THE BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF TRYPSINS AND CHYMOTRYPSINS FROM MIDGUTS OF *BACILLUS THURINGIENSIS* -SUSCEPTIBLE AND -RESISTANT *HELIOTHIS VIRESCENS* LARVAE (LEPIDOPTERA: NOCTUIDAE).

by

LOHITASH KARUMBAIAH

(Under the Direction of MICHAEL J. ADANG)

ABSTRACT

The onset of *Bacillus thuringiensis* (Bt) toxin resistance is a major threat to the long term use of Bt toxins for insect control. The Bt toxin mode of action involves a multi-step process, and the proteolytic cleavage of protoxin to toxin by midgut serine proteinases is an important step in this process. While altered toxin binding to midgut receptors is the most widely reported mechanism of resistance, the occurance of other mechanisms of resistance should not be discounted.

In this dissertation I investigated the possible role of a proteinase-related mechanism of resistance in Cry1Ac and Cry2Aa selected strains of *Heliothis virescens*. Enzymes in gut extracts of YHD2-B and CXC processed Cry1Ac and Cry2Aa protoxins slower than enzymes in gut extracts of the susceptible strain, YDK. The reduced rate of protoxin processing correlated with the absence of a ~32 kDa trypsin and ~35 kDa chymotrypsin from the gut extracts of the YHD2-B and CXC strains, respectively. Quantitative and qualitative differences in caseinolytic activities, as well as differences in pH optima of trypsin- and chymotrypsin-like proteinases in

gut extracts from the different strains, suggest that these differences may contribute to toxin insensitivity in the Bt resistant strains.

To characterize changes in proteinase expression I identified four trypsin-like (HvT) and ten chymotrypsin-like (HvC) transcripts from midguts of YDK and CXC larvae fed on diet alone or diet containing Cry1Ac or Cry2Aa toxins. Phylogenetic analyses, as well as pairwise sequence alignments, indicated that HvT sequences were highly similar while HvC sequences showed considerable sequence diversity. SNP analyses found mostly synonymous nucleotide changes; however, one non-synonymous change observed in the HvT1 loop-1 region, results in an amino acid change that could lead to altered substrate specificity. Gene expression analyses demonstrate that HvT3 expression is knocked out in toxin-fed YDK and CXC. Chymotrypsins were generally up-regulated in CXC; however, HvC6 expression was knocked out in Cry2Aa-fed CXC, indicating a toxin-specific down-regulation.

I conclude that a proteinase-related mechanism may be a resistance factor in the Bt resistant strain, CXC, and should also be considered in addition to altered toxin binding in the Bt resistant strain, YHD2-B.

INDEX WORDS: *Bacillus thuringiensis*, *Heliothis virescens*, trypsin, chymotrypsin, proteinase, resistance, gene expression, 3' RACE

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DEDICATION

I dedicate this work to my mother and role model Krishna Brahmanandam. I am eternally grateful to her for all the love, encouragement and support that she has given me in spite of having faced so much hardship in her own life. Ma, your mental strength and sheer determination will always be a source of inspiration to me as I strive to achieve greater heights in the future.

I also dedicate this work to my wife Sunaina, whose love and understanding have greatly contributed to the completion of this work. To my brother Vikram and my dear friends Shivakumar , Bruce and Justin, each one of you through your own lives have inspired me to pursue dreams of my own. This work bears testimony to your individual contributions.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Insecticidal crystal proteins produced by the spore forming soil bacterium *Bacillus thuringiensis* (Bt) have been increasingly used in insect control over the last decade. The alternative use of transgenic crops expressing Bt insecticidal proteins has led to a significant reduction in use of chemical insecticides. One of the major concerns accompanying the growing use of Bt toxins in insect pest control is the eventual onset of Bt resistance. It is therefore vitally important to address and manage insect resistance to Bt in the future. Ongoing efforts at understanding the multiple mechanisms of Bt resistance using laboratory-reared insects selected on toxin-contaminated diet are aimed at enhancing the efficacy and durability of these toxins in order to achieve maximum pest control.

1. *Bacillus thuringiensis*

Bacillus thuringiensis is a soil-borne gram-positive spore forming bacteria that is found ubiquitously in soil, stored grain and on plant material (de Maagd et al., 2001). One of the first incidences of Bt infection was observed in 1901 by Ishiwata in carcasses of the silkworm, *Bombyx mori* (Ishiwata, 1901). Berliner in 1915 observed similar infections among Mediterranean flour moths, *Ephestia küehniella*, which were brought from the central German region of 'Thüringen', hence the name *Bacillus thuringiensis* (Berliner, 1915).

B. thuringiensis produces parasporal crystals containing large amounts of a number of Crystal (Cry) and Cytolytic (Cyt) toxins that are also known as δ-endotoxins (Bravo et al., 2007; de Maagd et al., 2003). The crystal inclusions containing the various insecticidal proteins accumulate in the mother cell of the bacterium and account for nearly 25% of the dry weight of the sporulated cell (Agaisse and Lereclus, 1995). The crystal toxins and the accompanying

spores when ingested by the insect results in insect death and ensures bacterial survival as a result of spore germination (de Maagd et al., 2001).

Cry toxins are relatively host-specific and are known to be active against members of several insect orders including Lepidoptera, Coleoptera, Diptera, Hymenoptera, Homoptera, Orthoptera and Mallophaga (de Maagd et al., 2003; de Maagd et al., 2001; Schnepf et al., 1998), as well as nematodes belonging to phyla Platyhelminthes and Nemathelminthes (Bravo, 1997). Due to their narrow host-specificity, Bt toxins do not have any adverse affects on beneficial insects and non-target organisms, such as humans, making them ideally suited for use as insecticides (Clark et al., 2005). The development of resistance to chemical pesticides, coupled with their environmentally harmful properties, have prompted the extensive use of genetically modified crops expressing Bt toxins (e.g. corn and cotton) for insect control (Romeis et al., 2006).

Cry toxins were originally classified into CryI, CryII, CryIII and CryIV δ-endotoxins and the Cyt toxins by Höfte and Whiteley (1989). Since then, various other Bt genes having other host specificities have been discovered, necessiting a revision of nomenclature on the basis of evolutionary divergence rather than experimental determination of host-specificity. The revised nomenclature uses ‘Cry’ followed by a series of numbers and alphabets to designate toxin families and subfamilies (Crickmore et al., 1998). The listing of currently recognized Cry proteins is available at: http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/index.html

2. Cry toxin structure and function

Crystallographically resolved structures of activated forms of Cry1Aa, Cry2Aa, Cry3Aa, Cry3Bb1 and Cry4Ba toxins have provided insights into toxin structure and helped elucidate specific domain functions (Boonserm et al., 2005; Galitsky et al., 2001; Grochulski et al., 1995;

Li et al., 1991; Morse et al., 2001). Although these toxins exhibit different host-specificities and possess structural differences, they all nevertheless share a common three-domain toxin structure (Fig. 1.1). In the majority of toxin structures, domain-I consists of seven alpha-helices with six of them encircling the fifth α-helix (Fig. 1.2). Domain-II is made up of β-sheets which are arranged in the form of a 'Greek Key' motif. Domain-III consists of two anti-parallel β-sheets arranged in the form of a 'Jelly Roll' (Richardson, 1977).

2.1 Role of domain-I in membrane insertion and pore formation

The Cry toxin domain-I has characteristic features resembling the pore-forming domains of bacterial toxins, such as the diphtheria toxin secreted by *Corynebacterium diphtheriae* and colicin A secreted by *Citrobacter freundii* and *Escherichia coli* (Choe et al., 1992; Parker and Pattus, 1993). Colicin A pore-forming domain consists of α-helices 8 and 9 surrounded by eight other anti- parallel α-helices (Parker and Pattus, 1993). The diphtheria toxin undergoes a pH-induced change in conformation which enables a hydrophobic hairpin consisting of two α-helices to form pores (Schnepf et al., 1998).

The Cry toxin domain-I is made up of seven α-helices with six of them encircling α-helix 5 (Grochulski et al., 1995). Alignments of domain-1 sequences from the different Bt toxins indicates that α-helices 5 and 6 are the most conserved. The presence of the most number of hydrophobic residues on these two α-helices and the fact that they were the farthest away from the plasma membrane prompted Hodgman and Ellar (1990) to propose the "Penknife" model (Hodgman and Ellar, 1990; Knowles, 1994). According to this model, toxin-binding to a membrane-associated receptor causes a helical hairpin consisting of α-helices 5and 6 to "flip out" of domain-I and insert into the membrane.

The "Umbrella" model was based upon a similar model proposed for colicin A (Lakey et al., 1991; Li et al., 1991). According to this model either pair of α-helices 6 and 7 or α-helices 4 and 5 are inserted into the membrane as a result of their close proximity. This induces a change in conformation of the rest of the toxin molecule causing the remaining α-helices to open up on the membrane surface around the central helical hairpin like an umbrella (Knowles, 1994; Lakey et al., 1991). In both models, further oligomerization of toxin molecules or membrane insertion of other α-helices ultimately results in the formation of an aqueous pore (Knowles, 1994).

Sequence alignments of various Cry toxins resulted in the identification of five conserved sequence blocks (Hofte and Whiteley, 1989). A similar exercise by Schnepf et al. (1998) led to the identification of three additional blocks that lay outside of the toxin core in the C-terminal region of the protoxin (Schnepf et al., 1998). In all Cry toxin sequences compared, conserved sequence blocks-1 and -2 consist of α-helices 5 and 7 of domain-I. These two helices are implicated in maintaining structural integrity of the helical bundle and in retaining the shape of the protein during solubilization and activation (Schnepf et al., 1998).

2.2 Role of domain-II in receptor binding and specificity

Domain-II consists of three anti-parallel β-sheets, which have similar topologies. β-sheets 1 and 2 consist of β-strands 2 to 5 and 6 to 9, respectively, that are connected together in a 'Greek Key' motif. β -sheet 3 consists of β-strands 10, 11 and 1 along with the last α-helix from domain-I (Grochulski et al., 1995). The 'Greek Key' motif of β-sheets in domain-II was first described in the crystal structure of Cry3Aa (Li et al., 1991). This structure in domain-II of Cry3Aa and Cry2Aa resembles vitelline membrane outer layer protein-I from hen egg, as well as plant lectins, such as Jacalin and *Artocarpus hirsuta* lectin, which have insecticidal properties (Morse

et al., 2001; Rao et al., 2004; Sankaranarayanan et al., 1996; Shimizu et al., 1994). These lectins are known to bind N-acetylgalactosamine (GalNAc) residues, which are described to determine Cry1Ac binding in *Heliothis virescens, Manduca sexta* and CF1 cells (Knowles et al., 1991).

Apical loops present in three β-sheets of domain-II resemble immunoglobin antigen-binding sites and were consequently proposed as the regions involved in receptor binding (Li et al., 1991; Schnepf et al., 1998). Sequence variation observed in domain-II in comparisons of Cry1Aa and Cry3Aa toxins suggest that this region could be involved in determining binding specificity (Grochulski et al., 1995). Domain substitutions and mutagenesis of domain-II loop residues in Cry3A, Cry1A and Cry1C demonstrated that altering loop residues could directly affect binding specificity and insecticidal toxicity (Rajamohan et al., 1998; Schnepf et al., 1998; Smith and Ellar, 1994; Wu and Dean, 1996).

2.3 Role of domain-III in toxin stability, ion channel regulation and receptor binding

Domain-III consists of β-sheets arranged in a 'Jelly Roll' conformation. Receptor binding analyses and mutagenesis of domain-III residues indicate that alteration of key residues influenced Cry1Ac toxin binding and toxin specificity to midgut receptors in *H. virescens* and *M. sexta* (Aronson et al., 1995; Ge et al., 1991; Schnepf et al., 1990). Mutation of two arginine residues in Cry1Aa conserved block-4 to glutamates, glycines or lysines resulted in poor *E.coli* expression, indicating a disruption of structure (Chen et al., 1993). Li et al. (1991) reported that the Cry3Aa structure is stabilized at this position by a salt bridge that connects domain-I to domain-III (Li et al., 1991). A similar structure in Cry1Aa was disrupted by replacement of arginine with lysine, supporting the role of this domain in toxin stability (Chen et al., 1993; Li et al., 1991).

In a second finding, Chen et al. (1993) reported that the replacement of arginine residues R1 and R4 by lysines resulted in no alteration in structure, receptor binding, trypsin activation or CD-spectra. The mutant toxin, however, showed lower toxicity and reduced inhibition of short-circuit current across *B. mori* midguts indicating that domain-III could play a role as an ion channel regulator (Chen et al., 1993).

Purified aminopeptidase-N (APN) from the midgut of gypsy moth (*Lymantria dispar*) was found to bind with high affinity to Cry1Ac but not Cry1Aa (Valaitis et al., 1995). Receptor binding studies using slot blot and ligand blot assays showed that hybrid toxins containing Cry1Ac domain-III residues bound specifically to APN while toxins containing Cry1Aa domain-III residues instead bound to a 210 kDa protein, thus providing direct evidence of the role of domain-III in receptor binding and specificity (Lee et al., 1995b). Domain-swapping experiments with domains II and III of Cry1Aa and Cry1Ac revealed alterations in toxin specificity (Ge et al., 1989; Schnepf et al., 1990). Mutation of Cry1Ac GalNAc-binding residues in domain-III resulted in reduced binding to a 120 kDa APN and to *M. sexta* BBMV in general (Burton et al., 1999). The mutant toxin had a slower rate of pore formation but toxin binding was not inhibited by GalNAc, thus indicating that domain-III possibly has a weaker role relative to domain II in determining toxicity. Taken together, these analyses of domain-III function suggest that in addition to providing structural stability, domain-III could also play a role in receptor binding and specificity.

3. The lepidopteran midgut

Lepidopteran larvae are voracious feeders and can regularly ingest food quantities greater than their average body weight. Their digestive system consists of a short foregut, long midgut

and a short hindgut. The midgut is lined by a sac-like chitinous membrane called the 'peritrophic matrix' which serves to protect the midgut lining and also acts as a sieve, allowing the passage of molecules <800 nm in size (Adang and Spence, 1983; Terra, 2001).

Nearly all of the digestion is conducted in the long midgut region, which is lined by columnar cells that have finger-like projections called 'microvilli.' These cells facilitate absorption of amino acids along with K^+ ions via the microvilli, and also secrete digestive enzymes, which are mainly serine proteinases in lepidopteran larvae (Nation, 2002; Purcell et al., 1992; Terra WR, 1994). In between the columnar cells are goblet cells, which enable the transportation of K^+ ions from the hemolymph to the midgut lumen. The goblet cell membrane houses a V-ATPase H^+ pump which hydrolyzes ATP releasing H^+ into the goblet cell cavity. An H^+/K^+ antiport mechanism enables the exchange of protons for K^+, which is released back into the gut lumen, thus maintaining the highly alkaline environment of the lepidopteran midgut (Giordana and Parenti, 1994). The alkaline midgut in lepidopteran larvae is thought to have evolved from the need to extract hemicelluloses from plant cell walls (Terra, 1988). The base of the epithelial membrane is lined by regenerative cells whose function is not entirely characterized (Terra and Ferreira, 1994).

4. Mechanism of Cry1 toxin action

When susceptible lepidopteran larvae ingest Bt crystals, the crystals dissolve in the alkaline lumen of the midgut, releasing the protoxin form of Cry proteins. Solubilized protoxin forms of Cry proteins are non-toxic and hence need to be activated to a proteinase-resistant core by the action of gut proteinases, such as trypsins and chymotrypsins (Choma and Kaplan, 1990a; Choma et al., 1990b; Lightwood et al., 2000). Processing of the 130-160 kDa Cry1 protoxins

result in the sequential cleavage of most of the C-terminal region, as well as 28 residues from the N-terminal region (Choma et al., 1990b; Rukmini et al., 2000). The resulting activated toxin has a size of ~60 kDa and is able to bind with high affinity to midgut receptors present on the insect brush border membrane (BBM) (Hofmann et al., 1988). The process of toxin activation by midgut proteinases can affect toxin specificity as observed in the case of Bt *aizawai* IC1 strain. Protoxin from this strain, when activated by lepidopteran proteinases, is active against lepidopteran larvae. Further processing by dipteran proteinases releases amino acids 558-595 making the toxin dipteran-specific (Haider et al., 1986).

Recent efforts at determining the precise mechanism of Bt toxin action have culminated in two alternate hypotheses. Studies on *M. sexta* BBMV using Cry1Ab toxin indicate that toxin oligomerization after receptor binding, resulting in a pre-pore complex lacking domain-I α-helix-I. The pre-pore complex is capable of membrane insertion (Gomez et al., 2002). A subsequent report (Bravo A, 2004) proposed that activated toxin monomers initially bind the midgut cadherin Bt-R_1 inducing a conformational change that facilitates the cleavage of α-helix-1 by a membrane-associated proteinase. The resulting cleavage promotes toxin oligomerization resulting in a pre-pore structure that binds APN and subsequently inserts into the membrane.

Zhang et al. (2005) used cultured insect cells expressing cadherin Bt-R_1 to determine a novel mechanism of toxin action (Zhang et al., 2005). Those authors suggest that specific binding of toxin monomers to Bt-R_1 results in cell death, while membrane insertion of toxin oligomers is a non-specific interaction with lipids and does not result in cell death. According to this model, receptor-mediated insertion of toxin induces cytotoxicity only in the presence of magnesium, which triggers a cell signaling pathway that culminates in cell death (Zhang et al., 2005). A continuation of this study revealed that toxin binding to Bt-R_1 resulted in the stimulation of a G-

protein (G_α), adenylate cyclase (AC) pathway, activation of protein kinase A (PKA) and increased levels of cAMP (Zhang et al., 2006). Inhibition of G_α, AC and PKA protected the cells from Cry1Ab-induced cytotoxicity, while enhancement of cAMP resulted in increased cytotoxicity. In a revised model, the authors proposed that binding of toxin monomers to Bt-R_1 results in triggering of a signaling pathway that is characterized by G-protein and AC stimulation leading to accumulation of cAMP and activation of PKA. Activated PKA leads to destabilization of the cytoskeleton and ion channels, which ultimately results in cell death (Zhang et al., 2006).

In a third mechanism of action describing Cry1Ac toxicity in *H. virescens*, Jurat-Fuentes and Adang (2006a) integrate the sequential binding model of Bravo et al. (2004) and the cell signaling model proposed by Zhang et al. (2006). According their model, binding of monomeric Cry1Ac to a *H. virescens* cadherin-like protein (HevCaLP) triggers an intracellular signaling pathway that is regulated by phosphatases. Alternatively, the signaling process may also be triggered by interaction of Cry1Ac toxin with actin. Toxin oligomers then bind glycosylphosphatidylinositol (GPI)-anchored proteins such as *H. virescens* alkaline phosphatase (HvALP) and APN, and the resulting complex is driven into membrane lipid rafts. Aggregation of toxin oligomers in the lipid rafts ultimately leads to membrane insertion and activation of intracellular phosphatases, which regulate apoptotic processes leading to cell death (Jurat-Fuentes and Adang, 2006a; Pigott and Ellar, 2007).

5. Cry toxin receptors

The term cry toxin ‘receptor’ specifically refers to toxin-binding proteins that cause cell toxicity upon binding, thus differentiating them from toxin-binding proteins in general. Identification of receptors is conducted by probing one-dimensional (1D) and two-dimensional

(2D) blots of resolved brush border membrane vesicle (BBMV) proteins with labeled toxins (Garczynski et al., 1991; McNall and Adang, 2003). This process, called 'ligand blotting', is popularly used to derive an estimate of the diversity of toxin-binding proteins in BBMV. The Cry toxin receptors discovered from insects and nematodes have been described variously as N-aminopeptidases (Knight et al., 1994; Sangadala et al., 1994), cadherins (Vadlamudi et al., 1993), glycolipids (Griffitts et al., 2005) and alkaline phosphatases (McNall and Adang, 2003). For a recent review of Cry toxin receptors see Pigott and Ellar (2007).

5.1 N-Aminopeptidases

N-Aminopeptidases (APN's) are metallopeptidases that cleave amino acids from the N-terminus of peptides. These endopeptidases are ubiquitous in cells and tissues as soluble and membrane-bound forms. They function as virus receptors, elicitors of tumorigenesis, and influence pain and immune responses in humans (Luan and Xu, 2007).

APNs in insects are distributed along the insect BBM as indicated by APN activity in *M. sexta* BBMV preparations and immunocytochemistry of dissected midguts (Chen et al., 2005; Knight et al., 1994). APN was first identified as a toxin-binding protein and putative receptor by Knight et al. (1994) and Sangadala et al. (1994). This APN is a 120 kDa membrane-bound protein that is tethered to the insect midgut epithelium by means of a GPI anchor (Garczynski and Adang, 1995). Since the initial identification and characterization of APN as a toxin receptor in *M. sexta*, it has been similarly characterized in midguts of *B. mori* (Yaoi et al., 1997), *Helicoverpa armigera* (Sivakumar et al., 2007), *L. dispar* (Lee et al., 1996; Valaitis et al., 1995), *Spodoptera litura* (Rajagopal et al., 2002), *H. virescens* (Luo et al., 1997), *Trichoplusia ni* (Lorence et al., 1997) and *Plutella xylostella* (Denolf et al., 1997). In more recent reports,

controversy with regard to the role of APN as a receptor in toxin mode of action stems from suggestions that binding to APN's may be a non-specific interaction that does not culminate in cell death (Zhang et al., 2005; Zhang et al., 2006).

5.2 Cadherins

Classical cadherins are calcium-dependent cell-cell adhesion glycoproteins that are located at epithelial cell junctions. The structure of cadherins is characterized by an extracellular domain consisting of multiple cadherin repeats (CR's), a transmembrane domain and an intracellular domain. The structural and functional variation of these proteins necessitated their classification into classical, desmosomal, flamingo, FAT-family, T-cadherins and protocadherins (Angst et al., 2001; Bel and Escriche, 2006). Cadherins play an important role in tissue maintenance, tissue repair and morphogenesis, and are also involved in cell-signaling pathways (Angst et al., 2001; Takeichi, 1995).

In insects, a 210 kDa cadherin-like protein, Bt-R_1, was first identified by Vadlamudi et al. as a Cry1Ab-binding protein in BBMV prepared from *M. sexta* (Vadlamudi et al., 1993; Vadlamudi et al., 1995). Immunolocalization studies using anterior, posterior and middle sections of the *M. sexta* midgut suggest that Bt-R_1 is located along the entire microvilli in the middle section of the midgut and co-localizes with Cry1Aa and Cry1Ab toxin-binding regions (Chen et al., 2005). In cytotoxicity assays using *Drosophila* S2 cells, Hua et al. (2004) determined that cadherin repeat-12 (CR12) alone was sufficient for Cry1Ab binding and toxicity. A retrotransposon-mediated disruption of the gene encoding *H.virescens* cadherin-like protein (HevCaLP) was associated with Cry1Ac toxin resistance in *H. virescens* strain YHD2, thus implicating the role of HevCaLP as a functional receptor in this insect (Gahan et al., 2001). In other supporting evidence, Cry1Ac

resistance was linked to mutations in three alleles of a cadherin-encoding gene found among field populations of the pink bollworm (*Pectinophora gossypiella*) (Morin et al., 2003).

5.3 Alkaline Phosphatase

Alkaline phosphatases were identified as BBM markers by Wolfersberger (1984). Studies of the midgut of the silkworm, *B. mori,* led to the characterization of membrane-bound and soluble forms of this protein (Okada et al., 1989). While the GPI-anchored membrane bound alkaline phosphatase (mALP) exhibits digestive and absorptive functions, the soluble form (sALP) is thought to regulate ionic balance and possesses ATPase activity (Eguchi et al., 1990).

Ligand blots of *M. sexta* BBMV proteins resolved by 2D-electrophoresis and probed with Cry1Ac toxin enabled the identification of an alkaline phosphatase as a toxin-binding protein (McNall and Adang, 2003). In the *H. virescens* strain YHD2, reduced toxin binding as a resistance trait was correlated with altered glycosylation of 63 kDa and 68 kDa proteins (Jurat-Fuentes et al., 2002) Subsequent detection of alkaline phosphatase activity, crossreactivity with αALP antisera and presence of a GPI anchor on a 68 kDa protein indicated that this was a membrane-bound alkaline phosphatase and was called HvALP (Jurat-Fuentes, 2004a).

5.4 Glycolipids

Caenorhabditis elegans mutants lacking a glycosyltransferase gene, *bre5,* were reported to be resistant to Cry5B. These mutant worms also showed resistance to the nematicidal and insecticidal toxin Cry14A, indicating that carbohydrate modifications could lead to Bt resistance in nematodes, as well as insects (Griffitts et al., 2001).

Based on the role of *Drosophila* glycosyltransferase homologs EGGHEAD and BRANIAC in carbohydrate chain synthesis of glycosphingolipids, it was hypothesized that the *C. elegans* glycosyltransferases, BRE3 and BRE5, could have similar properties. Thin-layer chromatographical (TLC) analysis of lipids, as well as toxin overlays of immobilized glycolipids from mutant and wild-type worms, showed that the mutant worms lacked the glycolipid species required to bind Cry5B, thus leading to Bt resistance (Griffitts et al., 2005).

Glycolipids as toxin- binding molecules and potential receptors in insects were shown by toxin overlays where Cry1Ac, Cry1Ab and Cry1Aa bound to *M. sexta* glycolipids in a specific manner (Griffitts et al., 2005).

6. Midgut proteinases

6.1 Proteinase Secretion

Lepidopteran digestive proteinases are mainly serine proteinases, which are characterized by the presence of a serine, histidine and aspartic acid catalytic triad (Applebaum, 1985; Terra and Ferreira, 1994). The secretion of these proteinases can occur in a 'constitutive' manner wherein synthesis and release occurs simultaneously, or in a 'regulated' manner wherein proteinases are synthesized and stored as inactive 'zymogens' until required (Lehane et al., 1995). The secretion of digestive enzymes occurs either by 'prandial' or 'paracrine' control mechanisms. In prandial control, proteins in the ingested food directly stimulate midgut cells to secrete digestive enzymes. In paracrine control, the endocrine cells are stimulated to produce release factors that stimulate the secretion of digestive enzymes (Lehane et al., 1995)

Digestive enzyme secretion can take place by any one of three processes, namely holocrine, apocrine or merocrine secretions (Nation, 2002). In holocrine and apocrine secretions, enzymes

stored in the cytoplasm are released either by the disruption of the entire midgut cell (holocrine) or by disruption of small parts (apocrine) of the cell. In merocrine or exocytotic secretions, digestive enzymes are processed in the columnar cell golgi and subsequently packaged into vesicles. The enzyme-laden vesicles then fuse with the columnar cell membrane to release their contents into the gut lumen. In a variation of apocrine secretions called microapocrine secretion, enzyme-laden vesicles appear to be pinched off from the sides of columnar cells. This type of secretion in widely observed in the anterior midgut, whereas merocrine secretions are observed in the posterior midgut (Nation, 2002; Santos et al., 1986).

In lepidopteran larvae, trypsin-like enzymes are packaged into small vesicles within the columnar cells. These enzymes are processed to soluble forms within the vesicles and transported to the cell membrane inside the vesicles. As the vesicles bud out of the microvillar surface, trypsin-like enzymes are released into the gut lumen either through fusion of the vesicular membrane with the plasma membrane or through disruption of the vesicles by the highly alkaline pH of the gut lumen (Nation, 2002; Santos et al., 1986; Santos and Terra, 1984).

6.2 Serine Proteinases

Trypsin and chymotrypsin-like proteinases are the most abundant serine proteinases in lepidopteran midguts (Ahmad et al., 1976; Applebaum, 1985; Christeller et al., 1992; Purcell et al., 1992). The lepidopteran midgut is highly alkaline with a pH range of 10-12 (Dow, 1984). Consequently, these proteinases have evolved the ability to remain active under these conditions presumably due to the presence of a large number of arginine residues, which keep the enzyme protonated under these conditions (Peterson et al., 1994).

Serine proteinase specificity depends on the interaction of the S1 binding pocket with the amino acid side chains between the P1 and P1' sites of the substrate. The combination of amino acids in the S1 binding pocket is important in determining serine proteinase specificity. In trypsins for example, the presence of D^{189}, G^{216} and G^{226} residues creates a negatively charged S1 pocket, thus attracting the side chains of positively charged amino acids, such as arginine and lysine.In the case of chymotrypsins, S^{189}, G^{216} and G^{226} create a larger S1 pocket, thereby facilitating the entry and cleavage of large side chains of aromatic amino acids such as phenylalanine and tryrosine (Srinivasan et al., 2006).

6.2.1 Trypsins

Trypsins are serine proteinases that cleave the carboxyl termini of the positively charged amino acids arginine and lysine (Terra and Ferreira, 1994). Lepidopteran trypsins have molecular sizes in the range of 25-35 kDa, and differ distinctly from vertebrate trypsins by their ability to remain active in the highly alkaline conditions of the midgut. These trypsins are largely conserved and share a high degree of homology with mammalian trypsins. However, key differences exist between lepidopteran and mammalian trypsins. When compared to mammalian trypsins, lepidopteran trypsins have different activation peptides, do not need to be stabilized by calcium ions, are insensitive to mammalian inhibitors, and are not active in acidic pH (Davis et al., 1985; Johnston et al., 1991; Sakal et al., 1989).

Trypsin-like enzymes synthesized in the columnar cells are packaged into vesicles and transported to the apical membrane where they are processed into soluble forms and released into the gut lumen. In the absence of inactive trypsins, the secretion of these enzymes is controlled by

retaining the vesicles to the cell membrane until they are required to be released (Terra and Ferreira, 1994).

6.2.2 Chymotrypsins

Chymotrypsins cleave the C-terminal side of the peptide bond of aromatic amino acid residues such as phenylalanine and tyrosine (Terra and Ferreira, 1994). Lepidopteran chymotrypsins have molecular sizes ranging from 20–30 kDa and, similar to lepidopteran trypsins, have a large number of arginines that stabilize the substrate binding pocket. These chymotrypsins are different from mammalian counterparts in that they are unstable in acidic pH and are strongly inhibited by soybean trypsin inhibitor (Terra and Ferreira, 1994). Although insect chymotrypsins are reported to be quite similar to mammalian chymotrypsins in structure, they show a greater extent of variation in S1 binding pocket residues implying a broader specificity in recognition and proteolysis of diverse substrates (Peterson et al., 1995; Srinivasan et al., 2006).

7. Cry toxin resistance

With the growing use of transgenic crops, such as Bt corn, cotton and potato, the threat of insects developing resistance to Cry toxins in the near future is a major concern. The first report of Cry toxin resistance was reported in a laboratory-selected strain of the Indian meal moth, *Plodia interpunctella,* which developed a 100-fold resistance within 15 generations of selection on a Bt var.*kurstaki* formulation called Dipel® (McGaughey, 1985). Resistance to sprayable Bt formulations was observed in the Phillipines and Hawaii among field populations of *P. xylostella,* and more recently in greenhouse populations of the cabbage looper, *T. ni* (Janmaat

and Myers, 2003; Kirsch and Schmutterer, 1988; Tabashnik, 1990). Since these first incidences of toxin resistance, laboratory selection of insects on toxin-treated diet has been routinely used to study mechanisms of toxin resistance in Lepidoptera (Ferre and Van Rie, 2002).

Laboratory-selected strains of *P. xylostella* were found to be highly resistant to most Cry1A toxins, but remained susceptible to other Cry1 and Cry2 toxins (Tabashnik et al., 1994; Tabashnik et al., 1993; Tabashnik et al., 1997; Tabashnik et al., 1996). These results supported the development of a 'Gene Stacking' approach that is aimed at delaying the onset of resistance by transgenically expressing dual toxins having different mechanisms of action (Gould, 1998). The success of such an approach will depend entirely on the inability of the insect to develop resistance to both toxins simultaneously.

7.1 Proteinase-related mechanisms of resistance

Although reduced binding to midgut receptors has been reported as the major resistance-causing factor among field insects, additional mechanisms have been observed among laboratory-selected insects (Ferre and Van Rie 2002). Mechanisms of resistance involving altered proteinases have been reported in *P. interpunctella*, *Ostrinia nubilalis, H. virescens, H. armigera, P. xylostella, Melolontha melolontha* and *Leptinotarsa. decemlineata* (Chandrashekar and Gujar, 2004; Forcada et al., 1999; Forcada et al., 1996; Li et al., 2004a; Liu et al., 2000; Loseva et al., 2002; Oppert et al., 1997a; Wagner et al., 2002).

The role of proteinases in insect resistance to Bt Cry proteins is complex. Alterations in gut proteinases in Cry1Ab-resistant *P. interpunctella* were first reported by Oppert et al. (1994). Loss of a specific midgut trypsin in Cry1Ab-resistant *P. interpunctella* strain 198^{r} was associated with resistance to both protoxin and toxin forms of Cry1Ab (Oppert et al., 1996). Analysis of

midgut extracts using *ρ*-nitroanilide substrates demonstrated reduced proteolytic activity along with a reduced ability to process Cry1Ac protoxin to toxin when compared to the susceptible strain (Oppert et al., 1996). Further analysis established a genetic linkage between the lack of a major trypsin-like proteinase and toxin resistance in this strain (Oppert et al., 1997). A similar proteinase-related Bt resistance mechanism was reported for resistant *O. nubilalis* (Li et al., 2004b). In both insects, resistance was almost completely reversed when insects were fed activated toxin (Herrero et al., 2001; Li et al., 2005b), providing strong evidence that activation of protoxin is compromised in these insect strains.

Resistance also may be associated with degradation and elimination of toxin, as has been reported in *H. virescens* and *H. armigera* (Chandrashekar and Gujar, 2004; Forcada et al., 1996). Enzymes present in the gut extracts of the *H.virescens* resistant strain, CP73-3, processed Cry1Ab protoxin slower and degraded activated toxin faster than the susceptible strain. In another study of *H.virescens*, comparison of proteolytic activities between the susceptible CPN strain gut extract and the resistant KCB strain gut extract revealed qualitative differences in bands of proteolytic activity that could contribute to resistance in the KCB strain (Forcada et al., 1999). In the American bollworm, *H. armigera*, a 31-fold resistance to Cry1Ac was attributed to binding site alterations as well as the faster proteolytic degradation of Cry1Ac toxin, suggesting dual mechanisms of resistance in this strain (Chandrashekar and Gujar, 2004).

In other examples of proteinase-related resistance, the *P. xylostella* strain NO-95C exhibited a 2.5-fold greater resistance to Cry1Ca protoxin than toxin (Liu et al., 2000). Additional examples of proteinase-related Bt resistance were also reported for the coleopterans *M. melolontha* and *L. decemlineata* (Loseva et al., 2002; Wagner et al., 2002).

7.1.1 Regulation of insect proteinases

Digestive proteolysis in lepidopteran larvae is a complex process and regulation of secreted proteinases is hormonally controlled (Lehane et al., 1995). The differential regulation of midgut proteinases has been extensively studied in midguts of insects ingesting plant proteinase inhibitors. Instances of proteinase inhibitor resistance due to altered gut proteinases have been observed in *H. armigera*, *Helicoverpa zea, Spodoptera exigua* and *H. virescens* as well as in some Coleoptera, such as *Diabrotica undecimpunctata*, *L.decemlineata* and *Callosobruchus maculatus* (Bown et al., 1997; Brito, 2001; Broadway, 1986; Fabrick et al., 2002; Gruden et al., 1998; Jongsma et al., 1995; Volpicella et al., 2003; Zhu-Salzman et al., 2003). The cDNAs encoding a number of digestive proteinases from lepidopteran species, such as *B. mori, S. exigua, Lonomia obliqua, Sesamia nonagrioides, M. sexta, O. nubilalis, Agrotis ipsilon, H. zea, H. armigera, P. interpunctella, H. virescens, Scirpophaga incertulas, Choristoneura fumiferana* and *T. ni,* have been cloned and characterized (Borovsky et al., 2006; Bown et al., 1997; Diaz-Mendoza et al., 2005; Kotani et al., 1999; Li et al., 2005a; Mazumdar-Leighton et al., 2000; Mazumdar-Leighton and Broadway, 2001; Peterson et al., 1994; Peterson et al., 1995; Thompson et al., 2003; Veiga et al., 2005; Wang et al., 1993; Zhu et al., 2000a; Zhu et al., 2000b).

In *H. armigera*, alterations in the substrate-binding site residues of trypsin- and chymotrypsin-like enzymes are responsible for the loss of proteinase inhibition (Bown et al., 1997). Inhibitor-insensitive proteinases arise from genes expressed at low levels under normal conditions and overexpressed only after the ingestion of proteinase inhibitors (Bown et al., 2004). These proteinases are related to the serine proteinase class, but have distinct substrate specificities. One such class of proteinases is the ‘diverged serine proteinase’ class, whose members are predicted not to possess enzymatic activity due to lack of the characteristic serine

proteinase catalytic triad (Bown et al., 1997). The high abundance of these mRNA transcripts, however, suggests a function for these atypical proteinases. These 'diverged serine proteinases' may serve to bind and sequester plant proteinase inhibitors.

In other studies, *H. armigera* feeding on proteinase inhibitors were shown to resist proteinase inhibition by inducing the production of inhibitor-insensitive proteinases (Bown et al., 1997; Bown et al., 2004). For example, larvae fed on the soybean kunitz trypsin inhibitor (SKTI) produce inhibitor-insensitive variants of existing serine proteinases (Bown et al., 1997). Sequence analyses of cDNAs encoding inhibitor-insensitive proteinases revealed amino acid differences in regions that presumably are in contact with the inhibitor. Northern blots demonstrated increased levels of chymotrypsin-encoding mRNAs in SKTI-fed larvae. Probing midgut cDNA libraries of control and SKTI-fed larvae with a trypsin encoding probe demonstrated that the initial up-regulation of all trypsin genes was succeeded by the subsequent down-regulation of inhibitor-sensitive trypsins (Bown et al., 2004). While the exact mechanism of proteinase gene regulation in not known in *H. armigera*, it is believed that the secretion of major proteinases is regulated by peptide hormones (Broadway, 1997; Gatehouse et al., 1997; Lehane et al., 1995). According to this model, proteinase requirements are assessed by the rate of cleavage of a 'monitor peptide', which is a substrate for major proteinases. The inhibition of 'monitor peptide' cleavage results in a sustained increase of cholecystokinin (CCK) which in turn leads to increased proteinase production (Lehane et al., 1995).

In other instances of proteinase gene regulation, 4th instar larvae of the cowpea bruchid *C. maculates* (Cm), fed diet containing soybean cysteine proteinase inhibitor, soyastatin N (scN), were able to adapt to inhibitor selection by differentially regulating cysteine proteinases (CP) (Zhu-Salzman et al., 2003). Cloning and sequencing of digestive enzymes from the midguts of

larvae fed on scN led to the identification of two subfamilies of cathepsin-L-like cysteine proteinases (CP), CmCPA and CmCPB. Northern blot and real-time polymerase chain reaction (PCR) analysis of CP gene expression demonstrated a 116-fold over-expression of genes encoding CmCPB compared to only a 2.5-fold increase in those encoding CmCPA. Of all the proteinases present in this multigenic family, CmCPB had the highest proteolytic activity and was the most abundant, which ultimately led to the degradation of scN (Ahn et al., 2004).

In Cry toxin-resistant *P. interpunctella*, quantitative reverse transcription PCR (QRT-PCR) was used to analyze the gene expression of three trypsinogen-encoding genes *Pi*T2a, *Pi*T2b and *Pi*T2c, in susceptible and resistant strains (Zhu et al., 2000a). Levels of trypsinogen mRNA *Pi*T2a and *Pi*T2c were higher in the susceptible strain, while that of *Pi*T2b was higher in the resistant strain.

In *O. nubilalis*, resistance to Dipel® was associated with reduced midgut trypsin activity leading to inefficient protoxin activation (Li et al., 2005b). Real-time quantitative PCR and northern blots were used to analyze the expression of three trypsin encoding cDNAs, *On*T2, *On*T23 and *On*T25. The results demonstrated that reduced expression of *On*T23 in the Bt-resistant *O. nubilalis* was related to reduced trypsin activity.

7.2 Alternate mechanisms of resistance

Additional mechanisms of Bt resistance were suggested from scanning electron microscopy (SEM) studies of susceptible and resistant *H. virescens* midguts (Forcada et al., 1999). Toxin ingestion resulted in equal damage to midguts from susceptible and resistant strains. Toxin feeding affected growth and the ability to absorb nutrients in the susceptible strain but had no affect on the midgut epithelia of resistant strains, KCB and CP73-3. These strains

recovered much faster than the susceptible strain after being administered the same dose of Cry1Ac and Cry1Ab (Forcada et al., 1999; Martinez et al., 1999). A separate study showed that toxin feeding can increase the activity of stem and differentiating cells, thus facilitating the quick replacement of damaged midgut cells (Loeb et al., 2001)

Gut extracts and hemolymph from Bt-resistant *H. armigera* showed a higher immune response and greater extent of melanization when compared to susceptible insects (Ma et al., 2005). The immune response induced production of a glycoprotein called hexamerin which apparently binds and sequesters toxin by forming insoluble aggregates. Reciprocal crosses of resistant and susceptible insects resulted in transmission of the immune elicitor by a maternal effect (Ma et al., 2005).

8. *Heliothis virescens*

Heliothis virescens, the tobacco budworm, is a major pest of cotton, tobacco and soybean in the United States (Burris et al., 1995). The adults deposit eggs mostly in the buds or squares of cotton plants (King and Jackson, 1989), which hatch in 2 to 3 days of warm weather. The larva, which is the biological stage most damaging to crops, begins feeding and molts five to six times before burrowing into the soil to pupate for a period of two weeks. After a period of pupation, adults emerge and lay eggs after several days, completing the life cycle of about 30 days (Metcalf and Metcalf, 1993).

H. virescens larvae are now controlled mostly by transgenic cotton plants engineered to express Bt Cry proteins. However, the continued success of Bt cotton depends on methods to detect and delay insect resistance to the Cry proteins (Ferre and Van Rie, 2002). Toxin binding to

the brush border epithelium is a critical step in toxin action, and alteration of binding sites is a mechanism by which insects become resistant to Cry toxins.

In *H. virescens*, toxin binding studies have led to a model of three distinct populations of binding sites (Jurat-Fuentes and Adang, 2001; Van Rie, 1989). Receptor class-A molecules, comprising APNs and a cadherin-like protein called HevCaLP, bind the Cry1A toxins and other toxins with domain II loop homology (Jurat-Fuentes and Adang, 2001; Jurat-Fuentes, 2004b; Luo et al., 1997). Receptor class-B consists of a 130 kDa unidentified protein that binds Cry1Ab and Cry1Ac. Receptor class-C consists of a group of proteins <100 kDa in size, including HvALP that bind Cry1Ac (Jurat-Fuentes and Adang, 2001; Jurat-Fuentes, 2004a). Expression of HevCaLP in *Drosophila* S2 cells showed that Cry1Ac binding was specific. Cry1Ac binding to cells expressing HevCaLP resulted in toxicity, while Cry1Fa did not, thus indicating that HevCaLP is a functional Cry1A receptor in this insect (Jurat-Fuentes and Adang, 2006b). Recent proteomic analyses of *H. virescens* BBMV proteins led to the identification of V-ATP synthase subunit A, actin and a novel phosphatase as Cry1Ac-binding proteins and demonstrated the value of using a proteomic approach to identify toxin-binding proteins (Jurat-Fuentes and Adang, 2007; Krishnamoorthy et al., 2007).

8.1.Cry1 toxin resistance in H. virescens

Cry toxin resistance in *H. virescens* is extensively studied in three resistant strains that display different resistance mechanisms. The YHD2 strain, which was selected on Cry1Ac crystals, developed a 10,000-fold resistance when compared to the susceptible YDK strain. By 1995, the YHD2 strain, which is highly resistant to Cry1Ac, had lost Cry1Aa binding, and yet retained Cry1Ab- and Cry1Ac-binding (Lee et al, 1995a). A single gene, *BtR-4*, encodes

HevCaLP and is responsible for the majority of Bt resistance in the YHD2 strain. The *BtR-4* gene in YHD2 has a retrotransposon insertion which consequently disrupts the HevCaLP it encodes. HevCaLP functions as a toxin receptor and the predicted HevCaLP encoded by the retrotransposon disrupted *BtR-4* gene, lacks transmembrane and toxin binding domains (Gahan et al., 2001). Continued selection of YHD2 larvae for Cry1Ac resistance resulted in strain YHD2-B with >73,000-fold resistance to Cry1Ac toxin (Jurat-Fuentes et al., 2002), one of the highest reported resistance levels to Bt in insects. Additional mutations or adaptive events in YHD2-B have led to the loss of Cry1Ab- and Cry1Ac-binding (Jurat-Fuentes, 2004b). Reduced toxin binding and increased resistance is correlated with loss of a specific membrane-bound alkaline phosphatase in this strain (Jurat-Fuentes and Adang, 2007; Jurat-Fuentes, 2004a).

The Cry1Ac- and Cry2Aa-resistant KCBhyb and CXC strains were selected initially on diet using inclusion bodies composed of Cry1Ac protoxin and subsequently on diet with Cry2Aa inclusion bodies. Loss of HevCaLP accounts for Cry1Ac resistance in KCBhyb, but not CXC larvae (Jurat-Fuentes, 2004b). KCBhyb and CXC larvae, like YHD2-B, have reduced levels of alkaline phosphatase (Jurat-Fuentes and Adang, 2004a). Although the resistance mechanism for CXC is unknown (Jurat-Fuentes et al., 2003), the parental CP73-3 strain (selected on Cry1Ac and cross-resistant to Cry1Ab and Cry2A) exhibited slower Cry1Ab protoxin processing and faster toxin degradation (Forcada et al., 1996). A mechanism of resistance involving proteinases has been suggested in the Bt-resistant strain, CXC (Jurat-Fuentes et al., 2003).

9. Dissertation rationale

The rationale for my research was based upon previous evidence suggesting a proteinase-related mechanism of resistance in Bt-resistant *H. virescens*. In the lepidopteran midgut, protoxin

is converted to toxin by the action of serine proteinases (Rukmini et al., 2000). Differences in proteolytic activity and qualitative differences in proteinase composition reportedly contribute to Bt resistance in *H. virescens* (Forcada et al., 1999; Forcada et al., 1996).There were two major objectives to my study. The first objective was to examine whether midgut proteinases contribute to Bt resistance in *H. virescens*. To explore this possibility I conducted a comparative biochemical analysis of proteinases in larval gut extracts of three Bt-resistant strains (YHD2-B, CXC and KCBhyb) and one susceptible strain (YDK). The second objective addressed two specific questions:(1) Does toxin feeding induce production of new or altered trypsin and chymotrypsin encoding cDNA?; (2) Does toxin feeding result in the differential expression of trypsin and chymotrypsin encoding cDNA? I undertook the cloning, sequencing and gene expression analyses of midgut trypsin and chymotrypsin cDNA prepared from midguts of control and toxin-fed strains of susceptible YDK and Bt-resistant CXC to address these questions.

The three Bt-resistant strains YHD2-B, CXC and KCBhyb used in this study have different mechanisms of resistance (Jurat-Fuentes et al., 2003). Genetic linkage studies indicate that separate linkage groups are responsible for Cry1Ac and Cry2Aa resistance in the CXC parental strain, CP73 (Gahan et al., 2005). Based on evidence from these studies, we hypothesized that altered proteinases, as well as differences in midgut proteinase composition, could contribute to Bt resistance in *H. virescens*. In order to test this hypothesis we conducted protoxin activation assays using Cry1Ac and Cry2Aa protoxins. These results along with results from casein zymograms, microplate assays, and activity blots using enzyme specific substrates, helped identify qualitative and quantitative differences in proteinases from gut extracts of resistant strains when compared to the susceptible strain. The details of these studies are presented in Chapter Two of this dissertation.

While the loss of HevCaLP has contributed to resistance in the strains YHD2-B and KCBhyb (Gahan et al., 2005), the presence of this protein in midguts of CXC indicates that resistance in this strain could be due to other mechanisms (Jurat-Fuentes, 2004b). Evidence from studies on Bt-resistant strains of *P. interpunctella* and *O. nubilalis* indicate that trypsin-like proteinases are down-regulated in these insects (Li et al., 2004b; Oppert et al., 1996). In *H. armigera*, differential regulation of proteinase genes was observed in midguts of insects fed on diets containing SKTI and SBBI proteinase inhibitors (Bown et al., 1997, Bown et al., 2004). These studies imply that insects produce a diverse array of proteinase genes that are differentially regulated when fed on proteinase inhibitors or Bt toxins.

From results of studies described in Chapter Two, I hypothesized that toxin feeding could induce the production of altered proteinases and differentially express specific proteinases in toxin-fed YDK and CXC larvae. To test this hypothesis I undertook the sequencing and identification of individual trypsin and chymotrypsin cDNAs. Differences in the 3' UTR were used as the basis to identify individual proteinase transcripts while sequence comparisons and SNP analyses were conducted to identify sequence variations. Sequence analysis of trypsin and chymotrypsin-like transcripts from midguts of diet-only control and toxin-fed strains helped identify four trypsin-like and ten chymotrypsin-like cDNAs. Phylogenetic analyses indicated that the trypsin-like sequences are possibly allelic variants of a single gene type, while chymotrypsin-like sequences derive from multiple gene types. Gene expression analyses of individual trypsin and chymotrypsin-like genes were conducted in a quantitative manner using relative quantitative real-time polymerase chain reaction (rqRT-PCR). I discuss the results from these studies in Chapter Three of this dissertation.

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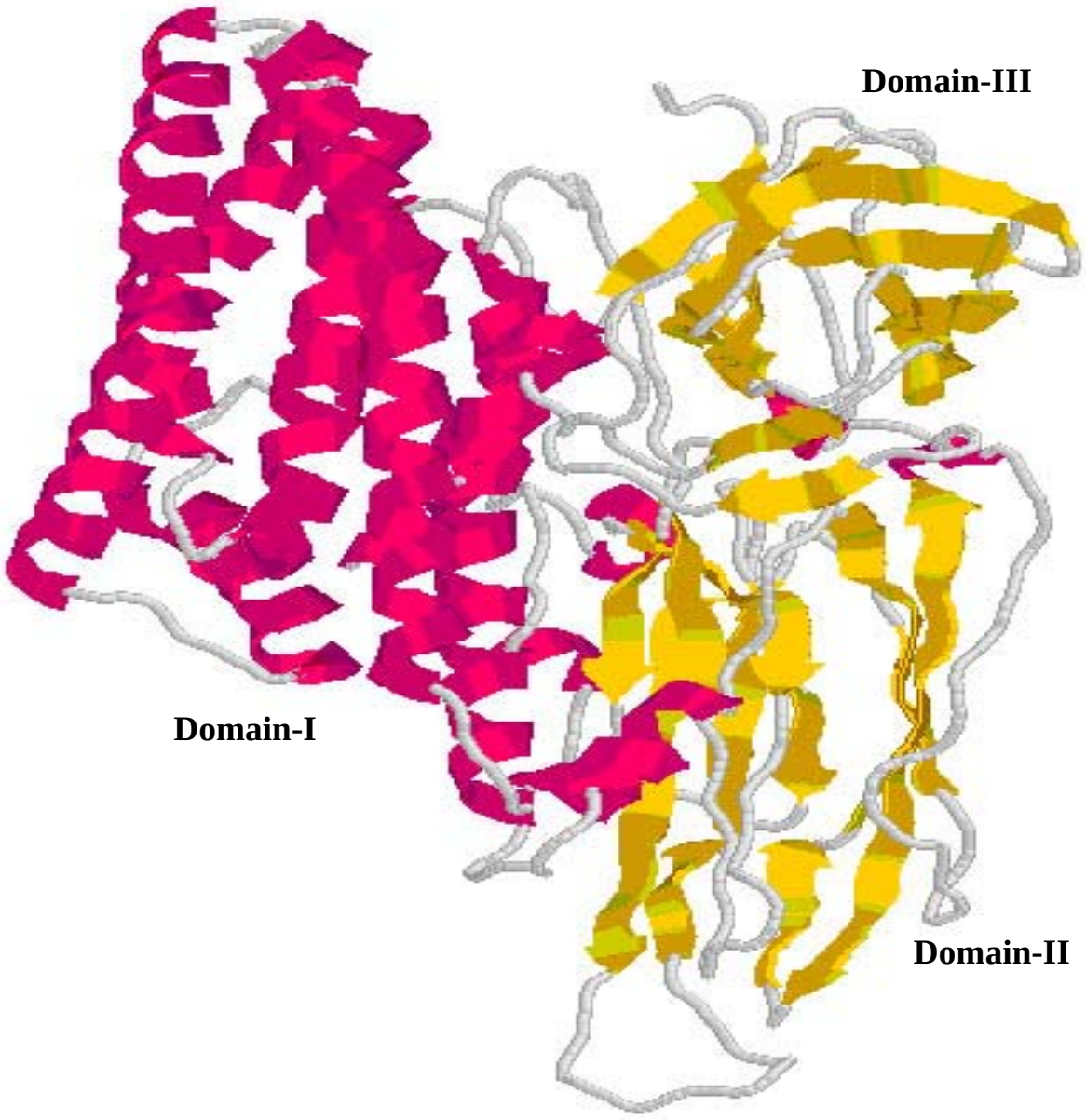

Figure 1.1 – Side view of the three domain structure of Cry1Aa toxin (Grochulski, et al., 1995).

Image created by Protein Explorer V.2.80-http://proteinexplorer.org (Martz, 2002)

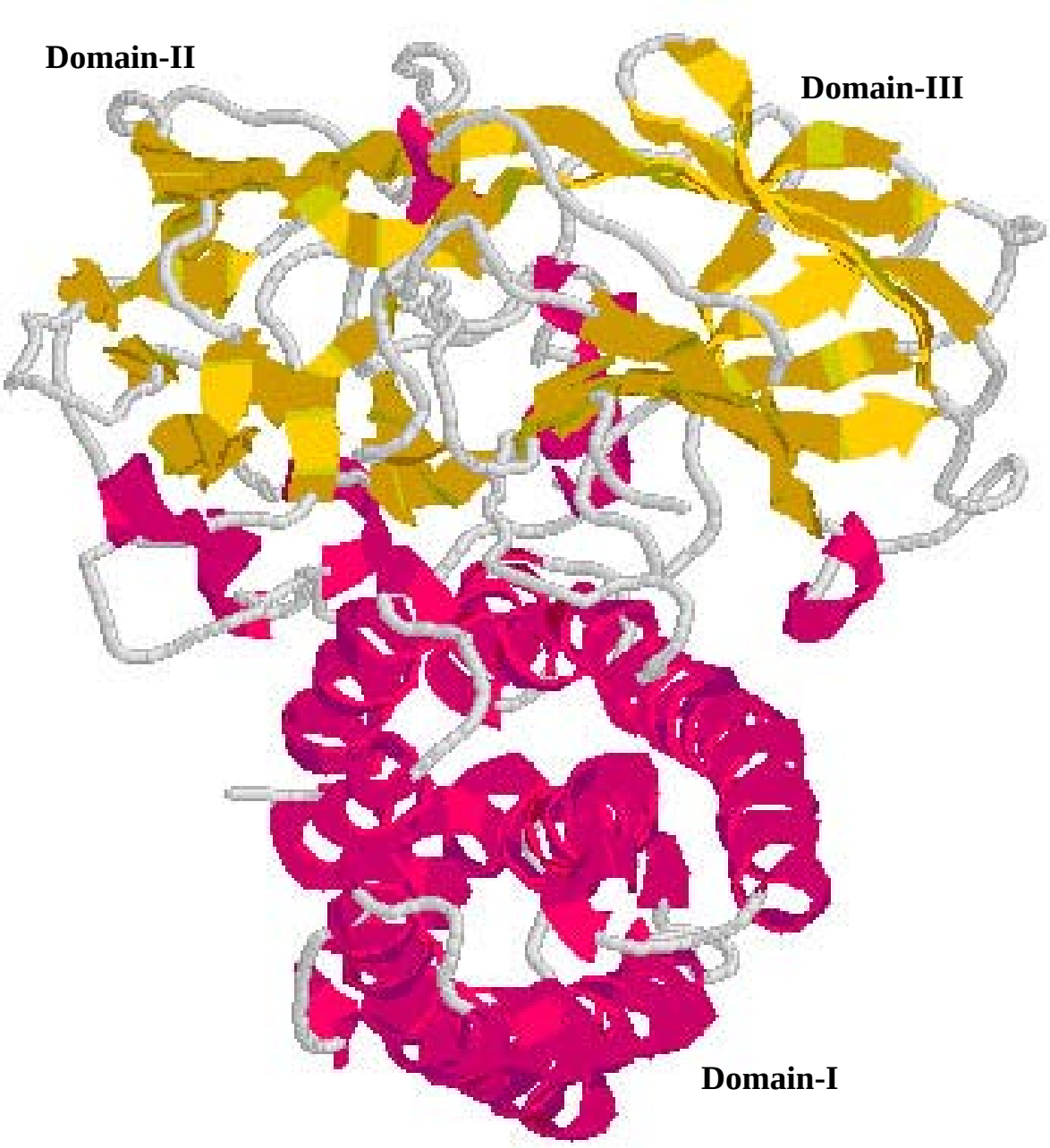

Figure 1.2 – Overhead view of the three domain structure of Cry1Aa toxin (Grochulski, et al., 1995). Image created by Protein Explorer V.2.80-http://proteinexplorer.org (Martz, 2002)

CHAPTER 2

ANALYSIS OF MIDGUT PROTEINASES FROM *BACILLUS THURINGIENSIS*-SUSCEPTIBLE AND -RESISTANT *HELIOTHIS VIRESCENS* (LEPIDOPTERA: NOCTUIDAE) [1]

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ABSTRACT

Insects with altered proteinases can avoid intoxication by *Bacillus thuringiensis* (Bt) toxins. Therefore, proteinase activities from gut extracts of Bt-susceptible (YDK) and -resistant (YHD2-B, CXC and KCBhyb) *H. virescens* strains were compared. The overall pH of gut extracts from YDK and CXC were statistically similar (9.56 and 9.62, respectively), while the pH of extracts from KCBhyb and YHD2-B were significantly more alkaline (9.81 and 10.0, respectively). Gut extracts from YHD2-B and CXC larvae processed Cry1Ac and Cry2Aa protoxin slower than extracts from YDK larvae, suggesting that differences in proteolysis contribute to resistance in these strains. Casein zymogram analysis of gut extracts revealed both qualitative and quantitative differences in caseinolytic activities among all strains, but the overall caseinolytic activity of YHD2-B gut extract was lower. Kinetic microplate assays with a trypsin substrate (L-BApNA) demonstrated that proteinases in YDK gut extract had increased alkaline pH optima compared to resistant strains YHD2-B, CXC and KCBhyb. Gut extracts from YHD2-B had reduced trypsin-like activity, and activity blots indicated that YHD2-B had lost a trypsin-like proteinase activity. In assays with a chymotrypsin substrate (SAAPFpNA), enzymes from all Bt-resistant strains had increased pH optima, especially those from KCBhyb. Activity blots indicated that CXC had lost a chymotrypsin-like proteinase activity. Because serine proteinases are a critical component of Bt toxin mode of action, these differences may contribute to decreased toxicity in the Bt-resistant strains.

1. INTRODUCTION

Heliothis virescens, the tobacco budworm, is a major pest of cotton in the United States, and is currently controlled by cotton plants engineered to express *Bacillus thuringiensis* Cry1Ac toxin. Second generation Bt cotton plants expressing Cry1Ac and Cry2Ab toxins display increased budworm control as well as improved control of alternative lepidopteran pests (Chitkowski et al., 2003). However, the continued success of Bt cotton depends on methods to detect and delay insect resistance to Cry proteins (Ferre and Van Rie, 2002).

When susceptible lepidopteran larvae ingest Bt crystals, the crystals dissolve in the alkaline lumen of the midgut, releasing the Cry protoxin form. Released protoxin is cleaved by midgut proteinases to a stable toxin core (Lightwood et al., 2000) that is then capable of binding to membrane-associated receptor proteins. Toxin receptor binding leads to oligomerization of toxin molecules, which results in binding to secondary receptors, including proteins and glycolipids, and toxin insertion into the epithelial membrane, forming pores that culminate in insect death (Bravo et al., 2004; Griffitts et al., 2005; Schnepf et al., 1998).

The role of proteinases in insect resistance to Bt Cry toxins is complex due to the distinct routes by which proteinases may affect toxin function. Loss of a specific midgut trypsin in *Plodia interpunctella*, necessary for converting protoxin to toxin, accounted for partial resistance to Cry1A toxins (Oppert et al., 1997). A similar proteinase-related Bt resistance mechanism was reported for Bt-resistant *Ostrinia nubilalis* (Li et al., 2004). In both insects, resistance was almost completely reversed when insects were fed activated toxin (Herrero et al., 2001; Li et al., 2005), providing compelling evidence that activation of protoxin is compromised in these insects. Resistance also may be associated with degradation and elimination of toxin (Forcada et al., 1996). Additional examples of proteinase-related Bt resistance have been reported for the

coleopterans *Melolontha melolontha* and *Leptinotarsa decemlineata* (Loseva et al., 2002; Wagner et al., 2002).

The Bt-resistant strains of *H. virescens* analyzed in this study are listed in Table 2.1. After selection with Cry1Ac crystals, the YHD2 strain (the precursor to YHD2-B) had become highly resistant to Cry1Ac, had lost Cry1Aa binding, and yet retained Cry1Ab and Cry1Ac binding (Lee et al., 1995). This resistant phenotype was accounted for by retrotansposon-mediated disruption of a single gene, *BtR-4*, that encodes the HevCaLP cadherin protein. HevCaLP was predicted to function as a toxin receptor, and the genetic alteration in resistant insects resulted in a form of HevCaLP that lacked transmembrane and toxin binding domains (Gahan et al., 2001). Jurat-Fuentes et al. (2004b) confirmed that "knock-out" of *BtR-4* resulted in loss of Cry1Aa, but not Cry1Ab or Cry1Ac binding. Continued selection of YHD2 larvae with Cry1Ac resulted in strain YHD2-B, which is >73,000-fold resistant to Cry1Ac toxin (Jurat-Fuentes et al., 2002), one of the largest reported resistance levels to Bt in insects. Additional mutations or adaptive events in YHD2-B have led to the loss of Cry1Ab and Cry1Ac binding (Jurat-Fuentes et al., 2004b). Reduced toxin binding and increased resistance in YHD2-B correlated with reduced levels of a specific membrane-bound alkaline phosphatase in larvae from this strain (Jurat-Fuentes and Adang, 2004a).

The Cry1Ac and Cry2Aa resistant KCBhyb and CXC strains were generated from the *H. virescens* Bt-resistant strains KCB and CP73, respectively, by selection with diet containing Cry2Aa inclusion bodies. Loss of HevCaLP accounted for Cry1Ac resistance in KCBhyb, but not in CXC larvae (Jurat-Fuentes et al., 2004b). Although the resistance mechanism for CXC is unknown (Jurat-Fuentes et al., 2003),the parental CP73 strain, which was resistant to Cry1Ac and Cry2Aa, exhibited slower protoxin processing and faster toxin degradation (Forcada et al.,

1996).Unfortunately, the CP73 strain no longer exists, and comparisons with this strain are not possible.

The goal of this study was to investigate the potential role of proteinase alteration in resistance to Cry toxins in these strains. We analyzed the profiles of digestive proteinases in Bt-resistant strains of *H. virescens*, including YHD2-B, KCBhyb, and CXC, relative to Bt-susceptible YDK larvae. Analyses included a time-course study of Bt protoxin activation, casein zymograms, kinetic microplate assays, and proteinase activity blots using trypsin and chymotrypsin specific substrates. Specific differences in serine proteinases, both quantitative and qualitative, were detected for the resistant strains compared to the susceptible strain. The relevance of these results to Bt-resistance management is discussed.

2. MATERIALS AND METHODS

2.1 Insect strains.

H. virescens larvae were kindly supplied by Dr. Fred Gould (North Carolina State University, Table 2.1). The Bt-susceptible strain, YDK, was the parental strain for selection of the YHD2 strain on artificial diet containing Cry1Ac protoxin (MVP, Dow AgroSciences, Indianapolis, IN), that resulted in 10,000-fold resistance to Cry1Ac (Gould et al., 1995). Continuous selection of this strain with Cry1Ac has led to strain YHD2-B, with increased levels of Cry1Ac resistance (>73,000 fold) (Jurat-Fuentes et al., 2002). The CXC strain, previously referred to as CPN X CP73-3 (Forcada et al., 1999), is about 289-fold resistant to Cry1Ac (Jurat-Fuentes et al., 2003) and resulted from the mating of Cry1Ac-resistant CP73-3 adults with susceptible insects and further selection on Cry2Aa protoxin produced in *E. coli* (Kota et al., 1999). Strain KCBhyb resulted from mating Cry1Ac resistant-KCB adults (Forcada et al. 1999) with susceptible moths and selection on Cry2Aa (Fred Gould, unpublished data), and is 187-fold

resistant to Cry1Ac (Jurat-Fuentes et al., 2003). Both CXC and KCBhyb have high levels of resistance to Cry2Aa, and no mortality was observed with doses up to 1000 μg/ml of toxin (Jurat-Fuentes et al., 2003). All insect strains were reared on artificial diet, under laboratory conditions as described previously (Gould et al., 1995).

2.2 Determination of midgut lumen pH.

Fourth instar larvae were chilled on ice and dissected between the 3rd and 4th prolegs. Larvae were held upright so as to prevent loss of midgut fluids. A pH microelectrode (Model M41410, Microelectrodes Inc. Bedford, NH) was inserted into the gut lumen and the pH value recorded. Measurements were taken from the midguts of five larvae from each strain. A one-way analysis of variance was conducted using the Fisher LSD (Systat Software, Inc., Point Richmond, CA).

2.3 Preparation of gut extracts.

Midguts from five late 4th instar larvae of each strain were dissected, five dissected guts pooled in 25 μl of sterile H_2O, and briefly vortexed. One gut equivalent (GE) was equal to 5 microliters of sample. The samples were centrifuged at 13,000 x *g* for 15 minutes at 4ºC, and the supernatant was carefully separated from the pellet. Protein in the supernatant, referred to subsequently as gut extract, was quantified by a dye binding assay (Bradford, 1976) and aliquots were stored at -80ºC. Each GE contained approximately 100 μg of protein.

2.4 Protoxin and toxin purification.

Bt strain HD-73 producing Cry1Ac toxin was obtained from the *Bacillus* Genetic Stock Collection (Columbus, OH). Cry1Ac toxin was purified as described elsewhere (Luo et al., 1999).

An *Escherichia coli* strain harboring the *cry2Aa* gene was obtained from Dr. Donald H. Dean (Ohio State University, Columbus, OH). Bacteria were cultured in LB medium containing 100 μg/ml ampicillin for three days at 37ºC. Bacterial cells were pelleted by centrifugation, the pellet was resuspended in lysis buffer (15% sucrose, 50mM EDTA, 50mM Tris pH 8.0, 10μg/ml of lysozyme) and Cry2Aa protoxin was purified according to methods previously described (Audtho et al., 1999). Purified toxin was quantified using a dye binding assay (Bradford, 1976) and aliquots were stored at -80ºC.

2.5 Temporal processing of Cry1Ac and Cry2Aa protoxin.

Proteolytic processing of Cry1Ac and Cry2Aa protoxin was evaluated by incubating 10 μg of purified protoxin with gut extract (total protein was 40 μg) in a final volume of 30 μl of pH 9.75 universal buffer (Frugoni, 1957) at room temperature for increasing periods of time. Proteolysis was terminated by heating samples at 100^{o}C for 5 min. Samples were cooled, loading buffer was added, and the samples were again heated at 100^{o}C for 5 min (Oppert et al., 1994) Samples were subjected to electrophoresis in 10% Tris-Glycine gels (Bio-Rad) and stained with Coomassie blue. Gels shown are representative of five independent assays.

To test the significance of the protoxin activation differences between strains, we quantified the intensity of the resultant toxin band in the gels shown in Figure 2.1. Band intensities corresponding to that of processed toxin were measured using the Alpha Ease FC

Densitometry software on the FluorChem ^{TM} IS-8900 imaging system (Alpha Innotech Inc.). Band intensity, which is represented by the area under the associated peak, was measured for each of the five replicates and the corresponding values fed into Sigma Stat 3.1 statistical software. One-way analysis of variance was conducted using the Fischer LSD and Tukey tests. When band alterations when compared to patterns of extracts from susceptible larvae were detected, the differences were statistically significant at the $P<0.001$ level.

Toxin band intensities in the gels as measured by densitometry were used to calculate the rate of toxin activation by gut extracts of the susceptible and resistant strains. Protoxin input (10 μg) was used to calculate the amount of toxin activated per minute for each sample. Because the majority of Cry1Ac toxin is activated within the first minute, and Cry2Aa is processed within 10 minutes of incubation, we used these time frames to calculate the corresponding toxin activation rates for each strain.

2.6 Casein zymogram analysis.

Gut extracts (total protein was 85 μg) were separated by electrophoresis in 10% Tris-Tricine gels at 4ºC (Schagger, 1987). After electrophoresis, the gel was incubated overnight at 4ºC in 50 mM Tris-HCl buffer (pH 7.5) containing 2% casein, followed by incubation at room temperature for 20 minutes (Garcia-Carreno et al., 1993). After Coomassie staining, clear bands of proteinase activity were visible against a dark blue background.

2.7 Microplate assays.

The enzymatic activity in gut extracts (40 μg total protein) from each strain was analyzed in a 96-well microplate assay (Oppert et al., 1997), in triplicate, using in buffers of increasing pH

(Frugoni, 1957). The microplate was preincubated in a microplate reader for 2 minutes at 37°C for temperature equilibration. To initiate the reaction, 50 µl of N_{α}-Benzoyl-DL-arginine ρ-nitroanilide (L-BApNA) or N-succinyl-ala-ala-pro-phenylalanine ρ-nitroanilide (SAAPFpNA) (2.2 mM final concentration) substrate solution was added, and absorbance was measured at 405 nm. Readings were taken at 11 sec intervals for 5 min at 37°C, during which time all increases in absorbance were linear. SigmaPlot software was used to calculate standard errors and plot graphs.

2.8 Activity blots.

To compare the number and relative activities of gut proteinases, gut extracts (85 µg total protein) from each strain were resolved in a 10% Tris-Tricine gel. Electrophoresis conditions were as for casein zymograms. Proteins were transferred to nitrocellulose membranes by electroblotting and then membranes were incubated in 200 mM Tris pH 9.75, 20 mM $CaCl_2$ containing 2.2 mM L-BApNA or SAAPFpNA at 37°C for 20 min (Oppert et al., 1996). The incubation was at pH 8.0 rather than the optimal pH for gut enzyme activity because of autohydrolysis of the substrate in higher pH buffers, resulting in indistinguishable bands of activity due to the higher background. Following the appearance of yellow bands of released nitroaniline, the product was diazotized to visualize activity bands, and membranes were placed in sealed plastic bags and stored at –20^{o}C until archived by photodocumentation.

3. RESULTS

3.1 pH of H. virescens larval midgut fluid.

Because Bt crystals are dissolved in the midgut lumen and activated by proteinases, factors in the midgut lumen are critical to toxin action. Therefore, the pH of midgut contents was measured for larvae from Bt-susceptible and resistant strains. The lumen pH was similar for YDK and CXC, 9.56 and 9.62, respectively. However, the gut pH was significantly more alkaline for KCBhyb and YHD2-B, measuring 9.81 and 10.0, respectively (Table 2.2). There was no significant difference in the midgut pH values of YHD2-B and KCBhyb.

3.2 Protoxin activation by H. virescens gut extracts.

A study of protoxin hydrolysis was conducted to determine if there were differences in protoxin activation by gut extracts from the different *H. virescens* strains. Cry1Ac and Cry2Aa protoxins were incubated with gut extracts for increasing periods of time, and products were analyzed by SDS-PAGE.

Enzymes in all gut extracts digested Cry1Ac protoxin through 74-, 72-, and 64-kDa intermediates, to a presumably active ~58-kDa toxin (Fig.2.1 A). Most of the activation process was observed to occur within one minute of incubation. In this time frame, gut extract from CXC larvae activated toxin more slowly (0.90 μg/min) than extracts from YDK larvae (1.23 μg/min). Gut extracts from YHD2-B (1.26 μg/min) had a rate of toxin activation similar to YDK extracts, while KCBhyb extracts displayed faster activation rates (2.01 μg/min). However, enzymes in extracts from resistant strains YHD2-B and CXC had less effective toxin activation than extracts from YDK larvae as evidenced by the presence of increased amounts of intermediates after 120 min of incubation when compared to YDK or KCBhyb extracts. A ~64

kDa intermediate remained in incubations with YHD2-B extracts after 15 h. Toxin degradation products of ~40 and 25 kDa were found in incubations with YHD2-B and CXC enzymes, and only the 25 kDa product was apparent in incubations with YDK and KCBhyb after 15 h. There was no apparent further proteolysis of the ~58 kDa form in extracts from any of the strains after longer incubation times (data not shown). Similar results were observed when Cry2Aa protoxin was incubated with *H. virescens* gut extracts (Fig.2 1 B). Enzymes from the susceptible YDK and the resistant KCBhyb larvae hydrolyzed the ~68 kDa protoxin to a 60 kDa intermediate form at the same rate (0.34 μg/min). Further proteolysis to a 55 kDa apparent toxin peptide, and a 48 kDa presumably non-toxic Cry2Aa peptide (Audtho et al., 1999), also was detected in the extracts. Gut extracts from resistant strains YHD2-B and CXC processed Cry2Aa protoxin at slower rates (0.30 mg/min and 0.28 mg/min respectively), than YDK or KCBhyb extracts, as an intermediate was observed only in these reactions after 10 min. Traces of protoxin were detected even after a 30 min incubation of Cry2Aa with YHD2-B gut extract (data not shown). All extracts produced detectable amounts of the presumably inactive 48 kDa Cry2Aa peptide. After 15 h, toxin degradation products of ~25 and 20 kDa were found in all samples, with an additional ~40 kDa product observed only in incubations with enzymes from KCBhyb larvae.

3.3 Zymogram analysis of gut extracts.

Because protoxin hydrolysis experiments suggested differences between strains in protoxin processing, proteinases in each strain were compared. A zymogram analysis was used to assess the relative number of soluble gut proteinases in Bt-susceptible and -resistant larvae. There were at least 11 caseinolytic activities discernable in extracts from the four strains (Fig.2.2). However, there were subtle differences in both quantitative and qualitative proteinase

activities in gut extracts from the different strains. Although the overall activity patterns were similar, enzymes in YDK, CXC, and KCBhyb gut extracts demonstrated an increased caseinolytic activity relative to that of YHD2-B. Certain activities (bands 3, 6, 7, 8, and 11) were found in all extracts. KCYhyb had the most distinct caseinolytic activities, with unique activities (bands 1, 2, and 5) that were not found in the other extracts. Other unique activities included band 9, only found in YDK, band 4, only found in CXC, and band 10, only found in YHD2-B and KCBhyb.

3.4 Determination of trypsin- and chymotrypsin-like proteinase activities at various pH.

Serine proteinases are prevalent in lepidopteran larvae and critical enzymes in the conversion of Bt protoxin to toxin. Therefore, substrates were used to evaluate the activities of typical serine proteinases in extracts from the four *H. virescens* strains. The pH optima for trypsin- and chymotrypsin-like enzymes in gut extracts were determined by the hydrolysis of L-BApNA and SAAPFpNA, respectively, using a kinetic microplate assay. These substrates were not hydrolyzed in acidic buffers by enzymes in gut extracts from either susceptible or resistant strains (Fig.2.3). Rates of hydrolysis of both substrates increased in buffers of pH greater than 6, with maximum hydrolysis detected between pH 8–11.2. At pH 9.6, the gut pH of larvae from strains YDK and CXC, the rate of BApNA hydrolysis by gut enzymes was greater than for KCBhyb and YHD2-B gut enzymes at their physiological pH, 9.9 (Fig.2.3 A). In assays with a chymotrypsin substrate (SAAPFpNA), enzymes in KCBhyb extracts at their physiological pHwere approximately 2-4 times more active than enzymes from the other gut extracts, with optimal activity at pH 10.6, but sharply reduced at pH 11.2 (Fig.2.3 B). In comparison, the

chymotrypsin-like activity of CXC enzymes at pH 9.6 was intermediate, whereas the activity of enzymes from YHD2-B at pH 9.9 and YDK at pH 9.6 was much lower.

3.5 Activity blot assays for trypsin- and chymotrypsin-like proteinases.

To test whether the quantitative differences in trypsin and chymotrypsin activities detected in the microplate assays were due to the gain or loss of specific proteinases, we performed activity blot analyses to study the molecular masses of trypsin- and chymotrypsin-like proteinases in gut extracts from all strains. Blots incubated in pH 8.0 buffer containing L-BApNA revealed similar patterns of trypsin-like enzymes in gut extracts from all four strains, with proteinases of ~48 and ~34 kDa common to all extracts (Fig.2.4 A). Weaker intensity bands were detected at ~100, ~98, ~97, ~77, and ~62 kDa, and may have resulted from protein aggregation in the extracts under the pH conditions used for electrophoresis. However, a trypsin-like proteinase of ~32 kDa was observed in YDK, CXC and KCBhyb extracts but was not detected in gut extracts from YHD2-B larvae. Activity blots with SAAPFpNA revealed that the most active chymotrypsin-like proteinases common to all strains were ~35, 37, and 47 kDa in mass (Fig 2.4 B). All strains had weak chymotrypsin-like activity at ~33 kDa. The activity of the 35 kDa band was barely detectable in CXC extracts, relative to extracts from the other strains. Additionally, faint bands of chymotrypsin-like activity were detected at ~66 and 73 kDa only in extracts from YDK and KCBhyb larvae.

4. DISCUSSION

In this study, gut proteinases from larvae of Bt-suseceptible and resistant *H. virescens* strains were examined for the relative hydrolysis of Cry1Ac protoxin, Cry2Aa protoxin, and casein.

Since trypsins and chymotrypsins are the two major classes of proteinases in this insect species (Johnston et al., 1995), and because these enzymes are involved in the activation of Cry protoxins (Rukmini et al., 2000), we examined gut extracts from larvae of the susceptible and resistant strains for differences in activities of these enzymes. Additionally, we compared the gut pH in larvae from all strains, because gut pH determines protein solubility, enzymatic activity and is a factor in toxin susceptibility. All of these studies identified differences among the strains that may contribute to reduced susceptibility to Bt toxins.

Larvae from the YHD2-B strain do not express the full length HevCaLP cadherin, a key Cry1A toxin receptor in *H. virescens* (Gahan et al., 2001; Jurat-Fuentes et al., 2004b). While reduced amounts of a Cry1Ac-binding alkaline phosphatase probably contribute to resistance (Jurat-Fuentes and Adang, 2004a), our results suggest that altered proteinase levels also should be considered as a potential resistance factor in this strain. The reduced rate of protoxin processing by YHD2-B extracts is consistent with the reduced activity of trypsin-like proteinases and the absence of a ~32 kDa trypsin-like protein. A similar reduction of specific trypsin-like proteinases was reported for Bt-resistant strains of *P. interpunctella* and *O. nubilalis* (Li et al., 2004; Oppert et al., 1996). Our results with YHD2-B larvae and published studies with other insect species support the contention that reduction of trypsin-like proteinases is an adaptive mechanism in lepidopteran larvae that delays processing of protoxin to toxin and, in this way, confers some degree of resistance to Bt crystals and protoxin. Certainly, insects have adaptive capacities to plant proteinase inhibitors and may incorporate this strategy for survival of other toxins. However, the relative contribution of proteinase alterations to resistance in this strain remains to be determined.

Mechanisms of altered toxin activation are generally associated with low levels of resistance (Ferre and Van Rie, 2002). In a Bt-resistant strain of *P. interpunctella*, the alteration of gut proteinases accounted for ~90% of the resistance (Herrero et al., 2001). The CXC strain of *H. virescens* is resistant to Cry1Ac and Cry2Aa toxins, yet the resistance has not been explained by reduced Cry1A toxin binding (Jurat-Fuentes et al., 2003). Therefore, the relative contribution of proteinase-mediated resistance in this strain may be more important than in YHD2-B. Previously, (Forcada et al., 1999) reported altered proteinase patterns in gut extracts from CXC, as well as enhanced midgut epithelium recovery after toxin challenge, compared to susceptible controls. Continuous selection with diet containing Cry2Aa toxin resulted in higher levels of resistance to this toxin in the CXC strain (Jurat-Fuentes et al., 2003). In our assays, we detected reduced levels of a 35 kDa chymotrypsin-like proteinase activity in gut extracts from CXC larvae when compared to extracts from larvae in other strains. We believe this protein is the same as the 36 kDa proteinase absent in gut extracts from CXC larvae in the previous study (Forcada et al., 1999). The elevated chymotrypsin activity levels detected in enzymes from gut extracts from this strain may represent compensatory alterations in the gut to counterbalance the reduced levels of the 35 kDa proteinase. In our study, extracts from CXC larvae processed Cry1Ac protoxin at a slower rate than YDK extracts, with protoxin intermediates remaining after the longest digestion time. A similar phenomenon was reported in the parental CP73-3 strain, which processed protoxin more slowly (Forcada et al., 1996). Those authors also reported that CP73-3 extracts degraded toxin more rapidly than a control susceptible strain. We were unable to confirm this observation in our experiments.

With respect to trypsin-like activity, CXC gut extracts had activities similar to YDK extracts in both kinetic and activity blot assays. While these results seem internally contradictory, it is important to consider that the activity blots provide qualitative estimations, while the kinetic assays present quantitative data. The CXC extracts also contained a 66 kDa protein with caseinolytic activity (band 4, Fig.2.2) that was not detected in the trypsin or chymotrypsin activity blots. Possibly, this 66 kDa protein represented a different type of proteinase that was not detected by the *p*-nitroanilide substrates. Overall, the CXC larvae had reduced amounts of a 35 kDa chymotrypsin-like enzyme, a reduced capacity to process both Cry1Ac and Cry2Aa protoxin, and an increased capacity to degrade Cry2Aa toxin. These results are consistent with previous reports on the proteinases of CXC and one of its parental strains (Forcada et al., 1996; Forcada et al., 1999). These data suggest that after continuous selection with Cry2Aa, proteinase alterations were conserved, indirect evidence that these alterations may correlate with resistance against Cry1Ac and Cry2Aa.

The KCBhyb larvae are characterized by Cry1Ac and Cry2Aa resistance and loss of Cry1Aa binding (Jurat-Fuentes et al., 2003). Even though the retrotransposon-mediated knockout was detected in this strain, this allele is not fixed in KCBhyb (Jurat-Fuentes et al., 2004b). Forcada et al. (1999) reported reduced levels of 34 and 44 kDa proteinases in gut extracts from larvae of the KCB strain, one of the parental strains for the KCBhyb strain. Chymotrypsin-like activity was much greater in KCBhyb gut extracts compared to extracts from susceptible larvae, and zymogram analysis detected several additional caseinolytic proteinases unique for KCBhyb extracts. Whether these proteinases have a role in Cry1Ac or Cry2Aa resistance is yet to be determined. However, no differences in protoxin activation or toxin degradation were observed in this strain when compared to YDK protoxin activation patterns.

Under natural conditions, the diversity of trypsin and chymotrypsin isoforms in gut extracts of lepidopteran larvae contribute to digestion of a complex plant diet and protect insects from harmful effects of plant resistance proteins. In *H. virescens*, selection for resistance to Bt Cry1Ac and Cry2Aa protoxins (for CXC and KCBhyb larvae) has resulted in resistant larvae with different qualitative and quantitative proteinase patterns. Additional research is needed to characterize the specific altered proteinases in Bt-resistant insects and investigate their potential role in resistance to Bt toxins.

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APPENDIX

L-BApNA - Nα-Benzoyl-$_{L}$-Arginine-$_{P}$-Nitroanilide; SAAPFpNA - N-Succinyl-Ala-Ala-Pro-Phe-ρ-Nitroanilide; Cry – Crystal; Bt – *Bacillus thuringiensis*; HevCaLP – *Heliothis virescens* cadherin like protein; EDTA - ethylenediaminetetraacetic acid; LB – Luria Bertani.

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FIGURES AND TABLES

Table 2.1- Toxicity, resistance ratios and resistance factors in Bt-susceptible and -resistant strains of *H. virescens.*

Strain	**Selection Toxin**	**LC_{50}[a]**		**Resistance Ratio[b]**		**Resistance Factors**		**Slower processing of Protoxin [c]**	
		Cry1Ac	**Cry2Aa**	**Cry1Ac**	**Cry2Aa**	**HevCaLP[d]**	**^{125}I-Cry1Ac Binding[e]**	**Cry1Ac**	**Cry2Aa**
YDK	None	0.73	4.30	NA	NA	+	+	-	-
YHD2-B	Cry1Ac	>2,000	138	73,700	9.5	-	-	+	+
CXC	Cry2Aa	211	>1,000	290	>250	+	+	+	+
KCBhyb	Cry2Aa	137	>1,000	188	>250	-	+	-	-

[a]LC_{50} values are expressed as µg of toxin/ml of diet (Jurat-Fuentes et al., 2002; Jurat-Fuentes et al., 2003; Kota et al., 1999).

[b]Resistance Ratio = LC_{50} of the resistant strain/ LC_{50} of YDK (Jurat-Fuentes et al., 2002; Jurat-Fuentes et al., 2003; Kota et al., 1999).

NA = Not Applicable.

[c] Slower rate of protoxin processing as observed in this study.

[d]The expression or absence of HevCaLP is indicated by + or – (Jurat-Fuentes et al., 2004b).

[e]BBMV binding assays (Jurat-Fuentes et al. 2002; 2003)

Figure 2.1- Proteolytic digestion of Cry1Ac (Panel A.) and Cry2Aa (Panel B) protoxins by gut extracts from larvae of Bt-susceptible YDK (lane 1) and -resistant YHD2-B (lane 2), CXC (lane 3) and KCBhyb (lane 4) *H. virescens* larvae. PT = Purified protoxin; T = purified trypsin activated toxin.

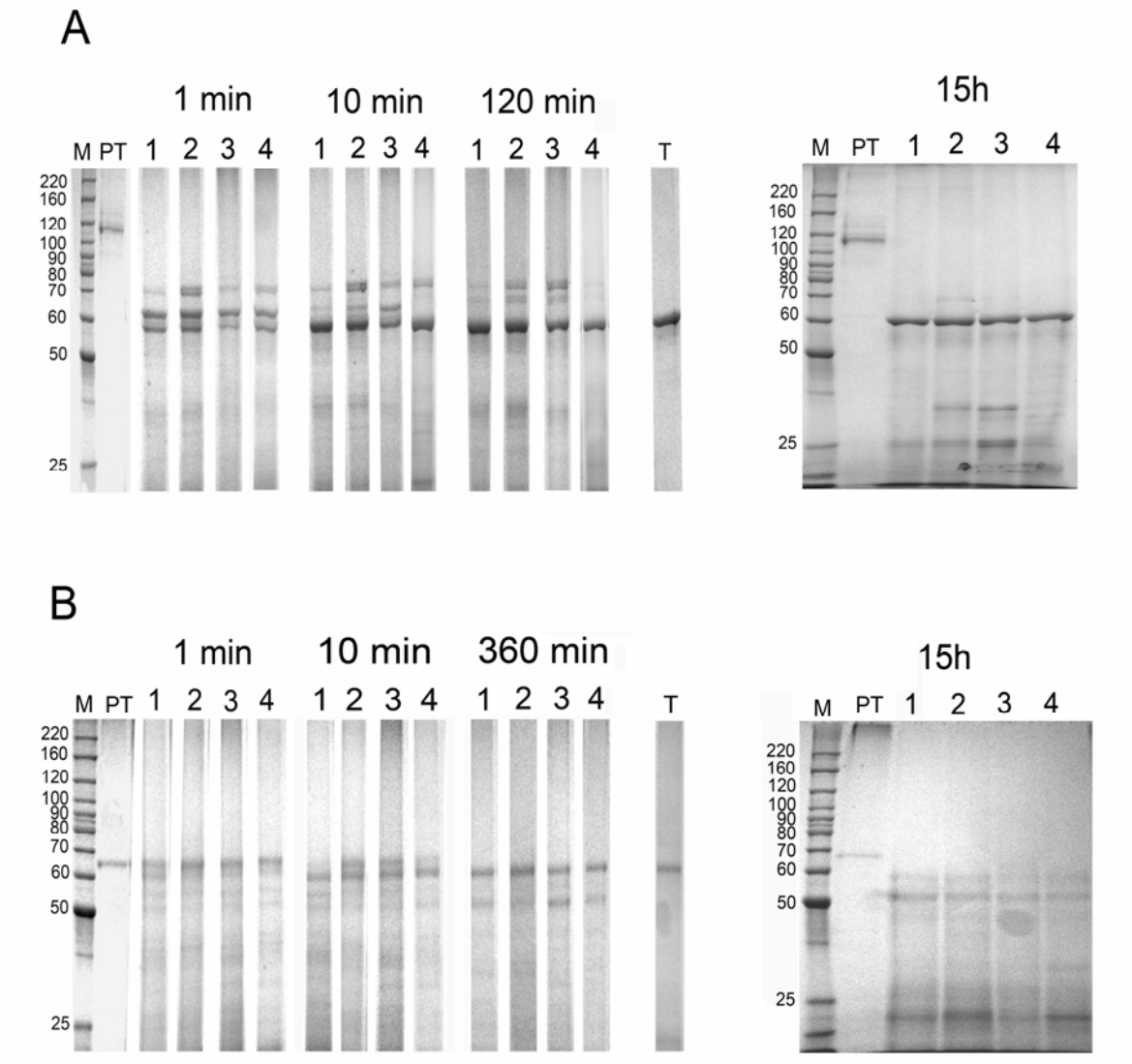

A
1 min
10 min
120 min
15h
M PT 1 2 3 4 1 2 3 4 1 2 3 4 T
M PT 1 2 3 4
220
160
120
100
90
80
70
60
50
25
B
1 min
10 min
360 min
15h
M PT 1 2 3 4 1 2 3 4 1 2 3 4 T
M PT 1 2 3 4
220
160
120
100
90
80
70
60
50
25

Table 2.2- Comparison of midgut lumen pH values in Bt-susceptible (YDK) and Bt-resistant (YHD2-B, CXC, and KCBhyb) *H.virescens* strains.

Strain	**Mean pH ± S.D.** [a]
YDK	9.62 ± 0.15 a
YHD2-B	10.0 ± 0.17 b
CXC	9.56 ± 0.06 a
KCBhyb	9.81 ± 0.16 b

[a]Comparisons based on triplicate measurements from five individual larvae from each strain (mean ± standard deviation). Means with different letters are statistically different as determined by Fisher LSD ($p < 0.05$).

Figure 2.2- Casein zymogram of gut extracts from YDK, YHD2-B, CXC and KCBhyb. Relative caseinolytic activities are numbered from slower to faster migrating on the left side of each sample, and unique activities are marked on the right with an asterisk. M: molecular mass markers.

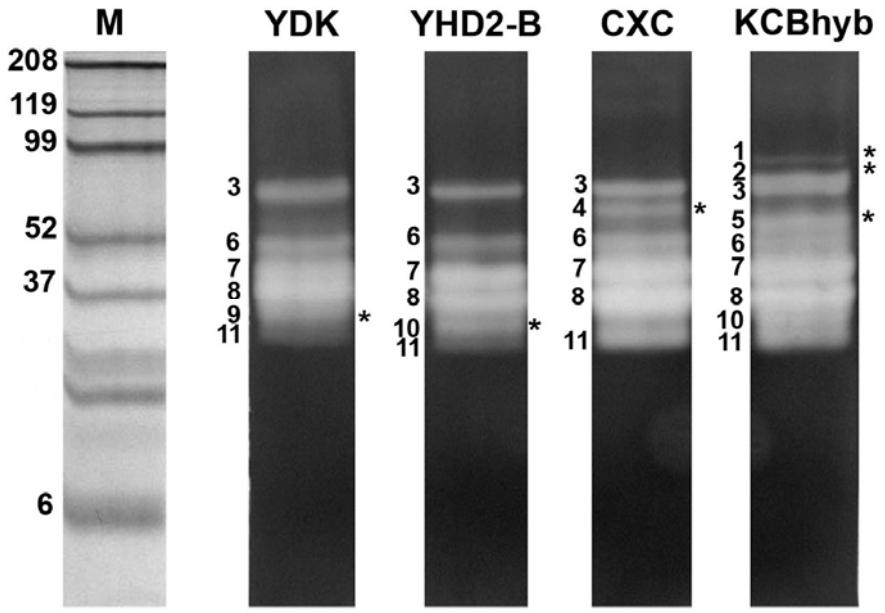

M
208
119
99
52
37
6
YDK
3
6
7
8
9
11
*
YHD2-B
3
6
7
8
10
11
*
CXC
3
4
6
7
8
11
*
KCBhyb
1
2
3
5
6
7
8
10
11
*
*
*

Figure 2.3- Activities of trypsin- and chymotrypsin-like gut proteinases from YDK, YHD2-B, CXC and KCBhyb larvae with L-BApNA (A) and SAAPFpNA (B), respectively, in buffers of increasing pH. A bar denoting the average physiological pH of the gut of YDK and CXC (9.6) and YHD2-B and KCBhyb (9.9) is noted on each graph.

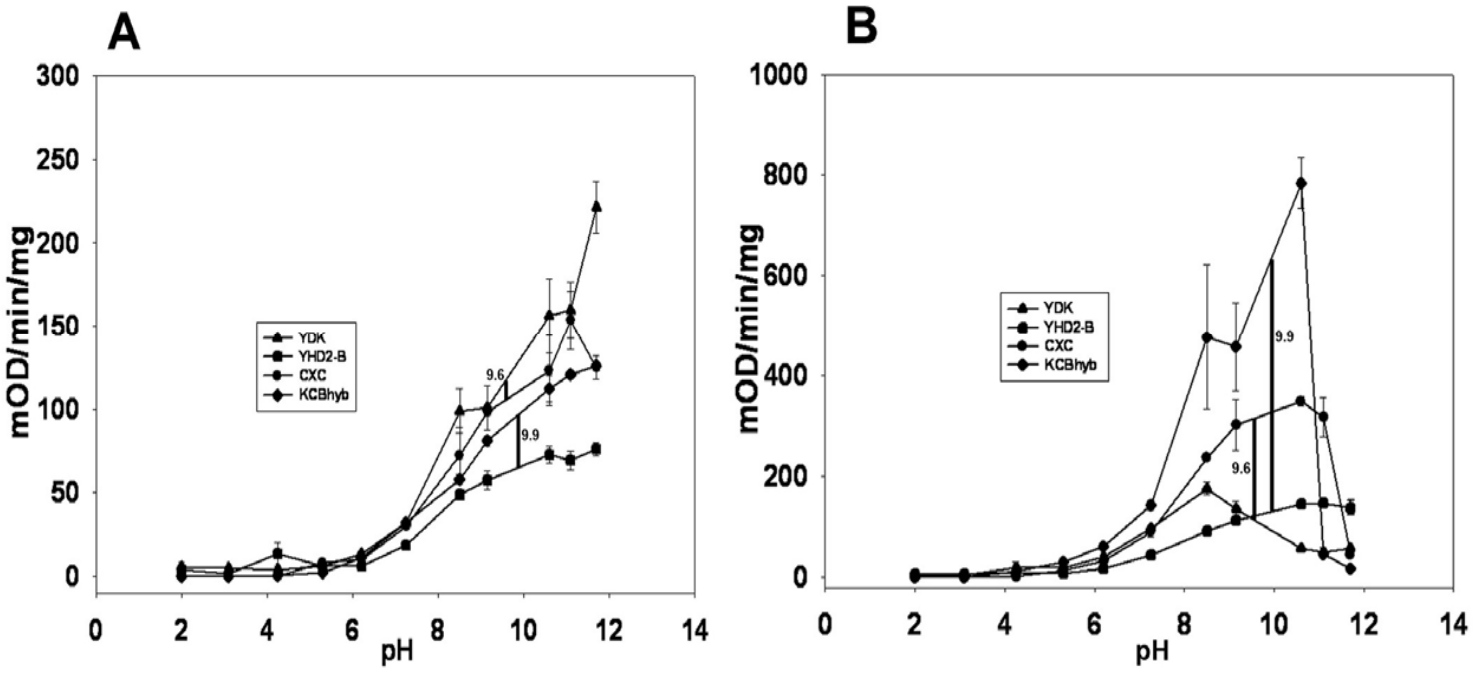
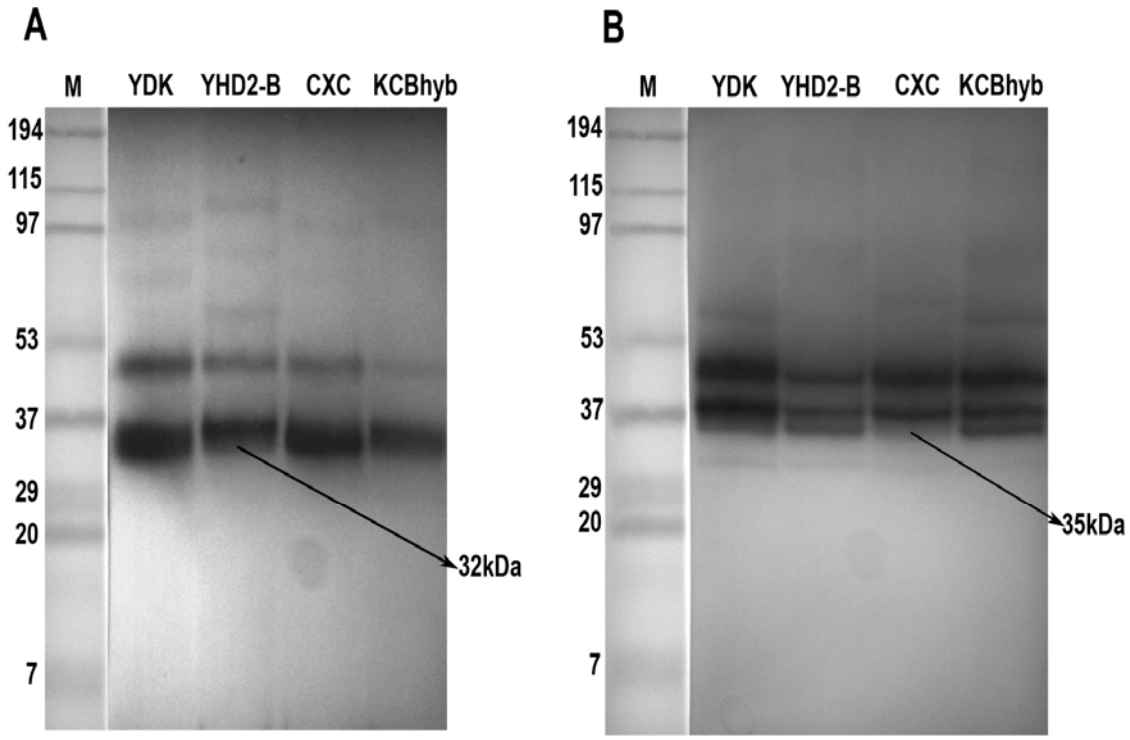

A
B
mOD/min/mg
pH
YDK
YHD2-B
CXC
KCBhyb
9.6
9.9

Figure 2.4- Activity blot analysis of trypsin- and chymotrypsin-like proteinases from YDK, YHD2-B, CXC and KCBhyb larvae using L-BApNA (A) and SAAPFpNA) (B), respectively. Faint or missing activities are denoted on the right.


A
M
YDK
YHD2-B
CXC
KCBhyb
194
115
97
53
37
29
20
7
32kDa
B
M
YDK
YHD2-B
CXC
KCBhyb
194
115
97
53
37
29
20
7
35kDa

CHAPTER 3

TOXIN FEEDING-INDUCED SEQUENCE POLYMORPHISMS AND ALTERED EXPRESSION OF TRYPSIN- AND CHYMOTRYPSIN-LIKE mRNAs IN MIDGUTS OF *HELIOTHIS VIRESCENS* (LEPIDOPTERA: NOCTUIDAE) [1]

[1]Karumbaiah L, Oppert B, Jurat-Fuentes JL, Adang MJ.
To be submitted to *Insect Mol Biol*.

ABSTRACT

Insect trypsin- and chymotrypsin-like proteinases play a major role in the solubilization and activation of Cry toxins from *Bacillus thuringiensis*. To characterize changes in proteinase expression in insects fed Cry toxins, we identified and compared cDNAs encoding trypsin- and chymotrypsin-like enzymes in *Heliothis virescens* larvae fed diet with or without Cry toxins. Four trypsin-like (HvT) and ten chymotrypsin-like (HvC) cDNAs were amplified by 3' RACE. The four HvT cDNAs were highly similar, while HvC cDNAs showed considerable sequence diversity. Phylogenetic analyses suggested that the HvT sequences are variant members of a single gene, while the separation of HvC sequences into three groups indicates the presence of multiple genes. Even though SNP analyses found mostly synonymous polymorphisms, one non-synonymous change was observed in the loop-1 region of HvT1, resulting in an amino acid change in trypsin from the toxin-fed larvae that may result in altered substrate specificity. To test the potential role of proteinase alterations in resistance to Cry toxins, we compared their gene expression profiles in larvae from susceptible (YDK) and resistant (CXC) strains of *H. virescens*. Only one trypsin-like cDNA (HvT3) had significantly reduced expression after toxin feeding in both YDK and CXC larvae. Chymotrypsins were generally upregulated in CXC when compared to YDK larvae, independent of the diet treatment. HvC6 was not expressed in YDK and in Cry2Aa-fed CXC larvae, suggesting a toxin-specific proteinase down-regulation. Because alterations in proteinase gene expression and sequence could lead to altered substrate specificities and hydrolysis efficiency, characterizing these differences is crucial to understanding the proteinase-mediated mechanisms by which insects may develop resistance to Cry toxins.

1. INTRODUCTION

Bacillus thuringiensis (Bt) toxins have been used to successfully control lepidopteran pests for over 40 years. The increasing use of transgenic crops expressing Bt toxins can be attributed to their host specificity, effectiveness and environmentally safe properties (Betz et al., 2000; Christou et al., 2006; James, 2005). Toxin specificity is determined by N-terminal proteolytic activation and subsequent specific binding of activated toxin to midgut receptors (Bravo A, 2002; Hofmann et al., 1988). The prevailing model of Bt toxin mode of action elucidates a series of steps beginning with toxin ingestion followed by solubilization and proteolytic activation in the alkaline midgut (Bravo A, 2004; Gringorten JL, 1992; Knowles, 1994). Activated toxin monomers bind to cadherin-like proteins on the brush border membrane (BBM). In one model, this binding step activates intracellular cell death pathways (Zhang et al., 2006). In another model, toxin binding facilitates a conformational toxin change that leads to cleavage of helix α-1 by a membrane associated proteinase, promoting toxin oligomerization (Bravo et al., 2004). Toxin oligomers bind to secondary receptors such as aminopeptidase-N (APN) and alkaline phosphatase (ALP) and migrate to membrane regions called lipid rafts, where they insert into the epithelial membrane ultimately causing cell death due to osmotic shock (Bravo A, 2004; Pigott and Ellar, 2007).

Considering the need for protoxin activation, proteolytic processing by serine proteinases is an important step in determining toxicity (Gill, 1992; Oppert, 1999). Lepidopteran midgut digestive fluids are mainly composed of serine proteinases, many of which are "trypsin-like" as determined by proteinase inhibition analyses (Applebaum, 1985; Purcell et al., 1992). In the case of Cry1Ac, trypsin-like proteinases sequentially degrade 130 kDa protoxin by introducing seven specific cleavages in the C-terminus to produce a 67 kDa active toxin core (Choma et al.,

1990). Chymotrypsin-like proteinases are thought to play more of a role in toxin degradation and inactivation of Cry1A and Cry2Aa toxins (Audtho et al., 1999; Indrasith LS, 1991; Miranda et al., 2001; Shao et al., 1998). Differences in the midgut proteinase composition and expression can thus potentially affect toxin activation and create differences in host specificity (Haider et al., 1986; Jaquet et al., 1987; Miranda et al., 2001). In *Plodia interpunctella* and *Ostrinia nubilalis* Bt resistance is linked to the slower processing of protoxin to toxin due to absence of trypsin-like proteinases (Li H, 2004; Oppert et al., 1997). In *Helicoverpa armigera*, chymotrypsin-like proteinases have been reported to cause degradation of activated toxin, making the insect insensitive to Bt treatment (Shao et al., 1998).

The tobacco budworm, *Heliothis virescens*, is a major pest of cotton and is effectively controlled by transgenic cotton expressing single (Cry1Ac) and dual (Cry1Ac and Cry2Ab) toxins (Adamczyk et al., 2001; Chitkowski et al., 2003; Stewart et al., 2001). Larvae have serine proteinases for food digestion, primarily trypsin and chymotrypsin (Johnston, 1995). Gut proteinases from larvae of two independently-selected Cry1Ac-resistant strains of *H. virescens* (YHD2-B and CXC) demonstrated a reduced ability to process Cry1Ac and Cry2Aa protoxins (Karumbaiah et al., 2007). This was consistent with reduced activity of trypsin-like proteinases and the absence of ~32 kDa trypsin-like and ~35 kDa chymotrypsin-like proteinases in the resistant strains.

Because the only described mechanism of resistance in the CXC strain of *H. virescens* is proteinase alterations (Jurat-Fuentes, 2004; Karumbaiah et al., 2007), in the current work we used larvae from this strain to compare alterations in expression of trypsins and chymotrypsins in digestive fluids of Bt-susceptible (YDK) or -resistant (CXC) larvae in response to feeding on diet alone, or diet containing either Cry1Ac or Cry2Aa toxins. We used 3' Rapid Amplification of

cDNA Ends (RACE) to recover nucleotide sequence of 3' untranslated regions (UTR's) as a means to discriminate between individual trypsin and chymotrypsin gene products. Sequence analysis identified four trypsin-like and ten chymotrypsin-like cDNAs, as well as sequence polymorphisms in the form of synonymous and non-synonymous changes. We conducted gene expression analyses of these proteinases using relative quantitative real time polymerase chain reaction (rqRT-PCR). The results showed a specific down-regulation of a midgut trypsin in toxin-fed YDK and CXC. A general up-regulation of chymotrypsin genes in the Bt-resistant strain CXC was observed in comparison to the susceptible YDK strain.

2. MATERIALS AND METHODS

2.1. Insect Rearing and Toxin Dose Response

H. virescens larvae (YDK and CXC strains) were provided by Dr. Fred Gould (North Carolina State University). The origin of these strains has been described elsewhere (Karumbaiah et al., 2007). Briefly, larvae from the YDK strain are susceptible while CXC larvae are resistant, to both Cry1Ac and Cry2Aa toxins. Larvae were reared under previously described laboratory conditions (Gould et al., 1995). Activated Cry1Ac or Cry2Aa toxins were incorporated into diet (Multi species diet-Southland Products, Lake Village, Ark.) at various concentrations to establish sub-lethal toxin concentrations that permitted larvae to grow to late fourth instars. The susceptible YDK strain was tested on Cry1Ac and Cry2Aa at concentrations of 1, 0.5, 0.2 and 0.1 μg/ml of diet. For toxin incorporation experiments, YDK larvae were reared on diet containing 0.1 μg/ml Cry1Ac or Cry2Aa toxin until late 4^{th} instars. The CXC strain larvae were tested at Cry1Ac toxin concentrations of 50, 10, 5 and 1 μg/ml of diet, and Cry2Aa at concentrations of 200, 100, 50 and 10 μg/ml of diet. CXC larvae were reared until

late 4th instar on a sublethal dose of 1 µg Cry1Ac/ml of diet and 200 µg Cry2Aa/ml of diet. Larvae fed diet without toxin were used as controls.

2.2. Tissue Isolation and Extraction of Total RNA

Midguts of late 4th instar *H. virescens* larvae fed diet only or diet with toxin were dissected and rinsed in phosphate-buffered saline (PBS) under RNase free conditions. Individual midguts were placed in RNA*later* (Ambion, Austin, TX) overnight at 4°C and stored at -80°C. Total RNA was extracted from frozen midguts using an RNeasy mini kit (Qiagen, Valencia, CA), and the isolated total RNA was subjected to DNase treatment to remove any residual DNA according to the manufacturer's instructions. Total RNA was quantified using RiboGreen® reagent (Molecular Probes, Carlsbad, CA) and readings were taken using a fluorescence microplate reader (FLUOstar GALAXY; BMG, Durham,NC). The integrity of total RNA was verified by the visualization of distinct bands corresponding to 18s and 28s rRNA resolved on a 1.2% formaldehyde agarose gel.

2.3. Cloning and Sequencing of 3' cDNA ends

For our PCR cloning strategy, we used specific trypsin and chymotrypsin forward primers and oligo dT as reverse primer. The trypsin-specific forward primer was designed from the conserved sequence "GTILNNR" found in alignments of trypsin-like sequences from *H. armigera, H. virescens, Lacanobia oleracea, Sesamia nonagrioides, Helicoverpa zea, Manduca sexta* and *O. nubilalis*. The chymotrypsin-specific forward primer was made to amplify nucleotides encoding "NNFNGAT" conserved in alignments of *H. armigera* and *H. zea* chymotrypsins. Other chymotrypsins from *H. armigera*, *Helicoverpa punctigera, H. zea,*

Spodoptera frugiperda, Agrotis ipsilon, P. interpunctalla, Bombyx mori and *O. nubilalis* had considerable diversity in this region, and therefore two additional degenerate primers were designed based on these sequences.

For amplification, 2 µg of total RNA pooled from 10 midguts of control or toxin-fed larvae were used to prepare 3' RACE-ready first-strand cDNA using a SMART ™ RACE cDNA Amplification kit (Clontech, Mountain View, CA) according to the manufacturer's protocol. First-strand cDNA served as template along with gene-specific trypsin and chymotrypsin forward primers in 3' RACE PCR reactions. Trypsin-specific forward primer 5' GAG GTA CCA TCC TCA ACA ACA GG 3', chymotrypsin-specific forward primer 5' CAA CAA CTT CAA CGG TGC CAC CGC 3', and chymotrypsin-degenerate forward primers 5'CTC AAC RAV MRM TNC RMC GGW KHH A 3' and 5'YBR YYR YSW SYG GMT DSG GMM SYA C 3' were designed to the specific protein sequences in alignments as previously described. The trypsin and chymotrypsin primers were designed to amplify partial fragments of sizes ~700bp and ~500bp, respectively. Touchdown PCR was performed with trypsin- and chymotrypsin-specific forward primers according to the manufacturer's protocol. PCR conditions for reactions using degenerate primers were modified and consisted of 5 cycles of 94°C for 30s, 58°C for 30s and 72°C for 3 min, followed by 5 cycles of 94°C for 30s, 55°C for 30s and 72°C for 3 min, followed by 30 cycles of 94°C for 30s, 50°C for 30s and 72°C for 3 min. PCR products were resolved in a 2% agarose gel containing ethidium bromide. The DNA bands were excised and purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA), cloned into pGEM-TEasy vector (Promega, Madison, WI), transformed into competent DH5α *E.coli* (Invitrogen, Carlsbad, CA) and plated onto LB agar plates containing 100µg/ml ampicillin. From each treatment, 96 clones were randomly picked and sequenced from T7 and SP6 promoter ends using BigDye

terminator chemistry (Applied Biosystems, Foster City, CA) on an ABI3700 capillary sequencer (Applied Biosystems, Foster City, CA). Sequencing in both directions resulted in sufficient sequence coverage to overcome failure or truncation of sequence due to the presence of PolyA tails (Yang et al., 2005).

2.4. Bioinformatic analyses

cDNA sequences were analyzed using an automated sequence processing package, MAGIC-SPP (Liang et al., 2006). Raw sequence reads were vector and adapter screened and PHRED base-called. High quality sequences with a PHRED quality score ≥ 16 and a minimum read length of 100bp were selected for assembly using CAP3 sequence assembly program (Huang and Madan, 1999). The consensus sequences were analyzed to confirm insect proteinase sequence homology using the BLASTX program (Altschul et al., 1997) with an e-value of $1e^{-10}$ to search the non-redundant (nr) database at NCBI.

The sequence assemblies, i.e. contigs, were subsequently analyzed for single nucleotide polymorphisms (SNPs) using the SNP analysis software, PolyBayes (Marth et al., 1999). The contigs were screened for SNP- containing sequences with a PolyBayes quality score ≥1 and a minimum number of ≥5 SNPs within each population in a given cluster. The translated protein sequences of trypsin and chymotrypsin mRNA were aligned using the multiple sequence alignment program, CLUSTALX (Thompson et al., 1997).

Neighbor-joining bootstrap phylogenetic analysis was conducted using multiple sequence alignments of *H. virescens* trypsin- and chymotrypsin-like amino acid sequences with other heliothine trypsin and chymotrypsin sequences obtained from GenBank. The trypsin-like sequences were: *H. armigera* -AF045138, AY437836, Y12270, Y12275, Y12277, Y12276,

Y12283, Y12271, Y12269, Y12278 ; *H. zea* -AF261986, AF261988, AF261982, AF261987, AF261981, AF261989, AF261985, AF261980, AF261983, AF261984, *H. virescens* AF237416, and bovine pancreatic trypsin XM_584594.3. The chymotrypsin-like sequences were: *H. armigera* - Y12273, Y12279, Y12281, Y12287, Y12284, Y12285, Y12280 *H. zea* – AF233733, AF233732, AF233731, *H. punctigera* AY618889, AY618890, AY618893, AY618895, AY618892, AY618891, *H. virescens* – AF237417, and bovine chymotrypsinogen precursor XM_608091.3.

2.5. Quantitative real-time PCR

First-strand cDNA was synthesized in reactions containing 2 µg of total RNA pooled from 5 midguts each of control and toxin fed larvae, using PowerScript™ reverse transcriptase (Clontech, Mountain View, CA), anchored oligo$(dT)_{20}$ primer (Invitrogen, Carlsbad, CA) and other reaction components in a 10µl reaction. The resultant first strand cDNA was diluted in a final volume of 100µl of tricine -EDTA buffer provided with the kit.

PCR amplification efficiencies were assessed using gene-specific primers (Table 3.1) and endogenous control ribosomal protein S18 (RBPS18) forward 5' ATG GCA AAC GCA AGG TTA TGT TT 3' and reverse 5' TTG TCA AGA TCA ATA TCG GCT TT 3' primers using cDNA equivalents of 100ng, 50ng, 20ng, 10ng, 5ng, 2ng and 1ng of total RNA. Forward primers were designed to specific regions and reverse primers were designed to highly variable regions in the 3' UTR to specifically amplify individual trypsin or chymotrypsin-like transcripts. Real-time PCR was conducted on an ABI 7900HT fast real-time PCR system (Applied Biosystems, Foster City, CA) in 12.5µl reactions. PCR reactions for each template and primer combination were conducted in triplicate and repeated three times with cDNA prepared from

separate batches of midguts dissected from 5 larvae per treatment. The reactions consisted of a cDNA equivalent of 20 ng of total RNA, 6.25 µl Power® SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and forward and reverse primers at 0.9 µM concentration. The PCR conditions consisted of an initial denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 15s, 58°C for 30s and 72°C for 30s. Data obtained were analyzed using the relative quantitation study option on the SDS 2.2.1 software (Applied Biosystems, Foster City, CA). The $2^{-\Delta\Delta CT}$ method was used to calculate transcript abundance, which was standardized to 1 for diet only-fed YDK and to which transcript abundances from other larval treatments were compared. Upon completion of a relative quantitation PCR run, a dissociation curve analysis was conducted to verify the presence of any nonspecific amplicons.

3. RESULTS

3.1. Sequencing and identification of proteinase encoding cDNA

Partial sequences encoding serine proteinases were obtained from *H. virescens* larvae of Bt-susceptible YDK and the Bt-resistant CXC strains. RNA was obtained from dissected larval midguts of both strains that were fed control or toxin diet, and cDNA libraries were made. Randomly selected clones were analyzed for sequences containing the 5' coding region and 3' untranslated region (UTR) of transcripts encoding proteins similar to trypsin and chymotrypsin. Sequencing of the cloned PCR fragments followed by PHRED base-calling and vector screening resulted in 1,854 high-quality partial-paired sequence reads. Sequence assembly using CAP3 resulted in 80 individual clusters. BLASTX searches of the NCBI nr database using the consensus sequences obtained from the assemblies indicated homology to insect trypsin- and chymotrypsin-like sequences with percentage identities ranging between 85 and 99%. Four and

ten partial sequences encoding trypsin- and chymotrypsin-like cDNAs, respectively, were thus identified based on sequence assembly and homology searches. Cloned sequences had an average length of ~660bp for trypsin-like and ~430bp for chymotrypsin-like cDNAs. Translated amino acid sequences were distinguished as trypsin-like by the conserved Asp_{189}, Gly_{212} and Gly_{222} residues and chymotrypsin-like by Ser_{189}/Gly_{189}, Gly_{216} and Gly_{224} residues in the S1 binding pocket (Srinivasan et al., 2006).

3.2. Sequence alignments and phylogenetic analysis

Multiple sequence alignments using CLUSTALW (Thompson et al., 1994) identified regions of similarity between *H. virescens* trypsin- and chymotrypsin-like cDNA sequences, and pairwise sequence comparisons enabled the calculation of percentage similarities between sequences using the bl2seq tool on NCBI (Tatusova and Madden, 1999). All trypsin- and chymotrypsin-like cDNA sequence ORF's were followed by dissimilar 3' UTR's (Fig.3.2) and a PolyA tail.

The nucleotide sequences of the truncated proteinase cDNAs were deposited into GenBank (Table 3.1). Pairwise alignments of *H. virescens* trypsin-like cDNA sequences HvT1, 2, 3, and 4 had 93-98% similarity among the sequences, with the highest percentage similarity between HvT3 and 4. Pairwise alignments of *H. virescens* chymotrypsin-like cDNAs placed HvC1-4 into group-1, HvC5-7 into group-2, and HvC8-10 into group-3, based on sequence similarities. Intra-group comparisons indicated that group-1 members share the highest overall percentage similarity of 92-96%, with HvC3 and 4 sharing 96% similarity. Members of group-2 were 87-92%, similar with HvC5 and 7 sharing the highest similarity of 92%. Members of group-3 had a percentage similarity range of 78-89% with HvC8 and 10 sharing the highest similarity of 89%.

Inter-group comparisons indicated that group-1 and group-3 members are 82-87% similar with HvC2 and 9 sharing the highest similarity of 87%. Groups-1 and 2, as well as groups-2 and 3, were 72-76% similar, with HvC2 and 6 sharing the highest similarity between group-1 and 2 (76%) followed by HvC5 and 8 of groups 2 and 3 having 75% similarity. Group-1 chymotrypsin-like sequences have an ORF of 350 bp, and group-2 consists of a 410 bp ORF (including the stop codon). HvC8, 9 and 10 have partial individual ORF lengths of 354bp, 365bp and 335bp, respectively, including stop codons.

Multiple sequence alignments of translated amino acid sequences identified regions that represent functional differences between the trypsin and chymotrypsin-like proteinases (Fig 3.1). Amino acids for the HvT proteinases were translated from a 603 bp region of the cDNA and compared to trypsin-like proteinases from other heliothine species and a bovine pancreatic trypsin (Fig 3.1 A). The four HvT sequences share a high degree of similarity (88-98%) with trypsins from *H. armigera* (Y12271, Y12278, Y12269, and Y12283), *H. zea* (AF261984) and a *H. virescens* trypsin-like protein precursor (AF237416). The catalytic triad, His_{57}, Asp_{102} and Ser_{195}, as well as the flanking binding pocket residues, D_{189}, Gly_{212} and Gly_{222}, characteristic of trypsin-like proteinases, were conserved in all sequences. Alignments of the truncated *H. virescens* trypsin sequences contained six conserved cysteine residues, although alignments with other insect trypsins indicate that there are probably two additional cysteines in the *H. virescens* sequences. Residue Cys_{191} is predicted to form disulfide bonds with Cys_{209} or Cys_{215} to stabilize the S1 binding pocket. All heliothine trypsins had an increased number of arginine residues and decreased number of lysine residues when compared to bovine trypsin, which has been proposed to increase their stability in the alkaline midgut (Peterson et al., 1994).

Alignments of the predicted amino acids for chymotrypsin-like proteinase sequences indicated a larger extent of variation among members than the trypsin-like sequences (Fig 3.1 B). HvC translated amino acid sequences were aligned with *H. armigera* (Y12284), *H. punctigera* (AY618892, AY618895) and *H. virescens* chymotrypsin precursor (AF237417). Only the Ser_{195} residue of the catalytic triad, Asp_{102}, $His_{57,}$ $Ser_{195,}$ was present in the partial sequences. The binding pocket residue, $Ser_{189,}$ present in group-1 and group-3, was substituted by Gly_{189} in group-2 chymotrypsin-like proteinases, indicating altered substrate binding specificity (Botos et al., 2000). Binding pocket residues Gly_{216} and Gly_{224} remained the same among members of all three groups.

A phylogenetic analysis of HvT and HvC translated amino acid sequences with 21 other heliothine trypsins and 17 chymotrypsins was conducted to depict genealogical relationships between the different enzymes (Fig. 3.3). The trypsin phylogenetic tree (Fig.3.3 A) branched into two distinct lineages, each having bootstrap values of 786 and 999 out of 1000 iterations. Bootstrap iteration values <65% were obtained for internal nodes corresponding to *H. zea* (AF261986, AF261988), *H. armigera* (Y12270, Y12283), HvT2, and HvT1, indicating uncertain relationships at those nodes. Overall, grouping of HvT1-4 in the phylogenetic analysis correlated with the high percentage similarities observed in pairwise sequence comparisons.

Bootstrap analysis of chymotrypsin alignments (Fig. 3.3 B), revealed two distinct lineages, as indicated by bootstrap values of 1000 and 906. As with the trypsin-like sequences, bootstrap iteration values <65% were found on several nodes of chymotrypsin-like sequences, indicating uncertain relationships between these sequences. Overall, the multiple sequence and pairwise sequence alignments largely correlated with the phylogenetic grouping of HvC1-4 into group-1

and HvC5-7 into group-2. Among members of group-3 however, HvC9 formed an out-group, while HvC8 and 10 clustered together.

3.3. SNP analysis.

SNP analysis of the proteinase nucleotide sequence assemblies using the PolyBayes prediction software (Marth et al., 1999) provided an approach to compare Cry toxin feeding treatments with sequence polymorphisms in the cloned cDNAs and relate nucleotide changes to amino acid sequence. Analysis of cDNA contigs for the presence of SNP's in control and Cry toxin fed larvae detected sequence polymorphisms in HvT1, HvT2, and HvC3 (Table 3.2). Overall, cDNAs with nucleotide substitutions were mostly present in the toxin-fed insects when compared to control diet-only fed larvae. Nucleotide substitutions were synonymous, except in the case of HvT1 position 442, in which an A/G transition resulted in the substitution of an Asn_{185} in the diet-only control with a Ser_{185} in the toxin fed larvae. Sequences of the SNP variants HvT1SNP (EU000256), HvT2SNP (EU000258) and HvC3SNP (EU000264) were deposited in Genbank and assigned independent accession numbers.

3.4. Gene expression analyses

Because elevated chymotrypsin activity correlates with toxin degradation (Audtho et al., 1999; Indrasith et al., 1991; Miranda et al., 2001; Shao et al., 1998), and because reduction in trypsin-like proteinases is an adaptive mechanism for Bt resistance in Lepidoptera (Huang et al., 1999; Li et al., 2004; Oppert et al., 1997), we conducted relative quantitative real-time PCR (rqRT-PCR) to measure the abundance of specific trypsin- and chymotrypsin-like transcripts in the midguts of diet-only control and toxin-fed Bt-susceptible and -resistant larvae. Since Cry1Ac

and Cry2A toxins are used in transgenic Bt cotton to control *H. virescens* in the field, we focused our analysis to larvae fed either of these toxins. The cDNA amplified along with the gene specific primers used are presented in Table 3.3**.** Transcript abundance of HvT1, 2 and 4 was significantly increased in CXC when compared to YDK larvae, regardless of treatment (Fig 3.4 A). In contrast, expression of HvT3 transcript was greatly reduced in larvae from both strains when fed toxin-treated diet.

In comparison to YDK levels, transcript abundance for chymotrypsin-like genes HvC2, 4, 5, 7 and 10 was increased significantly in CXC larvae independent of toxin treatment (Fig 3.4 B). HvC1 abundance increased marginally in toxin-fed CXC and YDK larvae fed Cry2Aa, but remained constant in CXC diet-only control and decreased in YDK larvae fed Cry1Ac. Transcript abundance of HvC3, 8 and 9, increased in toxin-fed CXC larvae when compared to CXC fed control diet or YDK fed with toxin. The HvC6 transcript was absent in all tested YDK larvae (diet-only, as well as toxin fed), so transcript abundance was standardized to levels of CXC larvae fed diet-only (Fig 3.4 C). The results show that while HvC6 expression remained constant in CXC larvae fed with Cry1Ac, expression was highly reduced in CXC larvae fed with Cry2Aa. A quantitative summary of the results from the gene expression analysis is presented in Table 3.4.

4. DISCUSSION

Serine proteinases play an essential role in the solubilization and activation of Cry toxins, and resistance to Bt toxins due to alterations in midgut serine proteinases have been noted (Li H, 2004; Oppert et al., 1997). Biochemical studies have demonstrated that these enzymes are the primary digestive proteinases in *H. virescens* (Johnston, 1995). Previously we reported

biochemical parameters of proteinase alterations in larvae from the CXC strain of *H. virescens* that correlated with resistance to Cry1Ac and Cry2Aa (Karumbaiah et al., 2007). In the present study, we further characterize these alterations by studying the expression of trypsin and chymotrypsin-like transcripts in susceptible and resistant larvae reared on diet with or without toxin. These studies have resulted in the identification of proteinase gene alleles that are differentially expressed in insects fed Bt toxin that may contribute to insensitivity to toxins.

We identified four distinct trypsin-like and ten chymotrypsin-like sequences that were used to study proteinase expression upon toxin feeding. Although differences did exist in the 3' UTR's of HvT cDNAs, their highly similar coding regions suggest that they are probably allelic variants. Each of the 4 trypsins and 10 chymotrypsins had numerous arginines and an overall reduction of lysines. This composition enables the stabilization of the active site under the highly alkaline conditions prevalent in the insect midgut (Bown DP, 1997; Hegedus et al., 2003; Mazumdar-Leighton S, 2000; Peterson AM, 1994). The Gly_{189} residue found in group-2 chymotrypsins is an unusual adaptation and possibly results in altered substrate specificity (Botos et al., 2000).

Pairwise alignments of *H. virescens* trypsin-like cDNAs indicated a high degree of similarity, also supported by the phylogenetic analysis. Interestingly, we did not identify trypsins from the second observed heliothine trypsin lineage. Therefore, there are likely additional trypsin isoforms in the *H. virescens* midgut that were not identified in this study.

Only one of the polymorphisms detected in control and toxin diet-fed larvae resulted in an amino acid change. This change of Asn to Ser in YDK and CXC toxin-fed larvae lies within the loop-1 region that determines substrate specificity (Bown DP, 1997; Hedstrom et al., 1992). Therefore, this alteration may have important implications for digestion in intoxicated larvae as

well as toxin susceptibility. The shorter neutral polar side chain of a serine residue may change either substrate preference and/or binding affinity.

In addition to substrate interactions, there may be other consequences of amino acid substitutions in proteinase genes. Although synonymous point-mutations present in the coding region were not predicted to have fitness costs, recent evidence suggests otherwise (Parmley and Hurst, 2007). Evidence from *Drosophila* suggests that codon choice is directly correlated to the most abundantly present isoacceptor tRNA species (Moriyama and Powell, 1997; Powell and Moriyama, 1997). In the human *Multidrug Resistance 1* (MDR1) gene, synonymous mutations in the anticodon wobble positions introduce a non-optimal codon reported to cause translational pausing. As a result, the MDR1 gene product (P-glycoprotein) has altered protein folding, resulting in drug resistance (Kimchi-Sarfaty et al., 2007). Further research is necessary to determine the effect of SNPs in resistance to Bt toxins.

Using rqRT-PCR, serine proteinase transcript abundances were found to be different in larvae fed toxin-treated diets. Results from these analyses reveal that Bt-susceptible, YDK, and Bt–resistant, CXC, larvae differ in their levels of expression of serine proteinases. Interestingly, when feeding on control diet, both trypsin and chymotrypsin proteinase transcripts were upregulated in CXC larvae when compared to YDK. Feeding on diet containing either Cry1Ac or Cry2Aa resulted in the up-regulation of transcript levels of HvT1, 2, and 4 in CXC when compared to levels in YDK larvae. It is important to note that the real-time PCR primers used were not designed to discriminate between the wild-type and SNP variants, and hence this observed up-regulation could be due to the presence of SNP variants. In contrast, HvT3 was greatly downregulated in both YDK and CXC larvae upon feeding on toxin. The lack of a trypsin-like proteinase is genetically linked to resistance in *Bt aizawai*- and *entomocidus*-

resistant *P. interpunctella* (Oppert et al., 1997). Reduced activity of trypsin-like proteinases and a reduced ability to process Cry1Ab protoxin also has been reported in the *O. nubilalis* strain KS-SC reared on Dipel® (Huang et al., 1999). Transcriptional profiling of *Choristoneura fumiferana* fed with sublethal doses of Cry1Ab showed reduced expression of a trypsin-like proteinase gene (van Munster et al., 2007). Our results of down-regulation of HvT3 in toxin fed YDK and CXC is in agreement with these studies and indicates a physiological response to intoxication.

Even though most chymotrypsins were similarly expressed in diet-fed YDK and CXC larvae, toxin feeding resulted in increased abundance of these transcripts in CXC compared to YDK larvae. In *H. armigera,* reduced susceptibility to HD-1 toxins compared to *B. mori* is attributed to excessive degradation of toxin due to the action of chymotrypsin-like proteinases (Shao et al., 1998). The degradation of HD-1 and HD-73 toxins by immobilized chymotrypsin reportedly resulted in reduced insecticidal activity (Indrasith et al., 1991). The up-regulation of chymotrypsin-like transcripts in our analyses may result in toxin degradation in CXC larvae and decreased susceptibility. Alternatively, increased expression of chymotrypsins may be a physiological response to compensate for knockdown or down-regulation of proteinases involved in toxin processing. HvC6 was not present in YDK and in Cry2Aa-fed strains, but is present at normal levels in CXC diet-only control and CXC-Cry1Ac-fed strains. Although changes in mRNA expression may not result in a corresponding increase in protein expression, our present data is in agreement with our previously reported data of proteinase activities in midguts of CXC larvae (Karumbaiah et al., 2007). However, we failed to detect the previously observed down-regulation of a chymotrypsin-like proteinase in CXC larvae. One possible explanation for this discrepancy may be the low levels of expression of this proteinase in susceptible larvae.

"Gene Stacking" of Cry1Ac and Cry2Ab toxin genes in transgenic cotton is designed to utilize their distinctly different modes of action as a strategy to combat the development of resistance (Gould, 1998). The CXC strain is resistant to both Cry1Ac and Cry2Aa and is therefore important from the perspective of understanding the different mechanisms of Cry1Ac and Cry2Aa resistance. Linkage analysis of Cry1Ac and Cry2Aa resistance suggests that genes (*BtR4* and *BtR5*) responsible for Cry1Ac resistance are distinctly different from those contributing to Cry2Aa resistance in this strain (Gahan et al., 2005). In our analyses of proteinase gene expression, we find that CXC larvae feeding on Cry1Ac and Cry2Aa elicit an overall up-regulation of proteinase genes, with a slightly higher response in Cry1Ac-fed compared to Cry2Aa-fed insects. Cry1Ac resistance in this strain is attributed to resistance allele, *BtR5,* and to a lesser extent due to the *BtR4* cadherin mutation, although biochemical evidence suggests that this does not result in altered toxin binding in this strain(Gahan et al., 2001; Gahan et al., 2005; Jurat-Fuentes et al., 2003; Jurat-Fuentes, 2004). Since neither *BtR4* and *BtR5* resistance alleles contribute to Cry2Aa resistance in this strain, and because Cry2Aa resistance is reportedly a result of cumulative contributions of multiple resistance genes (Gahan et al., 2005), the differential regulation of proteinase genes as observed in our study may contribute to both Cry1Ac and Cry2Aa resistance in this strain.

The regulation of proteinase genes in the insect gut is dependent upon the complex food source and toxic agents present therein. In this paper we have presented evidence of differences in sequence and expression of specific proteinases induced by Cry toxin feeding in susceptible and resistant *H. virescens* larvae. In another closely related heliothine, *H. armigera*, larval feeding on plant tissues containing proteinase inhibitors resulted in selective increased expression levels of inhibitor-insensitive proteinases (Bown et al., 1997; Bown et al., 2004).

This rapid response is facilitated by expression of multiple mRNA isoforms differing only in their 3' UTR region (Hughes, 2006; Moucadel et al., 2007). Although speculative, it is possible that the existence of a number of proteinase isoforms with distinct 3' UTR regions facilitates the broad changes in proteinase expression in resistant insects after feeding on Bt toxins. Our data demonstrates important physiological alterations upon feeding on Bt toxins in resistant larvae, supporting the potential role of these alterations in decreased susceptibility. Such a resistance mechanism would explain the CXC phenotype of resistance to Cry1Ac and Cry2Aa toxins, which supposedly have distinct modes of action. Considering that one of the strategies to delay the onset of resistance to Bt toxins in the field is the use of toxins with different mode of action, our data highlight the necessity to further characterize changes in digestive enzymes that may result in generalized resistance to Bt toxins.

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FIGURES AND TABLES

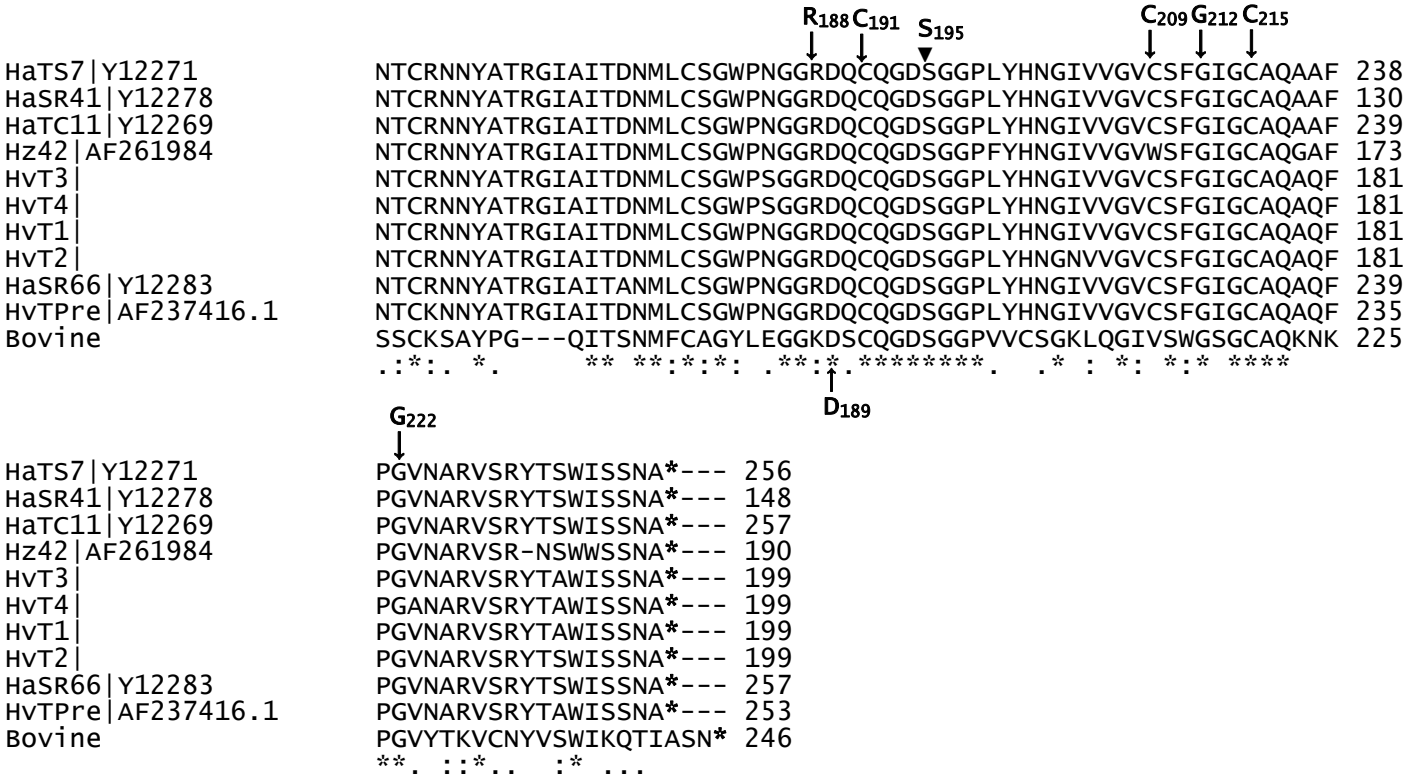
Figure 3.1 Alignments of translated *Heliothis virescens* trypsin- **(A)** and chymotrypsin-like **(B)** sequences using ClustalW. "*" below the alignments indicate identical residues across all sequences. ":" and "." symbols below the alignments indicate conserved and semi-conserved substitutions. Catalytic triad residues are represented by ▼ with the respective amino acid and number labeled above. S1 binding pocket residues are indicated by ↑↓. Stop codon at the end of the coding region is represented by *****.

A

```
HaTS7|Y12271          -NNMRILALVALCFAAVAAVPSNPQRIVGGSVTTIDQYPTIAALLYSWNLSTYWQACGGT 59
HaSR41|Y12278         ------------------------------------------------------------
HaTC11|Y12269         SNNMRILALVALCFAAVAAVPSNPQRIVGGSVTTIDQYPTIAALLYSWNLSTYWQACGGT 60
Hz42|AF261984         ------------------------------------------------------------
HvT3|                 ------------------------------------------------------------GT 2
HvT4|                 ------------------------------------------------------------GT 2
HvT1|                 ------------------------------------------------------------GT 2
HvT2|                 ------------------------------------------------------------GT 2
HaSR66|Y12283         FSNMRFLALLALCFAAVAAVPSNPQRIVGGSVTTIDRYPTIAALLYSWNLSAYWQSCGGT 60
HvTPre|AF237416.1     ---MRILALVALCFAAVAAVPSNPQRIVGGSVTTIDQYPTIAALLYSWNLSTYWQACGGT 57
Bovine                ---MKTFIFLALLGAAVAFPVDDDDKIVGGYTCGANTVP------YQVSLNSGYHFCGGS 51

                                    H57                                     D102
                                    ▼                                       ▼
HaTS7|Y12271          ILNNRAILTAAHCTAGDANNRWRIRLGSTWANSGGVVHNLNANIVHPSYNSRTMDNDIAV 119
HaSR41|Y12278         ----------------------------------------------NSRTMDNDIAV 11
HaTC11|Y12269         ILNNRAILTAAHCTAGDANNRWRIRLGSTWANSGGVVHNLNANIIHPSYNSRTMDNDIAV 120
Hz42|AF261984         ------IVTAAHCTAGDANNRWRIRLGSTWANSGGVVHNLNANIIHPSYNSRTMDNDIAV 54
HvT3|                 ILNNRAVLTAAHCTVGDANNRWRIRLGSTWANSGGVVHNLNANIIHPSYNSWTMDNDIAV 62
HvT4|                 ILNNRAVLTAAHCTVGDANNRWRIRLGSTWANSGGVVHNLNANIIHPSYNSWTMDNDIAV 62
HvT1|                 ILNNRAILTAAHCTAGDANNRWRIRLGSTWANSGGVVHNLNANIIHPSYNSRTMDNDIAV 62
HvT2|                 ILNNRAILTAAHCTAGDANNRWRIRVGSTWANSGGVVHNLNANIIHPSYNSRTLDNDIAV 62
HaSR66|Y12283         ILNNRAILTAAHCTAGDANNRWRIRVGSTWANSGGVVHNLAANIIHPSYNSRTMDNDIAV 120
HvTPre|AF237416.1     ILNNRAILTAAHCTAGDANNRWRIRLGSTWANSGGVVLNVNLNIVHPSYNSNTLNNDIAL 117
Bovine                LINSQWVVSAAHCYKSGIQVRLGEDNINVVEGNEQFIS-ASKSIVHPSYNSNTLNNDIML 110
                                                                ** *::*** :

HaTS7|Y12271          LRSATTFSFNNQVRAASIAGANYNLADNQAVWAAGWG-TTSSGGSSSEQLRHVQLVTINQ 178
HaSR41|Y12278         LRSATTFSFNNQVRAASIAGANYNLADNQAVWAAGWG-TTSSGGSSSEQLRHVQLVTINQ 70
HaTC11|Y12269         LRSATTFSFNNQVRAASIAGANYNLADNQAVWAAGWG-TTSSGGSSSEQLRHVQLVTINQ 179
Hz42|AF261984         VRSATTLSFNNQVRAASIAGANYNLADNQAVWAAGWG-TTSSGGSSCEQLRHVQLVTINQ 113
HvT3|                 LRSATTFAFNNNIRAASIAGANYNLADNQAVWAAGWG-TTSSGGASSEQLRHVQMVVINQ 121
HvT4|                 LRSATTFTFNNNVRAASIAGANYNLADNQAVWAAGWG-TTSSGGSSSEQLRHVQMVVINQ 121
HvT1|                 LRSATTFSFNNNVRAASIAGANYNLADNQAVWAAGWG-TTSAGGASSEQLRHVQVVTINQ 121
HvT2|                 LRSATTFSFNNNVRAASIAGANYNLADNQAVWAAGWG-TTSAGGSSSEQLRHVQMVVINQ 121
HaSR66|Y12283         LRSATTFSFNNNVRAASIAGANYNLADNQAVWAAGWG-TTSAGGSSSEQLRHVELRSINQ 179
HvTPre|AF237416.1     LHPPPT-SVQQQRASRPIAGSNYNLAANQFVWAAGWG-TISSGGAASEQLRHVQLIVINQ 175
Bovine                IKLKSAASLNS--RVASISLPTSCASAGTQCLISGWGNTKSSGTSYPDVLKCLKAPILSD 168
                      ::  .: :.:.     .*: ..   : .     :*** * *:* :  : *: ::   :.:
```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HaSR41|Y12278 NTCRNNYATRGIAITDNMLCSGWPNGGRDQCQGDSGGPLYHNGIVVGVCSFGIGCAQAAF 130
HaTC11|Y12269 NTCRNNYATRGIAITDNMLCSGWPNGGRDQCQGDSGGPLYHNGIVVGVCSFGIGCAQAAF 239
Hz42|AF261984 NTCRNNYATRGIAITDNMLCSGWPNGGRDQCQGDSGGPFYHNGIVVGVWSFGIGCAQGAF 173
HvT3| NTCRNNYATRGIAITDNMLCSGWPSGGRDQCQGDSGGPLYHNGIVVGVCSFGIGCAQAQF 181
HvT4| NTCRNNYATRGIAITDNMLCSGWPSGGRDQCQGDSGGPLYHNGIVVGVCSFGIGCAQAQF 181
HvT1| NTCRNNYATRGIAITDNMLCSGWPNGGRDQCQGDSGGPLYHNGIVVGVCSFGIGCAQAQF 181
HvT2| NTCRNNYATRGIAITDNMLCSGWPNGGRDQCQGDSGGPLYHNGNVVGVCSFGIGCAQAQF 181
HaSR66|Y12283 NTCRNNYATRGIAITANMLCSGWPNGGRDQCQGDSGGPLYHNGIVVGVCSFGIGCAQAQF 239
HvTPre|AF237416.1 NTCKNNYATRGIAITDNMLCSGWPNGGRDQCQGDSGGPLYHNGIVVGVCSFGIGCAQAQF 235
Bovine SSCKSAYPG---QITSNMFCAGYLEGGKDSCQGDSGGPVVCSGKLQGIVSWGSGCAQKNK 225
D189
G222
HaTS7|Y12271 PGVNARVSRYTSWISSNA*--- 256
HaSR41|Y12278 PGVNARVSRYTSWISSNA*--- 148
HaTC11|Y12269 PGVNARVSRYTSWISSNA*--- 257
Hz42|AF261984 PGVNARVSR-NSWWSSNA*--- 190
HvT3| PGVNARVSRYTAWISSNA*--- 199
HvT4| PGANARVSRYTAWISSNA*--- 199
HvT1| PGVNARVSRYTAWISSNA*--- 199
HvT2| PGVNARVSRYTSWISSNA*--- 199
HaSR66|Y12283 PGVNARVSRYTSWISSNA*--- 257
HvTPre|AF237416.1 PGVNARVSRYTAWISSNA*--- 253
Bovine PGVYTKVCNYVSWIKQTIASN* 246

B

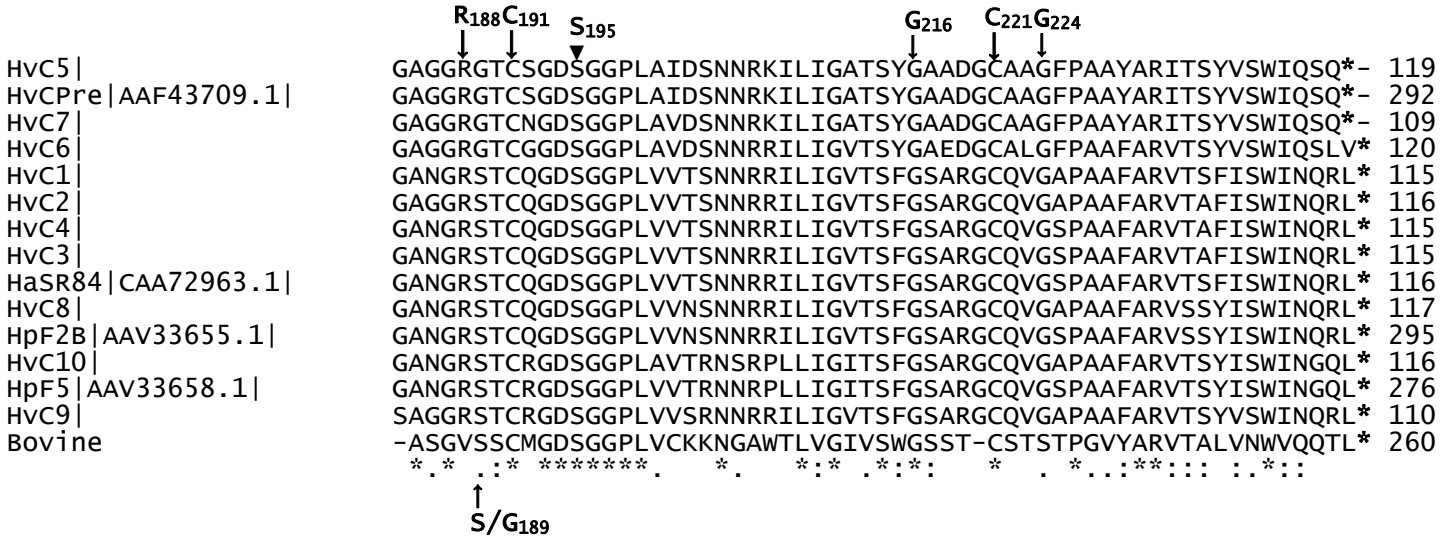
```
HvC5|                   ------------------------------------------------------------
HvCPre|AAF43709.1|      ----MKLFLGVCLALAVTVSAVEIGTPEADSPVFGYHAKFGIAEAARIKSMEEAQSFSGQ 56
HvC7|                   ------------------------------------------------------------
HvC6|                   ------------------------------------------------------------
HvC1|                   ------------------------------------------------------------
HvC2|                   ------------------------------------------------------------
HvC4|                   ------------------------------------------------------------
HvC3|                   ------------------------------------------------------------
HaSR84|CAA72963.1|      ------------------------------------------------------------
HvC8|                   ------------------------------------------------------------
HpF2B|AAV33655.1|       MKLLAVTLLAFAAVVSARNIDLEDVIDLEDITAYDYHTKIGIPLAEKIRAAEEEAERNPS 60
HvC10|                  ------------------------------------------------------------
HpF5|AAV33658.1|        ------------------VHLEDSIDLEDITAWGYLTKFGIPEAEKIRN--AEEASSAS 39
HvC9|                   ------------------------------------------------------------
Bovine                  --------------------MNFLWLLSYCALLGTAFGCGVPAIQPVLSGLSRIVNGEE 39


                                                          H57
                                                          ▼
HvC5|                   ------------------------------------------------------------
HvCPre|AAF43709.1|      RIVGGSITNIANVPYQAGLVITIFIFQSVCGASLISHNRLVTAAHCKFDNVMTANSFTVV 116
HvC7|                   ------------------------------------------------------------
HvC6|                   ------------------------------------------------------------
HvC1|                   ------------------------------------------------------------
HvC2|                   ------------------------------------------------------------
HvC4|                   ------------------------------------------------------------
HvC3|                   ------------------------------------------------------------
HaSR84|CAA72963.1|      ------------------------------------------------------------
HvC8|                   ------------------------------------------------------------
HpF2B|AAV33655.1|       RIVGGSTSSLGAFPYQAGLLASFASGQGVCGGSLLNVRRVLTAAHCWFDGRNQARSFTVV 120
HvC10|                  ------------------------------------------------------------
HpF5|AAV33658.1|        RIVGGSLSSVGQIPYQAGLVIDLAGGQAVCGGSLISASRVLTAAHCWFDGQNQAWRFTVV 99
HvC9|                   ------------------------------------------------------------
Bovine                  AVPG---------SWPWQVSLQDKTGFHFCGGSLINENWVVTAAHCGVTTSDVVVAGEFD 90
```

```
                                                  D102
                                                  ▼
HvC5|                  ---------------------------------------------------LN 2
HvCPre|AAF43709.1|     LGSNTLFFGGTRINTNDVVMHPNWSPSTVANDIAVIRISS-VSFSNVIQPIALPSGNEIN 175
HvC7|                  ---------------------------------------------------- 
HvC6|                  ---------------------------------------------------LN 2
HvC1|                  ---------------------------------------------------- 
HvC2|                  ---------------------------------------------------F 1
HvC4|                  ---------------------------------------------------- 
HvC3|                  ---------------------------------------------------- 
HaSR84|CAA72963.1|     ---------------------------------------------------N 1
HvC8|                  ---------------------------------------------------LN 2
HpF2B|AAV33655.1|      LGSVRLYSGGTRLNTASVVMHGSWNPNLVRNDIAMINLPSNVATSGNIAPIALPSGNELN 180
HvC10|                 ---------------------------------------------------H 1
HpF5|AAV33658.1|       LGSTTLFSGGTRIPTSNVVMHGSWTPSLIRNDVAVIRLGTNVATSNTIAIIALPSGSQIN 159
HvC9|                  ---------------------------------------------------- 
Bovine                 QGSSSEK--IQKLKIAKVFKNSKYNSLTINNDITLLKLSTAASFSQTVSAVCLPSAS--D 146



HvC5|                  EQYNGANALASGYGLTSDGGS--IGSNQQLSSVTLPVITNAQCAAVYGSAFVHNSNICTS 60
HvCPre|AAF43709.1|     NLFVGVNALASGYGLTSDGGS--IGSNQQLSSVTLPVITNAQCAAVYGSAFVHNSNICTS 233
HvC7|                  --------IATGWGRTSDSGS--IGTNQQLSSVTLPVITNAQCAAVYGPAFVHNSNICTS 50
HvC6|                  NRFTGANALASGFGLTSDNGN--IGSNQQLSSVTLPVITNAQCAAVYGPMFVHNSNICTS 60
HvC1|                  NNFNGATATASGFGLSRDGGS--VDGN--LRHVNLPVITNAVCTASFPG-LIQSTNICTS 55
HvC2|                  INFNGATATASGFGLSRDGGS--VDGN--LRHVNLPVITNAVCTASFPG-LIQSTNICTS 56
HvC4|                  NNFNGATATASGFGLSRDGGS--VDGN--LRHVDLPVITNAVCTVSFPG-IIQSTNICTS 55
HvC3|                  NNFNGATATASGFGLSRDGGS--VDGN--LRHVDLPVITNAVCTVSFPG-IIQSSNICTS 55
HaSR84|CAA72963.1|     NNFNGATAVASGFGLARDGGS--VDGN--LRHVNLPVITNAVCTVSFPG-IIQSSNICTS 56
HvC8|                  ENYTGDTATASGFGLARDGGS--IDGN--LRHVNLPVITNAVCSQSFPG-LIQASNVCTS 57
HpF2B|AAV33655.1|      NQFAGATATASGFGLARDGGV--IDGN--LRHVNLPVITNAVCSQSFPG-LIQASNVCTS 235
HvC10|                 KHFDGATATASGFGLARDGGS--IDGN--LRHVNLPVITNAVCSQSFPG-LIQASNVCTS 56
HpF5|AAV33658.1|       ENFAGETALASGFGLTSDTGS--ISSNQALSHVNLPVITNAVCRNSFPL-LIQDSNICTS 216
HvC9|                  --------TMSGWGRTGDASSCAITVNQFLSHVDVPVISNAVCRQSFV--IIQPSNICIN 50
Bovine                 DFAAGTTCVTTGWGLTRYTNA---NTPDRLQQASLPLLSNTNCKKYWGT-KIKDAMICAG 202
                                 :*:* :   .         *  . :*:::*: *   :    :: : :* .
```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HvC7| GAGGRGTCNGDSGGPLAVDSNNRKILIGATSYGAADGCAAGFPAAYARITSYVSWIQSQ*- 109
HvC6| GAGGRGTCGGDSGGPLAVDSNNRRILIGVTSYGAEDGCALGFPAAFARVTSYVSWIQSLV* 120
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HvC2| GAGGRSTCQGDSGGPLVVTSNNRRILIGVTSFGSARGCQVGAPAAFARVTAFISWINQRL* 116
HvC4| GANGRSTCQGDSGGPLVVTSNNRRILIGVTSFGSARGCQVGSPAAFARVTAFISWINQRL* 115
HvC3| GANGRSTCQGDSGGPLVVTSNNRRILIGVTSFGSARGCQVGSPAAFARVTAFISWINQRL* 115
HaSR84|CAA72963.1| GANGRSTCQGDSGGPLVVTSNNRRILIGVTSFGSARGCQVGSPAAFARVTSFISWINQRL* 116
HvC8| GANGRSTCQGDSGGPLVVNSNNRRILIGVTSFGSARGCQVGAPAAFARVSSYISWINQRL* 117
HpF2B|AAV33655.1| GANGRSTCQGDSGGPLVVNSNNRRILIGVTSFGSARGCQVGSPAAFARVSSYISWINQRL* 295
HvC10| GANGRSTCRGDSGGPLAVTRNSRPLLIGITSFGSARGCQVGAPAAFARVTSYISWINGQL* 116
HpF5|AAV33658.1| GANGRSTCRGDSGGPLVVTRNNRPLLIGITSFGSARGCQVGSPAAFARVTSYISWINGQL* 276
HvC9| SAGGRSTCRGDSGGPLVVSRNNRRILIGVTSFGSARGCQVGAPAAFARVTSYVSWINQRL* 110
Bovine -ASGVSSCMGDSGGPLVCKKNGAWTLVGIVSWGSST-CSTSTPGVYARVTALVNWVQQTL* 260
S/G189

Figure 3.2 Alignments of dissimilar 3′ UTR regions of *Heliothis virescens* trypsin- **(A)** and chymotrypsin-like **(B)** cDNA sequences using ClustalW. "*" below the alignments indicate identical residues across all sequences. ":" and "." symbols below the alignments indicate conserved and semi-conserved substitutions. Stop codon residues are represented in bold letters and underlined.

A

```
HvT3|          TAAAAAGTGA-----TTTGCATAGGTATCATTAAAA---AGTGTTA-GATGATTTTATAA
HvT1|          TAAAAAGTGA-----TTCGCAT----ATCATTAAAA---AGTGTTAAGATGATTTTCTAA
HvT4|          TAAAAAGTGA-----TTTGGA------------AAA---AGTGTTAAGATGATTTTCTAA
HvT2|          TAAGAAGGGAAGATTTTTTCTTTGGCACAATGTTGACTTAATGAATAAATATTTTTTTTA
               *** *** **     **                    *   * **     **  **** * *

HvT3|          AAAAATTAAAGCTATAGACTA
HvT1|          AAAAATAAAAGCTATAAATT-
HvT4|          AAAAATAAAAGCTATAAATTG
HvT2|          CAATGG---------------
                **
```

B

Group-1

```
HvC3|          TAAGAAACAT--AAAAACTTTGTCTGGAACTATGACGCAAACAAAACTTACTTAAAATAA
HvC4|          TAAGAAGCATAAAAAAACTTTGTCTGAAAATATAACGTAAATAAAATTTACTTGAAATAA
HvC1|          TAAGATGTTTATGGAAGACTTTATTTGTTTTG-ATCGGATTAAAATATGACTTAATACAA
HvC2|          TAAGATGTTTATGGAACACTTTACTGGTTTTG-ATCGG---AAAATATGACTTGATACAA
               *****:. :*  ..** . ** : * .:: *. .:**     ***: * ****.*:* **

HvC3|          AATATAAGCGTAACTCATTGCT
HvC4|          AATATAAGCGTAACTCATTGTT
HvC1|          TTTTAAAGTTTTTTTATC----
HvC2|          TTTT-----------------
               ::*:
```

Group-2

```
HvC5|          TAATCTCCTCCTCTAACAGTTAAAACAACTTAGAATTCTTAAATAAAAATAACTTTTTTT
HvC7|          TAATCTCCTCCTCTCTCA--------AACTTATAATGCCTCAATTAAATTTATTTTCCTG
HvC6|          TAATCTCTTTTTCTCACA--------AACTTATAATGCCATAATTAAATTTATTTCCTTC
               ******* *  ***  **        ****** *** *   *** *** * * **   *

HvC5|          CT-
HvC7|          CA-
HvC6|          AGC
```

Group-3

```
HvC9|          TGAATTTAA-CT--AGGTTTT--AATTGTTAACTACTAATG-ATACG---AATGAATAAA
HvC10          TAAATATCAGCATAACATTTTGCCAAACAGAGCTATTTAAGAATACGTTAATTAAAATAA
HvC8|          TGAAAAGCT------TTTTTAACTTGGCCTTTATAAAATATATAACG---GTTGATATAC
               *.**:: .:        ***:   :     : .** ::::  ::***   .:*.*:::*.

HvC9|          ATAATGAT--TTC
HvC10          ATATTTTTTATTC
HvC8|          -------------
```

Figure 3.3. Phylogenetic analyses of *Heliothis virescens* trypsin-like (HvT) **(A)** and chymotrypsin-like (HvC) **(B)** proteinases with other heliothine serine proteinases. Sequences were aligned and a neighbor joining bootstrap tree with 1000 bootstrap iterations was created using ClustalX v1.8. Phylogenetic trees were drawn using Tree View (Page, 1996). Genbank accession numbers of nucleotide sequences of *Helicoverpa armigera* (Ha), *Helicoverpa zea* (Hz), *Helcoverpa punctigera* (Hp) and *Heliothis virescens* (Hv) are presented next to the clone names.

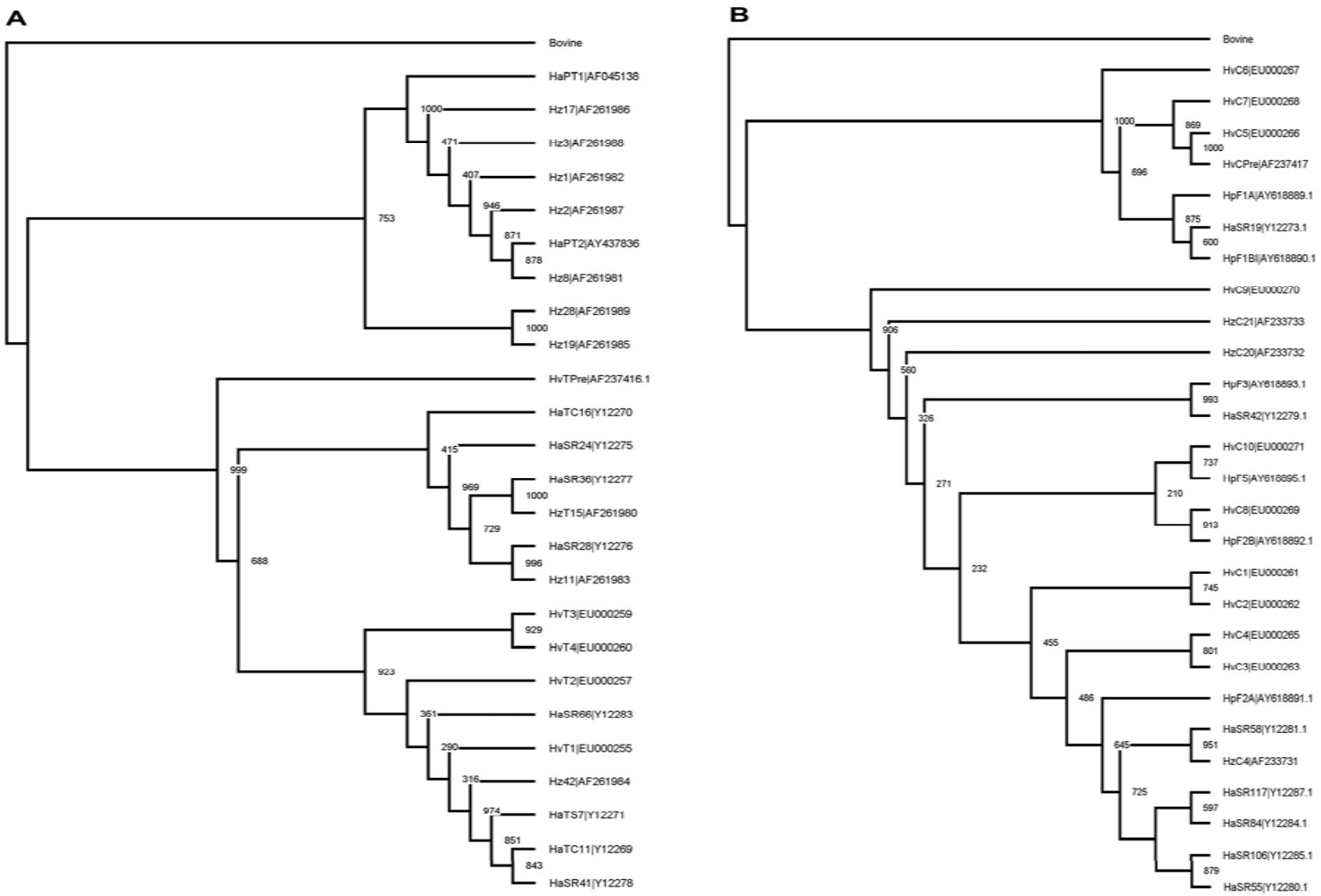
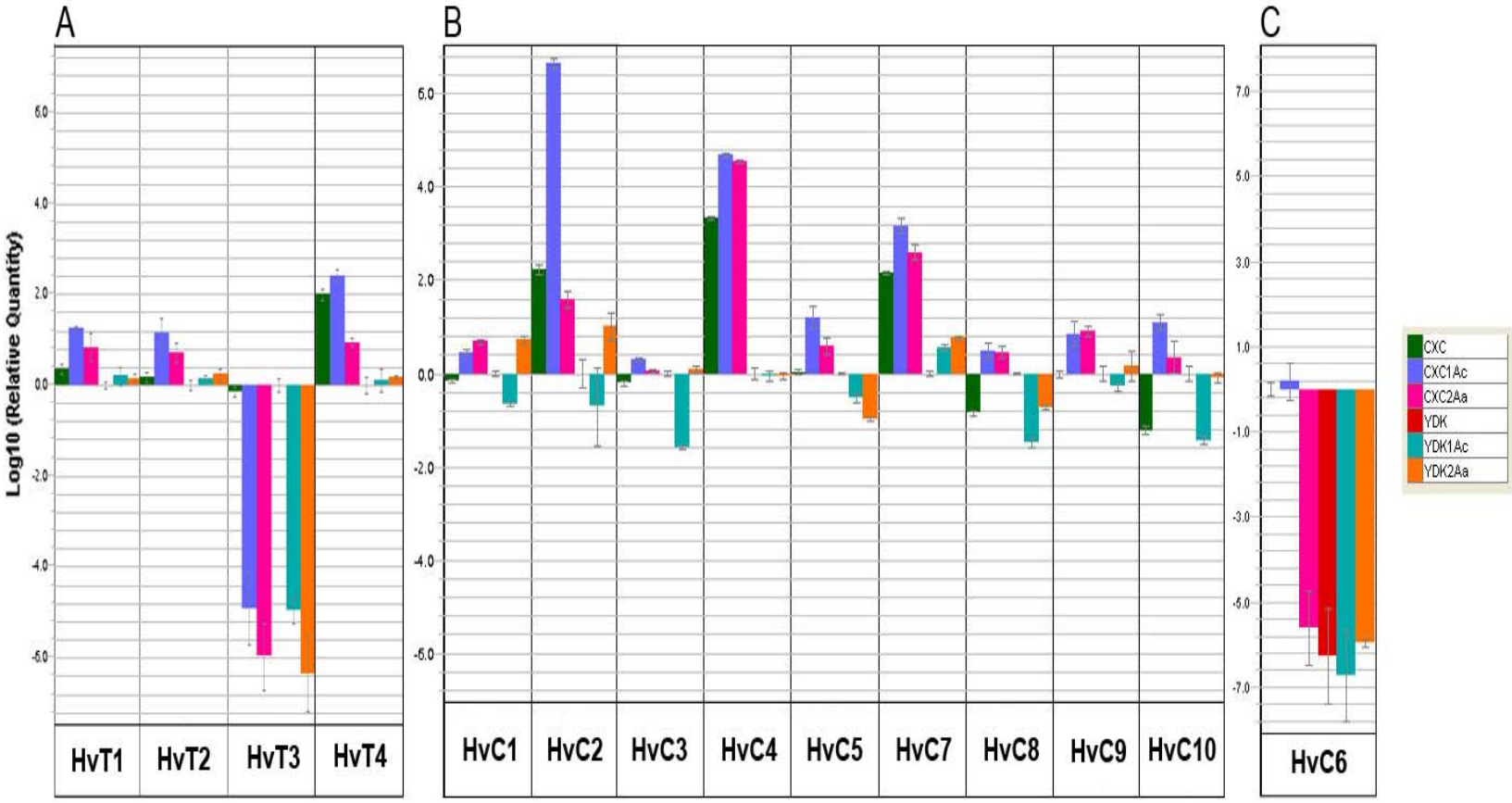

A
Bovine
HaPT1|AF045138
Hz17|AF261986
Hz3|AF261988
Hz1|AF261982
Hz2|AF261987
HaPT2|AY437836
Hz8|AF261981
Hz28|AF261989
Hz19|AF261985
HvTPre|AF237416.1
HaTC16|Y12270
HaSR24|Y12275
HaSR36|Y12277
HzT15|AF261980
HaSR28|Y12276
Hz11|AF261983
HvT3|EU000259
HvT4|EU000260
HvT2|EU000257
HaSR66|Y12283
HvT1|EU000255
Hz42|AF261984
HaTS7|Y12271
HaTC11|Y12269
HaSR41|Y12278
1000
471
407
946
871
878
753
1000
999
415
969
1000
729
996
688
929
923
361
290
316
974
851
843
B
Bovine
HvC6|EU000267
HvC7|EU000268
HvC5|EU000266
HvCPre|AF237417
HpF1A|AY618889.1
HaSR19|Y12273.1
HpF1Bl|AY618890.1
HvC9|EU000270
HzC21|AF233733
HzC20|AF233732
HpF3|AY618893.1
HaSR42|Y12279.1
HvC10|EU000271
HpF5|AY618895.1
HvC8|EU000269
HpF2B|AY618892.1
HvC1|EU000261
HvC2|EU000262
HvC4|EU000265
HvC3|EU000263
HpF2A|AY618891.1
HaSR58|Y12281.1
HzC4|AF233731
HaSR117|Y12287.1
HaSR84|Y12284.1
HaSR106|Y12285.1
HaSR55|Y12280.1
1000
869
1000
696
875
600
906
560
326
993
271
737
210
913
232
745
455
801
486
645
951
725
597
879

Figure 3.4. Quantitative RT-PCR analysis of *H. virescens* trypsin-like (HvT) **(A)** and *H. virescens* chymotrypsin-like (HvC) **(B & C)** mRNAs in midguts of control and toxin fed insects. The relative mRNA quantities are expressed in the Log_{10} scale and are normalized to YDK control diet treatment and ribosomal protein S18 (RBPS18) endogenous control except in **(C)** where relative quantities are normalized to CXC diet control and RBPS18. The error bars indicate standard deviations representing 99.9% C.I of three biological replicates and three technical replicates each.



SCALE: Log_{10} (2) = 100-fold difference

Table 3.1. Classification of *H.*virescens trypsin **(A)** and chymotrypsin-like **(B)** cDNA.

A

Trypsin-Like	cDNA Frequency[#]	Genbank Accession Number	Region in ORF
HvT1	0.6127	EU000255	~603bp
HvT2	0.2465	EU000257	
HvT3	0.0704	EU000259	
HvT4	0.0704	EU000260	

B

Chymotrypsin-Like	cDNA Frequency[#]	Genbank Accession Number	Region in ORF	Group
HvC1	0.4571	EU000261	~350bp	Group-1
HvC2	0.0539	EU000262		
HvC3	0.2754	EU000263		
HvC4	0.0758	EU000265		
HvC5	0.0110	EU000266	~410bp	Group-2
HvC6	0.0120	EU000267		
HvC7	0.0329	EU000268		
HvC8	0.0529	EU000269	~354bp	Group-3
HvC9	0.0130	EU000270	~365bp	
HvC10	0.0160	EU000271	~335bp	

[#] cDNA frequencies were calculated by dividing the total number of sequences for each transcript by total number of trypsin or chymotrypsin-like sequences multiplied by 100.

Table 3.2. SNP positions and allele frequencies in trypsin and chymotrypsin-like cDNA of diet-only control and toxin fed *H. virescens* populations

Transcript	Population[a]	SNP position	A 1[b]	A 2[c]	Codon and Amino acid Coded	A 1 [d] Frequency	A 2 [d] Frequency	Total Seq
HvT1	1a	63	G		GCG> 'Ala'	1	0	84
	2a	63	G		GCG> 'Ala'	1	0	92
	3b	63	A	G	GCA/GCG> 'Ala'	0.923	0.077	91
	4b	63	A	G	GCA/GCG> 'Ala'	0.706	0.294	95
	5c	63	A		GCA> 'Ala'	1	0	88
	6c	63	A	G	GCA/GCG> 'Ala'	0.944	0.055	72
	1a	327	G		TCG> 'Ser'	1	0	84
	2a	327	G		TCG> 'Ser'	1	0	92
	3b	327	T		TCT> 'Ser'	1	0	91
	4b	327	T		TCT> 'Ser'	1	0	95
	5c	327	T		TCT> 'Ser'	1	0	88
	6c	327	T		TCT> 'Ser'	1	0	72
	1a	333	G		CAG> 'Gln'	1	0	84
	2a	333	G		CAG> 'Gln'	1	0	92
	3b	333	A		CAA> 'Gln'	1	0	91
	4b	333	A		CAA> 'Gln'	1	0	95
	5c	333	A		CAA> 'Gln'	1	0	88
	6c	333	A		CAA> 'Gln'	1	0	72
	1a	440	A	G	AAC> 'Asn'	0.957	0.043	84
	2a	440	A		AAC> 'Asn'	1	0	92
	3b	440	G	A	AGC> 'Ser'	0.861	0.139	91
	4b	440	G	A	AGC> 'Ser'	0.811	0.189	95
	5c	440	G		AGC> 'Ser'	1	0	88
	6c	440	G	A	AGC> 'Ser'	0.652	0.348	72
	1a	471	T	C	TCT/TCC> 'Ser'	0.939	0.041	84
	2a	471	T		TCT> 'Ser'	1	0	92
	3b	471	C	T	TCC/TCT> 'Ser'	0.861	0.139	91
	4b	471	C	T	TCC/TCT> 'Ser'	0.774	0.189	95

	5c	471	C		TCC> ‘Ser’	1	0	88
	6c	471	C	T	TCC/TCT> ‘Ser’	0.870	0.087	72
HvT2	1a	90	T		GGT> ‘Gly’	1	0	47
	2a	90	C	T	GGC/GGT> ‘Gly’	0.6	0.4	27
	3b	90	C		GGC> ‘Gly’	1	0	34
	4b	90	C		GGC> ‘Gly’	1	0	38
	5c	90	C		GGC> ‘Gly’	1	0	29
	6c	90	C		GGC> ‘Gly’	1	0	35
	1a	381	C		AAC> ‘Asn’	1	0	47
	2a	381	T	C	AAT/AAC> ‘Asn’	0.571	0.428	27
	3b	381	C		AAC> ‘Asn’	1	0	34
	4b	381	C		AAC> ‘Asn’	1	0	38
	5c	381	C	T	AAC/AAT> ‘Asn’	0.889	0.111	29
	6c	381	C	T	AAC/AAT> ‘Asn’	0.595	0.404	35
	1a	522	T		GGT> ‘Gly’	1	0	47
	2a	522	C	T	GGC/GGT> ‘Gly’	0.571	0.428	27
	3b	522	T		GGT> ‘Gly’	1	0	34
	4b	522	T	C	GGT/GGC > ‘Gly’	0.972	0.027	38
	5c	522	T	C	GGT/GGC> ‘Gly’	0.882	0.118	29
	6c	522	T	C	GGT/GGC> ‘Gly’	0.548	0.452	35
HvC3	1a	308	T	C	GCT/GCC> ‘Ala’	0.941	0.059	81
	2a	308	C	T	GCC/GCT> ‘Ala’	0.986	0.014	82
	3b	308	C	T	GCC/GCT> ‘Ala’	0.895	0.105	31
	4b	308	C	T	GCC/GCT> ‘Ala’	0.5	0.5	27
	5c	308	T		GCT> ‘Ala’	1	0	26
	6c	308	C		GCC> ‘Ala’	1	0	29

[a] Diet-only control and toxin fed strains labeled as: 1a =YDK diet-only control; 2a = CXC diet-only control; 3b = YDK Cry2Aa fed; 4b = YDK Cry1Ac fed; 5c = CXC Cry2Aa fed ; 6c = CXC Cry1Ac fed.

[b] A 1 = Allele 1 frequencies

[c] A 2 = Allele 2

[d] Allele frequencies were calculated by dividing the total number of occurrences by the total number of sequences for that population multiplied by 100.

Table 3.3. Amplicon sizes and primers designed to amplify regions of *H.virescens* trypsin and chymotrypsin-like sequences in rqRT-PCR using SYBR green.

Primer	5' to 3' sequence orientation	Amplicon size (bp)
HvT1 –F	ATGCTCGCGTATCTCGCTACA	81
HvT1 –R	TCATCTAACACTTTTTAATGATACC	
HvT2 –F	TATTGGATGTGCTCAGGCTCAA	112
HvT2 –R	TATTTATTCATTAAGTCAACATTGTG	
HvT3 -F	TCAATGCTCGCGTATCTCGCT	90
HvT3 –R	TCATCTAACACTTTTTAATGATACC	
HvT4 –F	TCAATGCTCGCGTATCTCGCT	85
HvT4 –R	TTAGAAAATCATCTTAACACTTTTTC	
HvC1 -F	CTGCTGCCTTCGCCAGAGT	93
HvC1 -R	AATCCGATCAAAACAAATAAAGTC	
HvC2 -F	CTGCTGCCTTCGCCAGAGT	91
HvC2 -R	TCCGATCAAAACCAGTAAAGTG	
HvC3 -F	GCGGTTGCCAAGTTGGTTCA	104
HvC3 -R	TAGTTCCAGACAAAGTTTTTATGT	
HvC4 -F	GCGGTTGCCAAGTTGGTTCA	105
HvC4 -R	ATTTTCAGACAAAGTTTTTTTATGC	
HvC5 -F	CGGTTGCGCCGCTGGTTTC	109
HvC5 -R	AGAATTCTAAGTTGTTTTAACTGTT	
HvC6 -F	TGATCGGTGTTACGTCATACG	114
HvC6 -R	AAAGAGATTAAACAAGGGACTGA	
HvC7 -F	CGGTTGCGCCGCTGGTTTC	101
HvC7 -R	GGCATTATAAGTTTGAGAGAGGA	
HvC8 -F	CTGCTCGCGGTTGCCAAGT	107
HvC8 -R	TAAAGGCCAAGTTAAAAAAGCTTT	
HvC9 -F	CTCGCGGTTGCCAAGTAGGT	107
HvC9 -R	TAGTTAACAATTAAAACCTAGTTAAA	
HvC10 -F	CCGTGGTTGCCAGGTTGGA	103
HvC10 -R	TTTGGCAAAATGTTATGCTGATAT	

Table 3.4 Relative quantities of *H.virescens* trypsin and chymotrypsin like mRNA

	Log_{10} Relative Quantities †					
cDNA	**YDK**	**CXC**	**YDK (Cry2Aa)**	**YDK (Cry1Ac)**	**CXC (Cry2Aa)**	**CXC (Cry1Ac)**
HvT1	0.00	0.37	0.15	0.21	0.84	1.26 ↑
HvT2	0.00	0.18	0.24	0.14	0.70	1.14 ↑
HvT3	0.00	-0.14	-6.34 ↓↓↓	-4.95 ↓↓↓	-5.97 ↓↓↓	-4.92 ↓↓↓
HvT4	0.00	2.00 ↑↑	0.18	0.10	0.94	2.41 ↑↑
HvC1	0.00	-0.16	0.74	-0.66	0.68	0.46
HvC2	0.00	2.23 ↑↑	1.00 ↑	-0.70	1.59 ↑	6.66 ↑↑↑
HvC3	0.00	-0.20	0.07	-1.58 ↓	0.06	0.30
HvC4	0.00	3.34 ↑↑	-0.06	-0.04	4.56 ↑↑↑	4.70 ↑↑↑
HvC5	0.00	0.03	-0.99	-0.51	0.60	1.21 ↑
HvC6 *	-6.26 ↓↓↓	0.00	-5.97 ↓↓↓	-6.73 ↓↓↓	-5.60 ↓↓↓	0.18
HvC7	0.00	2.15 ↑↑	0.78	0.55	2.60 ↑↑	3.17 ↑↑
HvC8	0.00	-0.86	-0.74	-1.49 ↓	0.45	0.49
HvC9	0.00	-0.01	0.15	-0.28	0.90	0.85
HvC10	0.00	-1.21 ↓	-0.09	-1.43 ↓	0.36	1.11 ↑

† Relative quantities of mRNA are averages of three technical replicates and three biological replicates (5 Larvae each) at 99.9% C.I. All samples are normalized to YDK except where indicated otherwise.

* Samples are normalized to CXC.

Up (↑) and down (↓) arrows indicate up or down-regulation, with multiple arrows indicating strongly regulated transcripts.

CHAPTER 4

CONCLUSIONS

Toxin activation by midgut serine proteinases is an important step in the mode of action of Cry toxins. Studies on three different laboratory-selected Bt-resistant strains of *H. virescens* showed that toxin resistance in these strains occurs by multiple mechanisms (Jurat-Fuentes et al., 2003). While altered toxin-binding to receptors is the most widely reported mechanism of Bt resistance (Ferre and Van Rie, 2002), proteinase-related mechanisms of resistance have also been reported (Li et al., 2004; Oppert et al., 1997). This dissertation describes (1) The analysis of midgut proteinases from Bt-susceptible and -resistant *H. virescens*, and (2) The altered sequence and gene expression of trypsin and chymotrypsin-like mRNA.

1. The analysis of midgut proteinases from Bt susceptible and resistant *H. virescens*

Comparative studies of brush border membrane vesicle (BBMV) proteins from Bt-susceptible and -resistant strains of the tobacco budworm, *H. virescens,* have served to elucidate different mechanisms by which these insects develop toxin resistance in the laboratory. Although altered toxin binding to receptors is the dominant mechanism of resistance in these strains (Jurat-Fuentes and Adang, 2004a; Jurat-Fuentes et al., 2004b; Lee, 1995), alternate mechanisms of resistance have also been suggested (Forcada et al., 1999; Forcada, 1996; Jurat-Fuentes and Adang, 2006; Jurat-Fuentes et al., 2003).

In Chapter Two of this dissertation I discuss the potential role of proteinase alterations that may contribute to toxin resistance in these strains. Midgut pH and digestive proteinases from the Bt resistant strains YHD2-B, KCBhyb and CXC were analyzed and compared to the Bt susceptible YDK larvae. Because gut pH determines toxin solubility and enzymatic activity, I measured the gut pH of the susceptible and resistant *H.virescens* larvae. pH measurements indicated a significantly alkaline midgut in the resistant strains YHD2-B and KCBhyb when

compared to resistant strain, CXC, and the susceptible strain, YDK. A time-course incubation of Cry1Ac and Cry2Aa protoxins with gut extracts from the susceptible and resistant strains indicated differential rates of protoxin processing. Enzymes in CXC gut extract had the slowest rate of Cry1Ac toxin activation compared to gut extracts from susceptible or other resistant strains. Enzymes in both CXC and YHD2-B had slower rates of Cry2Aa toxin activation when compared to gut extracts from KCBhyb and YDK. Intermediates of protoxin processing observed in incubations with CXC and YHD2-B gut extracts indicated a less effective toxin activation process in both these strains compared to YDK and KCBhyb strains. Cry1Ac resistance in YHD2-B is attributed to altered toxin binding to HevCaLP and HvALP (Jurat-Fuentes and Adang, 2004a; Jurat-Fuentes et al., 2004b), whereas there is no such mechanism of altered toxin toxin binding evident in CXC. My results are evidence that a proteinase-mediated resistance mechanism could be more important in CXC than YHD2-B.

Zymogram analysis of proteinase activities in gut extracts from the different strains revealed 11 distinct caseinolytic activities and subtle qualitative and quantitative differences between the different strains. Kinetic and activity blot assays with trypsin and chymotrypsin-specific substrates were used to obtain quantitative and qualitative data for trypsin and chymotrypsin-like proteinase activities. Activity blots showed the absence of a ~32 kDa trypsin-like proteinase in gut extracts from the YHD2-B strain and a ~35 kDa chymotrypsin-like proteinase in gut extracts of the CXC strain. The down-regulation of trypsin-like proteinases is a mechanism adapted by lepidopteran larvae to reduce the rate of toxin activation (Li et al., 2004; Oppert et al., 1996) and so could confer some degree of Bt resistance in YHD2-B. Elevated chymotrypsin-like activity in gut extracts of CXC, as inferred from kinetic assays, may represent a compensatory alteration for the loss of the ~35 kDa proteinase.

Overall, my results suggest that the reduced toxin activating ability of enzymes in gut extracts of YHD2-B should be considered as a potential resistance causing factor in addition to altered toxin binding in this strain. The reduced ability to process protoxin has been suggested previously in the CXC parental strain CP73-3 (Forcada, 1996). The absence of a mechanism of resistance involving reduced toxin binding in the CXC strain implies that a proteinase-related mechanism as discovered in my analyses could contribute significantly to Cry1Ac and Cry2Aa resistance.

2. Altered sequence and gene expression of trypsin and chymotrypsin-like mRNA.

Genetic linkage analysis of the *H. virescens* resistant strains, YHD2-B and the CXC parental strain, CP73, shows that both *BtR4* and *BtR5* resistance alleles are responsible for Cry1Ac resistance in these strains (Gahan et al., 2005). Neither of these resistance alleles was found to contribute to Cry2Aa resistance in the CP73 strain or its descendent CXC (Gahan et al., 2005). Since Griffitts et al. (2003; 2001) showed multiple gene resistance to Cry5B in *Caenorhabditis elegans*, this was only the second study to show that resistance to a single toxin can be attributed to multiple genes (Gahan et al., 2005). In CXC, the lack of an altered toxin-binding related resistance mechanism, and the reported presence of multiple resistance genes contributing cumulatively to resistance, makes it an ideal candidate to study alternative mechanisms of resistance (Gahan et al., 2005; Jurat-Fuentes et al., 2003). In Chapter Two, we proposed that the slower processing of Cry1Ac and Cry2Aa protoxins could be an important resistance causing factor in the Bt resistant strain, CXC. The expression and regulation of serine proteinases in response to Bt toxins and proteinase inhibitors has been well studied in other lepidopteran insects (Bown et al., 1997; Bown et al., 2004; Coates et al., 2006; Diaz-Mendoza et al., 2005; Gatehouse

et al., 1997; Hegedus et al., 2003; Mazumdar-Leighton and Broadway, 2001; Volpicella et al., 2006; Zhu et al., 2000; Zhu et al., 1997). Since these enzymes are the most commonly occurring proteinases in the *H. virescens* midgut (Johnston, 1995), and because I found indirect evidence suggesting a possible role for a proteinase-related mechanism of resistance in CXC (Chapter Two), I designed experiments to identify individual trypsin- and chymotrypsin-like midgut cDNA and compare their gene expression patterns in insects fed on diet alone or diet containing either Cry1Ac or Cry2Aa toxins. In Chapter Three of this dissertation, the variation in proteinase sequence and their differential regulation in response to feeding on toxin are discussed.

The cloning and sequencing of partial sequences of trypsin- and chymotrypsin-like cDNAs from midguts of diet and toxin fed YDK and CXC insects led to the identification of four trypsin-like (HvT) and ten chymotrypsin-like (HvC) cDNAs. BLASTX searches indicated a high degree of homology to other insect serine proteinases, especially to other heliothine species. The highly similar coding regions and dissimilar 3' UTR's present in the four HvT sequences was also supported by phylogenetic analyses indicating that they are probably allelic variants of a single gene type. The possible presence of other trypsin isoforms, however, cannot be excluded since I did not use additional trypsin-specific primers to amplify these sequences. Translated amino-acid sequences of trypsin and chymotrypsin-like cDNAs show the presence of numerous arginines and an overall reduction of lysines. This adaptation, which is characteristic of insect proteinases, is thought to stabilize the S1 binding pocket in the highly alkaline insect midgut (Peterson et al., 1994). Group-2 chymotrypsin-like sequences show the presence of Gly_{189} instead of a Ser_{189} which is an unusual adaptation and could result in altered substrate specificity (Botos et al., 2000).

Sequence variations in the form of synonymous and non-synonymous nucleotide changes were observed in trypsin and chymotrypsin cDNA obtained from midguts of toxin fed insects. Although most of the changes were synonymous, one polymorphism results in the change of Asn to Ser in YDK and CXC toxin fed insects. This transition lies within the chymotrypsin loop-1 region that determines substrate specificity (Hedstrom et al., 1992). This alteration may therefore result in altered substrate binding affinity and hence have important implications for toxin susceptibility in intoxicated larvae. Although synonymous mutations do not result in amino-acid changes, they are known to alter codon choice which can affect downstream processes such as transcription and translation (Kimchi-Sarfaty et al., 2007; Parmley and Hurst, 2007). The effect of SNPs on the regulation of proteinase genes and their contribution to Bt resistance in general needs to be studied further.

Relative quantitative real-time PCR (rqRT-PCR) analyses of trypsin- and chymotrypsin-like transcript abundance revealed the differential expression of these transcripts in the Bt-susceptible, YDK and –resistant CXC larvae. Overall, trypsin- and chymotrypsin-like transcripts were generally upregulated in CXC irrespective of the diet they were fed. Although HvT1, T2 and T4 were upregulated in CXC fed on diet containing either Cry1Ac or Cry2Aa toxins, the up-regulation of HvT1 and T2 needs to be interpreted with caution as the PCR primers used were not designed to discriminate between the wild-type and SNP variants. The "knockdown" of HvT3 in toxin-fed YDK and CXC larvae is a physiological response to intoxication similar to that observed for other toxin-fed insects such as *P. interpunctella*, *O. nubilalis* and *C. fumiferana* (Huang et al., 1999; Oppert et al., 1997; van Munster et al., 2007).

Toxin feeding also induced the further up-regulation of chymotrypsin-like transcripts in CXC when compared to YDK. The up-regulation of chymotrypsins has been observed in *H. armigera*

feeding on proteinase inhibitors (Gatehouse et al., 1997). The degradation of Bt strain HD-1 and HD73 toxins by immobilized chymotrypsin (and by chymotrypsin-like enzymes present in gut extracts of *H. armigera*) reportedly results in reduced insecticidal activity (Indrasith et al., 1991; Shao et al., 1998). If similar mechanisms are present in CXC, the observed up-regulation of HvC transcripts may be responsible for toxin degradation and reduced susceptibility in toxin fed CXC larvae. Alternatively, the up-regulation of chymotrypsin-like transcripts could also be a result of the down-regulation of other proteinases more directly involved in toxin processing. HvC6 is absent in YDK (diet and toxin-fed) and is down-regulated in Cry2Aa-fed CXC indicating a toxin-specific response. Overall, the observed up-regulation of chymotrypsin transcripts in CXC correlates with proteinase activity data presented in Chapter Two. However, I could not detect the down-regulation of a chymotrypsin-like proteinase as stated in Chapter Two, possibly because of the low levels of expression of this proteinase.

Conclusions

The laboratory selection of *H. virescens* strains using Cry1Ac and Cry2Aa toxins has led to the development of multiple resistance mechanisms in these strains. The diverse trypsin and chymotrypsin-like proteinases present in the insect midgut have both digestive and protective functions. The results obtained from the biochemical and molecular analyses of midgut proteinases of Bt resistant strains of *H.virescens* demonstrates that altered proteinases may play a potential role in Bt resistance.

A comparison of midgut proteinases in YHD2-B, CXC and KCBhyb revealed evidence that altered proteinase levels should be considered as a potential resistance factor in the YHD2-B and CXC strains. The identification of trypsin- and chymotrypsin-like transcripts from diet and

toxin-fed YDK and CXC, as well as their gene expression analyses present evidence that the differential regulation of specific proteinase genes may contribute to the reduced susceptibility to Bt toxins in the CXC strain.

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