IDENTIFICATION OF NOVEL CRY1AC BINDING PROTEINS IN THE MIDGUT OF *HELIOTHIS VIRESCENS* USING PROTEOMIC ANALYSES

by

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(Under the Direction of MICHAEL J. ADANG)

ABSTRACT

Bacillus thuringiensis (Bt) toxins are specifically toxic to larvae in the taxa Lepidoptera, Diptera and Coleoptera. *Heliothis virescens*, commonly known as the tobacco budworm, is the major crop pest controlled by Bt cotton. In the past, brush border membrane vesicles of insects have been researched to identify toxin-binding proteins because of their significance in eliciting resistance to toxins. However several of the components are still unknown because of inadequate resolution of such complex samples on a single dimension polyacrylamide gel. In this study, we identify novel Cry1Ac binding proteins by the use of two-dimensional gel resolution. A subproteome of glycosylphosphatidyl inositol (GPI) anchored proteins was analyzed because several known Cry1Ac binding proteins are GPI anchored. Peptide mass fingerprints generated from the spots of interest were searched in protein databases. Alkaline phosphatase and V-ATPase subunits A and B were identified as novel Cry1Ac binding proteins, the former is GPI anchored, while the latter are not.

INDEX WORDS: *Bacillus thuringiensis*, *Heliothis virescens*, Cry1Ac, GPI anchored proteins, proteomics, alkaline phosphatase, V-ATPase

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DEDICATION

This work is dedicated to my parents, Gowri Krishnamoorthy and Krishnamoorthy. I would not be where I am today and would not have accomplished all the things I have without your constant love and support. There are no words to express the appreciation I have towards you for all you have given me and taught me.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Civilization began with agriculture. Human society was forever changed when our nomadic ancestors settled and grew their own food. Since then, crop productivity plagued by insect pests has been one of the greatest concerns of a country's economy. Though chemical insecticides have immensely contributed in this regard, undesirable residues in food, water, and the environment and toxicity to humans and other animals that accompany their use is a cause of concern. As an alternative, insecticidal crystal proteins of *Bacillus thuringiensis*, help achieve a safer approach to pest control.

Bacillus thuringiensis:

Bacillus thuringiensis, a gram-positive soil bacterium, has established itself as the cynosure of the agricultural biotechnology industry over the past two decades due its range of insecticidal activities. This bacterium synthesizes parasporal crystalline inclusion bodies during the stationary phase of its growth (sporulation), which are composed of Cry proteins (also called δ-endotoxins). Closely related species such as *Bacillus cereus* do not produce Cry proteins and hence Cry proteins differentiate these organisms (Schnepf et al., 1998). In addition, Bt produces virulence factors active against insects such as exotoxins, phospholipases, cytolytic toxins (Cyt toxins) and vegetative insecticidal proteins (VIPs) (Tanada and Kaya, 1993). VIPs are not classified as Cry proteins because they do not form crystals, are not homologous at the nucleic acid or protein levels and are secreted from the cell. Some Cry proteins such as Cry3Aa are produced during the vegetative and sporulation phases of Bt growth. Bt also produces antifungal compounds (Stabb et al., 1994); which can act to synergize Cry-induced intoxication of lepidopterans (Manker et al., 1994). Toxicity of Cry proteins is prominent over a wide range of

insects Orders including Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, and Phthiraptera. Bt strains with activity against Platyhelminthes, Nemathelminthes and Sarcomastigophora are reported (Feitelson, 1993). However Cry proteins apparently do not affect vertebrates (Delannay et al., 1989). Owing to their high specificity and environment friendly nature, Cry protein-based biopesticides have been widely accepted as an alternative to synthetic pesticides.

Insecticides in the form of sprays of Bt spores and crystals have limited field stability, mostly due to inactivation by sunlight. To overcome this problem, *cry* genes were introduced in plants to produce transgenic cultivars that synthesized their own insecticidal toxins (Adang et al., 1993; Perlak et al., 1991). Tobacco plants expressing a Cry1Ab toxin were the first transgenic Bt crops to be field-tested. Maize, cotton and potato have also been genetically engineered and since 1996 produced commercially in the United States (Betz et al., 2000; De Maagd et al., 1999). Transgenic crops offer a number of advantages in that they do not harm other species and are environment friendly (Betz et al., 2000). However the risk of horizontal and lateral gene transfer to other plants has created a wave of suspicion over Bt transgenic crops (De Maagd et al., 1999).

Cry toxin classification and structure:

Currently there are about 250 Cry proteins classified in the Bt toxin database (http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/). Originally Cry proteins were classified based on protein similarity and insecticidal specificity. This dual classification became cumbersome because some Bt Cry proteins kill different groups of insects. Crickmore et al.

(1998) established a nomenclature for Cry toxin classification based entirely on amino acid sequence identity. Cry proteins were assigned Arabic numerals for the primary rank, capital letters for the secondary rank and lowercase letters for the tertiary and quaternary ranks following the extent of amino acid sequence identity (Crickmore et al., 1998).

Despite having different insect specificities, Cry toxins have similar overall tertiary structures. X-ray crystallography has resolved the structure of Cry toxins: Cry3Aa (Li et al., 1994), Cry1Aa (Grochulski et al., 1995), and Cry2Aa (Morse et al., 2001) - each is comprised of three domains (Figure 1.5). The N-terminal domain I consists of a bundle of seven amphipathic α-helices wherein six of them surround a central hydrophobic helix. Domain II has three antiparallel β-sheets with a Greek key conformation arranged in a prism fold while the C terminal domain III contains two antiparallel β-sheets in a jellyroll topology (Li et al., 1991).

Domain I is constituted by amphipathic and hydrophobic helices that could render it responsible for membrane insertion and pore formation in the insect gut epithelium. According to the most accepted model, a central hydrophobic α-helical hairpin formed in the center of the alpha helical bundle inserts in the membrane of the host cell, leaving the rest of the helices to flatten out on the exterior of the membrane (Li et al., 1991). Comparison of the Cry3A domain I helices with other known classes of amphipathic helices suggests that many of the helices (in particular α1, α5, and α6) show features characteristic of lytic peptides (Thompson et al., 1995).

Domains II-III bound reversibly to insect midgut membranes, suggesting that domain I could be involved in irreversible binding while the other two in binding specificity (Flores et al., 1997). Domain II has three surface exposed loops that are highly variable (Grochulski et al., 1995). Site-directed mutagenesis and segment swapping experiments provided evidence that the loops of domain II are involved in receptor binding. Exchange of domain II segments between

closely related toxins led to changes in specificity, demonstrating the role of this domain in binding specificity (Schnepf et al., 1990; Ge et al., 1991). The β sheets of domain II have a Greek key structure that bears similarity with jacalin, a structure involved in binding carbohydrates via its exposed loops (Sankaranarayanan et al., 1996).

Domain III has a multifunctional role in toxin action. Li et al. (1991) suggested that domain III functions in maintaining the structural integrity of the toxin molecule, perhaps by protecting it from proteolysis within the gut of the target organism. Mutations in domain III of Cry1Ac resulted in a modified toxin with reduced binding and lower toxicity to brush border membrane vesicles (BBMV) prepared from *Manduca sexta* and *Heliothis virescens* (Aronson et al., 1995). Exchanges of domain II and domain III yielded toxins with altered specificity and increased activities against several lepidopterans (Ge et al., 1989; Ge et al, 1991).

An important feature of Cry1Ac binding is that N-acetylagalactosamine (GalNac) is required on the putative receptor aminopeptidase, for binding to occur (De Maagd et al., 1999). Burton et al. (1999) showed that addition of free GalNAc inhibits binding of Cry1Ac to intact membranes of *M. sexta* thus causing a reduction in pore formation. This indicated that GalNac on the receptor is involved in Cry1Ac binding and toxicity to insects. A specific pocket located on the external surface of Cry1Ac domain III of Cry1Ac binds GalNAc (Li et al., 1999; Burton et al., 1999).

To summarize, domain I is involved in pore formation, domain II in binding specificity and domain III in ion channel function and receptor binding. Interaction among these three domains is important for toxin activity (Rang et al., 1999).

Cry toxin processing and mode of action:

The mechanism of action of the *B. thuringiensis* Cry proteins is a multi-step process that involves solubilization of the crystal in the insect midgut, proteolytic processing of protoxin by midgut proteases, binding of the Cry toxin to midgut receptors, and insertion of the toxin into the apical membrane to create ion channels or pores (Schnepf et al., 1998). Fig 1.1 shows a diagram of the steps involved in Cry protein toxicity.

The crystal inclusions of the spores are solubilized under the alkaline conditions of the insect midgut releasing the protoxin form (Hofmann et al., 1988b). Lepidopteran midguts are characterized by high serine protease activity at pH optima of 10-11 (Purcell et al., 1992). The protoxin is activated by trypsin-like (Lecadet and Dedonder, 1966; Milne, 1993) or chymotrypsin-like (Johnston, 1995; Novillo, 1997) midgut proteases. Protoxin processing starts at the C terminus and proceeds toward the 55- to 65-kDa toxic core by removal of 28 amino acids from the N-terminus and 500 amino acids from the C-terminus (Milne and Kaplan, 1993). The toxin core is resistant to further proteolysis (Choma et al., 1990). N-terminal activation is essential for insecticidal activity and its absence leads to reduced toxin activity (Bravo et al., 2002).

Activated Cry toxins have two known functions, receptor binding and ion channel activity. They pass through the peritrophic matrix and bind readily to specific sites on the apical brush border of susceptible insects (Hofmann et al., 1986; 19988a; 1988b). Binding is a two-stage process involving reversible (Hofmann et al., 1986; 1988a) and irreversible (Ihara et al., 1993; Rajamohan et al., 1995; Van Rie et al., 1989) steps. The latter steps may involve a tight binding between the toxin and receptor, insertion of the toxin into the apical membrane, or both.

Toxin monomers insert into the membrane and aggregate in groups of fours (Aronson and Shai, 2001; Vie et al., 2001) leading to pore formation on the target membranes (Reviewed in Schnepf et al., 1998). This eventually results in disruption of membrane integrity (Carroll and Ellar, 1997) and osmotic cell lysis (Knowles and Ellar, 1987).

Toxin binding competition experiments portray a direct correlation between toxin binding and activity *in vivo* (Van Rie et al., 1989; 1990; Wolfersberger et al., 1990; Ferre et al., 1991; Karim et al., 2000). However, this is not always the case (Garczynski et al., 1991; Lee et al., 1996; Luo et al., 1999; Van Rie et al., 1990a; Wolfersberger et al., 1990).

Cry proteins behave as an ion channel in lipid bilayers and in the midgut epithelium. In the alkaline midgut, the toxin may function as a cation channel (Schwartz et al., 1993), taking advantage of the large K^+ gradient that exists in some insect midgut environments. As the pH falls due to cell lysis or leakage, the toxin may function as an anion channel (Schwartz et al., 1993), further harming the epithelial cells. In large amounts, the Cry protein may form very large pores, resulting in cell lysis and disruption of the midgut epithelium.

Cry toxin binding proteins and receptors:

There is disagreement in the Bt scientific community as to whether or not Bt Cry protein binding molecules should be called receptors. In a strict definition, molecules that bind ligand then transduce a response are defined as receptors. Unfortunately, the terms 'binding proteins' and 'receptors' are used interchangeably in the Bt literature. For practical purposes a toxin-binding protein that mediates Cry toxin pore formation is called a receptor.

It has been demonstrated that the apical brush border of columnar cells is the site of lesion (Percy and Fast, 1983; Singh et al., 1986) and that toxin binds to the microvillar region (Bravo et al., 1992; Ravoahangimalala and Charles, 1995). Consequently, the Bt receptor is thought to be localized in the microvilli of the columnar cells. Wolfersberger et al., (1987) isolated vesicles from the apical brush border membrane (BBMV) of midgut epithelial cells where toxicity occurs. These BBMV have since been used as a model to study *in vitro* interaction of Bt toxins with midgut receptors as substrates for binding assays. Hofmann et al. (1988b) first reported that a Bt toxin specifically bound in the brush border membrane vesicles of apical brush border columnar cells.

Toxin binding proteins in brush border membrane vesicles were first identified using ligand blots wherein total BBMV proteins are separated by SDS-PAGE, transferred to membrane filters and probed with Cry toxins (Garczynski et al., 1991; Knowles et al., 1991; Oddou et al., 1991, Martinez-Ramirez et al., 1994; Jurat-Fuentes et al., 2001). However due to the denaturing conditions under which ligand blots are carried out, toxin binding epitopes which are usually not found on the cell surface could get exposed (Daniel, 2002) and hence conflict with binding site models derived from membrane vesicle binding assays (Lee et al., 1996). Affinity chromatography (Banks et al., 2001; Gill et al., 1995; Lee et al., 1996; Valaitis et al., 2001) and immunoprecipitation (Luo et al., 1997) techniques have been used to isolate toxin-binding proteins from BBMV. However the former method is incompatible in the case of detergent-resistant membrane samples (Lu and Adang, 1996).

Cry toxin binding proteins basically fall under two major protein families: cadherin-like proteins and N-aminopeptidases (APN). Cadherins are considered a 'superfamily' of proteins with diverse structures and functions. The 'classical' cadherins mediate Ca^{2+} cell adhesion

forming intracellular junctions between cells. Cadherins also provide extracellular information to the cell, and have a role in morphogenesis and cell polarization (Truong and Ikura, 2002). Cadherin-like proteins mediate toxin binding in *M. sexta* (Vadlamudi et al., 1995; Keeton et al., 1998), *Bombyx mori* (Nagamatsu et al., 1998; 1999) and probably, *Heliothis virescens* (Gahan et al., 2001).

Aminopeptidases (APNs) are exopeptidases that catalyze the cleavage of N-terminal amino acid residues from proteins and peptides. Their C-termini are bound to the brush-border-membrane by a glycophosphatidylinositol (GPI) anchor (Garczynski and Adang, 1995; Yamamoto, 2000). APNs that bind Cry toxins have been reported in *Manduca sexta* (Sangadala et al., 1994; Gill et al., 1995, Banks et al., 2001), *Heliothis virescens* (Luo et al., 1997; Banks et al., 2001), *Bombyx mori* (Yaoi et al., 1997) and several other lepidopteran species.

Previous research on *H. virescens* showed the presence of a phosphatase that binds Cry1Ac (English and Readdy, 1989). In *M. sexta*, an alkaline phosphatase that binds Cry1Ac toxin has also been observed (Sangadala et al., 1994). Proteomic research on *M. sexta* BBMV also revealed the presence of alkaline phosphatases of 65- and 62-kDa that bound Cry1Ac (McNall and Adang, 2003). Yet another binding protein that has been recently found to belong to this class is a 68-kDa alkaline phosphatase in *H. virescens*, HvALP (Jurat-Fuentes and Adang, submitted). However, not much is known about the role of ALP in toxin binding and toxicity at this point.

Larval midgut:

When insects ingest Bt toxins, the midgut is disrupted in susceptible species. Bt Cry proteins are activated by proteinases and pass through the peritrophic matrix (PM). The PM surrounds the gut lumen separating the meal from the brush border epithelium and is composed of chitin and glycoproteins (Lehane et al., 1997). In Lepidopterans, the midgut is composed of a single layered epithelium resting on a continuous basal lamina or basement membrane. It serves as a conduit between the foregut and the hindgut and is composed of a unicellular layer of columnar and goblet cells. Its primary role is nutrient absorption and maintenance of an electrochemical gradient. The goblet cells are responsible in the creation and maintenance of an electrochemical gradient across the midgut epithelium (Knowles, 1994) while the columnar cells are involved in absorptive processes.

The movement of ions between the midgut lumen and hemolymph includes absorption of ingested ions from the midgut lumen, secretion of ions from hemolymph and the production of high luminal pH values (Reviewed in Dow, 1986). Three transport mechanisms are involved in the active and electrogenic K^+ secretion that occurs in the goblet cells. These are - (i) Basolateral K^+ channels (Dow and Harvey, 1988) (ii) Apical electrogenic $K^+/2H^+$ antiporters, responsible for secondary extrusion of K^+ from the cell into the gut lumen via the goblet cavity (iii) Apical V-ATPase proton pumps (Wieczorek, 1991) that create a membrane potential to drive K^+ transport into the midgut via electrogenic H^+/K^+ antiporters. The electrical gradient generated by these mechanisms helps drive most of the nutritive absorptive processes (Harvey et al, 1983) and also maintain high alkaline pH condition to assist in absorption of amino acids (Castagna, 1997).

Studies on the goblet cell apical membrane suggested that the K^+ pump consists of two molecular components: a primary H^+ pump (the V-ATPase) and a secondary K^+/H^+ antiport (Weiczorek, 1991; Lepier, 1994; Azuma, 1995). The V-type H^+-ATPase was first identified as an enzyme associated with endosomal membranes of lysosomes, clathrin-coated vesicles and vacuoles of yeast and plants (Nelson, 1992; Stevens and Forgac, 1997). The insect plasma membrane V-ATPase consists of at least 13 distinct subunits, morphologically divided into two components: a membrane bound domain V_0, that contains the ion channel, and an extrinsic domain V_1, in which ATP hydrolysis occurs (Graham et al., 2000; Bowman et al., 2000). Subunit stoichiometry is believed to be similar in all V-ATPases (Forgac, 1992). The two major subunits A and B, in a stoichiometry of A_3, B_3; contain the nucleotide binding sites and are connected to the V_0 part by the so-called stalk subunits C-H (Gruber et al., 2001). Figure 1.2 depicts the arrangement of the different subunits of the V_1 complex.

The goblet cell pumping system is Mg^{2+} and K^+ stimulated and translocates H^+ to generate a voltage greater than 240mV across the apical membrane of goblet cells. This voltage drives secondary $K^+/2H^+$ antiport and thus net active K^+ secretion (Wieczorek and Harvey, 1995). The resulting K^+ electrochemical potential energizes all secondary transport processes across the epithelium of the midgut. The combined action of the V-ATPase and $K^+/2H^+$ antiporter explains the alkalinity (pH>11) of the output side of a proton pump.

Although it is generally believed that V-ATPases transport protons by a rotary catalytic mechanism analogous to that used by F_1F_0-ATPases, the structure and subunit composition of the central or peripheral stalk of the multisubunit complex are not well understood. Elucidation of the physiological significance of protein-protein interactions is likely to further advance our understanding of this fundamental proton pump.

Another constituent of the ion-transport system are alkaline phosphatases. They are known to participate in cell adhesion and differentiation (Chang, 1993) and also have been proposed to function in active absorption of metabolites and transport processes (Eguchi, 1995). The insect midgut possesses two forms of alkaline phosphatase, a membrane bound form covalently bound to the apical membranes of columnar cells and a soluble form associated with the apical microvilli of goblet cells and the goblet matrix (Azuma and Eguchi, 1989; Azuma, 1991). The soluble form is an HCO^{3-} sensitive Mg^{2+}-dependent ATPase (Azuma, 1991). A very interesting observation is that all available amino acid sequences of V-ATPase subunits of *M. sexta* showed partial homology to *B. mori* ALP isozymes suggesting a functional relationship between them. This is particularly interesting because V-ATPase is a factor causing high alkalinity (pH 11) in midgut lumen. This agrees with the observation of Azuma et al. (1991) who detected HCO^{3-} stimulated-ATPase activity in purified sALP and proposed that sALP may play a role in maintaining the alkalinity of *B. mori* midgut. However, similar homologies were seen in mALP sequence and so this trait is not specific only to sALP.

***Heliothis virescens*:**

The tobacco budworm, *Heliothis virescens* (Fabricius), is found throughout the eastern and southwestern United States. Tobacco budworm is principally a field crop pest, attacking such crops as alfalfa, clover, cotton, flax, soybean, and tobacco. Larvae bore into buds and blossoms (hence the name), and also leaf petioles, and stalks. The tobacco budworm is one of the most important pests of cotton, *Gossypium hirsutum* in the United States, usually occurring in mixed populations with the bollworm, *Helicoverpa* zea. Bt toxins are effective against both

these larvae (Johnson et al., 1974; Stone and Sims 1993). *H. virescens* is the major crop pest controlled by Bt cotton.

Resistance to Bt toxins:

Because these toxins and insects have co-evolved, it was thought in the past that insects would not develop resistance to Bt toxins. Resistance can occur in 2 forms - natural and acquired. Cry1 toxins do not kill all lepidopteran species and hence there is a group of "tolerant" insects. There are some that do not bind toxin while there are others that bind toxin but are not killed. For instance, Cry1C toxin binds with very high affinity to the BBMV of *H. virescens*, but is not larvicidal (Van Rie et al., 1990). This anomaly can be accounted for by an inability to insert the toxin into the membrane. However in *Spodoptera frugiperda*, the toxin binds irreversibly to BBMV, but fails to permeabilize the membrane (Luo et al., 1999). The above cases fall under natural tolerance.

In the other mode of Bt toxicity failure namely, acquired resistance, insects are well equipped to adapt to the presence of Cry toxins. Though resistance in *Plutella xylostella* (diamondback moth) has been observed in various field locations, they have all been found to be due to the loss of a Bt toxin-binding site. Though this is the only insect that has accomplished resistance in the field (Tabashnik et al., 1990; Tang et al., 1997), it definitely issues a warning that such resistance patterns could occur in other insect species. Resistance against a toxin that has not been present in the selective environment or cross-resistance is yet another instance of Bt toxin failure (Tabashnik et al., 1996).

The first report on insect resistance to Bt was published in 1985 (McGaughey et al., 1985). This has been of concern especially after the observance of field resistance in *P. xylostella*. Since then, several such cases have been reported (Bauer et al., 1995; Ferre et al., 2000; Frutos et al., 1999; Schnepf et al., 1998; Tabshnik et al., 1994; Van Rie et al., 2000). Most of these colonies were selected for resistance under laboratory conditions. Though laboratory colonies usually have limited genetic variation and may not contain all resistance genes present in field populations, they serve as indicators of inheritance of resistance genes (Georghiou et al., 1994).

H. virescens has the ability to develop resistance and cross-resistance to Bt toxins after selection in the laboratory (Gould et al., 1992; MacIntosh et al., 1991). Over 10,000-fold resistance was obtained in a *H. virescens* (YHD2) colony by selection with Cry1Ac protoxin (Gould et al., 1995). Following continued selection on Cry1Ac, it acquired more than 230,000-fold resistant to this toxin. Larvae from susceptible and resistant colonies of *H. virescens* (KCB) showed midgut epithelium damage following Cry1Ac ingestion (Forcada et al., 1999). Hence it is possible that resistance is due to an efficient repair or replacement of damaged midgut cells.

Yet another reported mechanism of resistance is a retrotransposon-mediated disruption of a cadherin-superfamily gene that prevents full-length expression of the protein HevCadLP (Hv Cadherin-like-protein, Gahan et al., 2001). The most recent addition to resistance mechanisms is altered glycosylation of specific membrane vesicular proteins that bind Cry1 toxins, leading to decreased pore formation and resistance to Bt toxin (Jurat-Fuentes et al., 2002).

Sharing of receptors is also an important mechanism of cross-resistance (Jurat-Fuentes and Adang, 2001). Based on competition-binding studies (Lee et al., 1995; Van Rie et al., 1989) a model for Cry1A binding sites in *H. virescen*s was postulated: Cry1Aa, Cry1Fa and Cry1Ja

bind to receptor A; Cry1Ab binds to this receptor and also to receptor B; and Cry1Ac recognizes both of these sites, as well as receptor C. Thus, Cry1Ac and Cry1Ab also bind to the Cry1Aa binding site. Hence the altered Cry1Aa binding site causes resistance to all the Cry1A proteins and additional binding sites recognized by Cry1Ab and Cry1Ac may not be involved in toxicity (Jurat-Fuentes and Adang, 2001; Lee et al., 1995).

Genetic analysis has shown that resistance to Bt is autosomally inherited, although in some cases the sex of the parents had some influence on the resistance level of the offspring. Genes linked to Bt resistance, have been genetically mapped, but they need to be cloned to better analyze resistance mechanisms. One strategy for delaying evolution of resistance to Bt is gene stacking, by the production of multiple Cry toxins in each transgenic plant (Jurat-Fuentes and Adang, 2003). Detailed understanding of resistance mechanisms, with knowledge of pest biology and plant molecular biology could curb the growth of resistance. To increase efficiency of pest control, transgenic crops have been engineered (De Maagd et al., 1999). However, increase in selection pressure due to continuous exposure to Bt toxins carries with it the risk of resistance development. Careful implementation of resistance management methods such as crop rotation and Bt spray rotation, are required to safeguard the value of *Bt* for pest control. Acquired resistance by pest insects is the immediate threat to insect control using Bt proteins.

Proteomics:

In the past, proteomic tools have been employed in the characterization of the *Drosophila melanogaster* proteome, in the identification of novel Bt binding proteins of the insect gut (McNall and Adang, 2003) and also to determine potential resistance mechanisms by comparison

of Bt-susceptible and resistant insect proteomes (Candas et al., 2003). However, it still remains a nascent technology and is yet to be fully exploited in the case of insect systems.

Proteomics is the systematic analysis of the proteins expressed by a cell or tissue type at a given time or under certain environmental conditions (Nyman, 2001). A combination of sophisticated techniques, including two-dimensional electrophoresis (2DE), image analysis, mass spectrometry, amino acid sequencing, and bio-informatics is used to resolve, quantify, and characterize proteins. This electrophoretic separation of proteins and polypeptides in polyacrylamide gels has become the method of choice for the fractionation and characterization of complex mixtures of proteins and polypeptides at both the analytical and preparative level (O'Farell et al., 1975). Figure 1.4 shows a general approach to proteomic expression analysis.

Proteins are separated by two independent variables - isoelectric point (pI) and molecular weight - generating a "map" of hundreds to thousands of proteins (Gygi, 2000) visualized by Coomassie brilliant blue or by silver stain (Humphrey-Smith, 1997). Immunoblot analysis (Towbin, 1979) is the method of choice for the identification of proteins on 2D gels but is severely limited by the availability of specific antibodies. While 2DE gels provide information on the iso-electric point and approximate mass of the protein, this information alone is not sufficient to identify the majority of proteins and requires further investigation by techniques such as mass spectrometry.

Mass spectrometry determines the mass of peptide fragments generated by treatment of protein spots with proteolytic enzymes (Aston, 1933). Each digested protein yields a profile of peptides called a peptide-mass fingerprint (PMF). PMF has become the method of choice in protein identification (Henzel et al., 1993; Mann et al., 1993). A number of programs are available to automatically match peptide masses to protein databases. The tryptic masses are

evaluated using a PMF-tool such as Mascot, Prowl, MS-Fit or Peptide-Search which try to fit a user's mass spectrometry data to a protein sequence in an existing database and hence suggest the identity of the protein. Peptide mass mapping is most useful in analysis of proteins from organisms with completely sequenced genomes.

However it is frequently observed that not all of the experimentally determined peptide masses match a list of the possible peptide masses from the protein identified. Several explanations for these unassigned peptide masses are possible - the peptide mass database search might identify a homolog of the protein isolated, some peptides might be nonspecifically cleaved by a protease, or, more interestingly, some peptides might be post-translationally modified. Heavily modified proteins due to glycosylation will present a collection of peptides with molecular weights not predicted for proteins in the database. In such cases, confirmatory criteria such as complete or partial peptide sequences determined by Edman degradation (Vanderkerchove et al., 1985) or tandem mass spectrometry (Wilm and Mann, 1996) need to be employed. Antibodies specific to a protein of interest are also useful tools to confirm PMF identifications.

Tandem mass spectrometry of digested peptides yields d*e novo* amino acid sequence of the peptide. The sequence is used in a search tool such as MS-BLAST to identify proteins. This approach compliments or confirms the id obtained from PMF analyses.

Current methods have difficulties in identifying protein isoforms, proteins with posttranslational modifications, or variations in amino acid sequence due to mRNA splicing. Studies on the identification of major proteins of yeast have shown that there is an under representation of several proteins among the ones identified, including membrane proteins and glycoproteins (Garrels et al., 1997). The limitations posed by 2DE may be overcome in the near

future by advances in other methods of protein separation, but they present an important consideration when planning a proteomic approach. Proteome analysis will continue to play an important role in the understanding of biological systems in a wide variety of normal and pathological conditions.

Rationale and goals of the project:

In the past APNs, cadherins and alkaline phosphatases have been identified as Cry1Ac binding proteins. However, there are binding sites on the BBMV that do not match known binding proteins. Hence we decided to explore the possibilities of discovering new Cry1Ac binding proteins in the BBMV of *H. virescens* larvae using a proteomic approach that utilized the PMF of proteins of interest to search databases for a match. Cry1Ac was selected because it has the highest toxicity to this species and is the Bt protein expressed in the commercial Bt cotton. A sub-proteome of GPI-anchored proteins was studied, because known Cry1Ac binding proteins are GPI-anchored.

A secondary goal was to examine the complexity of the brush border proteome of *H. virescens* larvae with an emphasis on alkaline phosphatases. A 65-kDa protein in the midgut of *H. virescens* that binds Cry1Ac was recently identified as an alkaline phosphatase (HvALP, Jurat-Fuentes and Adang, submitted). Earlier studies on the ALP in *Bombyx mori* reveal the presence two different forms - soluble and membrane forms, so it is possible that this protein is present in multiple forms in the midgut of H. *virescens* also (Eguchi, 1995). I set out to identify the different forms of ALP present (if any) and if they bind Cry1Ac. PIPLC solubilization of the BBMV was used to enrich for the presence of ALP and also to study novel Cry1Ac binding

proteins residing in the GPI anchored fraction. Western blot analyses detected proteins that bind Cry1Ac (Towbin, 1979).

To achieve these goals, I used a proteomics based approach, the rationale being that it employs a high-resolution method of resolving complex samples. Two-dimensional electrophoresis coupled with mass spectrometry is a highly informative approach to answering questions concerning the identity of proteins. Though it is widely used in microbial systems, it has not been well exploited in insect systems. Recent research by Candas et al (2003) and McNall (2003), has demonstrated the potential of proteomic technology in identifying proteins in complex samples of insect systems. Those studies served to establish methodologies used in my research and suggested mass spectrometry based approaches to protein identification when a genome sequence is not available.

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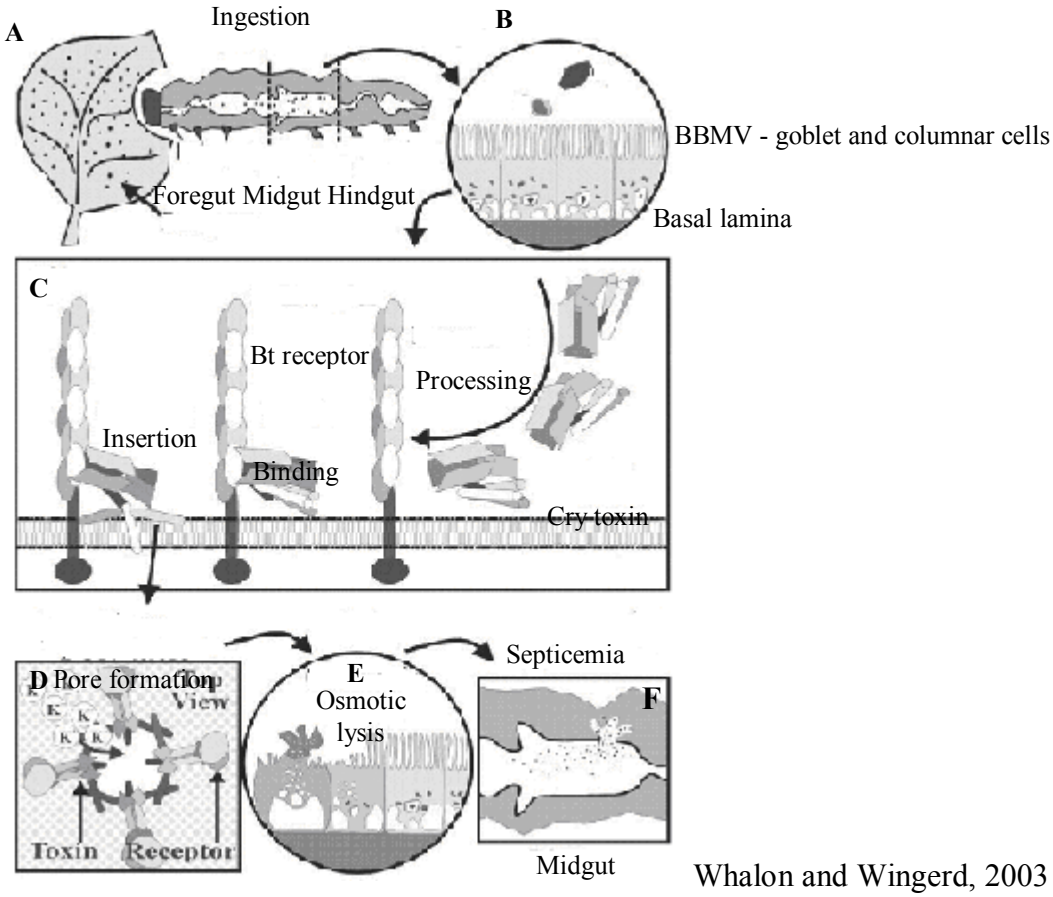


Whalon and Wingerd, 2003

FIGURE. 1.1 MECHANISM OF CRY PROTEIN TOXICITY

A: Ingestion of spores or recombinant protein by phytophagous larva.

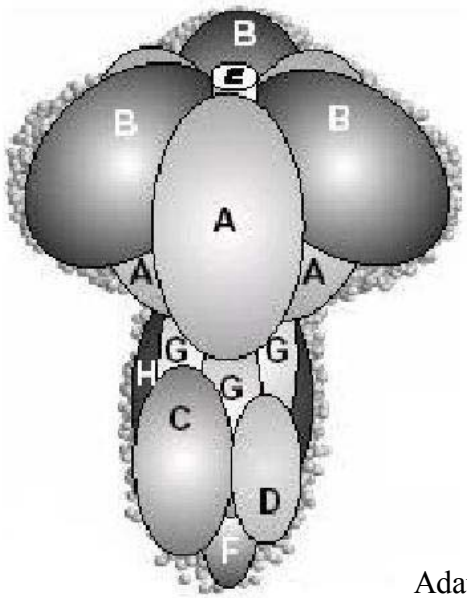
B: In the midgut, endotoxins are solubilized from Bt spores (s) and inclusions of crystallized protein. (cp).

C: Cry toxins are proteolytically processed to active toxins in the midgut. Active toxin binds receptors on the surface of columnar epithelial cells. Bound toxin inserts into the cellular membrane.

D: Cry toxins aggregate to form pores in the membrane.

E: Pore formation leads to osmotic lysis.

F: Heavy damage to midgut membranes leads to starvation or septicemia.



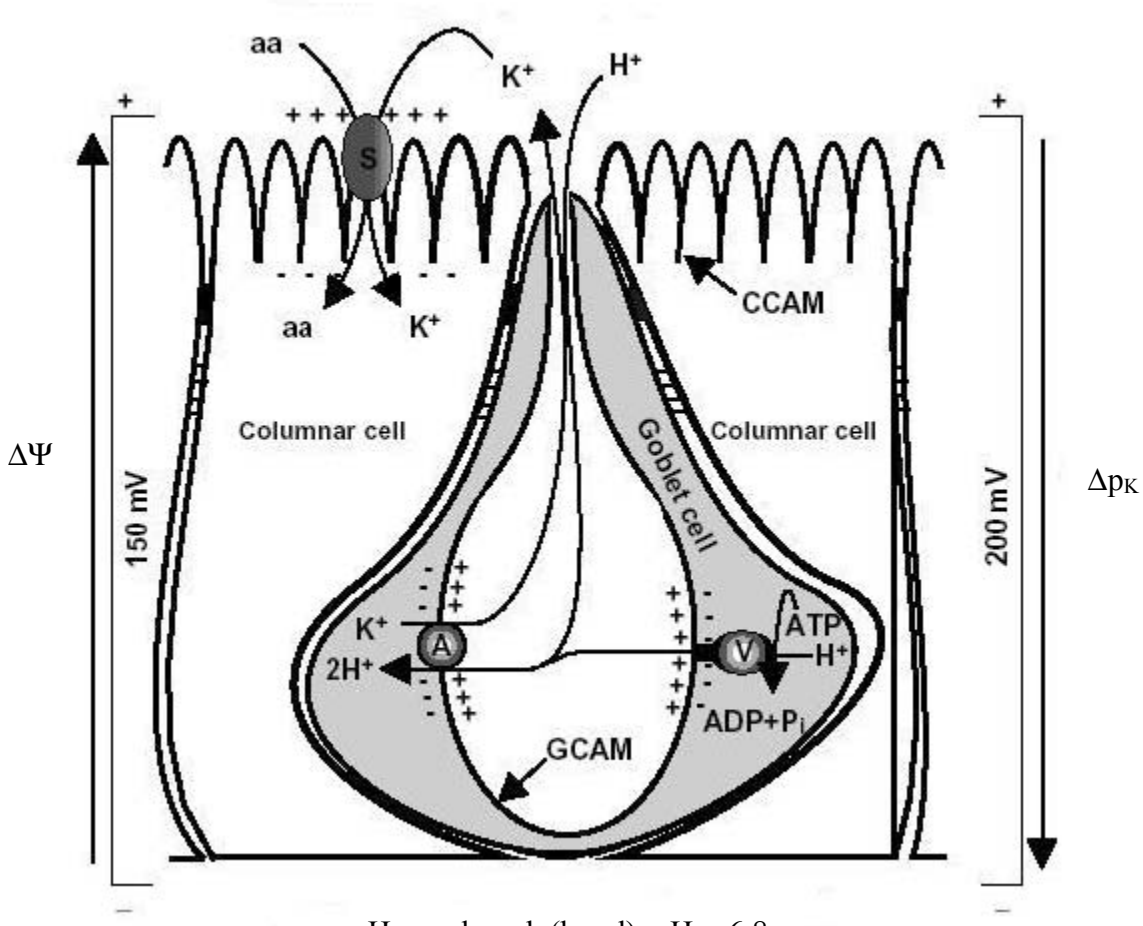
Adapted from Wieczorek et al., 2000

V_1 complex

FIGURE. 1.2 STRUCTURAL MODEL OF THE V1-ATPASE IN *M. SEXTA*

The subunits of V_1 (A–H) are placed within the molecular shape of *M. sexta* V_1 ATPase deduced from solution X-ray scattering (Svergun et al., 1998).

Gut lumen (apical) pH > 11



Haemolymph (basal): pH = 6.8

Adapted from Wieczorek et al., 2000

FIGURE 1.3 ENERGIZATION OF THE MIDGUT EPITHELIUM OF LARVAL *MANDUCA SEXTA* BY THE PLASMA MEMBRANE V-ATPASE
Abbreviations: Columnar cell apical membrane (CCAM), Goblet cell apical membrane (GCAM).

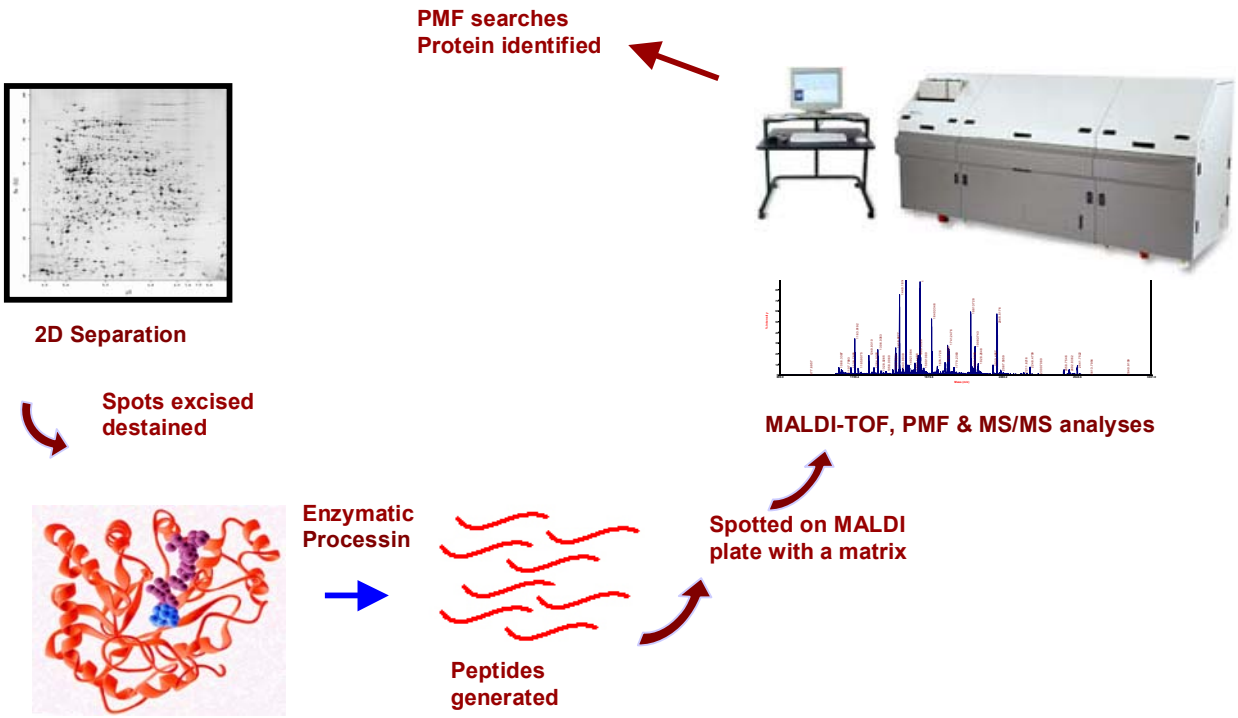


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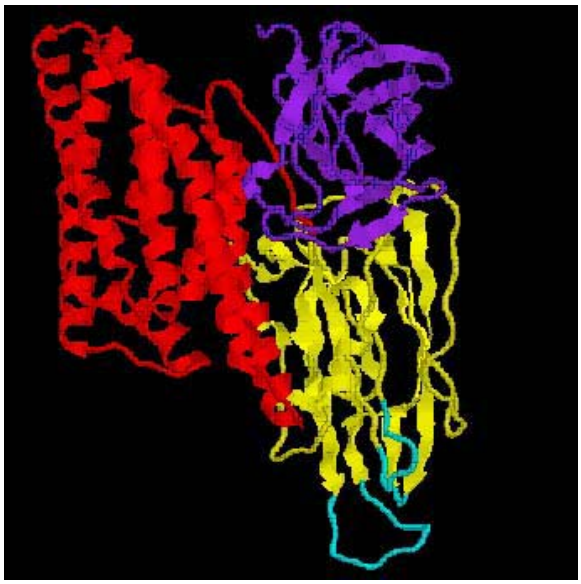
FIGURE: 1.4 GENERAL APPROACH TO PROTEOMIC EXPRESSION PROFILING USING PEPTIDE MASS FINGERPRINTING. Samples are separated on two dimensions based on pI and molecular weight. Proteins spots of interest are excised, destained and enzymatically processed to yield peptides, which are spotted along with a matrix on a MALDI-plate for time of flight analysis. The resulting fragmentation spectra are used for peptide mass fingerprinting to identify the protein.

Domain I



Domain III

Domain II

Grochulski et al., 1995

FIGURE 1.5 3D STRUCTURE OF THE INSECTICIDAL CRYSTAL TOXIN CRY1AA OF BACILLUS THURINGIENSIS

CHAPTER 2

IDENTIFICATION OF NOVEL CRY1AC BINDING PROTEINS IN THE MIDGUT OF *HELIOTHIS VIRESCENS* USING PROTEOMIC ANALYSES [1]

[1] Krishnamoorthy, M., J. L. Jurat-Fuentes, R. J. McNall, and M. J. Adang

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INTRODUCTION:

Bacillus thuringiensis (Bt) Cry1Ac protein expressed in transgenic cotton cultivars controls *Heliothis virescens*, the tobacco budworm. Due to its remarkable efficacy Bt cotton has been rapidly adopted by farmers since its commercial introduction in 1996. Consequently, Cry1Ac is uniquely valuable in transgenic cotton because Cry1Ac is the most active Cry protein against *H. virescens* larvae (http://www.glfc.forestry.ca/bacillus/).

When larvae ingest Cry1Ac, the 130-kDa protoxin form is cleaved by midgut proteinases to a 65-kDa-toxin core. After the 65-kDa toxin crosses the peritrophic matrix a final cleavage occurs upon contact with a receptor located on the midgut epithelium (Gomez et al. 2002). Toxin then oligomerizes into tetramers, and inserts into the midgut membrane at cell surface microdomains called lipid rafts (Zhuang et al., 2003). Insertion of toxin tetramers results in formation of pores in the epithelial cell membrane that cause cell death by osmotic shock and ultimately insect mortality (reviewed in Whalon and Wingerd, 2003).

The cadherin-like protein (HevCaLP) is hypothesized as a major Cry1A toxin receptor in *H. virescens* larvae that is responsible for mediating most of Cry1Ac toxicity (Gahan et al., 2001). 'Knock-out' of the gene encoding HevCaLP is associated with Bt resistance in *H. virescens* strain YHD-2 (Gahan et al., 2001). Although HevCaLP is critical to Cry1Ac toxicity, other binding sites and proteins are also involved in toxicity.

H. virescens larvae have three groups (A, B and C) of Cry1A binding sites (Van Rie et al., 1989; Jurat-Fuentes and Adang, 2001). Site A, which has the properties of Cry1Aa, Cry1Ab and Cry1Ac binding, is composed of HevCaLP and the 170-kDa aminopeptidase N (APN) (Luo et al. 1997; Jurat-Fuentes and Adang, 2001). The B binding site recognized by Cry1Ab and

Cry1Ac is associated with a 130-kDa protein, while the C binding site, recognized only by Cry1Ac, includes several proteins of less than 100-kDa in size. The recognition of multiple sites by Cry1Ac correlates positively with its high toxicity towards *H. virescens*. Also, there is evidence that successive reduction of toxin binding to fewer sites leads to increased resistance to Cry1 toxins in this insect (Jurat-Fuentes et al., 2002).

Alkaline phosphatases (ALPs) are biochemical markers for microvilli present on the surface of midgut epithelium brush border cells in lepidopteran larvae. In the silkworm (*Bombyx mori)* larvae, two forms of ALP have been identified. The 58-kDa membrane-bound form (mALP) is covalently tethered to the external membrane surface of columnar cells by a glycosylphosphatidyl inositol (GPI) anchor, while the soluble form (sALP) of 61-kDa is localized at the surface of goblet cells (Azuma and Eguchi, 1989; Okada et al., 1989). In brush border membrane vesicle (BBMV) proteins from *Manduca sexta* (tobacco hornworm), a 65-kDa ALP, which is GPI-anchored and binds Cry1Ac, was identified by proteomic analyses (McNall and Adang, 2003). In *H. virescens*, a 68-kDa glycoprotein proposed as part of the C binding site was identified as an ALP (Jurat-Fuentes et al., 2002; Jurat-Fuentes and Adang, submitted).

Recently, proteomic tools have been applied to discover novel Bt binding proteins and elucidate changes in midgut proteins associated with insect resistance. McNall and Adang (2003) used proteomic analyses to identify ALP and actin as novel toxin binding proteins in the brush border membrane of *M. sexta*. Bt susceptible and resistant larvae of *Plodia interpunctella* have also been compared using differential- in-gel (DIGE) analysis (Candas et al., 2003). Those authors detected several changes in midgut metabolism including increased glutathione and oxidative metabolism. The tools of proteomics have not been applied to the analysis of BBMV from *H. virescens* larvae.

The goal of this study was to discover and identify Cry1Ac binding proteins in *H. virescens* BBMV using a two-dimensional (2D) gel electrophoresis approach. Based on results from previous proteomic analyses (McNall and Adang, 2003) and the ability of Cry1Ac to bind multiple sites on *H. virescens* BBMV (Van Rie et al., 1989), we hypothesized that novel binding-proteins that had previously not been discovered using a one-dimensional (1D) approach would be discovered. Since several known Cry1Ac toxin-binding proteins are GPI-anchored, we focused on proteins released by phosphatidyl inositol-specific phospholipase C (PIPLC).

Binding proteins were detected by probing blots of separated proteins with biotinylated Cry1Ac. Novel Cry1Ac binding proteins were detected on blots and identified by peptide mass fingerprinting. Vacuolar-ATP synthase subunits A and B were found to bind Cry1Ac toxin. Another subunit of the same enzyme was identified as a BBMV constituent that does not bind Cry1Ac. Cry1Ac also bound to multiple spots of ALP.

MATERIALS AND METHODS:

Bacterial strains and toxin purification:

Bt strain HD-73 producing Cry1Ac was obtained from the *Bacillus* Genetic Stock Collection (Columbus, Ohio). Cry1Ac toxin was prepared from crystals of strain HD-73 as previously described (Luo et al., 1999). Purified toxin appeared as a single band after separation by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-10% PAGE). Cry1Ac (0.5 mg) was biotinylated by incubating a 1:30 molar ratio of toxin: EZ-Link^{TM} sulfo-NHS-LC-Biotin (Pierce) for 30 minutes at room temperature. Uncoupled biotin was removed by dialysis overnight in 20 mM Na_2CO_3, 200 mM NaCl at pH 9.6. Toxin preparations were quantified using the Bradford protein assay (1976) with BSA as standard and stored at -80°C until needed.

Insect rearing and midgut dissection:

H. virescens eggs were obtained from Stoneville, MS (USDA, ARS) and the larvae were reared on artificial diet (Southland Products, Lake Village, AR) at 25°C under a 12-hour day-night period. Midguts were dissected from fifth instar larvae, washed in ice-cold SET buffer (250 mM sucrose, 17 mM Tris [pH 7.5], 5 mM EGTA) and immediately frozen on dry ice and stored at -80°C until used.

BBMV preparation:

BBMV were prepared by a modification of the differential magnesium precipitation protocol of Wolfersberger et al (1987). Briefly, midguts were homogenized in SET buffer containing a protease inhibitor cocktail (Complete, Roche), and then one volume of 24 mM $MgCl_2$, 250 mM sucrose was added and the mixture placed on ice for 15 minutes. The homogenate was centrifuged at 2,500 x *g* for 15 minutes and then the supernatant was collected and centrifuged at 27,000 x *g* for 30 minutes. The resulting pellet was re-suspended in one-half SET volume per starting homogenate volume and the two centrifugation steps were repeated. The final BBMV pellet was suspended in ice-cold TBS (25 mM Tris [pH 7.5] 2 mM KCl, 135 mM NaCl) containing protease inhibitors, and protein concentration quantified as for toxin samples. BBMV were stored at -80°C until needed.

Aminopeptidase specific activity using leucine-ρ-nitroanilide as substrate served as an enzymatic marker for brush border membrane enrichment (Terra et al., 1994). BBMV preparations were enriched five-fold relative to the initial midgut homogenate.

Solubilization of GPI-anchored proteins:

Glycosylphosphatidylinositol (GPI) anchors of BBMV proteins were digested according to Luo et al (1997). BBMV proteins (1mg) in water were incubated with 1 unit of phosphatidylinositol-specific phospholipase C (PIPLC - Sigma) for four hours at 37°C with continuous mixing. After centrifugation at 15,700 x g for 10 minutes, solubilized proteins were recovered in the supernatant and used immediately for 2D sample preparation to avoid the risk of denaturation by freezing and thawing. Protein concentration was determined according to Bradford (1976) prior to preparing the samples for 2D electrophoresis.

Sample preparation for 2D electrophoresis:

Proteins to be analyzed by 2D gel electrophoresis (including BBMV and PIPLC-released proteins) were extracted and precipitated using the 2D Clean-up kit (Amersham, Biosciences). Precipitated proteins were dissolved in solubilization buffer (5 M urea [Plus-One; Amersham, Biosciences], 2 M thiourea [Sigma] and 2% CHAPS [Plus-One, Amersham Biosciences]. Proteins were forced into solution by sonication and vortexing. Samples were then centrifuged at 15,700 x g for 20 min. to pellet insoluble proteins and cell debris. Solubilized proteins in the supernatant were quantified using the Bradford (1976) assay.

Immobiline Drystrips (Amersham Biosciences) were rehydrated overnight with solubilized protein and rehydration buffer (solubilization buffer plus 0.002% bromophenol blue, 0.018 M dithiothreitol [DTT], and 0.5% ampholytes). Following rehydration, the strips were subjected to isoelectric focusing using the Multiphor II (Amersham Biosciences). Temperature

was maintained at 20°C throughout focusing. Voltages differed as strip lengths varied (7 cm and 18 cm strips). Focused strips were equilibrated for 20 minutes in equilibration buffer (6 M urea [Plus-One; Amersham Biosciences], 2% SDS, 30% glycerol, 0.05 M Tris [pH 8.8], 0.002% bromophenol blue) containing 1% DTT followed by a second equilibration for 20 minutes in equilibration buffer plus 4% iodoacetamide. For second dimension separation, equilibrated strips were overlaid on an SDS-8% PAGE gel and electrophoresed at 20 mA until the dye front reached a few millimeters from the bottom of the gel. Gels were silver stained to visualize protein spots.

Ligand and Western blotting:

BBMV proteins (15 µg) were separated by SDS-8% PAGE and transferred to polyvinylidene difluoride Q membrane filter (Millipore). Filters were blocked for one hour in TBST (25 mM Tris [pH 7.5], 3 mM KCl, 135 mM NaCl, 0.1% Tween-20) plus 3% BSA, and then cut into strips for the various treatments. Filter incubations and washes were in TBST plus 0.1% BSA.

For toxin blots, blocked filters were incubated with biotinylated Cry1Ac (5 nM) for one hour. After washing, filters were incubated for an hour with a 1:50,000 dilution of streptavidin serum conjugated to horseradish peroxidase (HRP) (Sigma). Binding proteins were visualized using ECL (Amersham Biosciences) following the manufacturer's instructions.

For immunoblots, blocked filters were probed with a 1:25000 dilution of rabbit sera against *B. mori* mALP (a gift from Dr. Masanobu Itoh, Kyoto Institute of Technology, Japan). Secondary antibodies were either anti-rabbit HRP conjugate, or anti-rabbit alkaline phosphatase

conjugate (both from Sigma). Alkaline phosphatase activity in the conjugate was visualized using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) substrates. Blots of BBMV proteins exposed directly to NBT-BCIP substrate had no endogenous alkaline phosphatase activity. To detect GPI anchored proteins we used antisera to the resulting cross-reacting determinant (CRD) after PIPLC digestion at 1:5000 (gift from Dr. Kojo Mensa-Wilmot, University of Georgia) and anti-rabbit HRP conjugate as the secondary antibody. All blots were repeated at least thrice to ensure consistency.

Mass spectrometry:

BBMV proteins were separated on an 18cm, pH 4-7 Immobiline Drystrip and subjected to 2D gel electrophoresis. The gel was then stained using Sypro Ruby (Molecular Probes) and imaged using a Typhoon Scanner (Amersham Biosciences) with excitation and emission wavelengths at 532nm and 610nm respectively. Quantitative analysis of spots on the gel was done using Decyder software (Amersham Biosciences). Spots of interest were excised and trypsin digested using an Ettan Spot Handling workstation (Amersham Biosciences). Selected gel plugs (1.4 mm^3) were treated with 140 ng of sequencing grade trypsin (Promega) in 20 mM ammonium bicarbonate at 37°C for 2 hours. Peptides were extracted by incubating the gel plugs with 50% acetonitrile, 0.1% trifluoro acetic acid for 20 minutes. The extract was saved and the procedure repeated on the same gel plug. The pooled extracts were then dried using a speedvac concentrator. Dried peptides were dissolved in 5 µl of 50% acetonitrile, 0.1% trifluoro acetic acid. An aliquot ($1/10^{th}$ of the sample volume) was spotted on a matrix associated laser desorption ionization (MALDI) plate using 50% saturated alpha-cyano-4-hydroxy-cinnamic acid

as a matrix. MALDI-time of flight (TOF) mass spectrometry was carried out using a 4700 Proteomics Analyzer (ABI) to obtain peptide mass fingerprints (PMFs). Spectral calibration was done using trypsin autolysis peaks of m/z 1045 and 2211.

MS Data analysis:

PMF searches to identify proteins were performed using two databases (NCBI and Swiss Prot) to increase the likelihood of protein identification. NCBI searches on metazoans and *Drosophila* were done using Profound (http://prowl.rockefeller.edu/). Tryptic autolysis peaks were deleted from the mass list prior to submission to the database. Mass accuracy of 0.1 Da, partial methionine oxidation and 1 missed trypsin cleavage were parameters considered in the search. The quality of scores was predicted using Z-score, which is a statistical measure of the probability that a candidate in a search is the protein being analyzed (http://129.85.19.192/prowl/help.html).

MS-MS using TOF/TOF analysis:

The remainder of the extracted trypsin-digest was spotted as described above on a MALDI plate and used for tandem mass spectrometry (MS/MS) analysis using the 4700 Proteomics Analyzer (ABI), which is a time-of- flight/time-of-flight instrument with a collision cell for peptide sequencing. It is optimized for mass analysis of peptides up to 6,000 Da.

MS/MS involves two stages of MS wherein ions of a desired m/z are first isolated from the rest of the ions created at the ion source. These isolated ions (termed parent ions or precursor

ions) are then collided with inert gas atoms in a collision cell, and a second analyzer separates the fragments generated by the collision. In *de novo* sequencing, mass differences between two peaks that correspond to amino acid masses help establish a sequence of amino acids. The *de novo* amino acid sequence was used to search a database using MS-BLAST (http://dove.embl-heidelberg.de/Blast2/msblast.html).

High-energy collision of 1 KeV was used to fragment the ions. Default calibration from the instrument based on immonium ions as standards was used. For several spots abundant ions from MALDI-TOF were used to generate spectra for *de novo* sequencing. The precursor mass windows were set at 10 and signal to noise threshold at 5, while the laser intensity and shots/second were optimized to obtain a sharp spectrum with minimal noise. The string of amino acids was then searched using MS-BLAST for matches to proteins in the NCBI nr (non-redundant) database.

RESULTS:

Detection of *Heliothis virescens* BBMV proteins by 1D and 2D electrophoresis:

The protein complexity of the brush membrane of *H. virescens* was compared by 1D and 2D gel electrophoresis. Figure 2.1A shows BBMV proteins that were either separated after the precipitation clean-up procedure (lane 1), or not (lane 2). The same-sized protein bands were present in both samples, except proteins of >100-kDa were relatively more abundant in the 'cleaned-up' protein preparation. In both samples, the silver-stained 1D-gels showed a pattern of

relatively abundant proteins separated in discrete bands against a continuous background of less abundant proteins.

The 2D gel shown in Figure 2.1B resolved more than 200 proteins ranging in size from 20-90-kDa and in pI from 3-10. Proteins of 100-kDa or greater were not detected on the 2D gel reflecting a selective bias of 2D gel electrophoresis towards smaller-sized proteins. Chains of protein spots, which could either represent distinct proteins, or a series of post-translational modifications of the same protein were detected.

Detection of Cry1Ac binding proteins and HvALP on blots of BBMV proteins:

Cry1Ac-binding proteins in BBMV were detected by probing blots of 1D and 2D gels with biotinylated toxin. This blot approach provides a direct method of detecting Cry toxin binding proteins, with the caveat that BBMV proteins on the blot are denatured. On the 1D blot, Cry1Ac bound proteins of 210-, 170-, 120-, 100-, 68- and 45-kDa among a background of minor bands (Figure 2.2A). The 170-, 120-, and 100-kDa proteins are mostly likely toxin-binding APNs (Gill et al., 1995; Banks et al., 2001). On the 2D blot, Cry1Ac toxin bound a series of proteins migrating at 70-, 68-, 50- and 42-kDa (Figure 2.2B). The most apparent difference between blots of 1D and 2D gels was the absence of the 100-kDa and larger toxin binding proteins on the 2D blot. Based on molecular size and pI we predicted that the 4 Cry1Ac-binding proteins migrating at 68-kDa were forms of ALP (Figure 2.2B; arrows 2 and 3). To test this possibility, BBMV proteins were separated by 2D gel electrophoresis, blotted and the filter probed with anti-mALP sera. Three groups of protein spots were detected in the 2D blot (Figure 2.2D, arrows 1-3), whereas a single band was detected on the 1D blot (Figure 2.2C). The ALP

spots were at the same gel coordinates as the Cry1Ac-binding spots, except that no binding was detected to chain 1. Cry1Ac bound to two spots in chain 2 and the most abundant spot in chain 3. We confirmed that the spots bound by Cry1Ac were ALPs by probing a 2D gel blot first with biotin-Cry1Ac, developing the blot, and then re-probing with anti-ALP serum (data not shown). The ALP spots bound Cry1Ac.

Analyses of GPI-anchored proteins:

GPI-anchored proteins were cleaved using PIPLC and separated from BBMV by centrifugation. The purpose was to obtain a protein fraction, i.e. sub-proteome, of GPI-anchored proteins that should include m-ALP and APNs, known GPI-anchored proteins. Figure 2.3A shows a 1D silver-stained gel containing PIPLC-released proteins (lane 3), untreated BBMV (lane 2) and PIPLC-treated BBMV (lane 4). Numerous proteins ranging in size from 220-kDa to <30-kDa were present in the PIPLC-released proteins. Figure 2.3B shows a silver-stained 2D gel containing proteins released by PIPLC treatment.

Cry1Ac binding proteins in the PIPLC-released fraction were detected by probing blots of 1D and 2D gels with biotinylated Cry1Ac (Figure 2.4A and 2.4B). After development for Cry1Ac binding, the same blot was probed with anti-mALP antibodies. On the 2D Cry1Ac blot a cluster of proteins at 120- and 110-kDa were detected. These proteins correspond in size to the Cry1Ac-binding APNs (Gill, et al., 1995; Luo et al., 1996; Oltean et al., 1999; Banks et al., 2001). Silver-staining (Figure 2.3B) only detected a group of proteins at 110- and not 120-kDa. Cry1Ac bound three chains of proteins in the 65 to 70-kDa-size range; two of which were detected by anti-ALP antibody. A single spot in the third Cry1Ac-chain also bound anti-ALP.

When 300-fold excess unlabelled Cry1Ac was included in the ligand blots, no biotin-Cry1Ac binding was detected.

Identification of Cry1Ac binding proteins by Peptide Mass Fingerprinting (PMF):

The 2D gel of PIPLC-released BBMV proteins used for protein identification by PMF analyses is shown in Figure 2.5. Spots 1 to 8 and spot 10 matched the coordinates of the Cry1Ac-binding proteins shown in Figure 2.4B. These spots plus spot 9 were picked, digested with trypsin and the resulting peptides were analyzed by MALDI-TOF MS. PROWL was used for searching protein databases (metazoan, *Anopheles* and *Drosophila* databases in NCBI). The results are shown in Table 1.

Based on molecular size, we anticipated that the PMF patterns of spots 1 to 3 (90 – 100-kDa) would match insect APNs. However, spots 1 and 2 matched predicted proteins in the *Anopheles gambiae* database and not APNs. The amino acid coding regions of the predicted *Anopheles* proteins were used in BLASTP searches against NCBI protein databases. Interestingly, the *Anopheles* match to spot 2 has cadherin repeat domains and is homologous to the calsyntenin proteins of the cadherin superfamily. The PMF of spot 3 matched a GPI-anchored apical gut membrane protein in the parasitic nematode *Haemonchus contortus*. Proteins at 65-kDa (spots 5-7 and 10) that bound both Cry1Ac and anti-mALP antibodies were analyzed by PMF. Spots 7 and 10 with pIs = 5.5-5.9, were identified as membrane bound alkaline phosphatases. Spots 5 and 6, also migrating at 65-kDa had pIs = 6.1, and PMF patterns distinct from the alkaline phosphatases. The spot 5 PMF spectra matched a protease in *B. mori*

with a Z score of 2.43 (>99% percentile score). Spot 6 matched a predicted *Anopheles* protein (Table 1) that has similarity to laminin-like proteins of extracellular matrices.

Cry1Ac-binding protein spots 4 (68-kDa) and 8 (55- kDa) gave 100% probability matches to V-ATP synthase subunits A and B. Spot 4, a 68-kDa protein, matched V-ATPase subunit A of *M. sexta*. Spot 8, the 54.8-kDa protein matched V-ATPase subunit B of *H. virescens*. Spot 9 was picked due to its proximity to spot 8, however it did not bind Cry1Ac. Surprisingly, spot 9 matched V-ATPase B subunit from *H. virescens* with a >99.9% probability.

MS/MS results:

As described above, several protein spots bound Cry1Ac, ALP antibody and the PMF patterns matched ALP. As final confirmation we subjected the ALP spots to MS-MS analyses. The ion with the highest intensity was picked for MS/MS analyses and the spectra analyzed by *de novo* sequencing. Although we obtained a sequence of 3 amino acids AYR, the length was too short to yield a meaningful MS-BLAST search result.

DISCUSSION:

We used a proteomic approach involving 2DE and mass spectrometry to identify Cry1Ac-binding proteins in the brush border membrane of *H. virescens*. Through this study, V-ATP synthase subunits A and B were identified as novel Cry1Ac binding proteins. A second form of V-ATPase subunit B was identified as a component of BBMV, but that specific subunit B did not bind toxin. A cluster of HvALP spots were identified as Cry1Ac binding proteins.

Additional spots that bound Cry1Ac had PMF patterns that matched Anopheles proteins with similarities to calsyntenin and laminin-like proteins.

In Lepidopteran larvae, ALP is a GPI-anchored protein of about 65-kDa in the brush border membrane (Eguchi, 1995). In *H. virescens*, ALP is an abundant protein associated with membrane microdomains call lipid rafts (Zhuang et al. 2003). The difference we observed for ALPs resolved on a 1D versus 2D gel illustrates the greater resolving power of 2D systems. Although migrating on a 1D gel as a single band at 65-kDa with a faint slightly smaller band (Figure 2.2C), the 2D gel system resolved proteins detected by anti-ALP antibody into three clusters of proteins ranging in size from 58-65-kDa (Figure 2.2D). Some of the diversity in ALP could be due to the association of both soluble and membrane ALPs with BBMV (Okada, 1989). Other possibilities are that individual spots within a chain or cluster may be isozymes or post-translational modifications of the same enzyme. One cluster of spots detected by anti-ALP antibody gave a strong signal when probed with Cry1Ac. Spots in that cluster were confirmed as ALPs through mass spectroscopy. This result, i.e. a single cluster of proteins bound by anti-ALP and Cry1Ac, is similar to that reported for BBMV from *M. sexta* (McNall and Adang, 2003). A difference is that in *H. virescens*, two additional protein clusters were detected by anti-ALP antisera, evidence that Cry1Ac binds a subset of ALPs in *H. virescens*. While Cry1Ac binds ALP on blots, does the toxin bind ALP in a non-denatured confirmation? Two studies support the assertion that Cry1Ac binds native ALP. Cry1Ac reduced ALP activity in BBMV prepared from *H. virescens* (English and Readdy, 1989). More convincingly, Cry1Ac inhibited ALP activity in a preparation of toxin binding proteins isolated from solubilized *M. sexta* BBMV (Sangadala et al. 1994).

GPI-anchored proteins, including aminopeptidases and ALPS comprise an important sub-set of proteins in the brush border membrane. We released GPI-anchored proteins with PIPLC then investigated the Cry1Ac binding proteins in that subset. Previously, this approach was used to enrich for GPI-anchored proteins in *Arabidopsis thaliana* (Sherrier et al., 1999) and in *M. sexta* (McNall and Adang, 2003). PIPLC release served to eliminate the bulk of BBMV proteins allowing the enrichment of proteins such as HvALP spots that were otherwise present in relatively small amounts.

APNs are GPI anchored and proteins that bind Cry1Ac toxin. Their sizes in *H. virescens* range from 110- to 170 kDa (Gill et al., 1995; Banks et al., 2001). We did not detect either 170-kDa or 120-kDa APNs in BBMV or PIPLC-released samples. The fact that membrane proteins are hard to resolve using IPG strips for 2DE can explain the absence of these proteins in BBMV samples without concentration by PIPLC digestion (Santoni et al., 2000). However, we expected spots 1-3 at 90-100-kDa to be APNs. Yet, the PMF spectra matched predicted *Anopheles gambiae* proteins with similarity to syntenins and a GPI-anchored protein from *H. contortus*.

V-ATP synthase subunits A and B of 68-kDa and 54.8-kDa were identified as Cry1Ac binding proteins. Both these subunits are part of the peripheral V_1 complex of vacuolar ATPase essential for assembly and catalytic function. It is known that V-ATPases occur ubiquitously in endomembranes (Harvey and Nelson, 1992). This enzyme is present in the apical membrane of goblet cells and represents the primary energy source for secretion and absorption by serving as an H^+/K^+ transporter across the insect midgut epithelium. V-ATPase is responsible for acidifying a variety of intracellular compartments in eukaryotic cells. It is possible that Cry1Ac binding to V-ATPase in the midgut interferes with the H^+/K^+ transport and hence destabilizes the ionic balance. This could lead to pH imbalance and cause the cell to collapse.

It was interesting to observe the presence of V-ATPase subunits that are non-GPI anchored, in the PIPLC solubilized BBMV sample. In agreement with this observation, lipid raft studies have shown that a 57-kDa V-ATPase (B subunit) partitioned exclusively into the soluble fraction (Zhuang, 2002). This could be due to weak binding that exists between these proteins and the brush border membrane. Alternatively, under certain temperature conditions, the V_1 component (containing the A and B subunits) may break off from the rest of the ion-transport machinery bringing them into the soluble phase. One other surprising observation was the presence of another subunit of V-ATPase (subunit B) of 54.8-kDa that does not bind Cry1Ac. Of the 2 B subunits, only one binds Cry1Ac while the other does not.

Whether V-ATPase is a functional receptor of Cry1Ac or a "null receptor" is yet to be classified. As proposed by Lee in 1995, "null receptors" accounted for Cry1Ab and Cry1Ac binding, but not killing in resistant insects. Besides, whether these proteins are modified in resistant insects and can confer resistance remains to be explored. Studies on Bt susceptible and resistant larva of *Plodia interpunctella* showed that the level of V-ATPase is increased in the resistant insects, probably reflecting the elevated cellular energy profile across the midgut epithelium that may be necessary to combat toxin stress and consequently to maintain resistance at the tissue level (Candas et al., 2001).

The present study demonstrates the usefulness of two-dimensional electrophoresis and peptide mass finger printing in identification of proteins in larval gut samples. Based on bioinformatics using current databases containing amino acid sequences of proteins, proteomics is a very useful tool in protein identification (Lester and Hubbard, 2002). This could result in new targets and new insecticides to combat the problem of resistance exhibited by insects. Further studies on the V-ATPase relating to Cry1Ac as functional receptors could prove useful in

determining the entire gamut of midgut proteins involved in Cry1Ac intoxication. Further studies involving glycosidase-mediated trypsin digestion followed by MS/MS analysis could prove useful to obtain information on HvALP sequence.

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FIGURES AND TABLES:

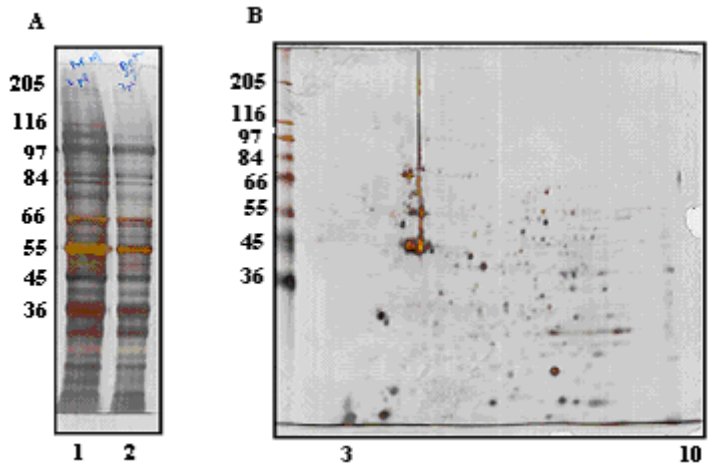

FIGURE 2.1 1D AND 2D POLYACRYLAMIDE GEL SEPARATIONS OF *H.VIRESCENS* BBMV PROTEINS

Gels were silver stained. (A) BBMV (10 µg) separated by 8% SDS-PAGE: Lane 1: BBMV proteins processed by precipitation and solubilization. Lane 2: BBMV proteins solubilized directly in SDS-PAGE sample buffer. (B) BBMV (45 µg) resolved on a pH 3-10 non-linear Immobiline DryStrip (Amersham Biosciences) for first dimension and an 8% SDS-PAGE for second dimension. Positions of molecular size markers are indicated on the side of each gel and pH range of isoelectric focusing are indicated at the bottom.

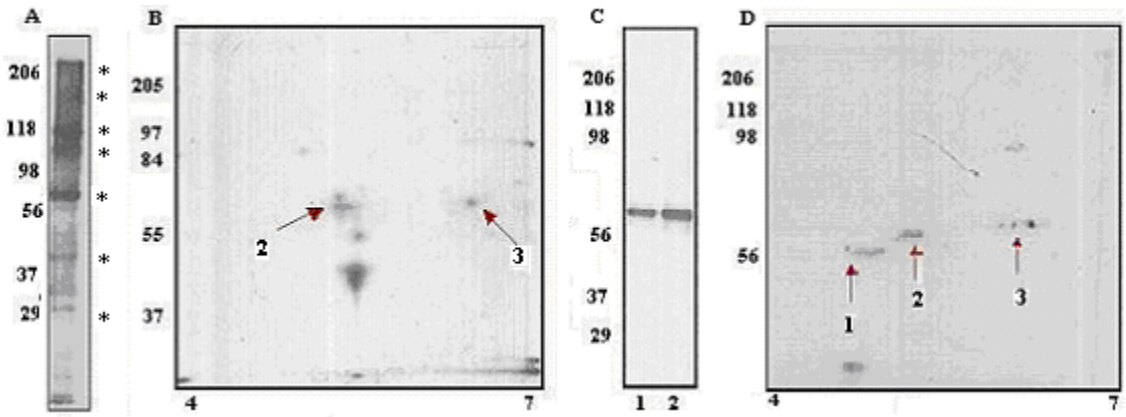

FIGURE 2.2 CRY1AC AND m-ALP BLOTS OF 1D AND 2D RESOLVED *H. VIRESCENS* BBMV PROTEINS

Blots A and B were probed with 5 nM of biotinylated Cry1Ac. Bound toxin was visualized by incubation with and streptavidin-HRP and detected by ECL. Blots C and D were probed with rabbit antibody against *B. mori* membrane ALP and detected by anti-rabbit antibody-HRP and ECL.

(A and C) BBMV proteins (10 µg) processed by precipitation and solubilization and separated on 1D on an 8% SDS-PAGE. Lane 1: BBMV proteins solubilized directly in SDS-PAGE sample buffer. Lane 2: BBMV proteins processed by precipitation and solubilization. (B and D) BBMV proteins (10 µg) resolved by 1D and 2DE using a pH 4-7 Immobiline DryStrip (Amersham Biosciences) for first dimension and 8% SDS-PAGE for second dimension. Positions of molecular size markers are indicated on the side of each gel and pH range of isoelectric focusing are indicated at the bottom. (*) denotes major Cry1Ac binding proteins. Numbers on the blots represent protein chains and the same number denotes spots that bound both Cry1Ac and anti-mALP antibody.

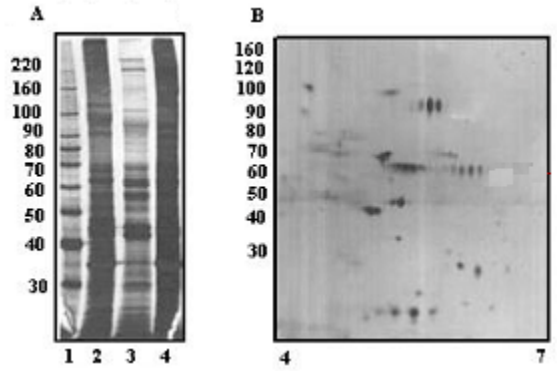

FIGURE 2.3 SILVER STAINED SDS-PAGE OF PIPLC-DIGESTED BBMV PROTEINS OF *H.VIRESCENS*

(A) BBMV proteins (10μg) resolved by 8% SDS-PAGE. Lane 1: Molecular size standard (Benchmark, Invitrogen). Lane 2: BBMV proteins processed by precipitation and solubilization. Lane 3: Supernatant obtained from the digestion of BBMV with PIPLC. Lane 4: Pellet that remained after digestion of BBMV with PIPLC. (B) Proteins (10μg) released from BBMV by PIPLC resolved by 2DE using a pH 4-7 Immobiline DryStrip (Amersham Biosciences) for first dimension and 8% SDS-PAGE for second dimension. Position of molecular size markers are indicated on the side of each gel and pH range of isoelectric focusing are indicated at the bottom.

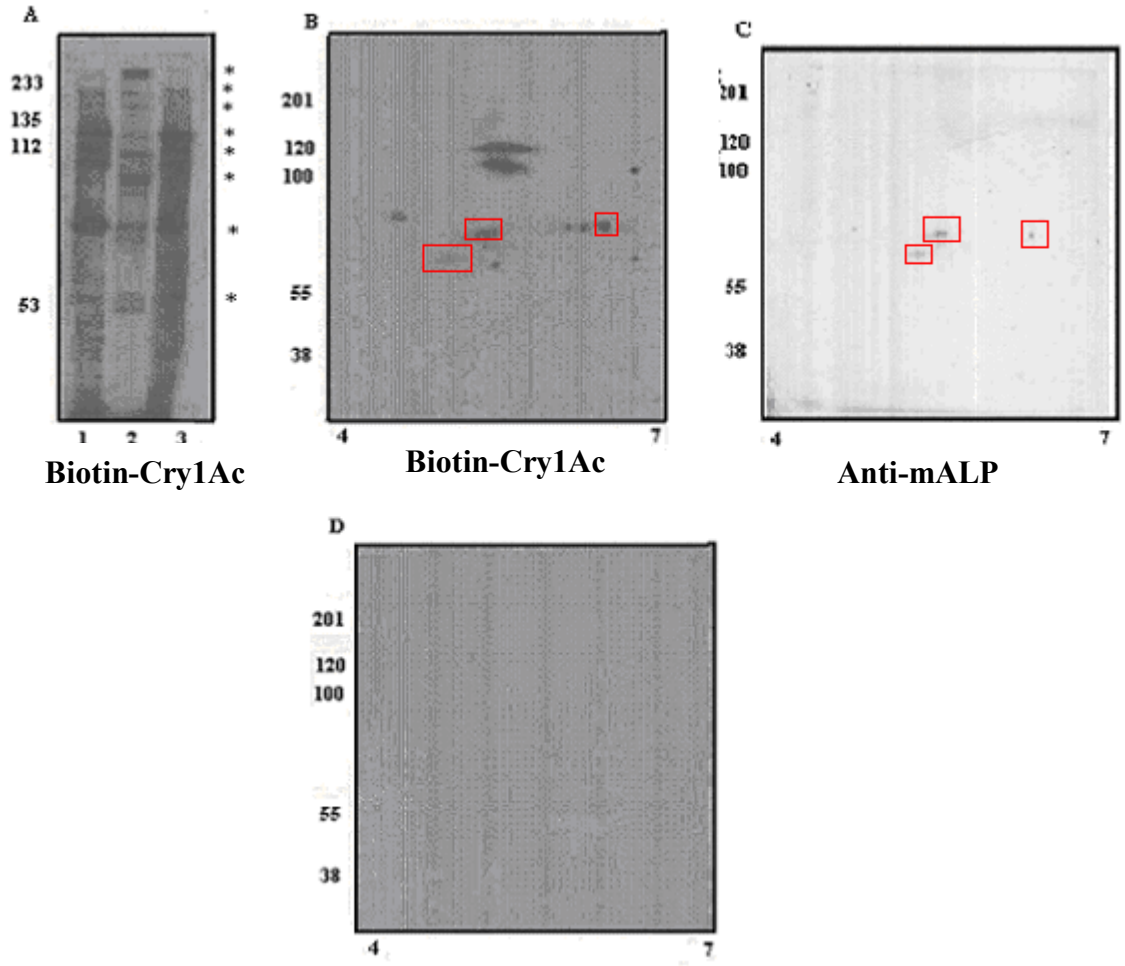

FIGURE 2.4 LIGAND AND WESTERN BLOTS OF PIPLC DIGESTED PROTEINS

(A) BBMV proteins (10 µg) resolved by 8% SDS-PAGE and blotted. Lane 1: BBMV processed by precipitation and solubilization. Lane 2: BBMV proteins released by PIPLC processed by precipitation and solubilization. Lane 3: Pellet that remained after digestion of BBMV with PIPLC. (B) BBMV proteins released by PIPLC (10 µg), resolved by 2-D electrophoresis on a 4-7 pH Immobiline DryStrip (Amersham Biosciences) for first dimension and 8% SDS-PAGE for second dimension. 5 nM of biotinylated Cry1Ac was used to probe blots. Bound toxin was visualized by incubation with streptavidin-HRP and detected by ECL. (C) 2D blot probed with

membrane anti-ALP and anti-rabbit -HRP. (D) Binding competition assay: A 2-D gel blot was incubated with 5 nM biotin-Cry1Ac in the presence of 1.5μM unlabelled Cry1Ac. Positions of molecular size markers are indicated on the side of each gel and pH range of isoelectric focusing are indicated at the bottom. (*) denotes Cry1Ac binding proteins detected in Lane 2 of panel A. Boxes indicate protein spots binding both the toxin and the antibody.

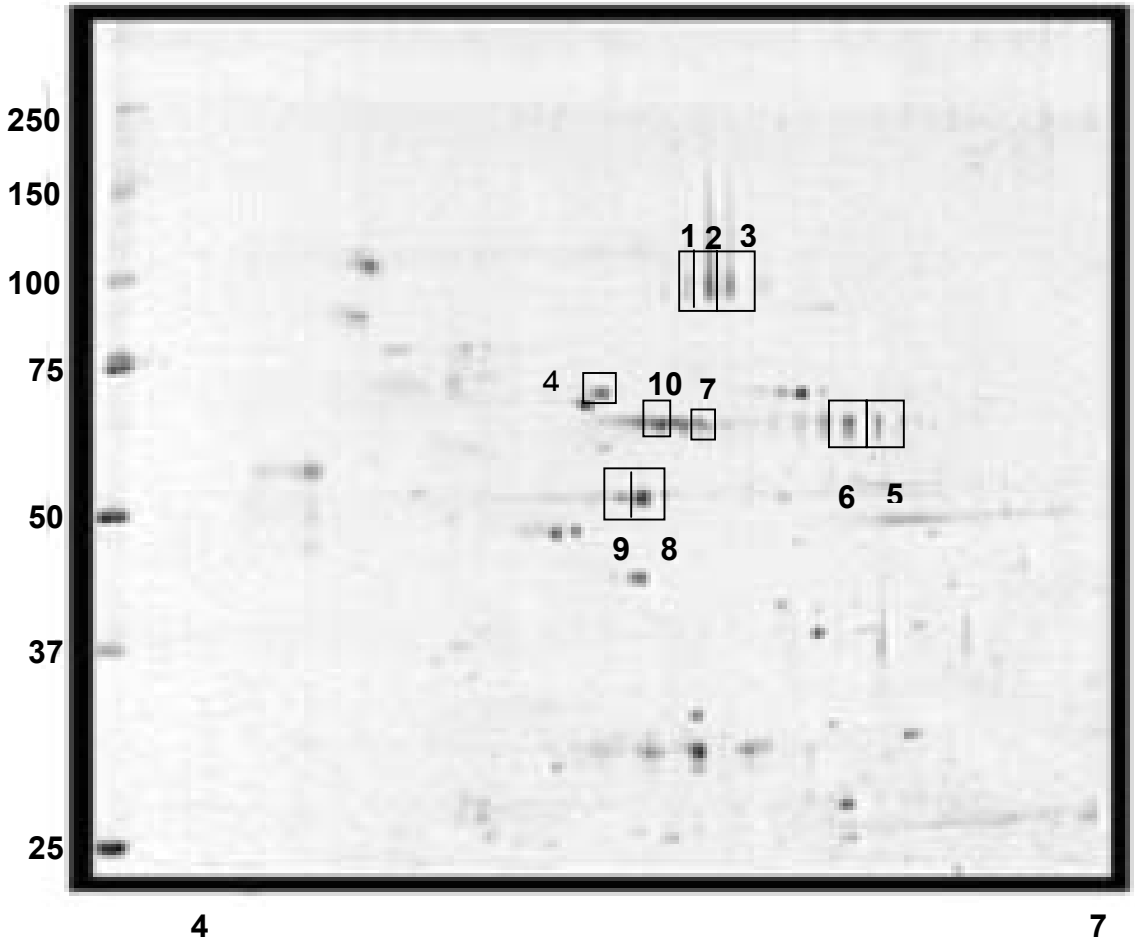

FIGURE 2.5 DEPICTION OF SPOTS ANALYZED BY PMF

BBMV proteins released by PIPLC (45 µg) separated on a 4-7 pH IPG strip and stained by SyproRuby. Numbers to the right of the gel represent spots that were picked for mass spectrometric analyses. Molecular weights of markers, pI and spot pattern were used to correlate spots on ligand and western blots (Figure 2.4B and 2.4C) with those on SyproRuby stained gel. Spots numbered 1 through 8 and 10 bound Cry1Ac on ligand blots.

TABLE 1: PEPTIDE MASS FINGERPRINT RESULTS USING PROWL

Spot number	Predicted Mass	Observed Mass/pI	Accession number	Species	Protein identified	% Coverage	Est'd Z
1	120	96.08/6.3	gi[31200245_	*Anopheles gambiae*	ENSANGP00000019537	7	0.42
2	120	98.76/6.2	gi[31209007_	*Anopheles gambiae*	ENSANGP00000011143	13	1.36
3	120	92.96/6.2	gi[1335976_	*Haemonchus contortus*	Apical gut membrane polyprotein	12	0.44
4	**68**	**68.15/5.1**	**gi[401323**	***Manduca sexta***	**Vacuolar ATP synthase A**	**32**	**1.97**
5	63-65	63.28/6.1	gi[84785_	***Bombyx mori***	Egg specific protein	9	2.43
6	63-65	58.37/6.4	gi[31231439_	*Anopheles gambiae*	ENSANGP0000002122	10	0.26
7	**63-65**	**59.13/5.8**	**gi[15128500_**	***Bombyx mori***	**Membrane-bound alkaline phosphatase**	**8**	**2.32**
8	**55**	**54.88/5.3**	**gi[401326_**	***Heliothis virescens***	**Vacuolar ATP synthase B**	**43**	**2.34**
9	**55**	**54.88/5.3**	**gi[401326_**	***Heliothis virescens***	**Vacuolar ATP synthase B**	**44**	**2.34**
10	**63-65**	**59.13/5.8**	**gi[15128500_**	***Bombyx mori***	**Membrane-bound alkaline phosphatase**	**10**	**1.19**

Spots were chosen from PIPLC released BBMV proteins against PROWL. Spot numbers correspond to Figure 2.5. Spots numbered 1 through 8 and 10 bound Cry1Ac on ligand blots. Highlighted search results denote good matches obtained from the searches.

CHAPTER 3

GENERAL DISCUSSION AND CONCLUSION

The primary goal of this research was to use a 2D gel-based proteomics approach to discover novel proteins in the brush border membrane of *H. virescens* that bind Bt Cry1Ac toxin. A secondary goal was to examine the complexity of the brush border proteome. *H. virescens* and Cry1Ac were selected as the pest insect and toxin, respectively, for this study because this economically important pest is controlled by Bt cotton expressing Cry1Ac (reviewed in Whalon and Wingered, 2003).

Protein separations of BBMV on one-dimensional electrophoresis (1DE) have been used to identify toxin-binding proteins by ligand blotting. Due to the complexity of proteins in BBMV, proteins of the same molecular size cannot be distinguished by 1DE. In a recent study McNall and Adang (2003) used a 2D-gel based approach to discover ALP and actin as Cry1Ac-binding proteins in *M. sexta*. This data is of value because identifying a toxin binding protein is the first step towards determining its potential as a toxin receptor.

Identification of Bt receptors and the protein components of BBMV can help in understanding resistance patterns exhibited by certain larva towards the toxin. 2DE has been used to compare Bt- susceptible and -resistant larval gut samples of *P. interpunctella*, to determine difference in levels of expression of proteins (Candas et al., 2003). Considering the usefulness of this approach, we decided to exploit the technology for the identification of novel Cry1Ac binding proteins in the BBMV of *H. virescens*.

In Chapter 2, I describe this approach to the discovery of two novel Cry1Ac binding proteins and information gathered about another potential Cry1Ac receptor. BBMV proteins were separated on Immobiline strips and then run on SDS-PAGE. Cry1Ac binding proteins (determined by ligand blotting with Cry1Ac) were excised from a gel, trypsin digested and the peptides extracted from the protein were then used for mass spectrometry analysis. This

generated a peptide mass fingerprint that was used to search a database for homology with other proteins. A probability score predicts the usefulness of the match, though many fingerprints come up with very poor matches. This could be due to the lack of the same or similar proteins in the database or modifications of the protein. Unsequenced genomes present a huge challenge in proteomic studies of such organisms, as is the case in insects. Western blotting is one way to confirm the identity of a protein with a low probability score. I employed the reverse of this logic; used mass spectrometry to confirm the identity of a series of proteins we identified as ALPs using antibodies to it.

A series of proteins of approximately 65-kDa in size were identified as ALPs using sera against m-ALP using western blot analysis (Fig: 2.2D and 2.4D). This correlated to several earlier findings such as; a 72-kDa alkaline phosphatase in *H. virescens* (English and Readdy, 1989), a 68-kDa protein that binds Cry1Ac (Jurat-Fuentes et al., 2002), alkaline phosphatase inhibition by Cry1Ac (English et al., 1989), and the presence of GPI anchored ALPs in *B. mori* (Eguchi et al., 1995). ALPs have been previously reported to interact with Cry1Ac toxin in ligand blots of BBMV from *M. sexta* (McNall and Adang, 2003). Collectively, the 68-kDa protein is a GPI anchored ALP, that binds Cry1Ac. Two spots (7 and 10) with pIs = 5.5-5.9, were identified as membrane bound alkaline phosphatases. However two other spots (5 and 6), also migrating at 65-kDa had PMF patterns distinct from the alkaline phosphatases. Because ALPs have been known to be localized in lipid rafts, Cry1Ac binding may play a crucial role in toxin oligomerization and pore formation. Spot 5 matched a protease in *B. mori* while spot 6 matched a predicted *Anopheles* protein that has similarity to laminin-like proteins of extracellular matrices. The importance of these identifications is not clearly understood at this time. Besides the PMF identifications, *de novo* sequencing was also attempted for the ALPs and putative

APNs. However no significant amino acid sequence could be established owing to poor fragmentation patterns detected on the mass spectrometer. This can be explained by various factors such as glycosylation, amino acid composition, or loss of ions in the spectra.

V-ATP synthase subunits A and B of 68- kDa and 54.8- kDa were identified as novel Cry1Ac binding proteins. Both these subunits are part of the peripheral V_1 complex of vacuolar ATPase essential for assembly and catalytic function. Though it seemed unusual that the non-GPI anchored V-ATPase subunits are found in the soluble fraction of GPI anchored proteins, it correlates to the detection of a 57-kDa V-ATPase subunit in the soluble fraction of lipid raft isolation (Zhuang et al., 2002). Of the 3 V-ATPase subunits identified, one of the subunits does not bind Cry1Ac.

The role of V-ATPase in Cry1Ac mediated toxicity is yet to be determined. Subunits of the enzyme used with and without Cry1Ac in an assay that offers a substrate for the enzyme and measures inorganic phosphate accumulation would be an ideal way to determine whether V-ATPase is a functional receptor of Bt toxin. I hypothesize that if the enzyme is a functional receptor, then presence of Cry1Ac in the sample should hamper ATPase activity, leading to the generation of low amounts of inorganic phosphate. Vice versa, equal amounts of inorganic phosphate accumulated in the presence and absence of Cry1Ac would establish that the enzyme merely binds the toxin, but does not have a functional effect and hence may not play a "direct" role in Cry toxicity. However this assay will not be able to reflect secondary effects of the toxin-enzyme binding that could contribute to toxicity. Based on the general function of V-ATPase, in figure 3.1, I propose the mode of action it may employ in Cry toxicity, if it were a functional receptor. Supporting the case that V-ATPase is involved in toxin action is the observation that a Bt-resistant strain of *P. interpunctella* (Indian meal moth) has an altered V-ATPase (Candas et

al. 2003). Although the mechanism is unknown, it is quite possible that V-ATPase is involved in Bt toxin activity and this subject should be the focus of further research.

Proteomics is not an oft-used technique in Lepidoptera. The biggest limitation of this technology in the study of lepidoptera is the lack of a well-stocked database. Very few insects have been sequenced and so obtaining matches for the peptide mass fingerprint from the database is challenging. Also the peptide mass fingerprint includes post-translational modifications that may not feature in the database and hence not generate high probability matches. Active persuasion of this technology to generate a wider database will help overcome this limitation, and hence offer a global view of the proceedings that occur upon Cry toxicity within an insect system.

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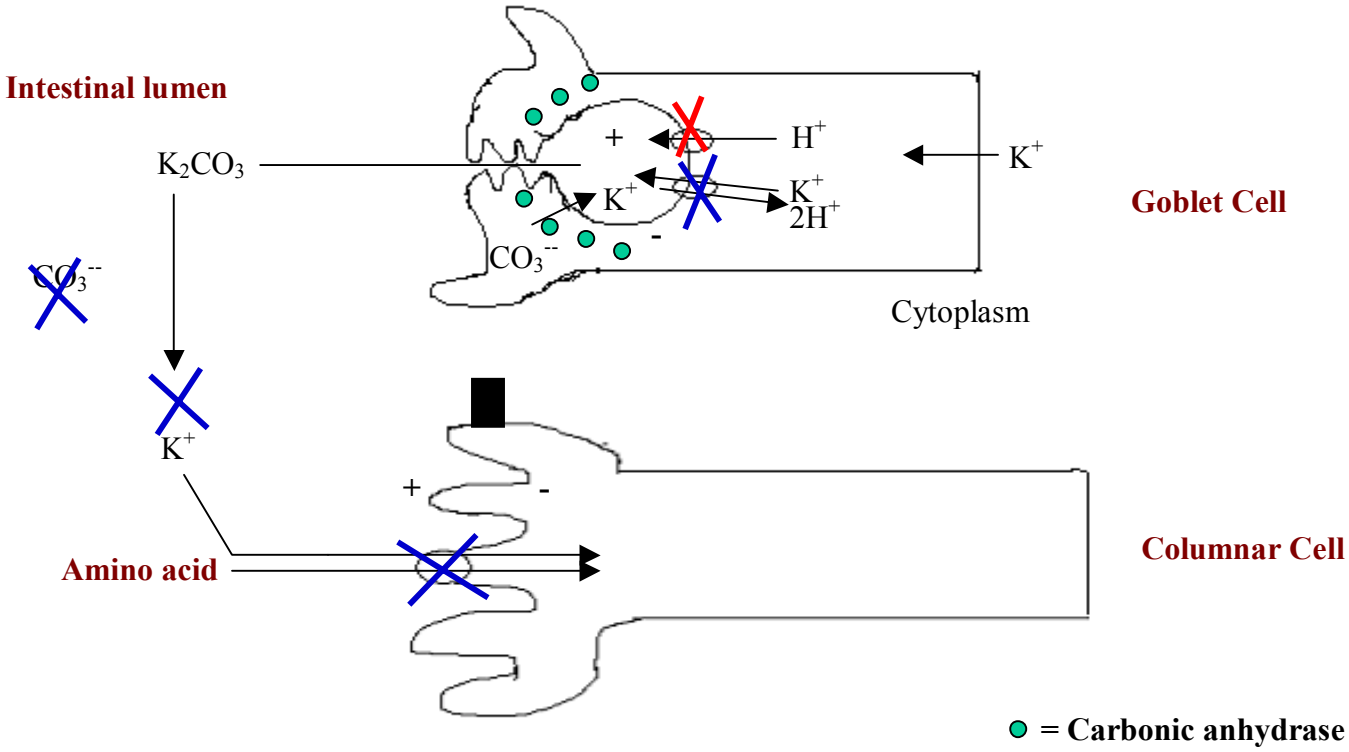

FIGURE 3.1 HYPOTHESIZED MODE OF ACTION OF A FUNCTIONAL V-ATPASE IN THE PRESENCE OF CRY1AC IN THE BBMV OF *H. VIRESCENS* (The red cross denotes impairment of V-ATPase by binding to the toxin and the blue crosses indicate processes that could potentially be hampered by this binding)