

CHARACTERIZATION OF CHITIN SYNTHASE AND CHITINASE GENE FAMILIES  
FROM THE AFRICAN MALARIA MOSQUITO

by

XIN ZHANG

M.S., Cornell University, 2007

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Entomology  
College of Agriculture

KANSAS STATE UNIVERSITY  
Manhattan, Kansas

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## Abstract

Chitin metabolism represents an attractive target site for combating insect pests as insect growth and development are strictly dependent on precisely toned chitin synthesis and degradation and this process is absent in humans and other vertebrates. However, current understanding on this process and the involved enzymes is rather limited in insects. In this study, two chitin synthase genes (*AgCHS1* and *AgCHS2* or *AgCHSA* and *AgCHSB*), and 20 chitinase and chitinase-like genes (groups I-VIII) presumably encoding the enzymes for chitin biosynthesis and degradation, respectively, were identified and characterized in African malaria mosquito, *Anopheles gambiae*. Immunohistochemistry analysis and developmental stage- and tissue-dependent transcript profiling by using reverse transcription PCR, real-time quantitative PCR, and in situ hybridization revealed new information on these genes. Current understanding on chitin synthases is extended by the expression profiles such as the localization of *AgCHS1* and *AgCHS2* transcripts in eggs, *AgCHS2* transcripts in the posterior larval midgut, *AgCHS1* and *AgCHS2* proteins in the compound eyes, and *AgCHS2* enzyme in pupal inter-segments. Chitinase and chitinase-like genes are highly diverse in their gene structure, domain organization, and stage- and tissue-specific expression patterns. Most of these genes were expressed in several stages. However, some genes are stage- and tissue-specific such as *AgCht8* mainly in pupal and adult stages, *AgCht2* and *AgCht12* specifically in foregut, *AgCht13* exclusively in midgut.

Functional analysis of each chitin synthase gene was conducted by using the chitosan/dsRNA nanoparticle-based RNA interference (RNAi) through larval feeding. The repression of the *AgCHS1* transcripts which are predominantly expressed in carcass initiated

from the mosquito larval feeding of dsRNA suggests the systemic nature of RNAi in mosquito larvae. In addition, silencing of *AgCHS1* increased larval susceptibilities to diflubenzuron, whereas silencing of *AgCHS2* enhanced the peritrophic matrix disruption and thus increased larval susceptibilities to calcofluor white or dithiothreitol. Furthermore, a non-radioactive method was adapted and optimized to examine the chitin synthase activity in mosquitoes. By using this method, diflubenzuron and nikkomycin Z show limited *in vitro* inhibition on chitin synthase at high concentration in cell free system, whereas no *in vivo* inhibition was observed.

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## Table of Contents

List of Figures .....	xii
List of Tables .....	xviii
Acknowledgements.....	xix
Dedication .....	xxi
CHAPTER 1 - Introduction .....	1
Chitin and chitin metabolism in insects .....	1
Insect Chitin synthases and chitinases .....	4
Diflubenzuron and its mechanism of action .....	7
RNA interference and its application in insect pest control .....	10
Specific aims and significance.....	13
References.....	15
CHAPTER 2 - Identification and characterization of two chitin synthase genes in African malaria mosquito, <i>Anopheles gambiae</i> .....	26
Abstract.....	26
Introduction.....	27
Materials and Methods.....	29
Mosquito rearing.....	29
Sequence analysis of <i>AgCHS1</i> and <i>AgCHS2</i> .....	29
RT-PCR analysis.....	30
Real-time quantitative PCR analysis .....	32
Heterologous expression and purification of antigens and antibody preparations .....	33
Immunohistochemistry .....	34
Fluorescence <i>in situ</i> hybridization (FISH).....	35
Data analysis for qPCR.....	37
Results.....	38
cDNA and deduced amino acid sequences .....	38
Stage-specific expression of <i>AgCHS1</i> and <i>AgCHS2</i> .....	39

Tissue-specific expression profiles of <i>AgCHS1/AgCHS2</i> and <i>AgCHS1</i> alternative splicing variants .....	40
Localization of <i>AgCHS1</i> and <i>AgCHS2</i> transcripts and proteins by FISH and immunohistochemistry.....	40
Discussion.....	41
Acknowledgements.....	45
References.....	46
CHAPTER 3 - Molecular characterization of chitinase genes in African malaria mosquito,	
<i>Anopheles gambiae</i> .....	62
Abstract.....	62
Introduction.....	63
Materials and Methods.....	65
Mosquito rearing.....	65
Genome-wide Blast search and sequence analysis .....	65
RT-PCR analysis.....	66
Real-time quantitative PCR analysis .....	67
Immunohistochemistry .....	68
Fluorescence <i>in situ</i> hybridization .....	69
Data analysis for qPCR.....	71
Results.....	71
Classification of chitinase and chitinase-like proteins in <i>An. gambiae</i> .....	71
Exon and intron organization of the chitinase genes .....	72
Domain architecture of chitinase genes .....	73
Developmental stage-specific expression profiles .....	74
Tissue-specific expression profiles of the <i>AgCht</i> genes .....	75
Localization of <i>AgCht8</i> in pupae by <i>in situ</i> hybridization .....	75
Localization of <i>AgCht5</i> and <i>AgCht8</i> in the pupae by immunohistochemistry.....	76
Discussion.....	76
Acknowledgements.....	81
References.....	82

CHAPTER 4 - Biochemical analysis of chitin synthases and its inhibition in African malaria	
mosquito, <i>Anopheles gambiae</i> .....	99
Abstract.....	99
Introduction.....	100
Materials and Methods.....	102
Materials .....	102
Mosquito rearing.....	103
Crude enzyme preparation, protein content assay, and pretreatment of the enzyme.....	103
CHS activity assays.....	103
<i>In vitro</i> and <i>in vivo</i> inhibition assay .....	105
Paraffin-embedded thin sections and chitin staining .....	105
Statistical analysis for enzyme activity.....	106
Results.....	106
Chitin synthase activity in mosquito pupae .....	106
Effects of dithiothreitol (DTT) and Mg <sup>++</sup> on CHS activity .....	107
Effects of UDP-GlcNAc and GlcNAc on CHS activity .....	107
Optimum pH and temperature for CHS activity.....	108
Proteinlytic activation of CHS activity.....	108
Effect of chitin synthesis inhibitors on CHS activity.....	108
Discussion.....	109
Acknowledgements.....	112
References.....	113
CHAPTER 5 - Chitosan/double-stranded RNA nanoparticle-mediated RNAi to silence chitin	
synthase genes through larval feeding in the African malaria mosquito ( <i>Anopheles gambiae</i> )	
.....	128
Abstract.....	128
Introduction.....	129
Results.....	131
Generation of chitosan/dsRNA nanoparticles for mosquito RNAi through larval feeding	131
RNAi for two chitin synthase genes in mosquito larvae.....	132
Effect of RNAi for <i>AgCHS1</i> on chitin content and larval susceptibility to DFB .....	134

Effect of RNAi for <i>AgCHS2</i> on larval susceptibility and PM permeability to DTT and CF .....	134
Discussion.....	135
Experimental procedures .....	139
Mosquito rearing.....	139
Preparation of chitosan/dsRNA nanoparticles .....	140
Preparation of mosquito larval food containing the chitosan/dsRNA nanoparticles .....	141
Larval feeding on food containing the chitosan/dsRNA nanoparticles .....	141
Atomic force microscopy (AFM) imaging .....	141
RT-PCR and qPCR analysis .....	142
Retention of dsRNA by chitosan/dsRNA nanoparticles.....	142
Chitin content assay .....	143
Chemical treatment and <i>in vivo</i> assay of PM disruption.....	143
Statistical analysis.....	144
Acknowledgements.....	144
References.....	146
CHAPTER 6 - Summary .....	159

## List of Figures

- Figure 1.1 Diagram of the cascade of chitin polymer chain production and subsequent deposition to form cuticle and peritrophic matrix in insects, and chitin degradation. .... 2
- Figure 2.1 Sequence analysis of *AgCHS* genes. (A) Gene structures of *AgCHS*s. *AgCHS1* has two splice variants with alternative splicing exons (6A and 6B). (B) Phylogenetic tree of insect chitin synthase. (C) Phylogenetic tree of the alternative exons of insect *CHS1*. *Ag*, *Anopheles gambiae*; *Ae*, *Aedes aegypti*; *Aq*, *A. quadrimaculatus*; *Dm*, *Drosophila melanogaster*; *Lc*, *Lucilia cuprina*; *Lm*, *Locusta migratoria*; *Ms*, *Manduca sexta*; *Px*, *Plutella xylostella*; *Tc*, *Tribolium castaneum*; *Se*, *Spodoptera exigua*; *Sf*, *Spodoptera frugiperda*; *Eo*, *Ectopis obliqua*; *Ce*, *Caenorhabditis elegans*. GenBank Accession numbers are listed in Table 2.2. Phylogenetic tree was generated using the software MEGA 4 after alignment with ClustalW method. .... 53
- Figure 2.2 Alignment of the conserved catalytic domain of the chitin synthases from three mosquito species: *Ag*, *Anopheles gambiae*; *Aq*, *An. quadrimaculatus*; *Ae*, *Aedes aegypti*. Seven characteristic motifs (M1-7) for insect chitin synthases are highlighted. Dashes are used to denote gaps introduced for a maximum alignment. .... 54
- Figure 2.3 Analysis of stage-specific expression of *AgCHS* genes. (A) RT-PCR analysis of each stage from egg to adult, and (B-D) the expression patterns confirmed by qPCR. Same letters on the error bars indicate no significant difference based on Fisher's LSD ( $P>0.05$ )..... 55
- Figure 2.4 RT-PCR analysis of stage-specific expression patterns of *AgCHS* genes in eggs (A) at 12, 24, 36, 48, and 60 h, respectively, and pupae (B) at 0, 10, 20, 30, and 34 h, respectively. .... 56
- Figure 2.5 Analysis of tissue-specific expression of *AgCHS* genes. RT-PCR analysis from four tissues: FG, foregut; MG, midgut; HG, hindgut; CA, carcass (A), and expression patterns confirmed by qPCR (B-D). Same letters on the error bars indicate no significant difference based on Fisher's LSD ( $P>0.05$ )..... 57
- Figure 2.6 Fluorescence *in situ* hybridization (FISH) analysis of *AgCHS* genes in mosquito gut. Localization of *AgCHS* gene transcripts in the midgut of the adult mosquito 24 h after a bloodmeal by using *AgCHS2* (A) and *AgCHS1* (B) probes, respectively. Localization of the

transcripts in the whole larval gut stained by <i>AgCHS2</i> (C) and <i>AgCHS1</i> (D) probes, respectively. The green color indicated by arrows shows positive staining. ....	58
Figure 2.7 Fluorescence <i>in situ</i> hybridization (FISH) analysis of <i>AgCHS1</i> and <i>AgCHS2</i> in the paraffin-embedded thin sections of the mosquito pupae (12-24 h after pupation). Green color indicated by arrows shows positive staining, and the blue color shows the nuclei by DAPI staining.....	59
Figure 2.8 Immunohistochemistry anti- <i>AgCHS1</i> (A, B) and anti- <i>AgCHS2</i> serum (C, D) in mosquito pupae. Paraffin-embedded thin sections of the 12-24 hour whole mount pupae were immunostained with primary antibodies and visualized by the reaction with Alexa 488-conjugated goat anti-mouse IgG. The epidermal cells in the adult cuticle (A) and the eyes (B) were immunoreactive (arrows indicate positive staining, arrow heads indicate negative staining). The abdominal inter-segmental region of the pupal cuticle (C) and the eyes (D) were immunoreactive (arrows indicate positive staining).....	60
Figure 2.9 Domain analysis of two chitin synthases by TMHMM program. Membrane-spanning $\alpha$ -helices are indicated by red columns. ....	61
Figure 3.1 Phylogenetic analysis of chitinase and chitinase-like proteins from four insect species based on catalytic domains. Ag: <i>A. gambiae</i> ; Ae: <i>Aedes aegypti</i> ; Tc: <i>T. castaneum</i> ; Dm: <i>D. Melanogaster</i> . Phylogenetic tree was generated with a distance neighbor-joining method by using the software MEGA 4 after alignment with ClustalW method. ....	88
Figure 3.2 Schematic diagram of the exon and intron organization of the chitinase and chitinase-like genes from <i>An. gambiae</i> . Boxes indicate exons. Lines indicate introns. ....	89
Figure 3.3 Schematic diagram of the domain architectures of chitinase and chitinase-like proteins from <i>An. gambiae</i> . ....	90
Figure 3.4 Analysis of stage-specific expression of <i>AgCht</i> genes as determined by RT-PCR (EG, eggs; L1-4, larvae from 1st to 4th instars; PU, pupae; AD, adults). ....	91
Figure 3.5 Relative expression of selected genes in different developmental stages as determined by qPCR. Same letters on the error bars indicate no significant difference based on Fisher's LSD ( $P>0.05$ ). ....	92
Figure 3.6 Analysis of tissue-specific expression of <i>AgCht</i> genes as determined by RT-PCR analysis (FG, foregut; MG, midgut; HG, hindgut; CA, carcass). ....	93

Figure 3.7 Relative expression of selected genes in different tissues of mosquito larvae as determined by qPCR. Same letters on the error bars indicate no significant difference based on Fisher's LSD ( $P>0.05$ ). The ribosomal S3 ( <i>AgRPS3</i> ) gene was used as a reference. ....	94
Figure 3.8 Fluorescence <i>in situ</i> hybridization (FISH) analysis of <i>AgCht8</i> in mosquito pupae. Green color indicated by arrows shows positive staining, and the blue color shows the nuclei by DAPI staining.....	95
Figure 3.9 Localizations of chitinase proteins expressed in mosquito pupae by using immunohistochemistry. The proteins were detected in the head and abdominal tip of a pupa by anti- <i>Manduca sexta</i> chitinase 5 polyclonal antibodies (Anti-MsCht5) and pupal compound eye by anti-sand fly ( <i>Lutzomyia longipalpis</i> ) chitinase 8 (Anti-sandy fly Cht8) polyclonal antibodies. Green color shows positive staining, and the blue color shows the nuclei by DAPI staining.....	96
Figure 3.10 RT-PCR analysis of developmental stage-specific expression patterns of <i>AgCht</i> genes in eggs. Developmental times of eggs include: 12, 24, 36, 48, 60 h after laying. The ribosomal S3 ( <i>AgRPS3</i> ) gene was used as an internal reference. ....	97
Figure 3.11 RT-PCR analysis of developmental stage-specific expression patterns of <i>AgCht</i> genes in pupae. Developmental times of pupae include: 0-, 10-, 20-, 30-, 34-h after pupation. The ribosomal S3 ( <i>AgRPS3</i> ) gene was used as an internal reference.....	98
Figure 4.1 Chitin staining in mosquito pupae. Paraffin-embedded thin sections of the 12-24 hour pupae were stained with FITC-CBD and visualized under fluorescent microscope. The epidermal cells in the cuticle were immunoreactive (arrows indicate positive staining) in two pupae (A and B). C and D: the amplified region (red box) in A and B, respectively..	120
Figure 4.2 Specific chitin synthase activity in mosquito pupae. Different volumes of crude enzyme were used for assay (5-, 10-, 20-, 40-, and 50 $\mu$ L). ....	121
Figure 4.3 The DTT and $Mg^{++}$ dependency of CHS activity in mosquito pupae. (A) Enzyme activity was dependent on the DTT in the enzyme extraction buffer. (B) The $Mg^{++}$ dependency of CHS activity. DTT and $Mg^{++}$ were added to the Tris-HCl buffer for crude enzyme preparation. Same letters on the error bars indicate no significant difference based on Fisher's LSD ( $P>0.05$ ).....	122
Figure 4.4 The UDP-GlcNAc and GlcNAc dependency of CHS activity from mosquito pupae. UDP-GlcNAc (A) and GlcNAc (B) were added in the reaction mixture and the	

concentrations in the figure represented the final concentration in the reaction system. Same letters on the error bars indicate no significant difference based on Fisher's LSD ( $P>0.05$ ). ..... 123

Figure 4.5 Effects of pH (A) and temperature (B) on the activity of CHS prepared from mosquito pupae. Same letters on the error bars indicate no significant difference based on Fisher's LSD ( $P>0.05$ ). ..... 124

Figure 4.6 Proteolytic activation of chitin synthesis in different enzyme preparations from the mosquito pupae. Specific CHS activity was measured in the presence (Trp+) and absence (Trp-) of trypsin in 500g supernatant and successive 40,000g pellet which was resuspended in proportional volume of the extraction buffer. Asterisks indicate significant difference based on Fisher's LSD ( $P>0.05$ ). ..... 125

Figure 4.7 Comparisons of chitin synthase activity in the crude enzyme preparations following incubation with various concentrations of three chitin synthesis inhibitors (A) and the crude enzyme preparations from the pupae exposed to the three chitin synthesis inhibitors (B). DF: diflubenzuron; PD: polyoxin D; NZ: nikkomycin Z. Same letters on the error bars indicate no significant difference based on Fisher's LSD ( $P>0.05$ ). ..... 126

Figure 4.8 Standard curve of chitin binding to WGA-coated wells ..... 127

Figure 5.1 Formation of chitosan/ double-stranded RNA (dsRNA) nanoparticles and the retention of dsRNA in the food gel. (A) Schematic representation of electrostatic interactions between chitosan and dsRNA. (B) Atomic force microscopy (AFM) image of the chitosan/dsRNA nanoparticles. (C) AFM image of chitosan solution without the addition of dsRNA. All the scan sizes of the images were  $1.0 \mu\text{m} \times 1.0 \mu\text{m}$ . (D) Retention of dsRNA by chitosan/dsRNA nanoparticles in the food gel. +NP: dsRNA was entrapped in nanoparticles; -NP: dsRNA was used directly without nanoparticles. The data are presented as means  $\pm$  SEM of triplicate samples. (E) Retention of dsRNA by the chitosan/dsRNA nanoparticles in the food gel was evaluated by agarose gel electrophoresis. .... 152

Figure 5.2 Designs of double-stranded RNA (dsRNA) for oral RNAi, and repressions of transcript levels of two chitin synthase genes *AgCHS1* and *AgCHS2* after RNAi in mosquito larvae. (A, B) Diagrams illustrating the regions for designing two dsRNA fragments (i.e., f1 and f2) from each of the two genes (*AgCHS1* and *AgCHS2*). Arrow heads indicate the primer regions for examining the transcript level of each gene by quantitative real-time

PCR. (C) Relative transcript levels of *AgCHS1* and *AgCHS2* in the larvae after continuously fed on each of the two *AgCHS1* dsRNA fragments (i.e., ds*AgCHS1*-f1 and ds*AgCHS1*-f2) or *green fluorescent protein (GFP)* dsRNA (ds*GFP* as controls) that were incorporated into the nanoparticles for 4 days (once a day). The data are presented as means  $\pm$  SEM of five replicate samples. (D) Relative transcript levels of *AgCHS1* and *AgCHS2* in the larvae after continuously fed on each of the two *AgCHS2* dsRNA fragments (i.e., ds*AgCHS2*-f1 and ds*AgCHS2*-f2) or *GFP* dsRNA (ds*GFP* as controls) that were incorporated into the nanoparticles for 4 days (once a day). The data are presented as means  $\pm$  SEM of three replicate samples. Same letters on the error bars indicate no significant difference based on Fisher's least significant difference test ( $P > 0.05$ ). ..... 153

Figure 5.3 Tissue-specific expression patterns of *Anopheles gambiae* chitin synthase gene (*AgCHS*) as analyzed by reverse transcription PCR in mosquito larvae. A ribosomal protein S3 gene (*AgRps3*) was used as an internal reference. .... 154

Figure 5.4 Effect of nanoparticles-mediated oral RNA interference (RNAi) of *Anopheles gambiae chitin synthase 1 (AgCHS1)* on chitin content and larval susceptibility to diflubenzuron (DFB) in mosquito larvae. (A) Effect of the RNAi on chitin content. The data are presented as means  $\pm$  SEM of four replications ( $n = 4$ ). (B) Effect of the RNAi on susceptibility of the larvae to DFB. The data are presented as means  $\pm$  SEM of three replications ( $n = 3$ ). Different letters on the error bars indicate significant difference based on Fisher's least significant difference test ( $P < 0.05$ ). ..... 155

Figure 5.5 Effect of nanoparticles-mediated oral RNAi of *AgCHS2* on larval susceptibility and peritrophic matrix (PM) permeability to calcofluor white (CF) and dithiothreitol (DTT) in mosquito larvae. (A) Disruption of larval PM by displaying blue gastric caecae (GC) following ingestion of blue dextran. The arrow shows the GC filling with the dye when the PM was disrupted by treating mosquito larvae with DTT or CF. (B) Effect of the RNAi on larval susceptibility to DTT. (C) Percentages of the surviving larvae with disrupted PM from the same treatments as in (B). (D) Effect of the RNAi on larval susceptibility to CF. (E) Percentages of the surviving larvae with disrupted PM from the same treatments as in (D). All the data are presented as means  $\pm$  SEM of three replications. Different letters on the error bars indicate significant difference based on Fisher's least significant difference test ( $P < 0.05$ ). ..... 156

Figure 5.6 Alignments and identities of cDNA sequences for generating dsRNA fragments used  
in RNAi experiments. .... 158

## List of Tables

Table 2.1 Sequences and relevant parameters of the primers used for RT- PCR and qPCR.....	51
Table 2.2 Pairwise comparison of <i>An. gambiae</i> chitin synthases with chitin synthases from other insect species and <i>C. elegans</i> .....	52
Table 3.1 Sequences and relevant parameters of the primers used for RT- PCR and qPCR.....	86
Table 3.2 Comparisons of the three new groups of chitinase and chitinase-like proteins from three insect species: <i>D. malenogaster</i> , <i>T. castaneum</i> and <i>An. gambiae</i> .....	87
Table 4.1 Toxicity of diflubenzuron to 3 <sup>rd</sup> -instar mosquito larvae.....	118
Table 4.2 Toxicity of diflubenzuron to 9-h Mosquito Pupae.....	119
Table 5.1 Sequences and relevant parameters of the primers used for dsRNA synthesis and quantitative real-time PCR analysis.....	151

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## **Dedication**

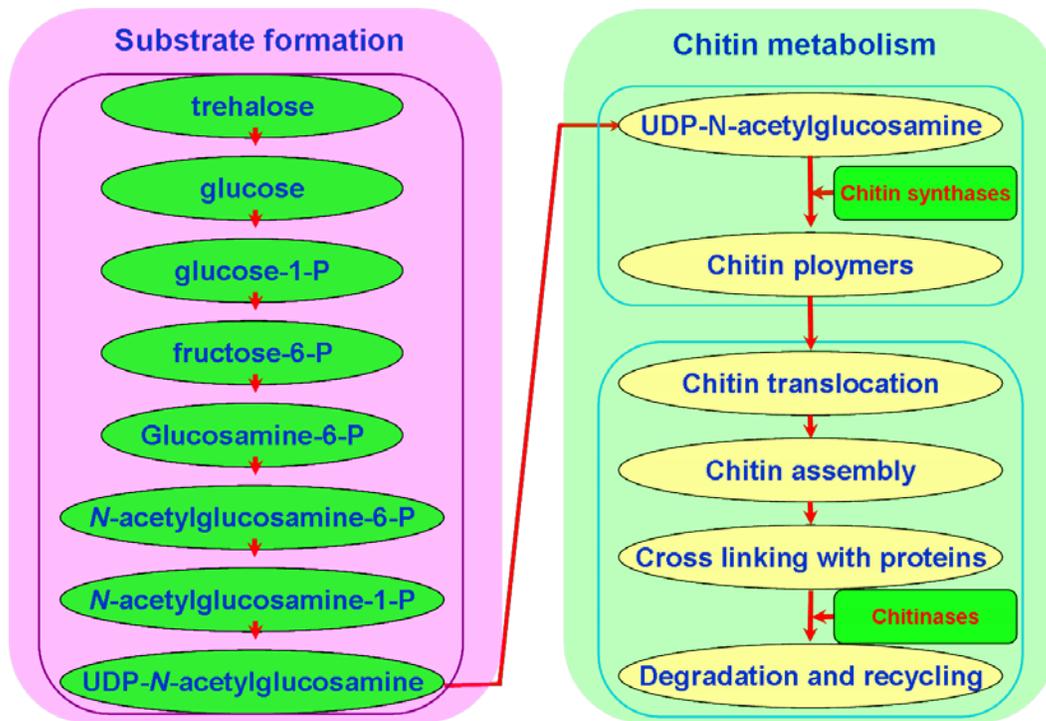
I dedicate this dissertation to my wife and my parents for their love, encouragement and support.

# CHAPTER 1 - Introduction

## Chitin and chitin metabolism in insects

Chitin, a linear polysaccharide of *N*-acetyl- $\beta$ -D-glucosamine residues joined by  $\beta$ -1, 4 glycosidic linkages, is the second most abundant biological polymer after cellulose (Merzendorfer, 2006; Kramer & Muthukrishnan, 2005). It is widely found in arthropods, fungi and nematodes. In arthropods, chitin is a vital component of the cuticular exoskeleton. Chitin synthesis is crucial for growth and development because chitin forms a major portion of the cuticular exoskeleton that is regularly shed and replaced by new cuticle (Merzendorfer & Zimoch, 2003). Chitin is also found in internal structures of many insects and other arthropods, including the cuticular linings of trachea and in the peritrophic matrix (PM) lining the gut epithelium (Richards, 1951; Hunt, 1970; Cohen, 2001). Chitin associated with the PM protects the intestinal epithelium from mechanical disruption, radical oxygen species and invasion by microorganisms (Lehane, 1997; Barbehenn & Stannard, 2004). In addition, chitin is also found in the salivary glands, mouthparts, foregut, and hindgut of some insects (Kramer & Muthukrishnan, 2005). In a recent study, the putative chitin-like material was identified in *Aedes aegypti* eggs, ovaries, and egg shells (Moreira et al., 2007). A more recent study confirmed that chitin was one of the constituents in the serosal cuticle (SC) and plays an important role in the desiccation resistance in *Ae. aegypti* eggs (Rezende et al., 2008).

Chitin production in arthropods is a complicated process including individual polymer biosynthesis and the subsequent chitin deposition. A series of biochemical pathways are involved in individual chitin polymer biosynthesis in which the terminal step is catalyzed by chitin synthase (Merzendorfer & Zimoch, 2003). While the chitin polymer is synthesized, it is



**Figure 1.1 Diagram of the cascade of chitin polymer chain production and subsequent deposition to form cuticle and peritrophic matrix in insects, and chitin degradation.**

extruded into the extracellular space and deposited as a protein-polysaccharide complex at the apical surface of epithelial or epidermal cells (Cohen, 2001; Kramer & Muthukrishnan, 2005).

The cascade of chitin production and degradation are summarized in Fig. 1.1. Briefly, these events include: a) sequential biotransformations of sugars (e.g., trehalose or glucose), amination and formation of the substrate UDP-GlcNAc; b) synthesis of chitin polymer by chitin synthase by adding one (or two) UPD-GlcNAc residue at each step to elongate the polymer chain; c) translocation of nascent chitin polymer across the plasma membrane and release of the polymer into the extracellular space; d) assembly of crystalline microfibrils by interchain hydrogen bonding; e) association of the microfibrils with proteins, glycoproteins and proteoglycans to form cuticles and PM in arthropods; and f) degradation of the chitin by enzymes such as chitinases, the degraded units might be recycled to form new chitin.

Despite decades of intensive research in chitin biosynthesis, many events associated with above cascade are still partially understood. In fact, most steps of the above cascade of reactions are still uncertain. For example, the stoichiometry of the chitin biosynthetic reaction (whether one or two GlcNAc residues are added in each polymerizing step) is not well understood because chitin synthase is a processive enzyme, meaning that the enzyme remains bound to the chitin polymer through many polymerization steps. The mechanisms associated with the translocation and assembly of chitin is largely unknown due to their extremely complex biochemical and biophysical events. Chitin biosynthesis starts intracellularly, but the product ends up at the apical surface of epidermal or epithelial cells and becomes part of exterior super molecular structures (cuticles or peritrophic matrices) (Cohen, 2001). In fact, a major portion of our knowledge about chitin synthesis in arthropods is actually based on results obtained from more advanced studies of the fungal chitin synthetic system (Cohen, 2001).

Two primary enzymes, chitinase (CHT) and  $\beta$ -*N*-acetylglucosaminidases (NAG), involve in the degradation of chitin in insects. Insect CHTs catalyze the endohydrolysis of chitin at random internal positions within the chitin polymer to generate  $\beta$ - (1-4) GlcNAc oligomers, whereas NAGs cleave the exohydrolysis of the chitin polymer from the nonreducing end and generate monomers of GlcNAc (Sahai & Manocha, 1993). In *M. sexta*, CHT and NAG coordinately catalyze chitin hydrolysis and these two enzymes showed a synergistic effect on chitin catabolism as compared with the sum of the rates observed with either enzyme alone (Fukamizo & Kramer, 1985a). Kinetic studies show that the short chitin oligomers produced by the action of CHT are used as the substrates for NAG to release GlcNAc monomers (Fukamizo & Kramer, 1985b; Sahai & Manocha, 1993). A recycling mechanism might exist for GlcNAc in insects as that has been characterized in *E. coli* (Park, 2001).

## Insect Chitin synthases and chitinases

Chitin synthase is a crucial enzyme catalyzing the transfer of sugar moieties from activated sugar donors to specific acceptors in all chitin-containing organisms including arthropods, nematodes, and fungi. The first cDNA encoding insect chitin synthase was isolated and sequenced from the sheep blowfly (*Lucilia cuprina*) in 2000 (Tellam et al., 2000). Since then, several cDNAs encoding chitin synthases have been isolated and sequenced from at least nine other insect species including the *Anopheles gambiae*, *Aedes aegypti* (Ibrahim et al., 2000), *Drosophila melanogaster* (Gagou et al., 2002), *Manduca sexta* (Zhu et al., 2002; Hogenkamp et al., 2005), *Tribolium castaneum* (Arakane et al., 2004), *Spodoptera frugiperda* (Bolognesi et al., 2005), *Plutella xylostella* (Ashfaq et al., 2007), *S. exigua* (Chen et al., 2007, Kumar et al., 2008), and *An. quadrimaculatus* (Zhang and Zhu, 2006). Furthermore, the completion of several insect genome sequencing projects has provided further information about relevant gene structures, and the analysis of gene expression patterns in different tissues and developmental stages.

Insects appear to possess two different chitin synthases encoded by two genes, *CHS1* and *CHS2*. Both genes are closely related but can be clearly grouped into two different phylogenetic classes (Merzendorfer, 2006). *CHS1* genes were exclusively expressed in the epidermis underlying the cuticular exoskeleton and related ectodermal cells such as tracheal cells; whereas *CHS2* genes were utilized for the synthesis of peritrophic matrix-associated chitin in gut epithelial cells (Merzendorfer & Zimoch, 2003; Arakane et al. 2004, 2005; Hogenkamp et al. 2005; Zimoch et al., 2005; Ashfaq et al., 2007). Alternative splicing exons have been documented in several insect chitin synthase 1 genes, leading to the production of two splicing variants which are differentially expressed in the epidermis and tracheae during development

(Arakane et al., 2004; Hogenkamp et al., 2005; Zimoch et al., 2005). In contrast, alternative splicing exons and splicing variants have not been reported for *CHS2* genes in insects.

In insects, chitin synthases are large transmembrane proteins and contain multiple transmembrane helices reflecting their association with either the plasma membrane or intracellular vesicles such as chitosomes (Tellam et al., 2000). Based on the amino acid sequence alignments of different insect chitin synthases, the enzyme has a tripartite domain structure with a central catalytic region that is flanked by two transmembrane domains (Merzendorfer, 2006). The N-terminal domain contains 9-10 transmembrane helices, whereas the C-terminal domain exhibits seven transmembrane helices of which five are located immediately next to the predicted central catalytic domain. In spite of the increasing amount of information on the primary structures of insect chitin synthases, knowledge of their actual structure is rather limited and is mainly based on predictive analyses of deduced amino acid sequences from insects and fungi.

During the chitin polymer synthesis, each step adds a single (or possibly two) *N*-acetylglucosamine (GlcNAc) unit to the non-reducing end of the growing polymer. Based on the recent hypothetical model proposed by Tellam et al. (2000) and Merzendorfer (2006), the growing chitin polymer passes the lipid bilayer of the plasma membrane through a central pore formed by the oligomerization of the transmembrane helices (5-TMS region) near the C-terminal of the enzyme. It is known that the catalytic reaction requires UDP-*N*-acetylglucosamine (UDP-GlcNAc) as the substrate, and divalent metal cations such as  $Mg^{2+}$  or  $Mn^{2+}$ . The initiation of chain assembly may involve a covalently bound primer to which the incoming sugar moiety is transferred (Merz et al., 1999). Presently, however, such a primer has not been identified. As a

consequence of a covalently linked primer, the complete reaction cycle would have to include the release of chitin by cleaving the bond linking the chitin polymer and the enzyme.

Insect chitinases belong to family 18 of the glycohydrolase superfamily. The typical architecture of a chitinase includes an N-terminal signal peptide, a catalytic domain, a chitin-binding domain (CBD), and a Ser/Thr-rich linker region between catalytic domain and CBD. The catalytic domain contains four highly conserved regions and the substitutions in these regions very possibly result in the loss of the catalytic activity (Kramer and Muthukrishnan, 1997; de la Vega et al., 1998; Zhu et al., 2004, 2008a). The CBD contains six conserved cysteine residue and their positions are highly conserved (Coutinho and Henrissat, 1999; Tellam, 1999).

Current understanding on chitinases supports the convention that insect chitinase or chitinase-like proteins involve in cuticle turnover, nutrition digestion, and PM degradation during molting. In the early works, single chitinase gene was studied in different insect species mainly from three orders: lepidopteran, dipteran and coleopteran (Kramer et al., 1993; Kim et al., 1998; Royer et al., 2002; Ahmad et al., 2003; Feix et al., 2000). In 2004, a large number of chitinase and chitinase-like genes was identified by a genome-wide search from a single insect species, *Drosophila melanogaster* (Zhu et al, 2004). After that, the chitinase gene family was reported from *Tribolium castaneum* and *Anopheles gambiae* after the whole genome sequences became available (Zhu et al, 2008a). Phylogenetic analysis classified the known chitinase and chitinase-like genes from *D. melanogaster*, *T. castaneum*, and *An. gambiae* into five groups (Zhu et al., 2008a). RNA interference (RNAi) of selected genes from each group performed in *Tribolium* supports the functional specialization of each chitinase gene (Zhu et al, 2008b). More information on more recent advances in the field of insect chitinases and chitinase-like protein is directed a recent review (Arakane and Muthukrishnan, 2010).

## **Diflubenzuron and its mechanism of action**

Dutch scientists discovered benzylphenolureas (BPUs) as chitin synthesis inhibitors when they attempted the synthesis of a novel herbicide in 1970s (Verloop and Ferrell, 1977). The resulting acylurea compounds turned out to be highly potent insecticides that selectively inhibit chitin synthesis in insects but not in fungi (Post and Vincent, 1973; Ishaaya and Casida, 1974; Post et al., 1974). Among these BPU derivatives, there are at least 13 available as biorational insecticides, such as diflubenzuron and lufenuron (Anonymous, 2003). These insecticides attack insects of different orders by inhibiting chitin formation, thereby causing abnormal endocuticular deposition and abortive molting (Ishaaya & Horowitz, 1998). Typical effects of chitin synthesis inhibitors on developing larvae are the rupture of malformed cuticle or death by starvation. Currently, the most widely used BPU insecticides include diflubenzuron under the trade name Dimilin® and lufenuron under the trade name Program®.

Diflubenzuron has been widely used to control various agricultural and public health pests such as mosquito and fly larvae since the 1970's. It is extremely toxic to young larvae of many mosquito species (Eisler, 1992, Baruah and Das, 1996; Ali et al., 1999; Zhang and Zhu, 2006). In contrast, diflubenzuron is practically non-toxic to higher animals. Its oral LD<sub>50</sub> for rats and mice is >4,640 mg/kg (Anonymous, 1996), which is even less toxic than table salt (4,000 mg/kg, Eaton & Klaassen, 2001). Diflubenzuron also shows low toxicity to birds and honey bees (Eisler 1992). Another human and animal health-related application of diflubenzuron is fly control. It is used as a highly palatable flow-through feed additive insecticide to control house fly, horn fly and stable fly in the manure of animals (Stringham & Watson, 2005). It can also be administered orally as a bolus to beef cattle for control of face fly and horn fly, two serious pests

of cattle in North America, whose immatures develop in fresh manure in open pasture (Scott et al. 1986).

The exact mechanisms of chitin synthesis inhibition are still elusive. Unlike the peptidyl nucleosides (i.e., nikkomycins and polyoxins which are substrate analogs) which are known to inhibit fungal chitin synthases *in vitro*, it is uncertain as to whether BPU's can directly inhibit insect chitin synthase because different studies have yielded inconsistent results. In cell-free chitin synthesizing systems, for example, the BPU's do not inhibit chitin synthesis (Cohen & Casida, 1980; Mayer et al., 1981) or block the chitin biosynthetic pathway between glucose and UDP-GlcNAc in intact larvae (Post et al., 1974). In contrast, Nakagawa et al. (1993) showed that diflubenzuron and polyoxin D clearly inhibited the incorporation of [<sup>3</sup>H]-*N*-acetylglucosamine into chitin in isolated intact integument from newly molted American cockroaches. Horst (1981) reported that diflubenzuron can dramatically inhibit chitin synthase activity (90% approximately) in the crude microsomes and membrane fractions prepared from brine shrimp larvae.

A recent study showed that diflubenzuron can significantly reduce the chitin content in the mosquito larvae in a concentration-dependent manner (Zhang and Zhu, 2006). More interestingly, the expression of the gene chitin synthase 1 was significantly increased in diflubenzuron-treated mosquito larvae. These results suggest these reduced chitin production may be due to the inhibition of chitin synthase 1 activity, whereas increased chitin synthase 1 gene expression (transcriptional level) may result from a feedback mechanism for compensation of the enzyme by diflubenzuron. Another possible mechanism to explain these observations is that diflubenzuron may cause increased translation of the enzyme resulting from the induction of gene transcription. The excessive amount of chitin synthase may not be properly translocated

and inserted into plasma membranes for normal function. Instead, these enzymes may accumulate inside the cells and competed for available substrates with the normal enzymes on the plasma membranes. Thus, even the chitin polymer is synthesized by these extra enzymes, the nascent chitin polymer cannot cross the plasma membrane to form extracellular microfibrils. Although no direct data available to support this hypothesis, one study in cat fleas has shown that lufenuron, one of the BPU insecticides, significantly inhibits endocuticle formation and decreases the amount of epidermal cytoplasm, and causes lytic changes in cytoplasmic organelles including mitochondria, and Golgi (Dean et al., 1999). Possibly, the reduced epidermal cytoplasm and the lytic changes in cytoplasmic organelles can affect either the appropriate translocation of chitin synthase enzymes or chitin translocation, and finally result in the reduced chitin microfibrils in the endocuticle.

More mechanisms have been proposed from early to current days. Early evidence of the insecticidal action of BPUs includes increased catalytic activities of cuticle chitinase, phenoloxidase, and enzyme involved in sclerotization of insect cuticle and other physiological processes including immunity (Ishaaya & Casida, 1974; Farlow, 1976). Increased chitinase and phenoloxidase activities result in a reduction of chitin content possibly a result of increased chitin degradation and softening of the endocuticle (Farlow, 1976). In addition, diflubenzuron was found to inhibit a serine protease, which may block the conversion of a chitin synthase zymogen into an active enzyme (Leighton et al., 1981; Cunningham, 1986; Muzzarelli, 1986). Several studies also indicated that BPUs might affect the hormonal balance in insects, thereby resulting in physiological disturbances such as inhibition of DNA synthesis (Deloach et al., 1981; Soltani et al., 1984), and suppression of microsomal oxidase activity (Van Eck, 1979; Ledirac et al., 2000). However, most of these hypotheses fail to adequately explain the

mechanism of action of BPU and may be only secondary effects of insecticide treatment (Cohen, 2001). Other researches suggested that the BPU might inhibit the exocytotic transport of chitin chains via vacuolar type of vesicles across the plasma membrane (Nakagawa & Matsumura, 1994; Cohen, 2001). Mauchamp & Perrineau (1987) proposed that BPU interrupt protein and chitin microfibril associations. More recently, Abo-Elghar et al. (2004) suggested that diflubenzuron which is structurally similar to sulfonylureas might interact with an insect homolog of the sulfonylurea as the key event for inhibiting the formation of chitin in fruitfly and German cockroaches. This conclusion was based on similar binding properties in competitive binding assays of diflubenzuron and glibenclamide; the later is known to interact with the sulfonylurea receptor in mammals. Because BPU have been shown to reduce  $Ca^{++}$  uptake by cuticular “vesicles” prepared from cockroach integuments, these authors proposed that ionic changes within the epidermal cells may interfere with chitin synthesis in these vesicles, which are predicted to contain sulfonylurea receptors.

### **RNA interference and its application in insect pest control**

RNA interference (RNAi) is a phenomenon of downregulation of gene expression by double-stranded RNA (dsRNA) or small interfering RNA (siRNA). RNAi is a post-transcriptional control mechanism involving degradation of a target mRNA mediated through the production of siRNAs from the dsRNA, which is cleaved by dsRNA-specific endonucleases known as dicers. The siRNAs are 21 bp dsRNA fragments carrying two base extensions at the 3' end of each strand; one strand of the siRNA is assembled into an RNA-induced silencing complex (RISC) in conjunction with the argonaute multi-domain protein, which contains an RNaseH-like domain responsible for target degradation (Price and Gatehouse, 2008). The natural mechanism of RNAi was firstly revealed in the nematode, *Caenorhabditis elegans* (Fire et al.,

1998), and now it has been found to be a conserved mechanism in eukaryotes including fungi, plants, insects and mammals (Mello and Conte, 2004).

As a breakthrough technique, RNAi has broadened our understanding of gene regulation and has revolutionized methods for genetic analysis, which has been widely used in model organisms such as *C. elegans* and *D. melanogaster*. RNAi has been widely used in genetic research in different insect orders at least including Diptera, Lepidoptera, Coleoptera, Hymenoptera, Orthoptera, Hemiptera, Blattodea, and Neoptera. Delivery of dsRNA to initiate RNAi in insects has predominately been via microinjection of nanogram amounts of long dsRNA into insect body cavity. In *C. elegans*, RNAi can be produced by feeding bacteria expressing dsRNA (Timmons et al., 2001), or even by soaking nematodes in dsRNA solution (Tabara et al., 1998). The RNAi responses to dsRNA molecules encountered in their environment is referred to as environmental RNAi (Whangbo and Hunter, 2008). Recently, orally-delivered RNAi has also been reported in the light brown apple moth, *Epiphyas postvittana* (Turner et al., 2006), bug *Rhodnius prolixus* (Araujo et al., 2006), and termite, *Reticulitermes flavipes* (Zhou et al., 2008). Most recently, plant-mediated RNAi has been demonstrated as a viable approach for control of insect pests in agricultural settings (Baum et al., 2007; Mao et al., 2007). These results show the great potential of applying RNAi in insect pest management in agriculture.

The phenomenon in which local administration of dsRNA (e.g., the gut through feeding) leads to an RNAi response in the whole body through the amplification and spread of silencing to other cells and progeny is known as systemic RNAi. Systemic RNAi has been well studied in plants and nematodes. The basis of systemic RNAi lies in the presence of an RNA-dependent RNA polymerase (RdRP) that is able to interact with the RISC complex and generate new

dsRNA based on the partially degraded target template by using the hybridized siRNA strands as primers (Sijen et al., 2001). To date, the identification and characterization of the RdRP have been documented in plants and *C. elegans* (Baulcombe, 2007). However, it appears that insects lack RdRP homology needed to drive this RNAi amplification in plants and *C. elegans* (Gordon and Waterhouse, 2007). In *C. elegans*, dsRNA in environment triggers strong responses in the whole organism. Orally-delivered RNAi provided information on how dsRNA molecules enter an organism from environment. The ingested dsRNA and resulting silencing signals spread systemically to distant cell within the animal. For spreading of silencing signals, the target gene is not required to be expressed in the intestine (Winston et al., 2007).

Although its genetic basis is still elusive as described above, systemic RNAi has been reported in several insect species (Tomoyasu et al., 2008). Insect systemic RNAi was first documented in the coleopteran insect, the red flour beetle, *T. castaneum* (Tomoyasu and Denell, 2004; Bucher et al., 2002). In addition to *Tribolium*, grasshopper also demonstrated systemic RNAi response in the eyes by injection of the dsRNA of a gene in the abdomen of the larvae (Dong and Friedrich, 2005). Similarly, the oral-delivered RNAi in several insect species also showed its systemic nature. These insect species include the light brown apple moth (*Epiphyas postvittana*) (Turner et al, 2006), *Plutella Xylostella* (Bautista et al., 2009), the bug *Rhodnius prolixus* (Hemiptera) (Araujo et al., 2006), and the termite *Reticulitermes flavipes* (Zhou et al., 2008), etc. The studies on oral-delivered RNAi are increasing very fast these days and the list of the successful examples of oral-delivered RNAi is getting larger.

## Specific aims and significance

As arthropod growth and development are strictly dependent on precise control (rate and timing) of chitin biosynthesis and degradation, disruption of chitin metabolism has been recognized as an attractive target for developing safe and effective insecticides as the chitin biosynthetic pathway is absent in humans and other mammals (Cohen, 2001). Although benzoylphenylurea (BPU) chitin synthesis inhibitors (CSIs) have been widely used as pesticides to control various agricultural and public health pests such as mosquitoes and flies for more than 30 years, the exact mechanism of action of this class of compounds is still elusive. The limited knowledge on chitin metabolism in insects and other arthropods and the unresolved mode of action of BPUs have become a key obstacle for developing other novel CSIs as safe and selective insecticides for agricultural and public health pest control. In this study, we use the important malarial mosquito, *Anopheles gambiae*, to study the mechanisms of chitin biosynthesis and degradation. Mosquitoes are very important vectors of different human diseases. To date, only limited insecticides could be used for control of these vectors due to high toxic properties to human beings and environmental concerns. Current studies show mosquito larvae are highly sensitive to BPU insecticides such as diflubenzuron (Zhu et al., 2007; Chen et al., 2008). Thus, mosquitoes might provide us a good system to study the mechanism of chitin biosynthesis and degradation. The specific aims for this study include

1. To identify and characterize the chitin synthase and chitinase gene families;
2. To analyze the enzymatic activity of chitin synthases and determine whether diflubenzuron and other CSIs directly inhibit chitin synthase activity or not;
3. To analyze the functions of chitin synthases by using RNAi.

This research will shed some new light on the enzymes involved in chitin synthesis and degradation in mosquitoes and will help us understand the mechanism of action of BPU's. Potentially, this research will help us discover novel target sites for developing new insecticides.

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## **CHAPTER 2 - Identification and characterization of two chitin synthase genes in African malaria mosquito, *Anopheles gambiae***

### **Abstract**

Current knowledge on chitin synthase (CHS), especially their structures, functions, and regulations in insects is still very limited. We report the identification and characterization of two chitin synthase genes, *AgCHS1* and *AgCHS2*, in African malaria mosquito, *Anopheles gambiae*. *AgCHS1* and *AgCHS2* were predicted to encode proteins of 1,578 and 1,610 amino acid residues, respectively. Their deduced amino acid sequences show high similarities to other insect chitin synthases. Transcriptional analysis showed that *AgCHS1* was expressed in egg, larval, pupal and adult stages whereas *AgCHS2* appeared to be expressed at relatively low levels, particularly during the larval stages as examined by RT-PCR and real-time quantitative PCR. Relatively high expression was detected in the carcass followed by the foregut and hindgut for *AgCHS1*, and the foregut (cardia included) followed by the midgut for *AgCHS2*. Fluorescent in situ hybridization (FISH) and immunohistochemistry analysis revealed new information including the localization two enzymes in the ommatidia of the compound eyes, and *AgCHS2* in the thoracic and abdominal inter-segmental regions of pupal integument.

**Key words:** chitin synthases, *Anophele gambiae*, expression pattern, in situ hybridization, immunohistochemistry.

## Introduction

Chitin, a linear polysaccharide of N-acetyl- $\beta$ -D-glucosamine residues joined by  $\beta$ -1,4 glycosidic linkages, is the second most abundant biological polymer after cellulose (Merzendorfer, 2006; Kramer & Muthukrishnan, 2005). It is widely distributed in arthropods, fungi and nematodes. In arthropods, chitin is a vital component of the cuticular exoskeleton and thus is crucial for growth and development (Merzendorfer & Zimoch, 2003). Chitin is also found in internal structures of many insects and other arthropods, including the cuticular linings of trachea and in the peritrophic matrix (PM) lining the gut epithelium (Richards, 1951; Hunt, 1970; Cohen, 2001).

Chitin synthase is a crucial enzyme catalyzing the transfer of sugar moieties from activated sugar donors to specific acceptors. The first cDNA encoding insect chitin synthase was isolated and sequenced from the sheep blowfly (*Lucilia cuprina*) in 2000 (Tellam et al., 2000). Since then, several cDNAs encoding chitin synthases have been isolated and sequenced from at least nine other insect species including the *Anopheles gambiae*, *Aedes aegypti* (Ibrahim et al., 2000), *Drosophila melanogaster* (Gagou et al., 2002), *Manduca sexta* (Zhu et al., 2002; Hogenkamp et al., 2005), *Tribolium castaneum* (Arakane et al., 2004), *Spodoptera frugiperda* (Bolognesi et al., 2005), *An. quadrimaculatus* (Zhang & Zhu, 2006), *Plutella xylostella* (Ashfaq et al., 2007), and *S. exigua* (Chen et al., 2007; Kumar et al., 2008). Furthermore, the completion of several insect genome sequencing projects has provided further information about relevant gene structures, and the analysis of gene expression patterns in different tissues and developmental stages.

Insects appear to possess two different chitin synthases encoded by two genes, *CHS1* and *CHS2* (also known as *CHS A* and *CHS B*, respectively). These genes are closely related but can

be clearly grouped into two different phylogenetic classes (Merzendorfer, 2006). *CHS1* is exclusively expressed in the epidermis underlying the cuticular exoskeleton and related ectodermal cells such as tracheal cells; whereas *CHS2* is responsible for the synthesis of the PM-associated chitin in gut epithelial cells (Merzendorfer & Zimoch, 2003; Arakane et al., 2004, 2005; Hogenkamp et al., 2005; Zimoch et al., 2005; Ashfaq et al., 2007). Insect chitin synthase 1 genes contain alternative exons which lead to the production of two splicing variants. These mRNA variants are differentially expressed in the epidermis and tracheae during insect development (Arakane et al., 2004, Hogenkamp et al., 2005, Zimoch et al., 2005). In contrast, alternative splicing variants have not been reported for *CHS2* in insects.

Current knowledge on chitin synthase function and its regulation in insects is rather limited. Functional analysis of two chitin synthases using RNA interference (RNAi) in different insect species such as *T. castaneum* and *Spodoptera exigua* showed that chitin synthases are required for survival, fecundity and egg hatching, and molting processes (Arakane et al., 2005, 2008; Merzendorfer, 2006; Tian et al., 2009). Chitin synthase presents an attractive target for combating insect pests and fungi-borne diseases as insect and fungus growth and development is dependent on precisely tuned expression of chitin synthase genes and this process is absent in vertebrates (Merzendorfer, 2006). For example, peptidyl nucleosides including polyoxins and nikkomycins are anti-fungi agents which competitively inhibit chitin synthases in fungi. Benzylphenolureas (BPUs) such as diflubenzuron are potent insecticides that inhibit chitin biosynthesis. However, it remains controversial whether chitin synthases are the direct targets for this group of insecticides. Interestingly, a recent study showed that up-regulation of chitin synthase 1 gene at transcriptional levels is associated with the exposure to diflubenzuron in *An. quadrimaculatus* (Zhang and Zhu, 2006).

*An. gambiae* is an important arthropod-borne disease vector in Africa (Hav et al., 2004). To date, very limited insecticides are available for control of mosquitoes and other human health-related arthropods. The BPU insecticides including diflubenzuron and lufenuron have shown a great potential for control of the mosquito populations (Moreira et al., 2007; Zhu et al., 2007). In this paper, we reported the identification and characterization of two chitin synthase genes of *An. gambiae*. The study is expected to facilitate the understanding of chitin synthase protein and potentially lead to the development of new insecticides targeting on chitin metabolic pathways in insects.

## **Materials and Methods**

### ***Mosquito rearing***

A colony of *An. gambiae* obtained from the Malaria Research and Reference Reagent Resource Center (MR4) (Manassas, VA) was maintained in the Department of Entomology at Kansas State University (Manhattan, KS) since 2007 as described by Zhang & Zhu (2006). Briefly, the larvae were fed with slurries of brewer's yeast and TetraMin Baby-E fish food, whereas adults were fed with 10% sucrose solution soaked into cotton balls. Two-day-old females were fed with pre-warmed, defibrinated horse blood (Colorado Serum Company, Denver, Colorado) in a membrane feeder made of a lubricated Naturalamb brand condom (Church and Dwight Co., Inc., Princeton, NJ), allowing the females to lay eggs.

### ***Sequence analysis of AgCHS1 and AgCHS2***

The online program GenomeScan (<http://genes.mit.edu/genomescan.html>) and UCSC Genome Bioinformatics program (<http://genome.ucsc.edu/>) were used to obtain the missing 5' end open reading frame (ORF) of *AgCHS2* and genomic organization of *AgCHS* genes,

respectively. By searching the GenBank, three cDNA clones putatively coding for two chitin synthases were identified. These three cDNA clones were named *AgCHS1A* (GenBank accession no. XM\_321337), *AgCHS1B* (GenBank accession no. XM\_321336) and *AgCHS2* (GenBank accession no. AY056833), coding for the two splicing variants of AgCHS1 (*AgCHS1A* and *AgCHS1B*) and *AgCHS2*, respectively, of *An. gambiae* chitin synthases. Two of these three *AgCHS* cDNA clones, *AgCHS1A* and *AgCHS1B*, contained a complete ORF, but *AgCHS2* from the GeneBank was a partial cDNA missing about 1000 bp at the 5' end of the ORF. To obtain the missing 5' end of the ORF in *AgCHS2*, the locations and exon-intron structures of *AgCHS2* in genomic sequences from *An. gambiae* were predicted using the program GenomeScan (<http://genes.mit.edu/genomescan.html>) by inputting the similar region of the amino acid sequence of *Aedes aegypti* chitin synthase 2, AeCHS2 (GenBank accession no. AF217249). Sequence analysis was performed using the computer software suite Lasergene (DNASar, WI). Genomic organizations of *AgCHS1* and *AgCHS2* were obtained by blast search of genome sequence of *An. gambiae* using UCSC Genome Bioinformatics program (<http://genome.ucsc.edu/>). Other software available from online servers was described in the results.

### ***RT-PCR analysis***

Total RNA was isolated from the insect sample representing each of seven developmental stages, including egg; first-, second-, third- and fourth-instar larva; pupa; and adult, by using TRIzol Total RNA Isolation kit (Invitrogen, Carlsbad, CA) for studying stage-specific expressions of *AgCHS* genes. The same method was used to isolate total RNA from insect sample representing each of five egg developmental times, including 12, 24, 36, 48, and 60 hour after egg laying, and five pupal developmental times, including 0, 10, 20, 30, and 34

hour after pupation, respectively. Similarly, total RNA was also isolated from the tissue sample representing each of four tissue types, including foregut, midgut, hindgut and carcass (the whole larva with the gut removed), for studying tissue-specific expressions. In brief, the fourth instar larvae were chilled on ice and dissected in cold 1×PBS to get the tissues. Mosquito larva was longitudinally opened by carefully cutting the integument from one side of the larva without damaging the gut. Then the whole intestine was gently pulled out and detached from other tissues including Malpighian tubules, trachea, and fat bodies. The midgut, foregut, and hindgut were carefully separated and immediately placed in TRIzol reagent. The foregut and midgut were separated in the junction area of the gastric caecum, and the gastric caecum was included in the midgut. All other tissues excluding the whole intestine were collected as carcass.

After total RNA was isolated and concentration was measured using NanoDrop ND-1000 (NanoDrop Technologies, Inc., Wilmington, DE). Aliquots of 2.5 mg of total RNA were then treated with DNase using DNase I kit (Fermentas, Glen Burnie, MD) and the first-strand cDNA was synthesized with First Strand cDNA Synthesis kit (Fermentas, Glen Burnie, MD) using an oligo (dT)<sub>12-18</sub> primer in a 20- $\mu$ l reaction following the manufacturer's protocol. Beacon Designer software from Primer Biosoft (<http://www.premierbiosoft.com>) was used to design the gene-specific primers for *AgCHS1A*, *AgCHS1B*, and *AgCHS2* that are shown in Table 2.1. PCR was performed using the PCR Master Mix (Fermentas) with the thermal cycle program consisting of an initial denaturation at 94°C for two min followed by 29 cycles of 94°C for 30s, 55°C for 30s and 72°C for 45s, and a final extension at 72°C for 10 min. The PCR products were resolved on a 1.8% agarose gel and visualized by staining the gel with ethidium bromide. If the gene expression level was low and the PCR products were not detected, five more cycles were run and the products were checked again. The mosquito ribosomal protein S3 (*AgRPS3*) gene

was used as a reference for RT-PCR analysis. At least three replicates were performed for each of two *AgCHS* genes and two splicing variants, *AgCHS1A* and *AgCHS1B*, for each developmental stage and each tissue type.

### ***Real-time quantitative PCR (qPCR) analysis***

To profile the developmental stage- and tissue-specific expression patterns of each *AgCHS* gene, cDNA prepared from above mentioned samples representing each of four developmental stages, including egg, 3<sup>rd</sup>-instar larva, pupa, adult, and from each of four tissues, including foregut, midgut, hindgut, and carcass, was used for qPCR analysis. The qPCR was performed in a 25- $\mu$ l reaction containing 10.5  $\mu$ l of 10-fold diluted cDNAs, 0.4  $\mu$ M of each primer, and 1 $\times$  Maxima SYBR Green qPCR Master Mix (Fermentas) using iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA). The optimized qPCR program was used for both *RPS3* and *AgCHS1*, which consisted of initial denaturation step at 95°C for 5 min followed by 40 cycles of 95°C for 15s, 55°C for 30s and 70°C for 30s. At the end of the PCR, amplification specificity was verified by obtaining the dissociation curve, in which the samples were cooled to 55°C after denaturing, and then melting curves were obtained by increasing 0.5°C/10 s for each cycle with a total of 80 cycles until reaching 95°C to denature the double-stranded DNA. The specificity of each reaction was evaluated based on the melting temperatures of the PCR products. The amplification efficiency of primer pairs was determined from the slope of the curve generated by amplification from serially diluted cDNA. The efficiency had to be at least 0.9 for a primer pair to be accepted. Relative expression values (REVs) for the tissue-specific gene expressions were then determined by dividing the quantity of the target sequence of interest with the quantity obtained for *AgRPS3* as an internal reference gene. We found that expression of *AgRPS3* fluctuated across the developmental stages we tested (data not

shown). Other genes including *RpS7*, ribosomal protein L32, elongation factor 2, and the ubiquitin-ribosomal protein L40 fusion protein were also tested. However, no one appeared to be suitable as a “housekeeping gene” to normalize our data across the developmental stages in *An. gambiae* as also noted in other insect species (Togawa et al., 2008). Therefore, we did not normalize the stage-specific gene expression using *AgRPS3*, but adopted very careful quantification of RNA using NanoDrop to standardize our samples. The qPCR for each gene was repeated with three biological replicates and two technical repeated measurements.

### ***Heterologous expression and purification of antigens and antibody preparations***

A cDNA fragment encoding a partial protein sequences of each of the two chitin synthases (AgCHS1 and AgCHS2) was amplified by RT-PCR with primers 5'-  
CCATGGGCAAACGACGGACGAGAAGGCGCA-3', 5'-  
GAATTCTTAGTGCGCAATACGTGCCTGTTTCCTC-3', 5'-  
CCATGGCCAGCGCCGAAAAGGAGCAAATCGCA-3' and 5'-  
GAATTCTTACTTCATCTCTTCCTTCTGCTTTTCCG-3', for AgCHS1 and AgCHS2, respectively, using total RNA (RNeasy Mini Kit, QIAGEN) of the *An. gambiae* 4<sup>th</sup> instar larvae as template, where underlining, double underlining, and dashed lines indicate the *Nco* I, *Eco*R I site, and stop codon, respectively. The amplified fragment (StrataScript® one-tube RT-PCR system with Easy-A® High-fidelity PCR cloning enzyme, Stratagene) (about 400bp) was inserted into PCR 2.1 vector of Topo TA cloning kit (Invitrogen) and sequenced to confirm their sequences. The plasmid DNA was digested with *Nco*1 and *Eco*R1 and the resulting DNA fragment was ligated into pET-32a (+) vector (Novagen, Madison, WI) that had been digested with *Nco*1 and *Eco*R1 to obtain the plasmid for expression of recombinant AgCHS1 and AgCHS2 proteins (rAgCHS1 and rAgCHS2) according to the manufacturer's instructions. In

brief, the plasmid encoded the fusion protein with a His-tag and a GST-tag was used to transform the BL21 (DE3) cells. The transformant cells were cultured at 37°C for 16 h in LB medium containing 100 µg/ml ampicillin. Two milliliters of the culture was added to 400 ml of LB medium and incubated at 37°C until OD<sub>600</sub> reached 0.5 followed by addition of IPTG at a final concentration of 0.4 mM and incubation of 4 h at 37°C. The cells were pelleted and resuspended in 10ml lysis buffer (8 M urea, 0.1 M sodium phosphate buffer, 0.01 M Tris-Cl, pH 8.0). The cells were lysed by gently shaking for 45 min at room temperature. After cell debris was removed by centrifugation at 5,000×g for 15 min, the recombinant proteins in the supernatant were purified by NTA-Ni<sup>2+</sup>-resin (Novagen) following the manufacturer's protocol. The eluted proteins were concentrated by using centriprep centrifugal YM-3 (Millipore, Billerica, MA), then digested with enterokinases (S-Tag™ rEK Purification Kit, Novagen) to cut the GST and His tag. The digested proteins were separated by 4-20% gradient SDS-PAGE. The band of the recombinant proteins without the tag was cut and the proteins were eluted by using Tris-NaCl buffer. The resulting recombinant proteins, rAgCHS1 and rAgCHS2, were used to immunize mice (Interdisciplinary Center for Biotechnology Research, University of Florida). The serum of the immunized mice was collected as the anti-AgCHS1 and AgCHS2 serums.

### ***Immunohistochemistry***

Paraffin-embedded thin sections were used for immunohistochemistry analysis of two chitin synthases. In brief, 12 to 24 h old pupae were fixed in 4% paraformaldehyde at 4 °C overnight and then washed three times ( each 5 min) with PBST (PBS and 0.1% Triton X-100). The samples were then dehydrated through a series of concentrations of ethanol (2× 30 min in each 70% and 96%, 2× 20 min in 100%), followed by 2× 1 hr in chloroform. The dehydrated samples were finally embedded in paraplast (56°C, Tyco Healthcare) after overnight penetration.

Histological sections (8 µm) were prepared by using microtome (Richard-Allan Scientific Microm) with a low profile microtome blade (Richard-Allan), straightened on Fisherbrand ColorFrost Plus microscope slides with 0.5% gelatin, and allowed to dry for 2 days at 40°C on the top of slide warmer. The sections were deparaffinized with two washes of 10 min xylene, rehydrated through successive baths of ethanol (100%, 96%, and 70% in water, 1x 5 min each), two water baths (5 min each), and finally PBST for 10 min.

To determine the localization of AgCHS1 and AgCHS2, the sections were blocked in 1% BSA (bovine serum albumin) in PBST for 15 min followed by incubation with a 1:100 dilution of anti-AgCHS1 and anti-AgCHS2 serums in PBST at 4°C overnight, respectively. Paraffin-embedded thin sections immunostained with a preimmune serum were used as negative controls. The sections were then washed with PBST three times with two min for each. The primary antibodies were detected by the reaction with Alexa 488-conjugated goat anti-mouse IgG (1:500 dilution in PBST) at 4°C overnight. After four time washes with PBS (10 min each), the sections were mounted in glycerol on a glass slide and the fluorescence was observed using Nikon Eclipse E800 fluorescence compound microscope equipped with appropriate filters. Photograph was taken with a Cool SNAP digital camera.

### ***Fluorescence in situ hybridization (FISH)***

To label the RNA probes, partial cDNA fragments of *AgCHS1* and *AgCHS2* were amplified by PCR using the sequence-specific primers with built-in restriction sites *Xho* I and *Xba* I as underlined: 5'- GTACACTCGAGATGTTGGTGGGTGCGTTC -3' (forward) and 5'- CTGCATCTAGAGATGATGGAGTAGAGGATGAGC -3'(reverse) for *AgCHS1*, and 5'- GTACACTCGAGCATGAAGAAGTACACTACCAAGTC -3' (forward) and 5'- CTGCATCTAGAGGAAGGAGGTCCAAATGTCG -3'(reverse) for *AgCHS2*. The PCR product

was inserted into pBlueScript SK (+) vector containing the same restriction sites. The plasmid DNA was then linearized with either *Xho* I or *Xba* I and used for transcription with T7 and SP6 RNA polymerases (Invitrogen) to generate anti-sense and sense probes, respectively. The anti-sense and sense (as negative control) probes were labeled with Fluorescence *in situ* hybridization (FISH) Tag™ RNA Multicolor Kit (Catalog No. F32956, Invitrogen) following the manufacturer's protocol. The probes were labeled with Alexa Fluor® 488.

Paraffin-embedded thin section preparation, deparaffinization and rehydration were described as above. For facilitating the penetration process of the probe into the sections of the tissues, the sections were incubated with proteinase K (10 µg/ml) (Sigma) in PBST for 10 min at room temperature and the reaction was stopped with 2 mg/ml glycine in PBST followed by rinsing with PBST two times. The sections were refixed in 4% paraformaldehyde in PBS at RT for 15 min, washed in PBST 5 min, PBST: hybridization buffer (1:1) 5 min, hybridization buffer (50% formamide, 5 X SSC, 50 µg/ml heparin, 0.1% Tween 20, and 100 µg/ml salmon sperm DNA) 5 min and prehybridized in hybridization buffer at 55°C for 1 h. Hybridization was performed with 1 µg/ml FISH probes in hybridization buffer at 55°C for 20 h following the manufacturer's procedures. Unbound probe was removed by a series of washing including hybridization buffer for 10 min at 55°C, hybridization buffer:PBST (1:1) for 5 min at RT, and PBST 4 times, 10 min per washing at RT. The sections were mounted in glycerol on a glass slide and the fluorescence was observed using a Nikon Eclipse E800 fluorescence compound microscope equipped with appropriate filters. Photograph was taken with a Cool SNAP digital camera attached to the compound microscope and pictures were edited using Adobe Photoshop 7.0.

For the whole mount *in situ* hybridization, the 4<sup>th</sup>-instar larvae were dissected in 1x PBS to get the whole gut and whole cuticle. Only half integument was used by longitudinal cutting of the whole integument into two equal sections. The tissues were fixed in 4% paraformaldehyde at 4 °C overnight, washed in PBST (0.1 M PBS + 0.2% Triton X-100) five min three times, treated with proteinase K (50 microgram/ml in PBST) for 10 min at room temperature. The reaction was stopped with PBST-glycin (2 mg Glycin/ml PBST) for 5 min followed by two washes in PBST (5 min each). The tissues were re-fixed in 4% formaldehyde for 1 hr followed by three washes in PBST, PBST:hybridization buffer (1:1), and hybridization buffer (5 min each) at room temperature. The tissues were pre-hybridized in hybridization buffer at 48 °C for 20 min, and then hybridized in the same hybridization buffer containing 10 µg/ml anti-sense probes for 20–30 h at 48 °C. Control samples were hybridized with the same amount of sense probes under the same conditions. After hybridization, the samples were washed with hybridization buffer for 2–4 hr at 48 °C, then three times with PBST at room temperature. The samples were mounted in a glycerol and visualized under a Leica M205 FA fluorescence stereomicroscope equipped with GFP filter. Photography was done with a Leica DFC 400 digital camera.

### ***Data analysis for qPCR***

For qPCR analysis, the percentage data of the relative *AgCHS* expression were obtained by dividing the REV of each developmental time point or tissue for each gene by the sum of REV throughout development or all tissues for that gene. The percentage data were then transformed using arcsine square root transformation before one-way ANOVA. Fisher's least significant difference (LSD) multiple comparisons were then used to separate the means among the samples.

## Results

### *cDNA and deduced amino acid sequences*

Two chitin synthase genes, *AgCHS1* and *AgCHS2*, were identified from *An. gambiae*. Chitin synthase 1 has two alternative splicing variants, *AgCHS1A* and *AgCHS1B*. *AgCHS1* and *AgCHS2* can be clearly grouped into two different phylogenetic groups (Fig. 2.1B). The open reading frame (ORF) of *AgCHS1A* cDNA is 4734 bp in length, encoding chitin synthase 1 of 1578 amino acid residues. Its predicted molecular mass and isoelectric point was 179.55 kDa and 6.43, respectively. The ORF of *AgCHS1B* cDNA is 4734 bp in length, encoding the chitin synthase 1B with 1578 amino acid residues. Its predicted molecular mass and isoelectric point was 179.64 kDa and 6.50, respectively. The ORF of *AgCHS2* cDNA is 4830 bp in length, encoding the chitin synthase 2 with 1610 amino acid residues. Its predicted molecular mass and isoelectric point was 184.44 kDa and 7.75, respectively. The ORF of each chitin synthase gene contains 9 exons and 8 introns. *AgCHS1* contains two alternate exons, 6A and 6B, which lead to the production of two splicing variants, *AgCHS1A*, and *AgCHS1B*, respectively (Fig.2.1A). Two alternative spliced exons are the same in size and each encodes a protein of 59 amino acid residues. These alternative exons are highly conserved both in size and amino acid identity in all CHS1 genes from several insect species (data not shown). It is clear that the two forms of this exon are separated into two groups (Fig. 2.1C).

The deduced amino acid sequences of these two chitin synthases exhibited the 7 highly conserved motifs (Fig. 2.2) which are the characteristic features of family 2 glycosyltransferases (GTF2) from fungi, insects and other organisms revealed (Merzendorfer, 2006). Each *Anopheles* chitin synthase contains 10 transmembrane helices at the N-terminal and seven transmembrane helices at the C-terminal, five of which are located immediately next to the predicted central

catalytic domain (Fig. 2.9A, B). These characters match the typical tripartite domain structure of insect chitin synthases with a central catalytic region that is flanked by two transmembrane domains (Merzendorfer 2006). The deduced amino acid sequences of two chitin synthases from *An. gambiae* showed high identities to those from two other mosquito species *An. quadrimaculatu* and *Ae. aegypti* (Fig. 2.2; Table 2.2), indicating the crucial functions of chitin synthases in mosquitoes. The identities between AgCHS1A/AgCHS1B and other insect CHS1s are much higher than the identities between AgCHS2 and other insect CHS2s (Table 2.2).

### ***Stage-specific expression of AgCHS1 and AgCHS2***

Stage-specific expression patterns of two *An. gambiae* CHS genes were determined in eggs, four different larval instars (1st, 2nd, 3rd and 4th), pupae and adults by using RT-PCR. *AgCHS1* was expressed in all seven life stages with some different expression levels and reached the maximum at pupal stage. We further examined the expression of the two alternative splicing variants of *AgCHS1* and revealed that the expression pattern of *AgCHS1A* was consistent with *AgCHS1*, whereas the expression of *AgCHS1B* was non-detectable by normal PCR cycles (Fig.2.3A). These RT-PCR results were also confirmed by qPCR (Fig. 2.3B, C). As the primer efficiencies were similar between *AgCHS1A* and *AgCHS1B* (more than 95% for each variant), these results suggested that *AgCHS1A* might be the predominant form of *AgCHS1* at all the seven stages examined. In contrast, both RT-PCR and qPCR revealed that *AgCHS2* was expressed at low level during the larval stages and high level at adult stage (Fig. 2.3A, D).

We further examined the stage-specific expression of the *AgCHS* genes in eggs and pupae at different times by RT-PCR analysis. Both *AgCHS1* and *AgCHS2* expressed in 24-h eggs and gradually increased their expressions after that and reached the maximum at 48 h. Expression of *AgCHS1B* was rather low and is only detected at 48 h eggs under normal RT-PCR

cycles (Fig. 2.4A). In pupae, relative high expressions of two *AgCHS* genes were found at early stages, for example, from 0 h to 20 h, and then gradually decreased afterwards (Fig. 2.4B). Similarly, *AgCHS1A* might be the predominant form of *AgCHS1* as *AgCHS1B* was almost non-detectable under normal PCR cycles. When the cycle number increased to 37, expression of *AgCHS1B* was detected only in 20-h pupae (Fig. 2.4B).

### ***Tissue-specific expression profiles of AgCHS1/AgCHS2 and AgCHS1 alternative splicing variants***

We examined the tissue-specific expression patterns of two exon-specific variants of *AgCHS1* by using RT-PCR and qPCR in each of four different tissues including the foregut, midgut, hindgut, and carcass (the insect body after its digestive canal was removed). *AgCHS1A* and *AgCHS1B* showed the same expression patterns and were predominantly expressed in the carcass. Low expression was detected in the foregut and hindgut, and almost no expression was found in the midgut (Fig. 2.5A, B, C). qPCR analysis of *AgCHS2* showed the consistent result with the RT-PCR analysis. More than 60% of transcripts of *AgCHS2* were found in the foregut (Fig. 2.5A, D).

### ***Localization of AgCHS1 and AgCHS2 transcripts and proteins by FISH and immunohistochemistry.***

We performed FISH in adult and larval gut by using *AgCHS1* and *AgCHS2* probes, respectively. In the guts of the female adults 24 h after a blood meal, *AgCHS2* transcripts evenly distributed in all midgut epithelium cells (Fig. 2.6A), whereas *AgCHS1* transcripts were not detected in the midgut epithelium cells (Fig. 2.6B). In a 4<sup>th</sup> instar larval gut, positive immunoreactive signals of *AgCHS2* was mainly detected in cardia and posterior midgut

(Fig.2.6C), whereas no positive signals of *AgCHS1* was detected (Fig. 2.6D). FISH analysis was also performed in the paraffin-embedded thin sections of the 12-14 h pupae. Strong positive signals were detected in the internal tissues within the head when probed with *AgCHS1* probes (Fig. 2.7). When probed with *AgCHS2* probes, positive signals were detected on the surface of the pupal legs, and tissues between two abdominal segments (Fig. 2.7).

Immunohistochemistry analysis was performed in the paraffin-embedded thin sections of the 12-24 h pupae and revealed that *AgCHS1* proteins were localized in the ommatidia of the compound eyes and epidermal cells of the adult integument newly developed underneath of the pupal integument (Fig. 2.8A, B). In contrast, *AgCHS2* proteins were localized in the ommatidia of the compound eyes, thoracic and abdominal inter-segmental regions of pupal integument (Fig. 2.8C, D).

## Discussion

Chitin synthases are highly conserved enzymes involved in the chitin biosynthesis pathway and the presence of chitin in the peritrophic matrix, exoskeleton, and trachea has been well documented in insects. Recent advances on chitin and chitin synthases in insects show that the distribution of either chitin or chitin synthases is beyond these tissues. For example, a recent study documents that the putative chitin-like material was identified in the eggs, ovaries, and egg shells from *Ae. aegypti*. The chitin-like material in the egg shells possibly comes from the female parents, whereas the chitin-like material from the eggs might be synthesized during embryogenesis (Moreira et al., 2007). A more recent study reported that chitin was synthesized in the serosal cuticle (SC) 11-13 hours after egg laying and played an important role the desiccation resistance for *Ae. aegypti* eggs. Very possibly, the chitin in the SC was synthesized by *AaCHS1A* as it was the sole variant specifically expressed during the SC formation (Rezende

et al., 2008). In *An. gambiae*, we found both *AgCHS1* and *AgCHS2* started to express 24 h after eggs laying (Fig. 2.4A), which may imply their roles in embryonic development for both of these enzymes in *Anopheles* eggs. However, the exact function for both enzymes remains elusive. Firstly, *Anopheles* eggs are not as resistant to desiccation as *Aedes* eggs. To some extent, this implies the difference of the profiles in chitin structure and functions between *Anopheles* eggs and *Aedes* eggs. Secondly, why *Anopheles* eggs need chitin synthase 2 as chitin synthase 2 is well documented to make PM associated chitin? Is it possible that the typical cycles of chitin biosynthesis and degradation occurring in larvae also occur in the eggs? A parallel study did show that several chitinase genes were detected to be expressed in 24 h eggs and the followup time frames before egg hatching (Fig. 3.10), showing very similar expression profiles as that for two chitin synthase genes. Embryonic molts have been documented in hemimetabolous insects such as grasshopper and crickets (Erezyilmaz et al., 2004). However, similar process has never been reported in dipterans to date. Further work is needed to address this issue.

Similar as what found in eggs, both *AgCHS1* and *AgCHS2* were strongly stained in the newly formed compound eyes in the pupae in immunohistochemistry analysis (Fig. 2.8B, D). This result implies some roles of chitin synthases in the structure and function in *Anopheles* visual system. This contention was supported by the fact that chitin was detected in both the ommatidial lenses and ocellar lenses in *Drosophila melanogaster* (Yoon et al., 1997; Faschinger 2010). What is interesting is the expression profile of *AgCHS2* in the pupal stage. Comparatively high level transcripts of *AgCHS2* were found in the pupal stage (Fig. 2.3A, D). This pattern is a kind of surprise to us, as we know that mosquitoes do not have PM in their pupal stage. Adult PM will not be formed until a blood meal as it has been well documented that mosquito adult PM is produced de novo in response to a bloodmeal (Shao et al., 2001; Hegedus et al., 2009). In

addition to the localization in the compound eyes, immunohistochemistry analysis also revealed the distribution of AgCHS2 in the abdominal inter-segmental regions of the pupal cuticle (Fig. 2.8C). Further work is needed to elucidate the biological implications of this special expression pattern for AgCHS2 in mosquito pupae.

As discussed above, current knowledge supports the notion that CHS2 enzymes are responsible for biosynthesis of the PM chitin (Arakane et al., 2005). Specially, two types of PM have been found in mosquitoes and other blood-feeding insects. The type 1 PM is thick (2-20 $\mu$ m) and is produced from all midgut epithelial cells and is produced in direct response to a blood meal, whereas type 2 PM is thin (less than 1  $\mu$ m) and produced continuously by a small group of highly specialized cells in the cardia in mosquito larvae (Shao et al., 2001; Kato et al., 2006). Consistent with this convention, FISH analysis in this study showed that *AgCHS2* transcripts are evenly distributed in all adult midgut epithelial cells (Fig. 2.6A). In the larval gut, we did find high level *AgCHS2* transcripts in the cardia. However, significant staining of *AgCHS2* was also detected in the posterior midgut (Fig. 2.6C). It is difficult to interpret this pattern at this time if PM associated chitin is exclusively produced in the cardia. One interesting issue needed to be addressed is whether the *AgCHS2* proteins could be translated from these transcripts. If so, what is the potential function of these enzymes localized at the posterior part of the midgut? Current studies support the notion that different part of the midgut serves different physiological function in mosquito larvae. Direct supporting evidence comes from the pH gradients in the gut contents. The pH of the luminal contents in mosquito larvae is near neutrality in the foregut, reaches to 10 in anterior midgut, and drops to 7.5 in the posterior midgut (Dadd, 1975; Okech et al., 2008). Further work is needed to address how *AgCHS2* involves in the physiological function of the posterior midgut. One issue to clarify here is that high level

*AgCHS2* transcripts detected by RT-PCR and qPCR in the foregut (Fig. 2.5A, D) is an artifact caused by the difficulty to separate the cardia from the foregut in dissection as the foregut is tiny and the cardia always tightly attached to the foregut. FISH analysis has revealed the high level expression of *AgCHS2* in the cardia but not in the foregut (Fig. 2.6C).

Current understanding on the structure and molecular constituents of the type 2 PM is rather limited. The chitin content was really low (7.2% of the weight of the matrix) in the type 2 PM from *Lucilia cuprina*, indicating chitin is a minor structural component of the type 2 PM in this insect (Tellam & Eisemann, 2000). We also failed to stain the PM associated chitin with high specificity chitin binding reagent FITC-CBD no matter the samples were pretreated with either proteinases or alkalines to release the PM associated proteins or not. Consistently, we found that the transcription level of *AgCHS2* during the larval stage was also pretty low. However, the low level *AgCHS2* transcripts and the possible low chitin content do not necessarily mean that chitin is not important for PM structure its physiological functions. Our recent study on the function of *AgCHS2* via RNA interference has revealed that *AgCHS2* is critical to maintain the integrity of type 2 PM in *Anopheles gambiae* larvae (Zhang et al., 2010). Type 1 PM has been considered as a potential barrier for malarial protozoon parasite before reaching the midgut epithelial cells (Shen and Jacobs-Lorena, 1998). The integrity of the adult PM is an important factor in regulating the parasites' passthrough of the PM barrier and the parasite development inside the gut (Dessens et al., 2001; Li et al., 2005). Disruption of the PM formation by RNA interference of chitin synthase gene results in the decreased infectivity of *Plasmodium gallinaceum* in *Aedes aegypti* (Kato et al., 2008). Thus, the study on chitin synthase and the PM structure and function in mosquitoes may provide deep insight on blocking the malaria parasites in mosquitoes.

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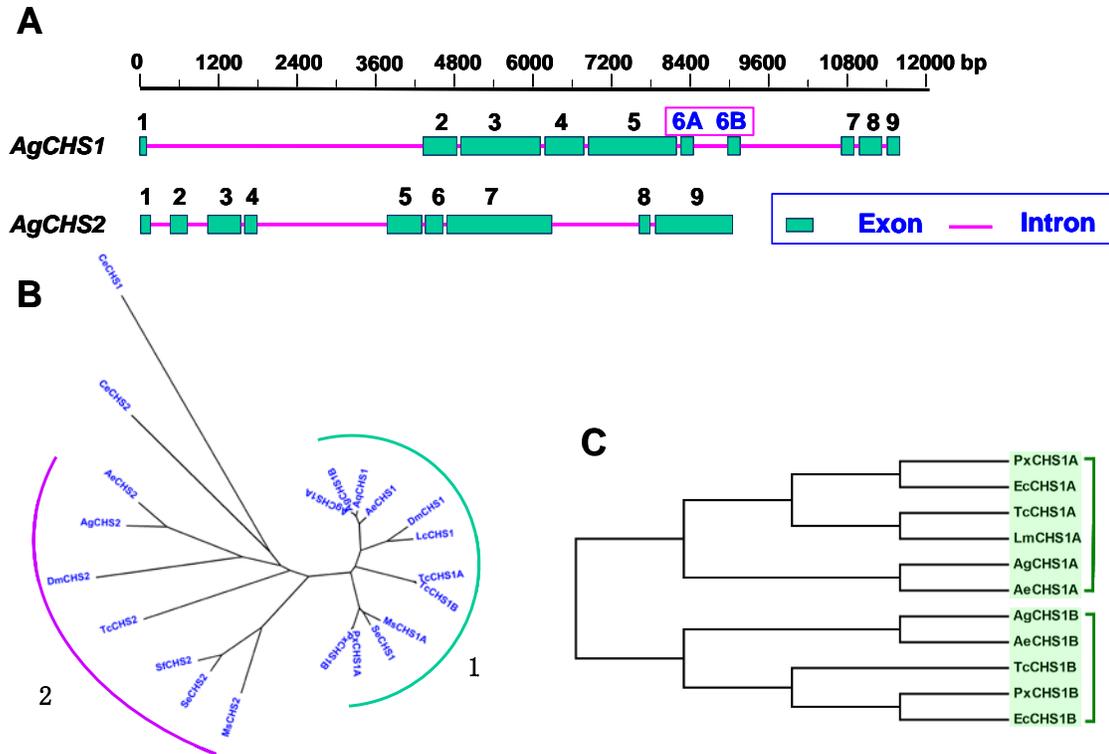
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**Table 2.1 Sequences and relevant parameters of the primers used for RT- PCR and qPCR**

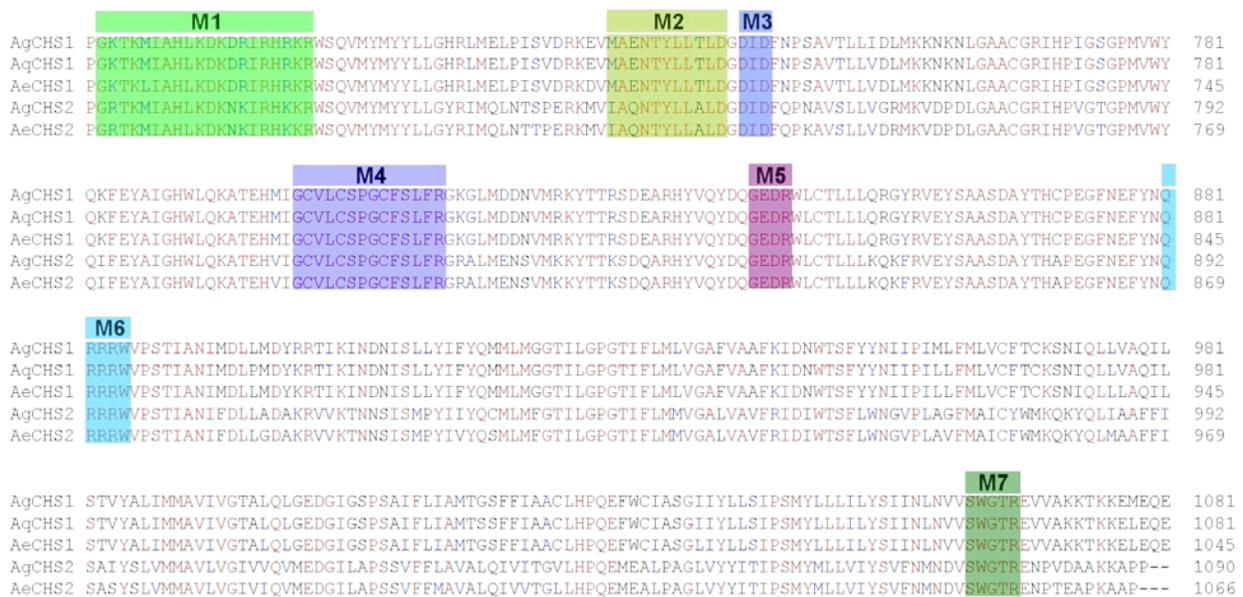
Gene	Primer name	primer sequence (5'-3')	Length	Tm (°C)
<i>AgCHS1</i>	AgCHS1F	ACCAACTGTCTGTGTGTTATAC	22	54.9
	AgCHS1R	CCGCAAGATGTTAGAAGAGC	20	54.6
<i>AgCHS1A</i>	AgCHS1AF	ACGAGCGCGACTTCCTCAC	19	55.4
	AgCHS1AR	GAGTCGCGCAACTCCTTGAG	20	55.9
<i>AgCHS1B</i>	AgCHS1BF	ACGAGCGCGACTTCCTCAC	19	55.4
	AgCHS1BR	GTCGACGGCGATTCTCGC	18	54.9
<i>AgCHS2</i>	AgCHS2F	CACCAGCAACGCCATCATC	19	53.2
	AgCHS2R	GAACACCAGCAGCAGAGTAAC	21	54.4
<i>AgRPS3</i>	AgRPS3F	GCTGGGCATCAAGGTCAAG	19	55.4
	AgRPS3R	ATCTCATCCTTCGGCTCAAC	20	54.4

**Table 2.2 Pairwise comparison of *An. gambiae* chitin synthases with chitin synthases from other insect species and *C. elegans*.**

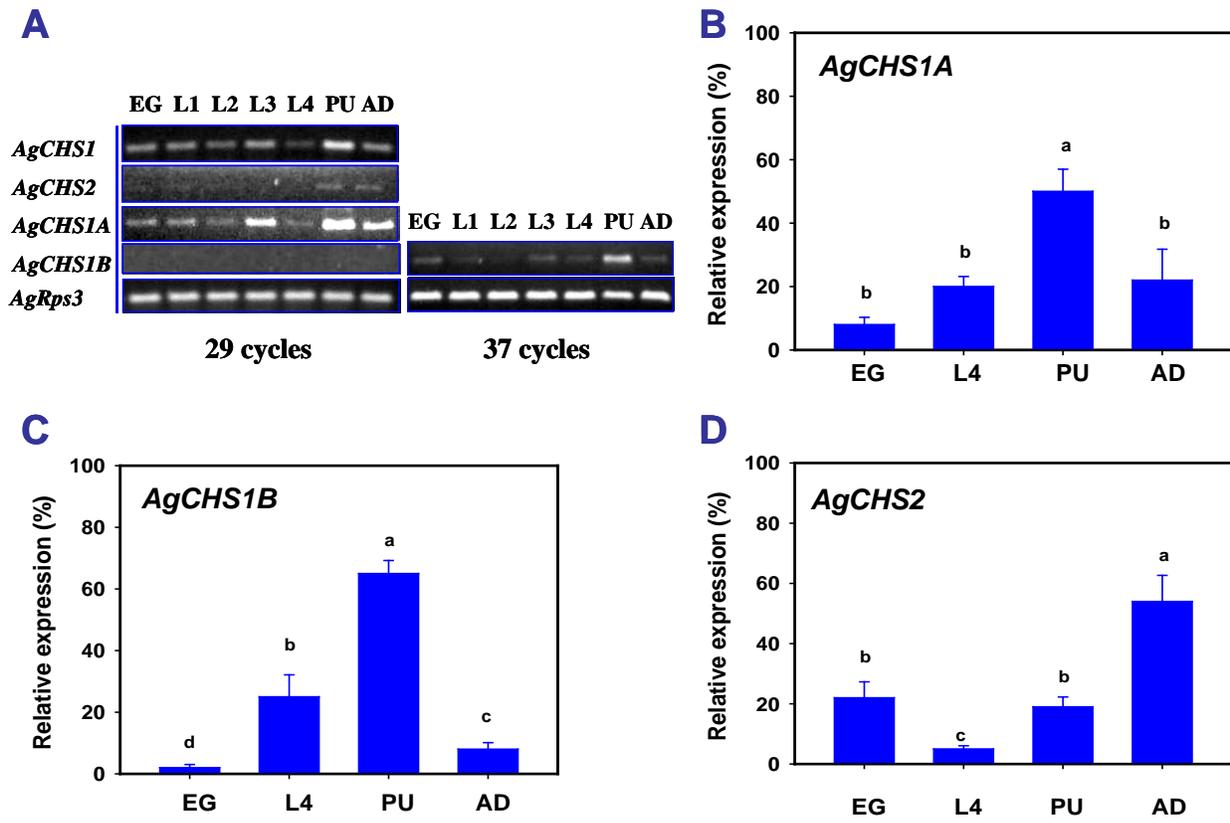
name	Identity (%)			Accession No.
	AgCHS1A	AgCHS1B	AgCHS2	
AgCHS1A	-	99.2	47.5	XM_321337
AgCHS1B	99.2	-	47.6	XM_321336
AqCHS1	97.0	97.8	47.7	DQ415985
AeCHS1	91.8	91.9	46.4	XM_001662150
LcCHS1	79.2	78.9	45.6	AF221067
DmCHS1	78.5	78.1	45.3	NM_079509
TcCHS1A	73.4	73.6	47.9	AY291475
TcCHS1B	73.3	73.5	47.4	AY291476
PxCHS1A	73.3	73.3	47.5	AB271784
PxCHS1B	72.9	73.2	47.2	AB281490
SeCHS1	72.7	72.7	48.5	DQ062153
MsCHS1	72.1	71.8	47.6	AY062175
CeCHS1	30.6	30.8	29.2	NP_492113
AgCHS2	47.5	47.6	-	N/A
AeCHS2	46.3	46.4	81.9	XM_001651113
DmCHS2	42.3	42.5	48.1	NM_079485
SeCHS2	52.5	52.6	43.3	EU622827
SfCHS2	51.3	51.4	43.3	AY525599
TcCHS2	48.0	48.0	43.0	AY291477
MsCHS2	52.6	52.6	42.8	AY82156
CeCHS2	40.9	40.9	35.2	NP_493682



**Figure 2.1** Sequence analysis of *AgCHS* genes. (A) Gene structures of *AgCHS*s. *AgCHS1* has two splice variants with alternative splicing exons (6A and 6B). (B) Phylogenetic tree of insect chitin synthase. (C) Phylogenetic tree of the alternative exons of insect *CHS1*. Ag, *Anopheles gambiae*; Ae, *Aedes aegypti*; Aq, *A. quadrimaculatus*; Dm, *Drosophila melanogaster*; Lc, *Lucilia cuprina*; Lm, *Locusta migratoria*; Ms, *Manduca sexta*; Px, *Plutella xylostella*; Tc, *Tribolium castaneum*; Se, *Spodoptera exigua*; Sf, *Spodoptera frugiperda*; Eo, *Ectropis obliqua*; Ce, *Caenorhabditis elegans*. GenBank Accession numbers are listed in Table 2.2. Phylogenetic tree was generated using the software MEGA 4 after alignment with ClustalW method.



**Figure 2.2 Alignment of the conserved catalytic domain of the chitin synthases from three mosquito species: Ag, *Anopheles gambiae*; Aq, *An. quadrimaculatus*; Ae, *Aedes aegypti*. Seven characteristic motifs (M1-7) for insect chitin synthases are highlighted. Dashes are used to denote gaps introduced for a maximum alignment.**



**Figure 2.3** Analysis of stage-specific expression of *AgCHS* genes. (A) RT-PCR analysis of each stage from egg to adult, and (B-D) the expression patterns confirmed by qPCR. Same letters on the error bars indicate no significant difference based on Fisher's LSD ( $P \geq 0.05$ ).

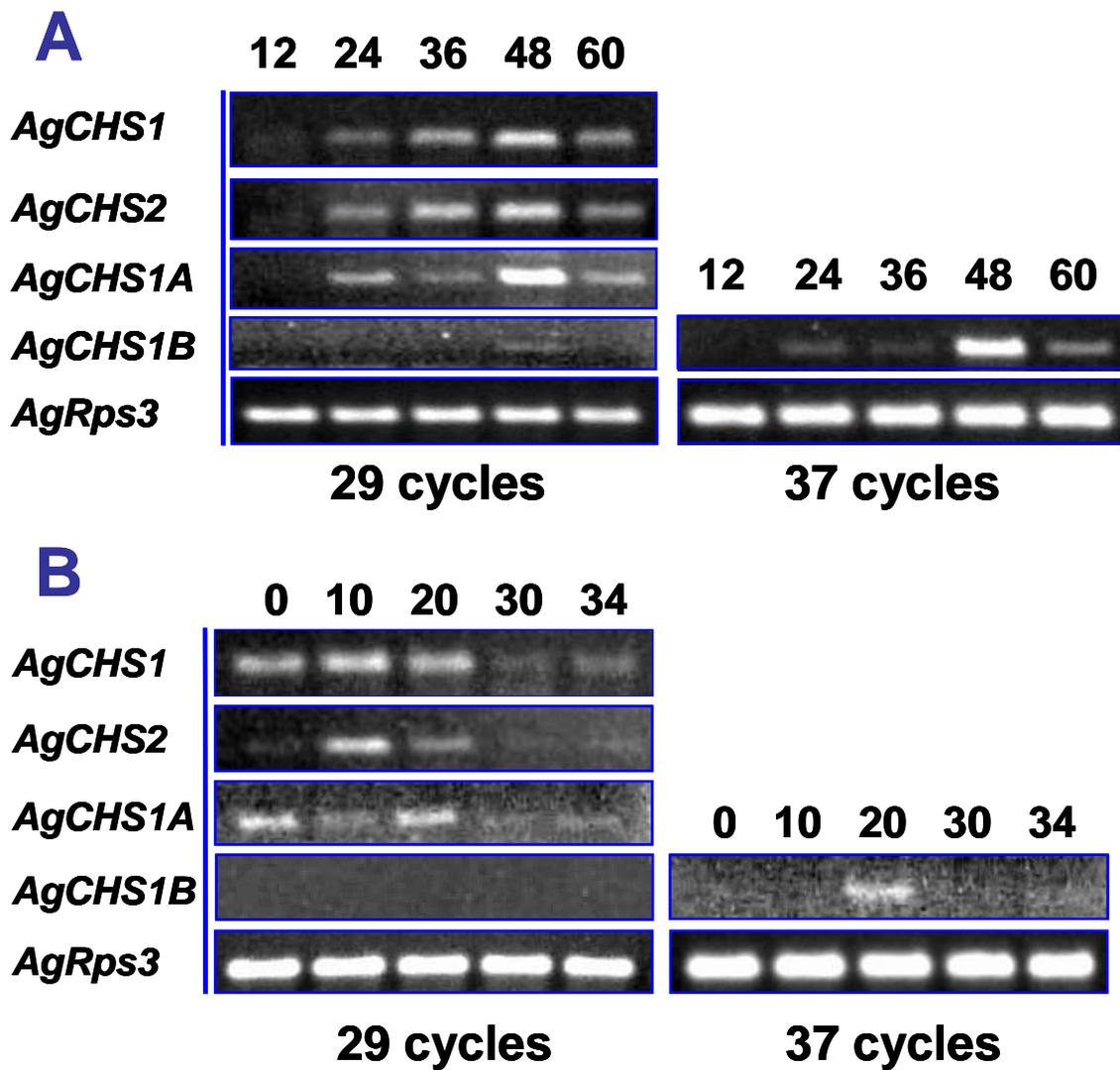
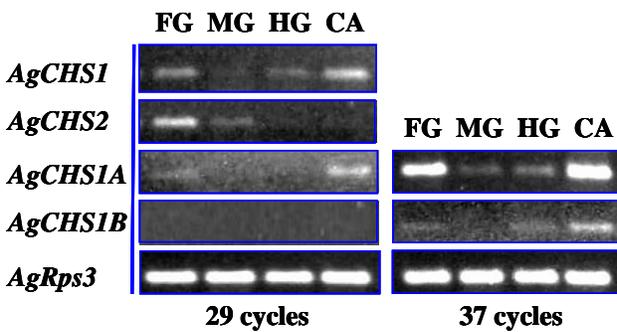
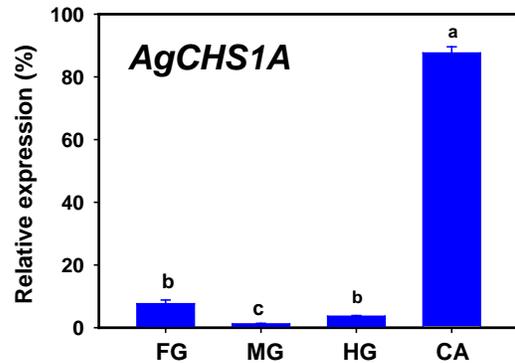
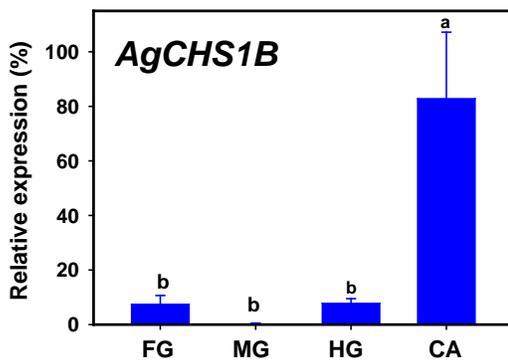
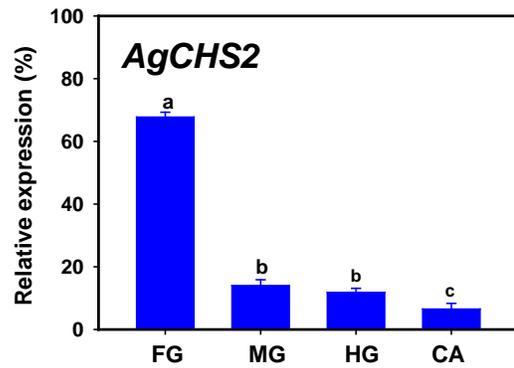
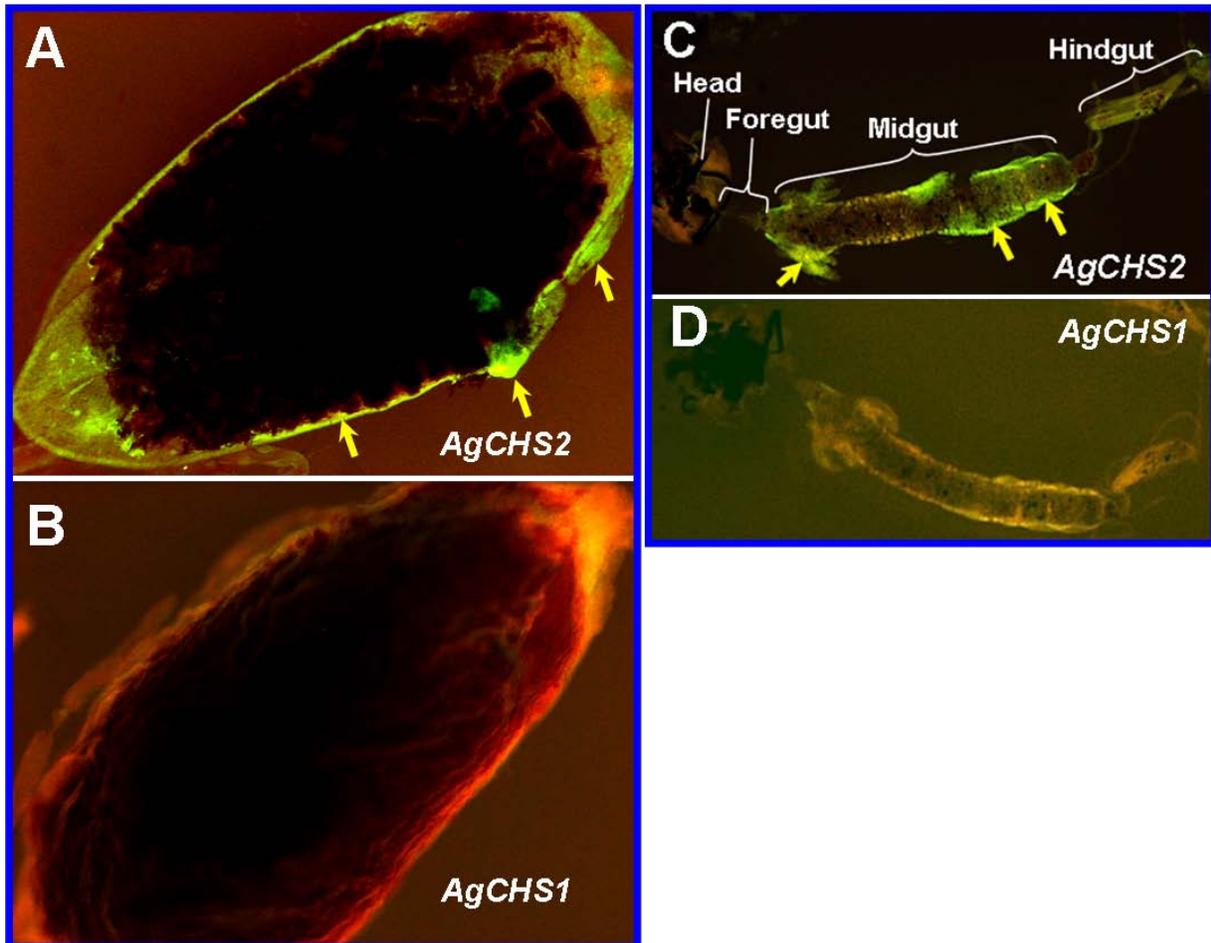


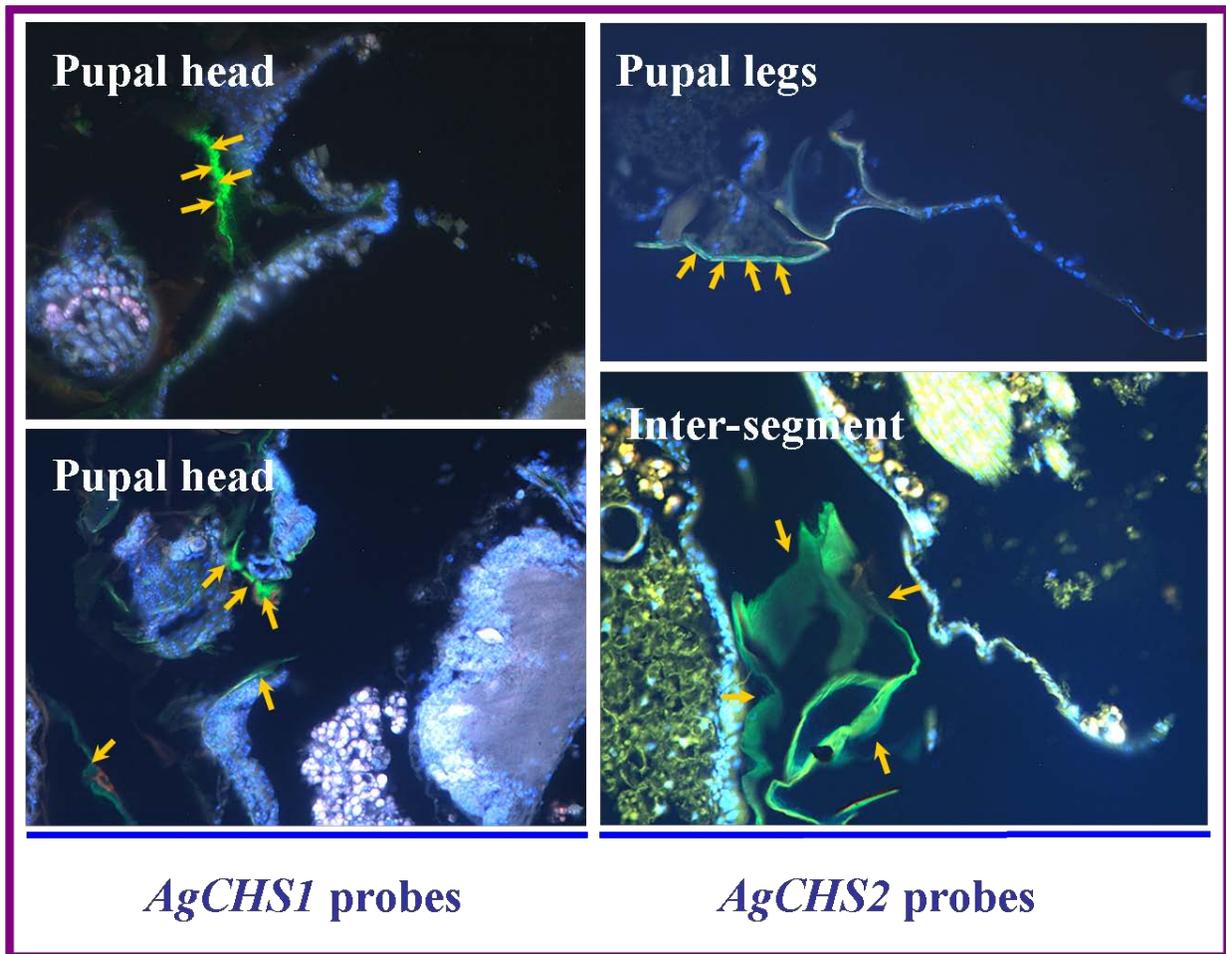
Figure 2.4 RT-PCR analysis of stage-specific expression patterns of *AgCHS* genes in eggs (A) at 12, 24, 36, 48, and 60 h, respectively, and pupae (B) at 0, 10, 20, 30, and 34 h, respectively.

**A****B****C****D**

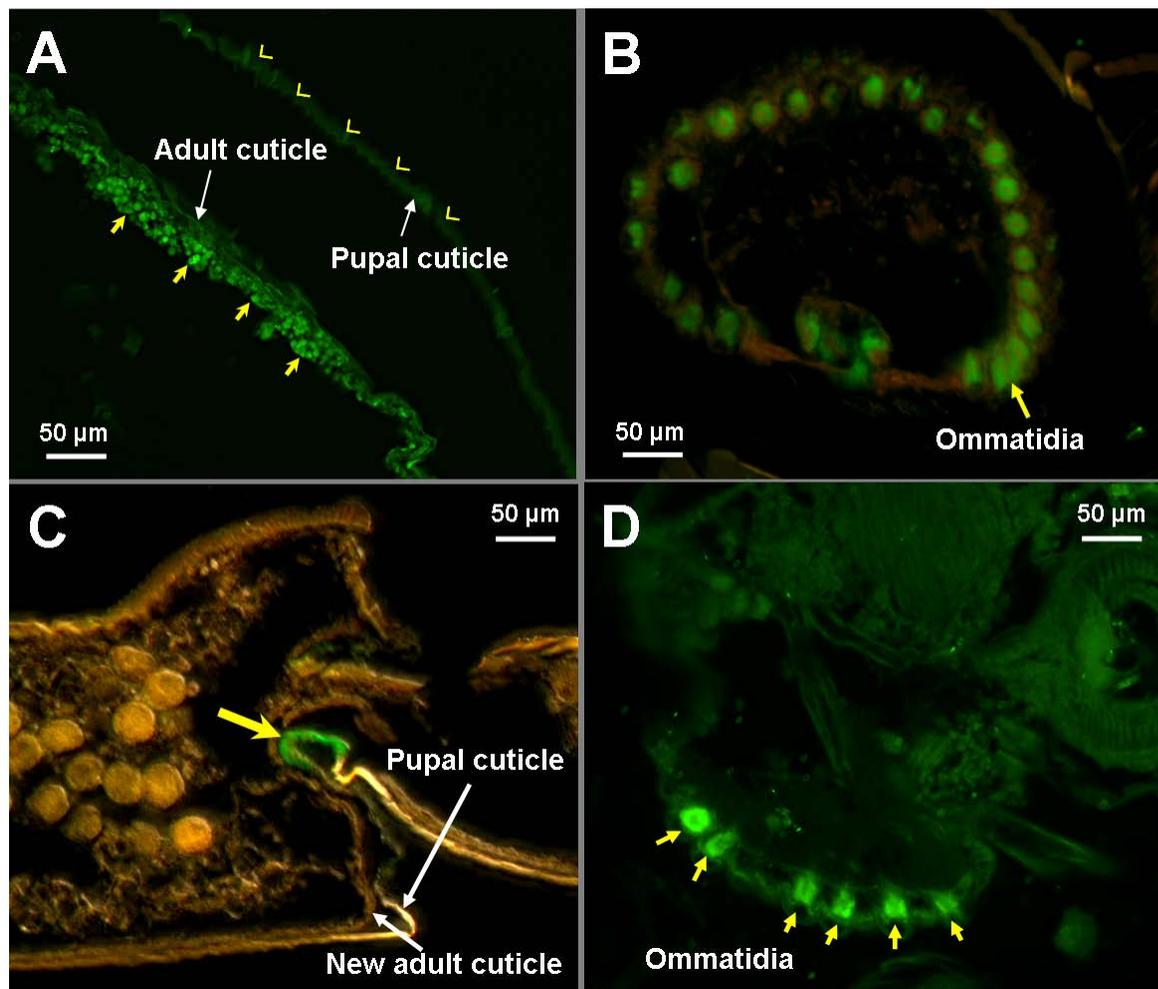
**Figure 2.5** Analysis of tissue-specific expression of *AgCHS* genes. RT-PCR analysis from four tissues: FG, foregut; MG, midgut; HG, hindgut; CA, carcass (A), and expression patterns confirmed by qPCR (B-D). Same letters on the error bars indicate no significant difference based on Fisher's LSD ( $P \geq 0.05$ ).



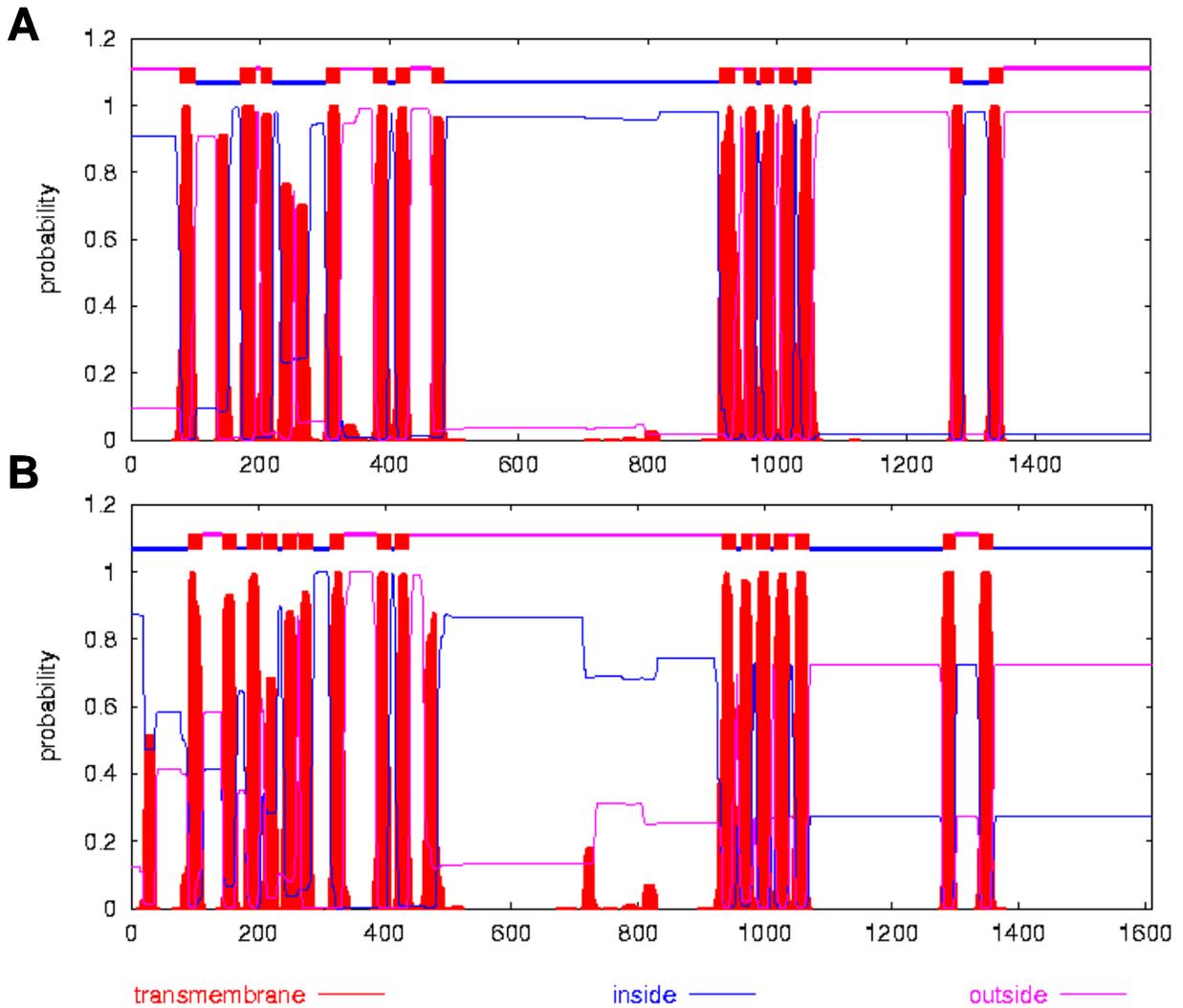
**Figure 2.6** Fluorescence *in situ* hybridization (FISH) analysis of *AgCHS* genes in mosquito gut. Localization of *AgCHS* gene transcripts in the midgut of the adult mosquito 24 h after a bloodmeal by using *AgCHS2* (A) and *AgCHS1* (B) probes, respectively. Localization of the transcripts in the whole larval gut stained by *AgCHS2* (C) and *AgCHS1* (D) probes, respectively. The green color indicated by arrows shows positive staining.



**Figure 2.7** Fluorescence *in situ* hybridization (FISH) analysis of *AgCHS1* and *AgCHS2* in the paraffin-embedded thin sections of the mosquito pupae (12-24 h after pupation). Green color indicated by arrows shows positive staining, and the blue color shows the nuclei by DAPI staining.



**Figure 2.8 Immunohistochemistry anti-AgCHS1 (A, B) and anti-AgCHS2 serum (C, D) in mosquito pupae. Paraffin-embedded thin sections of the 12-24 hour whole mount pupae were immunostained with primary antibodies and visualized by the reaction with Alexa 488-conjugated goat anti-mouse IgG. The epidermal cells in the adult cuticle (A) and the eyes (B) were immunoreactive (arrows indicate positive staining, arrow heads indicate negative staining). The abdominal inter-segmental region of the pupal cuticle (C) and the eyes (D) were immunoreactive (arrows indicate positive staining).**



**Figure 2.9** Domain analysis of two chitin synthases by TMHMM program. Membrane-spanning  $\alpha$ -helices are indicated by red columns.

## CHAPTER 3 - Molecular characterization of chitinase genes in African malaria mosquito, *Anopheles gambiae*

### Abstract

Chitinase is an important enzyme responsible for chitin degradation in insects and other chitin-containing organisms. However, current knowledge on chitinase, especially their structures, functions, and regulations in insects is still very limited. We identified 20 putative chitinase genes in the African malaria mosquito, *Anopheles gambiae*, through genome-wide search and assigned these genes into eight different chitinase classes (I-VIII). Domain analysis of chitinase and chitinase-like proteins showed that all the proteins contained at least one catalytic domain. However, only seven chitinases (AgCht4, AgCht5-1, AgCht6, AgCht7, AgCht8, AgCht10 and AgCht23) displayed chitin binding domain. Our analyses of stage- and tissue-specific gene expression showed that most of these genes were expressed in several stages. However, *AgCht8* was mainly expressed in pupal and adult stages. *AgCht2* and *AgCht12* were specifically expressed in foregut, whereas *AgCht13* was the only gene expressed in midgut. Immunohistochemistry analysis of selected chitinases in paraffin-embedded thin sections of *An. gambiae* revealed high expression of the proteins in certain body parts such as compound eyes. This study is expected to provide new insights into the functions of the diverse chitinase genes in insects.

**Key words:** chitinase and chitinase-like proteins, *Anopheles gambiae*, domains, expression patterns, immunohistochemistry

## Introduction

Chitin, a linear polysaccharide of N-acetyl- $\beta$ -D-glucosamine residues joined by  $\beta$ -1,4 glycosidic linkages, is the second most abundant biological polymer after cellulose (Merzendorfer, 2006; Kramer & Muthukrishnan, 2005). It is widely distributed in arthropods, fungi and nematodes. In arthropods, chitin is a vital component of the cuticular exoskeleton and thus is crucial for growth and development (Merzendorfer & Zimoch, 2003). Chitin is also found in internal structures of many insects and other arthropods, including the cuticular linings of trachea and in the peritrophic matrix (PM) lining the gut epithelium (Cohen, 2001). During insect growth and development, both the cuticle and PM must be degraded periodically and replaced to allow for growth, maturation and repair. Chitinolytic enzymes play important roles in shedding of the old cuticle and turnover of the PM.

Chitinase (EC 3.2.1.14, endochitinase) is an enzyme catalyzing the random hydrolysis of N-acetyl- $\beta$ -D-glucosamine  $\beta$ -1,4 glycosidic linkages in chitin and chitodextrins in a variety of organisms including insects, bacteria, fungi, plants, and marine and land animals. Chitinases are members of superfamily of O-glycoside hydrolases, which hydrolyze the glycosidic bond in polysaccharides or between a sugar and a noncarbohydrate moiety. All insect chitinases belong to family 18 of glycosylhydrolases, which have been found in a wide variety of organisms including bacteria, yeasts and other fungi, nematodes, arthropods, and even vertebrates such as mice, chicken, and human. The vertebrate proteins probably function as defensive proteins against chitin-containing pathogens (Yan et al., 2002).

The first insect chitinase gene was firstly cloned in *Manduca sexta* (Kramer et al., 1993). After that, single chitinase gene has been characterized from several insect species such as *Bombyx mori* (Kim et al., 1998), *Spodoptera litura* (Shinoda et al., 2001), *Choristoneura*

*fumiferana* (Zheng et al., 2002), *Anopheles gambiae* (Shen and Jacobs-Lorena, 1997), *Lutzomyia longipalpis* (Ramalho-Ortigao & Traub-Cseko, 2003), *Chelonas* sp. (Krishnan et al., 1994), and *Tenebrio molitor* (Genta et al., 2006). Multiple chitinase genes have been documented in *B. mori* (Daimon et al., 2005). With the completion of genome sequences, a rather large and diverse group of chitinase-like genes have been identified in insects. For example, 16, 16 and 13 putative chitinase-like genes were identified in the genomic databases of the red flour beetle, *Tribolium castaneum*, the fruit fly, *Drosophila melanogaster*, and the malaria mosquito, *An. gambiae*, respectively (Zhu et al. 2008a). Even though the genomes of *Apis mellifera*, *B. mori* and *Aedes aegypti* have not been completely analyzed, available data indicate that chitinases-like proteins are also encoded by multiple genes in these insect species (Zhu et al. 2008a).

Based on amino acid sequence similarity and phylogenetic analysis, insect chitinase family proteins have been classified into five groups (Zhu et al., 2004; Zhu et al., 2008a). Recently, the gene characterization and functional analysis of individual members by using gene-specific RNAi was performed in *T. castaneum* and revealed the functional specialization of insect chitinase gene families primarily during the molting process. These results provided a biological rationale for the presence of a large assortment of chitinase-like proteins (Zhu et al. 2008b). For example, the group I and group II enzymes are involved in molting by digesting cuticular chitin, whereas the group III enzymes have a morphogenetic role in development such as regulating abdominal contraction and wing expansion (Zhu et al. 2008b).

The mosquito, *An. gambiae*, is the important arthropod-borne disease vector in Africa. To date, only very few insecticides are available for control of the mosquitoes and other human health-related arthropods. Because insect growth and development depend on the precisely tuned chitin synthesis and degradation, chitinase presents an attractive target for combating

insect pests as chitin is not synthesized by vertebrates. We hereby reported the classification, developmental- and tissue-specific expression patterns of the chitinase-like genes in African malaria mosquito, *An. gambiae*. This study will facilitate the understanding of chitinase-like proteins in a diptera insect species and potentially lead to the development of new insecticides targeting on insect chitin metabolic pathways.

## **Materials and Methods**

### ***Mosquito rearing***

A colony of *An. gambiae* obtained from the Malaria Research and Reference Reagent Resource Center (MR4) (Manassas, VA) was maintained in Department of Entomology at Kansas State University (Manhattan, KS) since 2007 based on the method described by Zhang & Zhu (2006). Briefly, the larvae were fed with slurries of brewer's yeast and TetraMin Baby-E fish food, whereas adults were fed with 10% sucrose solution soaked into cotton balls. Two-day-old females were fed with pre-warmed, defibrinated horse blood (Colorado Serum Company, Denver, Colorado) in a membrane feeder made of a lubricated Naturalamb brand condom (Church and Dwight Co., Inc., Princeton, NJ), allowing the females to lay eggs.

### ***Genome-wide Blast search and sequence analysis***

TBLASTN was used to search chitinase-like genes from the *An. gambiae* genome database. Each protein sequence obtained was further searched by protein blast in NCBI. The protein sequences containing at least one of four signature sequences FDGXDLWEYP (highly conserved in all known insect chitinases) were considered as candidate chitinase-like proteins. The online program SMART (<http://smart.embl-heidelberg.de/>) and UCSC Genome Bioinformatics program (<http://genome.ucsc.edu/>) were used to obtain the domain architecture

and exon/intron organization of each gene. Sequence analysis was performed using the computer software suite Lasergene (DNASTar, WI). Phylogenetic tree was constructed based on domain amino acid sequences by Neighbor-joining algorithm using Mega 4.0 software. Other software available from online servers was described in the results.

### ***RT-PCR analysis***

Total RNA was isolated from the insect sample representing each of seven developmental stages, including egg; first-, second-, third- and fourth-instar larva; pupa; and adult, using TRIzol Total RNA Isolation kit (Invitrogen, Carlsbad, CA) for studying stage-specific expressions of *AgCHS* genes. To study the stage-specific expression in the egg and pupa stage, total RNA was isolated from insect sample representing each of five egg developmental times, including 12, 24, 36, 48, and 60 hour after egg laid, and each of five pupal developmental times, including 0, 10, 20, 30, and 34 hour after pupation. Similarly, total RNA was also isolated from the tissue sample representing each of four tissue types, including foregut, midgut, hindgut and carcass (the whole larva with the gut removed), for studying tissue-specific expressions. In brief, the fourth instar larvae were chilled on ice and dissected in cold 1×PBS to get the tissues. The larval was longitudinally opened by carefully cutting the integument from one side of the larva without damaging the gut. Then the whole intestine was gently pulled out and detached from other tissues such as Malpighian tubules, trachea, and fatbodies. The midgut, foregut, and hindgut were carefully separated and immediately put in TRIzol agent. The foregut and midgut were separated in the junction area of the gastric caecum. The larvae after their guts were removed were collected as carcasses.

After total RNA was isolated and concentration was measured using NanoDrop ND-1000 ( NanoDrop Technologies, Inc., Wilmington, DE), 2.5 mg of total RNA was then treated with

DNase I (Fermentas, Glen Burnie, MD) and the first-strand cDNA was synthesized with First Strand cDNA Synthesis kit (Fermentas) using an oligo (dT)<sub>12-18</sub> primer in a 20- $\mu$ l reaction following the manufacturer's protocol. Beacon Designer software from Primer Biosoft (<http://www.premierbiosoft.com>) was used to design the gene-specific primers for each gene that are shown in Table 2.1. Following PCR was performed using the PCR Master Mix (Fermentas) with the thermal cycle program consisting of an initial denaturation at 94°C for two min followed by 29 cycles of 94°C for 30s, 55°C for 30s and 72°C for 45s, and a final extension at 72°C for ten minutes. PCR products were resolved on a 1.8% agarose gel and visualized by staining the gel with ethidium bromide. The gene encoding an *An. gambiae* ribosomal protein S3 (*AgRPS3*) was used as a reference for RT-PCR analysis. At least three biological repeats were performed for each gene at each developmental stage and each tissue type.

### ***Real-time quantitative PCR (qPCR) analysis***

To confirm the stage- and tissue-specific expression patterns of each gene, cDNA prepared from above mentioned samples representing each of four developmental stages, including egg, fourth-instar larva, pupa, adult, and from each of four tissues, including foregut, midgut, hindgut, and carcass, was used for qPCR analysis. The qPCR was performed in a 25  $\mu$ l reaction containing 10.5  $\mu$ l of 1/10 diluted cDNAs, 0.4  $\mu$ M of each primer, and 1 $\times$  Maxima SYBR Green qPCR Master Mix (Fermentas, Glen Burnie, MD) using iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA). The optimized real-time qPCR program used for both *AgRPS3* and *AqCHS1* consisted of initial denaturation step at 95 °C for 5 min followed by 40 cycles of 95°C for 15 sec, 55°C for 30 sec and 70°C for 30 sec. At the end of the PCR, amplification specificity was verified by obtaining the dissociation curve, in which the samples were cooled to 55 °C after denaturing, and then melting curves were obtained by increasing

0.5 °C/10 s for each cycle with a total of 80 cycles until reaching 95 °C to denature the double-stranded DNA. The specificity of each reaction was evaluated based on the melting temperatures of the PCR products. The amplification efficiency of primer pairs was determined from the slope of the curve generated by amplification from serially diluted cDNA. Efficiency had to be at least 0.9 for a primer pair to be accepted. Relative expression values (REVs) for the tissue-specific gene expressions were then determined by dividing the quantities of the target sequence of interest with the quantity obtained for *AgRPS3* as an internal reference gene. We found that expression of *AgRPS3* fluctuated across the developmental stages we tested (data not shown). Other genes including *RpS7*, ribosomal protein L32, elongation factor 2, and the ubiquitin-ribosomal protein L40 fusion protein were also been tested. However, no one is a suitable “housekeeping gene” to normalize our data across the developmental stages in *An. gambiae*, as also noticed in other insect species (Togawa et al., 2008). Therefore, we did not normalize the stage-specific gene expression using *AgRPS3*, but adopted very careful quantification of RNA using NanoDrop to standardize our samples. The qPCR was performed with three biological replicates and two technical replicate for each gene.

### ***Immunohistochemistry***

Anti MsCht5 and anti-sand fly Cht8 serum, which were kindly provided by Dr. Muthukrishnan and Dr. Ramalho-Ortigao, respectively, were used for immunostaining of AgCht5 and AgCht8, respectively in mosquito pupae. Paraffin-embedded thin sections were used for immunohistochemistry. In brief, the 12-24 h pupae were fixed in 4% paraformaldehyde at 4 °C overnight followed by 3 × 5 min washing with PBST (PBS and 0.1% Triton X-100). The samples were then dehydrated through a series of grades of ethanol (2x 30 min in each 70% and 96%, 2x 20 min in 100%), followed by 2 x 1 hr in chloroform. The dehydrated samples were

finally embedded in paraplast (56 °C, Tyco Healthcare) after overnight penetration. Histological sections (8 µm) were prepared by using microtome (Richard-Allan Scientific Microm) with a low profile microtome blade (Richard-Allan), straightened on Fisherbrand ColorFrost Plus microscope slides with 0.5% gelatin, and allowed to dry for 2 days at 40°C on the top of slide warmer. The sections were deparaffinized with two washes of 10 min xylene, rehydrated through successive baths of ethanol (100 %, 96%, and 70% in water, 1x 5 min each), two water baths 5 min for each, and finally PBST for 10 min or more.

For localization of AgCht5 and AgCht8, above sections were firstly blocked in 1% BSA (Bovine serum albumin) in PBST for 15 min followed by incubation with a 1:100 dilution of the anti-MsCht5 and anti-sand fly Cht8 serum in PBST at 4°C overnight. Paraffin-embedded thin sections immunostained with preimmune serum were used as negative controls. The sections were then washed in PBST three times with two min for each. The primary antibodies were detected by the reaction with Alexa 488-conjugated goat anti-rabbit (for AgCht5) or anti-mouse (AgCht8) IgG (1:500 dilution in PBST) at 4°C overnight. After four times of washings in PBS (10 min each), the sections were mounted in glycerol containing 300 nM 4',6'-diamino-2-phenylindole (DAPI; 2 µg ml<sup>-1</sup>; Sigma) on a glass slide and the fluorescence was examined using a Nikon Eclipse E800 fluorescence compound microscope equipped with appropriate filters. Photography was done with a Cool SNAP digital camera.

### ***Fluorescence in situ hybridization***

To construct the probes, partial cDNA fragment of *AgCht8* was amplified by PCR using gene-specific primers with built-in *Xho* I and *Xba* I restriction sites as indicated by underlining: 5'- GTACACTCGAGGCTGTCGGTGGACTATGC -3' (forward) and 5'- CTGCATCTAGAAGCGGGTACTTCACATTGC -3' (reverse). PCR product was ligated into

pBlueScript SK (+) vector digested with the same restriction enzymes. The plasmid DNA was then linearized with either *Xho*I or *Xba*I and used for transcription with T7 and SP6 RNA polymerases (Invitrogen) to generate anti-sense and sense probes, respectively. The anti-sense and sense (as negative control) probes were labeled with Fluorescence *in situ* Hybridization (FISH) Tag™ RNA Multicolor Kit (Catalog No. F32956, Invitrogen) following the manufacturer's protocol. The probes were labeled in green by using the Alexa Fluor® 488.

Paraffin-embedded thin section preparation, deparaffinization and rehydration were described as above. For facilitating the penetration of the tissues, the sections were in pretreated with proteinase K (10 µg/ml) (Sigma) in PBST for 10 min at room temperature and the reaction was stopped with 2 mg/ml glycine in PBST followed by washing with PBST two times. The sections were refixed in 4% paraformaldehyde in PBS at RT for 15 min, washed in PBST 5 min, PBST: hybridization buffer (1:1) 5 min, hybridization buffer (50% formamide, 5 X SSC, 50 µg/ml heparin, 0.1% Tween 20, and 100 µg/ml salmon sperm DNA) 5 min and prehybridized in hybridization buffer at 55°C for 1 h. Hybridization was performed with 1 µg/ml FISH probes in hybridization buffer at 55°C for 20 h following the recommended procedure by the manufacturer. Unbound probe was removed by rinsing slides with hybridization buffer for 10 min at 55°C, hybridization buffer:PBST (1:1) for 5 min, PBST for 10 min 4 times at RT. The sections were mounted in glycerol on a glass slide and the fluorescence was examined using a Nikon Eclipse E800 fluorescence compound microscope equipped with appropriate filters. Photograph was taken with a Cool SNAP digital camera attached to the compound microscope and pictures were edited using Adobe Photoshop 7.0.

### ***Data analysis for qPCR***

For qPCR results, we obtained relative expression profiles in percentage rather than absolute expression levels in each replicate by dividing the REV of each developmental time point or tissue for each gene by the sum of REV throughout development or all tissues for that gene. The percentage data of the relative *AgCHS* expression were then transformed using arcsine square root transformation before the ANOVA. Fisher's least significant difference (LSD) multiple comparisons were then used to separate the means among the samples.

## **Results**

### ***Classification of chitinase and chitinase-like proteins in An. gambiae***

A recent work on chitinases by a bioinformatics-based investigation of the genome databases from three insect species identified 16, 16, and 13 putative chitinase-like genes in *T. castaneum*, *D. melanogaster*, *An. gambiae*, respectively (Zhu et al., 2008a). Based on the conserved catalytic domain, the chitinase-like proteins encoded by these genes were classified into five groups (I-V) (Zhu et al., 2008a). With our further investigation on the genomic database, more chitinase-like genes were identified in all these three insect species. Totally, 22, 17, and 20 chitinase and chitinase-like proteins encoded by these genes were revealed from *Tribolium*, *Drosophila*, and *Anopheles*, respectively.

Phylogenetic analysis was based on the catalytic domains of each chitinase-like protein. Results showed that these chitinase and chitinase-like proteins were assigned into eight groups (I-VIII) (Fig. 3.1). In addition to the five groups (I-V) which have already been reported (Zhu et al., 2004, 2008a), three new groups (VI-VIII) were identified. These three new groups, VI, VII, and VIII, were closely related but clearly distinct from group II, II, and V, respectively. Each of

these three new groups consisted of single chitinase protein: AgCht2 in group VI, AgCht6 in group VII, and AgCht11 in group VIII. The Cht6 proteins from all three insect species are large proteins which having 4498, 2369 and 3405 predicted amino acids for DmCht6, TcCht6 and AgCht6, respectively. A comparison of the three new groups of chitinase and chitinase-like proteins from three insect species was listed in table 3.2 including the number of predicated amino acid residues, number of the catalytic domain, and presence of chitin-binding domains.

Six of eight groups (I-III, VI-VIII) of chitinase and chitinase-like proteins consisted of single gene in each species, and the other two groups, IV and V, are encoded by multiple genes in each species. Group I chitinases consisted of chitinase 5. Multiple chitinase 5 proteins were identified in two mosquito species, five in *An. gambiae* and four in *Aedes aegypti*, possibly, originated from gene duplication during the evolutionary process, which will be discussed in other sections. In contrast, chitinase 5 gene duplication was not observed in *Tribolium* and *Drosophila*. Group II, III, VI, VII, and VIII chitinases consisted of chitinase 10, 7, 2, 6, and 11, respectively. Group IV chitinases are the most divergent and include 14, 6, and 3 chitinase proteins from *Tribolium*, *Anopheles*, and *Drosophila*, respectively. Group V proteins include the putative chitinase-like imaginal disc growth factors (IDGFs), which are encoded by several genes in each species. For example, *Drosophila*, *Tribolium*, and *Anopheles* possess 6, 2, and 2 IDGFs, respectively. Chitinase 12 is the only protein that is not consistently grouped into one group. TcCht12 falls into the divergent group IV, whereas AgCht12 is more closely related to group IV, and DmCht12 is relatively close to group I chitinases.

### ***Exon and intron organization of the chitinase genes***

The organization of the 20 chitinase and chitinase-like genes was shown in Fig.3.2. It is clear that the organization of chitinase genes has diverged among the genes within *An. gambiae*.

High variations can be observed from both the gene size and the number of exons/introns. For example, *AgCht16* and *AgCht5-5* each has only one exon and no intron, whereas *AgCht6* has as many as 19 exons and 18 introns. The sizes of the exon and the intron range from less than 100 bp to more than 2kb.

### ***Domain architecture of chitinase genes***

One of the four conserved motifs in the catalytic domain was used as signatures to identify chitinases or chitinase-like proteins for all three insect species. The consensus sequence, DWEYP, was considered as the essential trait for putative chitinase proteins. Those proteins are considered as chitinase-like proteins if their deduced amino acid sequences show similarity with chitinases but have the key residue substitution in DWEYP that is known to abrogate catalytic activity. Results of the analysis of the domain organization of the putative chitinase and chitinase-like proteins in *An. gambiae* were shown in Fig. 3.3. Most of them have one catalytic domain except *AgCht7*, *AgCht9*, and *AgCht10* which have 2, 2, and 4 catalytic domains, respectively. Six chitinase and chitinase-like proteins, *AgCht4*, *AgCht5-1*, *AgCht6*, *AgCht7*, *AgCht8*, and *AgCht23*, have one chitin binding domains (CBDs), whereas *AgCht10* has 4 CBDs and all other chitinase and chitinase-like proteins don't have CBD. Ten chitinases are predicted to be secreted proteins as they contain a cleavable signal peptide at the N-terminal of their sequences. Interestingly, *AgCht7* is found to have two predicted transmembrane segments at the N-terminal region. Similarly, at least one transmembrane segment was also found in *Cht7* from other species including *Drosophila*, *Apis* and *Tribolium* (Zhu et al., 2004). These results indicate that *Cht7* is a membrane-anchored protein.

### ***Developmental stage-specific expression profiles***

Stage-specific expression patterns of *AgCht* genes were determined in eggs, four different larval instars (1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup> and 4<sup>th</sup>), pupae and adults by using RT-PCR (Fig. 3.4). Among the 20 genes, two IDGF genes (*AgChtIDGF2* and *AgChtIDGF4*) were constitutively expressed in all developmental stages from egg through adult stage. Ten of the remaining 18 *AgCht* genes, *AgCht5-1*, *5-2*, *5-3*, *5-5*, *-10*, *-7*, *-16*, *-2*, *-6*, *-11*, showed various expression patterns in all the seven stages. *AgCht24* was also expressed in most of stages except in the eggs. Differently, *AgCht5-4*, *-4*, and *-9* are detected from egg to the fourth instar larval stage but not in the pupal and adult stage. In contrast, *AgCht8* was expressed in the pupal- and adult stage, whereas *AgCht12*, *AgCht13*, and *AgCht23* were almost exclusively expressed in the four larval instars, in which *AgCht12* was predominantly expressed in the fourth instar larvae. To confirm the RT-PCR results, expression patterns of the selected genes including *AgCht5-1*, *-7*, *-8*, and *-10* were evaluated by qPCR. The results from qPCR analysis (Fig. 3.5) were consistent with the RT-PCR results. These expression patterns may reflect their specific roles and functions during different developmental stages.

We further examined the expression patterns of the chitinase genes in different developmental times of eggs and pupae. RT-PCR analysis was performed in 12-, 24-, 36-, 48-, and 60-h eggs and in 0-, 10-, 20-, 30-h, and 34-h pupae. In the eggs, two IDGF genes were constitutively expressed in all the time points examined, *AgCht5-2* and *AgCht5-3* seemed to be expressed in all stages but showed different expression levels. Most of the remaining genes were expressed in the late eggs except for *AgCht11* which was detected in the early stage of the eggs and gradually decreased later on (Fig. 3.10). In the pupae, most of the chitinase genes showed various expressions in all the times of the pupae (Fig. 3.11). It was also revealed that *AgCht5-2*,

*AgCht5-5*, and *AgCht12* were only expressed in the early stage, whereas *AgCht23* was the only gene detected in the late stage of the pupae (Fig. 3.11).

### ***Tissue-specific expression profiles of the AgCht genes***

Expression patterns of the 14 *An. gambiae* *Cht* genes were analyzed in each of four different tissues including foregut, midgut, hindgut, and carcass by using semi-quantitative RT-PCR. Six of the 14 *AgCht* genes including *AgCht4*, -9, -16, -23, *AgIDGF2*, and *AgIDGF4*, were expressed in all tissues examined, although there were some significant variations in expression levels among different tissues (Fig. 3.6). In contrast, *AgCht2* and *AgCht12*, *AgCht13*, and *AgCht6* seemed to be exclusively expressed in the foregut, midgut, and carcass, respectively. Two *AgCht5* genes showed different expression patterns: *AgCht5-1* seemed to be gut-specific including fore-, mid-, and hindgut, whereas *AgCht5-4* was expressed in the foregut, midgut, and carcass. In addition, *AgCht7* was revealed to be expressed in the foregut and carcass, whereas *AgCht10* was expressed in the foregut, hindgut and carcass. Further analysis by qPCR revealed that *AgCht5-1* and *AgCht7* were predominantly expressed in the carcass, whereas *AgCht8* was predominantly expressed in the midgut, and *AgCht10* in the foregut, carcass and hindgut (Fig.3.7). The high variable expression patterns of all the *AgCht* genes may reflect their specialized roles in degradation of the chitin in different tissues in mosquitoes, which have been demonstrated in *Tribolium* (Zhu et al., 2008b).

### ***Localization of AgCht8 in pupae by in situ hybridization***

FISH was performed to localize *AgCht8*-containing tissues in mosquito pupae. *AgCht8* transcripts were detected in specific tissues inside the head. In addition, weaker signals of *AgCht8* transcripts were also detected in the tissues along side the compound eyes (Fig. 3.8).

### ***Localization of AgCht5 and AgCht8 in the pupae by immunohistochemistry***

Localization of AgCht5 and AgCht8 protein was determined by immunohistochemistry in paraffin-embedded thin sections of the mosquito pupae by using anti-*Manduca sexta* chitinase 5 polyclonal antibodies (Anti-MsCht5) and anti-sand fly (*Lutzomyia longipalpis*) chitinase 8 (Anti-sandy fly Cht8) polyclonal antibodies, respectively. Intensive staining was observed in the newly developed legs located at the base of the head and the abdominal tip of a pupa (Fig. 3.9) when anti-MsCht5 was applied. The high expression of AgCht8 proteins were detected in the pupal compound eyes (Fig.3.9) when anti-sandy fly Cht8 was used.

## **Discussion**

The availability of the whole genomic sequences in different insect species facilitates the genome-wide search of chitinase genes by using bioinformatics methods. Based on previous work by Zhu et al. (2008a), our further extensive search confirmed 22, 17, and 20 chitinase and chitinase-like genes in *T. castaneum*, *D. melanogaster*, and *An. gambiae*, respectively. This study and previous studies (Zhu et al., 2004, 2008a, 2008b) clearly demonstrate high degree of complexity of the chitinase and chitinase-like gene families in dipterans and coleopteran insects. Except *AgCht23*, *AgCht24*, *AgCht16*, and four *AgCht5* genes (apparently resulting from gene duplications of *AgCht5-1*), each of the 13 remaining genes identified its corresponding genes in *Tribolium* and *Drosophila* genome. RT-PCR analysis showed that transcripts of each of these genes were detected in all seven tested stages or only at some specific developmental stages (Fig. 3.4), suggesting that each of these genes may be functional.

The chitinase and chitinase-like proteins from three species are assigned into eight groups. If the apparent gene duplications of the mosquito Cht5 gene were not considered, six of eight groups has single member in each group, whereas group IV and V have multiple proteins

from each species. Group IV, the most divergent group, contains eight chitinases from *An. gambiae*, six of which are encoded by genes clustered on chromosome 2L, whereas the other two localized on chr3L and 3R, respectively. These results imply that gene duplications and functional divergence resulted in the current large number and high variety of the chitinase genes.

Domain analysis of the deduced proteins showed that all the proteins encoded by these genes have a multiple domain organization that includes 1 or 4 catalytic domains, 0, 1, or 4 chitin binding domains, 0 or 1 leader signal peptide or transmembrane-spanning domain, and linker regions. The domain organization of the chitinase and chitinase-like proteins from all eight groups from *An. gambiae* showed high similarity with that from *Tribolium* except some slight differences (Arakane & Muthukrishnan, 2010) as described following. The domain organization of AgCht5-1 from group I is the same as that from *Tribolium*. The differences were that 4 and 3 more Cht5 proteins were identified from *Anopheles* and *Aedes*, respectively, which were not observed in both *Tribolium* and *Drosophila*. The difference of the domain organization of the group II chitinases (Cht10s) between *Tribolium* and mosquitoes have been described in most recent review by Arakane & Muthukrishnan (2010). The group III AgCht 7 protein has two N-terminal transmembrane domains whereas only one in *Tribolium*. In contrast, AgCht11, the group VIII chitinase, lacks the N-terminal transmembrane domain which is found in *Tribolium* TcCht11. The most divergent chitinases in group IV also show high complexity in their domain organizations. In *Tribolium*, all 14 chitinases in group IV have the leader signal peptide; whereas only 4 of the 8 chitinases have their signal peptide in *Anopheles*. AgCht9, one member of group IV chitinases, has two catalytic domains but no signal peptide, whereas its counterpart in *Tribolium* has one catalytic domain and the signal peptide. Thus, *Anopheles* has three chitinases

which have more than one catalytic domains, whereas only two in *Tribolium*. The phylogenetic analysis and the high similarities of the domain organization for the chitinases from two insect species suggest that all these chitinase proteins evolve from a common ancestor and the distinctive groups of chitinases may predate the separation of the coleopteran and lepidopteran lineages of insects (Zhu et al., 2008a; Arakane & Muthukrishnan, 2010).

Stage-dependent expression of these chitinase genes demonstrated substantial differences in expression patterns of individual groups of chitinases (Fig. 3.4, 3.5). Chitinase genes encoding proteins belonging to groups I, II, III, V, VI, VII and VIII were expressed at almost all developmental stages from eggs through adult stages with the different expression levels, whereas the genes encoding the proteins belonging to group IV demonstrated high complexity of expressed patterns. For example, some genes were only expressed during the larval stages, whereas others expressed only during the pupal and adult stages such as *AgCht13* and *AgCht8*, respectively.

These chitinase genes also show differences in tissue specificity of expression (Fig. 3.6, 3.7). In *Tribolium*, it seemed that all of the group IV genes were expressed in the larval gut tissue, but not in the carcass (whole body after gut and head are removed) (Arakane & Muthukrishnan, 2010). However, similar pattern was not found for group IV genes in *Anopheles*. In *Anopheles*, group IV chitinase genes show diverse expression profiles. For example, *AgCht23* equally expressed in all tested tissues, *AgCht13* predominantly in the midgut, and *AgCht12* in the foregut (Fig. 3.6). Further work is needed to assign the expression of each chitinase gene into more specific tissues comprising the carcass including the fat body, trachea, muscle, etc. As we have known that chitin was the component of trachea and some other tissues, we do not know whether chitinases are needed to degrade the chitin in these tissues. The expression of certain

chitinase proteins in certain body parts, e.g. in compound eyes revealed by immunohistochemistry, implies the specific functions of specific chitinases in these tissues.

One interesting question on insect chitinases is that why one insect needs a rather larger number of chitinase and chitinase-like genes. In insects, chitin polymorphically occurs in three different crystalline forms:  $\alpha$ ,  $\beta$ , or  $\gamma$  chitin that differ in the degree of hydration, in the size of the unit cell and in the number of chitin chains per unit cell (Merzendorfer, 2006). It is possible that insects use different chitinases to efficiently degrade these different types of chitin and their modifications such as partial deacetylation. One supporting evidence come from the fact that substantial differences in the biochemical properties of chitinase-like proteins belonging to different groups have been documented (Zhu et al., 2008c; Matsumiya et al., 2006). On the other hand, different forms of chitin could occur in different extracellular structures at different developmental stages in insects. In addition to the chitin in the exoskeleton and PM, chitin and chitin-like material has recently been reported in mosquito eggshells, eggs, ovaries and eyes (Moreira et al., 2007; Yoon et al., 1997; Faschinger 2010). Consistent with these results, several chitinase and chitinase-like genes were identified in the eggs. The immunohistochemistry analysis of selected chitinase proteins showed the positive staining in the compound eyes. However, we still don't have good interpretation why insect need chitinases in these specific stages or tissues.

Mosquitoes are special as they have two types of PMs: type 1 PM lining the adult midgut which is blood-meal inducible, and type 2 PM lining the larval midgut which is constitutively expressed during the whole feeding stage. Type 1 and type 2 PM differ in their thickness and other physiological properties (Shao et al., 2001). Our result revealed two midgut-specific chitinase genes, *AgCht8* and *AgCht13*. The former was predominately expressed in the pupal and

adult stages, whereas the latter was exclusively expressed in the larval stage. Further work needed to address the hypothesis that these two chitinases are responsible for the turnover of the mosquito adult and larval PM, respectively.

As described above, the large number of chitinase and chitinase-like genes in mosquitoes differ in their size, gene organization, stage- and tissue-specific expression profiles. The proteins encoded by these genes differ in their domain organization. In addition, the chitinase and chitinase-like proteins from *Tribolium* and other organisms show their differences in their physical, chemical and enzymatic properties (Zhu et al., 2008c; Matsumiya et al., 2006). All these results support the hypothesis that genes belonging to different groups have distinctly different biological functions. Strong evidence came from the results of RNAi studies in *Tribolium* in which down-regulation of transcripts for chitinase genes belonging to different groups yielded quite different phenotypes. In that study, group I and group II enzymes have been revealed to be involved in molting, and the group III enzymes have a morphogenetic role in regulating abdominal contraction and wing expansion. Some of the members in group V have been shown to affect cell proliferation in imaginal disks (Zhu et al., 2008b).

As group VI, VII, and VIII are newly identified groups in this study, we performed RNAi experiments for the chitinase genes belonging to these three groups in *Tribolium*. Injection of *TcCht2* (group VI) dsRNA into *Tribolium* females results in the failure of egg development, whereas no any adverse effects were found when dsRNA was injected to the males (data not shown). No any observable defects were detected when dsRNA of *TcCht6* and *TcCht11*, belonging to group VII and VIII, respectively, were injected into *Tribolium* larvae. The phenotypical change triggered by depletion of *TcCht2* belonging group VI is unique compared with those caused by depletion of the each chitinase gene from other five groups which had been

reported previously (Zhu et al., 2008b). This result strongly support the functionally specialization of the chitinase gene in *Tribolium*. Each of three newly identified groups contains one ortholog from each of the three insect species including *T. castaneum*, *D. melanogaster*, *An. gambiae*, and *Ae. aegypti* (Fig. 3.1). Further work is needed to test the hypothesis that each of these orthologs performs similar functions as that in *Tribolium* from other insect species such as *An. gambiae*.

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**Table 3.1 Sequences and relevant parameters of the primers used for RT- PCR and qPCR**

Gene	Primer name	primer sequence (5'-3')	Tm (°C)	GC (%)	Product Length
<i>AgCht2</i>	F	CTGGATGAGGAGAAGAATGCC	55.4	52.4	129
	R	GAGCAGCACCTTCAGATGG	54.9	57.9	
<i>AgCht4</i>	F	AGGGATACTCGCCTACTACG	54.8	55	137
	R	TCTGCCCAATACTTTCCACAC	55.4	47.6	
<i>AgCht5-1</i>	F	TTCCGGCTACAAGGACTTTG	54.8	50.0	188
	R	TCGGGCTTTTCGATCAGTTTC	55.3	50.0	
<i>AgCht5-2</i>	F	ACGATAAGGACAACCTTTGTCTATC	54.4	37.5	152
	R	GTCAGCACTCTCGCACAG	54.6	61.1	
<i>AgCht5-3</i>	F	GCTGTGTGAAATGCTGAAGG	54.7	50.0	166
	R	TGCGTATATGCCACCCAATC	55.1	50.0	
<i>AgCht5-4</i>	F	TTCGCCAACCTGAAGAAGAC	55.1	50.0	146
	R	TGGAGGAACCAATCACACTG	54.8	47.6	
<i>AgCht5-5</i>	F	TTCATCGGCAGCGTGATC	54.4	55.6	197
	R	TCGACCGGCACCTGTATC	55.2	61.1	
<i>AgCht6</i>	F	ACTGGTTCAATCTGCTCTCC	54.2	50.0	138
	R	ACTTTACGCTGTAGTCAATGTTG	55.1	39.1	
<i>AgCht7</i>	F	AGTGGCTCAAGGAGGAAGG	55.2	57.9	172
	R	GGTCCGAACGACTCATAACG	54.6	57.9	
<i>AgCht8</i>	F	TGGAGTGTTAGTGCTAGTTGC	55.2	47.6	141
	R	ATGTCATACCGTCCGTTGC	54.7	52.6	
<i>AgCht9</i>	F	ATGGTGTGGTCTATTGAGTCTG	55.0	45.5	139
	R	TGGTCTTCGCAGTAGTTGTAG	54.7	47.6	
<i>AgCht10</i>	F	AACAAGGTCCTGTAACATCGG	55.2	47.6	120
	R	TACGCTGAGTGGTTGAAGTAG	54.7	47.6	
<i>AgCht11</i>	F	TGGCAACTACATCTACTCGAAG	55.1	45.5	168
	R	TTCAGGTGGGTGCAGAGG	55.1	61.1	
<i>AgCht12</i>	F	GTTAAATCGTTGGCTCAAAATGC	55.6	39.1	168
	R	ACAATCACCTGAAAGTCGTC	55.5	47.6	
<i>AgCht13</i>	F	GTCATCTACAACCTGGTTGGTATC	54.4	43.5	97
	R	TTGCCTATCGTCATATCCTATCC	54.8	43.5	
<i>AgCht16</i>	F	GACCTGAACCCGCACCTG	56.8	66.7	166
	R	CACCACCAACCGACACAAG	55.7	57.9	
<i>AgCht23</i>	F	GAACCTCCAGGACCAGTCAAG	54.5	55.0	96
	R	GTACCGAATCGTACTGTTTGC	54.7	47.6	
<i>AgCht24</i>	F	TGTTGACTATATCCTCCTGATGAG	54.9	41.7	150
	R	CCACCTATCCAATCGCTGAC	55.1	55.0	
<i>AgIDGF2</i>	F	GTAATCGCTGCTGAAGACG	55.2	57.9	172
	R	CTCCTCCCGATGCTCCTC	54.9	66.7	
<i>AgIDGF4</i>	F	GTACGACCAGCAGACACC	53.9	61.1	116
	R	GCCACGCCTTACCTTATC	55.3	57.9	
<i>AgRPS3</i>	F	GCTGGGCATCAAGGTCAAG	55.4	57.9	108
	R	ATCTCATCCTTCGGCTCAAC	54.4	50.0	

**Table 3.2 Comparisons of the three new groups of chitinase and chitinase-like proteins from three insect species: *D. malenogaster*, *T. castaneum* and *An. gambiae*.**

Group	Gene name	amino acid residues	catalytic domain	Chitin-binding domain
VI	<i>DmCht2</i>	484	1	-
	<i>TcCht2</i>	485	1	-
	<i>AgCht2</i>	485	1	-
VII	<i>DmCht6</i>	4498	1	+
	<i>TcCht6</i>	2369	1	+
	<i>AgCht6</i>	3045	1	+
VIII	<i>DmCht11</i>	432	1	-
	<i>TcCht11</i>	304	1	-
	<i>AgCht11</i>	428	1	-

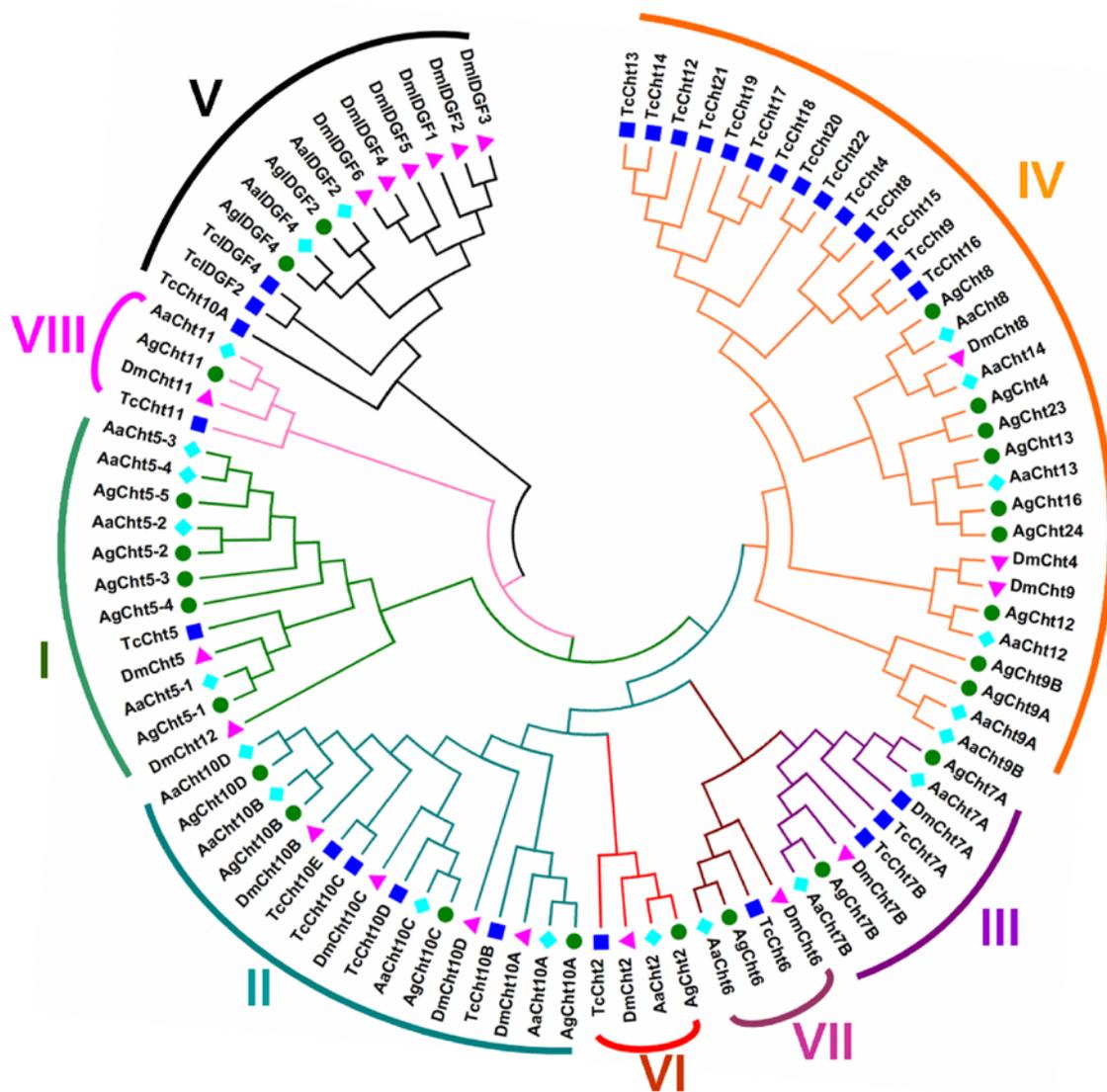
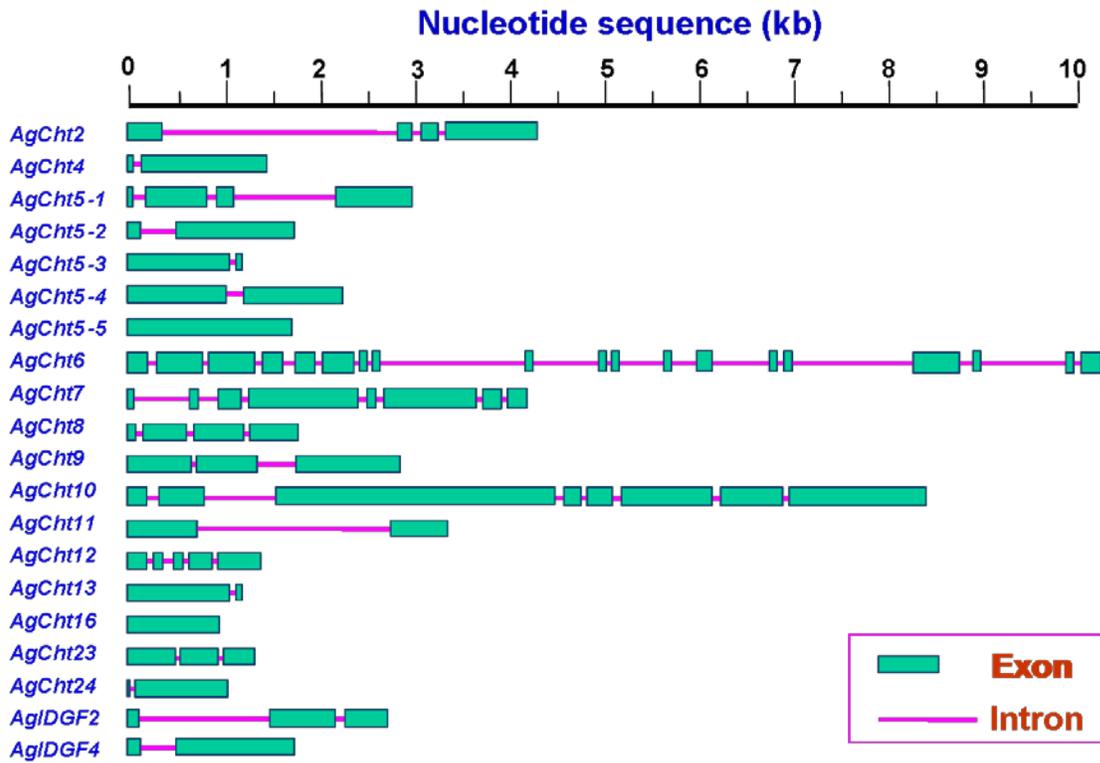
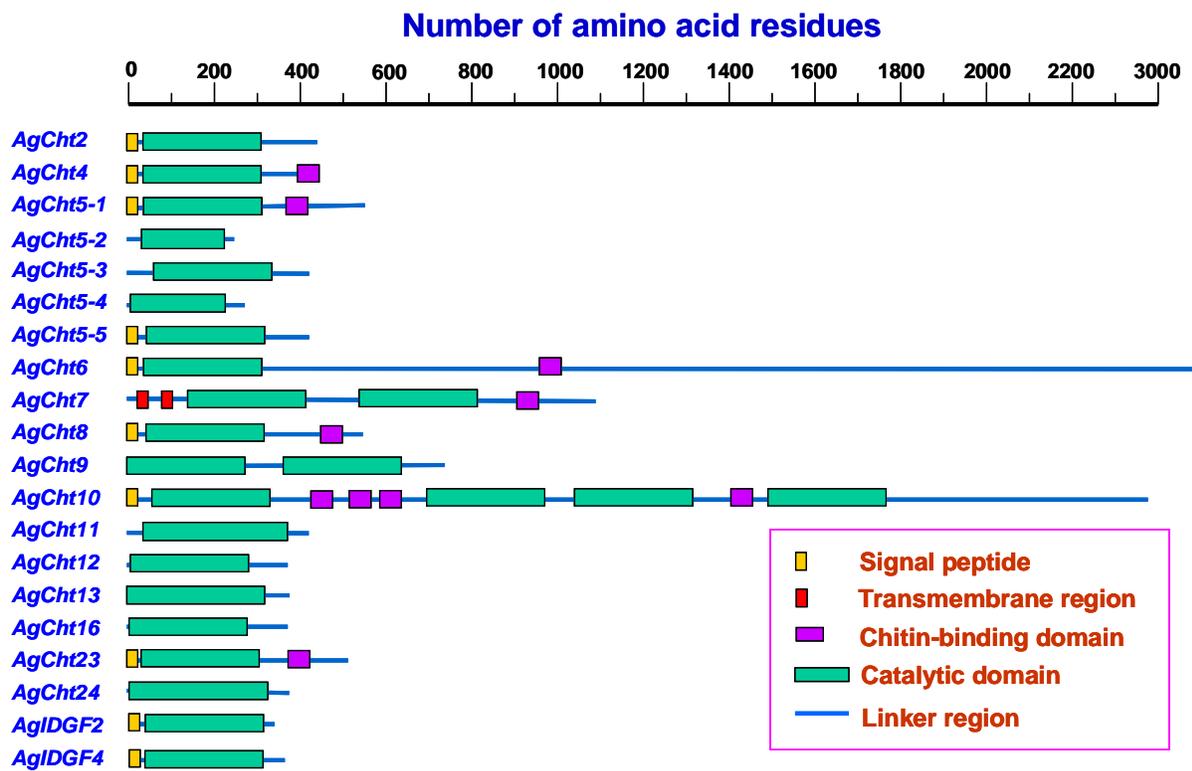


Figure 3.1 Phylogenetic analysis of chitinase and chitinase-like proteins from four insect species based on catalytic domains. Ag: *A. gambiae*; Ae: *Aedes aegypti*; Tc: *T. castaneum*; Dm: *D. Melanogaster*. Phylogenetic tree was generated with a distance neighbor-joining method by using the software MEGA 4 after alignment with ClustalW method.



**Figure 3.2** Schematic diagram of the exon and intron organization of the chitinase and chitinase-like genes from *An. gambiae*. Boxes indicate exons. Lines indicate introns.



**Figure 3.3** Schematic diagram of the domain architectures of chitinase and chitinase-like proteins from *An. gambiae*.

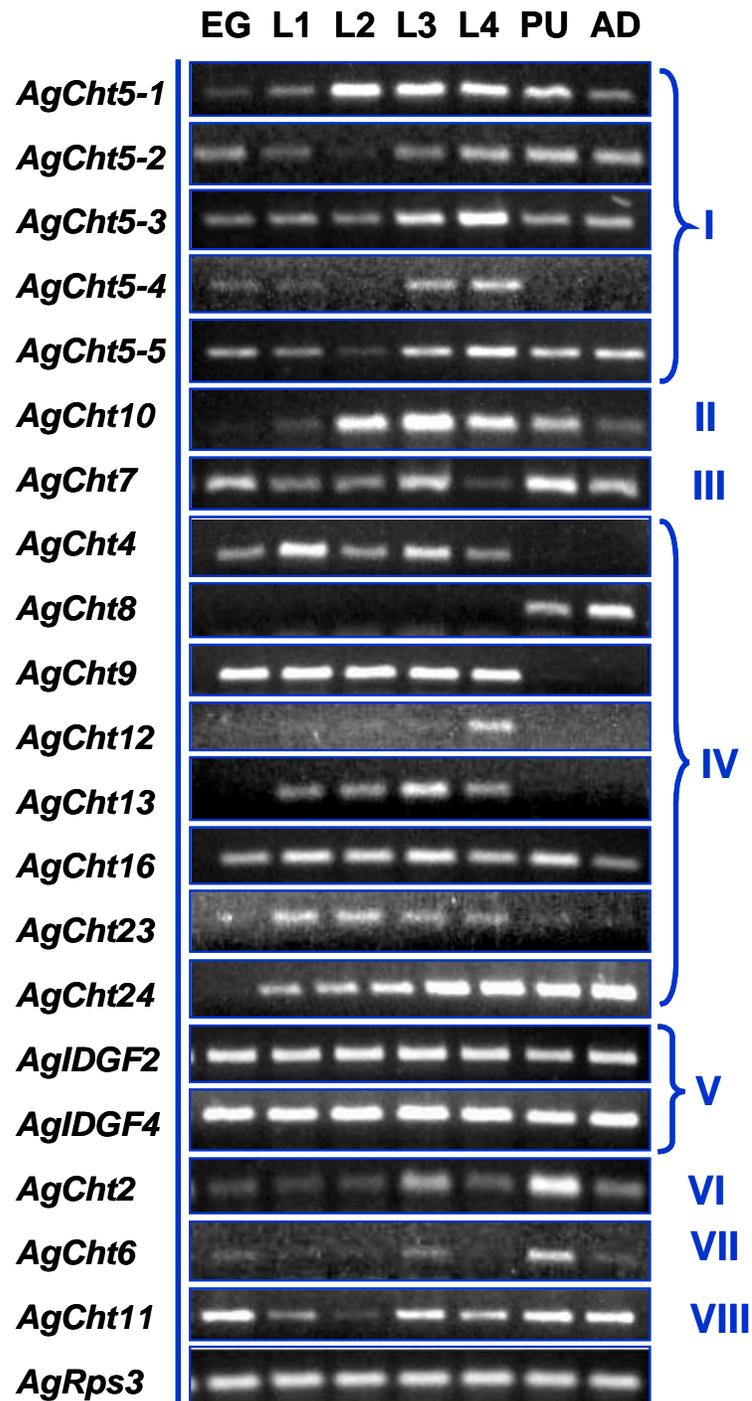
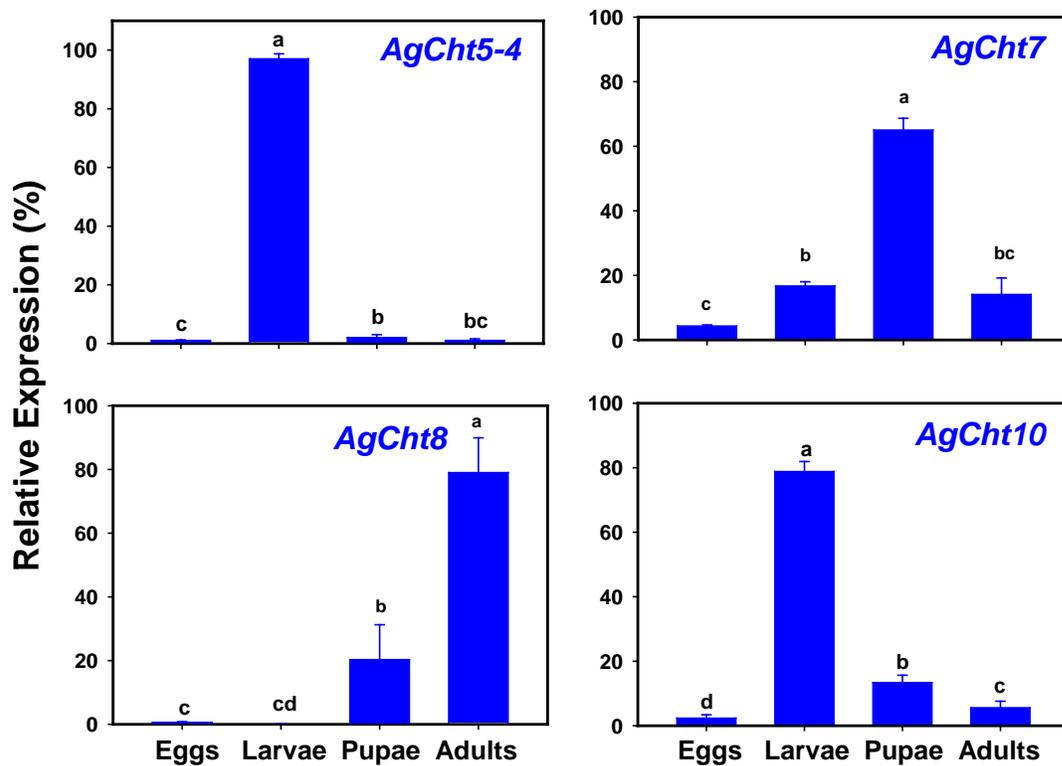


Figure 3.4 Analysis of stage-specific expression of *AgCht* genes as determined by RT-PCR (EG, eggs; L1-4, larvae from 1st to 4th instars; PU, pupae; AD, adults).



**Figure 3.5** Relative expression of selected genes in different developmental stages as determined by qPCR. Same letters on the error bars indicate no significant difference based on Fisher's LSD ( $P \geq 0.05$ ).

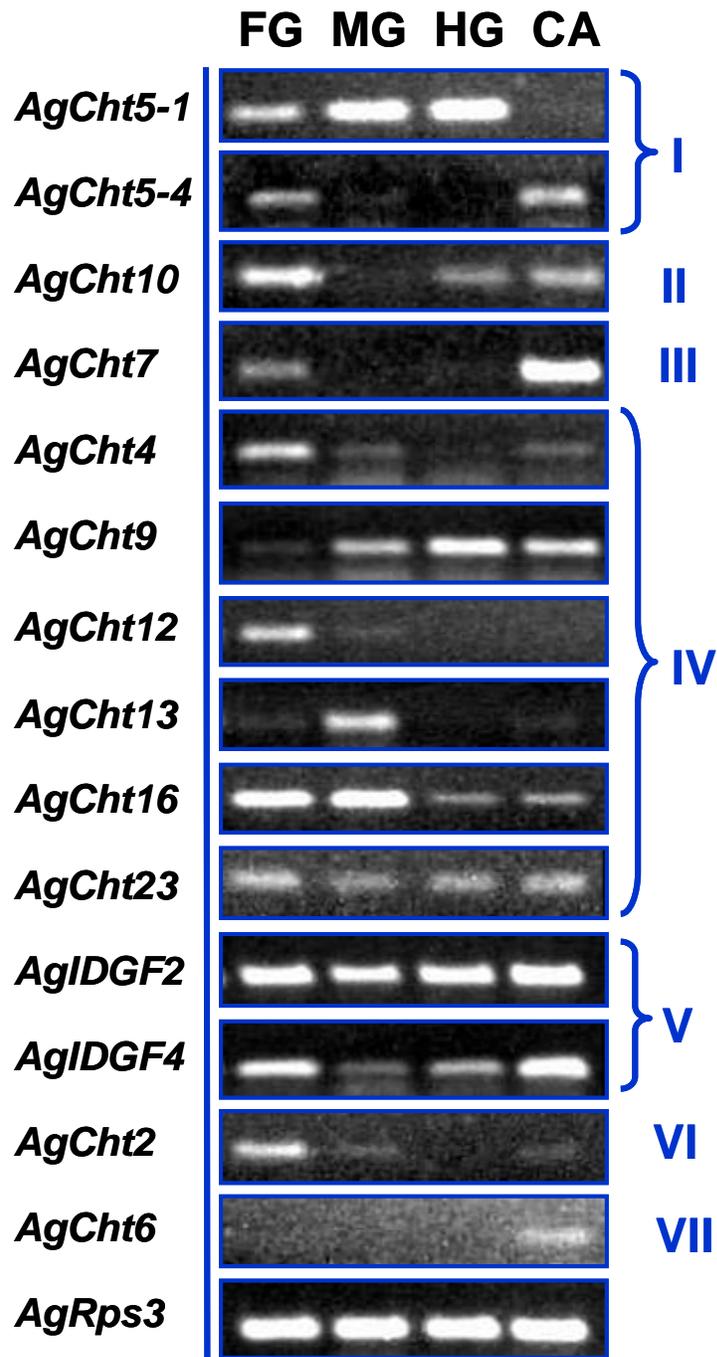
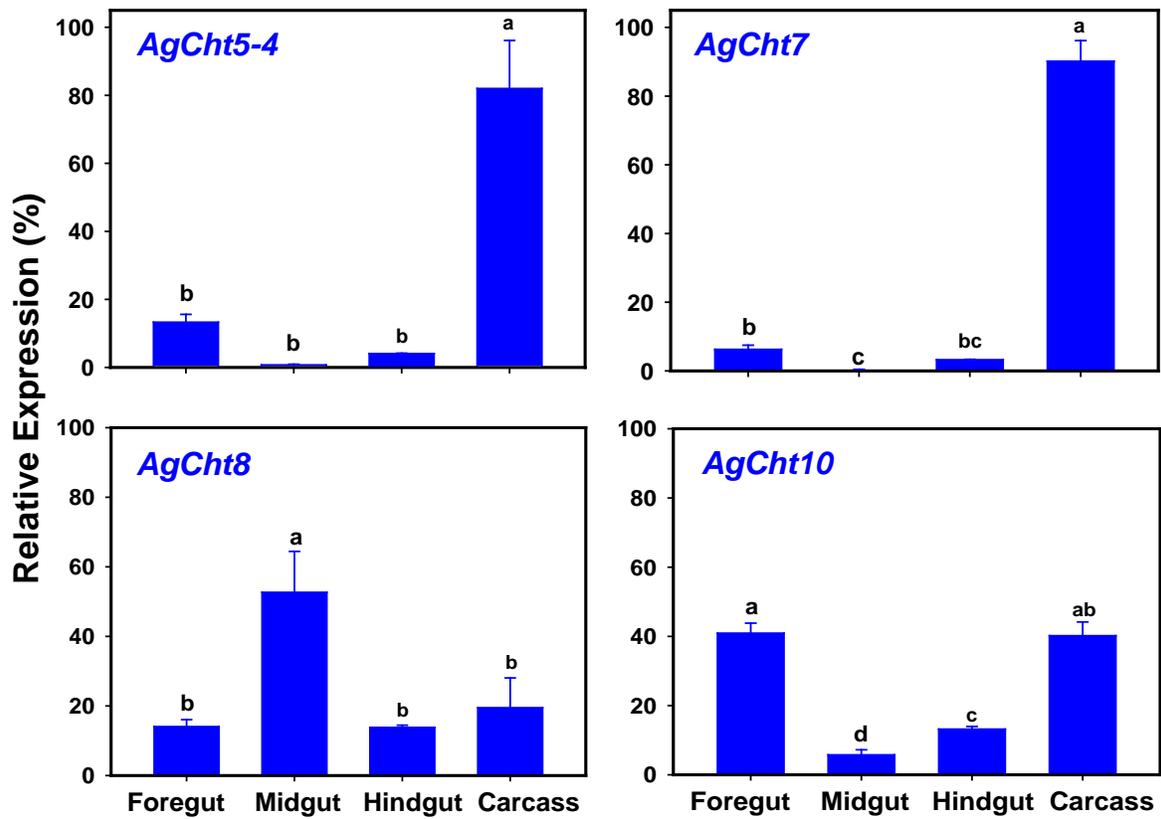
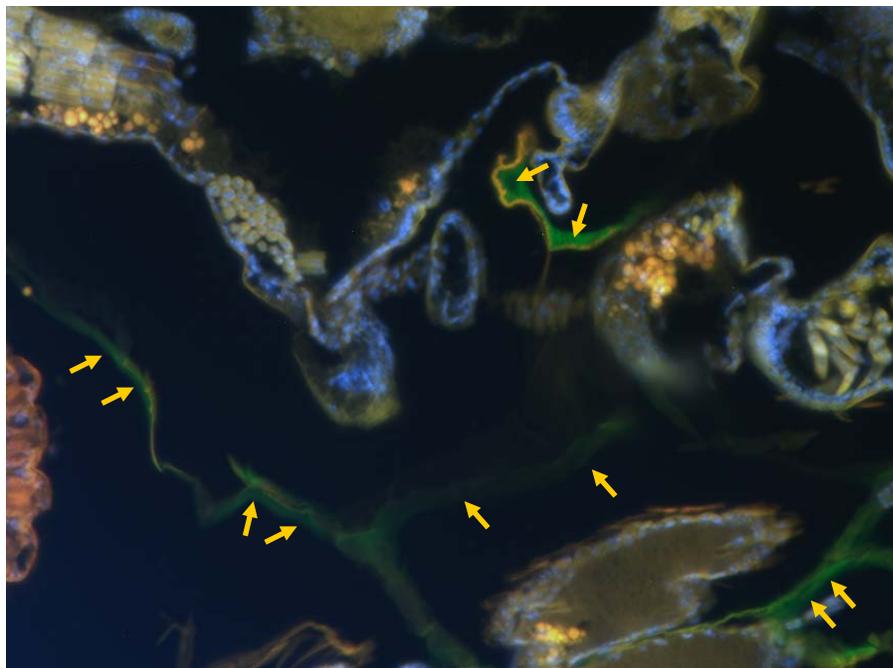


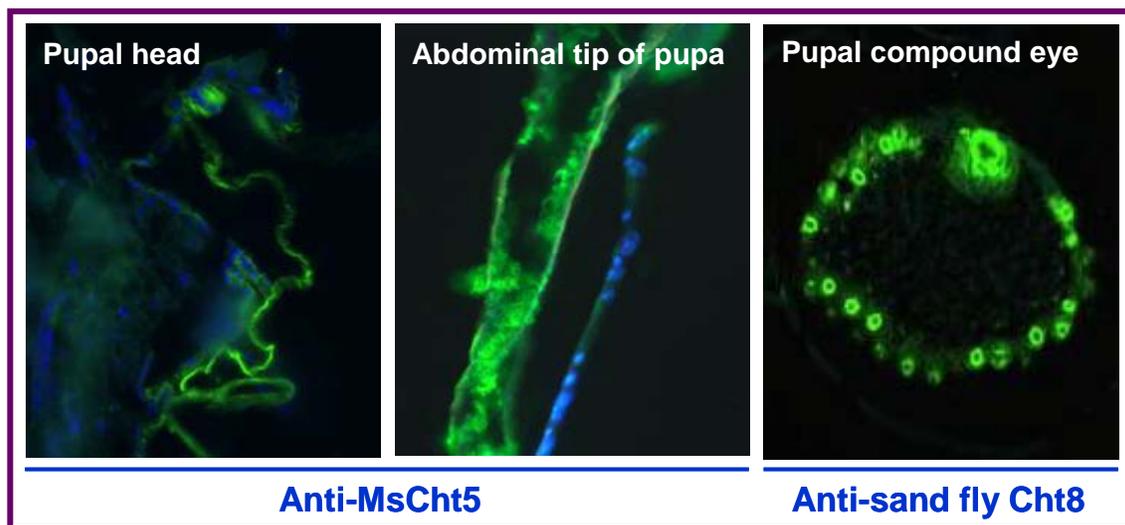
Figure 3.6 Analysis of tissue-specific expression of *AgCht* genes as determined by RT-PCR analysis (FG, foregut; MG, midgut; HG, hindgut; CA, carcass).



**Figure 3.7** Relative expression of selected genes in different tissues of mosquito larvae as determined by qPCR. Same letters on the error bars indicate no significant difference based on Fisher's LSD ( $P \geq 0.05$ ). The ribosomal S3 (*AgRPS3*) gene was used as a reference.



**Figure 3.8** Fluorescence *in situ* hybridization (FISH) analysis of *AgCht8* in mosquito pupae. Green color indicated by arrows shows positive staining, and the blue color shows the nuclei by DAPI staining.



**Figure 3.9 Localizations of chitinase proteins expressed in mosquito pupae by using immunohistochemistry. The proteins were detected in the head and abdominal tip of a pupa by anti-*Manduca sexta* chitinase 5 polyclonal antibodies (Anti-MsCht5) and pupal compound eye by anti-sand fly (*Lutzomyia longipalpis*) chitinase 8 (Anti-sandy fly Cht8) polyclonal antibodies. Green color shows positive staining, and the blue color shows the nuclei by DAPI staining.**

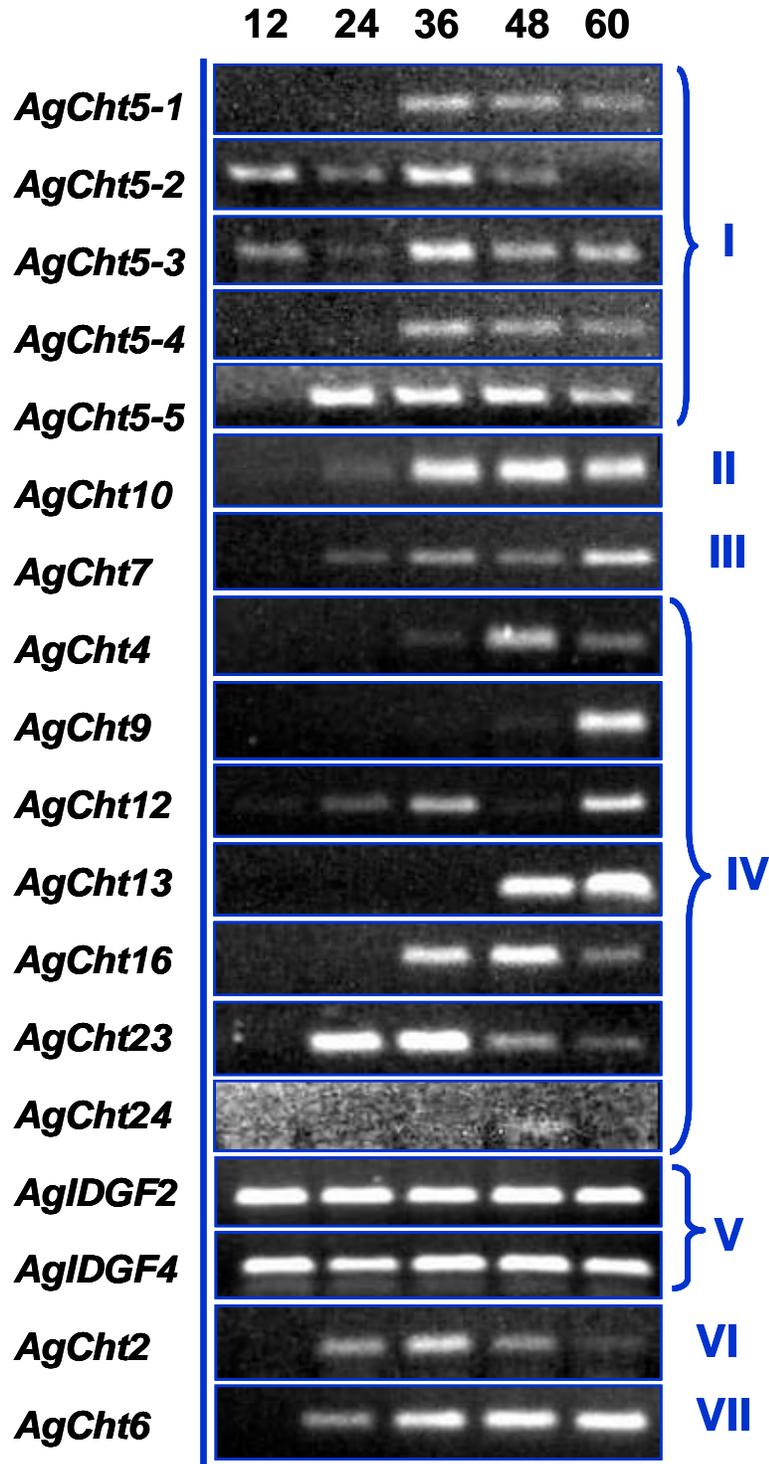


Figure 3.10 RT-PCR analysis of developmental stage-specific expression patterns of *AgCht* genes in eggs. Developmental times of eggs include: 12, 24, 36, 48, 60 h after laying. The ribosomal S3 (*AgRPS3*) gene was used as an internal reference.

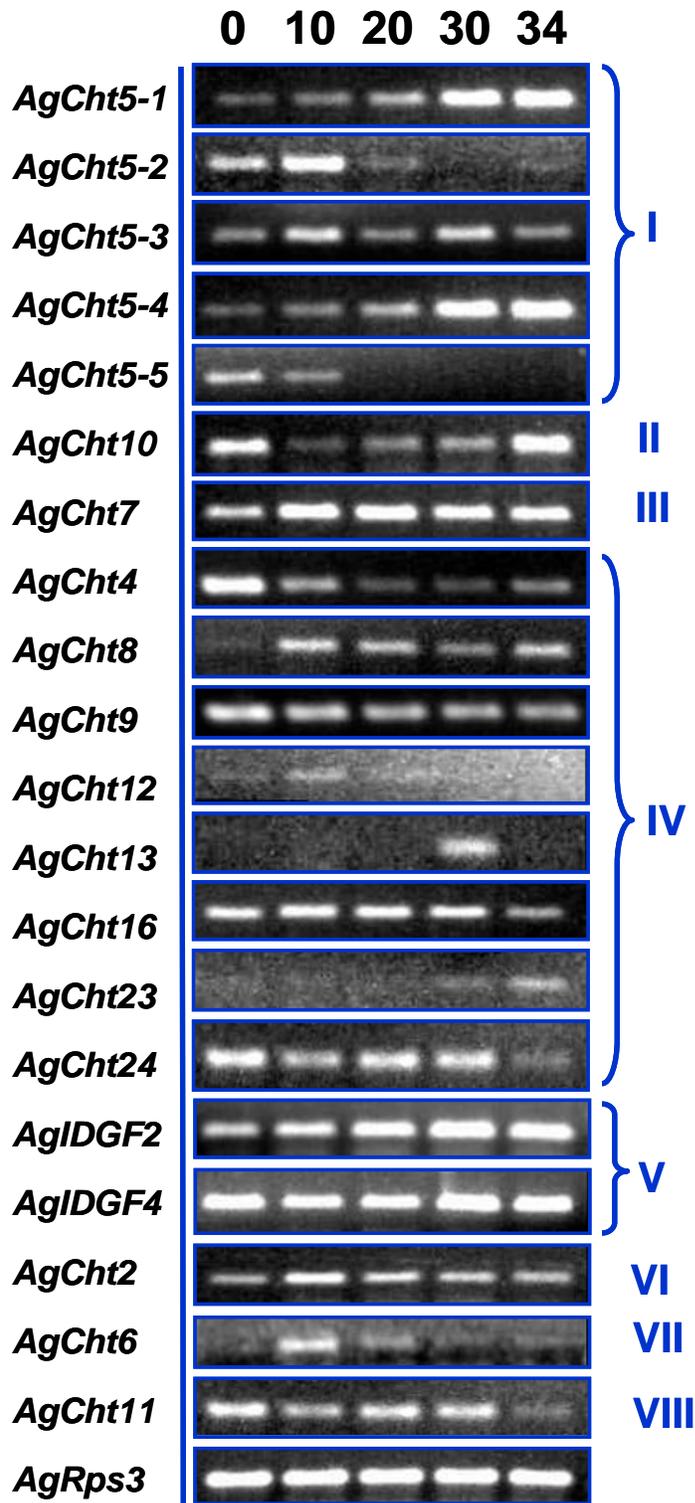


Figure 3.11 RT-PCR analysis of developmental stage-specific expression patterns of *AgCht* genes in pupae. Developmental times of pupae include: 0-, 10-, 20-, 30-, 34-h after pupation. The ribosomal S3 (*AgRPS3*) gene was used as an internal reference.

## **CHAPTER 4 - Biochemical analysis of chitin synthases and its inhibition in African malaria mosquito, *Anopheles gambiae***

### **Abstract**

Chitin synthase (CHS) is an important enzyme catalyzing the formation of chitin polymers in all chitin containing organisms and a potential target site for insect pest control. We report the enzymatic analysis of the chitin synthase and its inhibition in the African malaria mosquito, *Anopheles gambiae*, by using a nonradioactive method. Our results showed that this method can be effectively used for high throughput assay of chitin synthesis in the crude enzymes from insects. Optimum parameters for the enzyme activity were determined including concentration of the substrate UDP-GlcNAc and  $M^{++}$ . The optimal pH was around 6.5-7.0, and the highest activity was detected at 37°C and 44°C. Dithithreitol (DTT) is required for the activity to prevent melanization of the enzyme extract. Enzyme activity was enhanced at low concentration of GlcNAc, but inhibited at high concentrations. Proteolytic activation of the activity is significant both in the 500g supernatant and the 40,000g pellet. High concentration of diflubenzuron and nikkomycin Z showed marginal *in vitro* inhibitory effects on chitin synthase activity, whereas no *in vivo* inhibitory effects were observed in our experiments.

**Key words:** chitin synthases; *Anophele gambiae*; activity; diflubenzuron

## Introduction

Chitin, a linear polysaccharide of N-acetyl- $\beta$ -D-glucosamine residues joined by  $\beta$ -1,4 glycosidic linkages, is the second most abundant biological polymer after cellulose (Merzendorfer, 2006; Kramer and Muthukrishnan, 2005). It is widely distributed in arthropods, fungi, nematodes and other Phyla such as annelids, molluscs and coelenterates. In arthropods, chitin is a vital component of the cuticular exoskeleton and thus is crucial for growth and development (Merzendorfer and Zimoch, 2003). Chitin is also found in internal structures of many insects and other arthropods, including the cuticular linings of trachea and in the peritrophic membranes (PM) lining the gut epithelium (Richards, 1951; Hunt, 1970; Cohen, 2001).

Chitin production in arthropods is a complicated process and a series of biochemical pathways are involved in individual chitin polymer biosynthesis in which the terminal step is catalyzed by chitin synthase (Merzendorfer and Zimoch, 2003). Chitin synthase (CHS) (EC2.4.1.16) is a large transmembrane protein that belongs to the family of  $\beta$ -glycosyltransferases, and catalyzes the transfer of sugar moieties from activated sugar donors to specific acceptors in all chitin-containing organisms including arthropods, nematodes, and fungi. In insects, chitin synthases are large transmembrane proteins and contain multiple transmembrane helices reflecting their association with either the plasma membrane or intracellular vesicles such as chitosomes (Tellam et al., 2000).

As insect and fungi growth and development depend on precisely tuned expression of chitin synthase (Arakane et al., 2005, 2008; Merzendorfer, 2006) and chitin is not synthesized by vertebrates, chitin synthase presents an attractive target for combating insect pests and fungi-born diseases (Merzendorfer, 2006). For example, peptidyl nucleosides including polyoxins and

nikkomycins are anti-fungi agents which competitively inhibit chitin synthases in fungi and insects (Cohen and Casida, 1980b; Zhang and Miller, 1999; Ruiz-Herrera and San-Blas, 2003), whereas benzylphenolureas (BPUs) such as diflubenzuron are highly effective insecticides which inhibit chitin synthesis in insects (Post and Vincent, 1973; Ishaaya and Casida, 1974; Post et al., 1974). Diflubenzuron has been widely used to control various agricultural and public health pests such as mosquito and fly larvae since the 1970's. It is extremely toxic to young larvae of many mosquito species (Eisler, 1992, Baruah and Das, 1996; Ali et al., 1999; Zhang and Zhu, 2006). The exact mechanisms of chitin synthesis inhibition are still elusive. It is uncertain as to whether BPUs can directly inhibit insect chitin synthase because different studies have yielded inconsistent results. In cell-free chitin synthesizing systems, for example, the BPUs do not inhibit chitin synthesis (Cohen and Casida, 1980b; Mayer et al., 1981) or block the chitin biosynthetic pathway between glucose and UDP-GlcNAc in intact larvae (Post et al., 1974). In contrast, Nakagawa et al. (1993) showed that diflubenzuron and polyoxin D clearly inhibited the incorporation of [<sup>3</sup>H]-*N*-acetylglucosamine into chitin in isolated intact integument from newly molted American cockroaches. Horst (1981) reported that diflubenzuron can dramatically inhibit chitin synthase activity (90% approximately) in the crude microsomes and membrane fractions prepared from brine shrimp larvae.

Chitin synthase activity is traditionally measured by a radioactive assay using [<sup>14</sup>C] UDP-*N*-acetyl-D-glucosamine (GlcNAc) as a substrate followed by quantization of insoluble <sup>14</sup>C-labeled chitin after acid precipitation. The observation that WGA specifically binds to chitin polymer at multiple sites led to the development of a nonradioactive, high throughput screening for antimicrobial agents acting on chitin synthases (Lucero et al., 2002). The sensitivity of this new method was reported to be similar or even slightly higher than that of the radioactive assay. Also, the method was compatible with a variety of assay conditions, performed using low-cost,

widely available commercial reagents, and most helpful when multiple determinations of several samples are required.

The African malaria mosquito, *An. gambiae* is an important arthropod-borne disease vector in Africa. To date, very limited insecticides are available for control of the mosquitoes and other human health-related arthropods. Diflubenzuron is one of a few insecticides which are used for mosquito and fly control (Zhu et al., 2007). In this study, we examined the CHS activity by using the nonradioactive assay in *An. gambiae*. The optimal conditions for enzyme activity were determined and the effects of diflubenzuron, polyoxin D, and nikkomycin Z on the enzyme activity were examined.

## **Materials and Methods**

### ***Materials***

Reagents were listed followed by company and catalog number in parentheses: Trypsin (Sigma T-1426), soybean trypsin inhibitor (SBTI) (Bopchemika, WA13168), chitin (Sigma C-9752), protease inhibitor cocktail (Sigma P-8215), wheat germ agglutinin (Bector Lab, Inc. Buringame, CA, L-1020), wheat germ agglutinin peroxidase labeled (Sigma L-3892), *N*-acetylglucosamine (GlcNAc) (Sigma A-8625), UDP-GlcNAc (Sigma U-4375), BCA protein assay kit (Pierce 23225), high-sensitivity peroxidase substrate mixtures Colorburst Blue (Alercheck, Inc., 90101), 96-well microtiter plates (Corning 3595), diflubenzuron (Chem Service, West Chester, PA), Nikkomycin Z and polyoxin D (Calbiochem, San Diego, CA), etc.

### ***Mosquito rearing***

A colony of *An. gambiae* obtained from the Malaria Research and Reference Reagent Resource Center (MR4) (Manassas, VA) was maintained in Department of Entomology at Kansas State University (Manhattan, KS) since 2007 as described as by Zhang and Zhu (2006).

### ***Crude enzyme preparation, protein content assay, and pretreatment of the enzyme***

Fifty mosquito pupae were homogenized in 1.0 ml a Tris-HCl buffer (pH 7.5) containing 10 mM DTT and 1 mM MgCl<sub>2</sub> for 60s by using glass-pestle homogenizer. Another 0.5 ml same buffer was used to rinse the homogenizer and combined with the homogenate. The combined homogenate was then centrifuged at 500×g for 10 min to remove unbroken cells, nuclei and debris. The supernatant was carefully transferred to a new tube and used as crude enzyme for following analysis. To get the membrane fractions, the supernatant were centrifuged at 40,000×g for 10 min. The supernatant was carefully removed and the pellet was resuspended in the same buffer. All preparations were conducted on ice or at 4 °C. Protein determination was done in microtiter plate using bovine serum albumin as standard by the BCA (Sigma) method. To pretreat the enzyme, 10 µl of trypsin solution (2 µg/µl in buffer) was added to 250 µl enzyme preparations in a glass tube and incubate for 10 min at 30°C followed by addition of 10 µl of SBTI solution (3 µg/µl in buffer). Ten µl of buffer instead of 10 µl trypsin solution was used as control without trypsin treatment.

### ***CHS activity assays***

The assay was based on Lucero et al. (2002) with some modifications. In brief, 100 µl WGA solutions (50 µg/ml in deionized H<sub>2</sub>O) were added to each well of the microtiter plate followed by 16 h incubation at room temperature. WGA solutions were removed by vigorously

shaking of the plate content. To wash the plate, the empty plate was immersed in a basket of tap water followed by empty the water in the wells by shaking. This washing was repeated two more times to remove the unbound WGA completely. After washing, the wells were blocked by adding 300  $\mu$ l of bovine serum albumin (BSA) blocking buffer (20 mg/ml bovine serum albumin in 50 mM Tris-HCl, pH 7.5) and incubate for 3 h at RT.

After incubation, the blocking solutions were emptied by shaking. Fifty  $\mu$ l of reaction mixture (5 mM GlcNAc, 1 mM UDP-GlcNAc in 50 mM Tris-HCl buffer, 7.5) was added to the appropriate wells followed by the addition of buffer and pretreated enzyme (20  $\mu$ l) to a final volume of 100  $\mu$ l. For each assay the corresponding boiled enzyme preparations (95 °C for 10 min) was used to assess the assay background.

The plate was covered with a sealer and incubated at 37°C for 60 min by shaking at 100 rpm. To stop the reaction, the plate was emptied and washed as described above for five times, followed by the addition of 200  $\mu$ l WGA-HRP (0.5  $\mu$ g/ml, in blocking buffer) and incubation for 15 min at 30°C with gentle shaking at 100 rpm. The plate was emptied by vigorous shaking followed by five times washing as described above. Finally, 100  $\mu$ l peroxidase substrate reagents were added to each well and the optical density (OD) at 600 nm was read immediately for 3 min. The content of GlcNAc and the chitin synthase activity in the treatments were calculated by using the standard curve. The standard curve was prepared following the same procedure as described by Lucero et al. (2002). The specific enzyme activity was expressed as nmol GlcNAc.mg<sup>-1</sup>. hour<sup>-1</sup>. Each experiment was repeated 3-4 times, each with triplicate determinations.

### ***In vitro and in vivo inhibition assay***

For *in vitro* inhibition assay, diflubenzuron stock solution (1 mM) was prepared in acetone, whereas polyoxin D (1 mM) and nikkomycin Z (1 mM) were prepared in the solvent of acetone: water (1:1). Before use, diflubenzuron was further diluted 25  $\mu$ M, 5  $\mu$ M, 1  $\mu$ M and 0.2  $\mu$ M by using acetone, whereas polyoxin D and Nikkomycin Z were diluted to 25  $\mu$ M, 5  $\mu$ M, and 1  $\mu$ M by using acetone:water=1:1. Five  $\mu$ l of each solution was added to 45  $\mu$ l crude enzyme. The final concentrations of difubenzuron in the enzyme were 2.5  $\mu$ M, 0.5  $\mu$ M, and 0.1  $\mu$ M, 0.02  $\mu$ M, where as 2.5  $\mu$ M, 0.5  $\mu$ M and 0.1  $\mu$ M for polyoxin D and Nikkomycin Z. Same volume of its own solvent was used as control. The mixture was incubated at 37 °C (shaking at 100 rpm) for 20 min and the subsequent procedures were same as the enzyme activity assay.

For *in vivo* assay, a series of dilutions of diflubenzuron, nikkomycin Z, and polyoxin D were made using acetone. Twenty  $\mu$ l of each chemicals were added to a 500 ml glass beaker containing 15 9-hour old mosquito pupae in 100 ml distilled water and 1 ml fish food. The final concentrations of diflubenzuron were 25, 50, 100, 250, and 500  $\mu$ g/L, whereas 100 and 500  $\mu$ g/L for nikkomycin Z and polyoxin D. Same volume of acetone was used as control. After 24 h (L:D, 16:8) exposure at 25 °C, the pupal mortality was examined and the surviving pupae were collected for crude enzyme preparation followed by enzyme assays following the same procedure described as above. Each control and treatment was repeated 4 times.

### ***Paraffin-embedded thin sections and chitin staining***

Paraffin-embedded thin sections were used for chitin staining. In brief, the 12-24 h pupae were fixed in 4% paraformaldehyde at 4 °C overnight followed by 3  $\times$  5 min washing with PBST (PBS and 0.1% Triton X-100). The samples were then dehydrated through a series of grades of ethanol (2x 30 min in each 70% and 96%, 2x 20 min in 100%), followed by 2 x 1 hr in

chloroform. The dehydrated samples were finally embedded in paraplast (56 °C, Tyco Healthcare) after overnight penetration. Histological sections (8 µm) were prepared by using microtome (Richard-Allan Scientific Microm) with a low profile microtome blade (Richard-Allan), straightened on Fisherbrand ColorFrost Plus microscope slides with 0.5% gelatin, and allowed to dry for 2 days at 40°C on the top of slide warmer. The sections were deparaffinized with two washes of 10 min xylene, rehydrated through successive baths of ethanol (100 %, 96%, and 70% in water, 1x 5 min each), two water baths 5 min for each, and finally PBST for 10 min or more.

For chitin staining, 100 µL fluorescein-isothiocyanate (FITC-CBD, New England BioLabs, Beverly, MA) 100x (in PBST) diluted probe was applied to above deparaffinized sample and incubated overnight at room temperature. Then the sample was rinsed 4 x 10 min in PBS. Finally, the sample was mounted in glycerol for microscopic examinations.

### ***Statistical analysis for enzyme activity***

In each assay, the treatment showed the highest specific activity was used as reference and relative activity in other treatments were calculated. The relative activity in percentage was firstly transformed into arcsine square root before one-way ANOVA. Fisher's least significant difference (LSD) multiple comparisons were then used to separate the means of the transformed relative activity or specific enzyme activity among the samples.

## **Results**

### ***Chitin synthase activity in mosquito pupae***

Previous study showed that both two chitin synthase genes, *AgCHS1* and *AgCHS2*, are highly expressed in the pupal stage (Zhang et al., 2010), implying an intensive synthesis of chitin

in the pupal stage. Thus, we use mosquito pupae to prepare the enzyme for all assays in this study. Chitin staining with FITC-CBD in the paraffin-embedded thin sections of the 12-24 h old pupae showed that chitin is mainly distributed in the cuticle (Fig. 4.1C, D). Highly chitin staining was observed in the inter-segment regions in some pupae (Fig. 4.1A, B). To make the standard curve, homogeneous chitin suspension in acetic acid was used for the assay. A high linear correlation is observed when initial rate was plotted as a function of chitin amounts (Fig. 4.8). Using this standard curve, we examined the CHS activity in the crude enzyme. As shown in Fig. 4.2, the CHS activity linearly increased when low amount of enzyme used and reached a plateau phase as the amount of the enzyme increased further. Thus, the specific CHS activity of the crude enzyme could be determined by using the data within the linear phase.

#### ***Effects of dithiothreitol (DTT) and $Mg^{++}$ on CHS activity***

To prevent the enzyme oxidation, we added DTT into the buffer for enzyme extraction. Without using DTT, the crude enzyme turned black within a minute and no CHS activity was observed by using the oxidized enzyme in the subsequent assay. It is obvious that low concentration is not enough to inhibit the enzyme oxidation completely. However, decreased CHS activity was observed as high concentration of DTT was added (Fig. 4.3A). Divalent cations have been reported to stimulate CHS activity in insect and other systems. We found that low concentration of  $M^{++}$  at 1.0-4.0 mM significantly increased CHS activity, whereas 10.0 mM or higher concentration significantly inhibited CHS activity (Fig. 4.3B).

#### ***Effects of UDP-GlcNAc and GlcNAc on CHS activity***

The addition of 0.5 mM UDP-GlcNAc to the reaction mixture slightly increased the CHS activity, whereas high concentration of UDP-GlcNAc inhibited CHS activity significantly (Fig.

4.4A). Similarly, GlcNAc at low concentration enhanced CHS activity and inhibited CHS activity when the concentration was higher than 10 mM in the reaction mixture (Fig. 4.4B).

### ***Optimum pH and temperature for CHS activity***

To determine the optimum pH, we examined the CHS activity at different pH values using buffer Tris-HCl. Highest CHS activity was observed at pH 6.5-7.0 (Fig. 4.5A). Further, we examined the CHS activity under various temperatures. Maximum activity was observed at 37-44°C (Fig. 4.5B).

### ***Proteolytic activation of CHS activity***

To evaluate the effect of proteolysis, we measured the CHS activity in the presence of trypsin. In the presence of trypsin, the CHS activity was increased by about 1.2- and 1.7-fold in the 500g crude enzyme and the successive 40,000g fractions, respectively, as compared to control without addition of trypsin (Fig. 4.6).

### ***Effect of chitin synthesis inhibitors on CHS activity***

The larvae of *An. gambiae* were sensitive to diflubenzuron. Exposure of the third instar mosquito larvae to diflubenzuron at 50 µg/L resulted in about 60% mortality in 48 h, whereas the classical CHS inhibitors nikkomycin Z and polyoxin D, well established inhibitors of fungal enzymes, exhibited almost no mortality at 500 µg/L (Table 4.1). We further examined whether these chitin synthesis inhibitors can inhibit CHS *in vitro*. Very limited inhibition was observed at high concentrations for diflubenzuron and nikkomycin Z, whereas no inhibition on CHS activity was observed for polyoxin D (Fig. 4.7A). We further exposed the 9 h pupae to these three chemicals under the sublethal doses (Table 4.2). The surviving mosquito pupae were collected at

24 h and the crude enzyme were prepared from these pupae. In contrast to the *in vitro* assay, no *in vivo* inhibitory effects were observed in all these treatments (Fig. 4.7B).

## Discussion

Lucero et al. (2002) reported the first alternative to the radioactive assay for CHS activity since 1957 (Glaser and Brown, 1957) and successfully applied the assay for measurement of the fungal CHS activity. In current study, we adapted and applied this method for the measurement of insect CHS activity. The assay provides us a convenient, rapid, low costive and high throughput method for CHS activity assay by using the low cost, stable, nonradioactive reagents and microtiter plates. Also, the high sensitivity of the assay allows testing of multiple samples containing low amounts of active enzyme. A comparison between two methods showed that this chitin binding method is even more sensitive as compared with the conventional radioactive method (Lucero et al., 2002).

Wheat germ agglutinin (WGA) is a chitin-binding lectin with high affinity and specificity for GlcNAc. However, it has been reported that the specificity of this chitin binding assay is lower as compared that using in the radioactive assay (Kramer and Muthukrishnan, 2005). To avoid the effect brought by non-specific binding and the effect by endogenous chitin in the crude enzyme, same amount of the boiled enzyme was used as a valid control in this study. No significant differences were observed in the boiled control as the amount of the enzyme was increased. This result indicated that the effects of the endogenous chitin and the non-specific binding were negligible in the assay. Thus, the activity detected in the crude enzyme from mosquito pupae reflects the catalytic activity other than artifacts brought by non-specific bindings or endogenous chitin (Fig. 4.2).

Both  $Mg^{++}$  and UDP-GlcNAc stimulate CHS activity at low concentration and inhibit CHS activity at high concentrations (Fig. 4.3B, 4.4A). Relative high CHS activity was also observed even without the addition of  $Mg^{++}$  and UDP-GlcNAc (Fig. 4.3B, 4.4A). These results indicate that the endogenous  $Mg^{++}$  and substrate UDP-GlcNAc in the crude enzymes could sustain the CHS activity and thus produce chitin to some extent. Proper levels of  $Mg^{++}$  and substrate UDP-GlcNAc are required to maintain the active enzyme to make chitin. As one mechanism of enzyme activity regulation, substrate inhibition was not rare and has been found in other enzyme systems (Shafferman et al., 1992).

GlcNAc, a molecule that is described as an allosteric activator of fungal CHS activity (Merz et al., 1999) has been reported to inhibit enzyme activity at relatively low concentrations (1mM) for *Manduca sexta* (Zimoch et al., 2005) as well as for the stable fly (Mayer et al., 1980). Interestingly, we found that GlcNAc stimulates CHS activity at 2.5 mM and inhibits enzyme activity at higher concentrations (Fig. 4.4B). The mechanism underlying the stimulation by GlcNAc for *An. gambiae* CHS remains elusive. To date, the allosteric activation for CHS was only reported in fungal CHS but not in insect CHS.

High CHS activity was observed at 37-44°C (Fig. 4.5B). In *Manduca sexta*, high incorporation of UDP-GlcNAc was observed at about 30°C, and decreased at about 40°C (Zimoch et al., 2005). In general, CHS activity depends on the membrane fluidity, and thus, CHS activity decreased as temperature increased higher than 30°C. Our data at least suggested that the crude enzyme is relatively stable at about 37-44°C during the incubation time.

The addition of trypsin not only enhanced for the enzyme activity in the crude enzyme extracts but also in the 40,000g fractions (Fig. 4.6). To date, very limited information on CHS regulation is available. As a post-translational regulation, the addition of trypsin to cell-free

extracts leads to the stimulation of chitin synthesis in fungal and insect systems (Cabib and Farkas, 1971; Cohen and Casida, 1980a; Mayer et al. 1980; Ward et al., 1991; Zimoch et al., 2005), suggesting that inactive chitin synthase is synthesized as a zymogen. However, the *in vivo* activation factors of chitin synthase remained to be elusive. In some fungal systems, proteolytic fragments associated with chitin synthase activity have been identified (Kang et al., 1984; Machida and Saito, 1993; Uchida et al., 1996). In *Manduca*, trypsin stimulates chitin synthesis in crude midgut extracts but not in membrane fractions. Trypsin-dependent activation was recovered when the soluble fraction was added to the membrane fractions, suggesting that chitin synthase is not directly affected by trypsin but by an unknown soluble factor (Zimoch et al., 2005). Later, a chymotrypsin-like protease (CTLP1) that interacts with the extracellular carboxylterminal domain of CHS2 *in vitro* was identified. Highly conserved trypsin cleavage presented in the CTLP1 amino acid sequence, suggesting that the CTLP1 precursor is activated by trypsin CTLP1, although direct evidence is still missing that this CTLP1 is activated by trypsin and could stimulate chitin synthase activity (Broehan et al., 2007). Our results showed that the enzyme activity was enhanced by trypsin both in the crude enzyme preparations and the 40,000g fraction. In *M. sexta*, trypsin activation of CHS was not direct as trypsin failed to enhance the enzyme activity prepared from the microsomal membranes. The effect of trypsin on CHS was through a soluble factor in the supernatant as the activation was recovered after the supernatant was added to the microsomal preparations (Zimoch et al., 2005). It is challenging to reveal this soluble factor as the components of the supernatant are complicated. A very recent study showed that an active, oligomeric chitin synthase complex can be purified from the midgut of the tobacco hornworm (Maue et al., 2009). By using purified enzymes, it will be very helpful to study the properties of the CHS and its mechanisms of post-translational regulation.

Diflubenzuron showed limited *in vitro* inhibitory effect on chitin synthase activity at high concentration 2.5  $\mu$ M. With this same concentration, nikkomycin Z even showed limited inhibitory effect (Fig. 4.7A). Thus, it is not a surprise that no *in vivo* inhibitory effects were observed under the tested concentrations (Fig. 4.7B). To date, the only report of CHS inhibition by diflubenzuron in insects occurred in American cockroaches, in which the isolated intact integument from newly molted cockroaches was used for examination of the incorporation of [ $^3$ H]-N-acetylglucosamine into chitin (Nakagawa et al., 1993). It is interesting to know whether diflubenzuron inhibits incorporation of UDP-GlcNAc into chitin polymers by using the isolated intact integument in *An. gambiae*. Possibly, the inhibition of chitin synthesis by diflubenzuron is an indirect effect on chitin synthase enzyme and further work required to address this issue.

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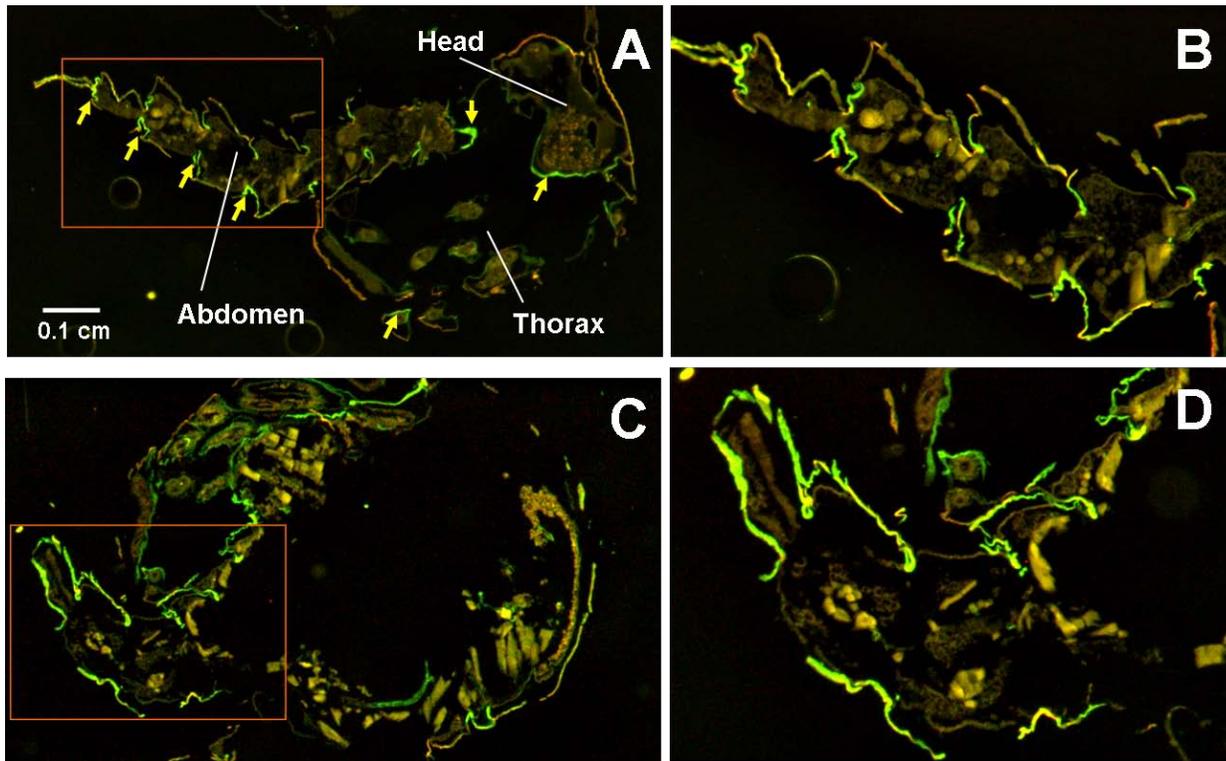
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**Table 4.1 Toxicity of diflubenzuron to 3<sup>rd</sup>-instar mosquito larvae**

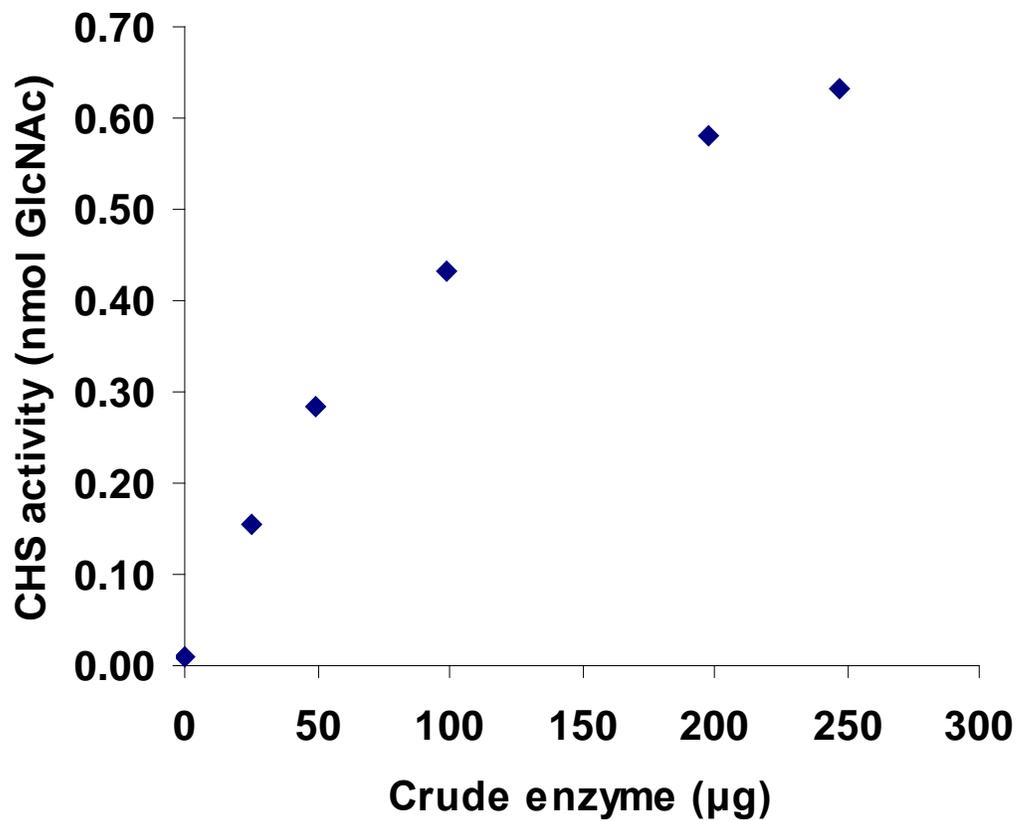
Treatment ( $\mu\text{g/L}$ )	24 h Mortality (Mean $\pm$ SE) (%)	48 h Mortality (Mean $\pm$ SE) (%)
Control	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00 c
Diflubenzuron 6.25	2.5 $\pm$ 1.67	4.9 $\pm$ 2.25 c
Diflubenzuron 12.5	26.3 $\pm$ 8.29	27.5 $\pm$ 8.66 b
Diflubenzuron 25	41.3 $\pm$ 5.95	47.5 $\pm$ 8.66 a
Diflubenzuron 50	57.5 $\pm$ 2.89	60.0 $\pm$ 2.36 a
Nikkomycin Z 50	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00 c
Nikkomycin Z 500	1.5 $\pm$ 1.70	1.5 $\pm$ 1.70 c
Polyoxin D 50	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00 c
Polyoxin D 500	2.1 $\pm$ 1.41	2.1 $\pm$ 1.41 c

**Table 4.2 Toxicity of diflubenzuron to 9-h Mosquito Pupae**

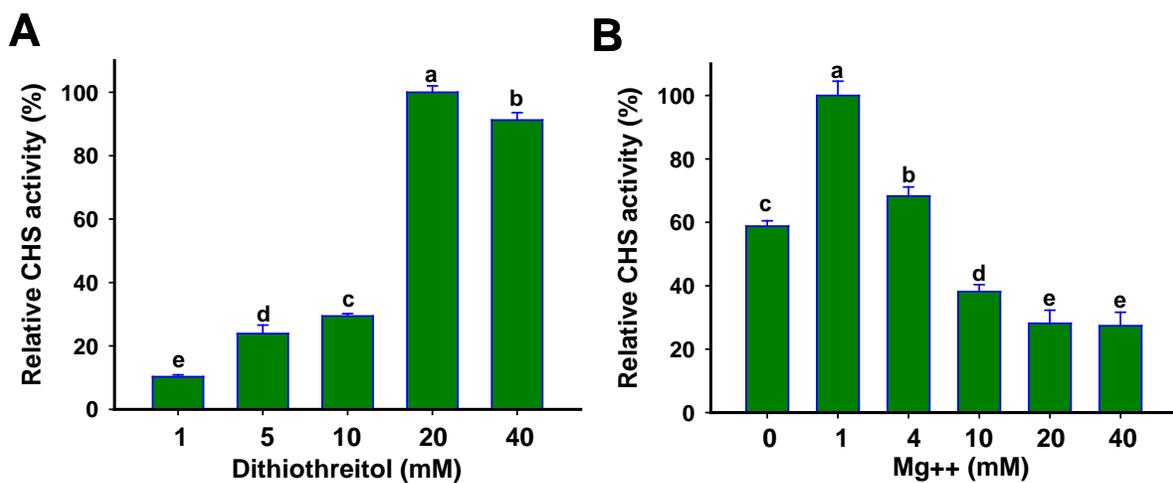
Treatment ( $\mu\text{g/L}$ )	24 h Mortality (Mean $\pm$ SE) (%)
Control	0.0 $\pm$ 0.00
Diflubenzuron 25	4.4 $\pm$ 2.72
Diflubenzuron 50	2.2 $\pm$ 2.72
Diflubenzuron 100	0.0 $\pm$ 0.00
Diflubenzuron 250	4.4 $\pm$ 5.44
Diflubenzuron 500	2.2 $\pm$ 2.72
Nikkomycin Z 100	0.0 $\pm$ 0.00
Nikkomycin Z 500	0.0 $\pm$ 0.00
Polyoxin D 100	4.4 $\pm$ 2.72
Polyoxin D 500	4.4 $\pm$ 5.44



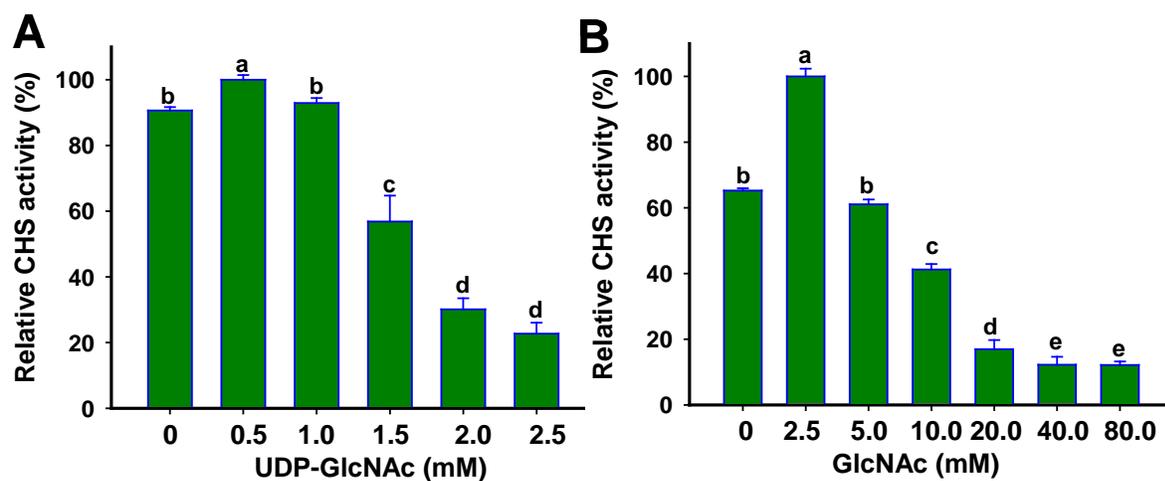
**Figure 4.1 Chitin staining in mosquito pupae. Paraffin-embedded thin sections of the 12-24 hour pupae were stained with FITC-CBD and visualized under fluorescent microscope. The epidermal cells in the cuticle were immunoreactive (arrows indicate positive staining) in two pupae (A and B). C and D: the amplified region (red box) in A and B, respectively.**



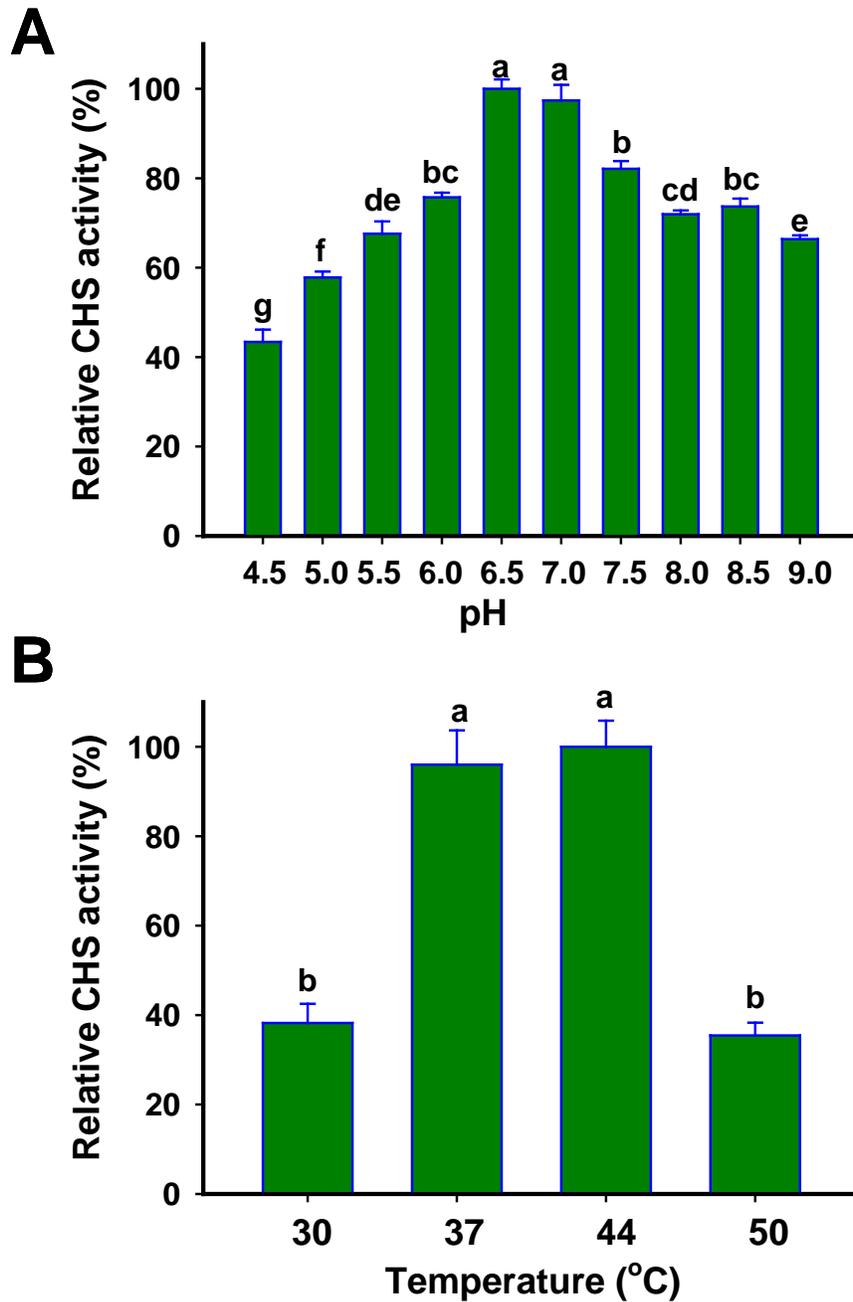
**Figure 4.2** Specific chitin synthase activity in mosquito pupae. Different volumes of crude enzyme were used for assay (5-, 10-, 20-, 40-, and 50 µL).



**Figure 4.3** The DTT and Mg<sup>++</sup> dependency of CHS activity in mosquito pupae. (A) Enzyme activity was dependent on the DTT in the enzyme extraction buffer. (B) The Mg<sup>++</sup> dependency of CHS activity. DTT and Mg<sup>++</sup> were added to the Tris-HCl buffer for crude enzyme preparation. Same letters on the error bars indicate no significant difference based on Fisher's LSD ( $P \geq 0.05$ ).



**Figure 4.4** The UDP-GlcNAc and GlcNAc dependency of CHS activity from mosquito pupae. UDP-GlcNAc (A) and GlcNAc (B) were added in the reaction mixture and the concentrations in the figure represented the final concentration in the reaction system. Same letters on the error bars indicate no significant difference based on Fisher's LSD ( $P \geq 0.05$ ).



**Figure 4.5** Effects of pH (A) and temperature (B) on the activity of CHS prepared from mosquito pupae. Same letters on the error bars indicate no significant difference based on Fisher's LSD ( $P \geq 0.05$ ).

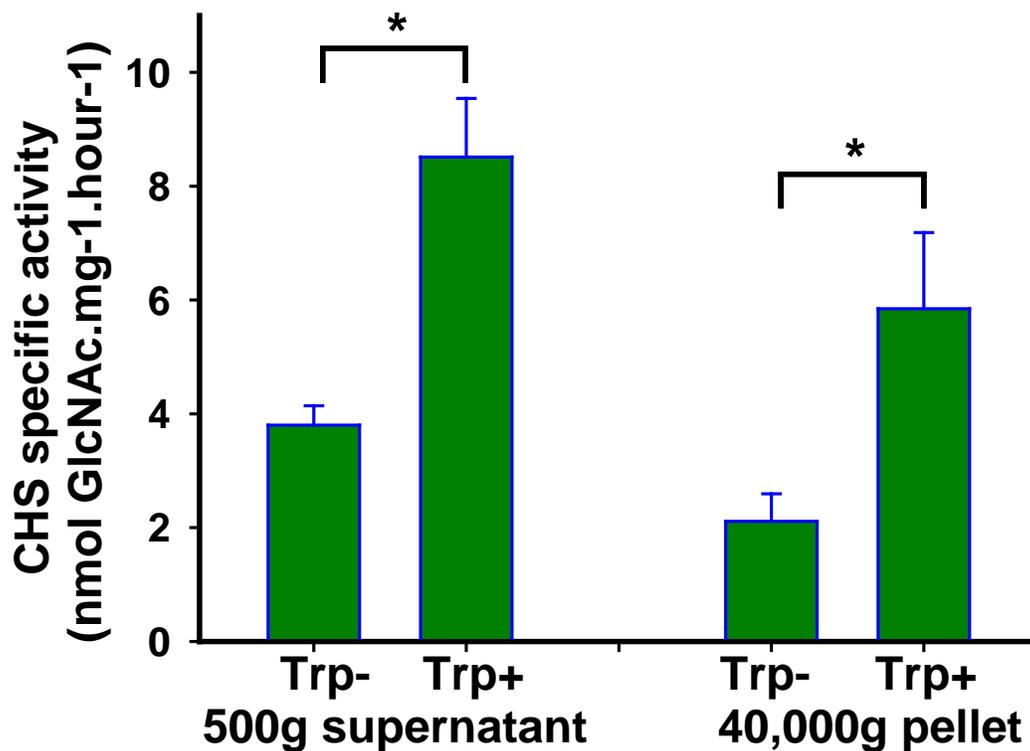


Figure 4.6 Proteolytic activation of chitin synthesis in different enzyme preparations from the mosquito pupae. Specific CHS activity was measured in the presence (Trp+) and absence (Trp-) of trypsin in 500g supernatant and successive 40,000g pellet which was resuspended in proportional volume of the extraction buffer. Asterisks indicate significant difference based on Fisher's LSD ( $P \geq 0.05$ ).

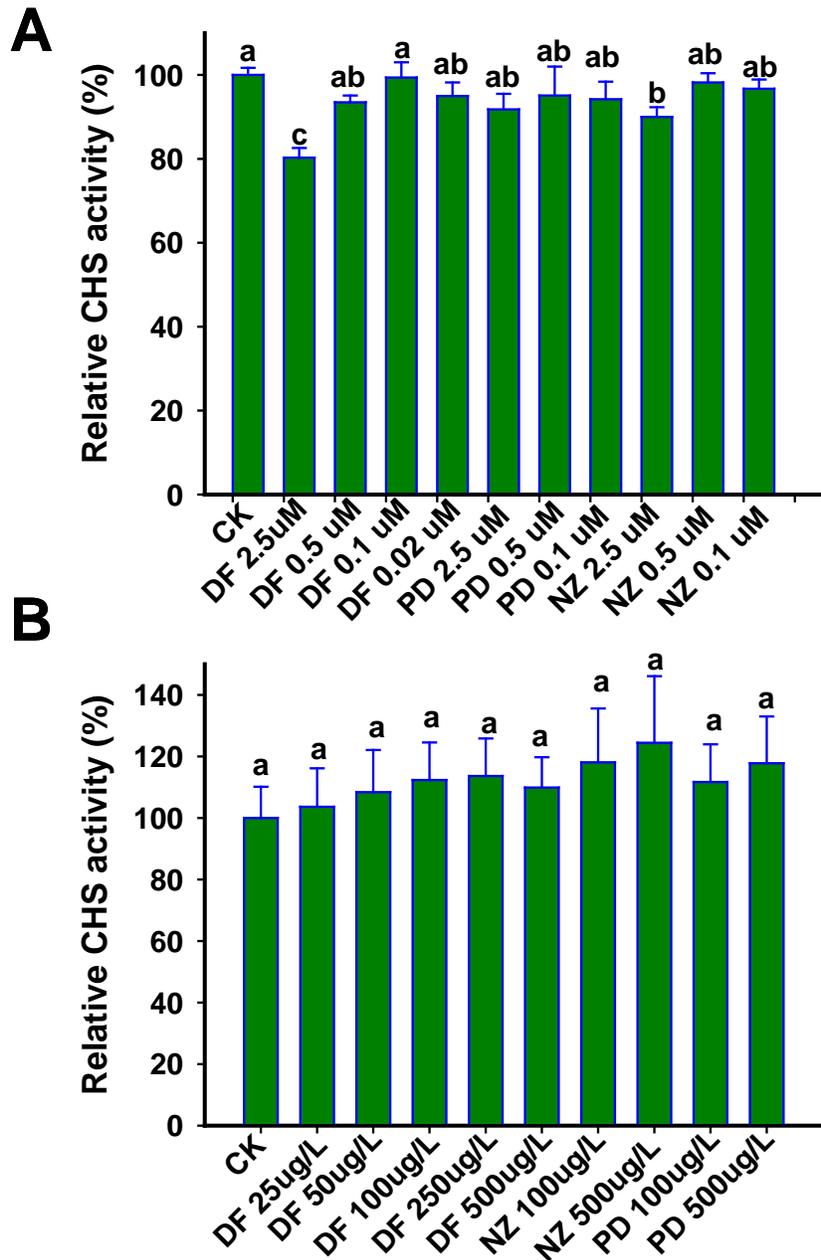
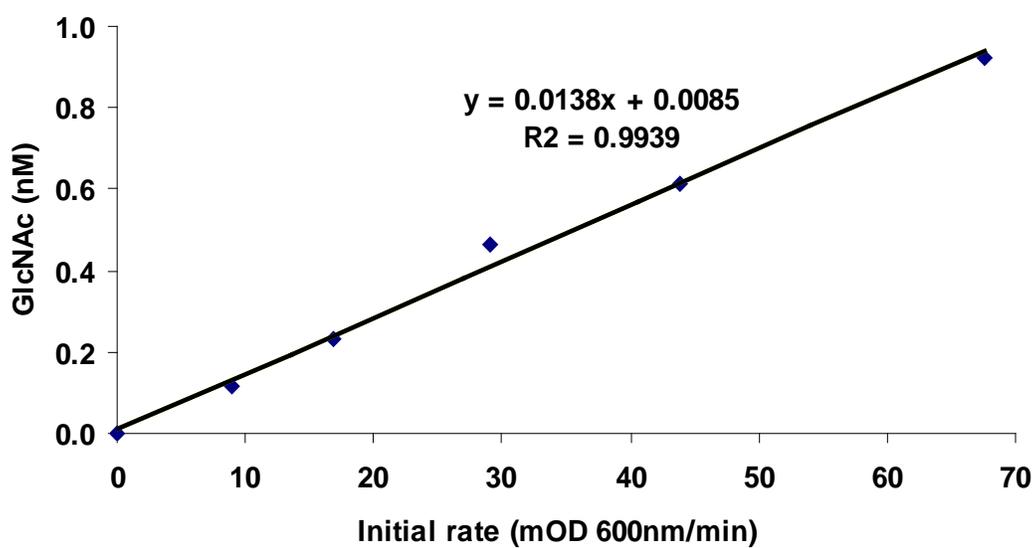


Figure 4.7 Comparisons of chitin synthase activity in the crude enzyme preparations following incubation with various concentrations of three chitin synthesis inhibitors (A) and the crude enzyme preparations from the pupae exposed to the three chitin synthesis inhibitors (B). DF: diflubenzuron; PD: polyoxin D; NZ: nikkomycin Z. Same letters on the error bars indicate no significant difference based on Fisher's LSD ( $P \geq 0.05$ ).



**Figure 4.8 Standard curve of chitin binding to WGA-coated wells**

## **CHAPTER 5 - Chitosan/double-stranded RNA nanoparticle-mediated RNAi to silence chitin synthase genes through larval feeding in the African malaria mosquito (*Anopheles gambiae*)**

**Insect Molecular Biology (2010) 19(5), 683-693**

### **Abstract**

The purpose of this study was to examine whether the expression of two chitin synthase genes, *AgCHS1* and *AgCHS2*, can be repressed by chitosan/*AgCHS* dsRNA-based nanoparticles through larval feeding in *Anopheles gambiae*. The *AgCHS1* transcript level and chitin content were reduced by 62.8 and 33.8%, respectively, in the larvae fed on chitosan/*AgCHS1* dsRNA nanoparticles compared with those of the control larvae fed on chitosan/GFP dsRNA nanoparticles. Our study suggested for the first time that RNA interference (RNAi) in mosquito larvae is systemic, and demonstrated that the larvae fed on the nanoparticles assembled from *AgCHS1* and *AgCHS2* dsRNA increased larval susceptibilities to diflubenzuron, and calcofluor white (CF) or dithiothreitol, respectively. These results suggest a great potential for using such a nanoparticle-based RNAi technology for high-throughput screening of gene functions and for developing novel strategies for pest management.

**Keywords:** African malaria mosquito, chitin synthase, nanoparticle, RNA interference, systemic RNAi.

## Introduction

RNA interference (RNAi) refers to the double-stranded RNA (dsRNA)- or small interfering RNA (siRNA)-triggered post-transcriptional gene silencing that destroys mRNA of a particular gene to prevent its translation into an active gene product (most commonly a protein) (Fire *et al.*, 1998; Mello & Conte, 2004). The discovery of RNAi has not only provided a breakthrough in the methodology for functional analysis of genes, but also opened a new avenue for treating human diseases and protecting crops against insect pest damages. Recent studies have shown some great potentials of using RNAi technologies to control agriculturally important insect pests (Baum *et al.*, 2007; Mao *et al.*, 2007).

Although RNAi is a conserved mechanism in eukaryotes including fungi, plants, insects and mammals, there have been great challenges for successful RNAi in some organisms or some stages of an organism (Miller *et al.*, 2008). Such difficulties may be attributed to the lack of effective delivery methods for dsRNA or siRNA, and the instability of these nucleic acids during and/or after the delivery. Currently, direct injection of dsRNA is the most commonly used delivery method for RNAi. However, the injection has some drawbacks such as high technical demanding and time consuming, and also some limitations in certain insect species (e.g., small size and aquatic living) (Walshe *et al.*, 2009; Nunes & Simões, 2009). In addition, direct injection of dsRNA into larvae in some insects such as *Drosophila melanogaster* often can not effectively trigger RNAi due to the lack of cellular uptake of dsRNA in most of the larval tissues except for hemocytes (Miller *et al.*, 2008).

In mosquitoes, RNAi is usually performed by the injection of dsRNA during the adult stage. Although mosquito larvae appear to process the core RNAi machinery when the RNAi of

a carbonic anhydrase gene was performed in a cell line prepared from mosquito larvae (Smith & Linser, 2009), RNAi in mosquito larvae has not been well established. To our knowledge, there has been only one report on mosquito larval RNAi by dsRNA injection to date (Blitzer *et al.*, 2005). The lack of information on mosquito larval RNAi is a result of the technical challenges associated with the larvae living aquatically and the unavailability of effective dsRNA delivery system for the larvae.

The success of RNAi is also largely determined by the stability of dsRNA or siRNA during and/or after the delivery. It has been reported that the half-life for naked siRNA in serum ranges from several minutes to about an hour (Bartlett & Davis, 2007). Such a short half-lives of the nucleic acids will not lead to an adequate RNAi response in an organism unless a high dose of dsRNA or siRNA is applied. To increase the stability of dsRNA or siRNA and enhance their cellular uptake, polymeric nanoparticles have been used for nucleic acid delivery in RNAi-based gene therapeutics. One of the most commonly used polymers to generate nanoparticles for delivery of therapeutic plasmids, and more recently for siRNA, is chitosan (Howard *et al.*, 2006). Chitosan is a virtually non-toxic and biodegradable polymer that can be prepared by deacetylation of chitin, a naturally occurring and second most abundant biopolymer after cellulose (Dass & Choong, 2008). Nevertheless, the application of chitosan/dsRNA-based nanoparticles for RNAi has not been reported in any insect species.

Chitin synthases are crucial enzymes responsible for chitin biosynthesis by catalyzing the transfer of sugar moieties from activated sugar donors to specific acceptors in all chitin-containing organisms such as insects. It has been well documented that insects possess two chitin synthase genes. *CHS1* (also known as *CHS-A*) is responsible for biosynthesis of the chitin found in the cuticular exoskeleton and other tissues that are ectodermal in origin, such as foregut,

hindgut and trachea, and is exclusively expressed in epidermal cells and other ectodermal tissues. By contrast, *CHS2* (also known as *CHS-B*) is responsible for biosynthesis of the chitin associated with peritrophic matrix (PM) and is specifically expressed in epithelial cells of the midgut (Merzendorfer, 2006). As insect growth and development depend on precisely tuned expression of chitin synthase genes (Arakane *et al.*, 2005, 2008; Tian *et al.*, 2009), chitin synthase is an ideal target for combating insect pests as chitin is not synthesized by vertebrates. However, current knowledge on these enzymes, especially their structures, functions, and regulations in insects is very limited, especially in mosquitoes (Zhang & Zhu, 2006).

In this paper, we report a non-invasive RNAi method by using chitosan/dsRNA self-assembled nanoparticles to mediate gene silencing through larval feeding in African malaria mosquito (*Anopheles gambiae*). We have demonstrated its potential applications for insect control by showing increased susceptibilities to diflubenzuron (DFB), calcofluor white (CF) and dithiothreitol (DTT) in the larvae fed on *AgCHS1*- or *AgCHS2*-based chitosan/dsRNA nanoparticles. Our research has not only established a novel RNAi method but also suggests for the first time the systemic nature of RNAi in mosquito larvae.

## Results

### ***Generation of chitosan/dsRNA nanoparticles for mosquito RNAi through larval feeding***

Owing to the unique chemical properties of chitosan and dsRNA molecules, the chitosan/dsRNA nanoparticles are formed by self-assembly of polycations with dsRNA through the electrostatic forces between the positive charges of the amino group in the chitosan and the negative charges carried by the phosphate group on the backbone of dsRNA (Fig. 5.1A). The

atomic force microscopy (AFM) images of the chitosan/dsRNA nanoparticles showed typical spherical- or ellipsoidal-shaped structures of similar sizes with diameter ranging from 100 to 200 nm (Fig. 5.1B). In contrast, the control samples in the absence of dsRNA did not form a significant number of nanoparticles. Instead, we found only a few smaller and spherical-shaped particles with an average diameter of 70 nm (Fig. 5.1C).

After slices of an agarose gel-coated mixture of food and dsRNA that was entrapped in chitosan-based nanoparticles were incubated in water for 24 h, only about 6% of dsRNA was released from the gel slices into water (Fig. 5.1 D, E). In contrast, >80% of dsRNA in the gel slices was released into water when dsRNA was not entrapped in the nanoparticles (i.e., dsRNA was directly mixed in the food gel). These results indicated that our chitosan/dsRNA nanoparticles can effectively protect dsRNA from releasing into water from the sliced food gel and can perhaps stabilize dsRNA that is incorporated into the chitosan/dsRNA complex as shown in other studies (Gao *et al.*, 2009).

### ***RNAi for two chitin synthase genes in mosquito larvae***

To evaluate the effectiveness of our nanoparticle-based RNAi method in silencing both *AgCHS1* and *AgCHS2* genes through mosquito larval feeding, two dsRNAs (i.e., ds*AgCHS1*-f1 and f2 or ds*AgCHS2*-f1 and f2) were *in vitro* synthesized for each gene and used to generate chitosan/dsRNA nanoparticles. One primer pair that did not overlap with the dsRNA regions was synthesized to examine the repression of gene transcript by quantitative real-time PCR (qPCR; Fig. 5.2A, B).

Our results clearly show that feeding third-instar mosquito larvae with either ds*AgCHS1*- or ds*AgCHS2*-based nanoparticles effectively triggered RNAi in the larvae. Specifically, ds*AgCHS1*-f1 and ds*AgCHS1*-f2 (both from *AgCHS1*) repressed the transcript levels of *AgCHS1*

by 62.8 and 52.4%, and *AgCHS2* by 48.4 and 57.9%, respectively (Fig. 5.2C). Thus, feeding the larvae with dsRNA of *AgCHS1* not only repressed *AgCHS1* expression but also *AgCHS2* expression, possibly because of the high sequence similarities of the two genes as shown in Fig. 5.6A, B.

In contrast, ds*AgCHS2*-f1 and ds*AgCHS2*-f2 (both from *AgCHS2*) specifically repressed the transcript levels of *AgCHS2* by 63.4 and 48.8%, respectively, but none of these *AgCHS2* dsRNAs repressed the transcript level of *AgCHS1* (Fig. 5.2D). As *AgCHS1* and *AgCHS2* also display comparably high sequence similarities in both ds*AgCHS2*-f1 and ds*AgCHS2*-f2 regions (Fig. 5.6C, D), the noncross repression was probably a result of RNAi triggered by one or more short but less conserved sequences in their dsRNAs.

To examine whether chitosan alone can affect *AgCHS* gene expression, a separate experiment in which chitosan/dsRNA nanoparticles were replaced by an equivalent amount of chitosan (0.02 mg in 100  $\mu$ l) showed no differences in expression of each gene as compared with the controls in which no chitosan was included in the food. These results indicate no effect of chitosan itself on *AgCHS* gene expression in our studies.

We further examined tissue-specific expression profiles of two chitin synthase genes by reverse transcription PCR (RT-PCR). As expected, *AgCHS1* was most abundantly expressed in larval carcass (i.e., the insect body after its digestive canal is removed), followed by the foregut and hindgut (Fig. 5.3). However, the expression of *AgCHS1* was not detected in the midgut. In contrast, *AgCHS2* was most abundantly expressed in the foregut followed by the midgut but expression was not detected in the hindgut and carcass (Fig. 5.3). The high expression of *AgCHS2* in the foregut as detected by RT-PCR is most likely to be a result of the fact that it is difficult to separate the cardia, a part of larval midgut, from the foregut during our dissection

because the larval foregut is very small and tightly connected to the cardia of the midgut. Thus, the high expression of *AgCHS2* detected in larval foregut might be an artifact of this.

### ***Effect of RNAi for AgCHS1 on chitin content and larval susceptibility to DFB***

After third-instar mosquito larvae were fed on chitosan/dsRNA nanoparticles, we evaluated the chitin content in the larvae. As expected, our RNAi reduced larval chitin content by 33.8% (Fig. 5.4A). Although such a reduction did not lead to larval mortality, the reduction of chitin content significantly increased the susceptibility of the larvae to DFB (Fig. 5.4B). Specifically, the mortality of the larvae fed on *AgCHS1* dsRNA increased by 26.5% as compared with that of the larvae fed on *green fluorescent protein (GFP)* dsRNA when the larvae were exposed to DFB at 200 µg/L. It appears that the increased mortality in the *AgCHS1* dsRNA-fed larvae was caused by reduced chitin content in larval cuticle as a result of the RNAi of *AgCHS1*.

### ***Effect of RNAi for AgCHS2 on larval susceptibility and PM permeability to DTT and CF***

We further investigated the effect of RNAi of *AgCHS2* on the survivorship of mosquito larvae and the permeability of their PM. The disruption of the PM by DTT or other reagents was expected to result in an increase of the PM permeability that could be visualized by distinct blue color in the gastric caecae when insects are fed on blue dextran (Fig. 5.5A). After mosquito larvae were fed on normal food without dsRNA (first control), the *GFP* dsRNA-based nanoparticles (second control), and *AgCHS2* dsRNA-based nanoparticles for four days, we exposed the larvae to CF or DTT. As expected, both the mortality and percentage of the larvae with a disrupted PM phenotype increased by 29.3% in the larvae fed on *AgCHS2* dsRNA-based nanoparticles as compared with those of the control larvae fed on *GFP* dsRNA-based

nanoparticles after the larvae were exposed to DTT at 2.5 mM (Fig. 5.5B, C). Similarly, after the larvae were exposed to CF at 3 and 4 mg/ml, their mortalities increased by 16.7 and 48.0%, respectively, and the percentages of the larvae with a disrupted PM phenotype increased by 31.1 and 24.4%, respectively, in the larvae fed on *AgCHS2* dsRNA-based nanoparticles as compared with those of the control larvae fed on *GFP* dsRNA-based nanoparticles (Fig. 5.5D, E). It appears that such increased larval susceptibility and PM permeability to CF and DTT in *AgCHS2* dsRNA-fed larvae resulted from the reduction of chitin biosynthesis in the midgut.

## Discussion

We have successfully developed a novel feeding-based RNAi method for mosquito larvae. The innovation of this method is the use of an oral delivery system for mosquito larvae by voluntarily feeding the agarose gel-coated mixture of food and dsRNA that was entrapped in chitosan-based nanoparticles. The major steps of this method include: 1) generation of chitosan/dsRNA nanoparticles; 2) preparation of agarose gel-coated larval food containing the nanoparticles; and 3) feeding mosquito larvae with the slices of agarose gel-coated mixture of food and chitosan/dsRNA-based nanoparticles. The critical step for the success of this method is the application of nanoparticles, which may serve two important functions. First, the retention of dsRNA by nanoparticles in the food gel may be significantly improved in feeding-based RNAi in an aquatic system. Second, the nanoparticles may dramatically stabilize dsRNA and enhance the efficacy of dsRNA delivery into larval gut epithelial cells, which has been evidenced in the use of siRNA for gene therapies in mammalian cells (Gary *et al.*, 2007; Huang & King, 2009).

In *Caenorhabditis elegans*, systemic RNA interference-deficient (SID-1), an RNA channel transporter, and a related RNA transporter, SID-2, are involved in dsRNA uptake in the gut lumen (Winston *et al.*, 2002, 2007). However, the mechanisms that facilitate the uptake of

dsRNA in insect gut remain elusive. Homologues of the *C. elegans sid-1* gene have been identified in several insect species such as *Tribolium castaneum*, *Bombyx mori*, *Apis mellifera* and aphids, but not in dipterans such as *Drosophila melanogaster* and mosquitoes. In addition, it appears that SID-1 may not serve the same role in insects as in *C. elegans* (Gordon & Waterhouse, 2007; Tomoyasu *et al.*, 2008). Recent studies in *C. elegans* and *D. melanogaster* suggest that receptor-mediated endocytosis may be a common mechanism for dsRNA uptake and may occur in different insect orders (Saleh *et al.*, 2006; Ulvila *et al.*, 2006). Thus, it is possible that the chitosan/dsRNA nanoparticles may facilitate epithelial uptake of dsRNA through an endocytosis pathway in the gut and enhance the effect of RNAi in mosquito larvae. This notion is supported by a recent study showing that certain transfection reagents such as lipofectamine can facilitate dsRNA uptake in *Drosophila* species (Whyard *et al.*, 2009).

The apparent differences in the susceptibility to RNAi in mosquito adults and larvae might be to the result of either the stability of dsRNA and/or efficacy of cellular uptake of dsRNA. In our laboratory, we took great effort in larval RNAi by injecting *AgCHS1* or *AgCHS2* dsRNA into larval bodies of the same mosquito species, but had very limited success. In contrast, consistent results were obtained when we used this nanoparticle-based approach for RNAi. Thus, our studies suggest that injection is not necessarily more efficient than ingestion for dsRNA delivery although this appears to be true in some insect species. In *Spodoptera litura*, for example, feeding the larvae with dsRNA targeting a gut-specific aminopeptidase fails to trigger RNAi, but injecting the same dsRNA into the larvae triggers a significant RNAi response (Rajagopal *et al.*, 2002).

The insect *CHS1* gene has been known to be exclusively expressed in epidermal and other ectodermal tissues. Indeed, our results support this notion by showing a high expression of

*AgCHS1* in the carcass. As the expressions of both *AgCHS1* and *AgCHS2* can be repressed by the ingestion of *AgCHS1* dsRNA in mosquito larvae, the reduction of total chitin content may be not only attributed to the reduction of chitin content in the cuticle and trachea, but also to the reduction of chitin content in the PM. However, research has shown that chitin content in Type 2 PM of *Lucilia cuprina* (a hematophagous insect) is very low (Tellam & Eisemann, 2000). In our study, we tried to stain chitin in the PM by using fluorescein isothiocyanate (FITC)-conjugated chitin-binding domain but were not able to show much chitin staining in the PM. As mosquitoes also possess Type 2 PM, it seems that chitin content in Type 2 PM is usually very low. All these suggest that decreased chitin content in mosquito larvae fed on *AgCHS1* dsRNA-based nanoparticles is mainly a result of the reduced chitin content in the cuticle and related ectodermal tissues.

Systemic RNAi is a phenomenon of which local administration of dsRNA (e.g. feeding) leads to an RNAi response in whole body through the amplification and spread of silencing to other cells and even to the progenies of an organism (Winston *et al.*, 2002; Tomoyasu *et al.*, 2008). Systemic RNAi in *C. elegans* and plants relies on the presence of an RNA-dependent RNA polymerase (RdRP) that can interact with the RNA-induced silencing complex and generate new dsRNA based on partially degraded target template by using hybridized siRNA strands as primers (Sijen *et al.*, 2001). Although systemic RNAi has been reported in several insect species including *Tribolium*, grasshoppers, and *Reticulitermes flavipes* (Tomoyasu & Denell, 2004; Dong & Friedrich, 2005; Zhou *et al.*, 2008), very limited information on its mechanisms is available in insects. To date, it appears that insects lack the RdRP necessary for driving this RNAi amplification in *C. elegans* and plants (Gordon & Waterhouse, 2007). Furthermore, mosquitoes lack not only RdRP but also SID-1, which is required for spreading

RNAi responses in *C. elegans* (Winston *et al.*, 2002). The success of feeding-based RNAi for *AgCHSI* gene, which is exclusively expressed in epidermal and related ectodermal tissues in our study strongly suggests a systemic nature of RNAi in mosquito larvae. This notion is mainly based on our results showing significant repression of *AgCHSI* gene expression and reduction of chitin content in the larvae carcass when *AgCHSI* dsRNA was delivered through larval feeding. Nevertheless, further studies are needed to elucidate the mechanism of systemic RNAi by showing any amplification and spread of silencing to other cells in mosquito larvae.

Our nanoparticle-based RNAi method can be applied for functional analysis of genes expressed in virtually any tissues if RNAi is systemic in an insect. As feeding is not restricted by insect size and developmental stage, this method holds great potential for high throughput screening of various genes for their functions. Our method also shows considerable potentials for insect control, in which ingestion of dsRNA is often required. For example, reduced chitin content in the cuticle by RNAi of *AgCHSI* can enhance the toxicity of DFB, a benzylphenolurea insecticide that inhibits chitin biosynthesis in insects (Merzendorfer, 2006; Zhu *et al.*, 2007). Such an RNAi-mediated effect could potentially be used as a strategy to enhance the toxicity of many insecticides for insect pest management.

In insects, the PM is a tubular film composed of proteins, chitin and glycosaminoglycans (Shao *et al.*, 2001), and plays important roles in protecting the epithelium from mechanical damage, facilitating digestion, serving as a barrier of pathogens, and filtration of toxins (Terra, 2001; Hegedus *et al.*, 2009). As the integrity of the PM is critical for maintaining normal physiological function for insect growth and development, the PM has been recognized as a potential target for insect pest control (Marian *et al.*, 2003). In lepidopteran insects, CF has been known to disrupt the PM by releasing proteins from the PM (Wang & Granados, 2000). In

mosquito larvae, DTT disrupts the integrity of the PM, resulting in increased PM permeability (Edwards & Jacobs-Lorena, 2000). Our results indicated that increased larval mortalities in DTT- or CF-treated mosquito larvae after RNAi of *AgCHS2* were caused by the reduction of chitin content because of the RNAi, along with the disruption of chitin-associated proteins because of the chemicals, both leading to the increased permeability of the PM in the larvae. Thus, our results further suggest that silencing of *CHS2* gene by RNAi may serve as a novel strategy for insect pest management

Although our nanoparticle-based RNAi of two target genes did not directly lead to larval mortality and further increase of nanoparticle concentration can not improve silencing effect (data not shown), our study has proved the concept of using oral RNAi for mosquito control. In addition, our results have shown a great potential for incorporating such a feeding-based RNAi method into a pest management programme to increase the efficacy of insecticides. As more genome sequences become available and more potential target genes are identified in insects, development of novel RNAi methods will not only facilitate functional studies of new genes but also revolutionize the technologies for insect pest management.

## **Experimental procedures**

### ***Mosquito rearing***

A colony of *An. gambiae* initially obtained from the Malaria Research and Reference Reagent Resource Center (MR4) (Manassas, VA, USA) was maintained in the Department of Entomology at Kansas State University (Manhattan, KS) based on the procedure as previously described (Zhang & Zhu, 2006).

### ***Preparation of chitosan/dsRNA nanoparticles***

To prepare dsRNA for each *CHS* gene, specific primers were designed based on the annotated *AgCHS1* (GenBank accession no. XM\_321337) and *AgCHS2* (GenBank accession no. AY056833) cDNA sequences. After total RNA was extracted from mosquito larvae with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), 3.5 µg of total RNA was used to synthesize the first strand cDNA using the First Strand cDNA Synthesis kit (Fermentas, Glen Burnie, MD, USA). To prepare GFP dsRNA for negative RNAi controls, specific primers were designed and a 684-bp fragment was amplified by using the plasmid 11335: GFP::L4440 (Addgene Inc., Cambridge, MA, USA) as a template. The sequences of the primers used for dsRNA synthesis are shown in Table 5.1. Each dsRNA was prepared by using MEGAScript RNA<sup>®</sup> kit (Ambion, Austin, TX, USA) based on the manufacture's procedure.

To generate chitosan/dsRNA nanoparticles, chitosan from crab shells (Cat. No. C3646-25G, ≥75 deacetylated; Sigma-Aldrich, Milwaukee, WI, USA) was dissolved in sodium acetate buffer (0.1 M sodium acetate–0.1 M acetic acid, pH 4.5) to make a 0.02% wt/vol working solution. A total of 32 µg of dsRNA in 100 µl of 50 mM sodium sulfate was added to 100 µl of chitosan solution. The amounts of dsRNA and chitosan should be balanced for their efficient electrostatic interactions between chitosan and dsRNA. After the mixture was heated at 55°C for 1 min, it was immediately mixed by vortexing for 30s by using a high-speed vortex (Model 232, Fisher Scientific, Pittsburgh, PA, USA) to allow the formation of nanoparticles (Sarathi *et al.*, 2008).

### ***Preparation of mosquito larval food containing the chitosan/dsRNA nanoparticles***

Once dsRNA had been entrapped in nanoparticles, the preparation was first centrifuged at 13,000 g for 10 min (Katas & Alpar, 2006) followed by mixing the resultant pellet with 6 mg of ground mosquito larval food consisting of TetraFin goldfish flakes (Tetra Holding, Inc., Blacksburg, VA, USA) and dry yeast (Universal Foods Corp., Milwaukee, WI, USA) at a ratio of 2:1. Both the goldfish flakes and yeast were ground to small particles (>300 µm as measured by NO. 50 USA standard test sieve). The mixture of the food and nanoparticles was then coated by thoroughly mixing with 30 µl of 2% pre-melted agarose (genetic analysis grade; Fisher Scientific) gel solution at 55 °C. After the solidified gel containing both the food and nanoparticles was cut into small pieces (approximately 1 mm thick) by using a razor blade, they were used to feed mosquito larvae in water.

### ***Larval feeding on food containing the chitosan/dsRNA nanoparticles***

A group of 15-20 third-instar mosquito larvae was transferred into a 500-ml glass beaker containing 100 ml of deionized water. One sixth of the gel slices that were prepared from 32 µg of dsRNA as described above were added into each beaker. Approximately an equal amount of the gel slices was used to feed the larvae once a day for a total of four days. Any potential phenotypic changes were visually examined in the larvae during the experiment. The transcript levels of *AgCHS1* or *AgCHS2*, chitin contents, and other phenotypic changes were assessed in the larvae at the end of the experiment (i.e., day 4).

### ***Atomic force microscopy (AFM) imaging***

To confirm the nanoparticle formation between chitosan and dsRNA, AFM was used to examine the nanoparticles by using a tapping mode with a high aspect ratio tip based on a

slightly modified method as previously described by Ganta *et al.* (2008). Briefly, 30  $\mu$ l of nanoparticle solution was placed onto freshly cleaved mica, washed with deionized water twice, and dried with N<sub>2</sub>. AFM images on different locations of the mica were then obtained using Nanoscope IIIa scanning probe microscope (Equipment for technology & Science Inc., San Jose, CA, USA).

### ***RT-PCR and qPCR analysis***

Total RNA was extracted from mosquito larvae with TRIzol reagent (Invitrogen, Carlsbad, CA) and 3.5  $\mu$ g of total RNA was used for first strand cDNA synthesis using the First Strand cDNA Synthesis kit (Fermentas, Glen Burnie, MD, USA). The first strand cDNA was then used as template for PCR and qPCR. A gene encoding ribosomal protein S3, *AgRPS3*, was used as an internal reference gene. PCR was performed with PCR Master Mix kit (Fermentas). The qPCR was performed by using Maxima SYBR Green qPCR Master Mix (Fermentas), and the  $2^{-\Delta\Delta CT}$  method was used to calculate the relative levels of *AgCHS1* and *AgCHS2* transcripts in the mosquito larvae fed on the food containing *AgCHS1* or *AgCHS2* dsRNA-based nanoparticles as compared with the control larvae fed on the food containing *GFP* dsRNA-based nanoparticles. The sequences of the primers used for RT-PCR and qPCR analyses are shown in Table 5.1.

### ***Retention of dsRNA by chitosan/dsRNA nanoparticles***

To determine whether the dsRNA entrapped in nanoparticles can be effectively retained in the slices of the food gel when added into water, 20  $\mu$ g of dsRNA entrapped in nanoparticles was mixed with food to make food gel as described above. Control food gel was prepared in the same way except that dsRNA was directly mixed with food without using nanoparticles. After the slices of the food gel were incubated in 1 ml water for 24 h, samples were centrifuged at

13,000 g for 10 min, and free dsRNA released from the food gel in the supernatant was extracted by phenol/chloroform. The extractant was then dissolved in 30 µl water, and dsRNA concentration was determined using an Ultrospec 3000 UV/visible spectrophotometer (Pharmacia Biotech, Ltd., Cambridge, UK) at 260 nm. For visual comparisons of dsRNA retention in the two samples, 4 µl of each extractant was examined on 1.2% agarose gel. The dsRNA bands were visualized by using ethidium bromide that was incorporated into the agarose gel and Tris-borate-EDTA running buffer.

### ***Chitin content assay***

Chitin content was determined by using a previously described method (Zhang & Zhu, 2006) except that the centrifugation force was increased from 1,800 to 4,000 g in all centrifugation steps.

### ***Chemical treatment and in vivo assay of PM disruption***

After mosquito larvae were fed with the food containing chitosan/dsRNA nanoparticles for four days, they (15-20 larvae) were transferred to a 500-ml glass beaker containing 100 ml deionized water. DFB (Cat. No. PS-1028; Chem Service, West Chester, PA, USA) stock solution in acetone was then added to each beaker to obtain a final DFB concentration of 200 µg/L. The larval mortality was assessed at 24 h. For CF (Fluorescent Brightener 28, Cat. No. F3545-5G; Sigma-Aldrich) and DTT (electrophoresis grade; Fisher Scientific) treatments and their subsequent assays of the PM permeability, we followed the method as described by Edwards & Jacobs-Lorena (2000). In brief, each group of untreated larvae (first control), *GFP*-dsRNA-fed larvae (second control), and *AgCHS2*-dsRNA-fed larvae were transferred into 5 ml of deionized water containing DTT at 2.5 mM, or CF at 3 or 4 mg/ml, and agar (Becton, Dickinson and

Company, Sparks, MD, USA) at 0.3 mg/ml. After the larvae were maintained overnight (17-18 h) at 25 °C, larval mortality was recorded and the surviving larvae were rinsed thoroughly with deionized water and transferred to 2 ml of 2% (w/v) blue dextran (MW 2 million Da, Cat. No. D5751-5G; Sigma-Aldrich) for 1 hr. The dye in the gut was examined under a Leica M205 FA stereomicroscope. Images were captured using a Leica DFC 400 digital camera (Vashaw Scientific Inc., Norcross, GA, USA) attached to the microscope. A blue gastric caecae following ingestion of blue dextran indicates a disruption of the larval PM.

### ***Statistical analysis***

For data obtained from qPCR, relative expression levels in percentage were calculated by dividing the relative expression value (REV) of each gene in the *AgCHS* dsRNA-treated larvae by the REV of the same gene in the *GFP* dsRNA-treated larvae. After the percentage data of the relative *AgCHS* expression were transformed using arcsine square root transformation, the transformed data were subjected to ANOVA followed by Fisher's least significant difference (LSD) multiple comparisons to separate the means among the treatments by using ProStat software (Poly Software International, Pearl River, NY, USA).

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Experiment Station, Kansas State University, Manhattan, Kansas, USA. The *Anopheles gambiae* voucher specimens (voucher No. 211) are located in the Kansas State University Museum of Entomological and Prairie Arthropod Research, Manhattan, Kansas.

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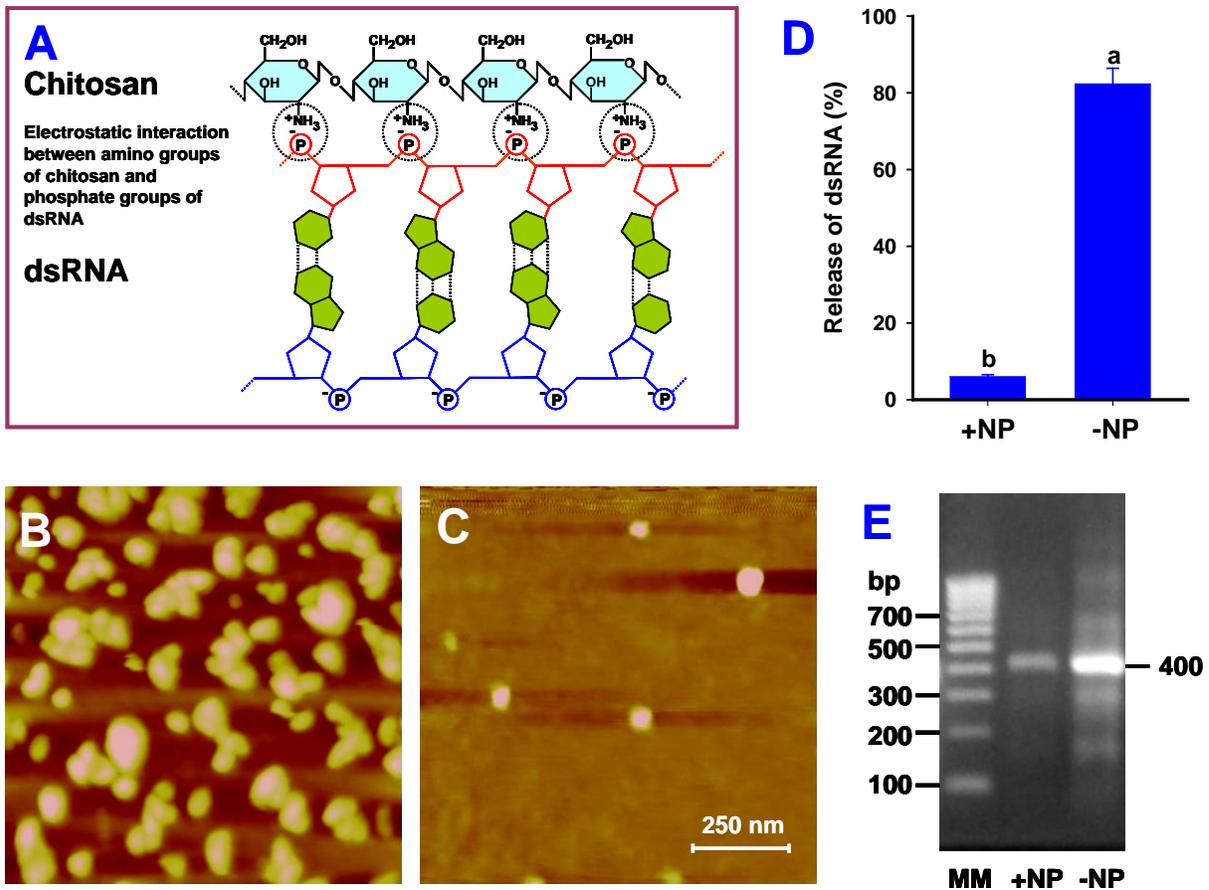
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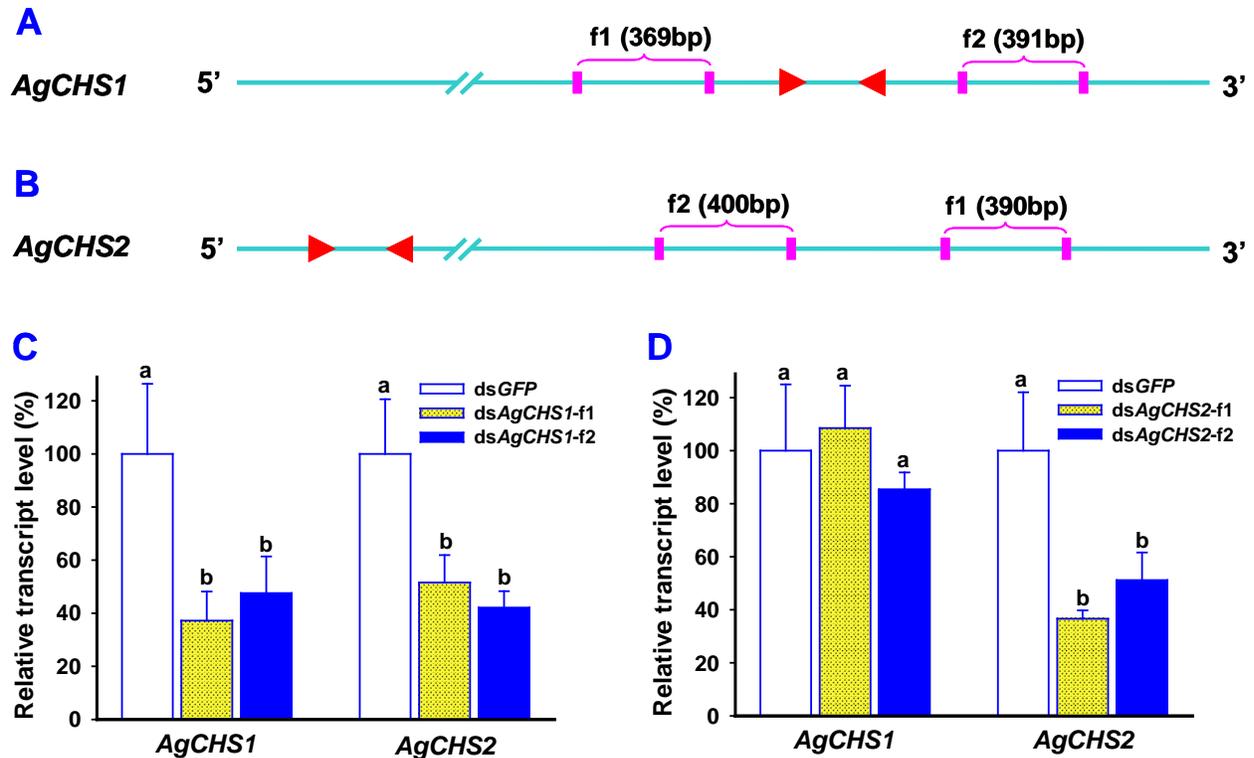
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**Table 5.1 Sequences and relevant parameters of the primers used for dsRNA synthesis and quantitative real-time PCR (qPCR) analysis**

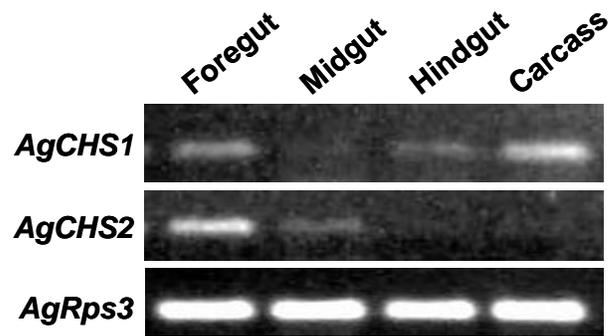
Application of primers	Gene	Primer name	Primer sequence (5'-3')	Length (base)	Tm (°C)
dsRNA synthesis	<i>AgCHS1-f1</i>	F	TAATACGACTCACTATAGGGTGAAACGCACATCTTCTTCG	40	65.5
		R	TAATACGACTCACTATAGGGAGCGTCAGCAGGTAGGTGTT	40	67.6
	<i>AgCHS1-f12</i>	F	TAATACGACTCACTATAGGGGGCAAACGACGGACG	36	66.7
		R	TAATACGACTCACTATAGGGGTGCGCAATACGTGCC	36	66.7
	<i>AgCHS2-f1</i>	F	TAATACGACTCACTATAGGGACACATCGAGTGGTGGTTCA	40	66.5
		R	TAATACGACTCACTATAGGGTTGTGCTGGTAGAGAATGCG	40	66.5
	<i>AgCHS2-f2</i>	F	TAATACGACTCACTATAGGGGCCAGCGCCGAAAAG	35	66.8
		R	TAATACGACTCACTATAGGGTCCGACAGATCGAGCG	36	66.7
	<i>GFP</i>	F	TAATACGACTCACTATAGGGGTGGAGAGGGTGAAGG	36	66.7
		R	TAATACGACTCACTATAGGGGGGCAGATTGTGTGGAC	37	66.7
qPCR analysis	<i>AgCHS1</i>	F	ACGAGCGCGACTTCCTCAC	19	55.4
		R	GAGTCGCGCAACTCCTTGAG	20	55.9
	<i>AgCHS2</i>	F	CACCAGCAACGCCATCATC	19	53.2
		R	GAACACCAGCAGCAGAGTAAC	21	54.4
	<i>AgRPS3</i>	F	GCTGGGCATCAAGGTCAAG	19	55.4



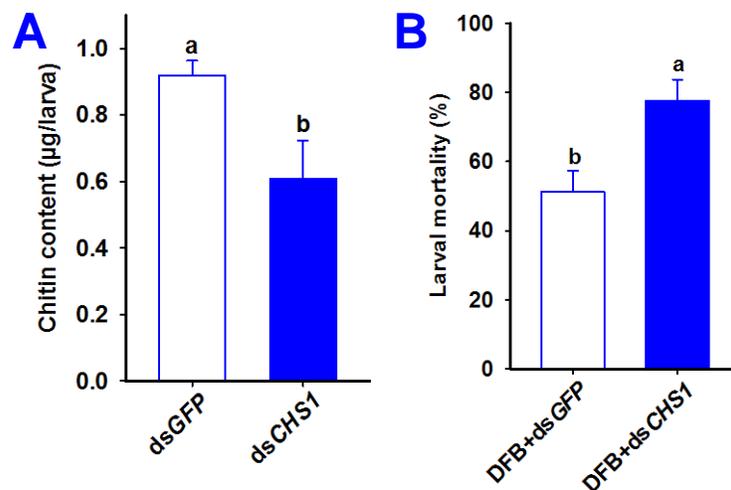
**Figure 5.1** Formation of chitosan/ double-stranded RNA (dsRNA) nanoparticles and the retention of dsRNA in the food gel. (A) Schematic representation of electrostatic interactions between chitosan and dsRNA. (B) Atomic force microscopy (AFM) image of the chitosan/dsRNA nanoparticles. (C) AFM image of chitosan solution without the addition of dsRNA. All the scan sizes of the images were  $1.0 \mu\text{m} \times 1.0 \mu\text{m}$ . (D) Retention of dsRNA by chitosan/dsRNA nanoparticles in the food gel. +NP: dsRNA was entrapped in nanoparticles; -NP: dsRNA was used directly without nanoparticles. The data are presented as means  $\pm$  SEM of triplicate samples. (E) Retention of dsRNA by the chitosan/dsRNA nanoparticles in the food gel was evaluated by agarose gel electrophoresis.



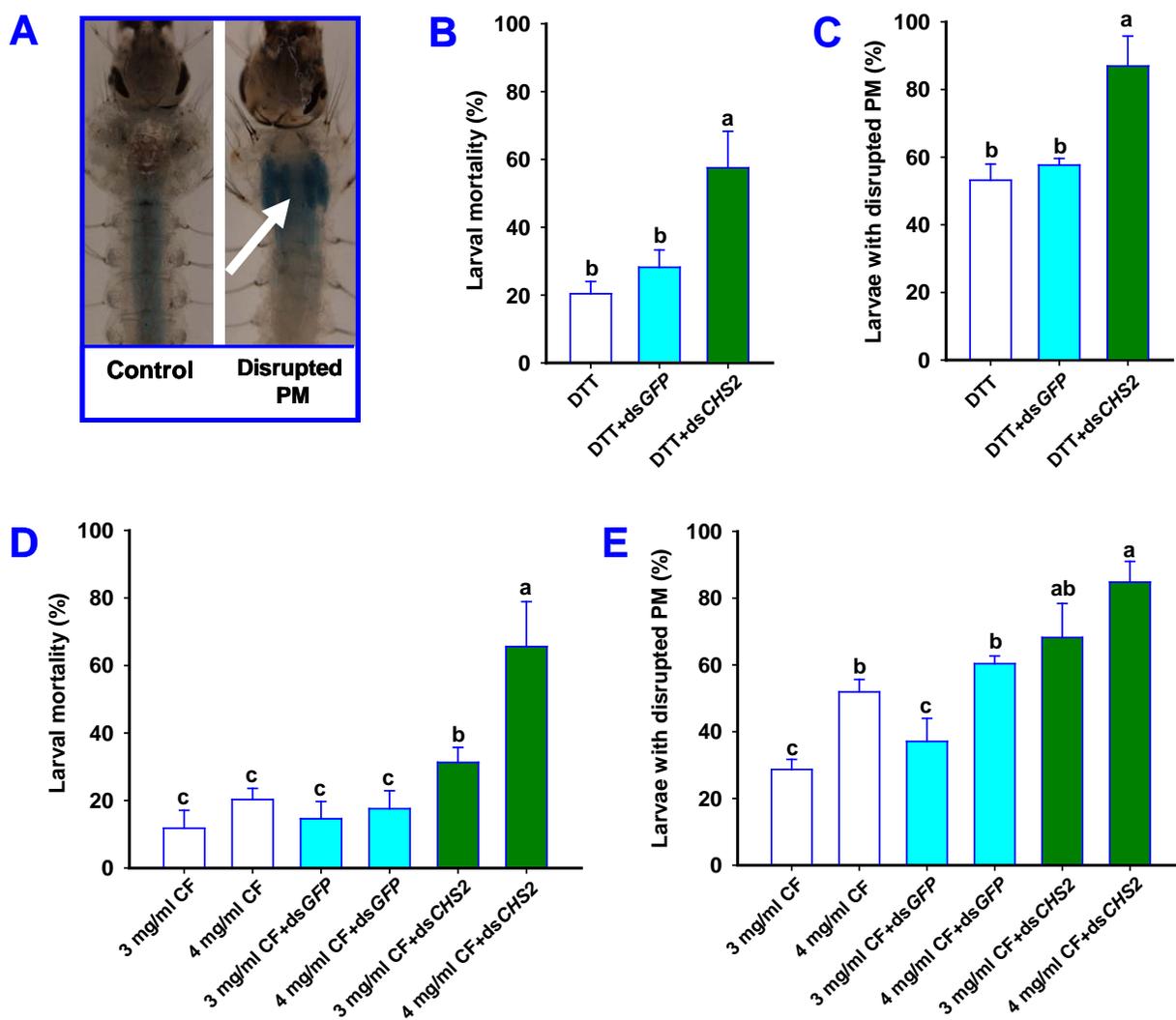
**Figure 5.2** Designs of double-stranded RNA (dsRNA) for oral RNAi, and repressions of transcript levels of two chitin synthase genes *AgCHS1* and *AgCHS2* after RNAi in mosquito larvae. (A, B) Diagrams illustrating the regions for designing two dsRNA fragments (i.e., f1 and f2) from each of the two genes (*AgCHS1* and *AgCHS2*). Arrow heads indicate the primer regions for examining the transcript level of each gene by quantitative real-time PCR. (C) Relative transcript levels of *AgCHS1* and *AgCHS2* in the larvae after continuously fed on each of the two *AgCHS1* dsRNA fragments (i.e., dsAgCHS1-f1 and dsAgCHS1-f2) or *green fluorescent protein* (GFP) dsRNA (dsGFP as controls) that were incorporated into the nanoparticles for 4 days (once a day). The data are presented as means  $\pm$  SEM of five replicate samples. (D) Relative transcript levels of *AgCHS1* and *AgCHS2* in the larvae after continuously fed on each of the two *AgCHS2* dsRNA fragments (i.e., dsAgCHS2-f1 and dsAgCHS2-f2) or GFP dsRNA (dsGFP as controls) that were incorporated into the nanoparticles for 4 days (once a day). The data are presented as means  $\pm$  SEM of three replicate samples. Same letters on the error bars indicate no significant difference based on Fisher's least significant difference test ( $P \geq 0.05$ ).



**Figure 5.3** Tissue-specific expression patterns of *Anopheles gambiae* chitin synthase gene (*AgCHS*) as analyzed by reverse transcription PCR in mosquito larvae. A ribosomal protein S3 gene (*AgRps3*) was used as an internal reference.



**Figure 5.4** Effect of nanoparticles-mediated oral RNA interference (RNAi) of *Anopheles gambiae chitin synthase 1 (AgCHS1)* on chitin content and larval susceptibility to diflubenzuron (DFB) in mosquito larvae. (A) Effect of the RNAi on chitin content. The data are presented as means  $\pm$  SEM of four replications ( $n = 4$ ). (B) Effect of the RNAi on susceptibility of the larvae to DFB. The data are presented as means  $\pm$  SEM of three replications ( $n = 3$ ). Different letters on the error bars indicate significant difference based on Fisher's least significant difference test ( $P \leq 0.05$ ).



**Figure 5.5** Effect of nanoparticles-mediated oral RNAi of *AgCHS2* on larval susceptibility and peritrophic matrix (PM) permeability to calcofluor white (CF) and dithiothreitol (DTT) in mosquito larvae. (A) Disruption of larval PM by displaying blue gastric caecae (GC) following ingestion of blue dextran. The arrow shows the GC filling with the dye when the PM was disrupted by treating mosquito larvae with DTT or CF. (B) Effect of the RNAi on larval susceptibility to DTT. (C) Percentages of the surviving larvae with disrupted PM from the same treatments as in (B). (D) Effect of the RNAi on larval susceptibility to CF. (E) Percentages of the surviving larvae with disrupted PM from the same treatments as in (D). All the data are presented as means  $\pm$  SEM of three replications. Different letters on the error bars indicate significant difference based on Fisher's least significant difference test ( $P \leq 0.05$ ).

(A) Comparison of the sequences between *AgCHS1-f1* and *AgCHS2* (identity 70.4%)

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AgCHS1-f1 TGAAACGCACATCTTCTTCGATGACGCGTTCG-AAATTTTCGGACC-----ACAGCGACGAGGACATTCACTGTAATC
AgCHS2 -GAAACTCACATTTTCTTCGACGACGCGTTCGTAATGATAAATCCAAGTGCAGAGTGCAGATGCCTCACCGCTCAATT

AgCHS1-f1 GGTTCGTGAAGATTCTTGTGACACCATCGACGAAAGCTGCCTCGGAAGTGCATCAGACCAACATTTCGATTGAGACCGCCC
AgCHS2 CCTACGTAAAGACGCTAATCAACCAATCGAAAGAGGCGGCCCTGGAAGTGTAACAACCAAGATGCGCGTAATTCGCCCC

AgCHS1-f1 AAAAAGTACCCAACAACCGTACGGTGGACGACTGGTGTGGACGCTGCCGGTAACACGAAGATGATTGCCACCTGAAGGA
AgCHS2 ACCAAGATTCGTCACCTCCATACGGTGGCCGATTGATCTGGACACTGCCGGGAAGGACGAATGATTGCAACCTCAAGGA

AgCHS1-f1 CAAGGATCGCATCCGGCACCGTAAGCGTTGGTCTCAGGTATGTACATGTACTATCTGCTCGGCACCCGGCTGATGGAGC
AgCHS2 CAATAACAAGATCCGACACAAGAAAGCGCTGGTCAAGGTTATGTACATGTACTACTCTGCTCGGCTACCGAATCATGACG

AgCHS1-f1 TGCCGATTTCCCTCGACCGTAAGGAAGGTGATGGCGGAAAACACCTACCTGCTGACGCT
AgCHS2 TGAACACATCGCCCGAGCGAAAGATGGTCACTCGCCAAAACACCTACCTGTTGGCACT

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(B) Comparison of the sequences between *AgCHS1-f2* and *AgCHS2* (identity 54.8%).

```

AgCHS1-f2 CAATAACGACGGACGAGAAGGCGCAGCTGATTCACATTGCCGATGCGCTGGACGCCATTACGAAGAAGATTGAGAACTGGG
AgCHS2 AAAGGCCAGCGCCGAAAGGAGCAAACTCGCAAAATTGCGGGCTTCGCTCAGCGAATAAGTGTAAAATGAAGGCACTGG

AgCHS1-f2 AAAAGCACATCGACCCGCACGGACATCATACGGGCAAGCGCACTGCGTCCGGCGGGTCAAGGATCACCACTCTCGGCTCG
AgCHS2 AAATGAA-ATTACCGGCAACGTCAACGTA-ATGAGATCAGACGACGAGATGATGACATCGGTAGTCTCGATATGCATC

AgCHS1-f2 GTGGCGGAGGATACGGAGGACGATGATGA-----GGATGAAGATTGGGAGACTTCGACGCTGCAG-----
AgCHS2 GTCCGGAAAGGTAACCGTGGCCCATCTCGCCATCTCCACCTCGGGCCCGCCCTCCCGATCCAGACGGTGAATAATGAT

AgCHS1-f2 ---CGCGACGAGCGGACTTCTCTCAACAAC-----CCGTACTGGATCGAGGATCCGGATCTGAAGAAGGCGAGGTGGA
AgCHS2 TCCCTCGAAAGAGCCCGAGAAAGCAATTAACATCTGCCGATTTGGTTGTACGACCTGGATTGAAAACGGCGACACTGA

AgCHS1-f2 CTTTATTTCCAGCCCGAGATCCAGTCTCGGAAGGACTGTATCGATAAGTACCTATACCCGATCGATCAAAAACAGGAGG
AgCHS2 GACGATCTCCGCTCGGAGGAGCAATCTCGGATTGAGCTGATCGAGAAGTATCTGAAGCCGCTCGATCTGTCGGAAGAGC

AgCHS1-f2 AACAGGCACGATTATGCG-CAC
AgCHS2 AGAAGGAAAGAGATGAAGTCAC

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(C) Comparison of the sequences between *AgCHS2-f1* and *AgCHS1* (identity 63.7%).

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AgCHS2-f1  ACACATCGAGTGGTGGTTCAACGTGAAGAACAGATCAGCTTTGACGAAAGCACGGTCGAGATCATGATCCGGCCGAGT
AgCHS1    CCACGTCAGTGGCCGTTGGGTGTTAAACTAACATTACCTATGATGAAGCCACGCAAGAGGTGCACATCTCCAAGGAGT

AgCHS2-f1  ACCTGGAGCTCGAACCGATCGGTTTGGTGTTTGTGATGTTCTTCGGTTTGATTCTCATCATTAGTTTGTGGCCATGCTG
AgCHS1    ATCTGCAGCTGGAACCGATCGGTCTGGTGTTTGTGTTCTTCTTCGCCCTGATTCTGATCATTAGTTTGTGGCAATGATG

AgCHS2-f1  ATGCATCGGTTCCGTACCATCTCCAGATTCTTGCCTCCACCGAGCTGAAGTGGTACTGTTTCAAGAAGGCTAAGGATAT
AgCHS1    TTCCATCGGTTCCGCACACTGTCCGACATTCTGGCCTCGACCGAGCTCAACTGGGCCTGCACCAAGAAGCCGGAGGAGCT

AgCHS2-f1  GTGACTCGATGCCAACTGCCGAGATGCCGTGAAATTGCACGTCGACTGCAGCGCC--CCAAACCGCGATGGG----
AgCHS1    TTCCGAGGATGCTCTCATAGACAGCACCGCTGTTGAGATAGTCAAGAAATTGCAACGACTGCAAGGCATCGATGGAGATT

AgCHS2-f1  ACGAGGAGGATCTAGAGGACGAGCAGAAAGCTATTGGACGTCGCGACACGATCCATCGCATTCTCTACCAAGCAAA
AgCHS1    ACGACATGATTGGGGCAGCGGTCCGGACCGCATCGACGCCGCCGCACGATCCA--GAACTCGAAAGGCACGG
  
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(D) Comparison of the sequences between *AgCHS2-f2* and *AgCHS1* (identity 53.8%).

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AgCHS2-f2  GCCAGCGCCGAAAAGGAGCAAATCG---CACAAATTGCGGCATCGCTCAGCGAA-----ATAAGTGTAAAAATGAA
AgCHS1    GGGTGGGATGAGGAGGGCTCGATCGACATCTCGATCGCGGGCTGTTCCGCTGTTTGCTCTGCACACAGCGCAAAAAGGAC

AgCHS2-f2  GGCACCTGGAATGAATTAACCGGCACGTCAGCGTAAATGAGATCAG--ACGACGAAGATGATGACATCGGTAG----TC
AgCHS1    GGCAGAGAAGGCGCAGCTGATTACATTGCCGATGCGCTGGAACCCATTACGAAGAAGATTGAGAACTTGGAAAAGCACA

AgCHS2-f2  TCGATATGCAT---CGTCGGAAGGTAACCGTGGCCCATCTCTCGCCATC-----CTCCACCTCGG-----GCGCCG
AgCHS1    TCGACCCGCACGGACATCATAACGCGCAAGCGCACTGCGTCCGGGGTCAAGGATCACCATCTCGGCTCGGTGGCGGAG

AgCHS2-f2  CCTCC-----CGATCCAGACGGTGAATAAT-GATTCCCTCGAG----AGCCGAGAAGCA-GATTAACTATCTG--CC
AgCHS1    GATACGGAGGACGATGATGAGGATGAAGATTCCGAGACTTCGACGCTGCAGCGGACGAGCGCGACTTCTCTGACCAACC

AgCHS2-f2  GGATTGGTTGTACGACGTGGATTGAAAACGGCGACACTGAGACGATCTCCGCTCGGAGGAGCAATTCTGGATTGAGC
AgCHS1    GTACTGGATCGAGGATCCGGATCTGAAGAAGGGCGAGGTGGACTTTATTTCAGCACCAGATCCAGTTCTGGAAGGACC

AgCHS2-f2  TGATCGAGAAGTATCTGAAGCCGCTCGATCTGTCGGAAAAGCAGAAGG
AgCHS1    TGATCGATAAGTACCTATACCCGATCGATCAAAACAAGGAGGAACAGG
  
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Figure 5.6 Alignments and identities of cDNA sequences for generating dsRNA fragments used in RNAi experiments.

## CHAPTER 6 - Summary

The availability of the genome sequences in *An. gambiae* makes it possible to identify all the genes involved in chitin metabolism. My dissertation represents a comprehensive study on two of the key gene families involved in chitin metabolism, chitin synthase (CHS) and chitinase (CHT), respectively. Knowledge on chitin synthases and chitinases in *An. gambiae* will result in a better understanding of chitin biosynthesis and degradation in mosquitoes and other arthropods. It may also lead to the development of new insecticides targeting on chitin metabolic pathway.

The identification and characterization of the two CHS genes in *An. gambiae* has significantly advanced our understanding on chitin synthesis in this model insect. Firstly, using RT-PCR and quantitative real time RT-PCR (qRT-PCR), it was determined that the both *AgCHS1* and *AgCHS2* were expressed in egg stage, indicating the involvement of CHSs in embryonic development. Secondly, using various gene expression analysis including RT-PCR, qRT-PCR, and in situ hybridization, it was determined that *AgCHS2* transcripts evenly distributed in the whole midgut epithelium cells in adult, whereas in the larval gut, *AgCHS2* transcripts localized in the cardia and the posterior midgut epithelium cells. This expression of *AgCHS2* in the posterior midgut implies new functions of *AgCHS2* besides producing the chitin associated with type 2 PM in mosquito larvae. Thirdly, immunohistochemistry analysis revealed that both *AgCHS1* and *AgCHS2* were highly distributed in the compound eyes in the pupae. In addition, *AgCHS2* was detected in the pupal inter-segments. These expression patterns reveal new information on the functions of each CHS in the pupal stage, although the exact biological meanings require further work.

With a genome-wide search, 20 chitinase and chitinase-like genes were identified and assigned into eight different chitinase groups (I-VIII) from *An. gambiae*. All these genes have been characterized to understand their developmental- and tissue-specific expression patterns. These 20 chitinase and chitinase-like genes showed highly diverse gene structures and domain structures. Our analyses of stage- and tissue-specific gene expression revealed that most of these genes were expressed in all tested stages and tissues. However, some genes were only expressed at certain stages and tissues. For example, *AgCht8* was mainly expressed in pupal and adult stages. *AgCht2* and *AgCht12* were specifically expressed in foregut, whereas *AgCht13* appears to be only expressed in midgut. Immunohistochemistry of selected chitinases in paraffin-embedded thin sections of *An. gambiae* indicated high expression of the proteins in certain body parts. This study is expected to provide new insights into the functions of the diverse chitinase genes in insects.

We report the enzymatic analysis and its inhibition of the chitin synthase activity by using a nonradioactive method. Our results showed that this method can successfully used to determine insect chitin synthase activity in the crude enzyme preparations . Optimal conditions for the enzyme activity were determined including the concentration of the substrate UDP-GlcNAc and  $M^{++}$ , pH, and temperature. Dithithreitol (DTT) was required for the activity to prevent melanization of the enzyme extract. Enzyme activity was enhanced at low concentration of GlcNAc, but inhibited at high concentrations. Proteolytic activation of the activity was significant both in the 500g supernatant and the 40,000g pellet. Diflubenzuron and one other chitin synthesis inhibitor nikkomycin Z showed very limited *in vitro* inhibition to chitin synthase activity at high concentrations in mosquito pupae by using a cell-free system, whereas no *in vivo* inhibition was observed.

Furthermore, the functional analysis of each CHS was studied by knocking down each transcript in the fourth instar larvae through the oral-delivered gene specific dsRNA/chitosan nanoparticles. Although RNAi is a conserved mechanism, it has been a great challenge in certain organisms or certain stages of an organism (e.g., mosquito larvae) as a result of the lack of delivery methods and/or possible lack of cellular uptake for dsRNA or siRNA. The *AgCHS1* transcript level and chitin content were reduced by 62.8 and 33.8%, respectively, in the larvae fed on the chitosan/*AgCHS1* dsRNA nanoparticles compared with those of the control larvae fed on the chitosan/GFP dsRNA nanoparticles. Our study suggested for the first time that RNAi in mosquito larvae was systemic, and clearly demonstrated that the larvae fed on the nanoparticles assembled from *AgCHS1* and *AgCHS2* dsRNA increased larval susceptibilities to diflubenzuron, and calcofluor white or dithiothreitol, respectively. These results suggest a great potential of using nanoparticle-based RNAi for high-throughput screening of gene functions and for developing novel strategies for mosquito control.

In conclusion, the characterization of all putative CHS and CHT genes in *An. gambiae* has led to an increased understanding of the biochemical processes behind the synthesis and breakdown of chitin in insects.