts in tissues. Methods of non-radioactive ISH are more popular, because such probes promise safely, stability, high resolution, and short development times as compared with radioactive probes (Miyazaki et al., 1994). In resent years, antisense RNA probes have

been used to increase detection sensitivity. On the other hand, they have some disadvantages, such as RNA degradation and contamination. Single stranded DNA (ssDNA) probes circumvent the problems of RNA probes. Moreover, ssDNA probes promise brighter signals and higher reliability than double stranded DNA (dsDNA) probes (Sanchez et al., 2004). Nevertheless, asymmetric PCR requires the optimization of conditions (Gyllensten and Allen, 1993; McCabe, 1999; Poddar, 2000). Once the disadvantages are overcome, using ssDNA probes for ISH can reliably detect mRNA transcript in tissue at a low level.

Spatial and temporal expression patterns of genes and their protein products have been characterized in a few insect species. The expression of *Drosophila melanogaster* AChE is found mainly in the central nervous system (CNS) of larvae and in the head and thorax of adults by using protein extraction and measurement (Zador, 1989). The mRNA transcript of *D. melanogaster* embryos was localized in the CNS and lateral chordotonal neurons as well. Before the first neuroblast differentiation, the AChE gene and protein expression were presented. ISH was performed by a cDNA probe in the embryo tissues (Zador and Budai, 1994). Two *ace* genes of the German cockroach (*Blattella germanica*) encoding two AChEs exist mainly in the CNS. *Ace*1 is the predominant gene to encode AChE1 of *B. germanica* associated with synaptic transmission. The study used dissected whole mount of adult males due to their larger size. A cDNA probe was used in the localization of *B. germanica* as well (Kim et al., 2006).

The African malaria mosquito, *Anopheles gambiae* (Diptera: Culicidae), is an important

vector of malaria (Von Seidlein et al., 1998; Arensburger et al., 2005). *A. gambiae* possesses two AChE genes (*Agace*1 *and Agace*2) (Weill et al., 2002). But, where and when the genes transcribe and the proteins express are still unknown. In this study, the spatial and temporal expression patterns of *Ag*AChE1 gene and protein were investigated by IHC and ISH from sections of paraffin-embedded *A. gambiae* adult, pupal, and larval specimens. We provide evidence for *Ag*AChE1 gene functions *in vivo*.

Materials and Methods

Chemicals and materials

Clear-Rite 3 (Richard-Allan Scientific, MI, USA), paraformaldehyde (Sigma, MO. USA), proteinase K (Sigma, MO, USA), formamide (J. T. Baker, NJ, USA), hybridization solution (Roche, IN, USA), bovine serum albumin (BSA) (Fisher Scientific, IL, USA), alkaline phosphatase (Sigma, MO, USA), nitro-blue tetrazolium (NBT) and bromo-chloro-indoryl phosphate (BCIP) (Bio-Rad, MO, USA), GeneScreen Plus Hybridization Transfer Membrane (GeneCreen, MA. USA), ProbeOn Plus Microscope Slides (Fisher Scientific, PA, USA) were directly purchased from the companies as quoted. *A. gambiae* cDNA pool was a generous gift from Dr. Susan Paskewitz at University of Wisconsin-Madison. *A. gambia* AChE1 EST plasmid (BM629847) was kindly provided by MR4/ATCC.

Mosquito specimen

The mosquito specimen of *A. gambiae* was kindly provided by Dr. Maureen Gorman at Kansas State University. The specimens include 40 adults (five day old males and females), 20 pupae, and 20 last instar larvae. For preliminary study, the fresh specimens of the Asian tiger mosquito, *Aedes albopictus*, were used. The adults, pupae, and larvae of *A. albopictus* were collected in Stillwater, Oklahoma.

Antibody preparation

500 µg purified *Ag*AChE1 was prepared for polyclonal antibody production. Rabbit polyclonal antibody was produced by Cocalico Biological (Reamtown, PA). The antibody titer was determined by immunoblotting analysis. The antibody specificity was determined using recombinant *Ag*AChE2, provided by Picheng Zhao in the laboratory. Because *Ag*AChE1 antibody cross-reacted with *Ag*AChE2, the cross-reacting antibodies were absorbed using recombinant *Ag*AChE2. In brief, 20 µl *Ag*AChE2 (38 µg/ml) solution was added to 500 µl 1:50 diluted *Ag*AChE1 antiserum at 4 °C overnight. The mixture was centrifuged at 10,000 *g* for 5 min to remove *Ag*AChE2-Ab complexes. Immunoprecipitation was repeated several times until *Ag*AChE2 signal disappeared. A proper dilution of the absorbed *Ag*AChE1 antiserum was determined for immunolocalization.

Single stranded DNA probe preparation

The 189 bp (nucleotides 131 ~ 319) probes of *A. gambia* actin (*Ag*actin, for use as a positive control) were prepared based on *Ag*actin sequence (BX063031) (Figure 6). *Ag*AChE1 probe is a 307 bp cDNA (nucleotides -1 ~ 306) designed based on *A. gambia*

AChE1 EST sequence (BM629847) (Figure 7). The primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). The forward primer of *Ag*actin was 5' GATGAGGCCCAGTCCAAGCGTGGTATC, nucleotides 131 ~ 157. The reverse primer of *Ag*actin was 5' CTTCTCGCGGTTAGCCTTCGGGTTCAG, reverse complement of nucleotides 293 ~ 319. The forward primer of *Ag*AChE1 was 5' GATGGAGATCCGAGGGCTG, nucleotides -1~ 18. The reverse primer of *Ag*AChE1 was 5' GTCCTCGGCTGACGATGACAG, reverse complement of nucleotides 285 ~ 306. To confirm *Ag*actin sequence, TA cloning and sequencing were carried out.

The first step was amplification of the cDNA pool for a 189 bp actin segment and of the EST plasmid (BM629847) for 307 bp segment of *Ag*AChE1. The thermal cycling conditions for *Ag*Actin were 40 cycles of 94 ºC for 30s, 61.5 ºC for 30 s and 72 ºC for 10 s. The thermal cycling conditions for *Ag*AChE1 were 40 cycles of 94 ºC for 30s, 55 ºC for 30 s and 72 ºC for 20 s. After gel electrophoresis, a gel slice containing the product (189 or 307 bp) was purified using the QIAquick Gel Extraction kit (Qiagen, MD).

The second step was the sense and antisense probes were synthesis using PCR DIG (digoxigenin) Probe Synthesis Kit (Roche, IN). Meanwhile, unlabeled sense and antisense strand DNAs were synthesized for *Ag*Actin and *Ag*AChE1 probe controls. PCR DIG Probe Synthesis Mix contains a mixture of nucleotides including DIG-dUTP. The thermal cycling conditions for labeled and unlabeled sense strand DNAs of *Ag*Actin were 32 cycles of 94 ºC for 30s, 61.5 ºC for 30 s and 72 ºC for 10 s. The thermal cycling conditions for labeled and unlabeled antisense strand DNAs of *Ag*Actin were 32 cycles of

94 ºC for 30s, 60.5 ºC for 30 s and 72 ºC for 10 s. The thermal cycling conditions for labeled and unlabeled sense strand DNAs of *Ag*AChE1 were 32 cycles of 94 ºC for 30s, 54 ºC for 30 s and 72 ºC for 20 s. The thermal cycling conditions for labeled and unlabeled antisense strand DNAs of *Ag*AChE1 were 32 cycles of 94 ºC for 30s, 55 ºC for 30 s and 72 ºC for 20 s.

The labeled and unlabeled single stranded DNAs were purified using the Wizard PCR Preps DNA Purification kit (Promega, WI) according to the standard protocol from Promega. Finally, the probe specificity was examined by dot blotting on GeneScreen Plus Hybridization Transfer Membrane (GeneCreen, MA) to assess labeling efficacy. The probes was dissolved in elution buffer and stored at -20 ºC.

Preparation of sectioned tissue slides

Fixation of mosquitoes: The wings and legs of adult mosquitoes were removed. Two gaps were made in the cuticle of each adult with a forcep, one from the thorax, and another one from the abdomen, to allow the fixative to get in. A hole was torn in the abdomen of each pupa and larva. The specimen was submerged in the fixation solution including 0.25% Trition-100, 4% paraformaldehyde, and 0.1 M sodium phosphate (pH of 7.2). It was fixed at room temperature for 3.5 h. Over-fixation may cause high background and low staining efficiency, whereas under-fixation decreases RNA hybridization signals. For the localization of protein by IHC, the use of a fixation with methanol/acetone/water (MAW) was an alternative.

Embedding and sectioning: The specimen was treated with 70% ethanol overnight, stored at 4 ^{o}C, and placed in melted paraffin in metal molds. Then molds were moved to a cold surface. The specimen then was pressed with a small weight until block comes out easily. Three stages of *A. gambiae*, larva, pupa, and adult, were embedded for IHC and ISH. Slides were treated by 2% TESPA (3-aminopropyltriethoxysilane, Sigma) carefully, or ProbeOn Plus Microscope Slides (Fisher Scientific) was used. Six µm sections were made and completely dried at room temperature. Then the slides were warmed at 40 °C overnight. Embedding and sectioning were done in Oklahoma Animal Diseases and Diagnosis Laboratory (OADDL) at Oklahoma State University.

Immunohistochemistry

Dewaxing and rehydration: The slides were washed 5 min twice in Clear-Rite 3 (Richard-Allan Scientific, MI) to remove the paraffin. Rehydration of the section was through ethanol series as follows: 30 s twice in 100% ethanol, 30 s once each in 95%, 90%, 70%, and 50% ethanol, respectively. Then the slides were washed 5 min twice in PBS (phosphate buffered saline)/0.1% Tween-20 (PTW).

Immunodetection: The sections were treated with 200 µl of blocking solution - PTW / 3% bovine serum albumin (BSA) (Fisher Scientific) and incubated at room temperature for 1 h., that is, 200 µl blocking solution was added to each slide, added coverslips, and placed in humid chamber at room temperature. The blocking solution was removed, and

excess solution was wiped off carefully.

The primary antibody (rabbit anti-*Ag*AChE1) solution (200 µl, 1: 500 antibody in the blocking solution) was added to each section and added coverslip. Negative controls for the immunohistochemical procedures used pre-immune serum (200 µl, 1: 500 diluted in the blocking solution). The slides were incubated at room temperature for 2 h. Coverslips were removed. The section was washed 10 min three times in PTW at room temperature with gentle shaking. The secondary antibody (goat anti-rabbit, 200 µl, 1: 1000 in the blocking solution) was added to each section, and the slides were incubated at room temperature for 2 h. The section was washed again 10 min three times in PTW at room temperature with gentle shaking.

Finally, the slides were ready to develop. The secondary antibody was conjugated to alkaline phosphatase (AP). The section was stained in NBT/BCIP (Bio-Rad Laboratories) solution according to the standard protocol. Slides were examined using a Zeiss Axiophot or an Olympus microscope BX51, and photographed using a digital camera on Olympus DP71 (Olympus America Inc., Melville, NY).

In situ hybridization

Pretreatment: The pretreatment included dewaxing, rehydration, fixation, deproteinization (proteinase K treatment), and dehydration. The slides were washed in Histo-Clear to remove the paraffin and rehydration of the section was through ethanol

series as described above. The section was washed with PTW and fixed with 4% paraformaldehyde in PBS for 20 min, washed with PBS, and treated with 10 µg/mL proteinase K (Sigma, MO) in PTW for 20 min. It was rinsed and fixed again. Then washed with PTW, the section was through methanol series as follows: 30 s once each in 30%, 50%, 70%, 90%, 95% methanol, and 30 s twice in 100% methanol, then air-dry.

Hybridization: *In situ* hybridization was performed on serially sectioned tissue slides. Before adding the probes, the sections were pre-incubated in DIG Easy Hyb solution (Roche) for 1 h. at 42 °C. The digoxigenin-labeled sense and antisense probe from *Ag*actin and *Agace*1 and unlabeled sense and antisense strand DNA were resuspended in hybridization solution, respectively, applied to sections, and incubated overnight. That is, 10 µl of the probe was diluted in 1 ml hybridization solution. A 30 µl hybridization solution was placed on each section with a coverslip on top without air bubbles. Slides were placed in a humidity chamber made with Kimwipe dampened with 5 ml 50% formamide /5 × SSC in a Petri dish. Hybridization was held at 48 °C overnight.

After slides were transferred to another Petri dish, they were washed in 50% formamide and 2 × SSC at 48 °C for 15 min and 30 min, respectively. Then they were washed twice in 25% formamide, 1 × SSC, and 0.5 × PBS at 48 °C for 30 min and washed in PBS at room temperature for 5 min. The final step was immunodetection and development. The procedure was the same as immunodetection in IHC above. The primary antibody was mouse anti-DIG, and the secondary antibody was goat anti-mouse.

All the methods for IHC and ISH were improved from what was described by Ingham and Jowett (1997), Franco et al. (2001), and Kim et al. (2006) to amplify signal and to reduce background noise. All controls were performed under the same conditions of the treatments without the primary antibody or the *Ag*AChE1 antisense probe.

Results

Immunohistochemistry

Antibody: The antibody titer was determined by immunoblotting analysis. Strong signals are obtained from 200 to 800 times *Ag*AChE1 antibody dilution (Figure 1). Therefore, 500 times *Ag*AChE1 antibody dilution was determined for immunolocalization.

Absorption: Since *Ag*AChE1 antibody cross-reacts with *Ag*AChE2, it is necessary to absorb the cross-reacting antibodies. Absorption is an efficient method (Figure 2). *Ag*AChE2 signal disappears in the immunoblotting after absorption seven times.

***Ag*AChE1 is predominantly in the CNS:** The temporal and spatial distribution of *Ag*AChE1 was determined from *A. gambiae* tissues. The presence of *Ag*AChE1 antigen was found in the brain of *A.gambiae* larvae and pupae, the thoracic ganglion of *A.gambiae* adults, the brain and thoracic ganglion of the Asian tiger mosquito (*Aedes*

albopictus) adults, and the abdominal ganglion of *A. albopictus* larva (Table 1, Figure 3, 4). IHC demonstrated that *Ag*AChE1 antigen is mainly localized in the CNS.

Although *Ag*AChE1 protein is present mainly in the CNS, it is also found in the peripheral nervous system (PNS), for example, the base of antennal and midgut nerves of *A. gambiae* adults (Figure 5). The signal from the base of the antenna is intense. Unfortunately, it is difficult to separate the signal of *Ag*AChE1 protein expression from the nerves of compound eyes, because of the pigment background.

Color precipitation showed that *Ag*AChE1 is expressed in the center of three brain lobes – protocerebrum (forebrain), duetocerebrum (midbrain), and tritocerebrum (hindbrain). *Ag*AChE1 antigen was in the neuropile of ganglia as well, but not in the cell bodies (Figure 3, 4). It is reasonable, because interneurons connect sensory neurons and motor neurons in the neuropile of ganglia as an interneuron bridge to form lots of synapses (Romoser and Stoffolano, 1994). These results suggested that the main function of *Ag*AChE1 is in the cholinergic synapse of the CNS.

In situ hybridization

Probes efficiency and the positive control: Because the template of the *Ag*actin sequence was an *A. gambia* cDNA pool, the confirmation of the *Ag*actin sequence is essential. TA cloning and sequencing analysis of *Ag*actin showed 99% identities between the PCR product and the EST sequence. The E-value was 9e-54. After optimizing the

conditions, the DIG labeled sense and antisense probes of *Aga*ctin and *Agace*1 were successfully generated by asymmetric PCR (Figure 8, 10). The sense and antisense probes of *Ag*actin can recognize 43 ng *Ag*actin dsDNA PCR products clearly with a 21.8 ng sense probe and a 22.5 ng antisense probe (Figure 9), while the 20.2 ng sense and 26.8 ng antisense probes of *Agace*1 can generate signals from at least 26.1 ng *Agace*1 dsDNA (Figure 11). Signals of *Ag*actin gene were obviously higher from DIG-labeled antisense probe compared to those from DIG-labeled sense probe at larval cuticle and brain (Figure 12).

***Agace*1 mRNA transcript is mainly in the CNS:** *In situ* detection of *Agace*1 gene expression demonstrated that signals were much higher from a DIG-labeled antisense probe compared to those from a DIG-labeled sense probe (Figure 13, 14, 15). These results showed that the antisense probe produced strong color precipitation in the target tissues while the color from the sense probe was very weak, and the sections treated by unlabeled sense or antisense probe were totally negative. The strong signals from an antisense probe were predominantly observed from the subset of neurons, or the neuropile of ganglia (Figure 14D) and three brain lobes – protocerebrum (forebrain), duetocerebrum (midbrain), and tritocerebrum (hindbrain) (Figure 14B, 15B), while *Agace*1 signals from cell bodies of the brain were observed from both sense and antisense probes (Figure 13A and B, 15A and B). Although *Agace*1 expresses predominantly in the CNS, it is also found in the PNS. For instance, the larval abdominal nerve obviously showed signals from antisense probe hybridization (Figure 13D) compared with the signals from sense probe hybridization (Figure 13C). The results of ISH are consistant

with IHC results. The findings indicated that *Agace*1 encodes *Ag*AChE1 and predominantly in the CNS. *In situ* hybridization confirmed that main function of *Ag*AChE1 may be in the cholinergic synapse of the CNS as well.

Discussion

Localization of protein and gene expression offers important information. In this study, the spatial and temporal expression patterns of *Ag*AChE1 and *Agace*1 were localized from the sections of paraffin-embedded *A. gambiae* adult, pupal, and larval specimens by IHC and ISH. The study yielded the main findings as follows. *Ag*AChE1 expression mainly localizes in the CNS including the brain, the thoracic ganglion, and the abdominal ganglion. *Ag*AChE1 is functional to hydrolyze ACh in the CNS. At same time, minor expression appears in the PNS, such as base of antenna and midgut. Furthermore, ISH confirmed that *Agace*1 encodes *Ag*AChE1 that is functional in the cholinergic synapse of the CNS. The signal sensitivity of ISH is lower than IHC, but ISH can provide mRNA transcript information. Utilizing both IHC and ISH to localize protein and gene expression can provide information of temporal and spatial expression patterns.

IHC technique has been used successfully in AChE studies (Girard et al., 2004). AChE1 in *C. pipiens, B.germanica* and some other insects also plays a fundamental role for the hydrolysis of ACh at synapses (Bourguet *et al*., 1996; Kim et al., 2006). This study concluded that *Ag*AChE1 is a true functional AChE. Encoded by *Agace*1, the

protein was expressed mainly in the CNS such as three lobes of brain, the neuropile of thoracic and abdominal ganglia. *Ag*AChE1 plays an essential role in synaptic transmission of *A. gambiae*, and probably involves in the organophosphorus and carbamate insecticide resistance. Nonetheless, the findings showed that *Ag*AChE1 exists the peripheral nervous system (PNS) as well (Figure 5).

The Asian tiger mosquito (*Aedes albopictus*) is maninly a vector for dengue fever and other diseases (Novak, 1992). *A. albopictus* were first found in North America in 1985 (Sprenger and Wuithiranyagool, 1986). Its AChE has not been studied. Interestingly, the AChE antigen of *A. albopictus* was recognized from the sections of paraffin-embedded *A. albopictus* adult, pupal, and larval specimens by using *Ag*AChE1 antibody. The signals were investigated in the brain and thoracic ganglion of the *A. albopictus* adults and the abdominal ganglion of *A. albopictus* larva by IHC. The AChE antigen should be AChE1 of *A. albopictus.* This indicated that the sequence of *Ag*AChE1 shares high similarity with the AChE of *A. albopictus*.

When an antibody localizes the protein expression in tissues, the specificity of the antibody is critically important (Burry, 2000). The specificity of antibody binds to appropriate epitopes of the protein. Therefore, the specificity must be determined. Immunoblotting analysis is the best way to evaluate the specificity of an antibody (Burry, 2000). The study showed that absorption is an efficient method to absorb the cross reacting antibodies. Alternatively, the specific antibody can be synthesized from the specific epitopes. For example, *Ag*AChE1 has 11 epitopes (see chapter IV Figure 2).

After *Ag*AChE1 aligns with some related proteins, the epitopes from less similar regions can be used to synthesize specific antibodies.

*Agace*1 mRNA transcripts mainly localize in the neuropile and cell bodies. The findings suggested that *Agace*1 encodes active *Ag*AChE1 and the gene expression mainly associated with synaptic transmission at cholinergic synapses. Spatial expression patterns of *Agace*1 in the CNS were almost the same as many other insect species, such as, *B. germanica*, *P. xylostella*, in where more *ace* genes are transcribed (Baek *et al*., 2005; Kim et al., 2006). Moreover, based on the observations of IHC and ISH in this study, it was clear that the brain is an ideal tissue and dominant location for determining the *Ag*ace1 and *Ag*AChE1 expression patterns.

Sense and antisense probes are becoming increasingly popular with *in situ* hybridization. Antisense provides excellent specificity and high sensitivity (Wang et al, 1998). It promises to detect low abundance mRNAs in tissue section, while sense probe is a good negative control. Most researchers have achieved excellent results with RNA probes, which need to avoid RNA contamination and degradation. In contrast, single-stranded DNA as a probe has many advantages without above problems. The hybridization procedure is easy to operate by using sense and antisense strand DNA probes. Even so, few published papers used this method in insect AChE ISH due to the difficult optimization of asymmetric PCR conditions to increase the yield and efficiency. Once some critical techniques are grasped, then it is a broad way to manage ISH. This study shows the successful experience that researchers could employ single strand DNA probes

to look into mRNA transcript locations.

In eukaryotes, there are endogenous antisense RNAs, which regulate gene expression (Knee and Murphy, 1997; Røsok and Sioud, 2003). Specifically, they compete with sense transcripts or affect splicing, RNA interference, RNA transport, mRNA stability, even translation (Li and Murphy, 2000; Vanhee-Brossolet and Vaquero, 2000). Antisense RNAs disturb the hybridization. It is why some signals appear from sense probes. In some cases, the levels of mRNA are even lower than the levels of antisense transcripts (Murphy and Knee, 1994). Therefore, the most important strategies of ISH are the inclusion of several controls as described below.

ISH is a long procedure with complex conditions. To deal with potential problems, there are some controls to include. For example: (1) Hybridizing the actin mRNA is an ideal positive control, because it is a housekeeping gene abundantly expressed in most living cells. If there is no signal detected using actin probes, problems may be from hybridization procedure, mRNA degradation from tissues, or techniques of tissue preparation. (2) Unlabeled sense and antisense DNA probes as controls check the background noise. (3) In all process of ISH, a labeled sense probe for use as a negative control. (4) The slides treated by RNase before application of the labeled sense and antisense probes. This is also a good negative control to test the background.

The research showed that DIG-labeled sense and antisense strand probes were successfully used for ISH. To increase ssDNA probe efficiency, some methods have been

tried in this study. (1) Optimizing PCR conditions are strongly needed. Asymmetric PCR requires extensive optimization from primer design to the amount of template, activity conditions, and number of amplification cycles so that one can generate reasonable amounts of products. (2) It is important to identify the proper primer ratios. It allows mainly production of the sense or antisense strand DNA at certain starting materials and conditions. From this experiment, a 1000:1 molar ratio of the two primers changed to 2000:1 molar ratio of primers, resulting in much less dsDNA but increased efficiency. (3) Optimizing probe denature temperature is a key to avoid dsDNA denaturation but allow ssDNA to denature. (4) Adding 1% unlabeled sense/antisense strand DNA into labeled antisense/sense probes help reduce background noise. (5) Based on my experience, in order to increase hybridization efficiency and decrease the background noise, it is necessary to purify the probes to remove DIG-dUTP and other non-probe materials. (6) Using higher probe concentration or longer hybridization time compensates the low probe concentration or efficiency.

In summary, *Agace*1 appears to encode active *Ag*AChE1 mainly in the CNS, *Ag*AChE1 associates with synaptic transmission. The sense and antisense strand DNA probes successfully localize *Agace*1 mRNA transcripts. The techniques could be employed in other insect ISH studies. The information gained from *Agace*1 and *Ag*AChE1 expression patterns provides direct evidence for its functions *in vivo*.

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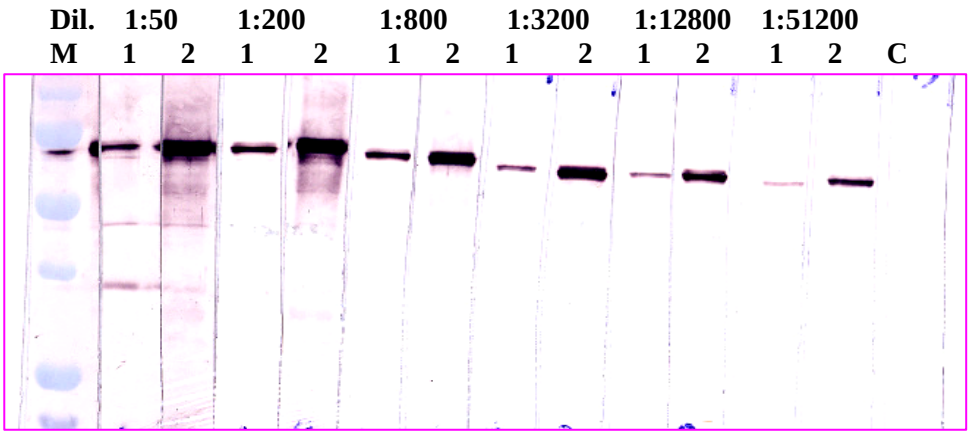


Figure 1. The rabbit anti-*Ag*AChE1 polyclonal antibody work efficiency determination. Dil.: dilution. M: marker. 1: 1st boost. 2: 2nd boost. C: Pre-immunization. 100 ng *Ag*AChE1 per lane. Development time 3.5 min.

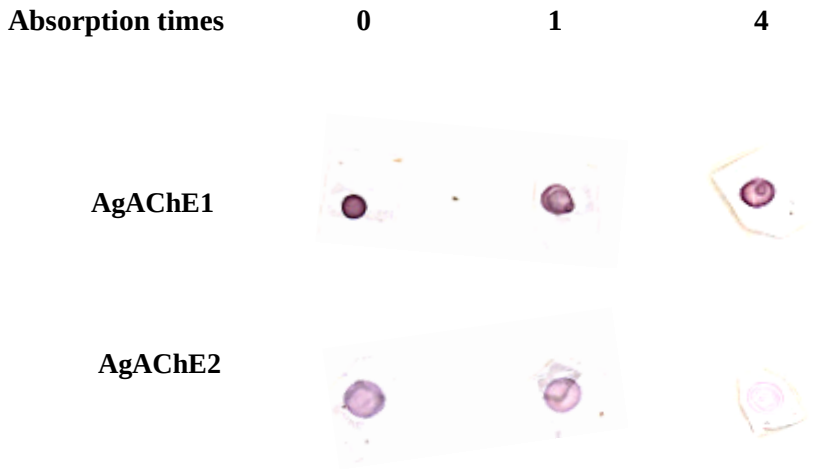


Figure 2. Dot blotting analysis of the rabbit anti-*Ag*AChE1 antibody absorption. The antibody was absorbed by *Ag*AChE2. 38 ng *Ag*AChE1 or *Ag*AChE2 per dot. Development time 5 min.

Table 1. The spatial and temporal expression patterns

A. gambiae	Brain	Ganglion	PNS
Adult	#	+ #	+
Larva	+ #		#
Pupa	+ #		

+: The signals were showed from IHC. #: The signals were showed from ISH.

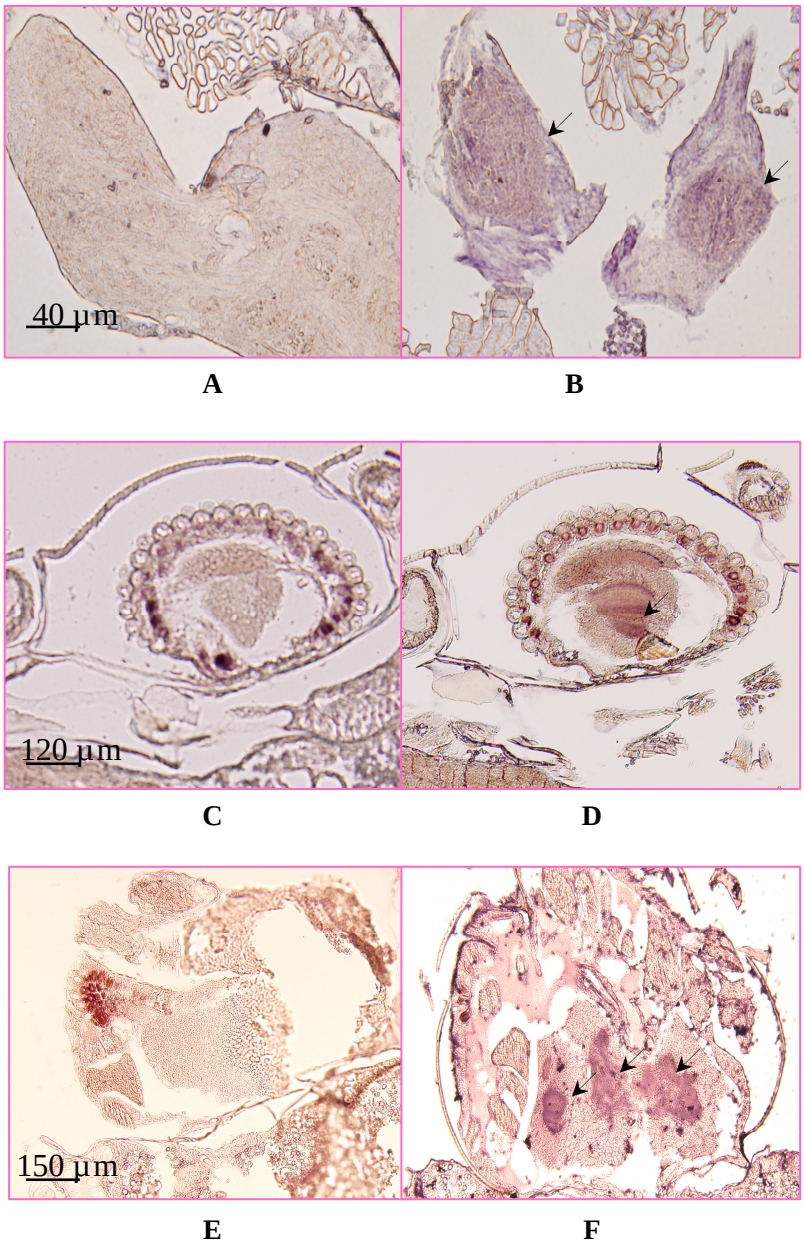


Figure 3. *Ag*AChE1 expression detection by IHC. *Ag*AChE1 antigen was stained in blue or brownish-black in the tissues. The section is 6 µm from the paraffin-embedded mosquito specimens. A: Thoracic ganglia of adult - control. B: Thoracic ganglia of adult - treatment. C: Brain of pupa – control. D: Brain of pupa – treatment. E: Brain of larva –

control. F: Brain of larva – treatment. Arrows indicate the signals.

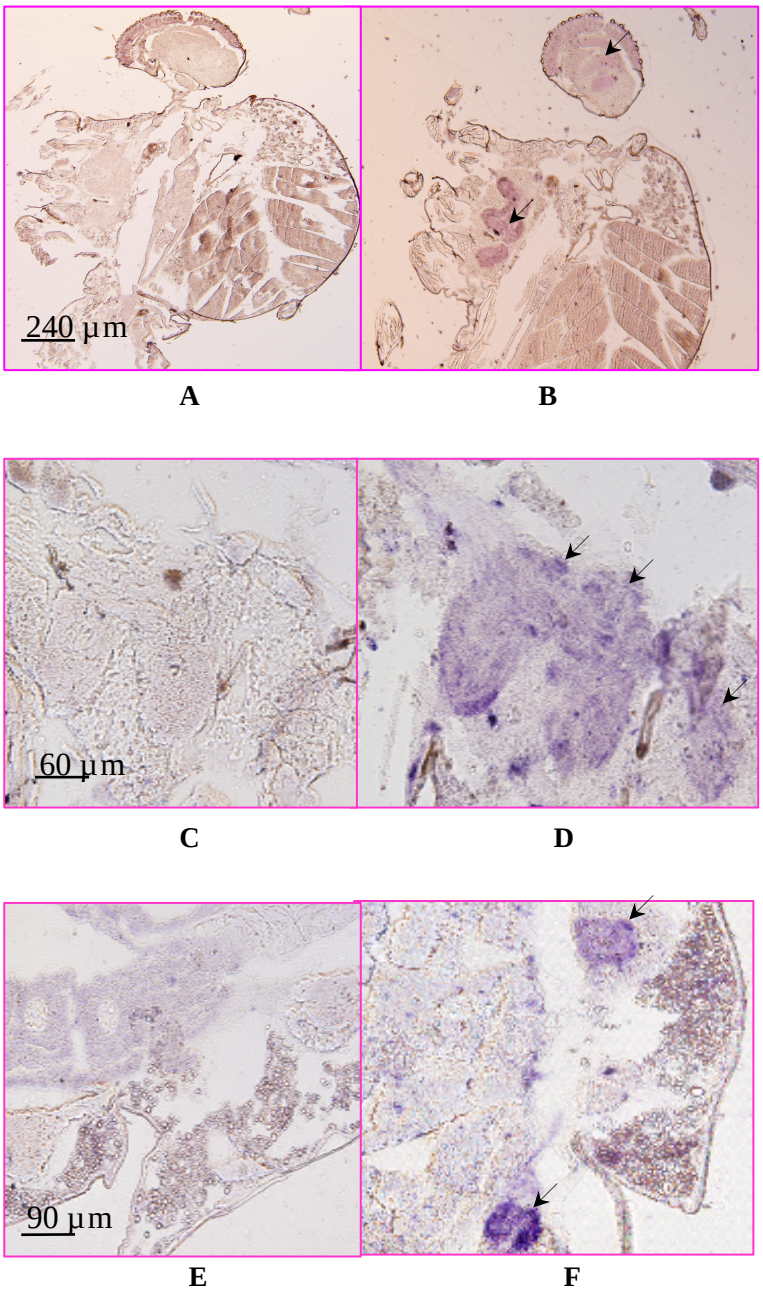
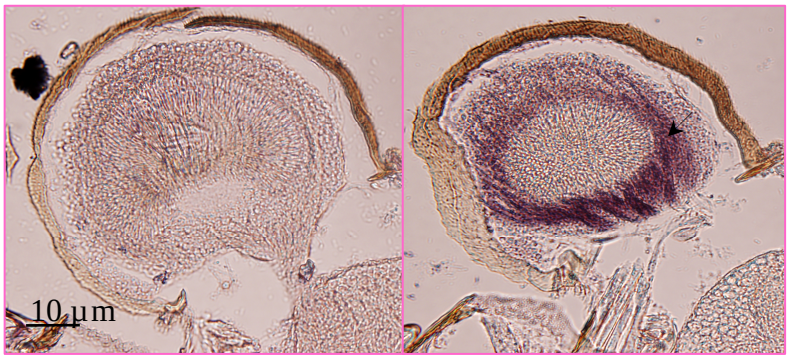
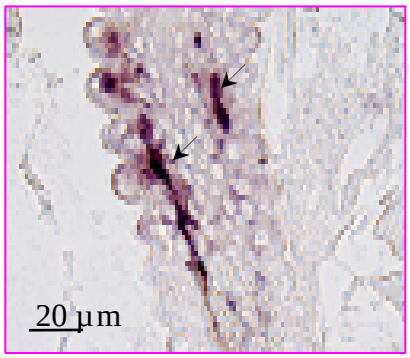


Figure 4. The *Ag*AChE1 antibody took effect in other mosquito tissues. IHC was performed on the Asian tiger mosquito (*A. albopictus*) in paraffin-embedded mosquitoes (6 µm) by using *Ag*AChE1 antibody. A: Brain and thoracic ganglia of adult – control. B:

Brain and thoracic ganglia of adult - treatment. C: Thoracic ganglia of adult - control. D: Thoracic ganglia of adult - treatment. E: abdominal ganglia of larva – control. F: abdominal ganglia of larva – treatment. Color precipitation is in the neuropile of ganglia, but not in the cell bodies. The antigen was stained in blue or brownish-black. Arrows indicate the signals.



A **B**



C

Figure 5. . *Ag*AChE1 expression detection at antennal and gut nerve by IHC. *Ag*AChE1 antigen was stained in blue on the tissues. The section is 6 µm from the paraffin-embedded mosquitoes. A: Base of antenna of adult – control. B: Base of antenna of adult – treatment. C: Signal in the stomatogastric nervous system. Arrows indicate the signals.

TGTTGGCTACTGGGTGCAGGGCGGATTCGCCGGTGATGACGCCCCACGTGCCGTCTTCCCGTCCATTGTTG
GCCGTCCCCGTCACCAGGGTGTGATGGTCGGTATGGGCAACAAGGACTCGTACGTCGGTGATGAGGCCCAG
TCCAAGCGTGGTATCCTCACCCTGAAGTACCCGATCGAGCACGGTATCATCACCAACTGGGATGATATGGA
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AGGCCCCGCTGAACCCGAAGGCTAACCGCGAGAAGATGACTCAGATCATGTTTGAGACCTTCAACTCGCCG
GCCATGTACGTCGCCATCCAGGCCGTGCTGTCGCTGTACGCTTCCGGTCGTACCACCGGTATTGTGCTGGA
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CCATTGGCAACGAGCGTTTCCGCTGCCCGGAGGCTCTGTTCCAGCCGTCGTTCCTGGGTATGGAATCGTCG
GGCATCCACGAGACCGTCTACAACTCGATCATGAAGTGCGACGTCGACATCCGTAAGATCTGTACGCCAAC
ACTGTCCTGTCCGGTGGTACACCATGTACCCGGGTATTGCCGATCGTATGCAGAAGGAAATCACTGCCCTT
GCCCCGTCCACCATCAAGATCAAGATCATCGCTCCCCCAGAGCGTAAGTACTCCGTCTGGATCGGTGGATC
CATCCTGGCCTCGCTGTCCACCTTCCAGGCCATGTGGATCTCCATTTTAGGAGTACGACGA

Figure 6. *Ag*actin probes for ISH from the nucleotide sequences of *Ag*actin EST (BX063031). The full-length cDNA cloned from *A. gambiae* adult (Gomez et al., 2005). The open reading frame (ORF) is from nucleotide 95 to nucleotide 967. The start codon and stop codon are shaded in pink. The probes are 189 nucleotides long in red. The forward primer is from nucleotide 131 to 157 in red underlined. The reverse primer is from nucleotide 293 to 319 in red underlined.

-32
ATGCTTATGCAATGCGCTCTCCGCCCGTGCCGATGGAGATCCGAGGGCTGCTGATGGGTAGACTACGGTTA
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TACCAGGGCCAGCACCGCCTAGTGAACCGTGCGAAAGCAGCGCATTTTTTTACCGACCTGATCTGATCGTG
CTGCTGGTGTCGCTGCTTACGGCGACCGTCAGATTCATACAATAATTA 2217

Figure 7. *Ag*AChE1 probes for ISH from the nucleotide sequences of *Ag*AChE1 EST. The EST (BM629847) has 4,106 bp (nucleotide -232 to 3874). The forward primer is from nucleotide -1 to 18 in red under lined. The reverse primer is from nucleotide 285 to 360 in red under lined. The antisense and sense probes are 307 oligonucleotides in red color in 3rd exon, which is between the two single shaded nucleotides at nucleotide 215 and 572 in green. The start codon and stop codon are shaded in pink. The probe segment is chosen from the beginning of the ORF. This region has less identical nucleotides between *Ag*AChE1 and *Ag*AChE2.

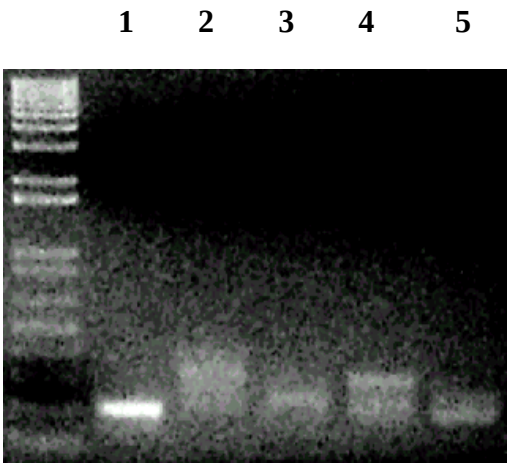

Figure 8. Analysis of *Ag*actin probes by gel electrophoresis. 1: Before labeling – dsDNA. 2-5: ssDNA. 2: DIG-Labeled sense probe. 3: DIG-Labeled antisense probe. 4: Unlabeled sense strand DNA. 5: Unlabeled antisense strand DNA.

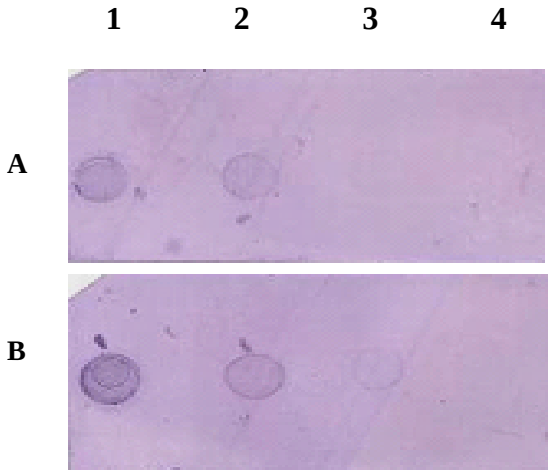

Figure 9. The efficiency determination of *Ag*actin probes by dot blotting.

A: DIG-labeled sense probe, 21.8 ng. B: DIG-labeled antisense probe, 22.5 ng.

(1) 430 ng, (2) 43 ng, (3) 4.3 ng, (4) 0.43 ng dsDNA PCR products.

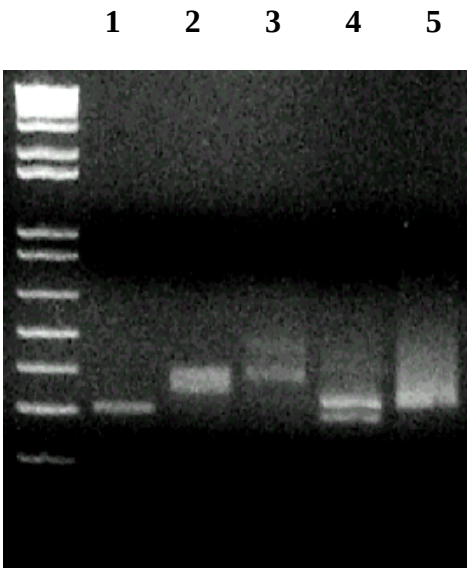

Figure 10. Analysis of *Ag*AChE1 probes by gel electrophoresis. 1: Before labeling – dsDNA. 2-5: ssDNA. 2: DIG-Labeled sense probe. 3: DIG-Labeled antisense probe. 4: Unlabeled sense strand DNA. 5: Unlabeled antisense strand DNA.

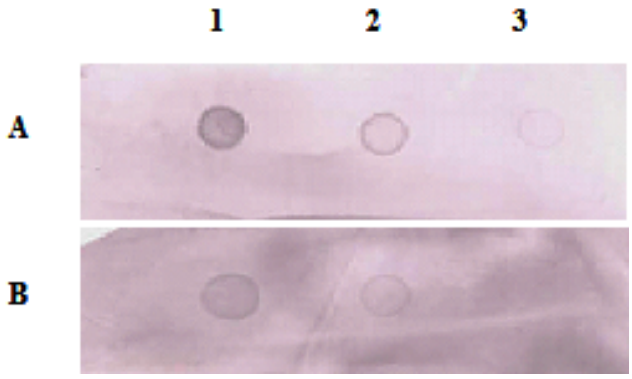

Figure 11. The efficiency determination of *Ag*AChE1 probes by dot blotting. A: DIG-labeled sense probe, 20.2 ng. B: DIG-labeled antisense probe, 26.8 ng. (1) 261 ng, (2) 26.1 ng, (3) 2.61 ng dsDNA PCR products.

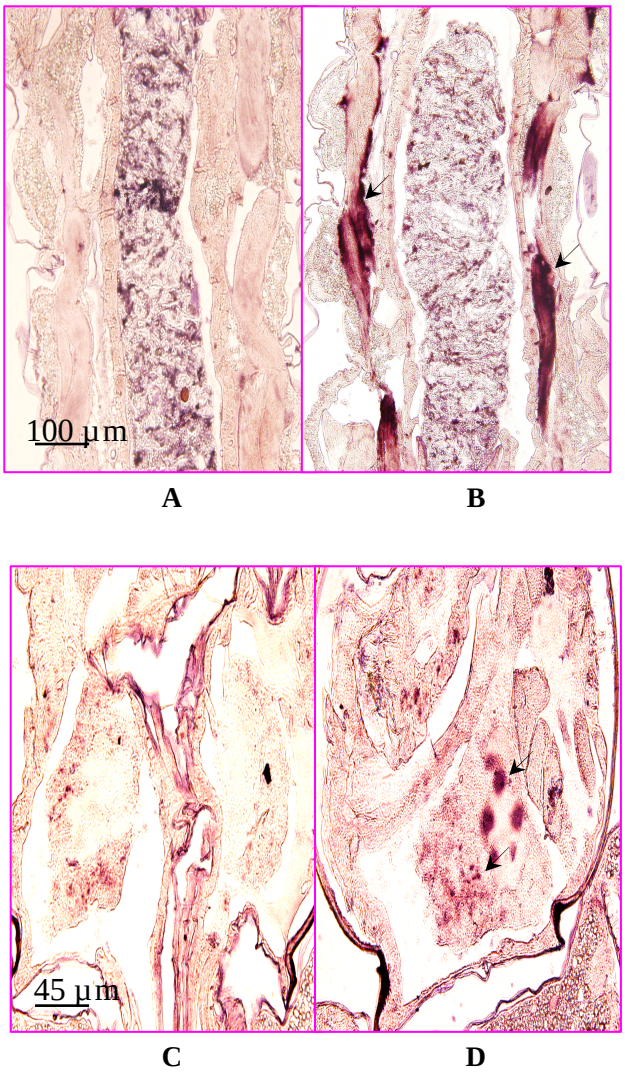

Figure 12. Localization of *Ag*actin mRNA transcript in larval tissues by ISH. The sections were hybridized with DIG-labeled sense and antisense probes. A: Larval cuticle treated with sense probe as a negative control. B: Larval cuticle hybridized with antisense probe. C: Larval brain treated with sense probe as a negative control. D: Larval brain hybridized with antisense probe. *Ag*actin antigen stains dark-blue on the tissues. Arrows indicate the signals.

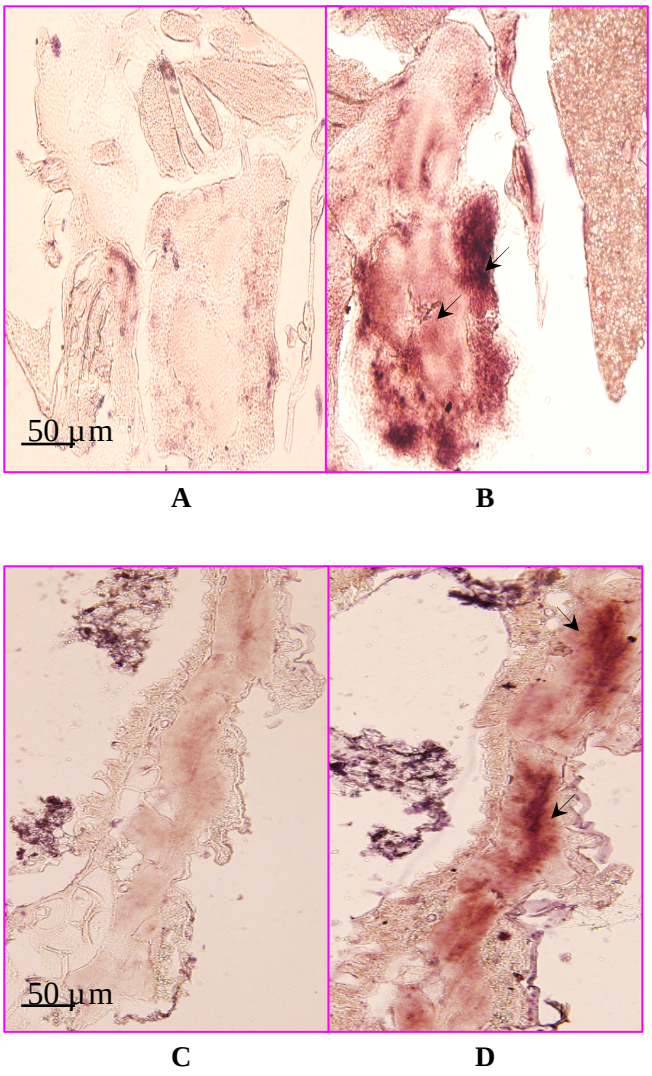

Figure 13. Localization of *Agace*1 mRNA in larval tissues by ISH. The sections were hybridized with digoxigenin-labeled sense and antisense probes. A: Larval brain treated with sense probe as a negative control. B: Larval brain hybridized with antisense probe. Color precipitation is on the neuropile and the cell bodies of the brain from antisense probe hybridization. The signal from sense hybridization only showed on the cell bodies. C: Larval abdominal nerve treated with sense probe as a negative control. D: Larval abdominal nerve hybridized with antisense probe. AgAChE1 antigen stains dark-brown on the tissues. Arrows indicate the signals.

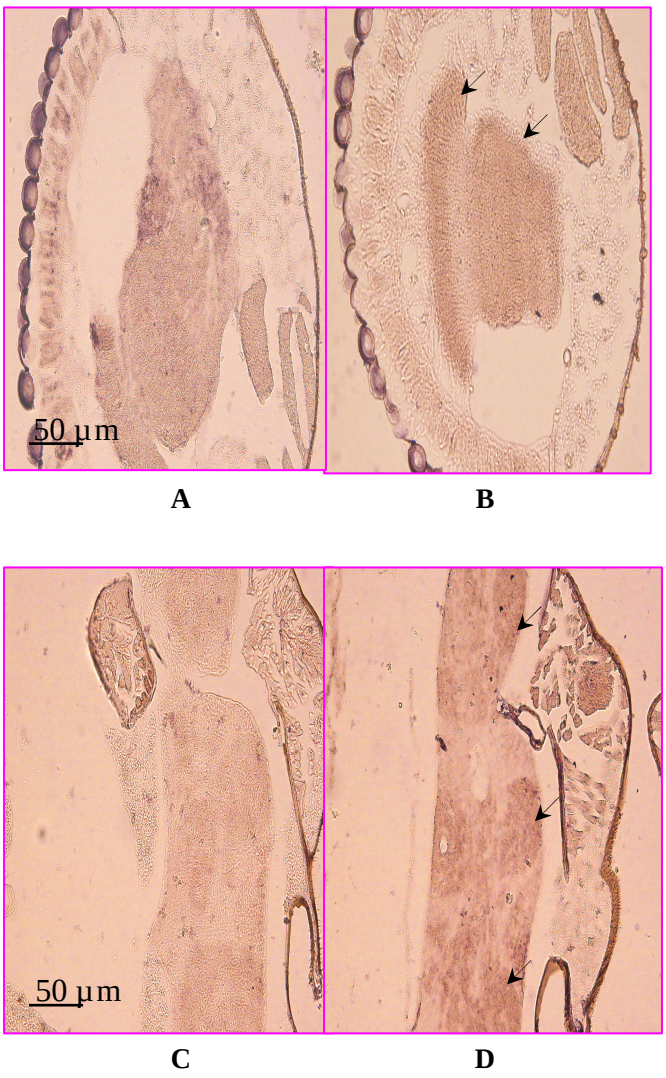

Figure 14. Localization of *Agace*1 mRNA in adult tissues by ISH. ISH using a DIG-labeled *Agace*1 sense or antisense probe on an adult section. A: Adult brain treated with sense probe as a negative control. B: Adult brain hybridized with antisense probe. Color precipitation is from three brain lobes – protocerebrum (forebrain), duetocerebrum (midbrain), and tritocerebrum (hindbrain). C: Adult thoracic ganglia treated with sense probe as a negative control. D: Adult thoracic ganglia hybridized with antisense probe. AChE1 antigen stains brown on the neuropile of the ganglia. Arrows indicate the signals.

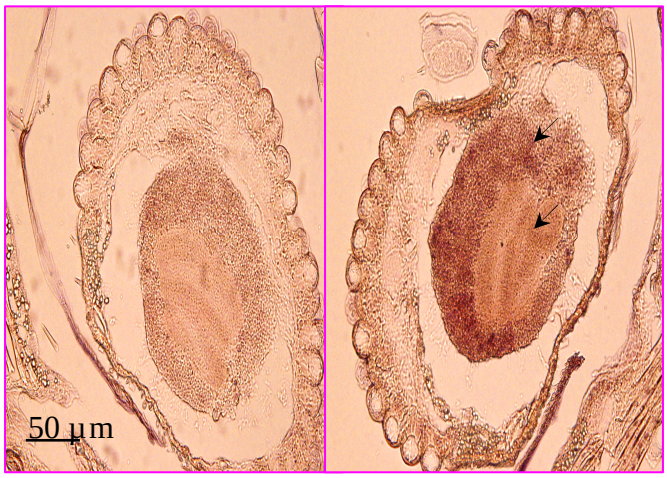

Figure 15. Localization of *Agace*1 mRNA in pupal tissues by ISH. ISH using a DIG-labeled *Agace*1 sense or antisense probe on an adult section. A: pupal brain treated with the sense probe as a negative control. B: pupal brain hybridized with the antisense probe. Color precipitation is strongly on the cell bodies and three lobes of the brain from antisense probe hybridization. The signals from sense probe hybridization showed on the cell bodies and three lobes of the brain as well, but weaker compared with the signal from antisense probe hybridization. Arrows indicate the signals.

CHAPTER VI

SUMMARY

Vertebrate and invertebrate acetylcholinesterases (AChEs, EC 3.1.1.7) terminate acetylcholine action at cholinergic synapses. Being the target enzymes of organophosphorus and carbamate pesticides, AChEs and their genes have been isolated from susceptible and resistant insects to study the molecular basis of target site insensitivity. Recombinant expression and characterization of highly purified wild-type and mutant AChEs serve as a reliable platform for studying structure-function relationship.

The African malaria mosquito, *Anopheles gambiae* (Diptera: Culicidae), possesses two AChE genes (*ace*1 and *ace*2). The molecular and biochemical properties of *A. gambiae* AChEs remain unknown. The existence of complex resistance mechanisms makes it difficult to correlate directly a mutation with the phenotype. These issues negatively impact the development of highly selective insecticides against this principal vector of malaria.

In this study, a cDNA fragment of AChE1 was subcloned from an *A. gambiae* EST and expressed in *Spodoptera frugiperda* (*Sf*21) cells. The purification scheme was optimized. After baculovirus amplification and expression, the final concentration of *Ag*AChE1 reached 56 μg/ml and was purified 2.5×10^3 folds in a three-step purification procedure including ion-exchange and affinity chromatography. This process took approximately eight hours and yielded 51% of the protein near homogeneity. From 600 ml culture supernatant, 476 μg of the protein was obtained with a specific activity of 523.1 U/mg.

*Ag*AChE1 biochemical properties were characterized. I analyzed the *Ag*AChE1 sequence and derived useful information about its properties. The best range for *Ag*AChE1 reaction with acetylcholine is pH 7.0-8.5. The enzyme migrated as a single band at 65 and 130 kDa positions on SDS-polyacrylamide gels under reducing and nonreducing conditions, respectively. While K_M's of *Ag*AChE1 for ATC, AβMTC, PTC, and BTC were comparable, the V_{max}'s were substantially different: 209, 122, 84 and 15 μM/min/mg, in the order given. *Ag*AChE1 hydrolyzes ATC much faster than BTC, suggesting that the enzyme is a true AChE. This is consistent with results from the inhibition assays. The IC_{50}s showed that *Ag*AChE1 was highly sensitive to inhibition by eserine and BW284C51, but was less sensitive to it by ethopropazine. The k_i and the K_d of six inhibitors for *Ag*AChE1 were $4.00 \times 10^3 \sim 2.19 \times 10^6$ M^{-1} min^{-1} and $1.19 \times 10^{-3} \sim 9.90 \times 10^{-8}$ M. The k_2 ranges from 0.06 ~ 53.62 min^{-1}. *Ag*AChE1 is most sensitive to malaoxon and BW284C51, least so to carbaryl and ethopropazine. The affinity of BW284C51 was about 1.21×10^4 -fold greater than the affinity of carbaryl. The data may help to better understand the development of insecticide resistance in the African malaria mosquito.

To characterize spatial and temporal expression patterns of a gene and to detect its protein product, immunohistochemistry (IHC) and *in situ* hybridization (ISH) are commonly used, respectively. In this work, IHC showed that *Ag*AChE1 protein predominantly exists in the central nervous system (CNS) (brain and ganglia) of *A. gambiae* larvae, pupae, and adults. The protein is found in the peripheral nervous system (PNS) as well, such as the base of antennal and mid-gut nerves of *A. gambiae* adults. We prepared the sense and antisense DNA probes by asymmetric PCR under optimized conditions. ISH indicated

that *Agace*1 transcripts exist primarily in the CNS. These results suggested that the main function of *Ag*AChE1 is in cholinergic synapses of the CNS to hydrolyze acetylcholine. The hybridization signals were clearly detected on the neuropile or three brain lobes and cell bodies of the CNS using the antisense probe. Signals were much weaker when sense probe was applied. *Ag*AChE1 is a physiologically relevant enzyme for ACh hydrolysis at cholinergic synapses. These findings further support that *Ag*AChE1 should be considered as a target enzyme for selective anticholinesterase agent design to suppress the African malaria mosquito.

VITA

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Findings and Conclusions:

Acetylcholinesterases (AChEs) play an essential role in neurotransmission at cholinergic synapses in vertebrates and invertebrates. *Anopheles gambiae* has two AChE genes, *ace*1 and *ace*2. The properties of *Ag*AChEs remain unknown, and the complex resistance mechanisms can cause the problem to directly correlate a mutation with the resistance phenotype. The purposes of this study are to express and purify AChE1, characterize it in vitro, and localize its expression in vivo. In this study, a cDNA fragment of AChE1 from an *A. gambiae* EST was subcloned and expressed. The optimized three-step purification scheme took approximately eight hours and yielded 51% of the protein with a specific activity of 523U/mg. A pH of 7.0-8.0 is the best range for *Ag*AChE1 reaction with acetylcholine. The enzyme size is 65 kDa and 130 kDa on SDS-polyacrylamide gels under reducing and nonreducing conditions, respectively. *Ag*AChE1 hydrolizes ATC 14-fold faster than BTC. The IC_{50}, k_i, and K_d demonstrated that *Ag*AChE1 is highly sensitive to inhibition by BW284C51 instead of ethopropazine, and the affinity of BW284C51 is greater than that of ethopropazine. These findings indicate that *Ag*AChE1 is a true AChE, which exerts the physiological function of ACh hydrolysis at cholinergic synapses. *In situ* hybridization and immunohistochemistry showed that *ace*1 is expressed mainly in the central nervous system. The procedures of *Ag*AChE1 purification and asymmetric PCR for making ISH probes could be used for similar studies in other insect species. The data are useful for understanding *Ag*AChE1 and for developing selective insecticides to control the African malaria mosquito.

Key word: Neurotransmission, Insecticide resistance, Mosquito, Malaria, Acetylcholine, *In situ* hybridization, Immunohistochemistry.

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