

THE MOLECULAR TARGET AND MODE OF ACTION OF THE ACYLUREA  
INSECTICIDE, DIFLUBENZURON

by

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B.S., Vinoba Bhave University, India, 2002  
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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Graduate Biochemistry Group

KANSAS STATE UNIVERSITY  
Manhattan, Kansas

2015

## Abstract

In this study, I have used dsRNA-mediated down-regulation of transcripts/proteins of several potential targets in the model beetle, *Tribolium castaneum* to identify the molecular target of the “chitin inhibitor”, diflubenzuron (DFB). The elytron of the red flour beetle, *T. castaneum*, was chosen as the model tissue for studying the mode of action of DFB and its molecular target(s). We have standardized the protocol for topical administration of DFB on precisely aged prepupae to achieve the desired level of mortality (90-95%) on day 5 of the pharate adult stage. Exposure of prepupae to DFB at 1000 ppm results in a near complete prevention of chitin in the newly forming adult procuticle of the elytron and the body wall. Whole-body analysis of transcripts by RNA Seq was carried out to look for differential expression of several critical genes of cuticle assembly in these insects compared to mock-treated controls. Interestingly, genes directly involved in the biosynthetic pathway of chitin were not among those affected by DFB. However, immunolocalization studies have shown that several proteins of chitin metabolism including chitin synthase A, which is involved in the synthesis of cuticular chitin are present in near normal amounts but are mislocalized in DFB-treated insects. Assays for chitin synthase using elytral extracts have indicated that the enzyme preparations from DFB-treated insects are inactive. By using RNA interference and competition studies with fluorescently tagged glibenclamide (a sulfonylurea compound) or DFB, we have identified a long-sought molecular receptor of DFB as an ABCC class transporter. DFB-treatment or RNAi of a specific ABCC-type transporter gene lead to identical phenotypes including loss of chitin, loss of laminar architecture of the cuticle,

and mislocalization of CHS from its normal plasma membrane location to intracellular locations presumably by affecting vesicular transport. Further, using an *in vitro* chitin synthesizing system consisting of microsomes prepared from elytral tissue, which exhibits all of the hallmarks of cuticular chitin-synthesizing epidermal cells including sensitivity to DFB, further insights into the mechanistic details of how this class of insecticides inhibits chitin synthesis have been obtained.

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Dr. Subbaratatnam Muthukrishnan

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locations presumably by affecting vesicular transport. Further, using an *in vitro* chitin synthesizing system consisting of microsomes prepared from elytral tissue, which exhibits all of the hallmarks of cuticular chitin-synthesizing epidermal cells including sensitivity to DFB, further insights into the mechanistic details of how this class of insecticides inhibits chitin synthesis have been obtained.

## Table of Contents

List of Figures .....	xii
List of Tables .....	xv
Acknowledgements .....	xvi
Chapter 1 .....	1
What is insect cuticle and what are its components? .....	2
Chitin metabolism proteins associated with cuticle .....	4
Chitin and chitin synthase .....	5
Benzoylphenylureas: chitin synthesis inhibitor-group of insecticides .....	10
Various effects of DFB .....	14
Other benzoylphenyl ureas .....	15
The ABC-transporter gene family .....	15
References .....	21
Chapter 2 .....	25
Introduction .....	26
Materials and Methods .....	29
<i>Tribolium</i> strains: .....	29
RT-PCR method .....	29
DFB treatment .....	29
Chitin content analysis .....	30
Immunohistochemistry and confocal analysis .....	30
Staining with Bodipy-DFB or Bodipy-Glibenclamide .....	30
Transmission electron microscopy .....	31
Vesicle preparation .....	31
Real Time PCR .....	32
Immunoblot analysis .....	32
Results .....	33
Validation for topical application of DFB to <i>T. castaneum</i> .....	34
Reduction of chitin and chitosan content in DFB-treated insects at pharate adult stage .....	36



DFB-treatment led to loss of laminar organization in the cuticle .....	38
DFB-treatment resulted in mislocalization of TcKnk protein .....	39
DFB treatment also resulted in mislocalization of TcCDA-1 protein .....	42
DFB treatment did not result in mislocalization of Chitinase-5 .....	43
Down-regulation of CHT-5 and 10 did not restore the chitin levels in the procuticle .....	45
Localization of TcCHS-A protein in DFB treated insects .....	47
DFB treatment reduced the apical plasma membrane associated CHS-A protein....	49
TcCHS-A protein processing is not affected by DFB.....	51
RNAi of different ABCC transporters identifies the molecular target of DFB .....	53
RNAi of ABC-C9A RNAi causes depletion of chitin in the procuticle .....	56
ABCC-9A RNAi and DFB treatment lead to abnormal structural organization of the procuticle /Body wall.....	58
CHS-A protein is mislocalized following RNAi of TcABCC-9A and DFB-treatment .....	59
Bodipy-Glibenclamide and DFB compete for the same receptor .....	61
<i>TcABCC-9A</i> gene encodes the DFB receptor protein in midgut.....	64
Loss of Bodipy-DFB staining after ABCC-9A RNAi in Ventral abdominal epidermal cells/Body wall.....	67
Transcript levels of chitin metabolism genes are not significantly altered after DFB treatment .....	70
Discussion .....	71
References.....	75
Chapter 3 .....	78
An in vitro system for chitin synthesis using vesicles from elytra of <i>Tribolium</i> <i>castaneum</i> .....	79
Materials and Methods: .....	82
<i>Tribolium</i> strains .....	82
DFB treatment.....	82
Vesicle preparation .....	82
Measurements of chitin synthesis .....	83

$^{45}\text{Ca}^{2+}$ uptake assay .....	83
Results.....	84
Membrane preparations from <i>T. castaneum</i> elytra have chitin synthase activity .....	84
Vesicle-mediated CHS activity of different supernatant fractions.....	84
Light microscopy of different vesicle preparation showing presence (A and B) and absence of (C) vesicles.....	87
Nikkomycin (chitin synthase inhibitor) inhibits <i>in vitro</i> chitin synthesis.....	88
Chitin synthase activity of vesicles is influenced by the concentration of $\text{K}^+$ ions in the reaction mixture. ....	89
Effect of various concentrations of $\text{CaCl}_2$ on the synthesis of chitin <i>in vitro</i> .....	91
Vesicle-mediated CHS activity is sensitive to inhibition by DFB and glibenclamide .....	93
Vesicular integrity is required to maintain susceptibility to DFB .....	94
Inhibitory effect of DFB was reversed by addition of KCl or $\text{CaCl}_2$ .....	96
Inhibitory effect of DFB is reversed by the $\text{K}^+$ and $\text{Ca}^{2+}$ ionophores .....	98
The role of $\text{K}^+$ is to allow entry of $\text{Ca}^{++}$ into vesicles.....	99
$\text{Ca}^{++}$ is absolutely required for CHS activity .....	101
CHS activity of vesicles is sensitive to ATP/ADP ratio .....	103
$^3\text{H}$ -Chitin product synthesized was sensitive to degradation by <i>M. sexta</i> chitinase and the chitin product is secreted out of the vesicles.....	105
The loss of CHS activity of vesicles following <i>in vivo</i> DFB treatment or RNAi of <i>ABCC-9A</i> was not reversed by 100 mM KCl or 2 mM $\text{CaCl}_2$ .....	107
Isolated vesicles do take up $^{45}\text{Ca}^{2+}$ from the medium.....	109
$^{45}\text{Ca}^{2+}$ Uptake by vesicle is inhibited by DFB in a dose-dependent manner .....	110
Reversal of DFB effects on $\text{Ca}^{++}$ uptake by valinomycin, A23187 and KCl .....	112
ADP reversed the inhibitory effect of ATP on $\text{Ca}^{2+}$ uptake .....	114
Discussion.....	115
Proposed mechanism of chitin synthesis in microsomal vesicles.....	120
Proposed mechanism of inhibition of chitin synthesis by DFB in microsomal vesicles .....	122
References.....	124

Chapter 4 .....	126
General Discussion .....	127
DFB causes mislocalization of enzymes of chitin metabolism .....	127
Identification of the target receptor of DFB .....	129
Mode of action of DFB .....	133
A model of regulation of CHS intact vesicle .....	135
Model depicting how DFB affects vesicular transport of chitin metabolism protein and chitin synthesis via its action on SUR/K <sub>ir</sub> channels <i>in vivo</i> . The model presented below is representing DFB treated condition of epidermal cell ( <i>Tribolium</i> <i>castaneum</i> ). .....	138
Future Directions .....	139
References .....	143

## List of Figures

Figure 1.1 Structure of insect cuticle .....	4
Figure 1.2 Alternative models of chitin formation in insects .....	7
Figure 1.3 Schematic representation of the domain structures of insect enzymes involved in chitin synthase A and chitin synthase B. ....	10
Figure 1.4 Structure of diflubenzuron.....	12
Figure 1.5 ABC full transporter structure .....	17
Figure 1.6 Pictorial model for showing mode of action of sulfonyl urea receptor .....	18
Figure 2.1 Validation for topical application of DFB to <i>T. castaneum</i> .....	35
Figure 2.2 Chitin and chitosan analysis of DFB-treated pharate adults. ....	37
Figure 2.3 DFB treatment led to loss of laminar organization in the cuticle.....	38
Figure 2.4 DFB treatment resulted in mislocalization of TcKnk protein .....	41
Figure 2.5 DFB-treatment also resulted in mislocalization of TcCDA-1 protein.....	42
Figure 2.6 DFB treatment did not resulted in mislocalization of chitinase-5 .....	44
Figure 2.7 Down-regulation of CHT-5 and 10 did not restore the chitin levels in procuticle.....	46
Figure 2.8 Localization of TcCHS-A protein in DFB treated insects.....	48
Figure 2.9 DFB treatment reduced the apical plasma membrane associated CHS-A protein .....	50
Figure 2.10 TcCHS-A protein processing is not affected by DFB. ....	52
Figure 2.11 RNAi of different TcABCC transporters identifies the molecular target of DFB.....	55
Figure 2.12 RNAi of ABCC-9A cause's depletion of chitin in the procuticle .....	57
Figure 2.13 ABCC-9A RNAi and DFB treatment led to abnormal organization of the procuticle of Body wall.....	58
Figure 2.14 CHS-A protein is mislocalized following RNAi of TcABCC-9A and DFB treatment .....	60
Figure 2.15 Bodipy-Glibenclamide and DFB compete for same receptor .....	63
Figure 2.16 TcABCC-9A gene encode the DFB receptor protein in midgut .....	66

Figure 2.17 Loss of BODIPY-DFB staining after <i>TcABCC-9A</i> RNAi in ventral abdominal epidermal cells .....	69
Figure 3.1 Vesicle-mediated CHS activity of different supernatant fractions.....	85
Figure 3.2 Light microscopy of different vesicle preparation showing presence and absence of vesicle.....	87
Figure 3.3 Nikkomycin inhibits <i>in vitro</i> chitin synthesis.....	89
Figure 3.4 Chitin synthase activity of vesicle is influenced by the concentration of K <sup>+</sup> ions in the reaction mixture.....	90
Figure 3.5 Effect of various concentration of CaCl <sub>2</sub> on the synthesis of chitin <i>in vitro</i> ...	92
Figure 3.6 Vesicle-mediated CHS activity is sensitive to inhibition by DFB and Glibenclamide .....	93
Figure 3.7. Vesicular integrity is required to maintain susceptibility to DFB.....	95
Figure 3.8 Inhibitory effect of DFB was reversed by addition of KCl or CaCl <sub>2</sub> .....	97
Figure 3.9. Inhibitory effect of DFB is reversed by K <sup>+</sup> and Ca <sup>2+</sup> ionophores .....	98
Figure 3.10 The role of K <sup>+</sup> is to allow entry of Ca <sup>2+</sup> into vesicles.....	100
Figure 3.11 Ca <sup>2+</sup> is absolutely required for CHS activity.....	102
Figure 3.12 CHS activity is sensitive to ATP/ADP ratio.....	104
Figure 3.13 <sup>3</sup> H-Chitin product synthesized was sensitive to chitinase and chitin product is secreted out of the vesicles.....	106
Figure 3.14 The loss of CHS activity of vesicles following <i>in vivo</i> DFB treatment or RNAi of <i>ABCC-9A</i> was not reversed by 100 mM KCl or 2 mM CaCl <sub>2</sub> .....	108
Figure 3.15 Isolated vesicles do take up <sup>45</sup> Ca <sup>2+</sup> from medium .....	109
Figure 3.16 <sup>45</sup> Ca <sup>2+</sup> uptake by vesicle is inhibited by DFB in a dose-dependent manner	111
Figure 3.17 Reversal of DFB effects on Ca <sup>2+</sup> uptake by Valinomycin, A23187 and KCl .....	113
Figure 3.18 ADP reversed the inhibitory effect of ATP on Ca <sup>2+</sup> uptake.....	114
Figure 3.19 Proposed mechanism of chitin synthesis in microsomal vesicles .....	120
Figure 3.20 Proposed mechanism of inhibition of chitin synthesis by DFB in microsomal vesicle .....	122
Figure 4.1 A model of regulation of chitin metabolism proteins in <i>T.castaneum</i> .....	137

Figure 4.2 The model representing DFB treated condition of epidermal cell ( <i>T. castaneum</i> ) .....	138
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## **List of Tables**

Table 1.1 Various effects of DFB .....	14
Table 2.1 Transcript levels of chitin metabolism genes after DFB-treatment .....	71
Table 4.1 Read for different ABCC transporters .....	131

## **Acknowledgements**

I am extremely grateful to Dr. Subbaratnam Muthukrishnan for accepting me as his student and for constantly guiding me all the time of my Ph.D. It was a very memorable experience working with him where he constantly took interest to correct me in research and tried hard to improve my approach towards experiments. I feel immensely lucky to get guidance from him for my doctoral study. His approach and enthusiasm towards science has always inspired me to achieve higher and higher. I am also indebted to him for his constant affection all through my PhD. He has been instrumental in solving problems at critical points that were vital for research. I will remain obligated to him my entire life for being critical at some points which were necessary for success in my research.

I am also extremely grateful Dr. Karl J. Kramer and Dr. Richard W. Beeman for constantly guiding me in each research steps that I took, and for providing critical inputs for any improvements needed in the experimental design. I want to thank Dr. Michael Kanost who was always there for me whenever needed and towards whom I constantly looked for guidance. At this moment, it will be highly appropriate to express my extreme gratitude towards Dr. Kun Yan Zhu who accepted to be my committee member and always helped me in the research.

Another two very important persons who always supported and helped me during my PhD are Dr. Hans Merzendorfer and Dr. Yasuyuki Arakane. They constantly discussed and provided critical inputs for my results and experiments.

I also thank my husband Dr. Amit Kumar, my parents, and In-laws for their constant support throughout my Ph.D. I thank my friend Aashima for being my



friend who was always there for me whenever needed. I have no words left to thank my fellow lab members, Sujata Suresh Chaudhary and Sinu Jasrapuria who always guided and helped in in the lab. Last but not the least, I also want to thank Dr. M. M. Chengappa, Department Head of Diagnostic Medicine/Pathobiology at College of Veterinary Medicine and Dr. T. G. Nagaraja, professor in the Department of Diagnostic Medicine/Pathobiology for their emotional support as a family during my entire period of stay in Manhattan, Kansas.

# Chapter 1

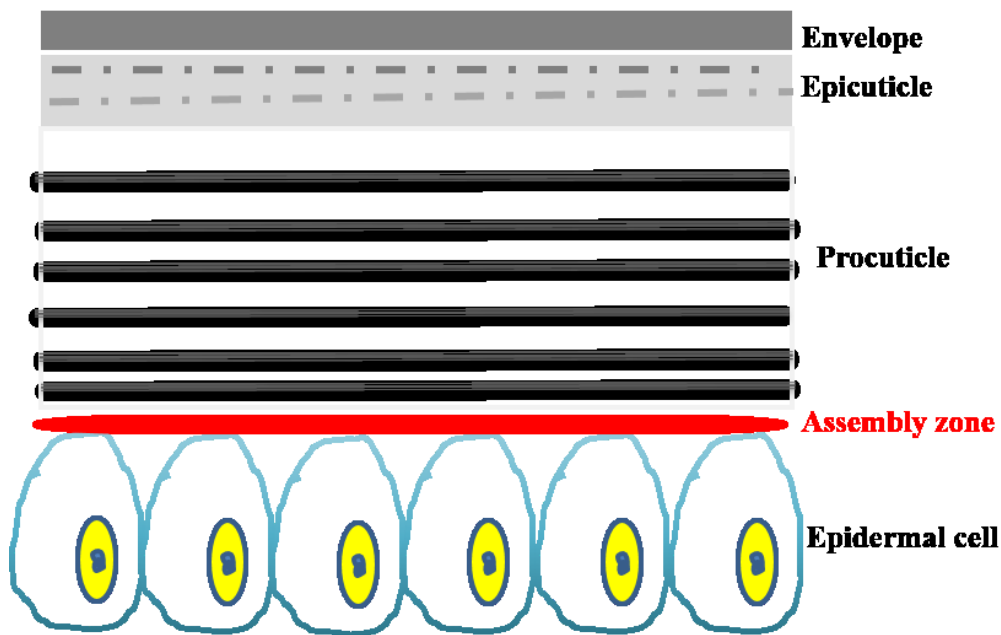
## Literature review

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## **What is insect cuticle and what are its components?**

Insect cuticle an exoskeleton serves not only as a physical barrier against mechanical and biological injury, but it also fulfills other physiological functions including the maintenance of structural integrity of the underlying epidermal cells while allowing sensory interactions with the environment and muscle attachment. The cuticle is a composite extracellular matrix consisting of an outermost layer of waterproofing envelope, below which there is a thin layer of epicuticle consisting of proteins cross-linked via quinones. Situated between the epicuticle and the apical plasma membrane of the underlying epidermal cells is the laminar procuticle consisting of several parallel layers of chitin complexed with cuticular proteins. A thin layer of amorphous fibers in the process of becoming laminae is often found between the apical plasma membrane and the laminar procuticle. We arbitrarily call this region the “assembly zone” (Locke, 1961; Moussian, 2010; Willis 2012). These cuticular proteins have two major types of chitin-binding motifs. The first motif is found in a group of proteins named Cuticular Proteins Analogous to Peritrophins (CPAPs) with a six-cysteine containing sequence motif known as the peritrophin-A domain (ChtBD2; CAZY), shown to bind to chitin (Tellam et al., 1999; Jasrapuria et al., 2010). The proteins of the second, larger group of cuticular proteins contain the histidine-rich (Rebers & Riddiford) consensus motif that has also been proposed to confer affinity for chitin (Willis 2012). The procuticle consists of a highly variable number of laminae (depending on developmental stage and location within the body plan) made up of coaxial bundles of chitin-protein complexes stacked parallel to the cell surface. The laminae are stacked on one another in helicoidal stacks or in orthogonal stacks (Cheng et al., 2009; Raabe et al., 2006). The stacks of laminae are

also interrupted by “pore canals” (also made of chitin and protein) that traverse the stacks of laminae in a perpendicular fashion and reach the epicuticle layer. In this study, I have worked with a model epidermal tissue, the coleopteran elytron, a modified forewing structure (Tomoyasu et al., 2009), which is capable of making a thick dorsal cuticle. The major differences between the elytron, which is comparatively hard, rigid, thick and highly pigmented, and the more ancestral hindwing is that the latter is soft, flexible, much less pigmented and has a much thinner cuticle (Tomoyasu et al., 2009). We have chosen to work primarily with the elytron as a model cuticle-forming tissue, because it can be separated from other tissues easily and cleanly, enabling us to follow precisely the sequential processes that occur during development of a well-defined cuticular structure. These processes include dynamic deposition and modification of chitin fibers and proteins, and formation of laminae containing chitin fiber bundles in the procuticle simultaneously with degradation of the cuticle of the previous developmental stadium by molting fluid enzymes (Locke & Huie 1979; Muthukrishnan et al., 2012). The elytron/hindwing model system is amenable to chemical/biochemical analysis for chitin/chitosan and proteins, and also is suitable for TEM, SEM and confocal microscopic studies using immunological and other probes to delineate various aspects of the ultrastructure of the cuticle



**Figure 1.1** Structure of insect cuticle

### **Chitin metabolism proteins associated with cuticle**

There are many chitin metabolism proteins such as chitin synthases, chitinases, and chitin deacetylases, as well as other chitin-organizing proteins with chitin-binding potential, including Knickkopf (Knk) and several members belonging to the family of Cuticular Proteins Analogous to Peritrophins (CPAPs) (Jasrapuria et al., 2010). They all are important for the formation and organization of cuticle. Two classes of chitin synthases (CHSs) have been described in most orders of insects with the exception of it hemiptera and Anapleura. Chitin synthase-A (CHS-A) has been shown to be the enzyme responsible for cuticular chitin synthesis in epidermal cells in several insect species and nematodes by RNAi studies ( Arakane et al., 2004; 2005; 2009; Tian et al., 2009; Zhang et al., 2010; Zhang et al., 2005). CHS-B is responsible for the synthesis of chitin

associated with the peritrophic matrix (Arakane et al., 2005; Hogenkamp et al., 2005; Zimoch et al. 2005; Chen et al., 2007). Degradation of chitin is carried out by a large family of enzymes that have been grouped into several classes. The *Tribolium castaneum* enzyme TcCHT-5 is the major chitinase found in the molting fluid and it is needed for turnover of the old cuticle (Zhu et al., 2008). Chitin deacetylase-like proteins belonging to five different groups have also been identified in several insect genomes. CDA1 and CDA2 groups are needed for maturation and for maintenance of proper tracheal tubule diameter and shape during embryonic development of *D. melanogaster* (Wang et al., 2006; Luschnig et al., 2006). Another cuticle-associate protein called Knickkopf (KNK) has been shown to bind to and organize chitin into laminae and to protect it from degradation by chitinases in the molting fluid. KNK (as well as its parlogs, KNK2, KNK3 and another protein, Retroactive) , CDA1 and CDA2 are essential for molting and all are needed for the arrangement of chitin as parallel stacks of laminae in the procuticle (Moussian et al., 2010 Chaudhari et al., 2011; 2013; 2014; Arakane et al., 2009).

### **Chitin and chitin synthase**

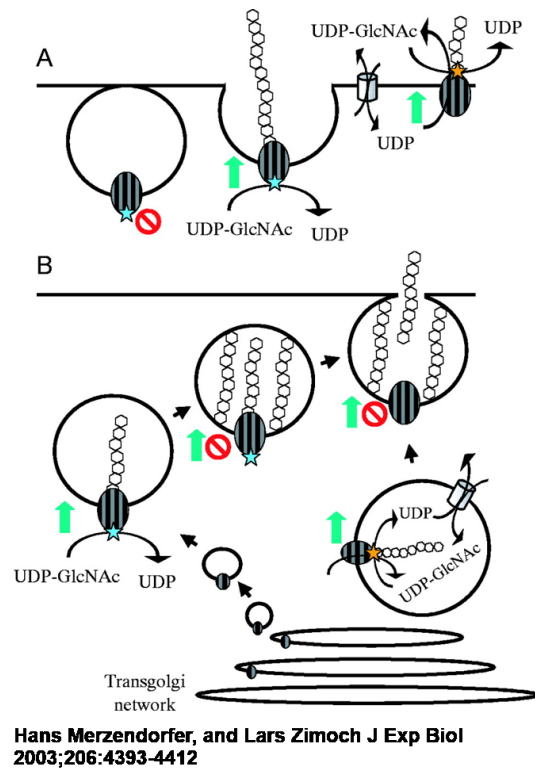
Chitin is a linear polymer of N-acetyl- $\beta$ -D-glucosamine. It is mostly present in the exo- and endocuticle or in newly secreted, unsclerotized procuticle but is absent from the outermost part of the cuticle. Chitin is also present in insect's peritrophic matrix, which acts as a permeability barrier between the food bolus and the midgut epithelium. It is an N-acetylated amino polysaccharide, which is, mostly present in fungal cell wall, arthropod exoskeleton and insect egg shell or nematode eggshells. Chitin is formed by linking N-acetylglucosamine residues, by the  $\beta$ -(1-4) - glycosidic bond. Chitin chains

self-associate to form microfibrils with ~20 chains that are antiparallel to form co-axial  $\alpha$ -chitin bundles, with a diameter of ~3 nm. The bundles are stabilized by both interchain and intrachain H-bonds involving the acetylamino side chains and C6-hydroxyl groups. Through x-ray diffraction analysis, three different forms of chitin were found. They are named  $\alpha$ -,  $\beta$ -, and  $\gamma$  which differ by degree of hydration number of chitin chains per unit cell and chitin orientation.

**$\alpha$ :** all chains are in antiparallel orientation. It is prevalent in chitinous cuticle and are tightly packed with multiple inter-chain hydrogen bonds.

**$\beta$ :** Chains are arranged in a parallel orientation and are less tight adjacent chains are held together by van der Waals forces and are hydrated. It is present in the peritrophic matrix.

**$\gamma$ :** consists of two parallel strands that alternate with a single antiparallel strand and is found in cocoons. The packing is less tight than that of that of  $\alpha$ -chitin.



**Figure 1.2 Alternative models of chitin formation in insects**

Chitin synthase (CHS) catalyzes the synthesis of chitin. CHS belongs to  $\beta$ -glycosyltransferase family and is a large integral membrane protein. CHS-A is expressed in the epidermis, and tracheae, and CHS-B is mainly expressed in gut epithelial cells.



(Arakane et al., 2004; Merzendorfer and Zimoch, 2003; Tellam et al., 2000). The specific mechanism by which chitin is produced is still not clear. There are two different possibilities of chitin formation in insects (Hans Merzendorfer, and Zimoch J Exp Bio 2003).

(A) The first speculative model suggests a constitutive secretory pathway in which CHS-loaded vesicles are transported from the trans-Golgi network to apical parts of epithelial cells where they fuse with the plasma membrane. After fusion with the plasma membrane, chitin synthase may get activated by molting fluid-associated or gut lumen proteolytic enzymes.

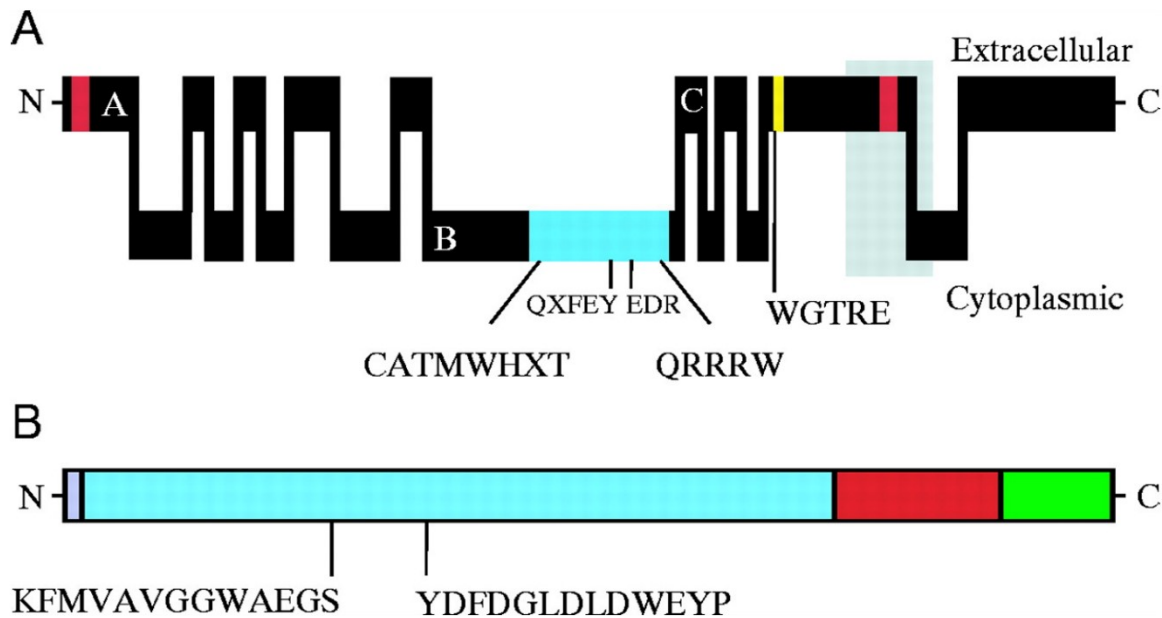
(B) The other speculative model suggests that an active chitin synthase is present in vesicles and that they make chitin before they fuse with the plasma membrane. After fusion with the plasma membrane, either they continue to make chitin or stop making the chitin.

Insect chitin synthases are a transmembrane protein whose size varies from 160 kDa to 180 kDa, with isoelectric points of pH 6.1 to 6.7 a tripartite domain structure.

Schematic representation of the domain structures of insect enzymes involved in chitin metabolism. (A) Domain structure and membrane topology of the *Manduca sexta* chitin

synthase 1 (accession no. AY062175) with all the three domains Domain A is present at N-terminal region and has varying number of transmembrane helices. Domain B is present at the center of CHS containing ~400 amino acids and contains the catalytic center of the protein. The B domain contains the EDR and QRRRW motifs which are highly conserved in CHSs from fungi and arthropods. Domain C is present at C-terminal part of the enzyme. It contains seven transmembrane helices and is far less conserved than the catalytic domain.

**Schematic representation of the domain structures of insect enzymes involved in chitin metabolism.**



Hans Merzendorfer, and Lars Zimoch J Exp Biol  
2003;206:4393-4412

**Figure 1.3** Schematic representation of the domain structures of insect enzymes involved in chitin synthase A and chitin synthase B.

B. Schematic representation of insect chitinases Blue, N-terminal signal peptide; Teel, catalytic domain with  $\beta_8 \alpha_8$  barrel structure; red, serine-threonine-rich linker domain; green, C-terminal peritrophin-A type chitin-binding domain with 6 cysteines.

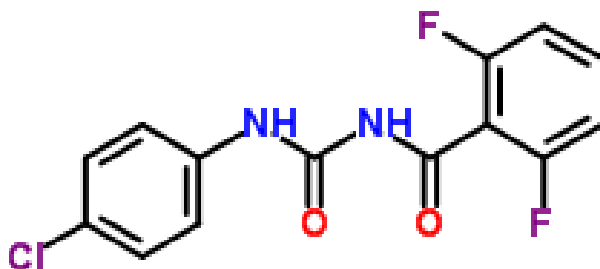
**Benzoylphenylureas: chitin synthesis inhibitor-group of insecticides**

Insect growth regulators are used to control insect pests, and they interfere with the growth of insects. Some ecdysone agonists like tebufenozide, methoxyfenozide, and juvenile hormone interfere with molting and metamorphosis. On the other hand,

benzoylphenyl urea compounds such as diflubenzuron [DFB: 1-(4-chlorophenyl)-3-(2, 6-diflubenzoyl) urea] and its derivatives reduce cuticular chitin content. DFB comes under the benzoylphenyl urea group of compounds. Benzoylphenylureas are a class of compounds with a central urea moiety. The diflubenzuron benzoyl moiety has two ortho-fluorine substitutions and para-chloride substitutions occurs on the phenyl moiety. DFB and other acylurea derivatives have been used effectively for insect control for over 40 years. Oral and topical administration of acylureas has been shown to have a variety of detrimental effects on insect growth, development and fecundity, accompanied by a reduction in cuticle thickness and loss of laminar organization, reduction in total chitin content, leading to molting arrest and death (Cohen 2001; Cohen & Casida, 1980; Gangishetti et al., 2009; Matsumura, 2010; Matsumura, 2010; Mayer et al., 1981; Merzendorfer et al., 2012; Sun et al., 2015; Soltani et al., 1984; Zhang & Zhu, 2006). Mature *Drosophila* females fed a diet containing DFB, laid normal numbers of eggs but they failed to hatch due to the inability of developing larvae to break open the eggshell (Wilson & Cryan 1977; Merzendorfer et al., 2012). Even though numerous cellular processes are known to be affected by acylureas, the precise mechanisms of disruption of the chitin metabolic pathway leading to reduced cuticular chitin have not been elucidated in any insect species in spite of many attempts to do so (Cohen 2001; Cohen & Casida, 1980; Mayer et al., 1981; Soltani et al., 1984; Gangishetti et al., 2009; Zhang & Zhu, 2006; Matsumura, 2010; Merzendorfer et al., 2012). Studies using several different insect species have led to the conclusion that transcript levels for *Chs-A*, the enzyme responsible for chitin synthesis in epidermal cells, appeared to be either unaffected or marginally elevated in insects treated with DFB (Zhang & Zhu 2006;

Merzendorfer et al., 2012). In fact, a substantial increase in CHS activity was reported using microsomal membrane preparations from *T. brevicornis* two days following DFB administration, even though the dropped to normal levels two days later (Cohen & Casida, 1980). While levels of CHS protein have not been analyzed systematically, attempts to show a direct effect of DFB on CHS activity in *in vitro* enzyme assays using solubilized membrane preparations of *Stomoxys calcitrans*, *T. castaneum* or *Manduca sexta* larvae did not demonstrate any inhibition of CHS activity even at high (>100  $\mu$ M) concentrations of DFB (Cohen & Casida 1980; Zimoch et al., 2005). On the other hand, low concentrations (100 ppm or less) DFB were quite effective in inhibiting incorporation of chitin precursors into chitin in *in vivo* experiments that utilized incubations of intact integument with radioactive chitin precursors (Nakagawa & Matsumura, 1993, 1994). However, there is one report of inhibition of chitin synthesis by DFB (1  $\mu$ g/ml) using a “vesiculated” microsomal preparation from the brine shrimp, *Artemia salina* (Horst, 1981). For inexplicable reasons this work did not attract much attention.

#### Structure of diflubenzuron



**Figure 1.4** Structure of diflubenzuron

Chitin synthesis occurs in polarized epidermal cells, with sugar precursor, trehalose entering through the basal lamina from the hemolymph. Chitin synthesis takes place in the apical plasma membrane. Vesicle preparations from the German cockroach, *Blattella germanica* showed inhibition of  $^{45}\text{Ca}^{++}$  uptake by DFB and stimulation by ATP (Matsumura, 2010). The vesicles carrying CHS are probably formed in endoplasmic reticulum and then they move to Golgi for maturation followed by migration to the plasma membrane and exocytosis to the tip of microvilli, where nascent chitin microfibrils are extruded. In the case of DFB treated vesicles exocytosis occurs before chitin is formed and the process comes to halt. This hypothesized that diflubenzuron inhibits the transport of chitin synthase has been suggested many years ago. Some other studies have suggested that chitin synthase enzyme is not directly inhibited by diflubenzuron but that the processing of the enzyme itself gets inhibited. However, processing of fungal chitin synthase was not affected by DFB because it was processing differently.

## Various effects of DFB

Inhibition of incorporation of $^3\text{H}$ -N-acetylglucosamine in isolated newly molted American cockroach integument	Nakagawa et al., (1993)
Inhibition of incorporation of $^3\text{H}$ -thymidine into DNA of imaginal disks from epidermal histoblast tissue from <i>Stomoxys calcitrans</i>	Mayer et al., (1983)
Detrimental effects on insect growth, development and fecundity, accompanied by reduction in cuticle thickness and loss of laminar organization, reduction in total chitin content, leading to molting arrest and death	Gangishetti et al., (2009); Merzendorfer et al., (2012); Mulder & Gijwijt (1973); Verloop & Ferrell (1977)
Failure of egg hatch in <i>Drosophila</i> due to the inability of developing larvae to break open the eggshell	Wilson & Cryan (1977); Merzendorfer et al., (2012); Mansur et al., (2010)
No reduction in transcript levels for Chs-A	Zhang & Zhu 2006; Ashfaq et al., 2007; Merzendorfer et al., 2012
Increase in CHS activity in microsomal membrane preparations from <i>T. brevicornis</i> followed by drop to normal levels two days later	Cohen & Casida (1980)
No inhibition of CHS activity	Zimoch et al., 2005
Inhibition of incorporation of chitin precursors into chitin in <i>in vivo</i> experiments using intact integuments or partially chopped integuments	Nakagawa & Matsumura, 1993; 1994
Inhibition of chitin synthesis using a “vesiculated” microsomal preparation from the brine shrimp, <i>Artemia salina</i>	Horst (1981)
Inhibition of calcium uptake in intracellular vesicles prepared from the homogenate of newly molted American cockroaches	Nakagawa & Matsumura (1994)
Inhibition of the pupal-adult development in <i>T. molitor</i>	Soltani, et al., (1984)
Alterations in mechanical penetrability and chitin content of the elytra of <i>Leptinotarsa decemlineata</i>	Grosscurt (1978)
Disrupted production of peritrophic membrane	Becker (1980); Clarke (1977)

**Table 1.1 Various effects of DFB**

### Other benzoylphenyl ureas

**Novaluron:** Novaluron was discovered in 1990. It is mostly effective on several lepidopteran, coleopteran, and homopteran and dipteran pests.

**Lufenuron:** It is more active than DFB. It is mostly used in controlling the mushroom sciarid fly and *Lycoxiella ingenua*. It has larvicidal, ovicidal and ovi-larvicidal effects.

**Flucycloxurons:** This is a second generation benzoylphenyl urea. It has shown to have ovicidal and ovi-larvicidal effects. It has a wider spectrum than DFB. Flucycloxurons toxicology profile is similar to that of diflubenzuron.

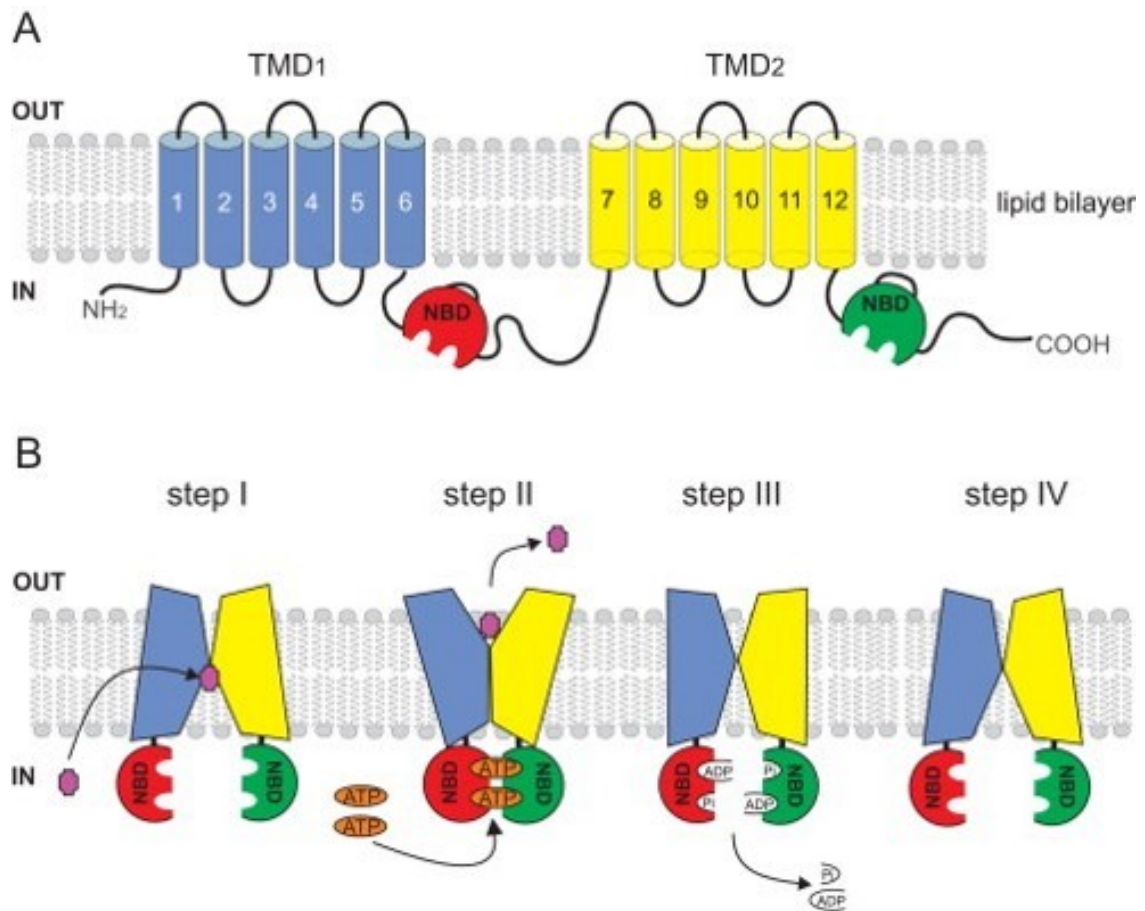
### The ABC-transporter gene family

Cells and organelles are surrounded by lipid membranes and they transport a wide variety of compounds across these membrane with the help of transporters. The transporter family members are present in all kingdoms of life and contain one or two ATP-binding cassettes (ABC). Most of the ABCs act as a primary active transporters; they require ATP binding and hydrolysis to transport substrate across the lipid membrane. ABC transporters consists of two cytosolic nucleotide binding domain (NBD) that bind and hydrolyze ATP and two integral transmembrane domains (TMD). The NBD contains walker A and walker B motifs that are highly conserved, Q loop, H-motif and ABC signature motif (LSGGQ). Five or six transmembrane helices are present in the transmembrane domain, which provide substrate specificity. These four domains can be present in either one protein (full protein) or can be present in two proteins separately. For example, NBD and TMD can be present on two separate proteins (each



called half proteins). In case of half proteins, a homo or heterodimer is required to form the full protein (Higgins, 1992, Higgins and Linton, 2004 ). The ABC protein family is divided into 8 subfamilies based on the sequence similarity of NBDs denoted by letters A- H. The ABC transporter proteins in eukaryotes mediate the efflux of compounds from cytoplasm to the outside of the cell or into organelles. Bacterial ABC transporters facilitate import of substances. In humans, ABC transporter proteins act as channel receptor or channel regulators.

**ATP switch model for the transport cycle of an ABC transporter (exporter-type-A) Typical structure of an ABC full transporter containing two TMDs, TMD1 (blue) and TMD2 (yellow) each containing 6 transmembranes.**



W. Dermauw, T. V. Leeuwen (2013)

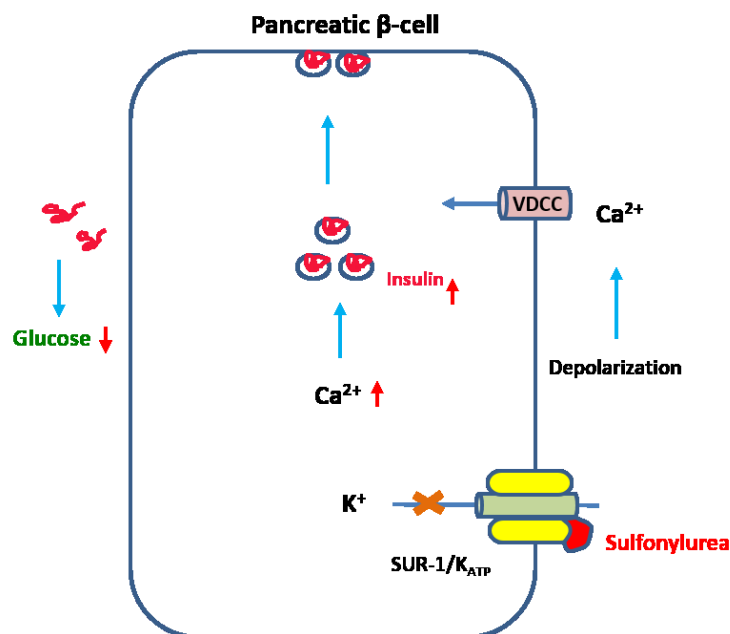
**Figure 1.5 ABC full transporter structure**

ATP switch model: The transport cycle starts after binding of substrate to the TMDs, which leads to a conformational change and this conformational change is transmitted to

NBDs which results in ATP binding and a closed NBD-dimer conformation.

Conformational change in NBDs result in change in conformation of TMDs, causing its rotation and opening towards outside which initiates substrate translocation. Finally, when ATP is hydrolyzed, it leads to the opening of the NBD dimer and a return of the transporter to its basal state.

### **Sulfonylureas regulate an ABCC-family transporter to regulate insulin secretion**



**Figure 1.6 Pictorial model for showing mode of action of sulfonyl urea receptor**

Sulfonylureas (SUs) are drugs used for treatment of type II diabetic patients (Proks et al., 2002). The proteinaceous receptors targeted by SUs in mammalian pancreatic  $\beta$  cells or cardiac myocytes or smooth muscles are SUR1, SUR2A and SUR2B respectively, which are members belonging to subfamily C of the ABC transporter superfamily. SURs regulate the activity of an ATP-sensitive inward rectifying potassium channel,  $\text{K}_{\text{ATP}}$  (a

subclass of  $K_{ir}$  channels) with which they associate and form a large octameric complex consisting of 4  $K_{ATP}$  and 4 SUR subunits (Evans et al., 2005; Hibino et al., 2010). The binding of SUs to the octameric SUR/ $K_{ATP}$  ion channel complex causes local membrane depolarization and allows influx of  $Ca^{++}$  via a voltage-dependent  $Ca^{++}$  channel. The  $Ca^{++}$  signaling, in turn, promotes vesicular transport of insulin-containing granules leading to fusion of these vesicles to the apical plasma membrane of pancreatic  $\beta$ -cells and release of insulin into the blood stream.

Nasonkin et al. (1999) reported the identification and isolation of a full length cDNA clone encoding a “novel Sulfonyl Urea Receptor (SUR) family protein” by searching the EST database of *D. melanogaster* using the human SUR as the query and named this gene, *DmSur*. Nasonkin et al. (1999) expressed the DmSUR protein in *Xenopus* oocytes, which resulted in the appearance of a  $K^+$  current that was sensitive to inhibition by on a SU analog glibenclamide. Matsumura (2010) was the first to propose that benzoylphenylurea has some structurally similarity to SUs.

They further demonstrated that indeed glibenclamide had weak insecticidal activity against the German cockroach and *D. melanogaster* when compared with DFB. Based on the ability of DFB to compete for binding of  $^3H$ -labeled glibenclamide to putative receptors present in membrane fractions from those insects, Matsumura and coworkers (Abo-Elghar et al., 2004) proposed that both of these compounds bound to the same receptor in their membrane preparations. They further proposed that this DFB-binding protein is the same as the one encoded by *DmSur*. However, they did not analyze the membrane preparations from *DmSur* mutants in their competition assays. However, a

recent study demonstrated that a homozygous deletion mutant of this *DmSur* gene failed to show any alteration in the laminar architecture of the cuticle, chitin content or survival, making it unlikely that this gene is involved in cuticle biogenesis/organization. A detailed search of databases of completely sequenced insect genomes indicates that there are multiple members of the *ABC-C* subfamily of genes with substantial sequence similarity to human SURs (Broehan et al., 2013; Sturm et al., 2009). The group headed by our collaborator, Dr. Merzendorfer, has carried out a complete characterization of the ABC-transporter genes in *T. castaneum* genome and found that it has 73 members with 35 genes in the ABC-C-subfamily alone. An extensive RNAi study identified several members of this family involved in the transport of ecdysteroids, cuticle lipids and eye pigments (Broehan et al., 2013). Using phylogentic analyses and transmembrane prediction software and BLAST searches of ABC transporter family genes related to human SURs, we narrowed down the number of likely *Tribolium* candidates orthologous to human *Sur* to three that we arbitrarily call *TcABCC-9A*, *TcABCC-7B* and *TcABCC-4A*. We have tested these and several other closely related members of the C-family of ABC transporters as potential targets of DFB which will be described in the remaining chapters.

*Tribolium castaneum* is a very good model system for gene function related studies. It has all the qualities for good genetic model organism including small body size, short generation time and large brood size. It is easy to culture in the normal laboratory environment with minimal effort. Availability of a fully sequenced and annotated genome (Richards et al., 2008) allows extensive bioinformatics studies. Systemic RNAi by injecting of double-stranded RNA works nicely in *T. castaneum*.

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# Chapter 2

**Effects of diflubenzuron on development and cuticle maturation of *Tribolium castaneum* and identification of its molecular target.**

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## Introduction

Chitin is a linear polymer of N-acetylglucosamine and is an essential part of insect exoskeleton and the peritrophic matrix (PM). It provides mechanical stiffness and stress resistance to the exoskeleton. Nascent chitin chains self-assemble to form chitin microfibrils, which further associate to form a sheet-like structure called lamina that run parallel to the cell surface. Successive laminae are stacked helicoidally along the vertical axis of the procuticle that lies between the protein-rich and chitin-deficient epicuticle and the apical plasma membrane of the underlying epidermal cell (Locke, 2001; Cheng et al., 2009; Moussian et al., 2005). The cuticle provides a physical barrier and shapes the exoskeleton of arthropods. Chitin is produced by the glycosyltransferase, chitin synthase, which aggregates to form electron-dense structures known as plasma membrane plaques of insect epidermal cells (Locke 1991). Genetic analysis of mutants defective in cuticle differentiation led to identification of some factors such as Knickkopf (KNK) and Retroactive (RTV), which are two proteins required for correct organization of chitin microfibrils into laminae (Moussian et al, 2005, 2006; Chaudhari et al., 2011; 2013).

Benzoylphenylureas belong to a group of “chitin synthesis inhibitors”, a class of potent insecticides used for pest control. They cause reduction of chitin content in treated animals without directly affecting the activity of chitin synthase *in vitro*. Some examples of this class of inhibitors are diflubenzuron and lufenuron. Diflubenzuron (DFB), initially synthesized as a potential herbicide, was discovered in early 1972 to have insect controlling property (Mulder and Gijswijt, 1973). DFB, the prototype compound of the acylurea group of insecticides and its several derivatives have been used for insect control for over 40 years. Even though, a wide range of effects of DFB is known

(molting, loss of chitin, cuticle deformities, reduced activity of several enzymes, and accumulation of precursors), the precise molecular target and mode of action of DFB have remained elusive (Matsumura 2010; Cohen 2013; Becker 1978; Wilson & Cryan, 1997; Merzendorfer et al., 2012). The reason lies in the fact that DFB or any of its prototypes did not show any effect on any enzyme associated with chitin metabolism *in vitro* (Cohen, 2001). But there are many speculations on the mode of action of DFB such as direct and indirect effects on the activity on the glycosidase enzyme, chitinase, phenoloxidase, and microsomal oxidase (DeLoach et al., 1981; Ishaaya and Ascher, 1977; Ishaaya and Cohen, 1974; Mitlin et al., 1977; Soltani et al., 1984). This insecticide, classified as a “chitin synthesis inhibitor”, is quite effective in reducing cuticular chitin as well as incorporation of labeled precursors into chitin *in vivo* as well as in chopped up or permeabilized segments of insect integuments *in vitro* (Nakagawa et al., (1993). The Peritrophic matrix (PM) also gets affected by DFB (Becker, 1978; Clarke et al., 1977; Soltani, 1984), However, DFB administration does not reduce the levels of transcripts for chitin synthase (*Chs*) in insects *in vivo* (Ashfaq et al, 2007; Zhang and Zhu., 2006) or affect the activity of this integral membrane enzyme *in vitro* in solubilized membrane preparations (Cohen & Casida, 1989; Kitahara et al., 1983; Mayer et al., 1981, Zimoch et al., 2005). However, DFB effects on CHS protein have not been investigated so far. Based on limited structural similarities between DFB and the type II diabetes drugs, sulfonylureas (SU's), and the ability of DFB to compete out the binding of a radiolabeled SU (<sup>3</sup>H-glibenclamide) to membrane preparations from integuments of cockroaches, Matsumura (2010) has proposed that DFB targets a putative *Drosophila* ortholog of a mammalian sulfonylurea receptor (SUR) named *DmSur* (Nasonkin et al., 1999). SUR is

a member of a large family of ABCC-type transporters involved in transport of a variety of compounds including drugs (Evans et al., 2005; Hibino et al., 2010). Previous studies have shown that *T. castaneum* genome encodes a large family of ABC-transporters with 73 members belonging to 12 subgroups (Broehan et al., 2013). The TcABCC subfamily (to which human SURs and DmSURs belong) is represented by 35 members. Most of the ABC proteins act as a primary-active transporters by binding and hydrolyzing ATP for the movement of substrates across the lipid membrane.

The mammalian SURs have been shown to form a complex with an inward rectifying K<sup>+</sup> ion channel complex that is sensitive to inhibition by ATP (K<sub>ATP</sub>) channels and to regulate this channel's open/close sensitivity to various ligands including ATP, ADP, SU's and K<sup>+</sup> ion channel openers such as diazoxide (Evans et al., 2005; Hibino et al., 2010; Proks et al., 2002; Moreau et al., 2000). The binding of SU's to SUR's results sequentially in the following cascade of events: closure of the K<sub>ATP</sub> channels; plasma membrane depolarization; influx of Ca<sup>++</sup> ions via a voltage-dependent Ca<sup>++</sup> ion channel; promotion of vesicular transport of insulin granules to the plasma membrane; vesicle fusion to the plasma membrane mediated by v-SNARES and t-SNARES; and release of insulin to the blood stream (Proks et al., 2002). A direct study was undertaken to verify this attractive hypothesis using larvae homozygous for the *DmSur* gene. However, cuticle from these mutant larvae exhibited no loss of chitin or any structural abnormalities of the cuticle after transmission electron microscopy (TEM) ruling out the involvement of DmSUR in inhibition of chitin synthesis by DFB (Meyer et al., 2013).

**Objective of this study:** To investigate the effects of DFB on chitin metabolism proteins in *Tribolium castaneum* and to identify the molecular target(s) of DFB.

## **Materials and Methods**

### ***Tribolium* strains:**

*Tribolium castaneum* GA-1 strain was reared on whole wheat flour containing 5% dried brewer's yeast at 30°C and 50% relative humidity. The insects were collected at different developmental stages. Life cycle of *Tribolium* is divided into four different developmental stages: embryonic (4-5 days), larval (~ 15 days), pupal (5 days) and adult (2 months to 2 years). For this study, mostly last instar (late or mature) larvae and pharate adults (one day before adult eclosion) were used.

### **RT-PCR method**

RT-PCR experiments were carried out to monitor the effects of dsRNA administration on levels of the targeted transcripts by using gene-specific primers. Total RNA was isolated from whole insect's 4-d post-injection of dsRNA using RNeasy Mini kit (Qiagen, Valencia, CA). Three insects were pooled for each RNA extraction. cDNA synthesis and RT-PCR were performed using gene-specific primers. cDNA prepared from RT-PCR as template and a pair of primers for the ribosomal protein S6 were used as internal loading controls.

### **DFB treatment**

Insects were collected on pupal day zero. DFB (1000 ppm) in acetone or acetone alone as solvent control was applied topically. Insects were kept at 30°C for five days after the DFB or solvent application and collected 5 days later at the pharate adult stage.

### **Chitin content analysis**

DFB and acetone-treated insects were collected at the pharate adult stage. Chitin and chitosan content of these insects was measured using a modified Morgan-Elson method as described previously (Arakane et al., 2005b). Acetone-treated insects were used as controls (n=5).

### **Immunohistochemistry and confocal analysis**

Pupae were collected after DFB treatment at the pharate adult stage (day 5 pupa) and fixed in 4% paraformaldehyde at 4 °C overnight followed by treatments with a series of solutions with increasing sucrose concentrations (12%, 15%, 18%, and 20% of sucrose). The fixed pharate adult bodies were cryosectioned (20 µm thick) and the body wall samples were stained for KNK, CHS-A, or CDA proteins using *D. melanogaster* KNK rabbit antiserum (1:100), *T. castaneum* CHS-A rabbit antiserum (1:50), and *T. castaneum* CDA rabbit antiserum (1:500) as primary antibodies, respectively. Alexa Fluor- 488 tagged goat anti-rabbit IgG (1:1,000 dilution) was used as the secondary antibody to detect the fluorescence of the respective proteins. Chitin and nuclei were stained using rhodamine-conjugated chitin binding probe (1:100 dilution; New England Biolabs) and DAPI (1:15) respectively. A Zeiss LSM 510 META laser scanning confocal microscope equipped with lasers capable of 405 nm, 488 nm, and 543 nm excitation was used for confocal microscopy using an oil objective (40 ×, 1.3 N.A.) with 8× zoom.

### **Staining with Bodipy-DFB or Bodipy-Glibenclamide**

*In vivo* DFB-treated and dsRNA-injected insects (dsVer, dsABCC-9A, dsABCC-4A and dsABCC-7B) were collected five days later at the pharate adult stage and fixed in 4% paraformaldehyde at 4°C followed by a series of treatments as described in the previous

paragraph. The fixed samples were cryosectioned (20  $\mu\text{m}$ ) and stained with Bodipy-DFB (1:2000; kindly provided by Yuxiu Liu, Nankai University) or with Bodipy-Glibenclamide(Thermo-Fisher Scientific, Waltham, MA) (1:1000) from the stocks of 1 mM for 1 h at 28°C followed by washing with PBST and staining with DAPI (1:15) a nuclear stain. Images were taken using a Zeiss LSM 510 Meta Laser Scanning Confocal Microscope as described in the previous paragraph.

### **Transmission electron microscopy**

Insects were collected and fixed in a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 24 h at room temperature. TEM analysis and immunogold labeling were performed as described previously (Noh et al., 2014).

### **Vesicle preparation**

Fifty pairs of elytra were isolated from insects on pupal day 5 and kept in CHS-A buffer (CHS-A buffer: 50 mM 3-(N-morpholino)-propanesulfonic acid (MOPS), 10 mM  $\text{MgCl}_2$ , 30 mM KCl, pH 6.5). The elytra were homogenized in 1 ml of CHS-A buffer in a mortar and pestle followed by centrifugation at 1,000g for 20 min. The supernatant was collected and centrifuged again at 12,000 g for 40 min. This supernatant was collected and again centrifuged at 100,000 g for 60 min. The pellet obtained was resuspended in 100  $\mu\text{l}$  of CHS-A buffer and was referred to as the 100,000 g vesicle fraction. It was used either immediately or stored frozen at -70°C until the following day and used for enzyme assays.



## **Real Time PCR**

Total RNA isolation, real-time PCR, and first strand cDNA synthesis were done as described previously [Noh, M, et al., 2014]. Total RNA was isolated from whole insects (n = 5 to 10 except for embryos). Transcripts for *T. castaneum* ribosomal protein S6 (*TcRpS6*) were measured for normalization of differences in concentrations of multiple cDNA templates. These analyses were performed by Miyoung Noh of Chonnam National University

## **Immunoblot analysis**

The proteins in tissue extracts were analyzed by SDS-PAGE on 4-12% Bis-Tris-Acrylamide gels. SDS- PAGE was performed according to the standard protocol. Western blotting was performed using the Mini-Trans-Blot® electrophoretic transfer cell (Bio-Rad). Proteins were transferred from the gel on to an Immobilon™-P membrane (Millipore, Billerica, MA) at 100V for 1 h. Blocking was performed using TBS containing 0.05% Tween-20 and 2% BSA. For immunodetection, a primary polyclonal antibody for a C-terminal fragment of TcCHS-A was used at 1: 200 dilutions followed by incubation with a goat anti-mouse IgG conjugated to HRP at 1: 500 dilutions. Colorimetric detection was achieved using the HRP/H<sub>2</sub>O<sub>2</sub> substrate according to manufacturer's protocol (Bio-Rad).

Target protein/fragment	Host
TcCHS-A C-terminal	Rabbit
MsCHS-B catalytic domain	Rabbit
TcCDA 1 (recombinant protein with His-tag)	Rabbit
DmKnk	Rabbit
MsCHT-5 (recombinant protein)	Rabbit

#### List of antibodies used in this work

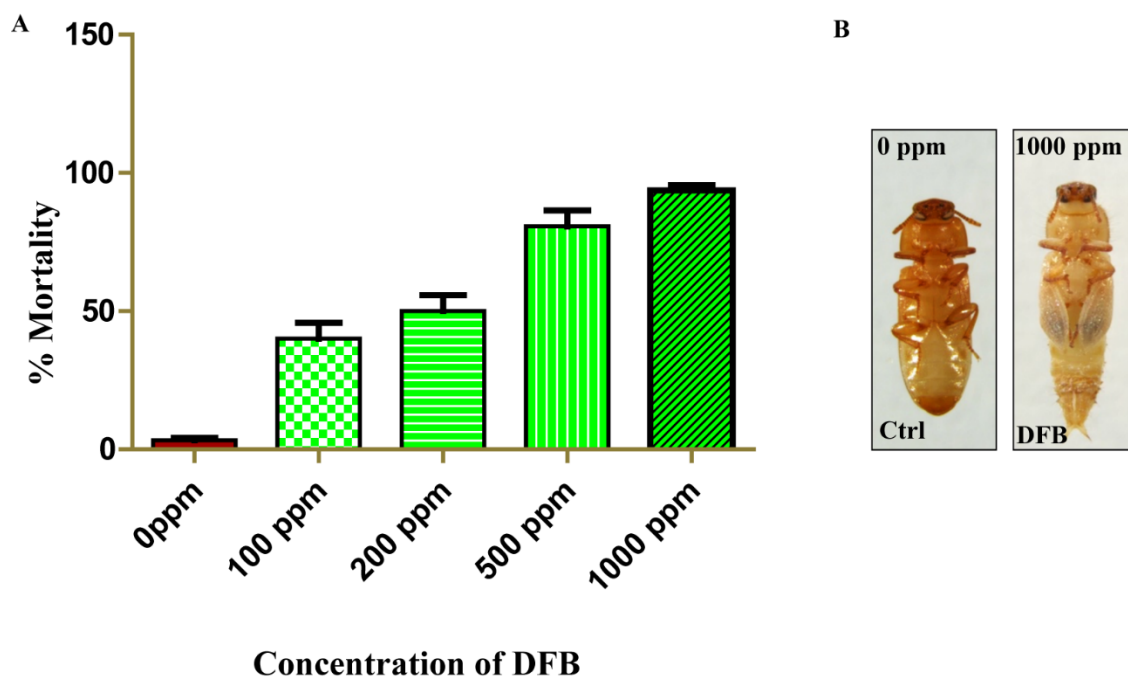
## Results

Diffubenzuron is a prototype member of the benzoylphenylurea group of insecticides, used for insect control over 40 years. DFB administration to insect has been shown to lead to molting defects, reduction in cuticular chitin, abnormal cuticle, and defective egg hatch, but its precise mode of action is not known yet. The objective of this study was to elucidate the mode of action of DFB. We first studied the effects of DFB on *Tribolium castaneum* development and mortality. We used *T. castaneum* as a model organism because of its unique combination of advantages, including facile and effective RNAi approach at any life stage, a high-quality whole-genome sequence assembly and annotation, and a sophisticated genetic toolkit that includes transgenics, balancer

chromosomes, and genetic maps and markers. For this study, we first standardized the dose of DFB and the mode application. We decided to apply DFB topically in our study as it was possible to administer a precise dose of DFB at a precise developmental stage.

### **Validation for topical application of DFB to *T. castaneum***

We have previously used DFB laced diet to young larvae to study the effects of this insecticide on molting and chitin content (Merzendorfer et al., 2012). But to obtain better control over the amount of DFB taken up by insects and to have all insects in identical developmental stages we utilized a topical administration route. In this study, we standardized topical application of DFB to *T. castaneum* using different concentration of DFB in acetone. DFB was applied topically to *T. castaneum* at pupal day zero and mortality dose response curve was assessed at the pharate pupae stage. The acetone alone was applied as control. The DFB-treated insects were significantly affected in comparison to control in a dose-dependent manner. 100 ppm of DFB caused less than 50% mortality, 200 ppm caused 50- 60% mortality, 500 ppm caused 70-90% mortality and 1000 ppm caused > 90% mortality. The pupae exposed to 1000 ppm DFB were arrested from pupal to adult molt as shown in Fig. **2.1.B**. Less than 10% of the insects molted into adults after 1000 ppm DFB treatment and even they did not survive more than seven days after adult eclosion.



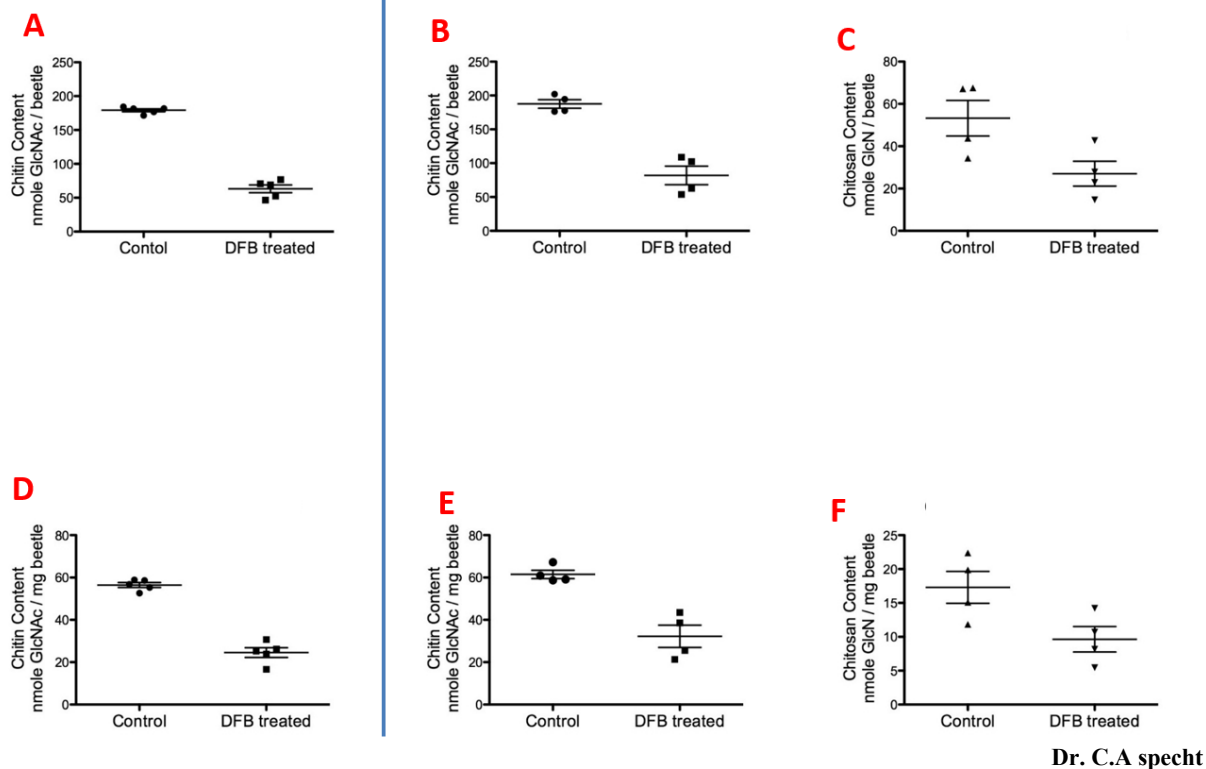
**Figure 2.1** Validation for topical application of DFB to *T. castaneum*

**(A)** Insects were collected on pupal day zero. Varying concentration of DFB (100 ppm, 200 ppm, 500 ppm and 1000 ppm) dissolved in acetone or solvent alone (control) was applied topically using a total volume of 1  $\mu$ l. Insects were kept at 30°C for five days after the DFB application and collected 5 days later at the pharate adult stage and mortality was monitored from pupal to adult molt.  $n = 3$  in triplicate assays. The value were calculated as an average of ( $\pm$ SD) of three independent experiment.

**(B)** 1000 ppm of DFB was applied topically to pupa day zero and phenotype was observed during pupal to adult molt. .

## **Reduction of chitin and chitosan content in DFB-treated insects at pharate adult stage**

Once we established and validated the DFB topical application method, we designed experiments to find out the effect of DFB on *T. castaneum*. We measured the total chitin content in DFB-treated insects as previous studies had reported that administration of DFB in a diet to the insect has resulted in reduction of chitin (Matsumura 2010; Merzendorfer et al., 2012; Cohen, 2013). Hence, we performed this experiment to know the effect of topically administered DFB (1000 ppm) on chitin content. This concentration had been shown to result in more than 90% mortality in the previous experiment (Fig. 2.1). Quantitative analysis of total body chitin content by a modified Morgan-Elson method confirmed significant reduction in the amount of chitin and chitosan in DFB treated insects from pupal to adult molt in comparison to untreated insects. Collectively these data suggested that DFB treatment affected molting by modulating the level of chitin and chitosan predominantly in newly forming cuticle.



**Figure 2.2 Chitin and chitosan analysis of DFB-treated pharate adults.**

*Quantitative analysis of chitin and chitosan from whole insect. Insects were collected at pupal day zero, 1000 ppm of DFB or solvent only (control) was applied topically on pupal day zero and five insects from each treatment were collected at pharate adult stage. Total chitin content was analyzed by using a modified Morgan-Elson method as described in Arakane et al., (2005) and reported as mean  $\pm$  SE (5). (A) Total chitin + chitosan (GlcNAc/beetle) (B) Chitin content (GlcNAc/beetle) (C) Chitosan content (GlcNAc/beetle) (D) chitin + chitosan content (GlcNAc/mg of beetle) (E) Chitin content (GlcNAc/mg of beetle) (F) Chitosan content (GlcNAc/mg of beetle). n=5*

### DFB-treatment led to loss of laminar organization in the cuticle

As the quantitative analysis of total body chitin content by a modified Morgan-Elson method confirmed significant reduction in the amount of chitin in DFB-treated insects (Fig 2.2), we analyzed the cuticular organization based on the expectation that reduction in total chitin should also affect the cuticle organization. To find out the possible ultrastructural changes in body wall cuticle of DFB-treated insects, TEM analysis was performed. DFB treated and solvent treated (control) insects were fixed and subjected to ultrathin sectioning and thoracic body wall cuticle was examined as shown the Fig (2.3). Results showed that control pupae had characteristic laminae with numerous layers while the pupae treated with 1000 ppm DFB appeared completely amorphous and with a thinner cuticle in comparison with that of control pupae.

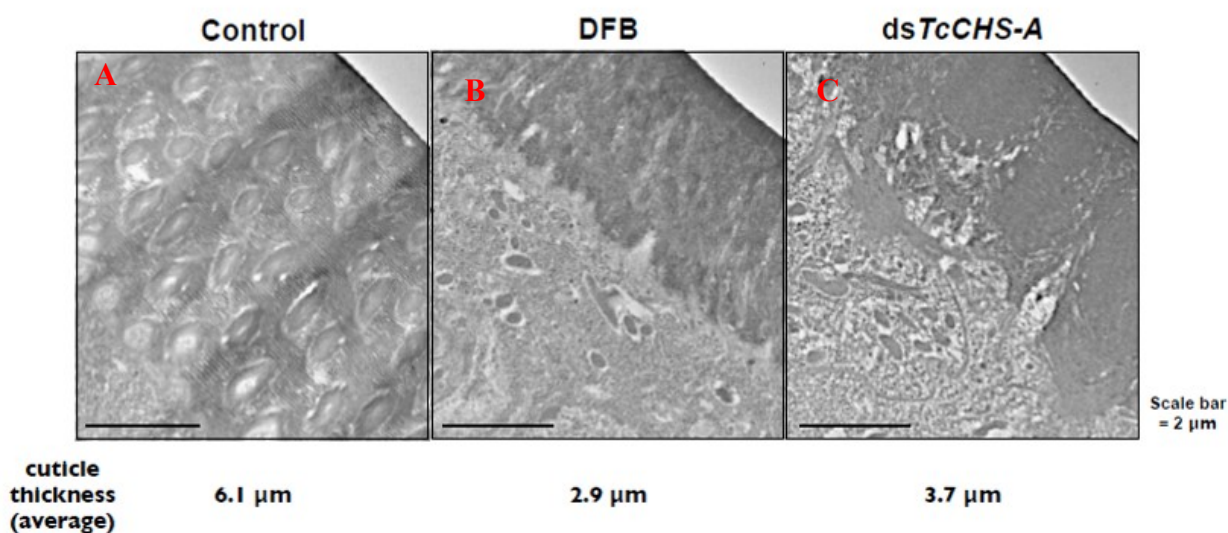


Figure 2.3 DFB treatment led to loss of laminar organization in the cuticle.

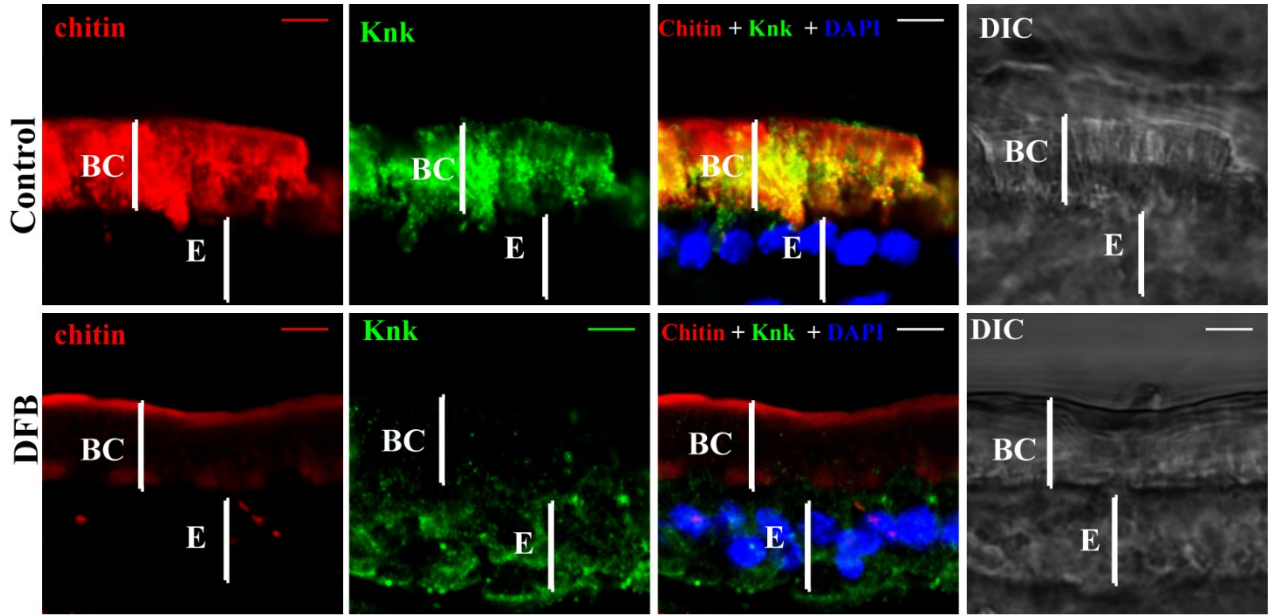
*Insects were collected on pupal day zero and DFB (1000 ppm) dissolved in acetone or solvent alone (control) was applied topically. Insects were kept at 30°C for five days after the DFB application and collected 5 days later at the pharate adult stage. Collected insects were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2 to 7.5) overnight at RT with constant rotation. Samples were washed with 0.1 M sodium cacodylate buffer. A series of dehydration steps with increasing ethanol concentrations with constant rocking at room temperature (50%, 60%, 70%, 80%, 90%, 95% and 100%) for 20 min, followed by resin infiltration with LR white in series with different combination of resin and ethanol (2:1 ethanol: resin for 4 hr, 1:1 ethanol :resin for 4 hr, 1:2 ethanol :resin for 4 hr, 100% resin for 4 hr and 100% resin for 16 hr). Vacuum infiltration was done for each sample for 2 hr followed by embedding the tissues in gelatin capsule and polymerization at 55-60° C for 24 hr in an oven and ultrathin sectioning. Ultrastructure of pharate adult elytral cuticle (A) Positive control (B) 1000 ppm DFB-treated (C) dsChs-A knock-down insects (negative control). The sections were analyzed by transmission electron microscopy (TEM); Scale Bar = 2  $\mu$ m.*

### **DFB-treatment resulted in mislocalization of TcKnk protein**

In the process of finding the effects of DFB in treated insects, we observed substantial reduction of chitin (Fig. 2.2) and loss of laminar organization in the newly forming procuticle (Fig. 2.3). These observations led to hypothesize that the effect of chitin could be due to effect on expression of genes involved in chitin metabolism.



To explore this possibility, we first checked the transcript levels of different chitin metabolism genes in RNA prepared from elytra of control and DFB-treated insects (1000 ppm topical administration and collected 5 days later) by RNA Seq analysis. However, the transcript levels of most of the proteins of chitin metabolism were not affected by DFB treatment (Table 2.1). In particular, we did not see significant changes in transcript levels for Chs-A and chitinases and chitin deacetylases 1 and 2. We then investigated the presence and localization of chitin metabolism proteins using immunolocalization studies. We first checked the localization of Knk protein that plays a role in protection of cuticular chitin from degradation by molting fluid associated chitinases as determined in a study conducted in our lab previously (Chaudhari et al., 2011). DFB treatment resulted in the loss of cuticular chitin and laminar organization as expected, but unexpectedly it also caused mislocalization of the TcKnk protein within the epidermal cell rather than in the procuticle. This led to conclusion that the absence of chitin in the procuticle may have prevented the secretion and incorporation of TcKnk into the newly forming cuticle as the protein was retained inside the epidermal cell as observed by confocal microscopy.



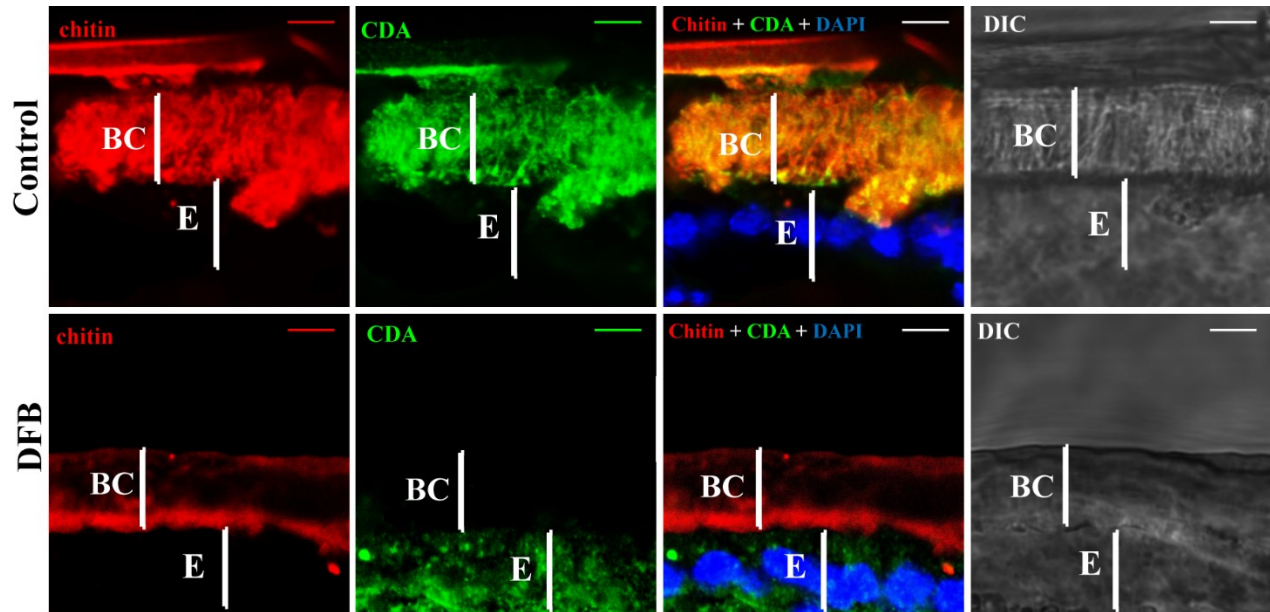
**Figure 2.4 DFB treatment resulted in mislocalization of TcKnk protein**

*Insects were collected on pupal day zero. DFB (1000 ppm) was applied as mentioned in Fig 2.1. Five days after the DFB application and collected 5 days later at the pharate adult stage (day 5 pupa) and fixed in 4% paraformaldehyde at 4 °C overnight followed by treatments with a series of solutions with increasing sucrose concentrations (12%, 15%, 18%, and 20% of sucrose). The fixed pharate adult bodies were cryosectioned (20  $\mu$ m thick) and the body wall samples were stained for KNK protein using a *D. melanogaster* Knk antiserum (1:100 raised in rabbits) to this protein as the primary antibody (kindly provided by Bernard Moussian, Tübingen University). Alexa Fluor-488 tagged goat anti-rabbit IgG (1:1,000 dilution) was used as the secondary antibody to detect the fluorescence of the respective proteins. Chitin and nuclei were stained using rhodamine-conjugated chitin binding probe (1:100 dilution; New England Biolabs) and DAPI (1:15) respectively. A Zeiss LSM 510 META laser scanning confocal microscope equipped with lasers capable of 405 nm, 488 nm, and 543 nm excitation was used for*

*confocal microscopy using an oil objective (40×, 1.3 N.A.) with 8× zoom. Chitin (red); Proteins (green); DAPI (blue); BC, Body wall cuticle; E, epithelial cell. Scale bar = 5 μm.*

### **DFB treatment also resulted in mislocalization of TcCDA-1 protein**

To find out whether DFB specifically affected the localization of Knk protein only or whether the effects of DFB on the localization of chitin metabolism proteins was generalized, we checked the cellular distribution several other proteins involved in chitin metabolism following DFB-treatment. We found that the localization of a chitin deacetylase protein CDA1, was also affected by DFB treatment. Treatment of DFB resulted in TcCDA protein to remain confined to the epithelial cells and did not allow TcCDA to get incorporated into the newly forming cuticles (Fig 2.5).

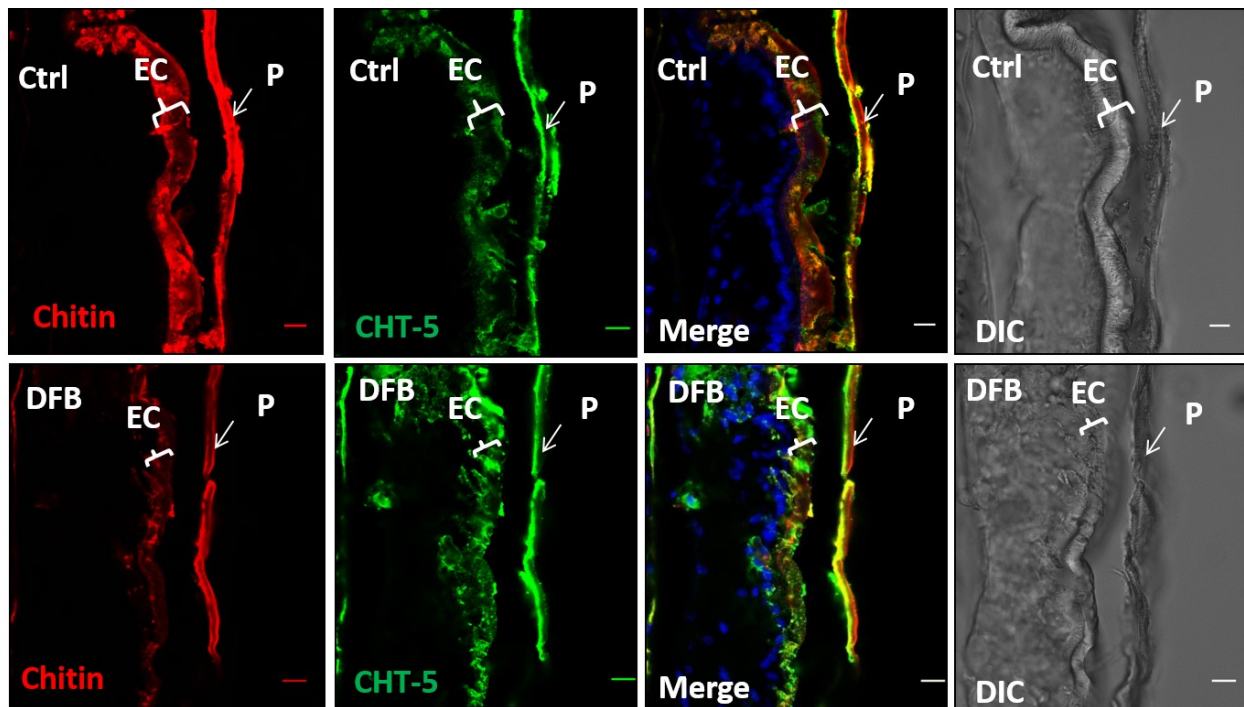


**Figure 2.5 DFB-treatment also resulted in mislocalization of TcCDA-1 protein.**

*Insects were collected on pupal day zero. DFB (1000 ppm) dissolved in acetone or solvent alone (control) were applied topically. Insects were kept at 30°C for five days after the DFB application and collected at the pharate adult stage (day 5 pupa) and processed as described in legend to Fig. 2.4. Proteins (green); DAPI (blue); BC, Body wall cuticle; E, epithelial cell. Scale bar = 5  $\mu$ m.*

### **DFB treatment did not result in mislocalization of Chitinase-5**

After checking the localization of TcKnk and TcCDA proteins, we further checked the localization of TcCHT-5 protein. CHT-5 is the major chitinase found in the molting fluid and it is needed for turnover of chitin in the old cuticle (Arakane et al., 2003; Zhu et al., 2008). DFB treatment produced no change in the localization of TcCHT-5 protein (Fig 2.6). TcCHT-5 protein was mostly present in the procuticle of the control insects. Likewise in the DFB treated insects also TcCHT-5 protein was present in the procuticle. Hence, Localization of the CHT-5 was not affected by DFB.



**Figure 2.6 DFB treatment did not result in mislocalization of chitinase-5**

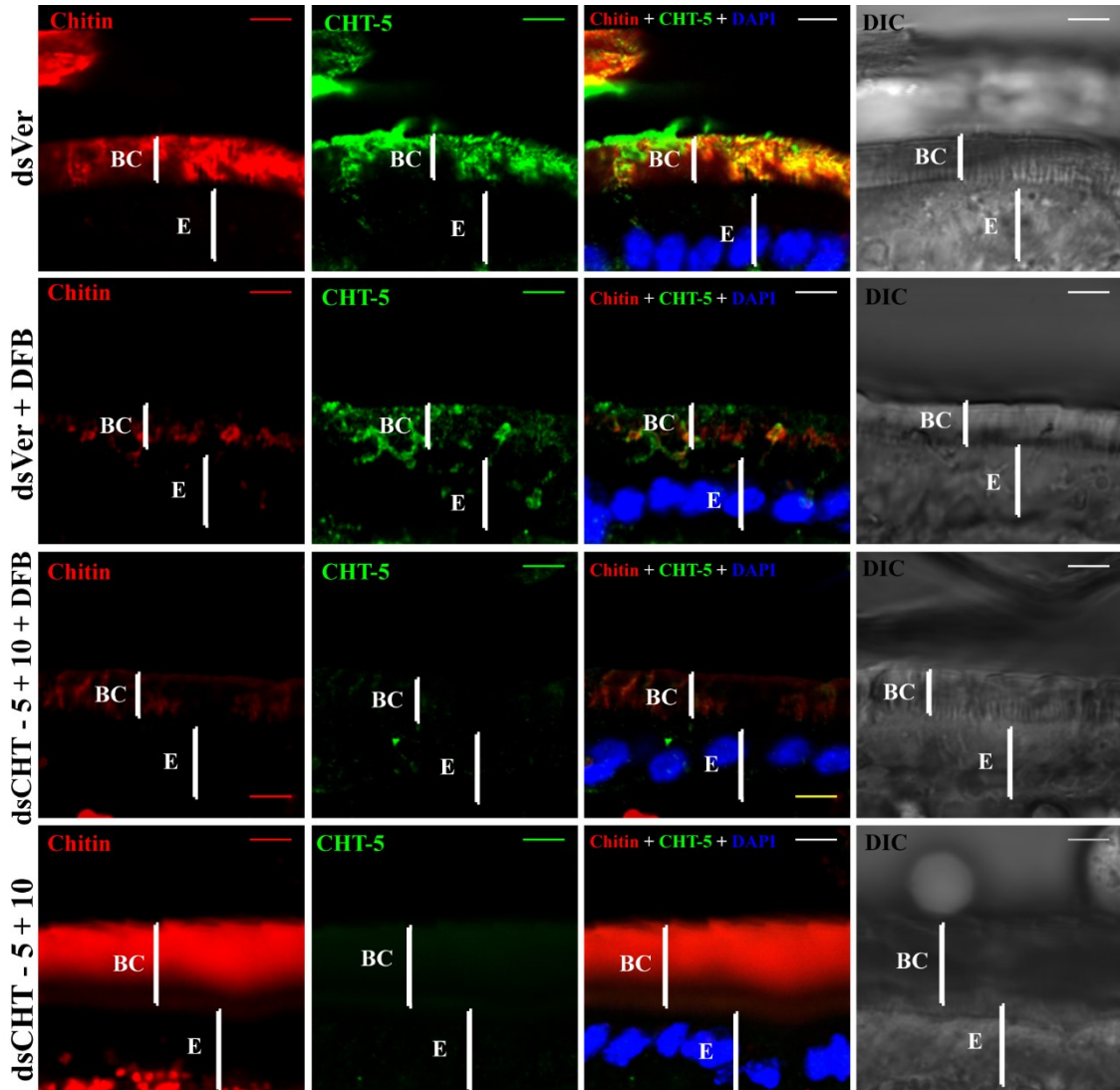
*Determination of location of chitinase-5 (CHT-5) in DFB-treated insects. Insects were collected on pupal day zero. DFB (1000 ppm) dissolved in acetone or solvent alone (control) was applied topically. Insects were kept at 30°C for five days after the DFB application and collected at the pharate adult stage (day 5 pupa) and fixed in 4% paraformaldehyde at 4 °C overnight followed by treatments with a series of solutions with increasing sucrose concentrations (12%, 15%, 18%, and 20% of sucrose). The fixed pharate adult bodies were cryosectioned (20  $\mu$ m thick) and the body wall sections were stained with *T. castaneum* CHT-5 rabbit antiserum (1:200). Alexa Fluor 488-tagged goat anti-rabbit IgG (1:1,000 dilution) was used as secondary antibody to detect the fluorescence of the respective proteins. Chitin and nuclei were stained using rhodamine-*

*conjugated chitin binding probe (1:100 dilution; New England Biolabs) and DAPI (1:15) respectively. A Zeiss LSM 510 META laser scanning confocal microscope equipped with lasers capable of 405 nm, 488 nm, and 543 nm excitation was used for confocal microscopy using an oil objective (40×, 1.3 N.A.) with 8× zoom. CHT-5 antibody (green), for chitin (red); DAPI (Blue); EC, Cuticle; E, epithelial cell: Scale bar = 5 μm.*

### **Down-regulation of CHT-5 and 10 did not restore the chitin levels in the procuticle**

As mentioned above, DFB treatment led to mislocalization of two proteins TcKNK (Fig. 2.4) and TcCDA (Fig 2.5) but not the localization of CHT-5. TcKnk protein protects the chitin from degradation in cuticle (Chaudhari et al., 2011) while chitinase protein (CHT-5) is responsible for degradation of chitin in the cuticle (Zhu et al., 2008). It was possible that in DFB-treated insects in which TcKnk was mislocalized inside the cell instead of being in the cuticle, chitinase had accelerated the degradation of chitin leading to loss of chitin in the procuticle. This possibility was tested by simultaneous down-regulation of chitinases 5 and 10 using RNAi method. These the two enzymes are mostly responsible for degradation of chitin in the cuticle (Zhu et al., 2008). However, the down-regulation of chitinase 5 and 10 did not restore DFB-mediated loss of chitin in body wall cuticle indicating that the DFB-mediated reduction in chitin is not due to accelerated chitin degradation. Chitin staining pattern was similar to DFB treated insects (dsVer + DFB) than that of control insects (dsVer/ Vermillion) or (dsCHT 5 + 10) treated insects.





**Figure 2.7 Down-regulation of CHT-5 and 10 did not restore the chitin levels in procuticle**

*Insects were collected on pupal day zero and at late larval stage. DFB (1000 ppm) dissolved in acetone was applied topically at pupa day zero. Late larval insects were injected with different dsRNAs (dsVer, dsCHT-5 + 10). Insects injected with dsCHT-5 + 10 were treated with DFB after two days when they became pupa day zero (dsCHT-5 + 10 + DFB). Insects were kept at 30°C and collected at pharate adult stage (day 5 pupa)*

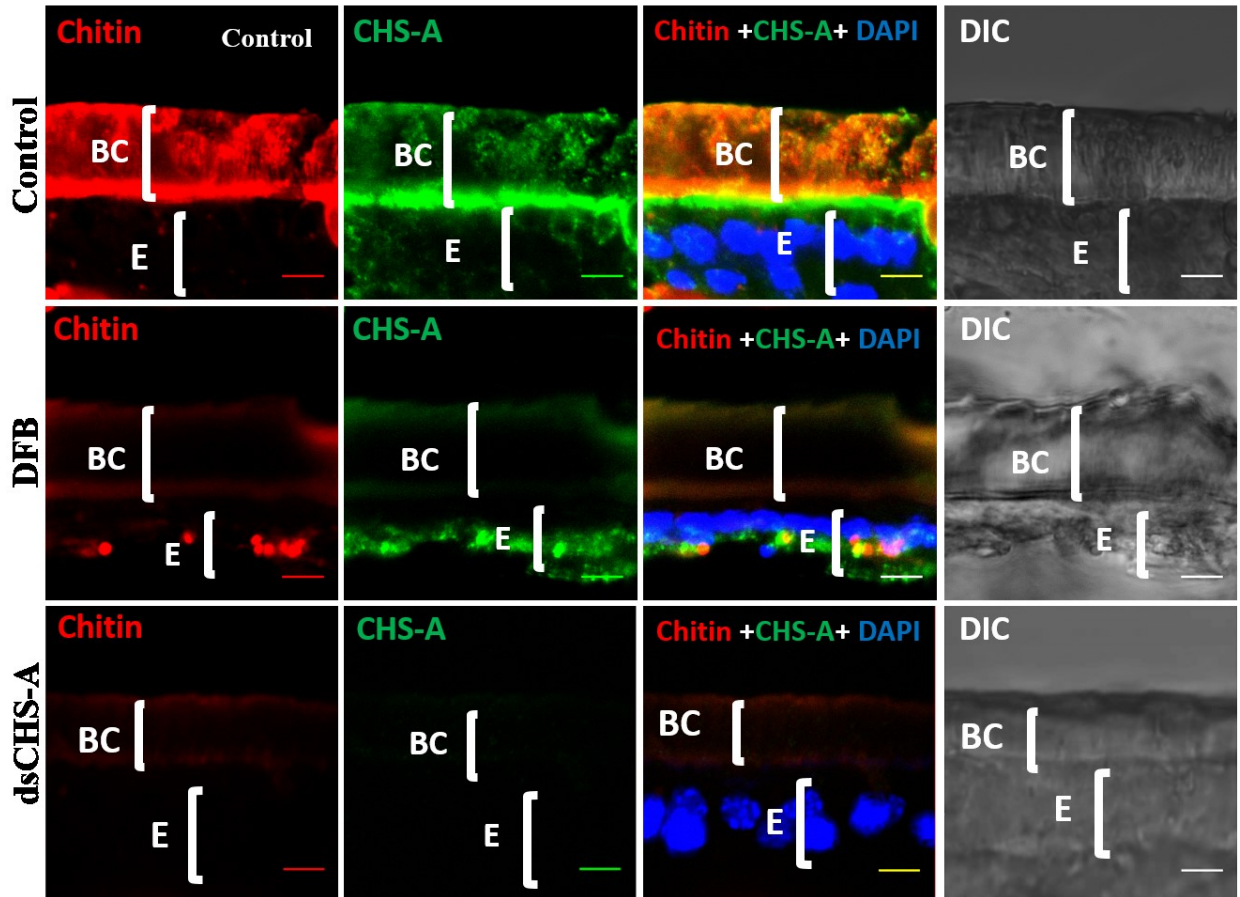
and fixed in 4% paraformaldehyde at 4 °C overnight followed by treatments with a series of solutions with increasing sucrose concentrations (12%, 15%, 18%, and 20% of sucrose). The fixed pharate adult body were cryosectioned (20  $\mu$ m thick) and the body wall samples were incubated with *T. castaneum* CHT-5 rabbit antiserum (1:200), followed by treatment with Alexa Fluor 48- tagged goat anti-rabbit IgG (1:1,000 dilution) secondary antibody to detect the respective proteins. Chitin and nuclei were stained using rhodamine-conjugated chitin binding probe (1:100 dilution; New England Biolabs) and DAPI (1:15) respectively. A Zeiss LSM 510 META laser scanning confocal microscope equipped with lasers capable of 405 nm, 488 nm, and 543 nm excitation was used for confocal microscopy using an oil objective (40 $\times$ , 1.3 N.A.) with 8 $\times$  zoom. CHT-5 antibody (green), for chitin (red); DAPI (Blue); BC, Body wall Cuticle; E, epithelial cell: Scale bar = 5  $\mu$ m.

### **Localization of TcCHS-A protein in DFB treated insects**

After eliminating the possibility of accelerated degradation of chitin by chitinases in DFB-treated insects, we focused on TcCHS-A protein, the sole contributor to epidermal chitin synthesis (Arakane et al., 2005). We found that transcript level of TcCHS-A protein was unaffected by DFB-treatment (Table 2.1). This led to further investigations to determine if the localization of TcCHS-A was affected by DFB treatment of *T. castaneum*. DFB treatment resulted in the mislocalization of CHS-A protein compared to control (dsVer). In control insects this integral membrane protein is present predominantly in the apical plasma membrane with a minority present in intracellular vesicles in transit to plasma membrane. However in the presence of DFB, CHS-A protein was retained inside cell presumably in vesicles and did not get



transported to the plasma membrane suggesting defective transport of vesicles with this enzyme to the plasma membrane.



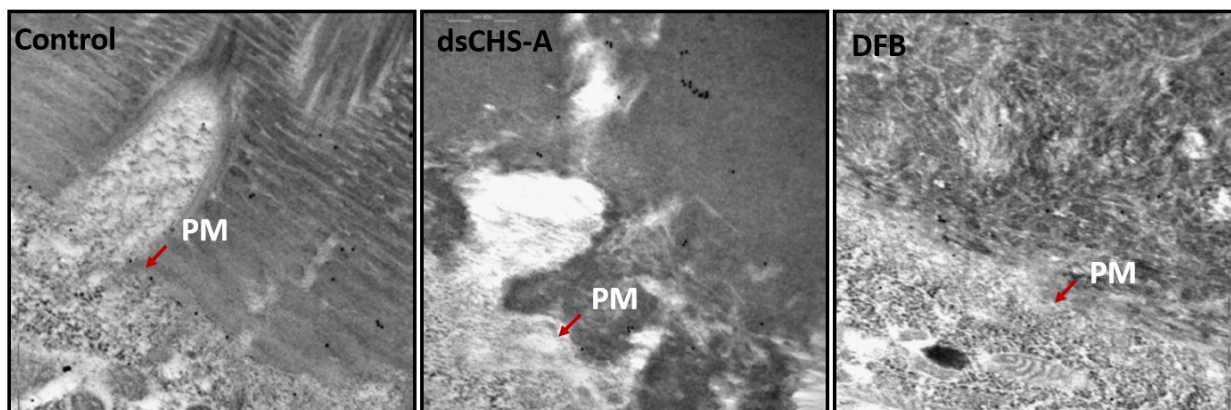
**Figure 2.8 Localization of TcCHS-A protein in DFB treated insects.**

*Localization of CHS-A protein in control (treated with solvent only), DFB treated and dsCHS-A knock-out insects. Insects were collected on pupal day and DFB (1000 ppm) dissolved in acetone was applied topically at pupa day zero. Late larval stage insects were injected with dsCHS-A. Insects were kept at 30°C and collected at pharate adult stage (day 5 pupa) and fixed in 4% paraformaldehyde at 4 °C overnight followed by treatments with a series of solutions with increasing sucrose concentrations (12%, 15%,*

18%, and 20% of sucrose). The fixed pharate adult bodies were cryosectioned (20  $\mu$ m thick) and the body wall sections were incubated with *T. castaneum* CHS-A rabbit antiserum (1:50), followed by staining with Alexa Fluor488-tagged goat anti-rabbit IgG (1:1,000 dilution) as secondary antibody to detect the respective proteins. Chitin and nuclei were stained using rhodamine-conjugated chitin binding probe (1:100 dilution; New England Biolabs) and DAPI (1:15) respectively. A Zeiss LSM 510 META laser scanning confocal microscope equipped with lasers capable of 405 nm, 488 nm, and 543 nm excitation was used for confocal microscopy using an oil objective (40 $\times$ , 1.3 N.A.) with 8 $\times$  zoom. CHS-A antibody (green), for chitin (red); DAPI (Blue); BC, Body wall Cuticle; E, epithelial cell: Scale bar = 5  $\mu$ m.

### **DFB treatment reduced the apical plasma membrane associated CHS-A protein**

As shown in the previous section (Fig. 2.8) DFB-treatment resulted in the mislocalization of CHS-A protein. To get more precise localization of TcCHS-A protein in the cuticle of *T. castaneum* after DFB treatment, immunogold localization was performed. For immunogold staining by electron microscopy, colloidal gold particles are attached to secondary antibody which in turn detects the primary antibody designed to bind to the specific antigen. TcCHS-A protein was predominantly localized in the apical plasma membrane in the epidermal cell underlying the procuticle. DFB treatment resulted in the complete loss of TcCHS-A protein from the apical plasma membrane. (Fig. 2.9)



**Figure 2.9 DFB treatment reduced the apical plasma membrane associated CHS-A protein**

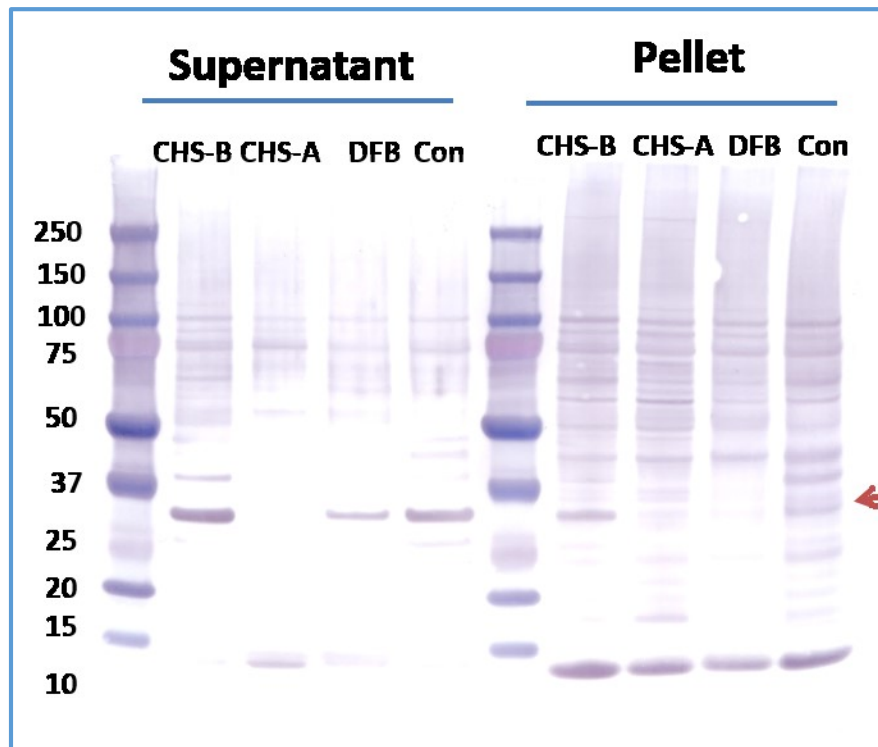
*Insects were collected on pupal day zero and at late larval stage. DFB (1000 ppm) dissolved in acetone was applied topically at pupa day zero. Late larval insects were injected with dsCHS-A. Insects were kept at 30°C and collected at pharate adult stage (day 5 pupa). Collected insects were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2 to 7.5) for overnight at RT with constant rotation. Samples were washed with 0.1 M sodium cacodylate buffer. Series of dehydration with ethanol with constant rocking at room temperature (50%, 60%, 70%, 80%, 90%, 95% and 100%) for 20 mins. Resin infiltration with LR white in series with different combination of resin and ethanol (2:1 ethanol :resin for 4 hr, 1:1 ethanol :resin for 4 hr, 1:2 ethanol :resin for 4 hr, 100% resin for 4 hr and 100% resin for 16 hr). Vacuum infiltration was done for each sample for 2 hr followed by embedding of the tissues in gelatin capsule and polymerization at 55-60° C for 24 hr in an oven at 54 °C. Ultrathin sections of dsCHS-A and DFB treated insects and dsVer-treated insects were incubated with anti TcCHS-A antibody. Anti TcCHS-A antibody was detected using goat*

*anti rabbits IgG conjugated with 10 nm gold particle and analyzed by transmission electron microscopy (TEM) Kindly performed by Miyoung Noh Chonnam National University; Ultrastructure of pharate adult elytral cuticle (A) Positive control; (B) 1000 ppm DFB-treated; (C) dsCHS-A knock out insects (negative control). Plasma membrane (PM); Scale bar = 500 nm.*

### **TcCHS-A protein processing is not affected by DFB**

As a change in the CHS-A protein level was not obvious using immunohistochemistry in cryosections (Fig. 2.8), we considered an alternative possibility involving processing of zymogenic CHS-A as the cause for its mislocalization and/or inactivity in chitin synthesis. To check the possibility of alteration in processing of TcCHS-A protein, we conducted a western blotting experiment where the elytral proteins were extracted from control and DFB-treated *T. castaneum*, subjected to western blotting and probed with anti-TcCHS-A C-terminal antibody. DFB treatment resulted in the loss of a 30 kD TcCHS-A fragment from 12,000g pellet fraction (red arrow) (lane labeled DFB) which is detectable in control (lane marked 'Con'). This band was also missing in TcCHA knockout (lane marked "CHS-A") but present in CHS-B knock-down insects (lane CHS-B). However, a small amount of this immunoreactive CHS-A protein band was also present in the 12,000g supernatant fraction in DFB-treated *T. castaneum* elytral protein (Supernatant fraction, DFB lane) compared to the total absence of CHS-A protein in the TcCHS-A knockout supernatant fraction (Supernatant fraction, CHS-A lane). It is worth pointing out that no new bands were obvious in the insects after DFB-treatment even though there is too much background in the western blotting. This lends support to support our previous observation that DFB-treatment resulted in mislocalization of CHS-

A rather than alteration in its processing. The absence of a large band corresponding to the full length CHS-A in the control in extracts from control insects suggests that CHS-A undergoes proteolytic processing under normal conditions. This process apparently is not compromised in DFB-treated insects because we do not see any larger bands in the lanes marked DFB relative to controls.



**Figure 2.10 TcCHS-A protein processing is not affected by DFB.**

*Insects were collected on pupal day zero and at late larval stage. DFB (1000 ppm) dissolved in acetone and solvent alone was applied topically at pupa day zero. Late larval insects were injected with dsCHS-A or dsCHS-B. Insects were kept at 30°C and collected at pharate adult stage (day 5 pupa). Fifty pairs of elytra were isolated from (DFB, dsCHS-A, dsCHS-B and control) insects on pupal day 5 and kept in CHS-A buffer*

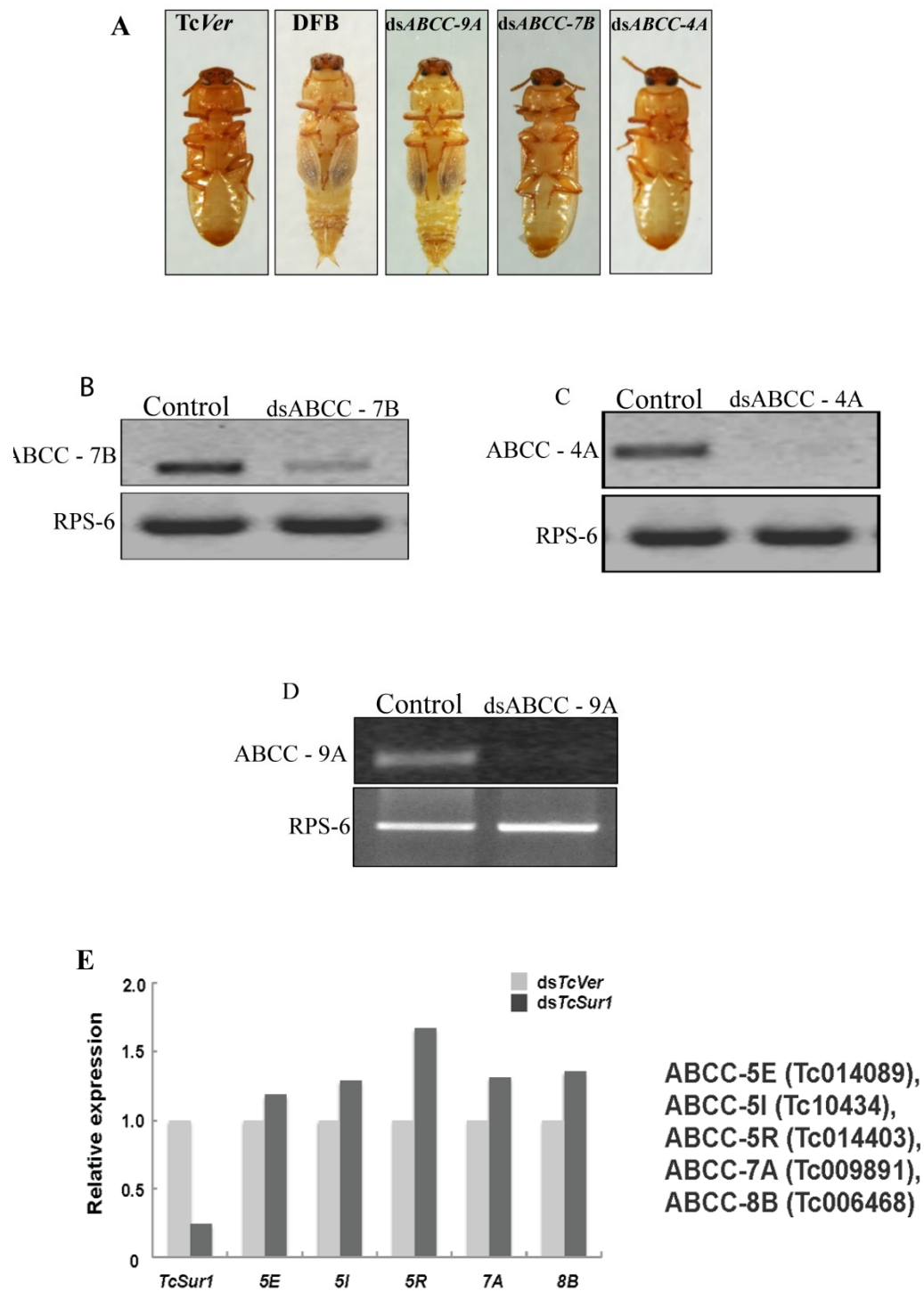
*(CHS-A buffer: 50 mM 3-(N-morpholino)-propanesulfonic acid (MOPS), 10 mM MgCl<sub>2</sub>, 30 mM KCl, pH 6.5). The elytra were homogenized in 1 ml of CHS-A buffer in a mortar and pestle followed by centrifugation at 1,000g for 20 min. The supernatant was collected followed by TCA precipitation for 1 hr on ice and centrifuged again at 12,000g for 40 min. The pellet was collected and resuspended in 100 µl of CHS-A buffer. The 12,000g pellets and supernatant fractions from each preparation were analyzed by SDS-PAGE on 4-12% Bis-Tris-Acrylamide gels. SDS-PAGE was performed according to the standard protocol. Western blotting was performed using the Mini-Trans-Blot® electrophoretic transfer cell (Bio-Rad). Proteins were transferred from the gel on to an Immobilon™-P membrane (Millipore, Billerica, MA) at 100V for 1 h. Blocking was performed using TBS containing 0.05% Tween-20 and 2% BSA. For immunodetection, a primary polyclonal antibody raised against a C-terminal fragment of TcCHS-A was used at 1: 200 dilution followed by incubation with a goat anti-mouse IgG conjugated to HRP at 1: 500 dilution. Colorimetric detection of immunoreactive bands was achieved using the HRP/H<sub>2</sub>O<sub>2</sub> substrate according to manufacturer's protocol (Bio-Rad). Control (Con), DFB treated (DFB), dsCHS-A knockdown (CHS-A) and dsCHS-B knockdown (CHS-B) of 12,000g Pellet (Pellet) and 12,000g supernatant (Supernatant).*

### **RNAi of different ABCC transporters identifies the molecular target of DFB**

After cataloging the various effect of DFB, our next goal was to identify the cellular receptor of DFB. Broehan et al., (2013) have reported that there are 73 members of the ABC family transporters in the *T. castaneum* genome with 35 members in the ABCC-family to which human SURs and DmSur belong. To find out the receptor for DFB in *T. castaneum*, we conducted “BlastP” at NCBI and identified several genes with the highest

sequence similarity to human SUR1 and SUR2 proteins or the DmSUR protein. Only seven of these genes are expressed in elytral tissue, which specializes in cuticle deposition. We decided to focus on these genes as potential targets of DFB. We carried out RNAi of these seven genes, including *ABCC-9A*, *TcABCC-4A* and *TcABCC-7B* which were expressed at high levels and four that were expressed at lower levels, namely *TcABCC-5R*, *TcABCC-7A*, *TcABCC-5T* and *TcABCC-7A* (See Table 2.1) along with a control dsRNA for *Vermilion* gene (dsVer; affects eye color pigment only; Arakane et al., 2005) and followed the effects of RNAi of this gene on mortality. RNAi of *TcABCC-9A* caused 100% mortality at the pharate adult stage and with that it also caused mortality from larval to pupal molt. All dsRNA's substantially down-regulated the target mRNAs (see Fig. 2.11 B, C and D) without significant effect on the other six *TcABCC*- family transcripts (See Fig 2.11/ E for results with *dsTcABCC-9A*). *TcABCC-9A* dsRNA-treated insects showed molting arrest similar to that of DFB-treated insects. However, insects treated with dsRNA for the other six *ABCC*-family genes including *TcABCC-4A* and *TcABCC-7B*, which were expressed at high levels did not show any molting arrest from pupa to adult molt or any significant amount of mortality. In our subsequent studies, we focused on the three genes with the highest expression in elytra, namely *ABCC-9A*, *TcABCC-4A* and *TcABCC-7B*.





**Figure 2.11** RNAi of different TcABCC transporters identifies the molecular target of DFB.

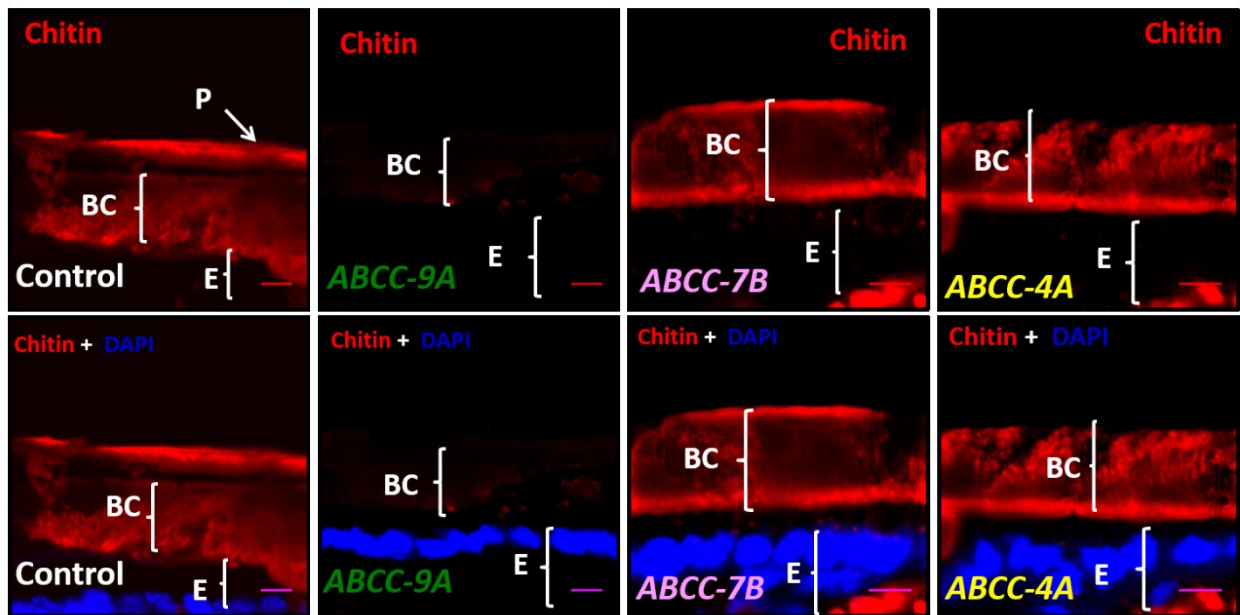


Approximately 400 ng of dsRNA Vermillion (*TcVer*) as control, *TcABCC-4A*, *TcABCC-7B* and *TcABCC-9A* were injected into last instar larvae and insects were collected at the pharate adult stage. Representative images of terminal phenotypes are displayed along with that of DFB-treated insects ( $n = 20$ ). The phenotypes are shown in panel A. Panels B, C and D; Effect of dsRNA-treatment on transcript levels was determined 4 days after injection by collecting dsRNA treated insects ( $n = 4$ ) from each treatment at pharate adult stage (pupa day 5). (B) *dsABCC-7B* (C) *dsABCC-4A* (D) *dsABCC-9A* from each treatment total RNA was extracted and depletion of transcript levels was determined by cDNA preparation followed by RT-PCR. *RPS6* was used as internal loading control.

Fig 2.11E. qPCR of transcripts from insects for multiple ABCC-family genes after administration of dsRNA for *TcABCC-9A*. See Materials and Methods section for experimental details.

### **RNAi of ABC-C9A RNAi causes depletion of chitin in the procuticle**

To confirm that DFB and ABCC-9A are acting in the same pathway and that only *TcABCC-9A* gene among the ABCC family genes tested shares this property, we investigated the chitin content of body wall procuticle in different RNAi treated insects (*dsABCC-4A*, *dsABCC-7B*, *dsABCC-9A* and *dsVer*) and compared the results with DFB treatment. The amount of chitin was highly reduced in *dsABCC-9A* injected insect in comparison to control (*dsVer*), which was similar to DFB treatment. However, in *dsABCC-4A* and *dsABCC-7B*-injected insects, the amount of chitin appeared normal compared to control.



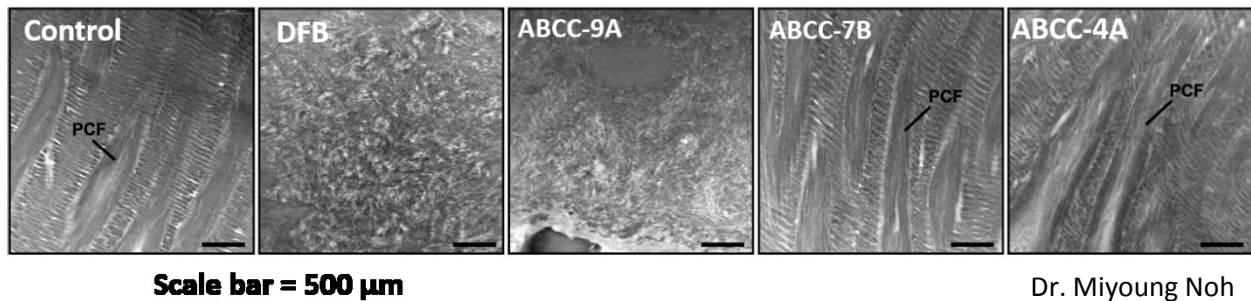
**Figure 2.12 RNAi of ABCC-9A cause's depletion of chitin in the procuticle**

*Approximately 400 ng of each dsRNA was injected into last instar larvae to perform RNAi of Vermillion (TcVer), TcABCC-4A, TcABCC-7B and TcABCC-9A at last instar larval stage. 1000 ppm of DFB was applied at pupa day zero, insects were kept at 30°C and insects were collected at pharate adult stage. Fixed in 4% paraformaldehyde at 4 °C overnight followed by treatments with a series of solutions with increasing sucrose concentrations (12%, 15%, 18%, and 20% of sucrose). The fixed pharate adult bodies were cryosectioned (20 µm thick) and the body wall samples were stained for chitin and nuclei by using rhodamine-conjugated chitin binding probe (1:100 dilution; New England Biolabs) and DAPI (1:15) respectively. A Zeiss LSM 510 META laser scanning confocal microscope equipped with lasers capable of 405 nm, 488 nm, and 543 nm excitation was used for confocal microscopy using an oil objective (40×, 1.3 N.A.) with*

8× zoom. Chitin (red); DAPI (Blue); BC, Body wall Cuticle; E, epithelial cell: Scale bar = 5  $\mu$ m.

### **ABCC-9A RNAi and DFB treatment lead to abnormal structural organization of the procuticle /Body wall**

We further investigated the effect of RNAi of ABCC family genes on laminar organization of procuticle in *dsABCC-9A*, *dsABCC-7B* and *dsABCC-4A* RNAi treated insects and compared them with the morphology of DFB-treated insects. RNAi of *ABCC-9A* resulted in an abnormal structural organization of the procuticle, which resembled that of DFB-treated insects while *dsABCC-9A* or *dsABCC-7B* treatment did not show any effect on structural organization of procuticle Fig (2.13).



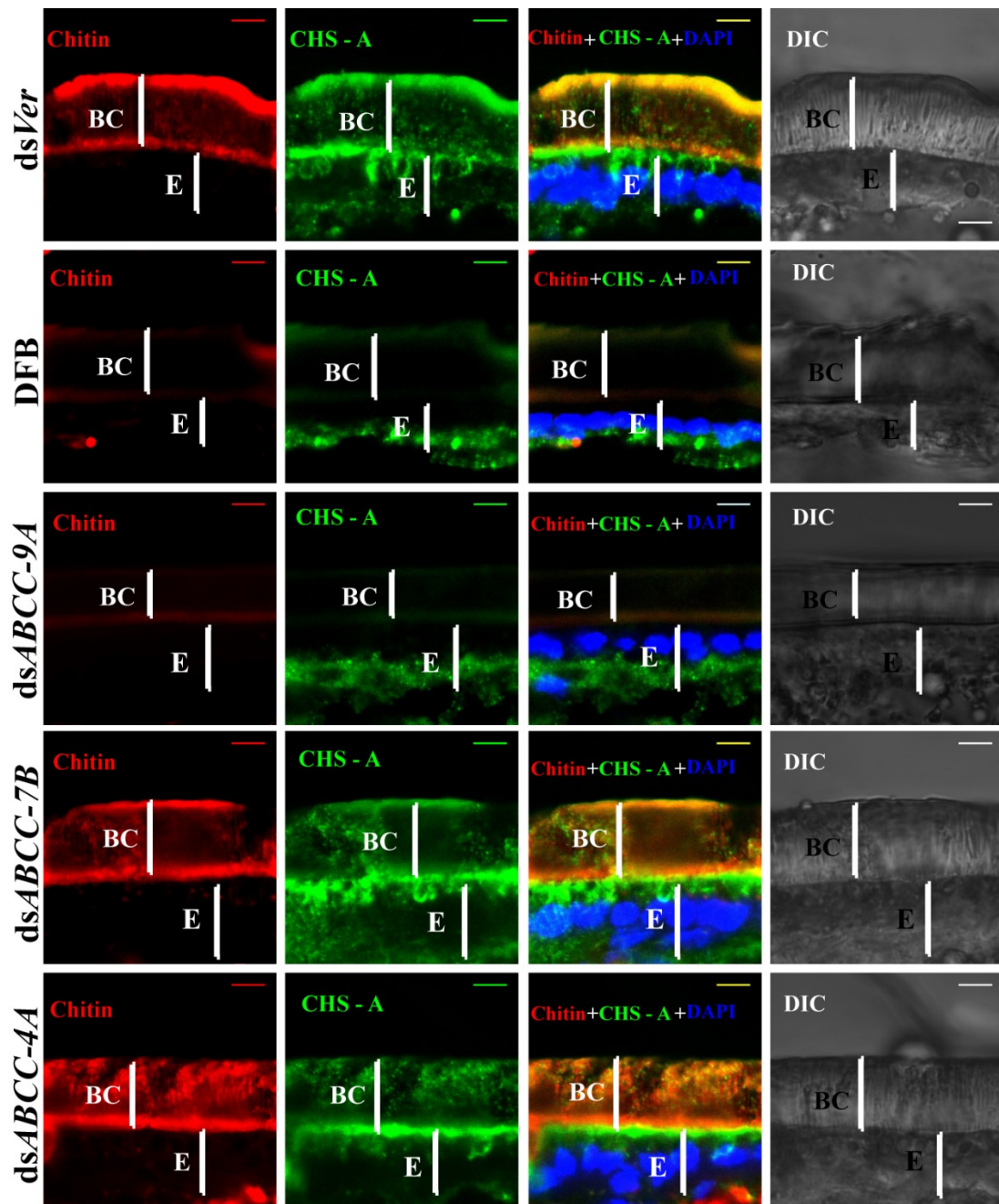
**Figure 2.13** ABCC-9A RNAi and DFB treatment led to abnormal organization of the procuticle of Body wall

Approximately 400 ng of dsRNA was injected to perform RNAi of Vermillion (*TcVer*), *TcABCC-4A*, *TcABCC-7B* and *TcABCC-9A* at last instar larval stage, 1000 ppm of DFB was applied at pupa day zero, insects were kept at 30°C for five days after the DFB application and collected at the pharate adult stage. Collected insects were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2

to 7.5) for overnight at RT with constant rotation. Samples were washed with 0.1 M sodium cacodylate buffer. Series of dehydrations with ethanol with constant rocking at room temperature (50%, 60%, 70%, 80%, 90%, 95% and 100%) for 20 min. Resin infiltration with LR white in series with different combination of resin and ethanol (2:1 ethanol: resin for 4 hr, 1:1 ethanol: resin for 4 hr, 1; 2 ethanol: resin for 4 hr, 100% resin for 4 hr and 100% resin for 16 hr). Vacuum infiltration was done for each sample for 2 hr followed embedding the tissues in gelatin capsule and polymerization at 55-60° C for 24 hr in oven. Ultrathin sectioning were made from control, DFB and dsRNA treated insect (*dsABC-C9A*, *dsABC-C7B* and *dsABC-4A*) at pupa day 5. The images were analyzed by using transmission electron microscopy (TEM). Scale bar = 500 µm; PCF, pore canal with pore canal fiber.

### **CHS-A protein is mislocalized following RNAi of TcABCC-9A and DFB-treatment**

As shown in Fig (2.8) DFB treatment caused mislocalization of TcCHS-A protein in body wall tissue. We wanted to know how the localization of TcCHS-A protein would be affected in *dsABCC-4A*, *dsABCC-7B*- and in *dsABCC-9A* RNAi treated insects. Knock down of *ABCC-9A*, but not of *ABCC-4A* or *ABCC-7B* transcripts resulted in the mislocalization of TcCHS-A protein inside the cells similar to that of DFB treated insects. In *dsABCC-4A* and *dsABCC-7B*-treated and control *dsVer*-treated insects TcCHS-A protein was localized predominantly in the apical plasma membrane (Fig. 2.13).



**Figure 2.14** CHS-A protein is mislocalized following RNAi of TcABCC-9A and DFB treatment

*Approximately 400 ng of each dsRNA was injected into last instar larvae to perform RNAi of Vermillion (TcVer), TcABCC-4A, TcABCC-7B and TcABCC-9A at last instar larval stage, 1000 ppm of DFB was applied at pupa day zero, Insects were kept at 30°C for five days and collected at the pharate adult stage. They were then fixed in 4% paraformaldehyde at 4 °C overnight followed by treatments with a series of solutions with increasing sucrose concentrations (12%, 15%, 18%, and 20% of sucrose). The fixed pharate adult bodies were cryosectioned (20 µm thick) and the body wall samples were stained for T. castaneum CHS-A rabbit antiserum (1:50), Alexa Fluor –488-tagged goat anti-rabbit IgG (1:1,000 dilution) was used as secondary antibody to detect the respective proteins. Chitin and nuclei were stained using rhodamine-conjugated chitin binding probe (1:100 dilution; New England Biolabs) and DAPI (1:15) respectively. A Zeiss LSM 510 META laser scanning confocal microscope equipped with lasers capable of 405 nm, 488 nm, and 543 nm excitation was used for confocal microscopy using an oil objective (40×, 1.3 N.A.) with 8× zoom. CHS-A antibody (green), chitin (red); DAPI (Blue); BC, Body wall Cuticle; E, epithelial cell: Scale bar = 5 µm.*

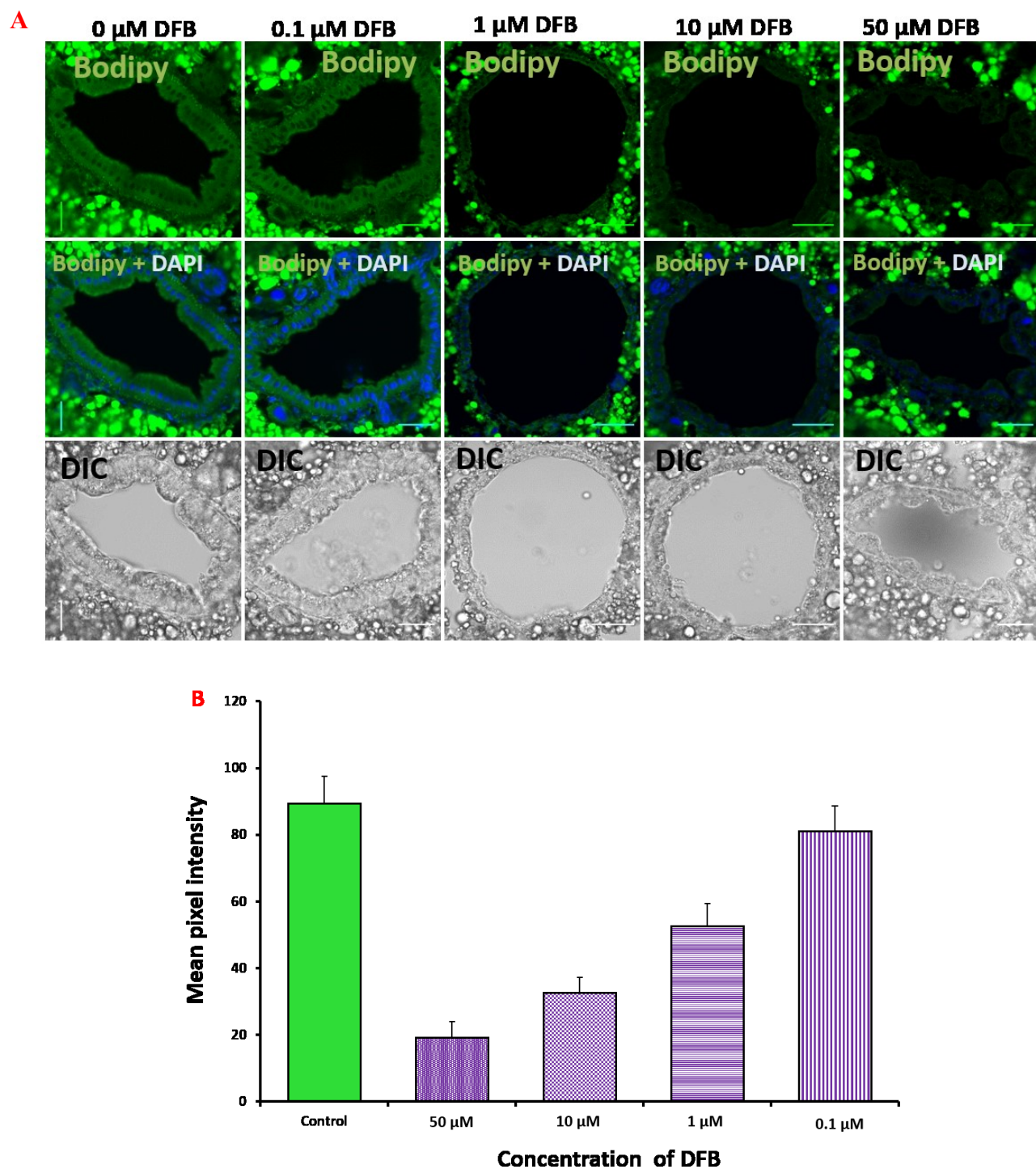
### **Bodipy-Glibenclamide and DFB compete for the same receptor**

When I started this work, we could only buy BODIPY-labeled glibenclamide for demonstrating the presence of a cellular target for DFB analogs. <sup>3</sup>H-labeled glibenclamide has been shown to bind to ABCC-group transporters in a variety of systems including mammalian and plant tissues (summarized in Moreau et al., 2005). Abo-Elghar et al. (2004) have reported that radioactively labeled glibenclamide can bind to membrane preparations from insects and that this binding could be displaced by unlabeled DBF suggesting that both glibenclamide and DFB bind to the same receptor.



We utilized a similar approach to identify molecular target of DFB except that we used fluorescent tagged glibenclamide in our analyses of cryosections of insect abdominal segments using confocal microscopy which allowed us to localize the putative receptor more precisely to intracellular locations. . We used commercially available BODIPY-tagged glibenclamide (sold as ER-tracker), which is known to bind human SUR to check whether it binds to target receptor in *T. castaneum*. Indeed, we could show strong binding of BODIPY-glibenclamide in the midgut epithelial cells. The labeling was found both in the basal membrane and the apical membrane of gut epithelial cells. There was also non-specific staining of fat body as BODIPY is known to be a lipid stain. However, in order to verify that BODIPY-glibenclamide labeling is to DFB-specific receptors in midgut epithelial cells of *T. castaneum*, we conducted a competition assay using progressively increasing concentrations of DFB while maintaining BODIPY-glibenclamide at 1  $\mu$ M. Figure 2.15 shows that Bodipy-glibenclamide binding to putative midgut receptors could be competed out by progressively increasing the concentration of DFB in the preincubation, suggesting that both BODIPY-glibenclamide and DFB bind to the same receptor in the midgut epithelial cell. Preincubation with equimolar concentration of DFB (1  $\mu$ M) significantly reduced the labeling of the membrane-bound receptors in both basal and apical locations. (Note the fat body staining is not visibly diminished as their saturation levels were not probably reached). Higher concentrations of DFB resulted in near complete loss of labeling of the membranes. Figure B is a graphical representation of the same experiment where the intensities of the Bodipy-glibenclamide –stained spots were quantified using the NIH Image J analysis verifying

that both BODIP-glibenclamide and DFB bind to a common receptor in a competitive manner. These results confirm the presence of a DFB- receptor in midgut epithelial cells.



**Figure 2.15** Bodipy-Glibenclamide and DFB compete for same receptor

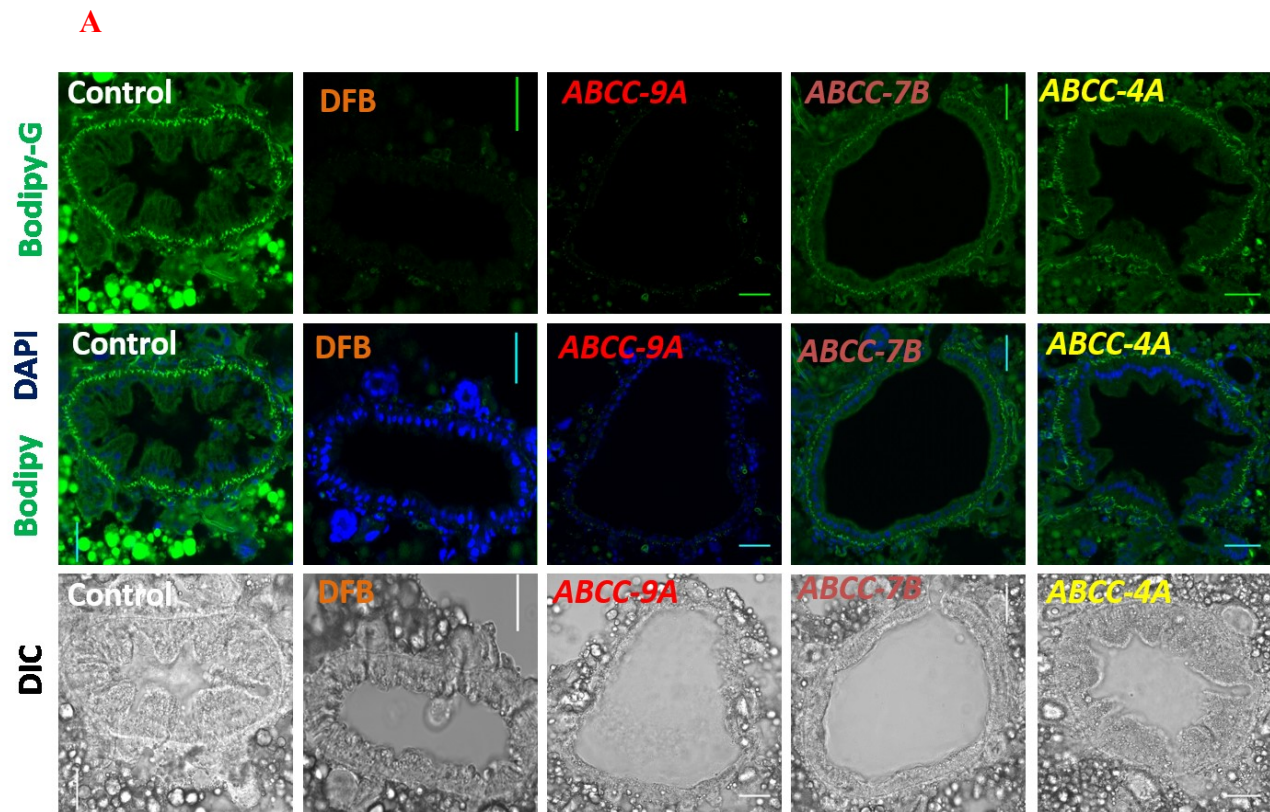


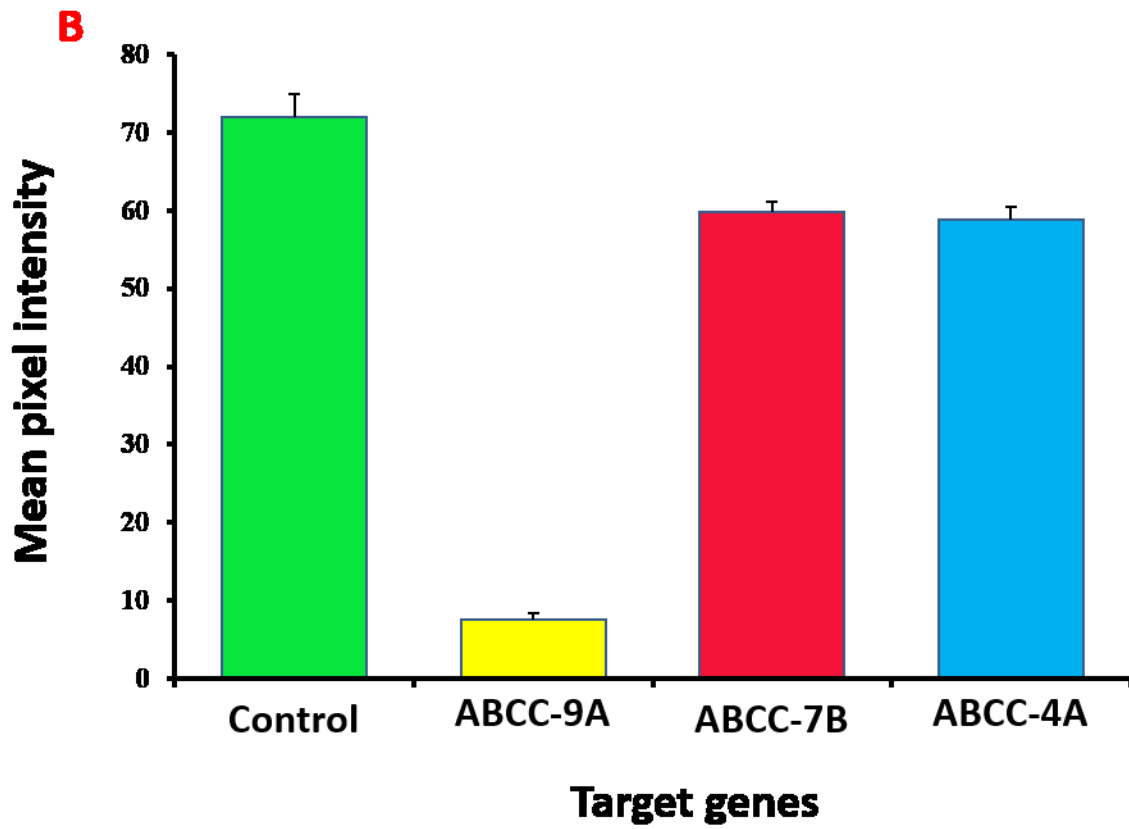
**A)** *Competition between glibenclamide and DFB by using BODIPY-tagged glibenclamide (1  $\mu$ M) stain. Insects were collected at the pharate adult stage. Fixed in 4% paraformaldehyde at 4 °C overnight followed by treatments with a series of solutions with increasing sucrose concentrations (12%, 15%, 18%, and 20% of sucrose). The fixed pharate adult bodies were cryosectioned (20  $\mu$ m thick) and sections were preincubated with different concentration of DFB (0.1, 1, 10 and 50  $\mu$ M) and then stained with Bodipy-glibenclamide (1  $\mu$ M) for 1 h at 28°C followed by washing with PBST and staining with DAPI (1:15), a nuclear stain. A Zeiss LSM 510 META laser scanning confocal microscope equipped with lasers capable of 405 nm, 488 nm, and 543 nm excitation was used for confocal microscopy using an oil objective (40 $\times$ , 1.3 N.A.) with 8 $\times$  zoom. BODIPY-glibenclamide (green), DAPI (Blue); BC, Body wall Cuticle; E, epithelial cell: Scale bar = 5  $\mu$ m. **B.** Quantification of BODIPY-glibenclamide staining of midgut epithelial cells was carried out by taking mean number of pixel from 5 different points by Image J software.*

### ***TcABCC-9A* gene encodes the DFB receptor protein in midgut**

Having established that DFB and BODIPY-tagged glibenclamide bind to same receptor, we used BODIPY-glibenclamide as a tag to identify the receptor for DFB. Our strategy was to knockdown transcripts for the putative receptor gene (*TcABCC-9A*) along with some negative controls (*ABCC-4A*, *ABCC-7B*) to down-regulate the protein corresponding to these genes and observe changes in BODIPY-glibenclamide staining of cryosections of midgut sections relative to sections from the untreated control and DFB-treated insects. Midgut sections from insects injected with dsRNAs for different *ABCC* family genes (*ABCC-4A*, *ABCC-7B* and *ABCC-9A*) were stained with BODIPY-

glibenclamide. Figure (2.16) shows that *ABCC-9A* RNAi resulted in greatly reduced binding of BODIPY-glibenclamide in the midgut epithelial cells. On the other hand *ABCC-4A* and *ABCC-7B* dsRNA-treated insects had only a marginal reduction in binding of BODIPY-tagged glibenclamide indicating that their affinity for BODIPY-glibenclamide was much weaker. Figure B is the graphical representation of the same experiment where the intensities of the BODIPY-glibenclamide were measured using Image J software verifying that *ABCC-9A* dsRNA- and DFB-treated insects had greatly reduced binding of BODIPY-glibenclamide in midgut epithelial cells.





**Figure 2.16** TcABCC-9A gene encode the DFB receptor protein in midgut

*Approximately 400 ng of dsRNA was injected to perform RNAi of Vermillion (TcVer), TcABCC-4A, TcABCC-7B and TcABCC-9A at the last instar larval stage, 1000 ppm of DFB was applied at pupa day zero and the insects were kept at 30°C for five days and collected at the pharate adult stage. They were then fixed in 4% paraformaldehyde at 4 °C overnight followed by treatments with a series of solutions with increasing sucrose concentrations (12%, 15%, 18%, and 20% of sucrose). The fixed pharate adult bodies were cryosectioned (20 µm thick) and then sections were stained with BODIPY-glibenclamide (1 µM) for 1 h at 28°C followed by washing with PBST and staining with DAPI (1:15,) a nuclear stain. A Zeiss LSM 510 META laser scanning confocal*

*microscope equipped with lasers capable of 405 nm and 488 nm excitation was used for confocal microscopy using an oil objective (40×, 1.3 N.A.) with 8× zoom. BODIPY-glibenclamide (green), DAPI (Blue); BC, Body wall Cuticle; E, epithelial cell: Scale bar = 5 μm (B) Quantification of BODIPY-glibenclamide binding to midgut epithelial cells was measured by taking mean number of pixel from 5 different points by Image J software.*

### **Loss of Bodipy-DFB staining after ABCC-9A RNAi in Ventral abdominal epidermal cells/Body wall**

To further confirm that ABCC-9A is the receptor for DFB in epidermal cells also, we used BODIPY-tagged DFB. We were fortunate to have this compound synthesized recently in spring 2015 in the laboratory of our collaborators Yuxiu Liu and Xingmin Wang, Nankai University. Staining was done on all knockouts (dsABCC-4A, dsABCC-7B and dsABCC-9A) as described in previous sections for detecting of BODIPY-glibenclamide binding to gut receptors. . In sections from control insects BODIPY-DFB stained the apical plasma membrane predominantly. There was punctate staining within the cell as well in a region that we speculate represents the basal plasma membrane. RNAi of ABCC-9A resulted in near complete loss of binding of BODIPY-DFB to the body wall epidermal cells in all locations as shown in the figure below. Treatment with DFB in vivo also resulted in a similar loss of BODIPY-DFB binding to epidermal cells. In contrast, sections from insects treated with dsRNAs for the other two ABCC genes or the dsVer gene did not affect the staining of both apical plasma membrane and the basal membrane of these body wall cells. Their staining looked similar to that of control, which suggested that the receptor for DFB is ABCC-9A. These results confirm that the protein

encoded by TcABCC-9A gene is the DFB receptor. Interestingly, the staining required two-fold lower concentration of BODIPY-DFB (0.5  $\mu$ M) compared to BODIPY-Glibenclamide.

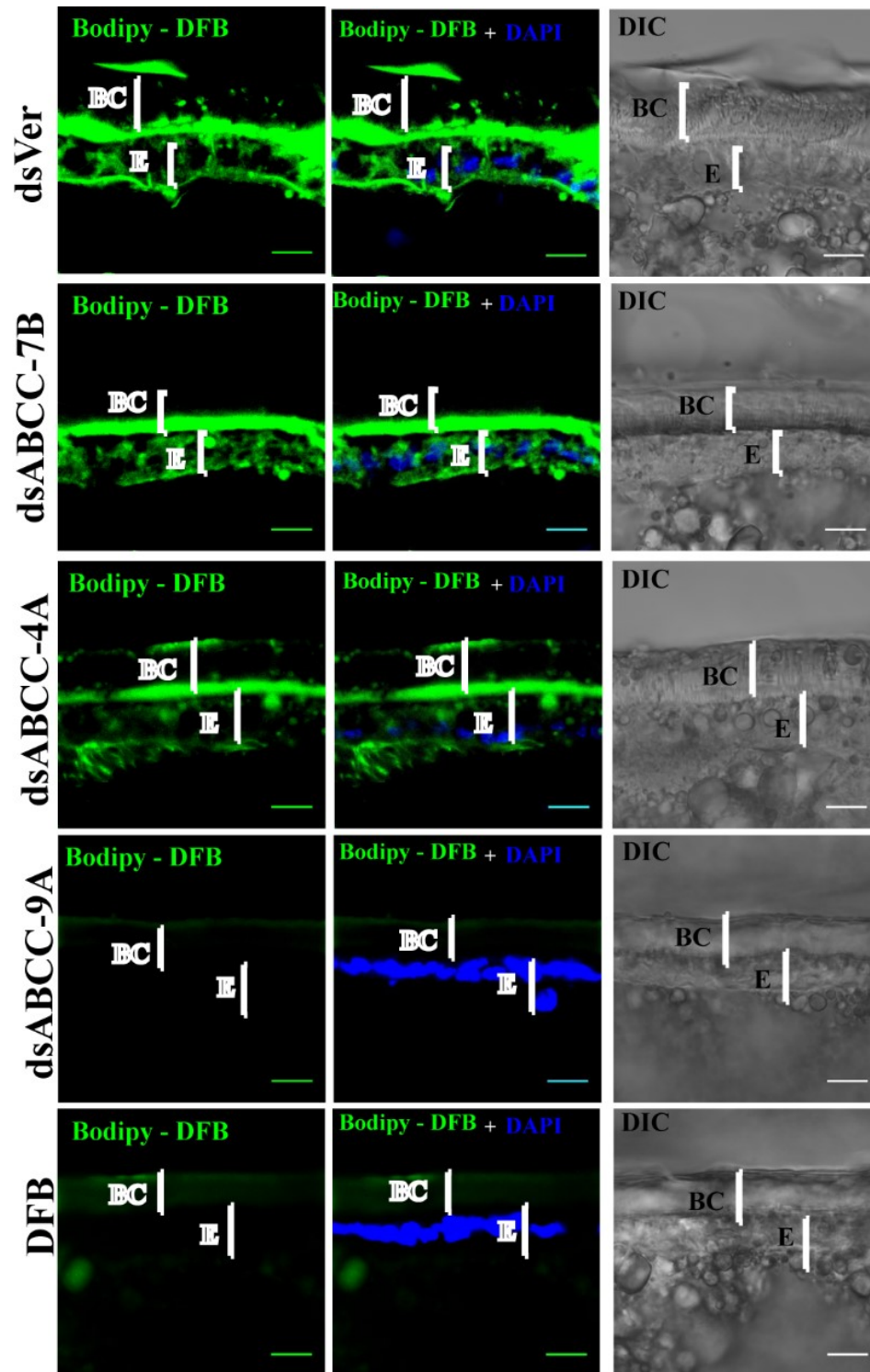


Figure 2.17 Loss of BODIPY-DFB staining after *TcABCC-9A* RNAi in ventral abdominal epidermal cells

*Approximately 400 ng of dsRNA was injected to perform RNAi of Vermillion (TcVer), TcABCC-4A, TcABCC-7B and TcABCC-9A at last instar larval stage, 1000 ppm of DFB was applied at pupa day zero, Insects were kept at 30°C and collected at the pharate adult. They were then fixed in 4% paraformaldehyde at 4°C overnight followed by treatments with a series of solutions with increasing sucrose concentrations (12%, 15%, 18%, and 20% of sucrose). The fixed pharate adult bodies were cryosectioned (20 µm thick). Sections were stained for with BODIPY-DFB (0.5 µM) for 1 h at 28°C followed by washing with PBST and staining with DAPI (1:15) a nuclear stain. A Zeiss LSM 510 META laser scanning confocal microscope equipped with lasers capable of 405 nm and 488 nm excitation was used for confocal microscopy using an oil objective (40×, 1.3 N.A.) with 8× zoom BODIPY-DFB (green), DAPI (Blue); BC, Body wall cuticle; E, epithelial cell: Scale bar = 5 µm.*

### **Transcript levels of chitin metabolism genes are not significantly altered after DFB treatment**

Table 2.1 shows the ratios of transcript level of chitin metabolism genes in DFB-treated elytra relative to control elytra as measured by Illumina RNA sequencing using RNA isolated from elytra (200) of control and DFB-treated insects. RNA-seq was performed to quantify the overall effect of DFB on transcript levels of all annotated genes in the elytra of *T. castaneum*. DFB-treatment resulted in no significant change in the transcript levels of several chitin metabolism genes in comparison to those of controls.

Gene name	Gene ID	RPKM Ratio ( DFB/Control )
TcCHS – A	TC014634	1.7
TcKnk - 1	TC010653	2.1
TcKnk - 3	TC010675	0.8
TcCDA - 1	TC014100	0.9
TcCDA - 2	TC014101	0.9
TcCDA - 3	TC005409	1.8
TcCDA - 4	TC007635	1.7
TcRTV	TC007384	2.0
TcCHT - 5	TC001770	1.0
TcCHT - 10	TC012734	0.8
TcCHT - 3	TC009176	2.1
TcABC – 5T	TC010434	3
TcABC – 5R	TC014403	0.7
TcABC – 7A	TC009891	0.8
TcABCC – 7B	TC009892	2.1
TcABCC – 9A	TC012253	1.1
TcABCC – 4A	TC008035	1.2
TcABC – 8B	TC006468	1.1

Dr. Y. Park

**Table 2.1** Transcript levels of chitin metabolism genes after DFB-treatment

## Discussion

DFB and other acylurea derivatives have been used effectively for insect control for over 40 years. Oral or topical administration of acylureas have been shown to have a variety of detrimental effects on insect growth, development and fecundity, reduction in cuticle thickness, loss of laminar organization, and reduction in total chitin content



leading to molting arrest and death (Gangishetti et al., 2009; Merzendorfer et al., 2012; Mulder & Gijwijt 1973; Verloop & Ferrell, 1977). Mature *Drosophila* females fed a diet containing DFB laid normal number of eggs but they failed to hatch due to the inability of developing larvae to break open the eggshell (Wilson & Cryan 1977; Merzendorfer et al., 2012; Mansur et al., 2010). In our study, topical application of DFB (1000 ppm) to the dorsal abdomens of pupae led to mortality (>90%), substantial loss of total chitin, and loss of the laminar architecture of the procuticle. This loss of chitin could not be correlated with alterations in the levels of transcripts for enzymes of chitin metabolism including CHS-A or chitinase 5 or 10 or chitin deacetylases (CDA) 1 and 2 in the elytron. Furthermore, simultaneous down-regulation of chitinases 5 and 10, the two major enzymes involved in degradation of chitin in the cuticle (Zhu et al., 2008) failed to restore DFB-mediated loss of chitin in body wall cuticle indicating that the DFB-mediated reduction in chitin is not due to accelerated chitin degradation. Confocal microscopic analysis of DFB-treated insects revealed that CHS-A protein, the sole contributor to epidermal chitin synthesis (Arakane et al., 2005) was mislocalized in the epidermal cells. CHS-A was found inside the epidermal cell on the basal side rather than in its normal location of the apical plasma membrane. There was no obvious evidence for reduced amounts of CHS-A protein. Other proteins such as CDA and Knickkopf (KNK) were also mislocalized (but not chitinase-5) in these tissues indicating that DFB-mediated interference of protein transport was not limited to CHS.

Even though DmSUR, which was presumed to be the ortholog of human SUR-1 by Matsumura and co-workers (Abo-Elghar et al., 2004), was ruled out as the target of DFB (Meyer et al., 2013), we were intrigued by the possibility that a gene closely related to

human *Sur*, a member of a large family of ABCC-type transporters involved in transport of a variety of compounds including drugs (Evans et al., 2005; Hibino et al., 2010; Dean et al., 2001) may be the real target of DFB. We have previously reported that *T. castaneum* genome encodes a large family of ABC-transporters with 73 members belonging to 12 subgroups (Broehan et al., 2013). The TcABCC subfamily (to which human SURs and Dm SURs belong) is represented by 35 members. We used a “BlastP” search to identify several genes with the highest sequence similarity to human SUR1 and SUR2 proteins that are expressed in the elytral tissue, which secretes copious amounts of cuticle at the pharate adult stage. We carried out RNAi of seven genes, named *TcABCC-9A*, *TcABCC-4A*, *TcABCC-7B*, *TcABCC-5E*, *TcABCC-5R*, *TcABCC-5I*, *TcABCC-7A* and *ABCC-8B* which are expressed in this tissue (Table 2.1) and followed the effects of RNAi of this gene on mortality, molting, chitin staining of the cuticle and eye color. We reasoned that that if one of these genes was the target of DFB, down-regulation of its activity would phenocopy the effects of DFB application. Only RNAi of *ABCC-9A*, mimicked all the effects of DFB administration including molting failure at both larval-pupal and pupal-adult molts, loss of cuticular chitin and death at each molt. Like DFB-treatment, RNAi for this gene caused mislocalization of CHS-A protein and loss of laminar organization of the procuticle further confirming that DFB and *ABCC-9A* are acting in the same pathway. Thus RNAi of *TcABCC-9A* reproduced all the effects of DFB that we tested for while the RNAi of the other six *TcABCC* genes tested did not bring about any molting or morphological defects or chitin loss or mortality. (RNAi of other *ABC* family genes produced phenotypes unrelated to molting: Broehan et al., 2013; and data not shown). These studies strongly suggest that *TcABCC-9A* is involved in the

same pathway as DFB in inhibiting chitin accumulation in the cuticle. We conclude that the molecular target of DFB is encoded by the *TcABCC-9A* gene.

Further confirmation that *ABCC-9A* gene encodes the DFB receptor was obtained by direct binding experiments with the fluorescently tagged BODIPY-DFB and a structurally related BODIPY-glibenclamide to membrane receptors in both epidermal cells and gut lining cells. This binding was abolished when transcripts for this gene were down-regulated by administration of dsRNA for this gene. Further, DFB-treatment also led to loss of binding of BODIPY-DFB to these receptors. Taking into consideration all the effects of RNAi of *TcABCC-9A* as well several other closely related *ABCC*-genes on chitin content, loss of laminar organization, cuticle morphology and mortality, and BODIPY-DFB and Bodipy-Glibenclamide binding and competition by DFB, we conclude that TcABCC-9A protein is the long-sought molecular target of DFB.

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# Chapter 3

***An *in vitro* system for chitin synthesis using vesicles from elytra of *Tribolium castaneum*.***

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## **An in vitro system for chitin synthesis using vesicles from elytra of *Tribolium castaneum*.**

One group of insect growth regulators labeled “chitin synthesis inhibitors” has been available commercially for insect control purposes for quite some time. The acylurea derivative, diflubenzuron (DFB), serves as the prototype member of this group. Even though more than 40 years have elapsed since its introduction as a pesticide, the precise mechanism(s) of disruption of the chitin metabolic pathway by acylurea compounds has not been elucidated in any insect species (Cohen 1987, 2001). Chitin synthetic pathway is an evolutionarily conserved set of reactions in which chitin synthase is the main and final enzyme of the pathway. Chitin synthases (CHS) are glycosyltransferases, which contain a central catalytic domain and two or three sets of transmembrane domains depending on the species (Merzendorfer and Zimoch, 2003). Chitin chains are synthesized and secreted to the extracellular matrix and are believed to be translocated across the membrane by the help a specialized set of membrane spanning helices known as the 5-TMS domain of chitin synthase enzyme. Basic properties of chitin synthase enzyme are still poorly understood. While the number of *Chs* genes in fungi varies widely, in most insects there are two genes encoding chitin synthases. They have been named either as *Chs-1* and *Chs-2* or as *Chs-A* and *Chs-B*. (Merzendorfer, 2006). In this work, I will use the latter nomenclature. The protein product of *Chs-A* is responsible for synthesis of chitin in the epidermal and tracheal cuticle, whereas *Chs-B* is responsible for making peritrophic matrix chitin (Arakane et al., 2005; Hogenkamp et al., 2005; Tellam 2000; Zimoch et al., 2005). *Chs-A* expression is upregulated during molt and somewhat down regulated during intermolt (Hogenkamp et al., 2005; Zimnoch et al.,



2005). Multiple peaks of *Chs-A* transcripts are observed during the pupal period when different cuticles are being deposited (Arakane et al., 2008). However *Chs-B* expression is higher during intermolt and lower during the molting period (Hogenkamp et al., 2005; Zimoch et al., 2005). It has been observed in various systems that trypsin treatment stimulates chitin synthase activity, which suggests that post-translational proteolytic activation is important for regulating chitin synthase activity (Duran and Cabib, 1978; Merz et al., 1999; Merzendorfer, 2006; Roncero, 2002). It has also been reported in yeast that chitin synthase (CHS-B) has a zymogenic character, which gets hyper-activated by an unidentified soluble yeast protease (Martinez-Rucobo et al., 2009).

Studies using different insects have concluded that transcript levels encoding CHS-A, the enzyme responsible for chitin synthesis in epidermal cells, appeared to be either unaffected or marginally elevated in insects treated with DFB (Zhang & Zhu 2006; Merzendorfer et al., 2012). In fact, a substantial increase in CHS activity was reported using microsomal membrane preparations from *T. brevicornis* two days following DFB administration, even though the enzyme levels dropped to normal levels two days later (Cohen & Casida, 1980). These results indicate that DFB does not directly affect the activity of *Chs-A* gene. While the levels of CHS proteins have not been analyzed systematically, attempts to show a direct effect of DFB on CHS activity in *in vitro* enzyme assays using solubilized membrane preparations of *Stomoxys calcitrans*, *T. castaneum* or *Manduca sexta* larvae did not demonstrate any inhibition of CHS activity even at high (>100  $\mu$ M) concentrations of DFB (Cohen & Casida 1980; Zimoch et al., 2005). On the other hand, low concentrations (100 ppm or less) of DFB were quite effective in inhibiting incorporation of precursors into chitin in *in vivo* experiments that

utilized incubations of intact integuments or partially chopped integuments with radioactive chitin precursors (Nakagawa & Matsumura, 1993, 1994). However, there is one report of inhibition of chitin synthesis by DFB (1 µg/ml) using a “vesiculated” microsomal preparation from the brine shrimp, *Artemia salina* (Horst, 1981). For inexplicable reasons this work has not attracted much attention.

Diflubenzuron inhibits chitin synthesis *in vivo* and so, is considered the main cause of insecticidal activity (Verloop and Ferrell, 1977). It has been also reported in many studies that benzoylphenylureas can block the process of chitin synthesis in intact tissues or even in cell culture. Some studies have shown that ionophores for  $K^+$ ,  $Ca^{2+}$ , and  $H^+$  inhibit the incorporation  $^3H$ -UDP-N-acetylglucosamine into chitin similar to DFB (Nakagawa, 1994). All of these studies indicate that an intact membranous system is required for chitin synthesis and that it can be regulated by ion movements. DFB could also directly or indirectly regulate the function of ion channels, which may be sensitive to this agent.

In this study, we used an *in vitro* chitin synthesizing system consisting of microsomes prepared from the modified wing, the elytron, which exhibits all the hallmarks of cuticular chitin synthesizing epidermal cells, to obtain insights into the mechanistic details of how this class of insecticides inhibits chitin synthesis in insects.

**Main Objective of this chapter:** To find out mode of action of DFB with the help of an *in vitro* system for chitin synthesis using vesicles from elytra of *T. castaneum*.

## **Materials and Methods:**

### **Tribolium strains**

*T. castaneum* GA-1 strain was reared on whole flour containing 5% dried brewer's yeast at 30°C and 50% humidity. The insects were collected at different stages. Life cycle of *T. castaneum* is divided into four different developmental stages: embryonic (4-5 days), larval (~ 15 days), pupal (5 days), and adult (2 month to 2 year). For this study, mostly last instar (penultimate instar or mature) larvae and pharate adult (one day before adult eclosion) were used.

### **DFB treatment**

Insects were collected on pupal day zero. DFB (1000 ppm) in acetone or solvent alone (used as control) was applied topically. Insects were kept at 30°C for five days after the DFB application and collected at the pharate adult stage.

### **Vesicle preparation**

Fifty pairs of elytra were isolated from pupal day 5 and kept in CHS - A reaction buffer (CHS-A buffer: 50 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 10 mM MgCl<sub>2</sub>, 30 mM KCl, pH 6.5). The elytra were homogenized in 1 ml of CHS-A buffer with a mortar and pestle followed by centrifugation at 1000g for 20 min. The supernatant was collected using a micropipette and centrifuged at 12,000g for 40 min. The supernatant thus collected, was again centrifuged at 100,000g for 60 min. The pellet obtained was resuspended in CHS-A (100 µl) buffer. The enzyme preparation was kept at 4°C and used the same day and kept in -20° for long term use.

### **Measurements of chitin synthesis**

The reaction was performed using 5 µl of vesicles (100,000 g pellet) containing ~ 1.5 to 3 µg of protein in a total volume of 100 µl and initiated by adding a master mix of CHS-A buffer containing 0.2 µCi UDP-N-acetyl- [6- <sup>3</sup>H] D-glucosamine ( specific activity) and 120 µM unlabeled substrate. Chitin synthesis was carried out for 90 min in 1.5-ml Eppendorf polypropylene tubes at 32°C. The reaction was terminated by adding 1 ml of 1.5 M KOH followed by incubation for 2 h at 100°C. A 2.5 cm glass fiber filter (GF/C Whatman) was used for filtering the undissolved material. The filtrate was finally washed with 10 ml of 98% ethanol, air-dried for 30 min at 50°C. The washed filters were transferred to scintillation vials containing 5 ml Econofluor scintillation fluid (ICN) and the radioactivity was measured using a Beckman liquid scintillation spectrometer and expressed as dpm.

### **<sup>45</sup>Ca<sup>2+</sup> uptake assay**

<sup>45</sup>Ca uptake by isolated vesicle preparations from elytral tissue of day 5 pupae was measured using a Beckman liquid scintillation spectrometer. The assay was performed in CHS-A buffer containing ~ 3 to 6 µg of protein, 120 µM UDP-N-acetyl-glucosamine in total volume of 50 µl. The reaction was started by adding 2 µCi of <sup>45</sup>CaCl<sub>2</sub> (>10Ci/gm). The incubation was in 1.5 ml Eppendorf polypropylene tubes for 5 min at 32°C. The reaction was stopped by filtering immediately through a 0.45 µm Millipore filter followed by washing with 2 ml of ice-cold CHS-A buffer. The washed filter was air-dried and the radioactivity was counted using a liquid scintillation counter and expressed as dpm.

## **Results**

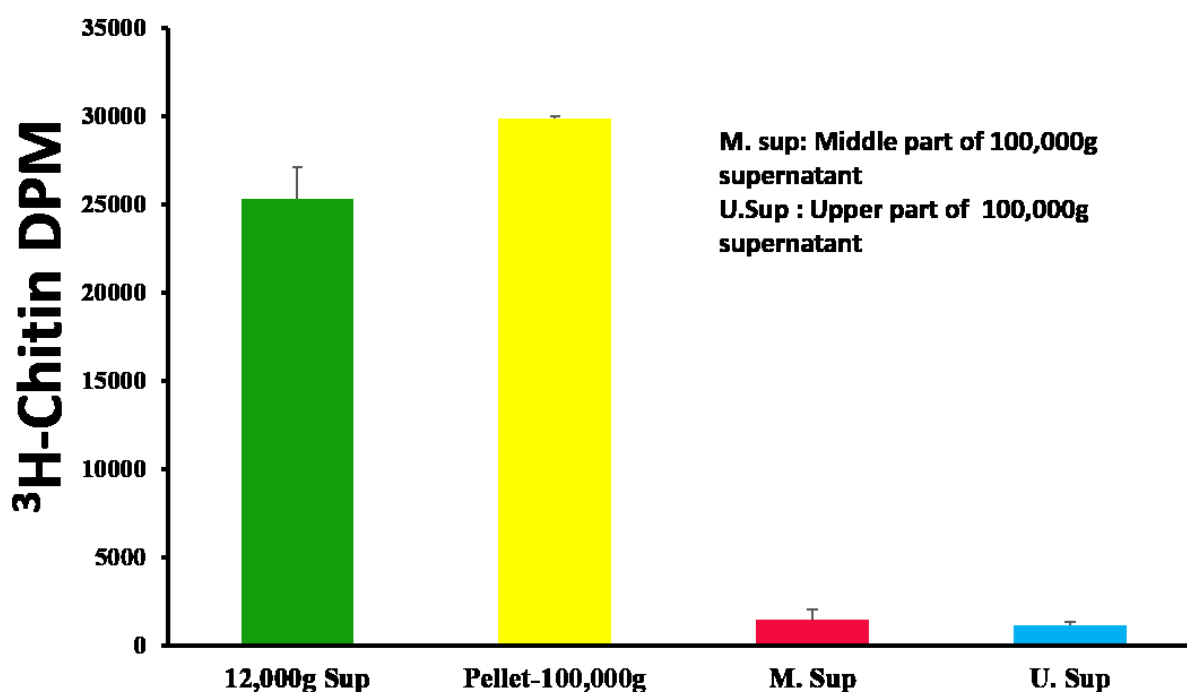
### **Membrane preparations from *T. castaneum* elytra have chitin synthase activity**

In view of the conflicting results on inhibition of CHS activity by DFB *in vitro* using membrane preparations from guts of *T. castaneum* larvae (Cohen & Casida, 1980) and that of the brine shrimp larvae (Horst, 1980), We decided to investigate whether similar membrane preparations from *T. castaneum* elytra would exhibit CHS activity and whether it will be sensitive to DFB. Extracts were prepared from elytral tissue (free of other body parts including hindwings) from 5-day old pupae. They were ground in CHS-A buffer and subjected to differential centrifugation at different g forces to obtain a 12,000g pellet, a 100,000g pellet and a 100,000g soluble supernatant fraction. These preparations were tested for CHS activity using the radioactive substrate, UDP-N-acetyl-D-glucosamine (6-<sup>3</sup>H). After a 90 min reaction, (when maximum <sup>3</sup>H-incorporation was observed in time course experiments) incorporation of label into hot KOH insoluble material (presumed to be chitin and devoid of glycoproteins that might have been labeled by this substrate) was determined as described in Materials & Methods section (see section 3. 4).

### **Vesicle-mediated CHS activity of different supernatant fractions**

As shown in Fig. 3.1, the 12,000g supernatant and the 100,000g pellet had CHS activity. The 100,000g soluble supernatant fraction was devoid of CHS activity, indicating that this enzyme was associated only with membrane preparations in expectation with CHS being a membrane-bound enzyme. To further confirm that the product was indeed chitin, we used inhibitors specific for either CHS (nikkomycin-Z, a competitive inhibitor of

CHS) or for the first enzyme involved in N-glycosylation of proteins (tunicamycin, a non-competitive inhibitor of the UDP-GlcNAc: dolichol-phosphate GlcNAc-1-phosphate transferase). As shown in Fig. 3.3, the incorporation of  $^3\text{H}$ - label into KOH-insoluble fraction was affected by nikkomycin-Z, but not by tunicamycin. These results indicated that the membrane preparations do synthesize chitin, and that our protocol is not complicated by N-glycosylation reactions. The vesicles were active in CHS assays even after storage for 7 days at  $-20\text{ }^{\circ}\text{C}$ . We could perform as many as 20 assays per vesicle preparation from 100 pairs of elytra. From these results we conclude that vesicular preparations from *T. castaneum* elytra do exhibit robust CHS activity *in vitro* and that this system is suitable for investigating the properties of CHS from this beetle.

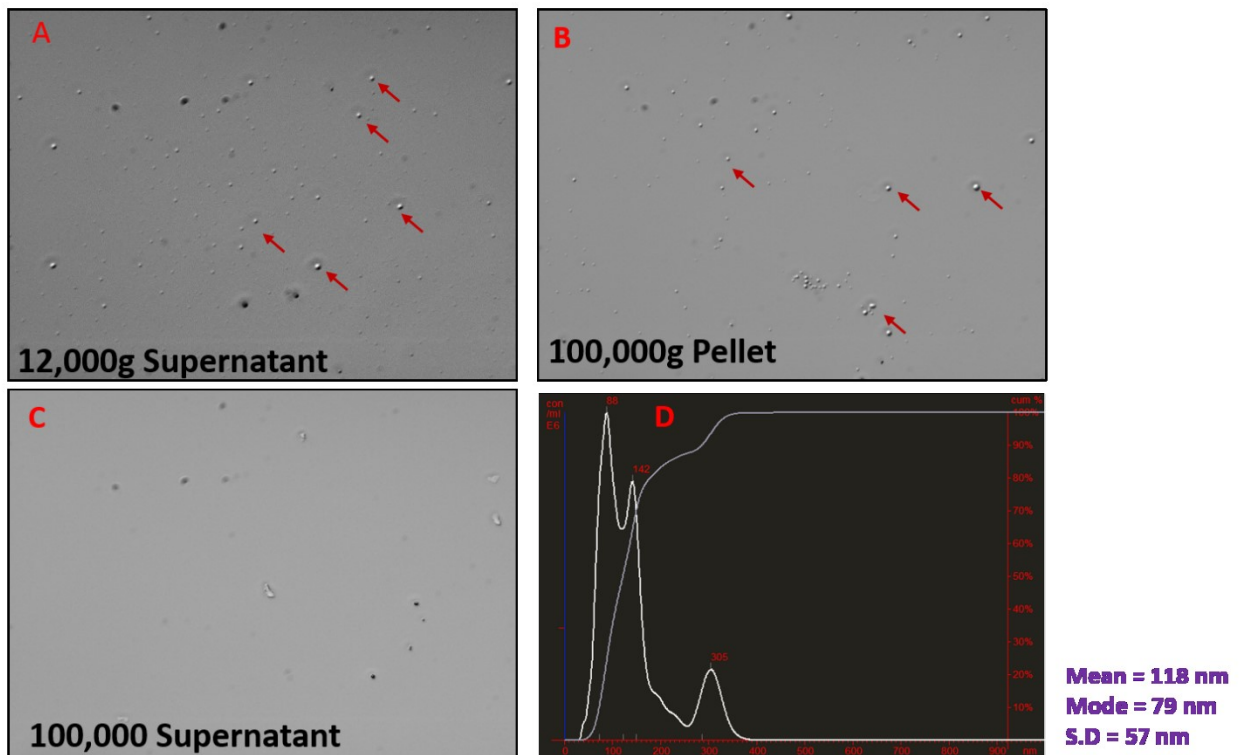


**Figure 3.1 Vesicle-mediated CHS activity of different supernatant fractions**

*Protein was extracted from the elytra (n=100) of T. castaneum at pharate adult stage. Elytra were ground in CHS-A buffer followed by differential centrifugation at different g forces to obtain 12,000 g pellet, 100,000g pellet and 100,000 g soluble supernatant fraction. 12,000 g pellet, 100,000g pellet and 100,000 soluble (M. Sup: Middle fraction of 100,000g supernatant, and U.Sup: upper fraction of supernatant) fraction were tested for CHS-A activity by using UDP-N-acetyl-D-glucosamine (6-<sup>3</sup>H) as a substrate. Differential centrifugation pellet fraction was resuspended in 100 µl of CHS-A buffer out of which 5 µl was used but in case of supernatant fraction 5 µl was used directly in CHS-A assay along with CHS-A buffer, 0.2 µCi UDP-N-acetyl-D-glucosamine (6-<sup>3</sup>H), 120 µM unlabeled UDP-N-acetyl-D-glucosamine and incubated for 90 min. Chitin synthase activity was measured by the incorporation of UDP-N-acetyl-D-glucosamine (6-<sup>3</sup>H) into KOH insoluble material as described in Materials & Methods section). The values were calculated an average (± SEM) of three independent experiments.*

### Light microscopy of different vesicle preparation showing presence (A and B) and absence of (C) vesicles

In addition, we further investigated and confirmed that the 12,000g supernatant (A) and 100,000g (B) pellets membrane preparations contain vesicles by direct microscopic observations using light-scattering conditions (Fig. 3.2). The 100,000g supernatant fraction (C), which was devoid of CHS activity was greatly depleted of vesicles. (D) Nanoparticle tracking analyses indicated the mean size of particle was 118 nm with a mode of 79 nm (analysis performed by NANOSIGHT, University of North Carolina).



**Figure 3.2 Light microscopy of different vesicle preparation showing presence and absence of vesicle**

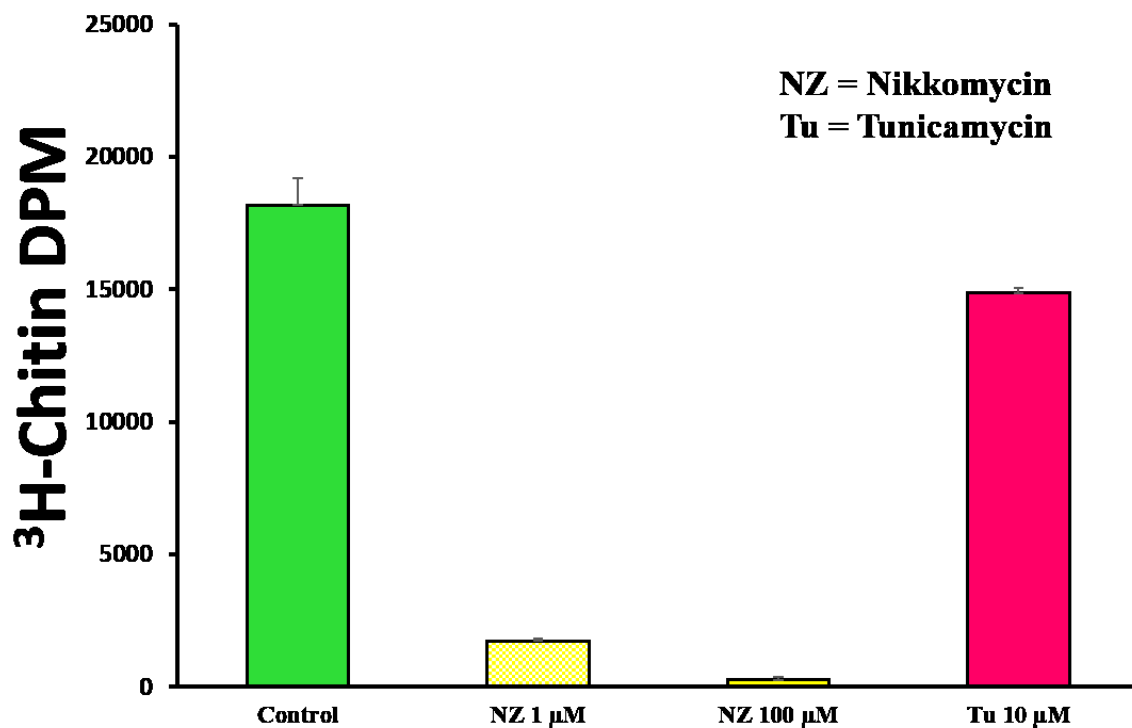
*12,000g supernatant and 100,000g pellet possessed capability to synthesize chitin in vitro as measured by using  $^3\text{H}$ -UDP-GlcNAc as the substrate for chitin formation. To find out*



if the protein fractions that had the capability to synthesize chitin *in vitro* also contain the microsomal vesicles, the fractions were examined using a fluorescent microscope. Both the active fractions (*A*) 12,000g supernatant and (*B*) 100,000g pellet contained microsomal vesicles.

### **Nikkomycin (chitin synthase inhibitor) inhibits *in vitro* chitin synthesis**

The 100,000g pellet fraction which was extracted from the elytra of *T. castaneum* and contained microsomal vesicles, also possessed the capacity to make chitin. To confirm that the synthesized product in the chitin synthesis reaction is indeed chitin, we incubated the reaction mixture with two different concentrations of nikkomycin-Z (1  $\mu$ M and 100  $\mu$ M) which is an inhibitor of chitin synthase (substrate analog) and with Tunicamycin 10  $\mu$ M, measured chitin formation using a scintillation counter. We found that there is no activity in 1  $\mu$ M or 100  $\mu$ M nikkomycin-Z incubated samples, which confirmed that the product was chitin indeed.



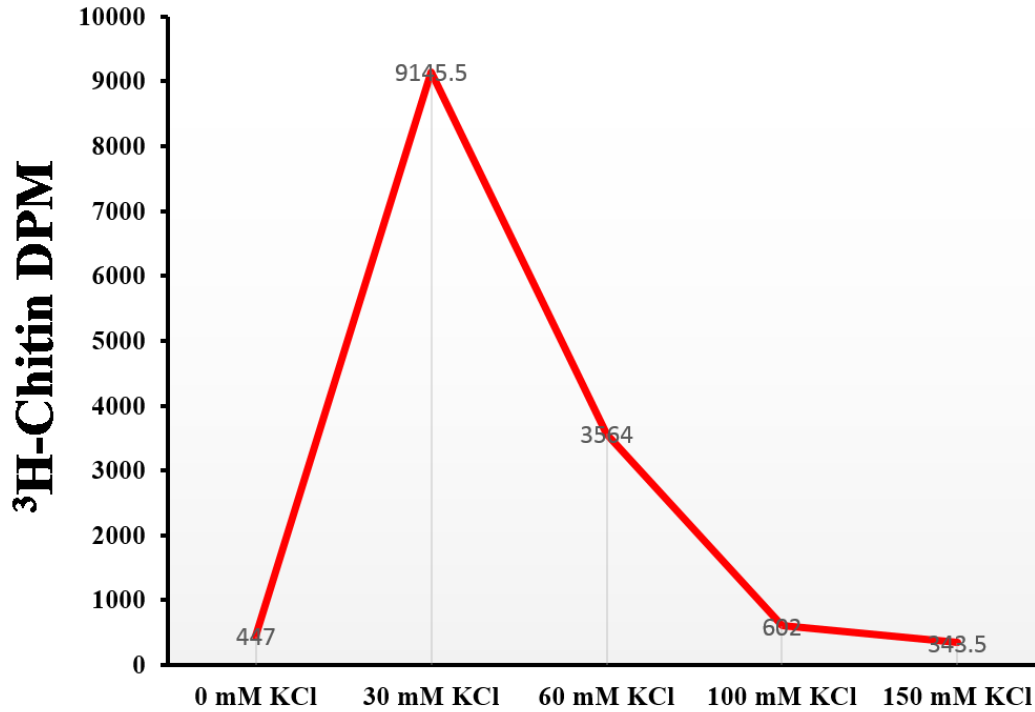
### **Figure 3.3 Nikkomycin inhibits *in vitro* chitin synthesis**

*Elytra (n=100) were isolated from T. castaneum at the pharate adult stage. The extracted elytra were ground with CHS-A buffer followed by differential centrifugation. After differential centrifugation the 100,000 g pellet fraction was resuspended in 100  $\mu$ l of CHS-A buffer out of which 5  $\mu$ l was used in CHS-A assay along with CHS-A buffer, 0.2  $\mu$ Ci UDP-N-acetyl-D-glucosamine (6- $^3$ H), 120  $\mu$ M unlabeled substrate, nikkomycin-Z (1  $\mu$ M or 100  $\mu$ M) or tunicamycin 10 $\mu$ M in the 100  $\mu$ l of reaction mix and were incubated for 90 min. Chitin synthase activity was measured by measuring the incorporation of UDP-N-acetyl-D-glucosamine (6- $^3$ H). The values were calculated as the average ( $\pm$  SEM) of three independent experiments. The amount of radioactivity incorporated was measured with a scintillation counter.*

### **Chitin synthase activity of vesicles is influenced by the concentration of K<sup>+</sup> ions in the reaction mixture.**

This experiment was conducted to study the influence of varying the extra-vesicular concentration of K<sup>+</sup> ions on *in vitro* chitin synthesis by using the 100,000 pellet fraction prepared from extracts of elytra of *T. castaneum*. In this assay system, we used 50 mM CHS-A buffer containing 50 mM MOPS, 10 mM MgCl<sub>2</sub> and 30 mM KCl to prepare the vesicles. To determine the optimum concentration of KCl in the buffer outside the vesicles, on incorporation of UDP-N-acetyl-[6- $^3$ H] D-glucosamine took place, the concentration of KCl in the reaction mixture was varied (while keeping concentrations of other components constant) and incorporation of UDP-N-acetyl-[6- $^3$ H] D-glucosamine

was measured. Highest incorporation was observed at 30 mM of KCl indicating that at other concentrations of external  $[K^+]$  (lower or higher than the intravesicular concentration of 30 mM) led to a decrease in chitin synthase activity.



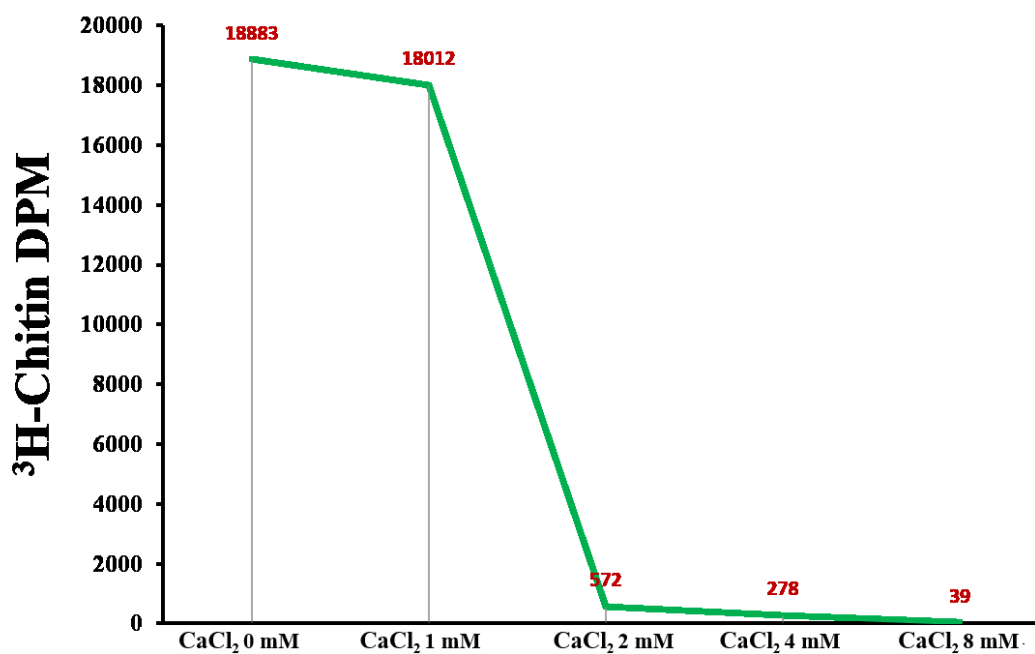
**Figure 3.4 Chitin synthase activity of vesicle is influenced by the concentration of  $K^+$  ions in the reaction mixture**

*Proteins were extracted from pharate adult elytra using the standard protocol as mentioned in (3.2.3) section of this chapter. In vitro CHS-A assay was performed using 50 mM CHS-A buffer, 0.25  $\mu$ Ci UDP-N-acetyl-[6-  $^3$ H] D-glucosamine (37 Ci/mmol Perkin-Elmer), 120  $\mu$ M unlabeled substrate and varying the concentrations of KCl (0 mM, 30 mM, 60 mM, 100 mM and 120 mM) for 90 min. The values were calculated as*

*average ( $\pm$  SEM) of three independent experiments. The amount of radioactivity incorporated was measured with a scintillation counter.*

### **Effect of various concentrations of $\text{CaCl}_2$ on the synthesis of chitin *in vitro***

The study from (Nakagawa and Matsumura., 1993) indicated that chitin synthesis in a vesicular system prepared from cockroaches is regulated by  $\text{K}^+$ ,  $\text{Ca}^+$  and  $\text{H}^+$  ions. Even though our *in vitro* assay did not include added calcium in the assay buffer, we wanted to know if addition of  $\text{CaCl}_2$  had an effect on chitin synthase activity. We added varying concentration of  $\text{CaCl}_2$  (0 mM, 2 mM, 4 mM, and 8 mM) to the reaction buffer (50 mM CHS-A buffer, containing (50 mM MOPS, 10 mM  $\text{MgCl}_2$  and 30 mM KCl), 0.25  $\mu\text{Ci}$  UDP-N-acetyl-[6-  $^3\text{H}$ ] D-glucosamine, 120  $\mu\text{M}$  unlabeled substrate and 5  $\mu\text{l}$  of vesicle preparation from elytra of *T. castaneum*, incubation for 90 min at 32°C the incorporation of label from the UDP-N-acetyl- -[6-  $^3\text{H}$ ] D-glucosamine substrate into chitin was measured. The synthesis of chitin was not affected when we increased  $[\text{Ca}^{2+}]$  from 0 to 1 mM. , Further additions of  $[\text{Ca}^{2+}]$  were inhibitory to chitin synthesis.



**Figure 3.5** Effect of various concentration of CaCl<sub>2</sub> on the synthesis of chitin *in vitro*

Proteins were extracted by using the standard protocol as mentioned in (3.2.3) section of this chapter from the elytra of *T. castaneum* (Pupa day 5). *In vitro* CHS-A assay was performed by using 50 mM CHS-A buffer, 0.25  $\mu$ Ci UDP-N-acetyl-[6- <sup>3</sup>H] D-glucosamine, 120  $\mu$ M unlabeled substrate and varying the concentration of CaCl<sub>2</sub> (0 mM, 1 mM, 2 mM, 4 mM and 8 mM). Effect of varying concentration of CaCl<sub>2</sub> on chitin biosynthesis was measured by using the incorporation of UDP-N-acetyl-[6- <sup>3</sup>H] D-glucosamine for 90 min. The values were calculated as average ( $\pm$  SEM) of three independent experiments. The amount of radioactivity incorporated was measured with scintillation counter.

## Vesicle-mediated CHS activity is sensitive to inhibition by DFB and glibenclamide

As reported above, the 100,000 g pellet fraction from the elytra of *T. castaneum* had the capacity to synthesize chitin. *In vitro* studies using membrane preparations from guts of *T. castaneum* larvae (Cohen & Casida, 1980) or from brine shrimp larvae (Horst, 1981), had not shown inhibition of CHS activity when DFB was included in the buffer in *in vitro* assays. So, we decided to investigate whether similar membrane preparations from *T. castaneum* elytra would inhibit CHS activity by DFB and the lowest possible concentration that would be inhibitory. The results shown in Fig. 3.6 demonstrated that chitin synthesis in the 100,000g vesicle from elytra of *T. castaneum* was inhibited by DFB in the pico molar (and higher) range and by Glibenclamide.

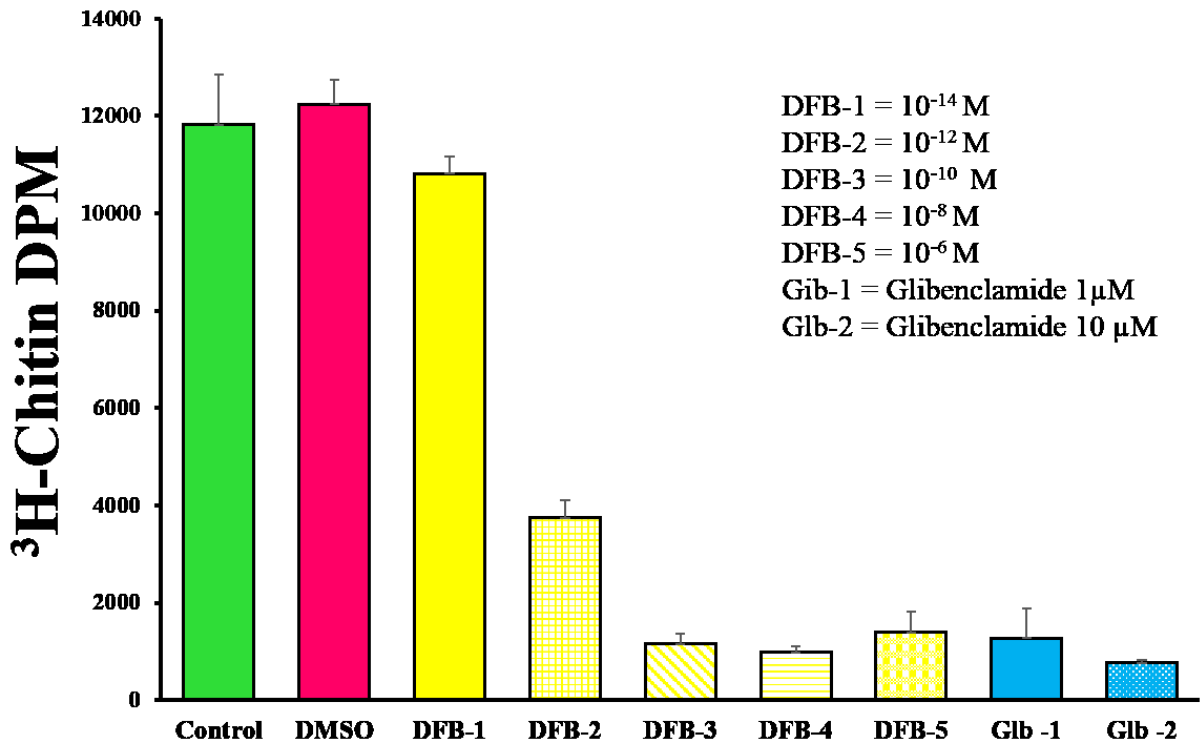
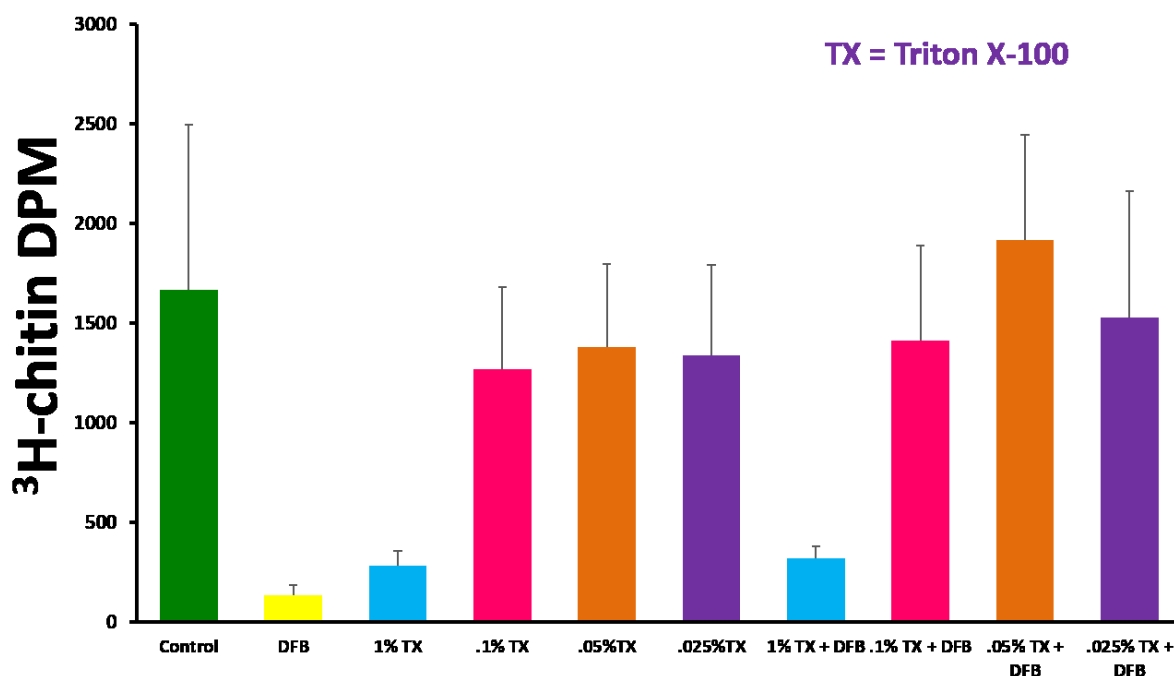


Figure 3.6 Vesicle-mediated CHS activity is sensitive to inhibition by DFB and Glibenclamide

*Protein was extracted using the standard protocol as mentioned in (3.2.3) section of this chapter from the elytra of T. castaneum (Pupa day 5). In vitro CHS-A assay was performed by using 5  $\mu$ l of 100,000 g pellet fraction ,different concentrations of DFB (  $10^{-15}$ ,  $10^{-14}$ ,  $10^{-12}$ ,  $10^{-8}$  and  $10^{-6}$ ), or by using Glibenclamide ( 1  $\mu$ M and 10  $\mu$ M ). 0.25  $\mu$ Ci UDP-N-acetyl-[6-  $^3$ H] D-glucosamine and CHS-A buffer for 90 min. At the end of the reaction, 1 ml of 1.5 M KOH was added and the tubes were kept at 90°C for 2 h (to hydrolyze proteins including glycoproteins which might receive the radioactive label; chitin is resistant to KOH treatment) and then filtered through a Whatman 2.5 cm GF/C filter, and washed extensively with ethanol. The filters were dried and radioactive decays were counted in a liquid scintillation counter after adding 5 ml of Econofluor. The values were calculated an average of ( $\pm$  SEM) of three independent experiments.*

### **Vesicular integrity is required to maintain susceptibility to DFB**

We confirmed that 12,000g or 100,000g pellet fraction, which had CHS-A activity also contained vesicles and that they were sensitive to DFB. The experiment was done to find out whether intact vesicles are important for the inhibitory effect of DFB. The inhibitory effect of DFB (1  $\mu$ M) was reversed by (0.1%, 0.05% and 0.025%) Triton X100, suggesting that intact vesicles were required for DFB to inhibit chitin synthesis *in vitro*. However, 1% Triton X100 by itself was inhibitory presumably because its concentration was above critical micelle concentration (CMC) and did not reverse the inhibition by DFB, presumably because at this concentration the membrane-bound CHS was no longer in its proper native conformation.



**Figure 3.7. Vesicular integrity is required to maintain susceptibility to DFB.**

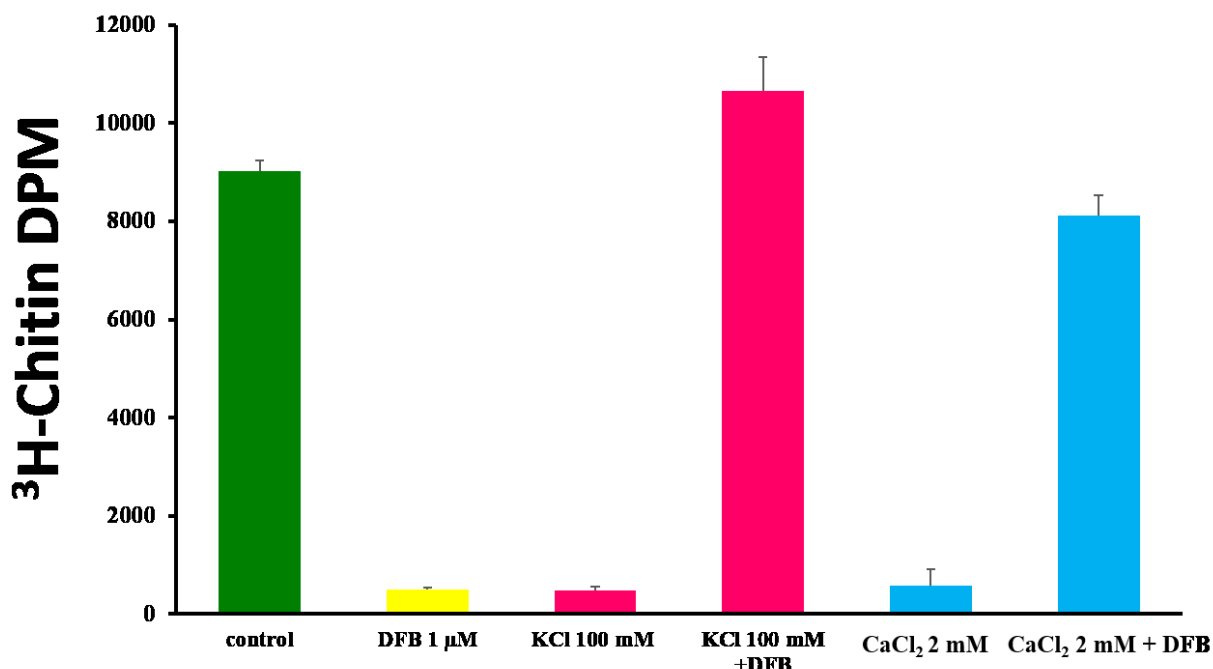
*Elytra (n = 100) were dissected out from T. castaneum at pupa day five. The elytra were ground in CHS buffer followed by differential centrifugation. The, 100,000 g pellet fraction was resuspended in 100  $\mu$ l of CHS buffer. Reaction mix was prepared containing 5  $\mu$ l of 100,000g resuspended fraction, 50 mM CHS-A buffer, 0.25  $\mu$ Ci UDP-N-acetyl-[6-<sup>3</sup>H] D-glucosamine, 120  $\mu$ M unlabeled substrate and varying the percentage of Triton X-100 (1%, 0.1%, 0.05% and 0.25%) with and without DFB(1 $\mu$ l). The reaction was carried for 90 min at 32°C. At the end of the reaction, 1 ml of 1.5 M KOH was added and the tubes were kept at 90°C for 2 h (to hydrolyze proteins including glycoproteins which might have received the radioactive label (chitin is resistant to KOH treatment) and then filtered through a 2.5 cm GF/C filter, and washed extensively with ethanol. The filters were dried and radioactive decays were counted in a liquid scintillation counter after*



*adding 5 ml of Econofluor. The values were calculated an average ( $\pm$  SEM) of three independent experiments.*

### **Inhibitory effect of DFB was reversed by addition of KCl or CaCl<sub>2</sub>**

Glibenclamide and DFB belong to the sulfonylurea group of chemicals. As glibenclamide has been found to bind SUR/K<sub>ir</sub> channel complex in humans and ions such as potassium and calcium play an important role in insulin secretion, we conducted an *in vitro* experiment to find out whether inhibition by DFB can also be counteracted by forcing extra potassium or calcium ions inside the vesicles. In this experiment, the concentration of KCl in the standard CHS-A buffer was increased from 30 mM to 100 mM or by adding 2 mM CaCl<sub>2</sub>. . It was observed that the inhibition of CHS activity by 1  $\mu$ M DFB in intact vesicles (i.e. in the absence of detergents) could be reversed by treatment with 100 mM potassium or 2 mM calcium ions.



**Figure 3.8 Inhibitory effect of DFB was reversed by addition of KCl or CaCl<sub>2</sub>**

*Effect of 100 mM KCl and 2 mM CaCl<sub>2</sub> on the inhibition of chitin synthase by DFB. Eytra (n = 100) were extracted from T. castaneum followed by differential centrifugation and 100,000g pellet fraction was collected. Reaction mix was made containing 5 μl of 100,000g pellet from resuspended fraction, 50 mM CHS-A buffer, 0.25 μCi UDP-N-acetyl-] D-glucosamine [6- <sup>3</sup>H), 120 μM unlabeled substrate, 100 mM of KCl with and without DFB (1 μM) and 2 mM CaCl<sub>2</sub> with and without DFB (1 μM). The reaction was carried for 90 min at 32°C. At the end of the reaction, 1 ml of 1.5 M KOH was added and the tubes were kept at 90°C for 2 h and then filtered through a 2.5 cm GF/C filter, and washed extensively with ethanol. The filters were dried and radioactive decays were counted in a liquid scintillation counter after adding 5 ml of Econofluor. The values were calculated an average (± SEM) of three independent experiments.*

### Inhibitory effect of DFB is reversed by the K<sup>+</sup> and Ca<sup>2+</sup> ionophores

In the previous experiment, we demonstrated that inhibition of CHS activity by 1  $\mu$ M DFB in intact vesicles could be reversed by treatment with 100 mM potassium or 2 mM calcium ions. We further investigated the reversal on the inhibitory effect of DFB on CHS activity by potassium and calcium ionophores that will allow free movement of these ions across the membrane. The potassium ionophore, valinomycin (10  $\mu$ M) or the calcium ionophore A23187 (10  $\mu$ M) were able to overcome the inhibition of CHS activity by DFB (1  $\mu$ M) in intact vesicles (i.e. in the absence of detergents). We conclude that calcium ionophore, potassium ionophore, excess calcium, and excess potassium ions, all showed similar results by overcoming the inhibition of CHS activity by DFB presumably by forcing the movement of the ions through the membrane.

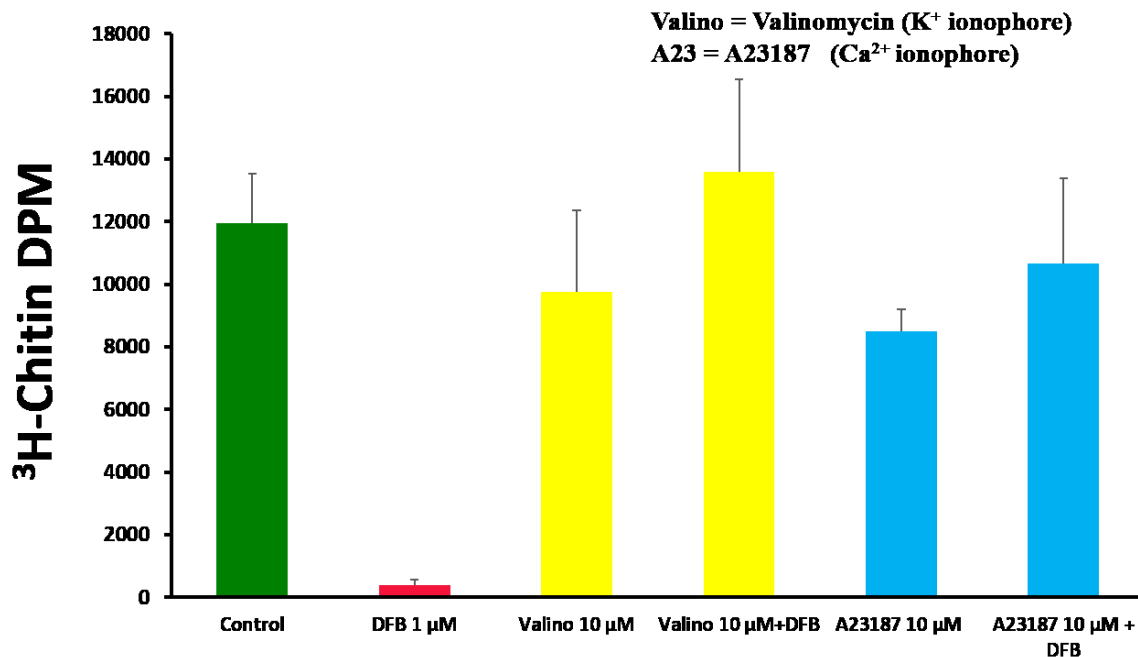


Figure 3.9. Inhibitory effect of DFB is reversed by K<sup>+</sup> and Ca<sup>2+</sup> ionophores

*Effect of calcium (10  $\mu$ M) and potassium (10  $\mu$ M) ionospheres on the inhibition of chitin synthase by DFB (1  $\mu$ M). In vitro CHS-A assay was performed by using 5  $\mu$ l of 100,000 g pellet fraction, 10  $\mu$ M of valinomycin ( $K^+$  ionophore) with and without DFB (1  $\mu$ M), 10  $\mu$ M A23187 ( $Ca^{2+}$  ionophore) with and without DFB (1  $\mu$ M), 0.25  $\mu$ Ci UDP-N-acetyl-[6- $^3$ H] D, 120  $\mu$ M unlabeled substrate, 5  $\mu$ l of resuspended 100,000g pellet fraction, and 50 mM CHS-A buffer for 90 min. At the end of the reaction, 1 ml of 1.5 M KOH was added and the tubes were kept at 90°C for 2 h ( to hydrolyze proteins including glycoproteins which might receive the radioactive label; chitin is resistant to KOH treatment) and then filtered through a 2.5 cm GF/C filter, and washed extensively with ethanol. The filters were dried and radioactive decays were counted in a liquid scintillation counter after adding 5 ml of Econofluor. The values were calculated an average ( $\pm$  SEM) of three independent experiments.*

### **The role of $K^+$ is to allow entry of $Ca^{++}$ into vesicles**

As indicated above, calcium ionophore, potassium ionophore, excess calcium, and excess potassium, all overcame the inhibitory effect of DFB on CHS-A activity. We further decided to investigate whether the requirement of  $K^+$  ions *in vitro* chitin synthesis by the vesicle prepared from the elytra of *T. castaneum* was a primary effect or a secondary effect. We found that requirement of  $K^+$  ions is indirect because when the permeability of the vesicle was compromised by low concentrations of detergent even in the absence of added  $K^+$  ions. CHS activity was similar to the control reaction with 30 mM KCl (compare green bar with yellow bar). In other words, there was no absolute requirement for 30 mM KCl in the assay buffer as long as the concentration of  $K^+$  ions on either side of the membrane was allowed to become equal either by balancing the concentration of

this ion across the vesicle membrane or by allowing membrane permeabilization by detergents or ionophores. We presume that with intact vesicles,  $K^+$  ions cannot freely move across the membrane except through designated  $K^+$  ion channels. Similarly, in the absence of added  $K^+$ , inclusion of 2 mM  $CaCl_2$  in the assay buffer allowed the vesicles to make the chitin, even though 2 mM  $CaCl_2$  in our standard assay conditions (30 mM KCl /control) was by itself inhibitory to chitin synthesis by the vesicles. Presumably, excess  $Ca^{2+}$  was able to enter the vesicles by diffusion and this process alone is sufficient to overcome a permeability barrier to this ion. The effect of the  $Ca^{2+}$  ionophore is also consistent with this interpretation. Thus the role of  $K^+$  ions is likely to be mainly to regulate the flow of  $Ca^{2+}$  ions across the membrane.

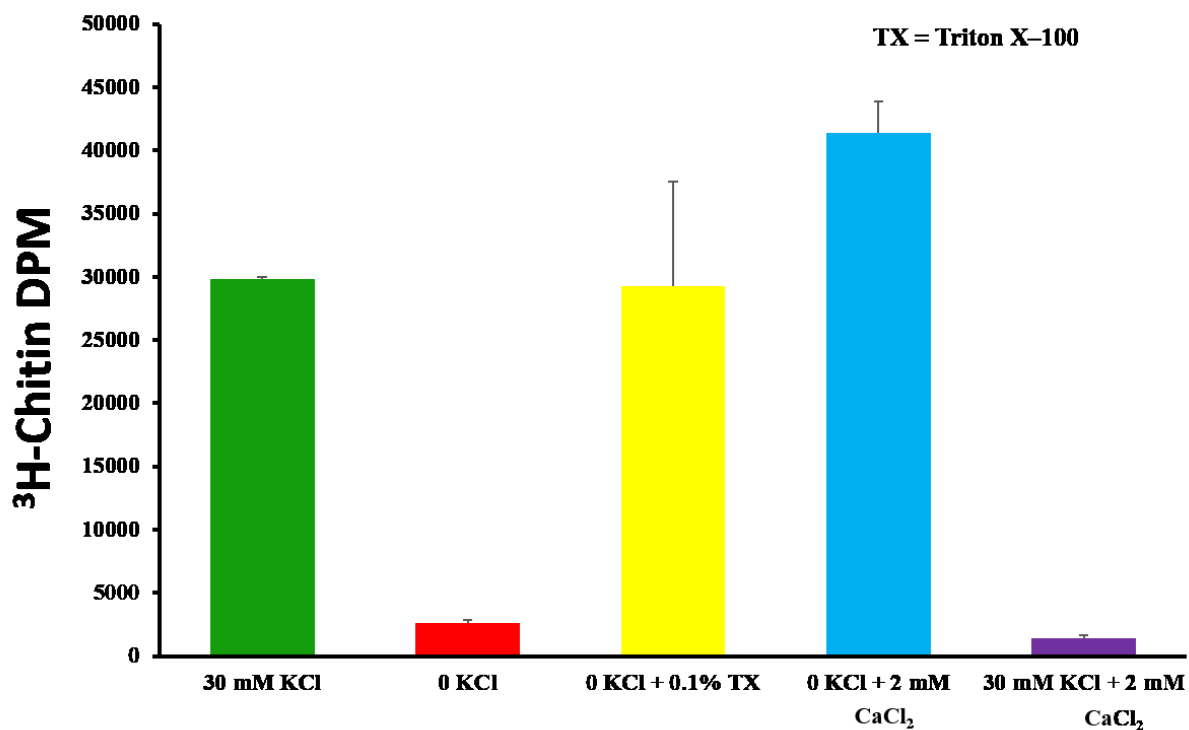


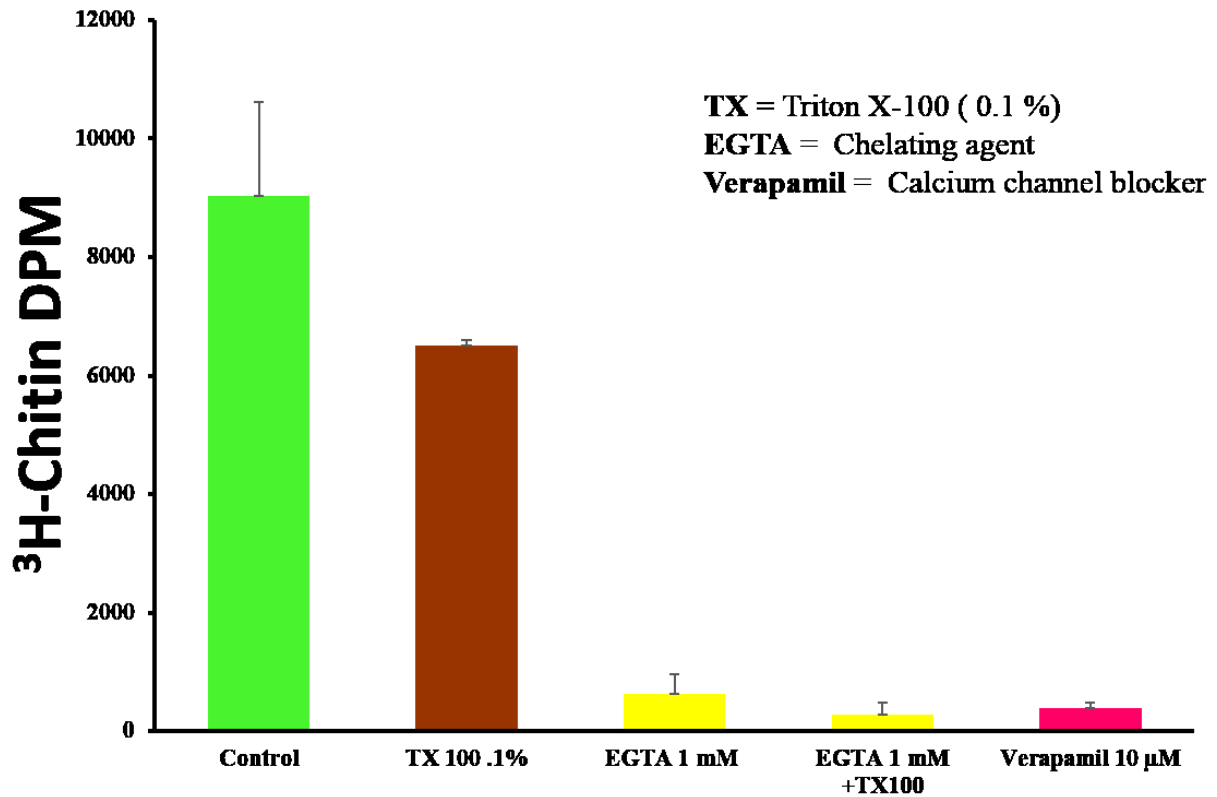
Figure 3.10 The role of  $K^+$  is to allow entry of  $Ca^{2+}$  into vesicles

*Elytra were extracted from T. castaneum at pharate adult stage followed by differential centrifugation through which 100,000 g pellet fraction was collected and resuspended in 100  $\mu$ l of CHS-A buffer. 100  $\mu$ l reaction mix was made containing 30 mM KCl (Control, green bar) or 0 mM KCl/ without KCl (red bar), or 0 mM KCl + 0.1% TX, (yellow bar) 0 mM KCl + 2 mM  $\text{CaCl}_2$  (blue bar) or 30 mM KCl + 2 mM  $\text{CaCl}_2$  (purple bar). 0.25  $\mu$ Ci UDP-N-acetyl-] D-glucosamine [6-  $^3\text{H}$ , 120  $\mu$ M unlabeled substrate, 5  $\mu$ l of resuspended 100,000g pellet fraction and 50 mM CHS-A buffer for 90 min. At the end of the reaction, 1 ml of 1.5 M KOH was added and the tubes were kept at 90°C for 2 h (to hydrolyze proteins including glycoproteins which might receive the radioactive label; chitin is resistant to KOH treatment) and then filtered through a 2.5 cm GF/C filter, and washed extensively with ethanol. The filters were dried and radioactive decays were counted in a liquid scintillation counter after adding 5 ml of Econofluor. The values were calculated an average ( $\pm$  SEM) of three independent experiments.*

### **$\text{Ca}^{++}$ is absolutely required for CHS activity**

So far we have concluded that several conditions such as addition of calcium ionophore, potassium ionophore, excess calcium, or potassium ions overcame the inhibitory effect of DFB on CHS-A activity by intact vesicles. We also found that the requirement for  $\text{K}^+$  ions is indirect as the reaction proceeded without any added  $\text{K}^+$  ions. So, we decided to investigate whether the requirement of calcium ions is absolute. We found that chitin synthesis was completely inhibited by 2 mM EGTA (which chelated  $\text{Ca}^{2+}$  ions preferentially) (yellow bar) and by verapamil (red bar) which blocks movement of  $\text{Ca}^{2+}$  ions across channels. The requirement for  $\text{Ca}^{2+}$  ions was evident even in the presence of low concentrations (0.1%) of the detergent, Tx100 (which is only mildly inhibitory;

brown bar) indicating an absolute requirement of  $\text{Ca}^{++}$  ions in the synthesis of chitin in vesicles even when its permeability is compromised.



**Figure 3.11  $\text{Ca}^{2+}$  is absolutely required for CHS activity**

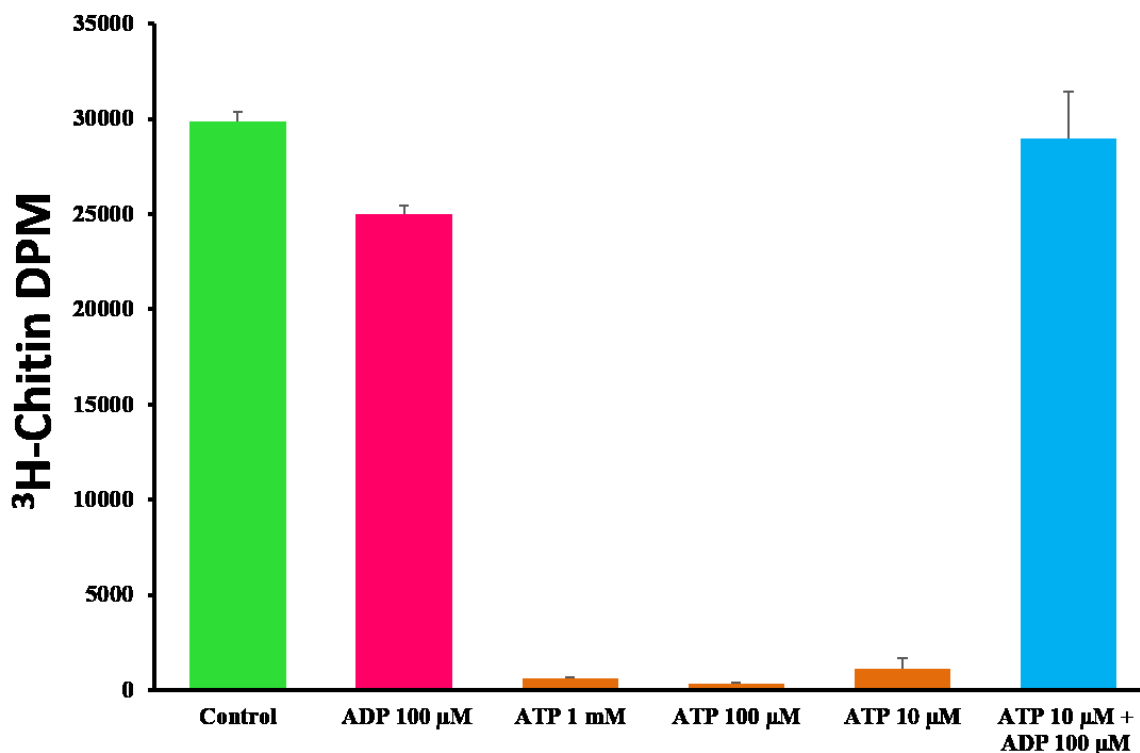
*Microsomal vesicles were prepared from the elytra *T. castaneum*. Hundred elytra were extracted from pupa day 5 of *T. castaneum* followed by differential centrifugation 100,000g pellet fraction were extracted and resuspended in 100  $\mu\text{l}$  of CHS-A buffer. Chitin synthase activity was measured in different buffer combinations {Triton X-100 (0.1%), EGTA (a calcium ion chelator), EGTA and Triton X-100, Verapamil (calcium channel blocker)} with 0.25  $\mu\text{Ci}$  UDP-N-acetyl D-glucosamine [6-  $^3\text{H}$ ], 120  $\mu\text{M}$  unlabeled substrate, 5  $\mu\text{l}$  of resuspended 100,000g pellet fraction and 50 mM CHS-A buffer for 90 min. At the end of the reaction, 1 ml of 1.5 M KOH was added and the tubes*

*were kept at 90°C for 2 h (to hydrolyze proteins including glycoproteins which might receive the radioactive label; chitin is resistant to KOH treatment) and then filtered through a 2.5 cm GF/C filter, and washed extensively with ethanol. The filters were dried and radioactive decays were counted in a liquid scintillation counter after adding 5 ml of Econofluor. The values were calculated an average ( $\pm$  SEM) of three independent experiments.*

### **CHS activity of vesicles is sensitive to ATP/ADP ratio**

The mammalian SUR/K<sub>ATP</sub> channel in pancreatic  $\beta$ -cells has been shown to be regulated by the ATP/ADP ratio (Proks et al., 2002). We designed an experiment to find out whether chitin synthesis in intact vesicles is also regulated by ATP/ADP ratio. Chitin synthesis by the vesicles was inhibited by 1 mM or 100  $\mu$ M or 10  $\mu$ M ATP (brown bars). The inhibition by 10  $\mu$ M ATP was reversed by simultaneous addition of 100  $\mu$ M ADP (blue bar) whereas 100  $\mu$ M ADP by itself was not significantly inhibitory (red bar). These results suggest that chitin synthesis by the intact vesicles is regulated by ATP/ADP ratio similar to its action on K<sub>ATP</sub> channels in pancreatic  $\beta$ -cells.





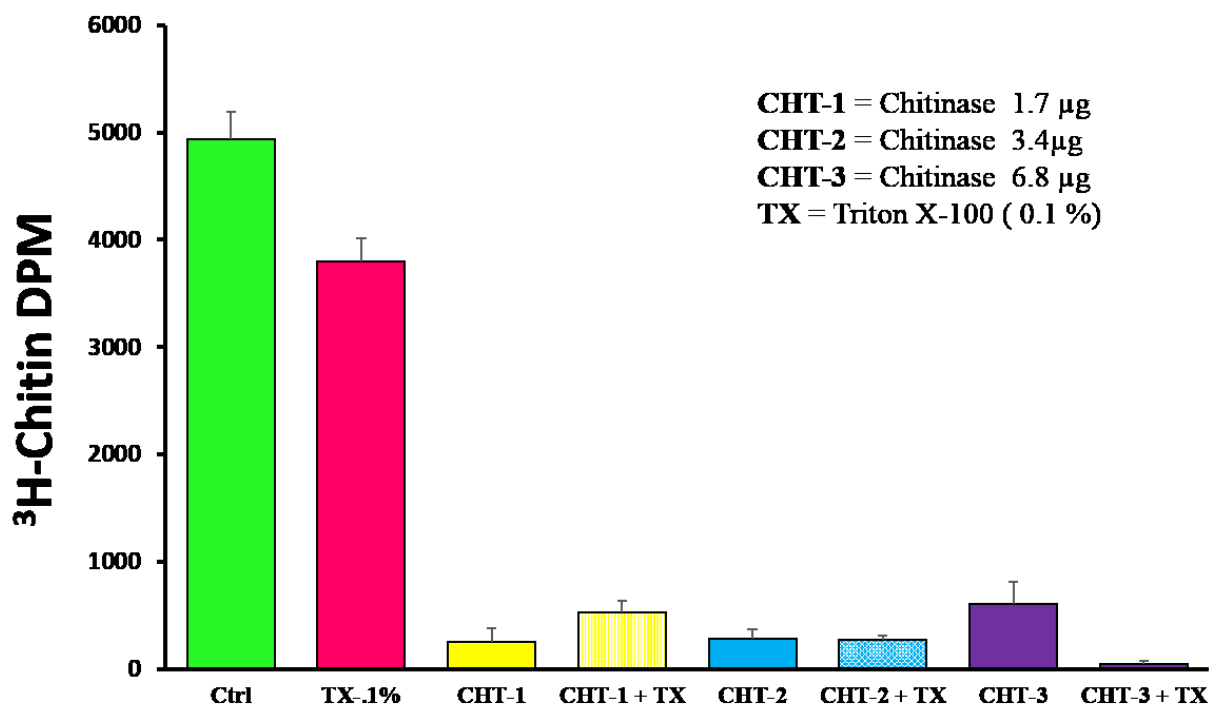
**Figure 3.12 CHS activity is sensitive to ATP/ADP ratio**

*To find out the effect of different concentration of ATP and ADP on CHS activity in vesicles. CHS assay was performed by using elytra (100) from *T. castaneum*. The elytra was ground in CHS-A buffer followed by differential centrifugation and collection of 100,000 g pellet fraction. The 100,000 g pellet fraction was resuspended in CHS-A buffer out of which 5 μl was used per reaction. Reaction mixture was made containing different concentration of ATP (10 μM, 100 μM and 1 mM), ADP (100 μM) or ATP (10 μM) + ADP (100 μM) with 0.25 μCi UDP-N-acetylD-glucosamine [6- <sup>3</sup>H], 120 μM unlabeled substrate, 5 μl of resuspended 100,000g pellet fraction and 50 mM CHS-A buffer for 90 mins. At the end of the reaction, 1 ml of 1.5 M KOH was added and the tubes were kept at*

*90°C for 2 h (to hydrolyze proteins including glycoproteins which might receive the radioactive label; chitin is resistant to KOH treatment) and then filtered through a 2.5 cm GF/C filter, and washed extensively with ethanol. The filters were dried and radioactive decays were counted in a liquid scintillation counter after adding 5 ml of Econofluor. The values were calculated an average ( $\pm$  SEM) of three independent experiments.*

**<sup>3</sup>H-Chitin product synthesized was sensitive to degradation by *M. sexta* chitinase and the chitin product is secreted out of the vesicles.**

To further investigate if the product synthesized by the vesicles in 100,000 g pellet fraction from the elytra of *T. castaneum* was indeed chitin we digested the product with a purified endochitinase prepared from Hi-5 cells/baculovirus expression system (Gopalakrishnan et al., 1995). This enzyme was added to chitin synthesis reaction mixture at the end of 90 min of incubation followed by followed by incubation for additional one hour period either in the presence of or absence of Triton-X100. We anticipated that if the chitin accumulated inside the vesicles, it will be insensitive to the enzyme unless the vesicles were disrupted by the detergent. All of the chitin product was degraded by added chitinase irrespective of whether the detergent was present or not. This experiment revealed that the chitin product was secreted outside the vesicle in this system. Since the extent of chitin digestion did not increase upon detergent addition, we conclude that all vesicles capable of chitin synthesis were in the “outside-out” configuration and not a mixture of “inside-out” and “inside-in” vesicles as has been reported for vesicle preparations synthesizing hyaluronan (Hubbard et al., 2012).



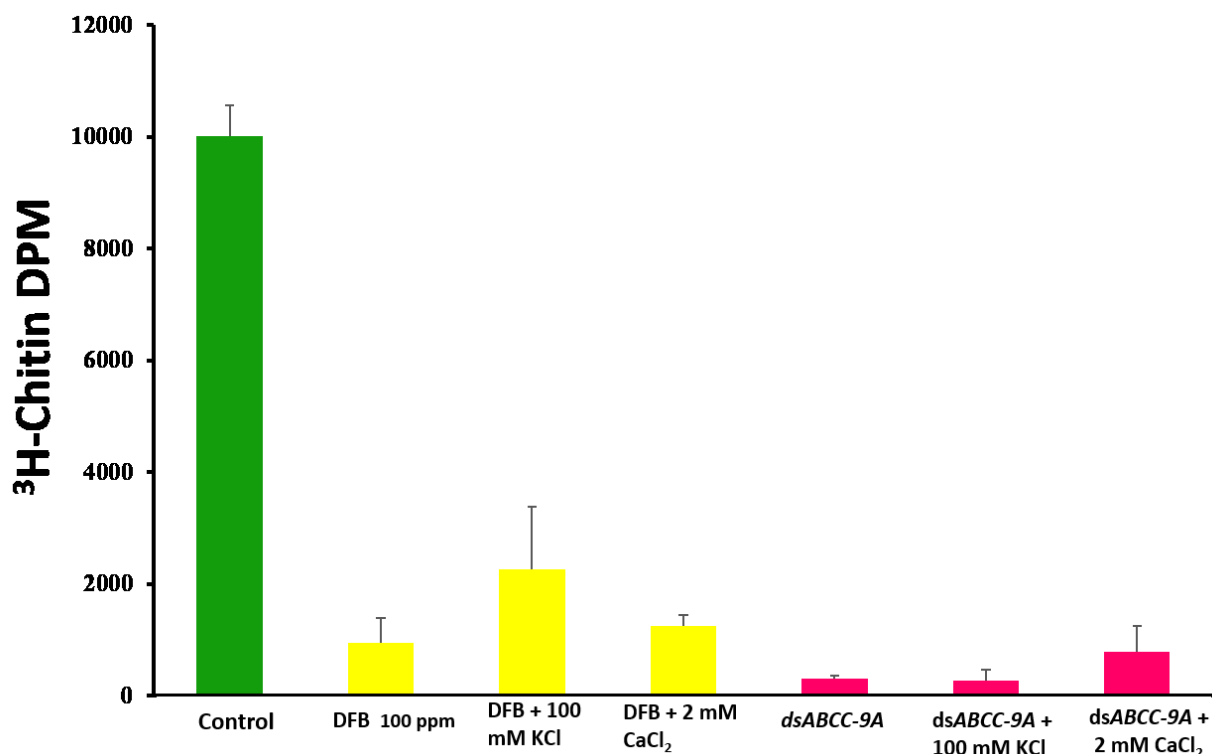
**Figure 3.13 <sup>3</sup>H-Chitin product synthesized was sensitive to chitinase and chitin product is secreted out of the vesicles**

*Effect of different concentration of Manduca sexta chitinase on the chitin formed by the intact vesicle or by disrupted vesicle was evaluated by using elytra from T. castaneum where 100 elytra were ground in a CHS-A buffer followed by differential centrifugation and 100,000 g pellet fraction was collected and resuspended in 100 µl of CHS-A buffer. 100 µl of reaction mix was made containing different concentration of Manduca sexta chitinase (1.7 µg, 3.4 µg, and 6.8 µg) with and without 0.1% Triton X-100, 0.25 µCi UDP-N-acetyl-D-glucosamine [6- <sup>3</sup>H], 120 µM unlabeled substrate, 5 µl of resuspended 100,000g pellet fraction and 50 mM CHS-A buffer for 90 min. At the end of the reaction, 1 ml of 1.5 M KOH was added and the tubes were kept at 90°C for 2 h (to hydrolyze proteins including glycoproteins which might receive the radioactive label; chitin is*

resistant to KOH treatment) and then filtered through a 2.5 cm GF/C filter, and washed extensively with ethanol. The filters were dried and radioactive decays were counted in a liquid scintillation counter after adding 5 ml of Econofluor. The values were calculated an average ( $\pm$  SEM) of three independent experiments.

**The loss of CHS activity of vesicles following in vivo DFB treatment or RNAi of *ABCC-9A* was not reversed by 100 mM KCl or 2 mM  $\text{CaCl}_2$**

The inhibition of CHS activity by DFB *in vitro* prompted us to investigate whether the reduction in chitin in cuticles of insects exposed to DFB *in vivo* might also have been inhibition of CHS activity at the apical plasma membrane of epidermal cells. To test this possibility we isolated vesicles from insects exposed to DFB *in vivo* as well as from insects in which the target of DFB namely ABCC-9A protein was depleted by RNAi as described in chapter 2. We could not detect any CHS activity in vesicle preparations from either treatment (yellow bars), unlike the control which had normal activity (green bar). Further, we could not restore the enzyme activity by adding excess  $\text{Ca}^{++}$  or  $\text{K}^+$  ions. This is unlike the *in vitro* inhibition by DFB of CHS-A activity in isolated Vesicles, which could be reversed by adding excel of these ions. (Fig. 3.8) This suggested a secondary effect of DFB in the pathway on post-translation activation and/or transport of CHS-A. It should be pointed out that both DFB treatment and RNAi of *ABCC-9A* resulted in mislocalization of CHS-A in epidermal cells. (Chapter 2, Fig 2.14).



**Figure 3.14** The loss of CHS activity of vesicles following in vivo DFB treatment or RNAi of *ABCC-9A* was not reversed by 100 mM KCl or 2 mM CaCl<sub>2</sub>

*Analysis of formation of chitin in the vesicles of 100,000g pellet fraction prepared from elytra of insects after exposure to 1000 ppm DFB (50 pupa; DFB applied on day 0 of pupal stage), dsABCC-9A (400 ng) and dsVer as control (200 ng) were injected into late larva and elytra were collected on pupal day 5. Collected elytra were ground in a CHS-A buffer followed by differential centrifugation and the 100,000 g pellet fraction was collected and resuspended in 100  $\mu$ l of CHS-A buffer. 100  $\mu$ l of reaction mix was made containing 5  $\mu$ l vesicles prepared from the above mentioned three methods under indicated conditions. Chitin synthase assays were conducted as described in Materials & Methods. The values were calculated an average ( $\pm$  SEM) of three independent experiments.*

### Isolated vesicles do take up $^{45}\text{Ca}^{2+}$ from the medium

Previous experiments (see Fig. 3.11) from CHS *in vitro* assays have established that  $\text{Ca}^{++}$  ions are required in the synthesis of chitin, in vesicle prepared from elytra of *T.*

*castaneum* and suggested that the inhibition of CHS activity by DFB may be mediated through inhibition of  $\text{Ca}^{2+}$  ion uptake. To obtain direct evidence that the inhibitory effects of DFB on CHS activity of microsomal vesicles is due to inhibition of  $\text{Ca}^{++}$  ion movement across the membrane, we measured the uptake of  $\text{Ca}^{++}$  ions (supplied as radiolabeled  $^{45}\text{CaCl}_2$ ) by the vesicles. The uptake of  $^{45}\text{Ca}^{++}$  ions was rapid using our standard assay conditions and seemed to saturate by about 1 min. Verapamil (a calcium channel blocker) inhibited the calcium uptake in the vesicles Fig (3.15).

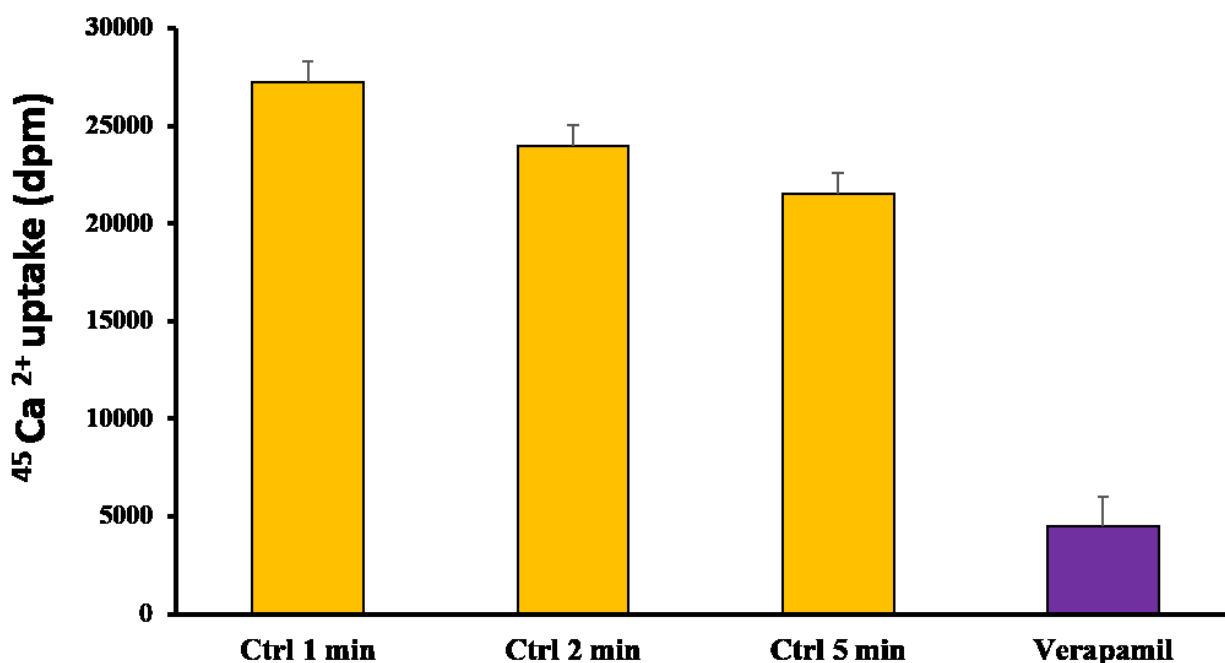
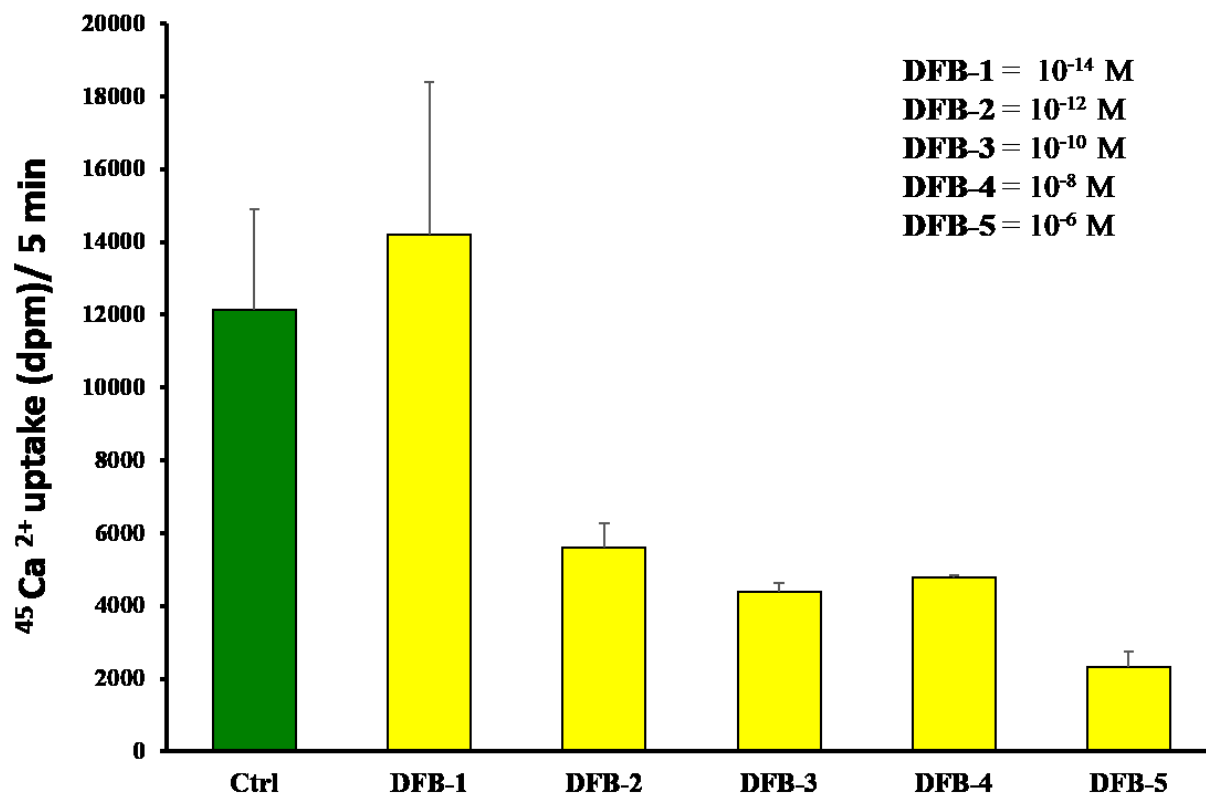


Figure 3.15 Isolated vesicles do take up  $^{45}\text{Ca}^{2+}$  from medium

*Elytra (n = 100) were collected from pharate adult stage of T. castaneum. Elytra were ground in CHS-A buffer followed by differential centrifugation and 100,000 g pellet fraction were collected and resuspended in 100 µl of CHS-A buffer 5 µl from resuspended 100,000g pellet were used per reaction. The assay was performed by using CHS-A buffer containing 5 µl (~ 3 µg) of protein, 120 µM UDP-N-acetyl-glucosamine in total volume of 50 µl. The reaction was started by adding (2 µCi) of  $^{45}\text{CaCl}_2$ . The incubation was carried out in 1.5 ml Eppendorf polypropylene tubes for 5 min at 32°C. The reaction was stopped at various time points (1min, 2 min and 5 min) by filtering immediately through a 0.45 µm nitrocellulose Millipore filter followed by washing four times with 0.5 ml of ice-cold CHS-A buffer. Verapamil (a calcium channel blocker) was used as a negative control to block uptake of  $\text{Ca}^{++}$ . The washed filter was air-dried and the radioactivity was counted using a liquid scintillation counter and expressed as dpm. The values were calculated as average ( $\pm$  SEM) of three independent experiments.*

#### **$^{45}\text{Ca}^{2+}$ Uptake by vesicle is inhibited by DFB in a dose-dependent manner**

To further obtain direct evidence that the inhibitory effects of DFB on CHS activity of microsomal vesicles is due to inhibition of  $\text{Ca}^{++}$  ion movement across the membrane as calcium was needed for chitin synthesis shown in previous experiment, we directly measured the uptake of  $\text{Ca}^{++}$  ions (supplied as radiolabeled  $^{45}\text{CaCl}_2$ ) by the vesicle in presence of different concentration of DFB. DFB inhibited the uptake of  $^{45}\text{Ca}^{2+}$  in a dose-dependent manner, and DFB inhibited calcium uptake in pico molar (and higher) range roughly paralleling the dose response curve of inhibition of CHS activity by DFB described in Fig. (3.16).



**Figure 3.16  $^{45}\text{Ca}^{2+}$  uptake by vesicle is inhibited by DFB in a dose-dependent manner**

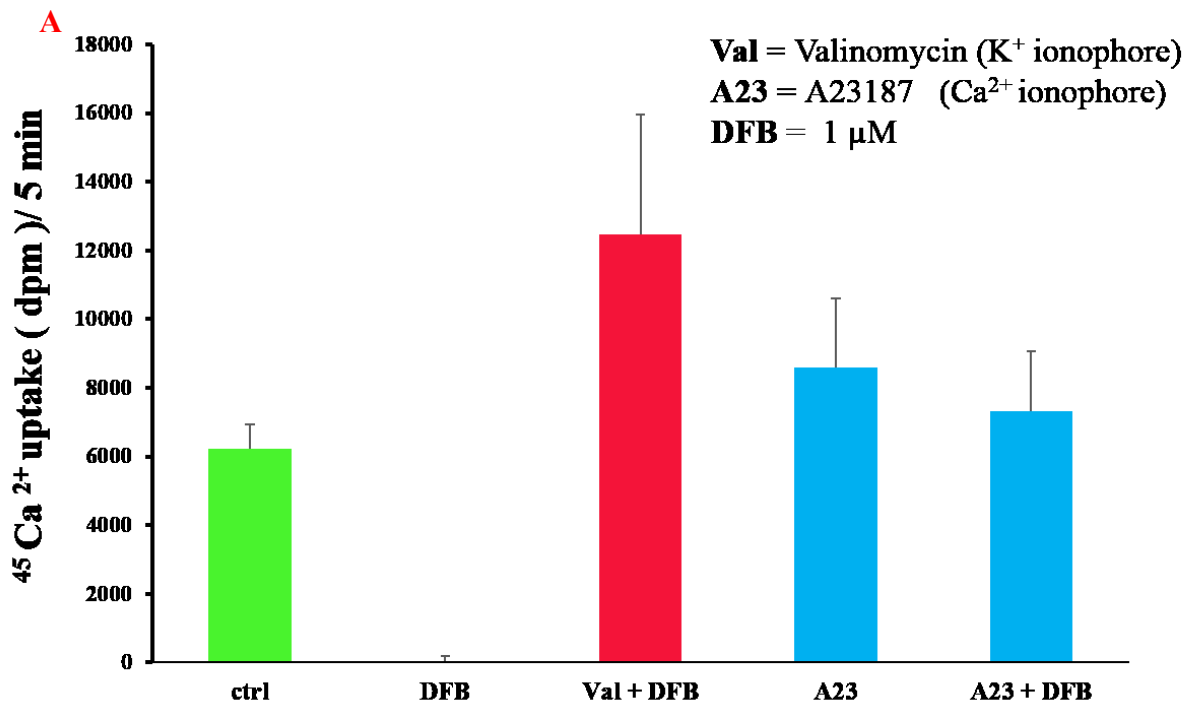
Inhibitory effect of varying concentration of diflubenzuron on  $^{45}\text{Ca}^{2+}$  uptake by the vesicles: *The assay was performed using CHS-A buffer containing 5  $\mu\text{l}$  ( $\sim 3\mu\text{g}$  of protein) of vesicle suspension from the elytra of *T. castaneum* (100,000 g pellet fraction), 120  $\mu\text{M}$  UDP-N-acetyl-glucosamine in total volume of 50  $\mu\text{l}$  containing different concentration of DFB ( $10^{-15}$ ,  $10^{-14}$ ,  $10^{-12}$ ,  $10^{-8}$  and  $10^{-6}$  M). The reaction was started by adding (2  $\mu\text{Ci}$ ) of  $^{45}\text{CaCl}_2$ . The incubation was carried out in 1.5 ml Eppendorf polypropylene tubes for 5 min at 32°C. The reaction was stopped by filtering immediately through a 0.45  $\mu\text{m}$  Millipore nitrocellulose filter followed by washing four times with 0.5 ml of ice-cold*

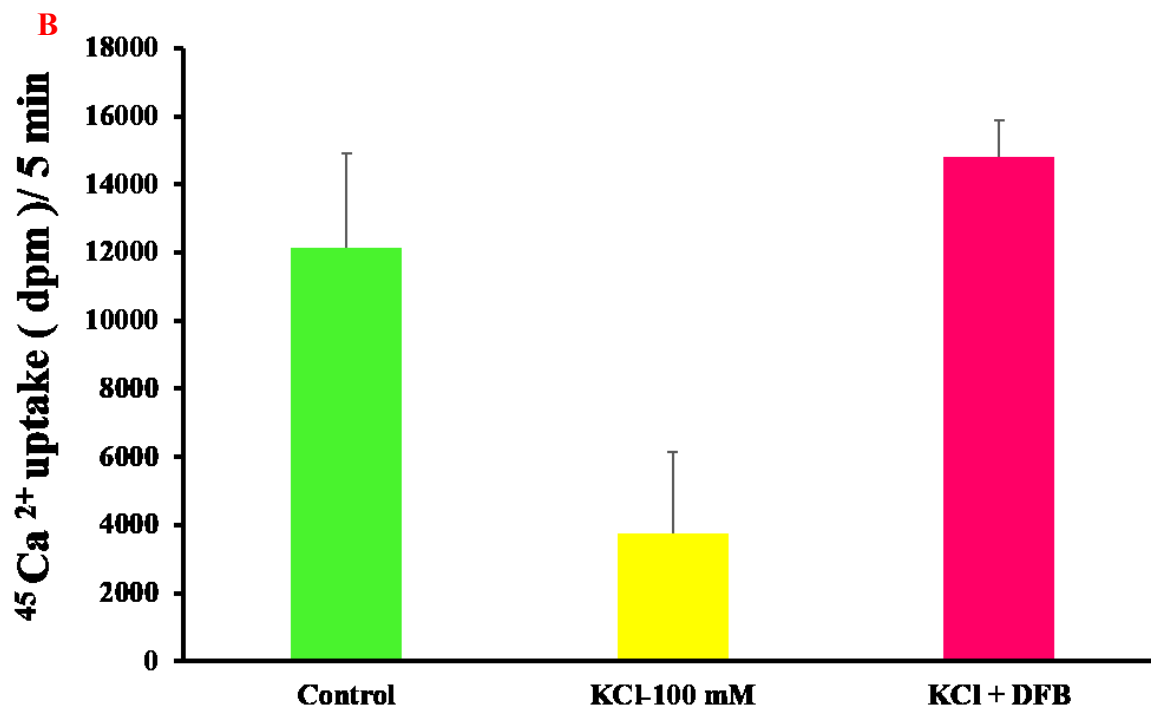


*CHS-A buffer. Verapamil (a calcium channel blocker) was used as a negative control. The washed filter was air-dried and the radioactivity was counted using a liquid scintillation counter and expressed as dpm. The values were calculated as average ( $\pm$  SEM) of three independent experiments.*

### **Reversal of DFB effects on $\text{Ca}^{++}$ uptake by valinomycin, A23187 and KCl**

In our previous experiments, we found that the inhibitory effect of DFB on chitin synthesis was reversed by adding of  $\text{K}^+$  ionophore {valinomycin (10  $\mu\text{M}$ )}, calcium ionophore {A23187 (10  $\mu\text{M}$ )}, or KCl (100 mM). An experiment was conducted to find out whether the addition of the  $\text{K}^+$  ionophore {valinomycin (10  $\mu\text{M}$ )}, calcium ionophore {A23187 (10  $\mu\text{M}$ )}, or KCl (100 mM) reverses inhibition of uptake of calcium by DFB. The results presented in Fig. 3.17 indicated that the inhibition of  $^{45}\text{Ca}^{2+}$  uptake by DFB in these vesicles was indeed reversed by ionophores and by adding 100 mM KCl, which facilitate the movement of  $\text{Ca}^{++}$  ions across the vesicles.





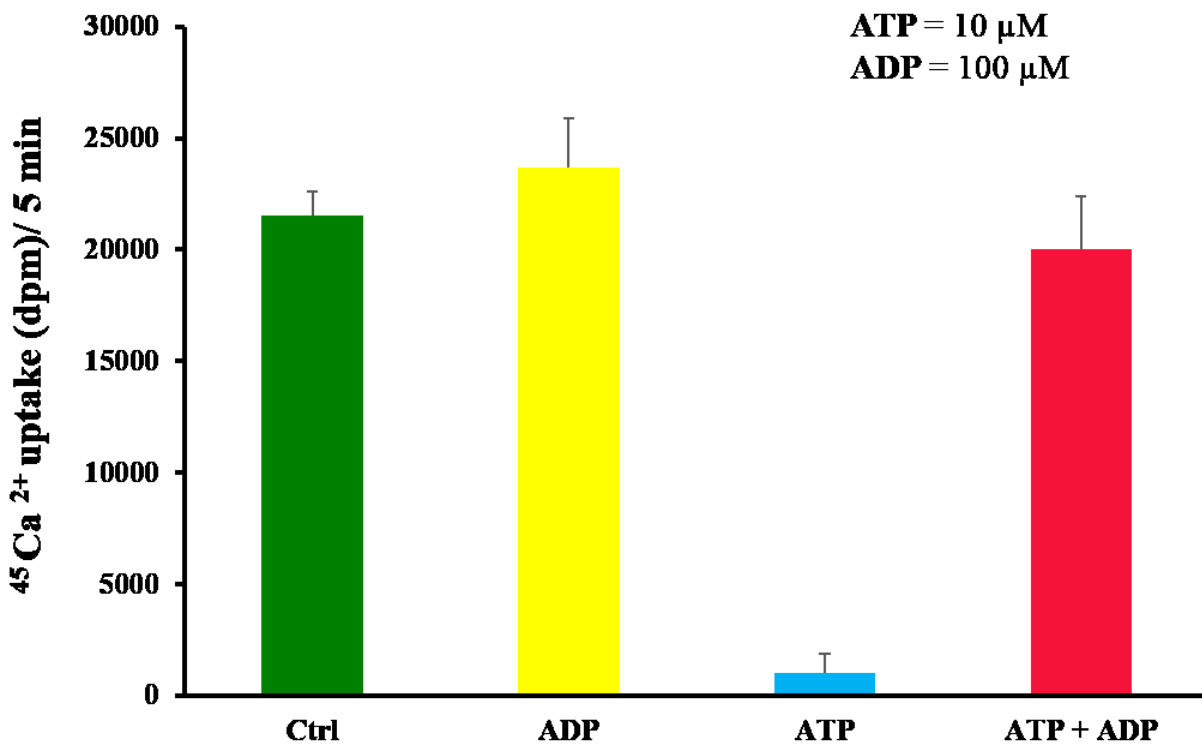
**Figure 3.17 Reversal of DFB effects on  $\text{Ca}^{2+}$  uptake by Valinomycin, A23187 and KCl**

(A) Effect of calcium ( $10\ \mu\text{M}$ ) and potassium ( $10\ \mu\text{M}$ ) ionophores and (B)  $100\text{mM}$  KCl on the inhibition of  $^{45}\text{Ca}^{2+}$  uptake by DFB ( $1\ \mu\text{M}$ ). The assays were performed using CHS-A buffer containing  $5\ \mu\text{l}$  vesicles ( $\sim 3\ \mu\text{g}$  of protein) from elytra of *T. castaneum* ( $100,000\ \text{g}$  pellet fraction),  $120\ \mu\text{M}$  UDP-N-acetyl-glucosamine in total volume of  $50\ \mu\text{l}$  and calcium ionophore ( $10\ \mu\text{M}$ ) with and without DFB, potassium ionophore ( $10\ \mu\text{M}$ ) with and without  $1\ \mu\text{M}$  DFB,  $100\ \text{mM}$  KCl with and without DFB. The reaction was started by adding ( $2\ \mu\text{Ci}$ ) of  $^{45}\text{CaCl}_2$ . The incubation was carried in  $1.5\ \text{ml}$  Eppendorf polypropylene tubes for  $5\ \text{min}$  at  $32^\circ\text{C}$ . The reaction was stopped by filtering immediately through a  $0.45\ \mu\text{m}$  Millipore filter followed by washing four times with  $0.5\ \text{ml}$  of ice-cold CHS-A buffer. Verapamil (calcium channel blocker) was used as a negative control. The washed filter was air-dried and the radioactivity was counted using a liquid scintillation

counter and expressed as dpm. The values were calculated as average ( $\pm$  SEM) of three independent experiments.

### ADP reversed the inhibitory effect of ATP on $\text{Ca}^{2+}$ uptake

To investigate whether calcium uptake is also regulated by ATP/ADP ratio as in the case of CHS activity of isolated vesicles, we carried out  $\text{Ca}^{2+}$  uptake assays in the presence of ATP alone or along with ADP. Results presented in Fig. 3.18 indicated that the  $^{45}\text{Ca}^{2+}$  uptake was inhibited by 10  $\mu\text{M}$  ATP (compare green and blue bars). This inhibitory effect of ATP on  $\text{Ca}^{2+}$  uptake was overcome by simultaneous addition of 100  $\mu\text{M}$  ADP (red bar). These results suggested that calcium uptake by the intact vesicles is regulated by ATP/ADP ratio just as in the case of ATP/ADP ratio on CHS activity.



**Figure 3.18** ADP reversed the inhibitory effect of ATP on  $\text{Ca}^{2+}$  uptake

Effect of ADP, ATP and ATP + ADP on the uptake of  $^{45}\text{Ca}^{2+}$  in the microsomal vesicles in 100,000g pellet fraction from the elytra of *T. castaneum* was measured. The assay was

*performed using CHS-A buffer containing 5  $\mu$ l vesicles ( $\sim 3\mu$ g of protein) from the elytra of *T. castaneum* (100,000 g pellet fraction), 120  $\mu$ M UDP-N-acetyl-glucosamine in total volume of 50  $\mu$ l containing either 100  $\mu$ M of ADP, 10  $\mu$ M of ATP, or 100  $\mu$ M of ADP + 10  $\mu$ M of ATP. Reaction was started by adding (2  $\mu$ Ci) of  $^{45}$ CaCl<sub>2</sub>. The incubation was carried in 1.5 ml Eppendorf polypropylene tubes for 5 min at 32°C. The reaction was stopped by filtering immediately through a 0.45  $\mu$ m Millipore filter followed by washing with four washes with 0.5 ml of ice-cold CHS-A buffer. Verapamil (a calcium channel blocker) was used as a negative control. The washed filter was air-dried and the radioactivity was counted using a liquid scintillation counter and expressed as dpm. The values were calculated an average of ( $\pm$  SEM) of three independent experiments.*

## **Discussion**

DFB and other acylurea derivatives have been used effectively for insect control for over 40 years. Oral or topical administration of acylureas has been shown to have a variety of detrimental effects on insect growth, development and fecundity, accompanied by reduction in cuticle thickness and loss of laminar organization, reduction in total chitin content, leading to molting arrest and death (Gangishetti et al., 2009; Merzendorfer et al., 2012; Mulder & Gijwijt 1973; Verloop & Ferrell, 1977). Even though numerous cellular processes are known to be affected by acylureas, the precise mechanisms of disruption of the chitin metabolic pathway leading to reduced cuticular chitin have not been elucidated in any insect species in spite of many attempts to do so (Cohen 2001; Cohen & Casida, 1980; Mayer et al., 1981; Soltani et al., 1984; Gangishetti et al., 2009; Zhang & Zhu, 2006; Matsumura, 2010; Merzendorfer et al., 2012). To solve this puzzle we developed a protocol for assaying CHS activity using

membrane fraction from the elytron, the tissue whose major role is to secrete the chitinous cuticle.

Zimoch et al., (2005) have previously optimized an *in vitro* system capable of chitin synthesis from midgut extracts and microsomal preparations of *Manduca sexta*. We used this protocol to prepare a similar microsomal preparation (100,000 g pellet) from extracts of isolated elytra of *T. castaneum*, a tissue that is predominantly made up of cuticle secreting cells. CHS activity in this preparation was assayed using  $^3\text{H}$ -UDP-GlcNAc as the substrate. The radioactive product was identified as chitin using the following criteria: its formation was sensitive to nikkomycin (an inhibitory substrate analog of CHS) but not to tunicamycin (an inhibitor of protein glycosylation); the product was susceptible to microbial (data not shown) and insect chitinases, but was resistant to KOH treatment for 2 h at 95°C. In contrast to previous studies with similar preparations from integument tissues or total body homogenates of larvae or pupae of insects, the CHS activity of the microsomal preparation from elytra of *T. castaneum* was susceptible to DFB at concentrations as low as  $10^{-12}$  M with stronger inhibition at higher concentrations. Even glibenclamide, a sulfonylurea, inhibited the reaction at 1  $\mu\text{M}$ . It is not clear why the vesicles prepared from *T. castaneum* exhibit sensitivity to DFB whereas microsomal preparations from larval guts of *T. castaneum* and *M. sexta* were not inhibited by DFB or ATP (Cohen & Casida 1980; Zimoch et al., 2005). One possibility is that the gut-specific CHS-B enzyme has properties that are different from those of CHS-A, which specializes in cuticular chitin synthesis. Alternatively, the gut tissue may be devoid of some of the channel proteins that are enriched in the epidermal cells. We have not so far localized any of the proteins in the vesicle preparations because of low amounts of proteins

associated with these preparations and the lack of antibodies for these proteins. A scale up of our isolation protocol with more elytra and western blot with appropriate antibodies and mass spectrometric analyses will be needed to sort out the answers to this important question.

This “microsomal” fraction was highly enriched in “vesicles” when analyzed by nano particle tracking with a mean particle size of 118 nm and mode of 79 nm. The 100,000g supernatant fraction lacking the vesicles was devoid of CHS activity. Since these vesicles were obtained from untreated control insects in which vesicular transport would have been unaffected, the inhibition of CHS activity by DFB in *in vitro* assays was unexpected and suggested a secondary effect of DFB in a post-vesicular transport event. This effect is in addition to the *in vivo* effects of DFB treatment on the localization of several enzymes of chitin metabolism, most importantly chitin synthase. Since these vesicle preparations described in this chapter appeared to mimic the behavior of whole epidermal tissue in their susceptibility of chitin synthesis by DFB *in vivo*, they were utilized for dissecting the effects of DFB in greater detail *in vitro*.

The integrity of the vesicles appeared to be important to maintain the inhibition by DFB treatment, because the DFB inhibition is overcome with 0.1% or 0.025% Triton X-100; or 0.1% digitonin. At these concentrations, the activity of CHS itself is not significantly affected. Inhibition of CHS activity of intact vesicles (i.e. in the absence of detergents) by 1  $\mu$ M DFB could be reversed by treatment with the potassium ionophore, valinomycin (10  $\mu$ M), or the calcium ionophore, A23187 (10  $\mu$ M). Alternatively, increasing the concentrations of  $K^+$  ions and  $Ca^{++}$  ions in the reaction buffer also reversed the inhibitory effect of DFB presumably by forcing the diffusion of these ions across the membrane

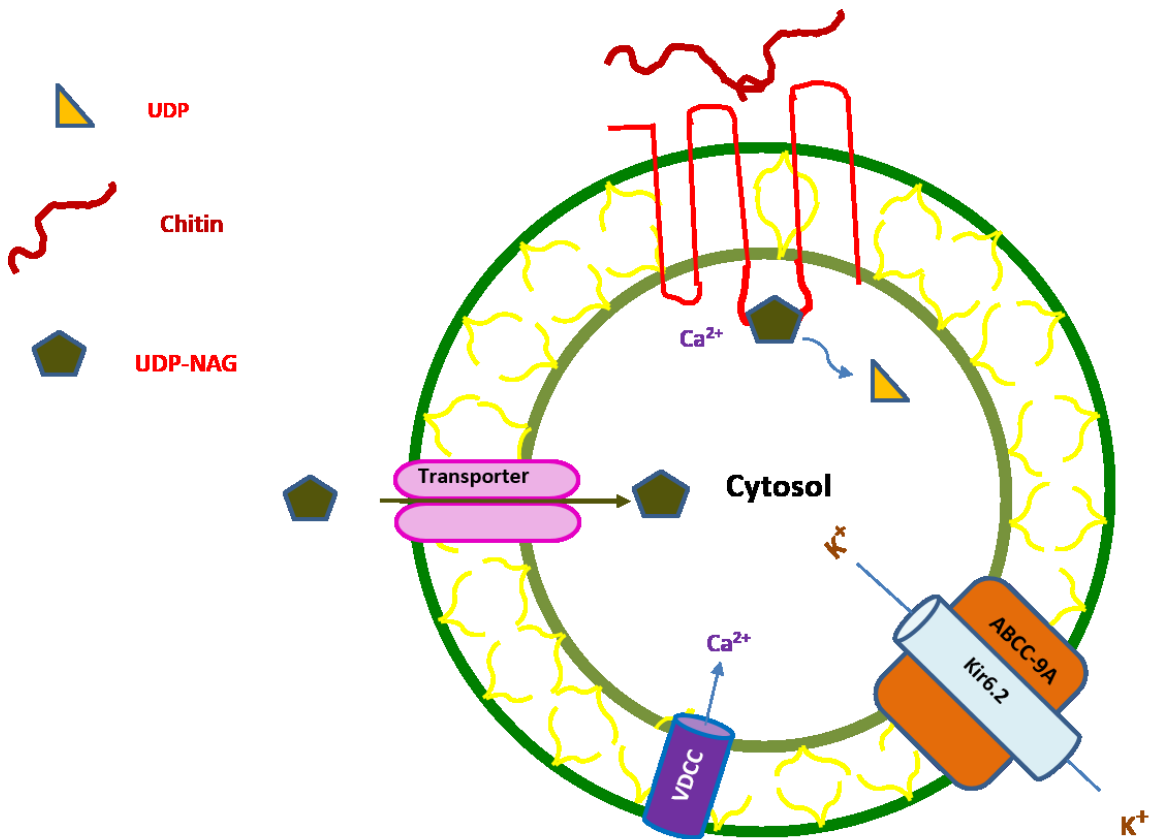
through other mechanism not involving the  $K_{ATP}$  channel. All of these data indicated that the DFB-sensitive CHS activity (but not the activity *per se*) was dependent on the integrity of vesicular preparations and their ability to regulate the movement of  $Ca^{++}$  ions and  $K^+$  ions through appropriate ion channels across the membrane in response to changing energy charge (ATP/ADP ratio) of the epidermal cell. Based on a partial analogy of the action of SUs on human SUR- $K_{ATP}$  complex, we postulate that DFB binds to its receptor, ABCC-9A, which associates with the  $K_{ATP}$  channel subunits and causes the closure of the  $K_{ATP}$  channels leading to membrane depolarization and flow of  $Ca^{++}$  across the membrane (Evans et al., 2005). However, the requirement for  $K^+$  ion movement is indirect because, when vesicles were permeabilized by low concentrations of detergents, there was no requirement for addition of  $K^+$  ions for CHS activity. In contrast, even after mild detergent treatment, CHS activity was inhibited by EGTA (1 mM) indicating an absolute requirement for  $Ca^{++}$  ions. Since our CHS assay buffer included 10 mM  $MgCl_2$ , the amount of EGTA used in this experiment would not have chelated more than 2 mM  $Mg^{++}$ . Therefore, the loss of CHS activity upon addition of EGTA is presumably due to chelation of  $Ca^{++}$  ions retained inside the vesicles during the enzyme preparation protocol. We postulate that the ABCC-9A-regulated  $K_{ATP}$  channel(s) regulates  $Ca^{++}$  ion movement across the membrane involving a voltage-dependent  $Ca^{++}$  ion channel as in the case of insulin secretion by pancreatic cells (Proks et al., 2002). Even when  $K^+$  ions were omitted from the assay buffer with intact vesicles, addition of 2 mM  $Ca^{++}$  ions restores the CHS activity indicating that the requirement for  $K^+$  ions is only to promote the movement of  $Ca^{++}$  ions across the vesicular membrane. The biochemical basis of the  $Ca^{++}$  requirement for chitin synthesis is unknown, but we

speculated that it may be related to polymerization of chitin and/or its extrusion through the active site of CHS or the 5-TMS region of CHS that has been postulated to be the channel through which chitin chains are extruded to the procuticle (Merzendorfer 2006).

To obtain direct evidence that the inhibitory effects of DFB and ATP on CHS activity of microsomal vesicles is due to inhibition of  $\text{Ca}^{++}$  ion movement across the membrane, we directly measured the uptake of  $\text{Ca}^{++}$  ions (supplied as radiolabeled  $^{45}\text{CaCl}_2$ ) by the vesicles. The uptake of  $^{45}\text{Ca}^{++}$  ions was rapid using our standard assay conditions and seems to saturate by about 1 min. In the presence of 1  $\mu\text{M}$  DFB or 10  $\mu\text{M}$  ATP or 10  $\mu\text{M}$  verapamil ( $\text{Ca}^{++}$  channel blocker), the uptake of  $^{45}\text{Ca}^{++}$  ions was inhibited substantially. In agreement with our hypothesis that ABCC-9A/ $\text{K}_{\text{ATP}}$  channel complex regulates  $\text{Ca}^{++}$  uptake in these vesicles, addition of the  $\text{K}^{+}$  ionophore, valinomycin (10  $\mu\text{M}$ ), or the calcium ionophore, A23187 (10  $\mu\text{M}$ ) reversed inhibition by DFB. These results indicated that the inhibition of CHS by ATP and DFB in these vesicles is reversed by ionophores by facilitating the movement of  $\text{Ca}^{++}$  ions across the vesicular membrane via the regulation of  $\text{K}^{+}$  ion movement. This was further supported by the finding that addition of 100 mM KCl to the reaction mixture, which reverses the inhibition of CHS activity by DFB, also reversed the inhibition of  $\text{Ca}^{++}$  uptake by DFB. Further, the dose response of inhibition of  $\text{Ca}^{++}$  uptake by DFB parallels the extent of inhibition of CHS activity by DFB being effective in the same concentration range. All of these finding strongly supported the notion that DFB acts by regulating the flow of  $\text{Ca}^{++}$  across the vesicular membrane.



### Proposed mechanism of chitin synthesis in microsomal vesicles

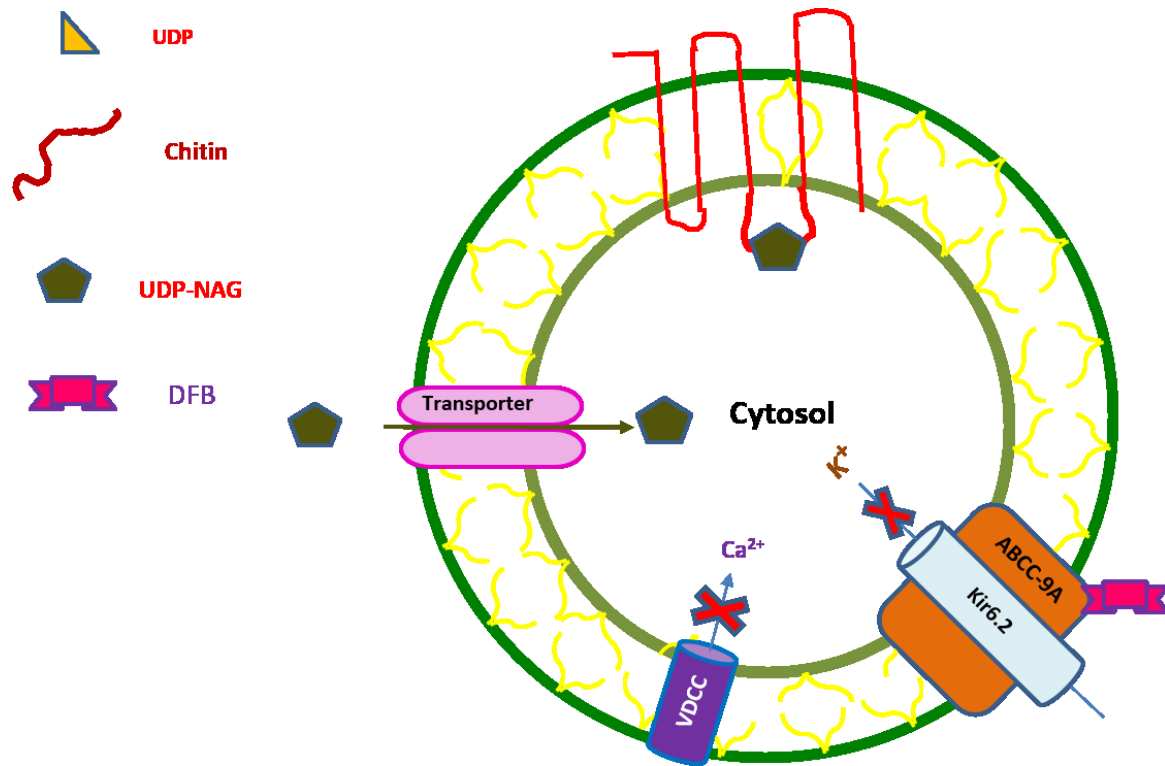


**Figure 3.19 Proposed mechanism of chitin synthesis in microsomal vesicles**

Microsomal vesicles prepared from the elytra of *T. castaneum* have the ability to synthesize chitin. Chitin synthesis in this system is robust and is sensitive to inhibition by the chitin synthesis inhibitor, nikkomycin. The reaction product is completely digested by addition of chitinase at the end of the reaction and the chitin product is secreted outside the vesicles. Chitin synthesis in this system is inhibited by DFB and this system is regulated by ATP/ADP ratio. Based on these findings, we propose a pictorial model for *in vitro* chitin synthesis. We propose that these microsomal vesicles also contain  $\text{K}_{\text{ATP}}$

channels under the control of regulatory ABCC-transporters with properties similar to those of mammalian Sur-K<sub>ATP</sub> ion channels that regulate the movement of potassium ions (Fig. 3.19). Since these vesicles have the same ionic composition inside and outside, the membrane potential is essentially zero which facilitates the movement of Ca<sup>++</sup> ions via the voltage-gated Ca<sup>++</sup> channels (VGCC), which are also postulated to be in the same vesicles. This inward flow of Ca<sup>++</sup> ions is essential to maintain the activity of chitin synthase which requires Ca<sup>++</sup> for activity. Because the vesicles are likely to be not freely permeable to nucleotides, it is likely a membrane bound antiporter for UDP-NAG which exchanges UDPGlcNAc for UMP known to be ubiquitous in eukaryotic cells (Hirschberg et al., 2001; Maszczak-Seneczko et al., 2011)

### Proposed mechanism of inhibition of chitin synthesis by DFB in microsomal vesicles



**Figure 3.20 Proposed mechanism of inhibition of chitin synthesis by DFB in microsomal vesicle**

When the movement of potassium ions is inhibited either by DFB or by an unfavorable ATP/ADP ratio, membrane potential builds up as chitin synthesis proceeds and negatively charged UDP accumulates inside the vesicles. The polarization of the membrane leads to inhibition of  $\text{Ca}^{++}$  movement through the voltage gated calcium channel, (Fig. 3.20). Binding of DFB or ATP to ABCC-9A subunits resulted in the closing of  $\text{K}_{\text{ATP}}$  channel associated with them. Closing of  $\text{K}_{\text{ATP}}$  channel resulted in the

buildup of membrane potential, which, in turn, affects the movement of  $\text{Ca}^{2+}$  ions.

Reduction of calcium flow into the vesicles inhibits chitin synthesis.

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# Chapter 4

## Discussion and Future Directions

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## General Discussion

Diflubenzuron (DFB), a prototype of benzoylphenylurea developed in 1970s by Philips-Duphar company, has been used as an effective insecticide over 40 years. This compound prevents formation of chitinous structures in many insects with no effect on fungi and other natural enemies of insects. The most obvious and visible effect of DFB is defective cuticle and loss of chitin. However, the precise mode of action of DFB has remained a mystery for a long time

In our study, we wanted to find out the mechanisms of DFB action as well as the precise molecular target of DFB in tissues that leads to adverse effects on chitin formation. We used the red flour beetle, *Tribolium castaneum*, as a model organism and standardized a topical application method which proved to be highly effective in delivering the desired dose and in producing the anticipated phenotypes. We determined that DFB application at a relatively high dose of 1000 ppm leads to high mortality ( $\geq 90\%$ ), substantial loss of chitin, and loss of laminar architecture of the procuticle.

### DFB causes mislocalization of enzymes of chitin metabolism

Chitin synthase-A (CHS-A) is an important enzyme needed for synthesis of chitin in the cuticle of insects. There are also several other enzymes that play important roles in chitin metabolism. We wanted to investigate the effect of DFB on these proteins as well, because they could potentially affect chitin levels in the cuticle. Previous studies have shown that DFB administration does not reduce the level of transcripts for chitin synthase (*Chs-A*) nor does DFB directly affect the activity of this integral membrane enzyme in assays with microsomal preparations or solubilized and partially purified preparations of CHS from several insects (Cohen & Casida, 1989; Kitahara et al., 1983; Mayer et al.,



1981, Zimoch et al., 2005). We conducted an RNA Seq analysis of elytra isolated from control and DFB-treated insects (instead of whole body) because this tissue is highly enriched for epidermal cells specializing in the formation of cuticle. These studies failed to show any significant differences in transcript levels for several enzyme of chitin metabolism including CHS-A and chitinase 5 or 10 or chitin deacetylase 1 and 2 upon DFB treatment. Analysis of global transcriptional changes using RNA isolated from control and DFB-treated elytra indicated that the loss of chitin in the insect cuticle could not be explained as due to a decrease in levels of transcripts of *Chs-A* gene or enhanced transcription of enzymes involved in chitin degradation or modification such as chitinases and chitin deacetylases. Down-regulation using RNAi of both chitinases 5 and 10 which degrade cuticular chitin also failed to restore DFB-mediated loss of chitin in body wall cuticle indicating that the DFB-mediated reduction in chitin is not due to accelerated chitin degradation. We then considered the possibility that the effect of DFB may be at a post-transcriptional step involving CHS-A enzyme protein, the sole contributor of cuticular chitin (Arakane et al., 2005). Confocal microscopic analysis of DFB-treated insects was carried out to examine the presence and localization of CHS-A protein. Surprisingly, immunoreactive CHS-A protein was indeed detectable in the epidermal cells after DFB-treatment. However, they were not in their normal cellular location. While CHS-A protein was predominantly in the apical plasma membrane of abdominal epidermal cells in control insects, it was absent from the apical plasma membrane but was instead mislocalized inside the epidermal cells in DFB treated insects. There was no obvious evidence for reduction in CHS-A protein content. We also discovered that other chitin metabolism proteins such as CDA and KNK proteins were also mislocalized

indicating that other proteins normally targeted to the extracellular matrix were also affected by DFB-treatment suggestive of an effect of DFB on vesicular transport of these proteins.

The failure of CHS-A to migrate to the apical plasma membrane can explain why chitin is not made in presence of DFB. There may also be another effect of DFB at the level of post-translational activation of CHS-A because vesicle preparations from DFB-treated or dsRNA *ABCC-9A* treated insects are inactive in *in vitro* assays for CHS-A. Perhaps some proteolysis or phosphorylation of the zymogenic form of CHS-A could be involved as reported previously (Zimoch et al., 2005; Maue et al., 2009). In fact, smaller peptides cross-reacting with CHS-A antibody have been found in western blots of SDS-PAGE gels and not with large protein bands corresponding to the full length CHS-A protein (Fig. 2. 10. Chapter 2). All these finding indicated that DFB does not affect the synthesis or turnover of the CHS-A, CDA and CHT-5 proteins but their processing or the movement of the proteins from inside the epidermal cell to their normal cellular (or cuticular) locations.

### **Identification of the target receptor of DFB**

Matsumura and co-workers (Abo-Elghar, 2004) have proposed that DFB affects chitin synthesis indirectly by targeting a homolog of a sulfonylurea receptor (SUR1), in *Drosophila melanogaster* named *DmSur*. The human homolog(s) of this gene, (*HsSur1* and *HsSur2*) have been identified previously, and their mode of action has been the subject of intensive studies (Moreau et al., 2000; Proks et al., 2002; Evans et al., 2005). However, another study of homozygous mutants of *DmSur* gene indicated no cuticular abnormalities including the laminar arrangement or thickness of chitin-containing

laminae in *D. melanogaster* larvae ruling out the involvement of DmSUR in inhibition of chitin synthesis (Meyer et al., 2013). This indicated a role for another potential receptor, perhaps closely related to DmSUR, in DFB-mediated effects on chitin synthesis. Hence, we assumed that the potential target(s) of DFB in *T. castaneum* may be an ABCC-type of transporter or multidrug resistance protein (MRP) similar to human SUR or MRP (Moreau et al., 2005). SURs and other ABCC proteins including cystic fibrosis transmembrane regulator (CFTR) bind to the sulfonylurea drug, <sup>3</sup>H-labeled glibenclamide and regulate the activity of ion channels. SURs in particular, associate with an ATP-sensitive inward rectifying potassium channel, K<sub>ATP</sub> (a subclass of K<sub>ir</sub> channels) with which they associate and form a large octameric complexes consisting of 4 K<sub>ATP</sub> and 4 SUR subunits (Moreau et al., 2005; Evans et al., 2005; Hibino et al., 2010). Previous studies have shown that glibenclamide also binds to membrane preparations from cockroaches and *Drosophila* (Abo-Elghar et al., (2004) and that DFB could compete for this binding.

Broehen et al., (2013) have reported that *T. castaneum* genome has a large family of ABC-transporters with 73 members. To simplify the identification of the gene(s) that encodes the target receptor or DFB, we wanted first to identify the genes belonging to the ABCC group of transporters (to which human SURs belong) that are indeed expressed in elytra to narrow down the search for the putative DFB receptor. We used “BlastP” search to identify the genes encoding *T. castaneum* proteins with the highest sequence similarity to HsSUR1 and HsSUR2 and picked a handful of genes predicted to encode proteins with greatest sequence similarity to human SUR1 and SUR2 proteins. We further wanted to carry out systematic and exhaustive expression studies of all *ABCC*

family genes after DFB-treatment. The RNA SEQ study showed expression of only 7 *ABCC* transporter family genes in the elytra of *T. castaneum*. A list all these seven *ABCC*-subfamily genes that are expressed in elytra of *T. castaneum* is listed in Table (4.1) RNA Seq provides very little evidence for transcriptional regulation of the *ABCC*-family genes following DFB treatment indicating that if these genes were involved in DFB-mediated inhibition of chitin synthesis, the effects of DFB are probably post-transcriptional.

Gene name	Control read	DFB read	RPKM ratio ( DFB/Control)
ABCC-5E	0	7	0
ABCC-5I	41	50	2.4
ABCC-5R	37	30	1.6
ABCC-7A	34	19	1.1
ABCC-7B	133	147	2.2
ABCC-9A	111	71	1.3
ABCC-4A	57	43	1.3
ABCC-8B	29	20	1.4

**Table 4.1 Read for different ABCC transporters**

We further investigated the potential role of all of these 7 genes of the *ABCC* family using RNAi. We anticipated that RNAi of only the real target gene will mimic the effects

of DFB. Only RNAi of *ABCC-9A* duplicated all the phenotypes of DFB application to *T. castaneum* such as molting defects, mortality at the pharate adult stage, loss of laminar organization of cuticle, mislocalization of CHS-A protein and loss of CHS-A activity in *in vitro* assays. Even the most closely related and highly expressed genes *ABCC-4A* and *ABCC-7B* failed to reproduce the DFB symptoms and vesicle preparations from the elytra of these insects had normal CHS-A activity (Fig. 3.14 Chapter 3).

We used a fluorescently tagged glibenclamide or DFB to detect binding to cognate receptors in *T. castaneum* tissues using sections of midgut and body wall. Both BODIPY-glibenclamide and Bodipy-DFB bind to putative receptors enriched in the apical and basal plasma membranes of gut lining cells and epidermal cells of the body wall (Figure 2.15). Competition experiments involving preincubation with increasing concentrations of DFB further established competitive displacement of the BODIPY-labeled ligands from the target receptor indicating that the binding of the tagged ligand is in fact due to the DFB part. As the concentration of the DFB in the preincubation was increased there was greater loss of binding of the fluorescent ligand indicating that the binding is reversible.

To identify the gene encoding the DFB-receptor, we down-regulated transcripts for *ABCC-9A*, *ABCC-4A* and *ABCC-7B* followed by binding to fluorescently tagged glibenclamide or BODIPY-DFB to identify which, if any, of these proteins was responsible for binding to DFB. RNAi of only *ABCC-9A* (but not the other two highly expressed *ABCC* genes, *ABCC-7B* and *ABCC-4A*) resulted in loss of BODIPY-glibenclamide and BODIPY-DFB binding further confirming that DFB directly bound to *ABCC-9A* protein, thus leading to unambiguous identification of the molecular target. If

the target of DFB had been some other protein (for example, another ABCC family member or even CHS-A), there should have been no loss of binding of BODIPY-DFB or BODIPY-glibenclamide upon RNAi of ABCC-9A. The results of these binding experiments are consistent with the observed changes in cuticle morphology, mortality and loss of chitin content and laminar organization of the procuticle only after RNAi of *ABCC-9A* gene.

DFB treatment *in vivo* also resulted in near complete loss of BODIPY-ligand binding to the apical or basal plasma membrane either due to loss of receptor or due to persistent binding of DFB to the target receptor several days past application of DFB. RNAi of *TcABCC-9A* also caused complete loss of BODIPY-DFB signal. All the evidence presented so far indicate that RNAi for *TcABCC-9A* mimics the effect of DFB administration, strongly suggesting that ABCC-9A protein is the receptor for DFB.

### **Mode of action of DFB**

The RNAi experiments combined with ligand-binding studies using confocal microscopy identified the molecular target of DFB as the protein encoded by *TcABCC-9A* gene. DFB causes mislocalization of CHS-A and other enzymes/proteins of chitin metabolism by an unknown mechanism. Based on the analogy of the effect of sulfonylureas on the secretion of insulin by pancreatic  $\beta$ -cells, we propose that the perturbation of normal regulation of  $K^+$  and  $Ca^{++}$  channels as a result of DFB binding disrupts vesicular trafficking in epidermal cells. We have unpublished data that indicate that knock down of syntaxin1A, a t-SNARE protein involved in vesicular fusion showed molting defects similar to DFB treated insects and *dsABCC-9A*-treated insects. In *Drosophila* also

homozygous syntaxin 1A mutants exhibit cuticular abnormalities and loss of laminar organization (Moussian et al., 2007).

The development of an *in vitro* system for chitin synthesis using vesicles prepared from elytral tissue and the finding that DFB inhibits chitin synthesis by these vesicles have provided further insights into the mode of action of DFB. Intact vesicles are important for DFB-mediated inhibition of chitin synthesis further emphasizing that regulation occurs at the level of membrane polarization and modulation of the membrane potential. The sensitivity of CHS-A to inhibition by ATP and reversal of this inhibition by ADP combined with the actions of ionophores suggests that the epidermal cell probably responds to changing ATP/ADP ratios by regulating the flow of  $K^+$  ions. ABCC-9A appears to be present in the apical plasma membrane along with CHS-A and  $K_{ATP}$  channels because all chitin synthesis is inhibited by DFB. We do not think that intracellular CHS-A contributes to CHS activity because we do not see chitin staining associated with intracellular vesicles. We propose that in addition to a role in vesicular transport, there is an additional step where  $Ca^{++}$  is required for chitin synthesis and extrusion. This is based on our finding that chelation of  $Ca^{++}$  ions results in total loss of CHS activity even after vesicles from control elytra (never exposed to DFB) have been permeabilized by low concentrations of detergents.

Our hypothesis that movement of  $Ca^{++}$  ions is essential for chitin synthesis by intact vesicles received experimental confirmation by the direct demonstration of uptake of  $^{45}Ca^{++}$  by the vesicles prepared from *T. castaneum* elytra. The inhibition of  $^{45}Ca^{++}$  uptake by DFB and the reversal of this inhibition by  $K^+$  and  $Ca^{++}$  ionophores and excess  $K^+$  and  $Ca^{++}$  provide further evidence that ion channels are definitely involved in

regulation of chitin synthesis. Our *in vitro* model system is also sensitive to  $K^+$  and  $Ca^{++}$  ionophores as well as DFB suggesting the presence of ABCC-9A protein and CHS-A and ion channels in the same vesicles. This represents a major breakthrough because we can now use this system as a miniature chitin synthesis machinery that can be analyzed biochemically *in vitro* for studies on the requirements and regulatory controls of chitin synthesis.

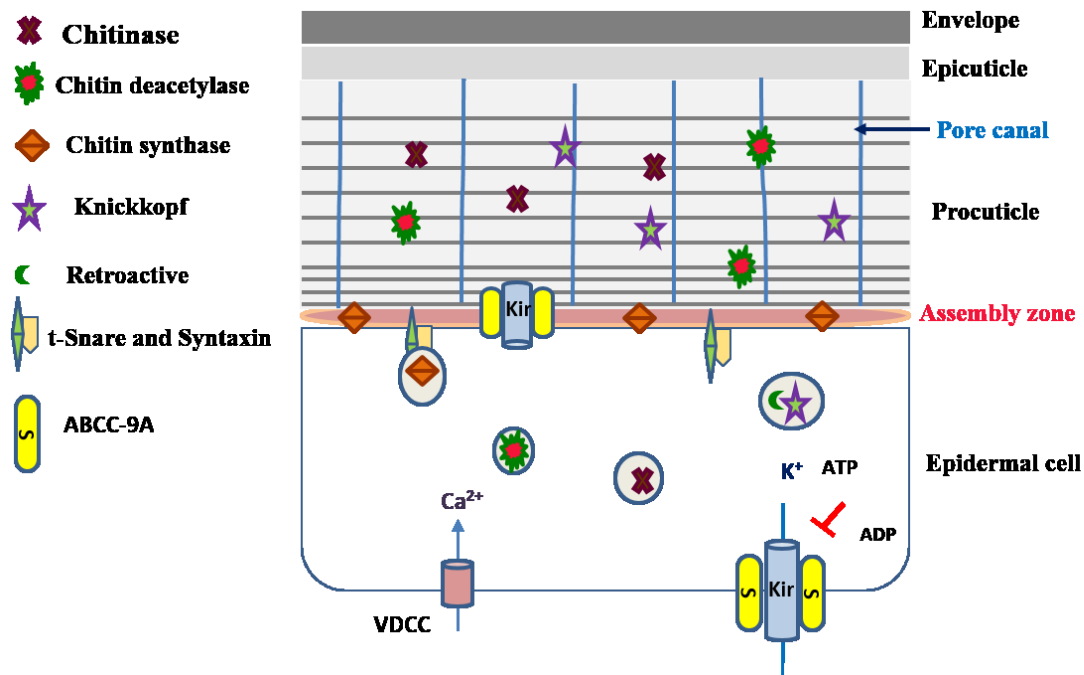
### **A model of regulation of CHS intact vesicle**

A model for regulation of CHS activity in intact vesicles explaining all of the obtained data is presented in Fig 4.1 and 4.2. This scheme is based largely by analogy to the mode of action of SU on human SUR in pancreatic  $\beta$ -cells (Proks et al., 2002) but with some notable differences. Both models envisage a central role for a  $K_{ATP}$  channel regulated by an ABCC-class regulatory protein and that this complex is sensitive to DFB (or SU in the case of pancreatic  $\beta$ -cells) and to ATP. Whereas both ATP and DFB lead to inhibition of  $K^+$  channels and the concomitant increase in  $Ca^{++}$  uptake by pancreatic  $\beta$ -cells and enhanced vesicular transport of insulin containing granules to the plasma membrane, the effects of DFB treatment on insect epidermal cells is quite different. Inhibition of  $K_{ATP}$  channels By DFB in isolated vesicles results in inhibition rather than stimulation of  $Ca^{++}$  uptake. This might have been due to the absence of membrane polarization in isolated vesicles. While we do not know the *in vivo* effects of DFB on  $Ca^{++}$  uptake, we know that transport of several proteins including CHS-A is inhibited rather than enhanced. We don't know the *in vivo* effects of elevated ATP /ADP ratios on CHS-A transport in normal epidermal cells or whether DFB influences the ATP/ADP ratio. Perhaps regulation of ATP/ADP may be the cue for regulating the movement of CHS-A to the



plasma membrane and controlling the rate of cuticle deposition. This is in contrast to the promotion of vesicular transport of insulin containing granules by SUs (Proks et al., 2002). Subtle differences in  $\text{Ca}^{++}$  requirement for vesicular transport of CHS protein compared to other proteins, and/or competition among different vesicular transport pathways may account for this fundamental difference between these two systems. We are currently investigating how different components of the vesicular transport pathway are affected by DFB treatment. In addition, our finding that DFB completely inhibits chitin synthesis by isolated vesicles suggests that CHS and TcABCC-9A and  $\text{K}_{\text{ATP}}$  are found in the same vesicles. Thus, there is an additional site(s) where DFB can also influence the activity of the enzyme in addition to its effects on vesicular transport of chitin metabolism proteins.

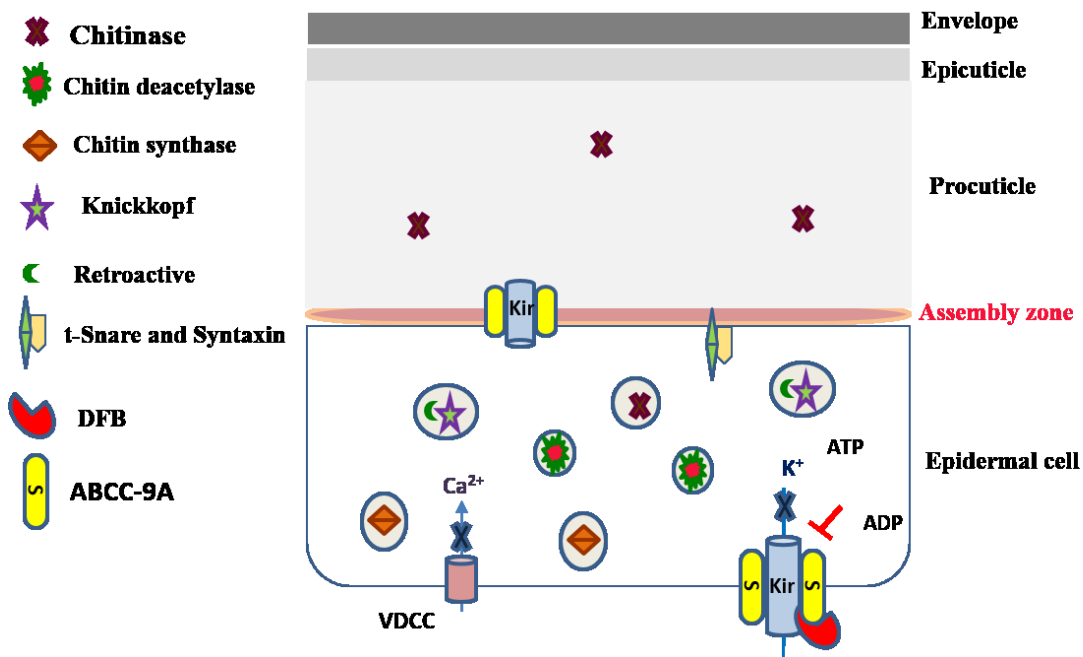
A model of regulation of CHS intact vesicle explaining all of the data is presented in figure below. The model is based largely by analogy to mode of action of DFB on HsSUR1 in pancreatic cell (Proks et al., 2002). The figure below represents the distribution of chitin metabolism proteins in a *T. castaneum* epidermal cell in the absence of any DFB.



**Figure 4.1 A model of regulation of chitin metabolism proteins in *T. castaneum***  
 This model is showing a complex process of cuticle biogenesis, which requires the transport of large quantities of chitin metabolism proteins from the epidermal cell to the procuticle. To transport of CHS-A is regulated by altering membrane polarization and selective transport of  $K^+$  and  $Ca^{++}$  ions across the plasma membrane of the epidermal cells. This hypothetical model is based on the mode of action of ATP and SU's on human SUR-1 (Evans et al., 2005; Hibino et al., 2010). The model above represents the normal cell conditions where ABCC-9A is present in the apical and basal plasma membranes and is regulated by ATP/ADP ratio. The function of ABCC-9A/ $K_{ir}$  complex is to regulate membrane polarization, which helps in regulating the flow of calcium. The regulation of flow of calcium is important for calcium-mediated vesicular transport of proteins from

cell to the plasma membrane or into the procuticle of insects. The presence (and possibly the movement) of it is also required for the activity of membrane-bound chitin synthase.

**Model depicting how DFB affects vesicular transport of chitin metabolism protein and chitin synthesis via its action on SUR/K<sub>ir</sub> channels *in vivo*. The model presented below is representing DFB treated condition of epidermal cell (*Tribolium castaneum*).**



**Figure 4.2** The model representing DFB treated condition of epidermal cell (*T. castaneum*)

This model is based on the mode of action of ATP and SU's on human SUR-1 (Evans et al., 2005; Hibino et al., 2010). The binding DFB to TcABCC-9A located on the plasma membrane is postulated to lead to the following sequence of events: Binding of ATP or

*DFB to ABCC-9A results in closure of a  $K_{ir}$  channel which is associated with the channel regulator, ABCC-9A. Closing of  $K_{ir}$  channel results in a change in membrane potential, which indirectly leads to alteration in the  $Ca^{++}$  ion influx via a VDCC. At present we don't know whether  $Ca^{++}$  uptake is increased or decreased in epidermal cells by DFB treatment. But this alteration in  $Ca^{++}$  ion influx inhibits the transport of several chitin metabolism proteins by  $Ca^{++}$ -dependent mediators of the vesicular transport pathway. Alteration in vesicular transport results in inhibition of fusion of vesicles to the apical plasma membrane due to which chitin metabolism proteins are not moved to apical plasma membrane or to the procuticle leading to accumulation of these proteins inside the cell. Reduction in intracellular calcium ions also causes inhibition of chitin synthesis as calcium is absolutely required for the activity of chitin synthase.*

### **Future Directions**

In order to further investigate the molecular mechanism of DFB-mediated inhibition of chitin synthesis, the following objectives can be evaluated, which will give us a deeper understanding of chitin metabolism and different protein components involved.

#### **1. To establish physical interaction between chitin synthase and ABCC-9A**

The microsomal preparation from elytra of *T. castaneum*, which has the capacity to make chitin (*in vitro* system) also gets inhibited by DFB. In our study, we have established that DFB binds directly to ABCC-9A and this complex is present in the apical plasma membrane presumably in association with  $K_{ir}$  channels. There is the possibility that microsomal vesicles which have the capacity to make chitin, contain both CHS-A and ABCC-9 together in the same vesicle and if this is true, there is a very strong chance that they are physically interacting with each other.

## **2. Measure change in membrane potential after treatment with DFB.**

In our studies we established that ABCC-9A is a target of DFB which forms a complex with  $K_{ir}$  and that  $K_{ir}$  regulates the movement of potassium ions. Binding of DFB to ABCC-9A-  $K_{ir}$  Complex results in the closing of  $K_{ATP}$  due to which membrane potential changes occur. This experiment can help to gain information whether membrane is hyperpolarized or depolarized in presence of DFB. This experiment will also help us to know the direction of movement of potassium and calcium in *in vivo* and *in vitro* experiments.

## **3. Identification of Tc $K_{ir}$ which associates with TcABCC-9A and identification of *TcVDCC*, *TcSyntaxin(s)* which are involved in the movement of CHS-A protein and other chitin metabolism protein**

In our studies we established that ABCC-9A is a receptor for DFB. In case of humans, four subunits SUR-1 form a complex with 4 subunits of  $K_{ir}$ . Similarly, our study showed a possible involvement of  $K_{ir}$  as a regulator of the synthesis of chitin, responsive to changes in ATP/ADP ratio. In later studies, we can find which Tc  $K_{ir}$  is involved in complexing with TcABCC-9A and which other downstream elements like *TcVDCC* and *TcSyntaxins* are involved in the regulation of movement of chitin metabolism proteins. We can evaluate the effect of RNAi of genes encoding *TcABCC-9A*, *TcK<sub>ir</sub>*, and voltage-dependent calcium channels *TcVDCCs*, *TcSyntaxins*, and putative *Tribolium* orthologs of human v-SNARES and synaptogamin on the distribution of chitin metabolism proteins and alterations in cuticle architecture. There are several *K<sub>irs</sub>*, (*VDCC*), and *syntaxin* orthologs in the *Tribolium* genome but only a few (3  $K_{ir}$ 's and 3-4 *syntaxins*) are

expressed in the pharate adult elytral tissue based on our RNA Seq analyses. To establish that the corresponding *Tribolium* proteins are indeed involved in the same pathway of  $\text{Ca}^{++}$  signaling initiated by ATP binding to the SUR-KIR complex, we can determine whether RNAi of the genes involved in regulation of membrane potential (ABCC-9A and  $\text{K}^{+}_{\text{ATP}}$ ) as well as those involved in  $\text{Ca}^{++}$ -mediated vesicular transport leads to chitin loss, molting defects, loss of laminar cuticle organization, and mislocalization of CHS or not.

Once we have identified the specific  $\text{K}_{\text{ir}}$  gene regulated by *TcSur-1* (as indicated by similarity in the RNAi phenotypes of these two genes), we will test whether its activity can be altered by  $\text{K}^{+}$  channel blockers or openers. The  $\text{K}^{+}$  channel opener, diazoxide, used in treatment of hyperinsulinism to inhibit insulin secretion (Hibino et al., 2010) as well as the  $\text{K}^{+}$  channel blocker glibenclamide (which promotes insulin secretion) will be topically administered (in acetone; along with or without DFB) to the prepupae to determine whether they mimic or antagonize the effects of DFB. The effect of these compounds on chitin content in the procuticle, localization of CHS-A and laminar architecture of the procuticle in elytra and body wall will be monitored. Similarly, we can determine the changes in procuticle architecture after administration of  $\text{Ca}^{++}$  ion channel blocker, verapamil. These experiments are expected to provide support for modulation of membrane potential as a normal mechanism for regulated cuticle deposition presumably by sensing intracellular ATP/ADP ratios. Alterations in intracellular  $\text{Ca}^{++}$  may affect proteins other than chitin metabolism proteins via the  $\text{Ca}^{++}$ -dependent vesicular transport pathway.

**4. To establish the role of calcium ions in the process of chitin synthesis**

In our *in vitro* studies we found that when we block the uptake of calcium, the synthesis of chitin is inhibited. It will be interesting to study the role of calcium in the process of chitin synthesis which will help to better understand the process of chitin synthesis.

**5.** It will be interesting to study whether other matrix polymerases such as hyaluronan synthase or cellulose synthase have a similar  $\text{Ca}^{++}$  requirement.

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