PROTEOMIC ANALYSES OF THE INTERACTION OF INSECT MIDGUT PROTEINS WITH *BACILLUS THURINGIENSIS* TOXINS

by

REBECCA JO MCNALL

(under the direction of Michael J Adang)

ABSTRACT

Brush border membrane vesicles (BBMV) from insect midguts are an *in vitro* model for studying *Bacillus thuringiensis* (Bt) toxins. Despite their widespread use, the protein components of these vesicles are mostly unknown. Two-dimensional electrophoresis (2DE) was used to analyze vesicles from *Manduca sexta* and establish two subproteomes: GPI-anchored proteins and Cry1Ac binding proteins. Through mass spectrometry and western blot analyses, two new Bt binding proteins were identified from 2DE blots: alkaline phosphatase and actin.

BBMV proteins from diet adapted Bt-susceptible and -resistant *Plutella xylostella* strains were tagged with fluorescent dyes and simultaneously compared by two dimensional difference gel electrophoresis (2D-DIGE). Although BBMV derived from whole animals are a suitable substitute for BBMV prepared from dissected guts, both preparation types were analyzed by 2D-DIGE to determine if protein differences between strains were observed. More proteins were resolved in whole animal preparations, but more differences in protein abundance were detected in dissected gut BBMV preparations between susceptible and resistant *P. xylostella*. Mass spectrometry analysis by MALDI-ToF/ToF of several altered spots resulted in the identification

of two proteins. In resistant animals, actin was identified as a less abundant protein and glucosinolate sulphatase (GSS) as a more prevalent protein. Western blot comparisons of diet-adapted and cabbage-reared susceptible and resistant animals revealed that GSS was more abundant in diet adapted strains and in resistant animals. Enzyme activity assays revealed that alkaline phosphatase levels were also higher in these populations. The amount of aminopeptidase protein was unchanged between the four *P. xylostella* BBMV preparations.

The identification of new Bt binding proteins and proteins implicated in Bt resistance broadens the understanding of Bt action in insect midgut epithelial cells, especially post-binding interactions that lead to the insect's death. This knowledge will help prolong the use of Bt as an effective biological control. Our findings realized the potential of 2DE-based proteomics as an approach for identifying proteins in insect tissues, specifically those involved in Bt action. Additionally, my studies are the first to describe the use of additional techniques, western blotting and bioinformatics analyses, to facilitate proteins identification by mass spectrometry and overcome the limitations of this technique.

INDEX WORDS: *Bacillus thuringiensis*, *Manduca sexta*, *Plutella xylostella*, resistance, proteomics, 2D-DIGE, alkaline phosphatase, glucosinolate sulphatase, actin, aminopeptidase, mass spectrometry

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

A. *Bacillus thuringiensis*

A.1. History of *Bacillus thuringiensis*

Bacillus thuringiensis (Bt) is a Gram positive, spore-forming bacterium that produces crystalline inclusion bodies (Schnepf et al., 1998). The crystals are comprised of Cry proteins that are toxic to certain species of insects, nematodes and protozoa. Over 250 different *cry* genes have been identified and categorized by amino acid similarity (http:www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/index.html) (Crickmore et al., 1998). Typically most closely related toxins affect insects in the same order.

Although the status of Bt as an insect pathogen had been known since the early 1900's, it wasn't until the mid-1950's that Angus proved that the crystalline inclusion body was responsible for toxicity (Angus, 1956). That finding led to the development of Bt spore – crystal mixtures as commercial biopesticides that have been used for the past 50 years. The use of Bt instead of synthetic pesticides has two main benefits: it does not get amplified through the food chain and is very specific for its target pests. Thus, it is both a feasible and environmentally friendly alternative to traditional chemical pesticides. In fact, Bt-based insecticides are the most widely used biological control agents (Betz et al., 2000).

While Bt in powdered or sprayable formulations is practical for small-scale use, it can be quite costly for large-scale growers. Ultraviolet light quickly degrades the Bt crystal protein, so formulations directly applied to leaves must be repeated for continuous insect control. Plants engineered to express Bt proteins have overcome limitations of conventional Bt formulations by providing insect control throughout the growing season (de Maagd et al., 1999). Since they were commercialized in 1996, Bt-corn and cotton have become an integral part of crop production.

A.2. Cry toxin structure and function

The crystal structures of four Bt cry toxins have been resolved: Cry3Aa (Li et al., 1991), Cry1Aa (Grochulski et al., 1995), Cry2Aa (Morse et al., 2001), and Cry3Bb (Galitsky et al., 2001). Although these four toxins are active against different insect orders and share low amino acid sequence identity (27-36%), their protein structures are virtually identical (Schnepf et al., 1998). Activated toxin consists of three structural domains: domain I consists of seven alpha-helices, domain II has three anti-parallel beta-sheets in a Greek key motif, and domain III is composed of two anti-parallel sheets in a jelly-roll motif.

Numerous mutational studies have demonstrated that initial contacts with molecules in brush border of the insect midgut occur primarily through the loops of domain II that connect the beta-sheets and extend from the bulk of the toxin structure (reviewed in Schnepf et al., 1998). Toxins that have high amino acid similarity in the loops of domain II share binding sites on the brush border membrane (Jurat-Fuentes et al., 2001), Additionally, similarity in domain II loop structure is correlated with cross-resistance in lepidopteran insects (Tabashnik et al., 1996), and alterations in these loops can make a toxin effective against previously non-target insects (Abdullah et al., 2003).

Domain III has an overall topology comparable to lectins (Burton et al., 1999). The sugar N-acetylgalactosamine (GalNac) is an important binding epitope for Cry1Ac (Knowles et al., 1984; Knowles et al., 1991; Garczynski et al., 1991). The GalNAc binding site is located in a pocket of domain III (Burton et al., 1999; Jenkins et al., 1999). Domain III also has a broader role in binding and insect specificity (de Maagd et al., 2001).

Once binding has occurred, toxins oligomerize and insert into the midgut epithelium forming a pore (Aronson et al., 1999; Soberon et al., 2000). Based on its structural similarity to

other bacterial toxins, domain I was first proposed as the pore-forming region (Parker and Pattus, 1993). Biophysical and mutational studies have identified that only helices 4 and 5 of domain I insert into the membrane and forming the pore with four other toxin molecules (reviewed in de Maagd et al., 2001). The rest of the helices lay spread out on the membrane surface in an umbrella-like fashion (Gazit et al., 1998). Domain III may also play a role in pore formation (reviewed in Schnepf et al., 1998) although its primary role seems to be toxin stability and specificity (Li et al., 1991).

A.3. Cry toxin mode-of-action

In Lepidoptera, after a susceptible insect ingests Bt crystals, the crystal protein is solubilized in the alkaline midgut of the animal. This solubilized protein, referred to as protoxin, exists as a 130 kDa form for Cry1 toxins. The C-terminal half of protoxin is removed sequentially by serine proteinases and additional cleavages occur near the N-terminus. The result is an activated toxin of about 65 kDa. Activated toxins bind specifically and with high affinity to the brush border membrane of the insect midgut epithelial cells (reviewed in Schnepf et al., 1998). There is evidence that contact with receptor allows an additional cleavage of the first a-helix in domain I (Soberon et al., 2000).

Research has focused on studies to identify the molecule(s) to which Bt toxins binds. The Bt field refers to molecules (primarily proteins) that have been shown to specifically bind Bt toxins as receptors, although the criteria used by endocrinologists have not in most cases been met. In Lepidoptera, identified Cry1A receptors belong to two main families: aminopeptidase N and cadherin-like proteins (reviewed in Whalon and Wingerd, 2003). Aminopeptidase N binding to Cry1Ac has been shown to be mediated by GalNAc interactions and the binding affinity for Cry1A toxins is 75 - 100 nM (Masson et al., 1995a; Jenkins and Dean, 2001). The affinity of the

cadherin-like protein is approximately 1 – 2.5 nM (Vadlamudi et al., 1995, Jenkins and Dean, 2001). Recently, these proteins' differences in binding affinity for Bt toxins were proposed to dictate the order of toxin binding, i.e., toxin binds the cadherin-like protein first (Zhuang et al., 2003). However, the difference in binding affinities is only significant if concentrations of each protein are determined. For example, if aminopeptidase N is present at 100-fold higher levels than cadherin, the differences in binding affinities are essentially negated. Future studies will be required to determine the protein concentration of each of these proteins in BBMV, and if the differences in concentrations or affinities are important in toxin mode-of-action.

Gomez et al. (2002) demonstrated that the binding of toxin to the cadherin-like protein mediated interactions, i.e. oligomerization, between Cry1Ab molecules. These interactions had been previously shown to be required for toxicity (Aronson et al., 1999; Soberon et al., 2000). Toxin-cadherin binding promotes the cleavage of helix-1 from domain I followed by the formation of toxin oligomers. This oligomeric form has been labeled the 'pre-pore' since it is much more efficient at forming pores than monomers of toxin (Gomez et al., 2002).

Whereas the cadherin-like protein appears to be a critical interaction for mediating successful toxicity, recent work has demonstrated that multiple interactions must take place for the oligomers to penetrate the midgut epithelium (Zhuang et al., 2003). Other pathogens, including bacterial toxins, such as clostridium and aerolysin utilize lipid rafts to mediate toxicity (Manes, 2003). It is important to note, however, the debate over biologically-relevant and experimentally created lipid rafts. Lipid rafts are often purified by the treatment of membranes with detergents. A concern is that these detergents induce proteins to associate with rafts or induce the assembly of the rafts themselves (reviewed in Zajchowski and Robbins, 2002). However, other methods have been used to isolate lipid rafts, suggesting that they are in fact not

an artifact of detergent-based purification schemes. The use of cross-linkers and immunofluorescently-labeled antibodies has demonstrated the localization of specific proteins within, and outside of, rafts (reviewed in Zajchowski and Robbins, 2002). Although the sum of this evidence suggests that lipid rafts exist *in vivo*, the continued debate over the true nature of these lipid rafts should be kept in mind especially in insects. Studies focused on lipid rafts may require purification of these rafts using detergent-based and detergent-free methods.

In *Manduca sexta*, as in mammals, lipid rafts are composed of cholesterol, GPI-anchored proteins, and sphingolipids (Zhuang et al., 2002). When purified with Triton X-100, these lipid rafts preferentially contain the toxin-binding 120 kDa aminopeptidase N (Zhuang et al., 2002). Cry1A toxins associate with these rafts and form pores only when lipid rafts are intact (Zhuang et al., 2002). Immunoprecipitation experiments demonstrated that cadherin only interacts with monomers of Cry1Ab, while aminopeptidase associates with toxin oligomers (Zhuang et al., 2003). In fact, the affinity of APN for toxin is greatly increased for the oligomeric form versus the monomeric form (0.75 nM vs 165 nM) (Zhuang et al, 2003). These findings suggest that toxin monomers bind to cadherin with high affinity and then oligomerize. Oligomers, having a high affinity for APN, bind to this molecule and pull the cadherin-like protein into lipid rafts. Once in these rafts, pore formation can occur.

After the prepore oligomer is in its proper configuration, there is an additional conformational change where domain I swings away from the rest of the molecule and inserts into the membrane. Vie et al. (2001) described a functional pore consisting of a toxin tetramer. This is in agreement with the tetrameric prepore described by Gomez et al. (2002). In biophysical assays, pores formed by oligomers are more stable than monomeric pores;

conductance measurements are highly stable indicating that these pores stay open almost exclusively, unlike monomeric pores which open and close (Rausell et al., 2004).

The insertion of toxin into the apical midgut epithelium results in both non-selective pores and anion and cation specific channels (Kirouac et al., 2002 and reviewed in Schnepf et al., 1998). The resulting osmotic lysis is thought to be the primary cause of cell destruction and eventual death of the insect. There has been, however, evidence that calcium levels are influenced by Bt toxins. Extracellular calcium enhances Cry1C activity against insect cell lines and results in increased release of calcium from intracellular stores (Schwartz et al., 1991; Monette et al., 1997; Potvin et al., 1998). The rise in cellular calcium early after toxin introduction may be an important part of the mode-of-action of Bt.

A.4. Resistance to Bt

Evolution of Bt resistance by target insects is the most serious threat to the continued successful use of this biopesticide. This fear is not unwarranted as numerous insects have developed resistance to a wide range of insecticides. Laboratory colonies of insects selected for Bt resistance provide models for studying the inheritance and mechanisms of resistance. The first strain selected was a resistant line of *Plodia interpunctella*, but resistant populations of other Lepidoptera (*Heliothis virescens*, *Spodoptera exigua*, *S. littoralis*, *Ostrinia nubilalis*, *Pectinophora gossypiella*, *Trichopulsia ni*), Coleoptera (*Chrysomela scripta*, *Leptinotarsa decemlineata*), and Diptera (*Culex quinquefasciatus*) have also been developed (reviewed in Ferre and Van Rie, 2002). So far only *Plutella xylostella* has developed resistance to Bt applied as a biopesticide in the field (reviewed in Ferre and Van Rie, 2002). To date, there have been no reports of insect populations that have developed resistance to Bt crops. Bt resistance has also been studied in the nematode, *Caenorhabditis elegans* (Marroquin et al., 2000).

A.5. Resistance in *Plutella xylostella*

The first population of Bt-resistant *P. xylostella* was found in Hawaii, but additional strains have been found worldwide (reviewed in Ferre and Van Rie, 2002). Although these populations have developed resistance to different commercial formulations of Bt (Dipel, Javelin, *Bta*-based), each typically shows resistance to each toxin in that formulation (reviewed in Ferre and Van Rie, 2002). Additionally, several populations have also evolved cross-resistance to toxins that are not part of these commercial formulations. Based on amino acid homology, Tabashnik et al. (1996) proposed that cross-resistance is due to altered interactions in the loops of domain II, particularly loop 1.

A.5.1. Mechanisms of Resistance

The primary mechanism for resistance in *P. xylostella* is related to alterations in binding sites in the insect's midgut. Toxins that are no longer effective against resistant populations show dramatically reduced binding to brush border membrane vesicles (BBMV). The compilation of competitive binding experimentsof toxins to BBMV, both homologous and heterologous, led to a model that also accounts for cross-resistance (Ferre and Van Rie, 2002). There are four proposed binding sites: site 1 is recognized by Cry1Aa alone, site 2 is shared by Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa, Cry1Ja, site 3 binds Cry1Ba, and site 4 binds Cry1Ca. Site 2 encompasses the phenomenon of cross-resistance, while sites 3 and 4 account for the remaining susceptibility of resistant insects to Cry1Ba and Cry1Ca.

Based on binding studies of BBMV from various resistant strains, two types of alterations have been proposed (Ferre and Van Rie 2002). Type I describes an alteration in binding site 2 where only Cry1Ab binding is drastically reduced. Examples of this kind of change are a resistant colony from the Philippines (PHI) and one from Malaysia (SERD3). The second type

of alteration, Type II, also describes a change in binding site 2, but this alteration causes a reduction in binding of Cry1Ab, Cry1Ac, and Cry1Aa. This type of resistance is exemplified by a Hawaiian strain (NO-QA), a Pennsylvania colony (PEN), and another strain from Malaysia (1AcSEL-MEL).

One obvious explanation for the changes in binding would be the elimination of a toxin binding molecule. Interestingly, different types of binding analyses have produced results that contradict soluble BBMV binding assays. Immunohistochemistry studies demonstrated Cry1A binding to midgut tissue sections from both susceptible and resistant *P. xylostella* (Escriche et al., 1995b). Similar results were generated from surface plasmon resonance studies using either immobilized toxin or BBMV (Masson et al., 1995b). Although association and dissociation constants did not differ between susceptible and resistant insects, there was a three-fold reduction in Cry1Ac receptors in the resistant strain. Finally, ligand blotting analysis determined that in both strains, Cry1Ac binds to a 120 kDa protein in BBMV that was subsequently identified as an aminopeptidase (Luo et al., 1997). These findings demonstrate that binding alone is not sufficient for toxicity and that a lack of binding may not be the determining factor for resistance.

Other resistance mechanisms have been described for several laboratory-generated resistant strains. In *P. interpunctella*, altered proteolytic processing of toxin has been well established through enzyme activity assays and genetic linkage studies (reviewed in Ferre and Van Rie, 2002). By incorrect, inefficient, or accelerated protoxin cleavage, downstream events (i.e. binding) in the mode-of-action cannot occur. In a Cry1Ca resistant population of *P. xylostella* (NO-95C), a 2.5 fold difference in toxicity was observed for Cry1Ca toxin vs protoxin (Liu et al., 2000). No changes in protease activity have been reported for any other strain of *P. xylostella*.

A recent study described differences in the lipid content of BBMV prepared from Bt-susceptible and -resistant *P. xylostella* (Kumaraswami et al., 2001). Total lipids were extracted into non-polar lipids, phospholipids, neutral glycolipids, and acidic glycolipids. The only significant differences detected were in the neutral glycolipid fraction. The overall abundance was lower (approximately half) in resistant animals compared to susceptible ones. Further separations by thin layer chromatography revealed 7-8 different species. Most were somewhat reduced in the resistant sample compared to the susceptible one, but a possible trisaccharylceramide and a possible hexasaccharylceramide were reduced by at least 50%. This may indicate a reduction in a specific transferase required to create these lipids.

Interestingly, there have been other reports of toxin interactions with glycolipids. Dennis *et al*. (1986) reported that several glycolipids and neutral glycosphingolipids isolated from *Calliphora vicina* were bound by toxin. Toxin-glycolipid interactions in *M. sexta* have also been detected (Garczynski and Adang, 2000). Although the significance of these differences is unclear, they might relate to membrane subdomains. Since glycosphingolipids and GPI-anchored proteins are enriched in membrane rafts, alterations of these molecules may inhibit proper oligomerization of toxin monomers or efficient pore formation. Future studies will be required to determine the role neutral glycolipids serve in Bt toxin mode-of-action.

A.5.2 Genetic Basis for Resistance

The identification of genes responsible for resistance in *P. xylostella* has been elusive. As mentioned earlier, no change in the levels of aminopeptidase N have been found, nor have disruptions or changes in post-translational modifications to this protein been identified. Linkage analysis through amplified fragment length polymorphism (AFLP) mapped the multi-toxin resistance locus within linkage group 7 that is 8.4 cM from the nearest AFLP marker

(Heckel et al., 1999). A sequence tag site (STS) was cloned and sequenced and can be used in other linkage studies to help identify resistance genes.

Another genetic linkage analysis used isozyme polymorphisms to identify resistance loci (Herrero et al., 2001). This technique with the PHI strain identified a strong correlation between Cry1A resistance and two mannose-6-phosphate isomerase isozymes. An STS was cloned and sequenced for use in future linkage studies. Interestingly, this linkage was also identified in a resistant *H. virescens* strain (YHD2) (Heckel et al., 1997), indicating a common basis for resistance in these two insects.

A.6. Resistance in other insects

As described above, many other insects have had populations selected for Bt resistance under laboratory conditions. There have been several different proteins identified as being responsible, at least in part, for the development of resistance. Aside from those cases where specific midgut proteases have been identified as missing or drastically reduced (reviewed in Ferre and Van Rie, 2002), identified altered proteins generally relate to toxin binding.

In two cases, *H. virescens* and *P. gossypiella*, the disruption of a cadherin-like protein has been linked to Cry1A resistance. In the YHD2 strain of *H. virescens,* genetic mapping experiments linked Cry1Ac resistance to a cadherin gene (*BtR4*), and further demonstrated that a retrotransposon insertion disrupted this gene (Gahan et al., 2001). The extracellular domain of cadherin contains repeated sequence and these repeats are referred to as ectodomains. The retrotransposon insertion occurs after ectodomain 8 resulting in a truncated, soluble form of the cadherin protein. In *P. gossypiella*, three resistant alleles (*r1, r2, r3*) of the *BtR* gene were identified from a Cry1Ac resistant colony (Morin et al., 2003). Each of these alleles has a major and unique deletion. The *r1* allele has two amino acid substitutions and is missing 8 amino

acids, all within ectodomain 8. The *r2* allele has a 202 bp deletion that causes a frameshift mutation which results in a stop codon within the last third of ectodomain 6. The *r3* allele is missing 42 amino acids within ectodomain 8 due to a deletion of 126 bp. Biphasic linkage analysis demonstrated that all resistant larvae were *rr* and all susceptible larvae were *rs* or *ss* for the BtR gene. While the *r2* allele is the most frequent in resistant insects, all six resistant genotypes were detected. Because resistance in both *H. virescens* and *P. gossypiella* fits the mode 1 profile (high resistance levels to at least one Cry1A toxin, recessive inheritance, reduced binding of at least one Cry1A toxin, and little or no cross-resistance to Cry1C) (Tabashnik et al., 1998), the finding that both share a disruption in a cadherin gene suggests other insects may develop this mode of resistance. Interestingly, a new study has shown no linkage between resistance and cadherin genes in *P. xylostella* (David Heckel, personal communication).

Another resistance mechanism detected in the YHD2 strain of *H. virescens* is a reduced amount of membrane alkaline phosphatase compared to a susceptible strain (Jurat-Fuentes and Adang, submitted). This 68 kDa protein contains a terminal GalNAc, as signified by its recognition by various lectins. Additionally, ligand blotting analysis determined that this carbohydrate moiety is required for Cry1Ac binding (Jurat-Fuentes and Adang, submitted). In this *H. virescens* strain there are at least two mechanisms of resistance, alkaline phosphatase reduction and cadherin truncation, making it likely that in other insects a similar pattern of multi-component resistance mechanisms will be found.

A.7. Resistance in nematodes

Finally, in *C. elegans*, *bre* (Bt-toxin resistant) mutants have been identified that confer resistance to Cry5B (Marroquin et al., 2000). The resistance gene *bre*-3 was identified as a orthologue to the *Drosophila* gene *egghead* (Griffitts et al., 2003). The *bre-5* gene which had

been identified as a β1,3 glycosyltransferase (Griffitts et al., 2001) also has homology to *Drosophila brainiac.* In *Drosophila*, *egghead* and *brainiac* function in a single pathway. The determination of *C. elegans* orthologs of these two proteins suggests a similar pathway in this animal. In fact, complementation analyses showed that *bre-5* could rescue the *Drosophila brainiac* mutant (Griffitts et al., 2003). Two other *bre* mutants are predicted to encode glycosyltransferases: *bre-2* a β1,3 glycosyltransferase and *bre-4* a β1,4 galactosyltransferase. All four *bre* genes were demonstrated to function in one pathway shown by the similar resistance levels between all single mutants and all double mutants tested.

The complex picture of resistance (multiple mechanisms in one strain, different mechanisms in different insects and different populations of the same insect, and the discrepancy between mechanisms in field- and laboratory-generated resistant populations) is increasingly challenging to decipher. The evolution of user friendly high-throughput techniques (microarrays, protein chips, and proteomics) should be more efficient at detecting multiple changes in response to Bt toxins.

B. Proteomics

The term proteome was first used to describe the complete set of proteins encoded by a genome (Wilkins et al., 1996a). Proteomics now refers to techniques that characterize not only all encoded proteins, but also their modifications and isoforms. This typically implies a comparative study investigating differences between control and experimental samples (e.g. cells, tissues). To some researchers, proteomics also includes studies designed to resolve all interactions between all proteins, and higher ordered complexes. For the sake of this review, the focus will be on gel-based (two-dimensional electrophoresis) and solution-based (mass spectrometry) techniques.

B.1. Evolution of Proteomics

Modern proteomic studies began in 1975 with the advent of two-dimensional electrophoresis (2DE) to examine the global protein composition of complex samples (Klose, 1975; O'Farrell, 1975; Scheele, 1975). The reproducibility of early 2DE separations were problematic and limited by a lack of adequate hardware and software for imaging and analyzing gels, sequencing spots, and processing large amounts of data. These constraints limited this technique to relatively simple biological studies as well as to only a few experts in the field. Despite these challenges, however, landmark findings were made including the interaction of the proto-oncogenes jun and fos (Rauscher et al., 1988).

An explosion of advances in the 1990's impelled proteomics to mainstream status. Interestingly, the advancement of proteomics was due in large part to the rapid growth of the field of genomics. Sequence databases were flooded with large-scale expressed sequence tag (EST) data from public and private institutions. Simultaneously, there were reports detailing the use of mass spectrometry as a protein identification method, which exploited the growing databases. These reports described peptide mass fingerprinting (PMF), where an isolated protein could be digested with an enzyme of known cleavage specificity, yielding various peptides. These peptides were fragmented via mass spectrometers yielding a series of masses that could uniquely identify a protein (Henzel et al., 1993; James et al., 1993; Mann et al., 1993; Pappin et al., 1993; Yates et al., 1993). In 1994, the term proteome was coined at the Conference on Genome and Protein Maps in Siena, Italy marking the beginning of proteomic analyses as we know them today (Wasinger et al., 1995; Wilkins et al., 1996b).

Two mass spectrometry methods were developed in the late 1980s that ionized peptides (proteins) at high sensitivity allowing for mass measurements of these molecules: electrospray

ionization (ESI) (Fenn et al., 1989) and matrix-assisted laser desorption ionization (MALDI) (Karas and Hillenkamp, 1988). These two methods led to the development of commercial instruments that became widely used by researchers. Combined with the evolution of fingerprinting algorithms, these methods of protein identification became more popular than N-terminal sequencing (through Edman degradation) at least in the proteomics field. By using an additional mass spectrometry step in tandem, the amino acid sequence of small peptides can be ascertained, providing sequence information that can be used to identify the protein.

B.2. Challenges of Proteomic Separations

One of the challenges of proteomic separations is the dynamic range of proteins in the sample being studied. As a working definition, 'dynamic range' is the ratio of the most abundant protein to the rarest protein detected. In cells, this range is 10^5-10^6, but in serum the range is near 10^{10} due to the difference between interleukins at 2 pg/ml and albumin at 50 mg/ml (Patterson and Aebersold, 2003). Adding to this problem is the unknown number of proteins in a sample. In theory, the minimum number of proteins in the proteome is defined by the number of genes in the genome. However, the number of proteins is much higher due to alternate splicing, various glycoforms, transient phosphorylation, and other posttranslational modifications. Protein turnover is also a factor due to the strict regulation of protein synthesis and degradation. It is quite obvious that only a portion of the protein complement can be detected at any given time. These challenges must be taken into consideration before choosing a proteomic analysis in order to maximize the number of proteins analyzed.

B.3 Solution Based Proteomic Analysis

Appella et al. (1995) reported one of the first gel-free proteomic analyses. In a series of studies, they identified the peptides bound to major histocompatibility complex (MHC). The

sample was proteolytically cleaved and the resulting peptides were fractionated with liquid chromatography (LC) followed by tandem mass spectrometry (MS/MS) (Appella et al., 1995). This process was repeated multiple times to further fractionate the samples allowing for successful protein identifications. This type of high throughput experiment can simultaneously identify a large number of proteins from a complex sample using a peptide as a representative of the whole protein. The term ‘shotgun proteomics’ was given to this solution based approach.

B.3.1. Multidimensional protein identification technology (MudPIT)

To maximize the utility of shotgun proteomics, efficient and reproducible methods needed to be developed. Link et al. (1999) described a multi-dimensional LC separation before MS/MS fragmentation. In a single run, 80 proteins were identified from the ribosomal complex of *Saccharomyces cerevisiae* including a novel component (Link et al., 1999). Additionally, more than 150 proteins were identified from whole cell lysate in one run. Wolters et al. (2001) described an improvement to this method which they named MudPIT (multidimensional protein identification technology). They replaced the LC separations described by Link et al. with one biphasic column composed of a strong-cation exchange (SCX) resin and a reverse phase resin at the other end. Since the peptides are separated by increasing pI from the SCX phase and then by hydrophobicity, the peptide should always elute in the same chromatographic space, allowing for reproducible separations. Additionally, the use of these types of resins combined with MS/MS scans can resolve up to 23,000 peaks (Wolters et al., 2001).

One of the most important factors in proteomic separations is the ability to detect low abundance proteins. With the MudPIT technique, a dynamic range of 10,000 was demonstrated for proteins in *S. cerevisiae* (Wolters et al., 2001). This means a protein present at 100 copies/cell can be detected in a background of protein at 1,000,000 copies/cell. This is a great

improvement over 2DE separations which have a limit of detection by silver staining of 1,000 copies/cell with the same amount of starting material (Gygi et al., 2000).

Although the power of MudPIT is the ability to analyze highly complex samples (i.e. whole cells, whole tissues), methods to somewhat reduce this complexity were developed. This was of particular importance with the shift from proteomic studies focused on profiling samples to comparative studies. The capture of specific populations of peptides (e.g. phosphopeptides, glycopeptides, cysteinyl peptides) (Spahr et al., 2000; Ficarro et al., 2002; Kaji et al., 2003; Zhang et al., 2003) is developing into a standard method of simplifying comparative analyses through shotgun proteomics. An additional goal of a comparative analysis is to be able to quantitate the observed differences. Quantitation is often difficult to achieve in a mass spectrometer. Peptides produce different signal intensities due to their chemical composition, the matrix environment, and other variables that are not understood (Patterson, 2003). Techniques that can both simplify and quantitate complex samples facilitate the extraction of relevant biological information from shotgun proteomic studies.

B.3.2. Isotope-coded affinity tag (ICAT)

Until the dramatic proliferation of proteomics, mass spectrometry had been primarily used for analyzing small molecules. De Leenher and Thienpont (1992) described the use of mass spectrometry to obtain accurate quantitative measurements based on isotope dilution. Essentially, two molecules that have the identical structure but differ in isotopic composition will differ in mass, but will fragment in an identical pattern (de Leenheer and Thienpont, 1992). Gygi et al. (1999) used this theory to create a quantitative, comparative proteomic technique called isotope-coded affinity tag (ICAT).

ICAT serves not only to provide a way to quantitate peptides, but also to reduce the complexity of the samples. The ICAT tag has three components: a biotin group, a linker group containing eight hydrogens or deuteriums, and a thiol-specific reactive group (Gygi et al., 1999). Two cell states are labeled with either the light (hydrogen) or heavy (deuterium) tag which will covalently attach to cysteines in every protein in the sample. The two samples are combined and proteolytically digested. The labeled peptides (containing cysteine) are recovered by passing the mixture over an avidin column and are then analyzed by LC-MS/MS (Gygi et al., 1999). The resulting MS spectra will show doublets at given mass-to-charge ratios signifying the light- and heavy-labeled peptide. A measurement of the peak areas is used to determine a ratio for quantitating the difference in abundance of that peptide in the two samples. Using the sequence information generated from the MS/MS scan allows for the identification of the protein from which the peptide was isolated. Proteomic analysis with the ICAT strategy has been used to identify proteins associated with hepatocellular carcinomas (Li et al., 2004), examine differences between *Mycobacterium tuberculosis* strains (Schmidt et al., 2004), and to identify proteins associated with tendon healing (Harris et al., 2003).

Variations on the ICAT technique have been used: stable isotope labeling with amino acids in cell culture (SILAC) (Ong et al., 2002), the use of ^{18}O transferred from water to peptides (Mirgorodskaya et al., 2000), labeling of carbohydrates (Hang et al., 2003), as well as many other tags specific for other chemical groups (reviewed in (Aebersold and Mann, 2003)). A related technique has been used to quantify proteins and phosphoproteins. AQUA (absolute quantification) was developed as an alternative to ICAT (Gerber et al., 2003). In this strategy, synthetic peptides corresponding to a protein of interest are synthesized using amino acids containing stable isotopes. The fragmentation of the peptide can be measured and used as an

internal standard (Gerber et al., 2003). This peptide is added to a sample and the proteins are separated by SDS-PAGE. This step is critical because unlike ICAT, the tag is only on one specific peptide from one specific protein. It is important to reduce the complexity of the sample to enrich for the protein of interest. A fragment of the gel is excised corresponding to the molecular size of the protein being studied. The proteins are digested with trypsin and the resulting peptides eluted and analyzed by LC-MS/MS. Since a known amount of labeled peptide is used, a direct comparison of labeled and unlabeled spectra can be made and the abundance of the peptide can be calculated. Using this method, quantitation of low-abundance yeast proteins, horse heart myoglobin (in a yeast background), and the change in phosphorylation of a human cell-cycle protein were conducted (Gerber et al., 2003).

B.3.3. Specific Applications

The use of MALDI for analysis of proteins directly from tissue sections was first described by Caprioli et al. in 1997. Proteins from different sections of mouse colon were analyzed with this technique revealing similarities and differences in protein expression patterns (Chaurand et al., 1999). Recently this technique has been used on histology slides to directly correlate MS spectra with various morphologies detected by microscopy (Chaurand et al., 2004). The power of this type of analysis could provide direct links between specific proteins and diseased tissues.

Recently a new approach was reported that measures the relative dynamic turnover of proteins in *Escherichia coli* (Cargile et al., 2004). A relative synthesis/degradation ratio is calculated based on the rate of ^{13}C incorporation as measured by shifts in MS/MS spectra. The analysis involves protein solubilization, separation by SDS-PAGE, band excision and trypsin digestion, and fragmentation in a shotgun approach (Cargile et al., 2004). Not only is this

method advancing quantitative proteomics and elucidation of important biological phenomena, but it also exemplifies the flexibility of proteomic techniques.

B.3.4 Advantages and Disadvantages

One of the distinct advantages of solution-based proteomic studies is that sample preparation is not a limiting factor. The use of different chromatography media (e.g. ion exchange, microcapillary, reverse phase) before mass spectrometry fractionation allows multiple runs of the same sample to fully analyze the complement of proteins within the sample. Unlike gel-based proteomics, factors such as sample solubility, extreme isoelectric point, and hydrophobicity (e.g. membrane bound proteins) are not a factor in these solution based methods. In turn, however, this becomes a disadvantage in that there are often too many peptides within a sample to adequately determine important proteins whether in a profiling or comparative experiment.

Although solution-based techniques have the advantage of rapidly providing an enormous amount of data over the entire proteome, the power of this shotgun approach is only useful when there is an extensive sequence database for the study organism. Performing such a study on an organism with minimal sequence information will simply result in a list of peptides/proteins of unknown identity. However, these data can be stored for future use when more sequences become available.

Finally, shotgun approaches require a high degree of technical skill and computer support. A single LC-MS/MS experiment can generate >10,000 peaks. One can envision multiple experiments or separations that generate too much data to be handled without the aid of databases and sophisticated searching algorithms. This is perhaps the prevailing reason why most solution-based proteomic searches are conducted only by dedicated mass spectrometry labs.

B.4. Gel-based Proteomic Analysis

The principles behind 2DE are relatively simple: solubilization of proteins, separation by isoelectric point, separation by molecular mass, imaging, and identification of spots. As described previously, initial 2DE experiments were challenging due to the difficulties manipulating the tube gels used for isoelectric focusing, imaging limitations, and lack of sophisticated protein identification techniques. In 1982, Bjellqvist et al. described the use of immobilized pH gradients (IPG) for isoelectric focusing. These polyacrylamide strips are analogous to gradient gels except that the pH is varied rather than acrylamide concentration. Instead of the carrier ampholytes used in tube gels, buffering monomers (Immobilines) covalently linked to acrylamide serve to establish the pH gradient within the gel matrix (Bjellqvist et al., 1982). The advantage of IPG strips is that they have a known ionic strength, uniform buffering capacity, and higher resolution and loading capacities (Bjellqvist et al., 1982). The standardization of these strips makes isoelectric focusing easily reproducible.

Although some problems had been encountered when strips were laid on a gel for second-dimension separations (e.g. poor solubility in SDS, electroendosomosis at the basic end of the strip), these challenges were quickly overcome with minor method modifications (Westermeier et al., 1983; Görg et al., 1985; Görg et al., 1988). With the commercialization of these strips, 2DE became a technique that was both affordable and available for most researchers. However, two limitations of this approach still existed: identification of individual spots in the resulting gels and sample preparation. Protein identifications became easier as mass spectrometry techniques were developed (described in section B.2). Appropriate sample preparation, however, is vital for achieving a thorough depiction of the proteome of study and is therefore in need of continual improvement.

B.4.1. Sample Preparation

Isoelectric focusing yields the highest resolution when performed under denaturing conditions. When each protein within a mixture is present in only one configuration, intermolecular interactions are minimized, therefore complete denaturation and solubilization are critical for maximal separations. The components of the solubilization buffer vary somewhat depending on the sample being analyzed, but generally include urea, detergent, and the presence of a reducing agent.

Urea is a neutral chaotrope and is the denaturant of choice for the isoelectric focusing step of 2DE. This compound solubilizes most proteins, unfolding them into their most random conformation with all ionizable groups exposed. Thiourea has been shown to improve solubility of membrane proteins (Rabilloud et al., 1997), but must be used in combination with urea due to its poor solubility in water. The optimal conditions are to maximize the concentrations of chaotropes and detergents to solubilize as many proteins as possible, however, different detergents have different compatibilities with high (7-8 M) urea concentrations.

The presence of detergent ensures sample solubilization and prevents hydrophobic interactions. Non-ionic or zwitterionic detergents must be used to avoid introducing charges that will interfere with isoelectric focusing. Initial 2DE experiments used NP-40 or Triton X-100 (Klose, 1975; O'Farrell, 1975; Scheele, 1975) during isoelectric focusing, but CHAPS was shown to be much more effective (Perdew et al., 1983). The use of alternate detergents has also been demonstrated to increase protein solubility. Linear sulfobetaines (e.g. SB3-10) were shown to enhance membrane protein solubility when used at 2% (Rabilloud et al., 1997). This detergent, however, is not compatible with high urea concentrations. To maintain the overall chaotrope concentration, SB3-10 is often used with 5 M urea, 2 M thiourea solutions. Combined

with 2% CHAPS, solubilization buffers containing both thiourea and SB3-10 provide an enhanced solubility scheme versus the traditional 8 M urea, 4% CHAPS buffer (Rabilloud et al., 1997).

Development of new solubilization buffers continues. Herbert et al. (1998) reported the use of tributyl phosphine as an alternative to dithiothreitol (DTT) as the reducing agent during isoelectric focusing. Protein losses, observed as streaking in the second dimension, have been observed and attributed to protein adsorption to the IPG matrix (Rabilloud et al., 1997). It has been suggested that protein insolubility is due to the loss of DTT during long isoelectric separations. The thiol groups can ionize which results in movement of DTT to the anode, concentration decreases along the strip, and proteins can fall out of solution due to reformation of disulfide bonds (Herbert et al., 1998). Because tributyl phosphine has no free thiol groups, it will not run off the end of the strips and it maintains protein solubility. Another alternative to DTT is hydroxyethyl disulfide which better resolves proteins in the basic range (Olsson et al., 2002). This compound was recently used on human adipose tissue and resulted in the identification of several proteins previously undetected (Corton et al., 2004). A review by Herbert (1999) discusses many of the choices in chaotropes, detergents, and reducing agents, including the benefits of each, and the importance of sequential extraction for reducing the complexity of the sample and thereby maximizing the solubilization of the components of each step.

B.4.2. Sample Fractionation

The use of a sequential extraction protocol for complex samples was first reported by Molloy et al. (1998). These investigators used a three step process on *E. coli* cell lysate to create several fractions based on relative insolubilities. The pellet from each step was subjected to a more powerful solubilizing solution. Cytosolic proteins are solubilized first, the bulk of the

protein mass second, followed by membrane proteins (Molloy et al., 1998). In this case, 94% of the proteins in the sample were solubilized. This type of separation may simplify protein identifications since some information about the sample is already known. For example, when the first fraction is analyzed, any subsequent identification as membrane proteins can be discarded because such proteins would not be present in this fraction.

In a series of papers, Fountoulakis et al. reported the enrichment of various proteins from *Haemophilus influenzae* before 2DE separations (Fountoulakis and Takacs, 1998a; Fountoulakis et al., 1997; Fountoulakis et al., 1998b; Fountoulakis et al., 1999). Affinity chromatography with heparin gels, chromatofocusing with ion-exchange columns, hydrophobic interaction chromatography, and hydroxyapatite chromatography were all used to reveal proteins that had not been previously observed in unfractionated samples. In each type of separation, many low abundance proteins were identified that had been masked by the more abundant protein in the total extract. A drawback to these approaches is the manipulations required to get eluted samples into appropriate buffers for isoelectric focusing. Additionally, these proteins will still spread the pI spectrum requiring the use of wide range IPG strips, which may still result in overlapping proteins spots and make identifications challenging.

Finally, the use of multicompartment electrolyzers has been reported (Herbert and Righetti, 2000). This apparatus fractionates a sample through multiple chambers separated by isoelectric membranes. The fractionated samples can be recovered from each chamber and separated by narrow IPG strips, allowing much more sample to be loaded and facilitating the observation of low abundance proteins. Additionally, the pI homogeneity of the sample results in less streaking and protein precipitation, thus yielding higher quality gels (Herbert and Righetti, 2000).

The use of appropriate sample fractionation and rigorous solubilization studies, as described above, is critical to observing low abundance proteins, membrane proteins, and basic proteins on 2DE gels. The successful application of these approaches was recently reported in a study of *S. cerevisiae* (Pedersen et al., 2003). Membrane proteins from intact cells were isolated after the three-step solubilization procedure described by Molloy (Molloy et al., 1998). Alkaline proteins of the membrane fraction were recovered after pI enrichment in a muticompartment electrolyzer. 2DE analysis resulted in the identification of 320 gene products and 780 total isoforms (Pedersen et al., 2003). Additionally, large percentages of the proteins identified were membrane proteins and low-abundance proteins. Although these types of treatments require more time and up-front manipulations, ultimately they will help develop a more complete description of a sample's proteome. Righetti et al. (2001) have recently reviewed the importance of prefractionation techniques and solubility schemes.

B.4.3. Comparative Applications

An inherent challenge to comparative analyses with 2DE lies with trying to match two samples run on different IPG strips and on different gels. Unlu et al. (1997) developed two structurally similar dyes (Cy3 and Cy5) that can be covalently attached to proteins. Two pools of samples can be labeled and run simultaneously on one strip and one gel. The gel is imaged at two different wavelengths yielding an image corresponding to each sample. Differences in spot abundance are easily quantified by computer software allowing for statistical determinations to be made. Alban et al. (2003) reported the use of an internal standard to assure equal protein loads. The pooled standard consists of equal percentages of all samples being compared and the total amount of protein equals the amount of each sample being analyzed. This standard is

labeled with another Cy dye (Cy2) and run as a third component in the 2DE separation (Alban et al., 2003).

One of the potential drawbacks of this technique is the fact that these dyes react with lysine residues and lysine is a trypsin cleavage site. Additionally, these dyes are hydrophobic, so labeling must be limited to avoid cause protein losses due to insolubility. Cy dye labeling, therefore, is restricted to 1-2% of available lysines. This may mean that the same lysines are not labeled in each sample or that the labeling cannot be detected in all proteins. Despite these factors, this technique has been successfully used to determine differences in a variety of samples. Friedman et al. (2004) recently compared tumor and normal colon samples from six patients and identified 50 proteins altered between the two disease states. Other example of the use of this technique are additional studies of cancer (Zhou et al., 2002; Gharbi et al., 2002), studies measuring the responses of yeast to metal stress (Hu et al., 2003), and quantification of the differences in sugar metabolism in *Pirellula*, a marine bacteria (Gade et al., 2003).

B.4.4. Advantages and Disadvantages

As discussed above, the primary disadvantages associated with 2DE are due to the evaluation of appropriate solubilization and fractionation schemes. Once a preparation has been selected, there are still inherent problems with isoelectric focusing using IPG strips. Because the pH ranges of these strips do not extend over the full pH spectrum, there will be proteins within a sample that will not resolve on the strip and simply run off the ends. This is true for sample loads that are sufficient to visualize the protein profile, but excessive loads result in a larger number of proteins that clog the strip.

Additionally, with current strip technology resolves only proteins with pI values between 3 and 10 will resolve in 2DE gels. Realistically, proteins with pI values >8 are not readily

separated by 2DE. Large molecular weight proteins are also not well resolved on 2DE gels due to their limited ability to enter IPG strips, although this can be somewhat overcome by applying a low voltage during the sample application (Görg et al., 1998).

The distinct advantage 2DE separations have over solution based proteomic approaches is the ability to target proteins of interest. Differentially expressed proteins (spots) detected in comparative separations can be specifically excised from a gel before mass spectrometry identification. Differences in isoforms related to posttranslational modifications, specifically those that are due to slight variations in charge, can easily be seen as charge trains on 2DE gels or by staining with glycoprotein or phosphoprotein specific stains. Additionally, western blots can be performed that allow for further protein characterization or identification. Because organisms with relatively few protein sequences in protein databases (<1000) are better suited to 2DE analyses than shotgun methods, western blotting can be invaluable for ascertaining protein identifications.

C. Dissertation Rationale

The goals of the research presented in this dissertation were to determine proteins implicated in Bt susceptibility and resistance in Lepidoptera through 2DE analyses. First, methods were developed for 2DE separations using brush border membrane proteins from *M. sexta*. The results of these separations were compared to one-dimensional separations that are routinely employed in Bt research. Two sub-proteomes of BBMV proteins: GPI-anchored proteins and Cry1Ac binding proteins were investigated. The second major goal was to compare BBMV proteins from Bt-resistant and –susceptible *P. xylostella* and identify altered proteins through 2D-DIGE and mass spectrometry.

The first objective was to determine a method for maximal solubilization of the protein components of BBMV. Because no 2DE experiments had been conducted on midgut proteins from lepidopteran larvae, models for membrane proteins had to be used. Fortunately much of the recent research focused on the development of new solubilization cocktails targeted the extraction of membrane proteins (Chevallet et al., 1998; Rabilloud, 1998; reviewed in Herbert, 1999). These protocols were used as a foundation and numerous schemes were tested with the best separations achieved with the combination of chaotropes and detergents described in Chapters 2 and 3 of this dissertation.

Once an appropriate solubilization method was achieved, a comparison of 1D and 2D methods was performed to ascertain the similarities and differences between the techniques. Ligand blotting has been the primary method for determining toxin binding proteins (Garczynski et al., 1991; Knowles et al., 1991; Vadlamudi et al., 1993), so it was important to visualize the proteins detected in 1D and 2D blots. To accomplish this, two sub-proteomes were examined: GPI-anchored proteins and Cry1Ac binding molecules. Garczynski and Adang (1995) demonstrated that the Cry1Ac binding 120 kDa aminopeptidase N has a GPI-anchor, therefore this protein served as a control in comparative studies.

To date, the only proteins identified in insects that may play a role in Bt toxin mode-of-action have been toxin binding proteins. Although pore-formation and osmotic cell lysis are steps in toxin action, there is a likelihood that part of the toxin molecule, particularly helix 4 and 5 of domain I, interacts with structural or intracellular proteins after pore formation. Loeb et al (2000) detected an increase in apoptotic markers in *H. virescens* cells upon exposure to Bt toxin. Additionally, several studies have reported that Bt affects calcium levels (Schwartz et al., 1991;

Monette et al., 1997; Potvin et al., 1998). Both of these findings suggest that second messenger pathways may be important in Bt mode-of-action.

I hypothesized that the use of 2DE in ligand blotting would elucidate new toxin – insect midgut protein interactions not detected in 1D separations. Because BBMV are routinely used as an *in vitro* model for the study of Bt mode-of-action, I wanted to determine some of the protein components of these vesicles as well as identify toxin binding proteins. Additionally, because this is a relatively new technique in the Bt field, I made comparisons between 1D and 2D separations throughout. GPI-anchored proteins and Cry1Ac binding proteins were identified through blotting analyses. Biotinylated Cry1Ac was used to probe both 1D and 2D separated *M. sexta* BBMV proteins and several proteins were detected only in 2DE blots. These protein spots were subjected to MALDI-ToF analysis and the resulting peptide mass fingerprints were used to search protein databases. Mass spectrometry identifications generally did not result in matches to *M. sexta* proteins or matched with very low probabilities. In hindsight, this was not unexpected. Lepidopteran proteins are generally underrepresented in protein databases. Because no genomes from Lepidoptera have been sequenced, or at least these sequences are not deposited in searchable databases, and there are relatively few researchers studying these organisms, compared to mouse, human, and yeast, identifications are challenging simply because there is a lower probability that a protein of interest is in the database. Additionally, experimentally derived masses are derived from peptides that may have post-translational modifications and may not match to database sequences that consist of amino acid sequence alone. As a result of the poor matches from the peptide mass fingerprinting experiments, I had to use additional techniques to confirm mass spectrometry identifications. The combination of these techniques

led to the identification of two novel Cry1Ac binding proteins. These results are presented in Chapter Two of this dissertation.

In Chapter Three, I report the results of comparative analyses between Bt-susceptible and –resistant strains of *P. xylostella*. Although resistance is primarily due to a lack of toxin binding in resistant animals, no molecule has been identified as being responsible for resistance in this insect (reviewed by Ferre and Van Rie, 2002). I chose a comparative proteomic approach to examine protein differences between susceptible and resistant *P. xylostella* larvae. The development of 2D-DIGE and creation of a proteomics facility at the University of Georgia facilitated this type of approach. However, an additional comparison also needed to be done. MacIntosh et al. (1994) had previously reported a method of *P. xylostella* BBMV preparation using whole animals instead of dissected guts. This was a welcome finding due to the intensive labor required for dissection of these small larvae. Whole animal BBMV were determined to be a suitable substitute for dissected gut BBMV because binding constants were similar in toxin binding assays with either preparation (Escriche et al., 1995a).

I compared diet-adapted susceptible and resistant *P. xylostella* BBMV for both whole and dissected preparations to ascertain if whole animal BBMV preparations are an appropriate model for elucidating differences in midgut proteins that may be related to Bt resistance. One dimensional separations indicated that whole animal derived BBMV had a slightly different banding pattern than dissected midgut BBMV. 2D-DIGE experiments also revealed substantial differences between the two types of BBMV preparations. Although a larger number of proteins were resolved in whole animal preparations, few differences were revealed between susceptible and resistant samples. However, comparisons of susceptible and resistant dissected gut BBMV revealed differences in the abundance of many proteins.

Charge trains are a series of horizontal spots that run very tightly together and typically represent different forms of one protein due to slight differences in post-translational modifications. However, urea can modify protein through carbamylation resulting in artificial charge trains. In this preliminary study, I targeted four charge trains for mass spectrometry identifications. Three of these were more prevalent in susceptible *P. xylostella*, while the other was more prevalent in resistant animals. Multiple spots within a charge train were used as replicates to maximize the probability of successful identifications. Peptide mass fingerprinting resulted in a high probability identification of one protein and two other tentative identifications including the more abundant protein in resistant animals. In an effort to confirm this protein, MALDI-ToF ToF sequencing was done. Unexpectedly, this analysis resulted in a different protein being identified: glucosinolate sulphatase (GSS) from *P. xylostella*.

Plant myrosinases are part of a protective pathway that is activated when plants are wounded by insect herbivory. Glucosinolates are processed into compounds that are toxic to insects and therefore inhibit damage. However, *P. xylostella* uses GSS to process glucosinolates into non-toxic compounds (Ratzka et al., 2002) essentially competing with the plant myrosinase. Because it seems unlikely that *P. xylostella* would require high levels of GSS when feeding on artificial diet, I hypothesized that the differences between susceptible and resistant animals was due to the different time intervals that had ensued since each strain was adapted to diet. The susceptible strain had been adapted in the early 1990's (Shelton et al., 1991), while adaptation of the resistant strain occurred in the late 1990's (Tabashnik et al., 2000). Production of GSS would be of little benefit for feeding on diet, the insect would shift its energy resources away from the synthesis of this enzyme.

To test this hypothesis, a 1D western blot comparison was made between susceptible and resistant *P. xylostella* that had been reared on either cabbage or diet. Surprisingly GSS was present in higher levels in the diet-adapted strains than the cabbage-reared strains. Both strains of resistant animals had higher GSS levels than their susceptible counterparts. I examined two other midgut enzymes, aminopeptidase N and alkaline phosphatase, to see if there were any alterations that would indicate inherent differences in protein composition that might account for the GSS differences seen in the western blots. Aminopeptidase levels were the same in all four strains as measured by western blots, but alkaline phosphatase enzyme activity were not. This activity differed between the four strains in a pattern similar to the differences determined for GSS. That is, diet-adapted strains and resistant strains had higher enzyme activities than their counterparts. I propose that these alterations signify that in *P. xylostella*, Bt resistance may involve intracellular pathways that have not yet been implicated in Bt action.

Taken together, the work presented in Chapters 2 and 3 indicate that there are a number of proteins implicated in Bt mode-of-action and development of Bt resistance. The continued elucidation of midgut proteins that interact with and respond to Cry toxins is critical for understanding the details of Bt action and the continued success of Bt crops.

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CHAPTER 2

IDENTIFICATION OF NOVEL *BACILLUS THURINGIENSIS* CRY1AC BINDING PROTEINS IN *MANDUCA SEXTA* MIDGUT THROUGH PROTEOMIC ANALYSIS[1]

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Abstract

The crystal proteins of *Bacillus thuringiensis* are widely used in transgenic crops and commercially available insecticides. *Manduca sexta*, the tobacco hornworm, is the model insect for *B. thuringiensis* studies. Although brush border vesicles prepared from larval *M. sexta* midgut have been used in numerous mode-of-action studies of *B. thuringiensis* toxins, their protein components are mostly unknown. Vesicles prepared from the brush border of *M. sexta* midgut were analyzed using one- and two-dimensional gel electrophoresis to establish a midgut brush border proteome. Sub-proteomes were also established for *B. thuringiensis* Cry1Ac binding proteins and glycosylphosphatidyl inositol anchored proteins. Peptide mass fingerprints were generated for several spots identified as Cry1Ac binding proteins and GPI-anchored proteins and these fingerprints were used for database searches. Results generally did not produce matches to *M. sexta* proteins, but did match proteins of other Lepidoptera. Actin and alkaline phosphatase were identified as novel proteins that bind Cry1Ac in addition to the previously reported aminopeptidase N. Aminopeptidase N was the only GPI-anchored protein identified. Actin, aminopeptidase N, and membrane alkaline phosphatase were confirmed as accurate protein identifications through Western blots.

Keywords: aminopeptidase, alkaline phosphatase, actin, GPI-anchored proteins, proteomics, *Bacillus thuringiensis*, Cry1Ac

Abbreviations: BBMV, brush border membrane vesicles; PMF, peptide mass fingerprint

1. Introduction

The Cry1 proteins of *Bacillus thuringiensis* are specifically toxic to lepidopteran larvae. These toxins are solubilized and enzymatically processed in the midgut of susceptible insects. Processed toxins bind to proteins in the midgut epithelium and a portion of the toxin inserts into the membrane forming a pore. The integrity of midgut epithelia cells is destroyed and the insect dies (reviewed in Schnepf et al. 1998). Details of the intoxication process, especially events downstream of binding, remain poorly understood.

The identification of proteins that bind *B. thuringiensis* toxin in insect midgut cells has been facilitated by the use of brush border membrane vesicle (BBMV) preparations. Toxin binding proteins identified so far fall into two types: aminopeptidase N (APN; EC 3.4.1.2) and members of the cadherin superfamily. In *Manduca sexta*, the tobacco hornworm, a 120 kDa APN (MsAPN1) was identified as a Cry1Ac toxin binding protein (Sangadala et al., 1994; Knight et al., 1994) and determined to be tethered to the brush border membrane with a glycosylphosphatidyl inositol (GPI) anchor (Garczynski and Adang, 1995). MsAPN1 has been found localized in lipid rafts presumably through this GPI-anchor (Zhuang et al., 2002). Expression of MsAPN1 in cultured Sf21 cells did not result in susceptibility to *B. thuringiensis* toxins (Luo et al., 1999). MsAPN1 was, however, recently transgenically expressed in *Drosophila melanogaster* midgut. Transformed larvae, unlike normal larvae, were susceptible to Cry1Ac toxin and pathogenic effects were observed in midgut tissue (Gill and Ellar, 2002).

The other family of candidate receptors is represented by BT-R_1 and BtR175. BT-R_1 is a 210 kDa cadherin-like protein that was originally identified as a Cry1Ab binding protein in *M. sexta* (Vadlamudi et al., 1995). Expression of BT-R_1 in vertebrate COS-7 cells resulted in Cry1Ab binding and gross morphological changes when cells were exposed to Cry1Ab (Dorsch

et al. 2002). When BT-R_1 is expressed in *Drosophila* S2 cells, Cry1Ab toxin is able to bind these cells and cell death is evident as measured by propidium iodide assays (Dr. Gang Hua, personal communication). BtR175 is a 175 kDa cadherin-like protein identified from *Bombyx mori* BBMV. When this protein is expressed in Sf9 cells, gross cell morphology and cell swelling occurred when exposed to Cry1Aa toxin (Nagamatsu et al., 1999). Additionally, exposure to toxin resulted in dramatic changes in membrane currents demonstrating that expression of BtR175 results in Cry1Aa induced osmotic flux across the membrane (Nagamatsu et al., 1999).

MsAPN1 and BT-R_1 were both initially identified as toxin binding proteins through ligand blot analysis. Protein separations of BBMV for ligand blots are done with traditional one-dimensional electrophoresis (1DE). These vesicles, however, have a complex protein composition consisting of proteins with overlapping molecular sizes that cannot be distinguished by 1DE. Candas et al. (2003) used two-dimensional electrophoresis (2DE) to compare differences between *B. thuringiensis*--susceptible and -resistant Indianmeal moth, *Plodia interpunctella.* Proteins present at different levels in either strain were identified through mass spectrometry and N-terminal amino acid sequencing. In resistant insects, increases in levels of several specific proteins were found that implicate alterations in oxidative metabolism (Candas et al., 2003). Resistant animals had higher levels of glutathione transferase, cytochrome c oxidase subunit I, and NADH dehydrogenase subunit 5 indicating that there is a shift in the redox state of the midgut epithelial cells in these animals (Candas et al., 2003). A decrease in chymotrypsin activity was detected that may affect processing of these altered proteins, processing of toxin, or both. Additionally, there was a shift in both charge and size of an ortholog of mitochondria F_1F_0-ATPase subunit δ between susceptible and resistant *P. interpunctella* (Candas et al., 2003).

The significance of this shift is unknown. These findings indicate that, at least in this insect, resistance to these toxins is multifaceted and also suggests that the mechanism of *B. thuringiensis* toxicity is complex.

Proteomic analysis has been used infrequently in the analysis of lepidopteran midgut proteins. In addition to the study by Candas et al. (2003), Bonfanti et al. (1992) used 2DE to analyze the cytoskeletal proteins of *M. sexta* midgut brush border. This technique allows for the more complete estimation of the number and types of proteins in biological samples. Despite the widespread use of BBMV in toxin mode-of-action studies, little is known about the protein composition of these vesicles. In this study, a proteomic approach was used to establish a proteome for *M. sexta* BBMV, as well as estimate the number of proteins in this tissue. Western blotting techniques were used in conjunction with 2DE to establish two sub-proteomes of midgut BBMV: Cry1Ac binding proteins and GPI-anchored proteins. Comparisons of *M. sexta* BBMV separated by 1DE and 2DE were conducted to establish potential differences between the two types of separations. A combination of mass spectroscopy and Western blotting was used to identify specific proteins of interest. The use of these techniques provided identification of two new *B. thuringiensis* Cry1Ac binding proteins.

2. Materials and Methods

2.1. Toxin preparation

B. thuringiensis HD73 was obtained from the *Bacillus* Genetics Stock Center (Ohio State University) and Cry1Ac protein was prepared as previously described (Garczynski et al., 1991). For labeling, purified toxin was incubated with NHS-biotin (Pierce) at a ratio of 22:1 (biotin:protein) for 2 hours at room temperature. Free biotin was removed by dialysis in a

10,000 MWCO Slide A-Lyzer (Pierce) against 20 mM Na_2CO_3 (pH 9.2), 200 mM NaCl for 2 hours at room temperature.

Protein concentration of this initial BBMV preparation was determined by the method of Bradford (1976) using BSA as a standard.

2.2. Insects and brush border membrane vesicle preparation

M. sexta eggs were obtained from Carolina Biologicals (Burlington, NC) and larvae were reared on artificial diet (Southland; Lake Village, AR) to second day, fifth-instar larvae at 26°C with a 12:12 (L:D) photoperiod. Midguts were dissected and BBMV were prepared by the precipitation method described by English and Readdy (1989) except that a protease inhibitor cocktail (Complete, Roche) was used in the grinding buffer instead of PMSF. BBMV were resuspended in 1x TBS (25 mM Tris, 2 mM KCl, and 127 mM NaCl) and protein concentration was determined as described above.

BBMV were subjected to protein precipitation using the Plus-One 2-D Clean-Up kit (Amersham) as described by the manufacturer. Recovered proteins were resuspended in solubilization buffer using a sonicating water bath and vortex. Solubilization buffer consisted of 5M Plus-One urea (Amersham Biosciences), 2M thiourea (Sigma-Aldrich), 2% Plus-One CHAPS (Amersham Biosciences), 2% caprylyl sulfobetaine (Sigma-Aldrich), and protease inhibitors (Complete, Roche). The protein solution was then clarified by centrifugation at 30,000 x *g* for 30 minutes. Protein concentrations were determined using the Plus-One 2-D Quant Kit (Amersham Biosciences).

2.3. Isolation of GPI-anchored proteins

Treatment of BBMV with phosphatidyl inositol-specific phospholipase C (PIPLC) and Triton X-114 (TX-114) phase separation were performed as described by Garczynski and Adang (1995). BBMV (1 mg) not subjected to precipitation by the Plus-One 2-D Clean-Up kit were solubilized with 1% Triton X-114 (v/v) in 0.5 ml Tris-saline (10 mM Tris, 150 mM NaCl, pH 7.4) for 10 min on ice. The mixture was centrifuged at 12,000 x *g* for 10 minutes. The supernatant was layered over a sucrose cushion (6% v/v sucrose, 0.06% TX-114, Tris-saline) incubated at 30 ˚C for 3 minutes, and centrifuged at 3000 x *g*. The upper phase (aqueous) was removed and brought up to 0.5% Triton X-114 and incubated on ice for 10 min. This material was layered over the same sucrose cushion, incubated, and centrifuged as above. The upper phase was removed and brought up to 2.0% TX-114 and treated as above. The final detergent-rich phase was diluted to 0.6 ml with Tris-saline. Protein concentration was determined by the method of Bradford (1976) using BSA as a standard.

Proteins from the detergent-rich phase were treated with *B. cereus* PIPLC (Sigma-Aldrich) using 1 U of enzyme for each 100 g of protein. The sample was incubated for 1 hr at 30˚ C with mixing every 15 min. Following the incubation, TX-114 phase separation was performed as above. Two sequential digests were done by adding 2 U PIPLC to the detergent-rich phase after each separation to assure complete digestion of the GPI-anchor. The PIPLC released proteins were pooled and stored at -20˚C until use.

2.4. Two-dimensional electrophoresis

Immobiline DryStrips (Amersham Biosciences) were rehydrated overnight with the recommended volume of liquid consisting of solubilized protein and rehydration solution

(solubilization buffer with the addition of bromophenol blue, 18 mM DTT, and 0.5% ampholines). The amount of protein and strip length used for each separation is described in figure legends. Following rehydration, strips were subjected to isoelectric focusing using a Multiphor II (Amersham Biosciences). The duration of the focusing varied with strip length: 6375 Vhr for 7 cm strips and 17000 Vhr for 13 cm strips. Focused strips were equilibrated 20 min with buffer (6M urea, 2% SDS, 30% glycerol, 50 mM Tris (pH 8.8), bromophenol blue) containing 1% DTT followed by a 20 min incubation in the same buffer containing 4% iodoacetamide instead of DTT. An equilibrated strip was overlaid on a 10% SDS-PAGE and run at 20 mA until the dye front reached the bottom of the gel. At least four different BBMV preparations were used for each type of separation.

2.5. Western and ligand blotting

Proteins separated by SDS-PAGE gels (either one- or two-dimensional) were transferred to polyvinylidene difluoride (PVDF) (Millipore) using the method of Towbin et al., (1979). Western blots were blocked with 5% BSA in 1xTBS/ 0.1% Tween-20 (TBST) for 2 hours then incubated with primary antibody for four hours at room temperature. For ligand blots, blocking in 5% BSA in TBST lasted 3 hours and was followed by overnight incubation with 5 nM of biotinylated Cry1Ac at 4˚C. All blots were washed with three changes of TBST for 15 minutes each. Washed blots were incubated with secondary antibody, conjugated to horse-radish peroxidase, for 1 hour at room temperature. After another set of washes, blots were developed with ECL or ECL+ chemiluminescence substrate (Amersham Biosciences) and exposed to film.

The primary antibodies used for immunoblots were: anti-cross-reacting determinant (CRD) antibody at 1:10,000 (gift from Dr. Kojo Mensa-Wilmot), anti-MsAPN1 antibody

(1:25,000 for 1D blots and 1:2,000 for 2D blots), anti- chicken actin antibody at 1:20,000 (Sigma-Aldrich), and anti- *Bombyx mori* membrane alkaline phosphatase at 1:40,000 (gift from Dr. Masanobu Itoh). Secondary antibodies used were goat anti-rabbit antibody (Sigma-Aldrich) at 1:20,000 and a monoclonal antibody against biotin (Sigma-Aldrich) at 1:250,000. Both secondary antibodies were conjugated to horseradish peroxidase.

All blots were repeated at least three times. Estimations of the sizes of detected proteins were determined from the migration of the standards.

2.6. Mass spectrometry

Mass spectrometry was performed by the Proteomics Resource Facility at the University of Georgia. To obtain enough material for mass spectrometry analysis, 75 ug of *M. sexta* BBMV proteins were separated on a 13 cm pH 4-7 Immobiline DryStrip and subjected to 2DE as described above except the iodoacetamide equilibration step was omitted.

The SDS-gels were fixed and stained with SyproRuby (Molecular Probes). After imaging, spots of interest were excised and trypsin digested using the recommended protocol for the Spot Handling Workstation (Amersham Biosciences). Briefly, 140 ng of sequencing grade trypsin (Promega) in 20 mM ammonium bicarbonate was added to a 1.4 mm^3 gel plug. Plugs were incubated at 37˚C for 1 hr. Digested peptides were extracted by incubating the plugs with 50% acetonitrile/0.1% TFA for 20 minutes. The extract was removed and saved. The extraction step was repeated, and the saved solutions pooled resulting in a final volume of 100 ul. The solution was dried, resuspended in 50% acetonitrile/0.1% trifluoroacetic acid and spotted to a MALDI plate with saturated alpha-cyano-4-hydroxy-cinnamic acid. MALDI-ToF mass spectrometry was conducted using a 4700 Proteomics Analyzer (Applied Biosystems) generating

peptide mass fingerprints (PMF). Spectra were calibrated internally using two trypsin autolysis peaks.

2.7. Computer Analyses

Correlative searching strategies were used to search the Metazoan subset of NCBI using ProFound (http://prowl.rockefeller.edu/) and a confidence level of 0.1 Da. All peptides produced as the PMF for a given spot were used in the searches. Methionine is commonly oxidized during electrophoresis; therefore the searches were conducted using methionine oxidation as a modification. Z-scores, used to indicate the quality of the search result, are generated when the search result and an estimated random match population are compared (http://129.85.19.192/profound/help.html). These scores are reflective of the percentage of random matches that could produce a higher Z-score than that of the resulting match.

In an attempt to understand why better matches were not obtained, we examined the PMF produced from two spots (1 and 9) using FindMod. All peptides of the PMF were entered into this program along with the protein sequence of the best matching *M. sexta* protein (i.e. highest Z-score). In the case where the only match identified was not a *M. sexta* protein, both the matching protein and the corresponding *M. sexta* protein were used. FindMod (http://us.expasy.org/tools/findmod/) is a web-based program that creates masses of peptides of a protein sequence by *in silico* trypsin digestion and then identifies potential sites of post-translational modifications on these peptides (Wilkins et al., 1999). The *in silico* generated peptide masses are compared to experimentally determined PMF from digested spots. FindMod then determines if post-translational modifications can account for any mass differences between the experimental and theoretical peptides according to "intelligent rules" constructed from

information in PROSITE, examination of annotations in Swiss-Prot, and examination of information in the literature. Additionally, FindMod determines if the mass differences can be attributed to single amino acid substitutions.

3. Results

3.1. Silver-stained SDS-PAGE

BBMV proteins separated by 2DE (Figure 1.1B) revealed approximately 10-fold more spots than detected in 1DE separations (Figure 1.1A). Approximately 450 individual proteins were detected in 2DE separations. These separations also showed many ‘chains’ of proteins that typically represent one protein with different degrees of post-translational modifications such as phosphorylation or glycosylation. Most proteins were smaller than 100 kDa and fell in the pI range of 4-7.

3.2. Ligand Blots of Cry1Ac binding proteins

Ligand blot analyses are a standard method of detecting Cry1 binding proteins in BBMV preparations. Therefore, blots of 1D and 2D gels were probed with Cry1Ac toxin. As shown in Figure 1.2A, detection of a 120 kDa protein was very strong on 1D blots, corresponding to the size of the reported Cry1Ac binding APN (Sangadala et al., 1994; Knight et al., 1994). Proteins of 82, 74, 70, and 45 kDa were also detected although not as strongly. The detection of a protein at 120 kDa was far less intense on the 2D blot than on 1D blots and was distributed across 3 spots (Figure 1.2B, arrow #1). A cluster of approximately 6 proteins that bound Cry1Ac was detected at 65-70 kDa (arrow #2). A protein of 45 kDa bound toxin on both blots (arrow #3). Binding was specific as no spots were detected in a competition experiment where 500 nM (100-

fold excess) unlabeled Cry1Ac was added with the labeled toxin (Figure 1.2C). Overall, the binding proteins detected on 2D blots corresponded to the size of the proteins detected on 1D blots, although the intensity of signals detected appeared to be different between the two separation methods.

3.3. Western Blots of GPI-Anchored Proteins

GPI-anchored proteins were isolated from other brush border proteins to facilitate their resolution and detection in 2D gels and blots. The combination of PIPLC treatment with TX-114 phase partitioning resulted in a sample of proteins with cleaved GPI-anchors. 1D and 2D blots of these proteins were probed with anti-CRD antibody. This antibody recognizes the cyclic inositol moiety that results when a GPI-anchor is cleaved by PIPLC (Hooper, 1992). Western blot analysis of 1D separated GPI-anchored proteins revealed four distinct bands (115, 83, 62, and 58 kDa), a cluster of proteins at 90-100 kDa, and a protein at the bottom of the gel (Figure 1.3A). A Western blot analysis of these same proteins separated by 2DE showed a slightly different pattern (Figure 1.3B). 115 and 62 kDa proteins (arrows #1 and 3 respectively) were clearly visible, however, the 58 kDa protein was present as a smear extending from 65-55 kDa (arrow #4). The 83 kDa protein was not detected. A group of proteins was detected at 90 kDa (arrow #2) that may correspond to the cluster of bands seen in a 1D blot at this same molecular size range. An additional protein at 32 kDa (arrow #5) was also detected in the 2D blot.

3.4. Peptide Mass Fingerprinting (PMF) and Computer Analyses

Protein spots identified from the Cry1Ac and anti-CRD blots were analyzed by mass spectrometry. By using the molecular size and approximate pI of proteins detected in these

blots, the corresponding spots were identified in Sypro stained 2D gels. The spots chosen for analysis are marked on Figure 1.4. Long spots on the 2D gel were suspected to be overlapping proteins. To test this possibility, spot 4 was picked as one entire long spot, while spots 5, 6, and 7 were picked as the top, middle, and bottom of a neighboring long spot.

The spectra generated from mass spectrometry of trypsin digested spots from 2DE gels were used to search the Metazoa subset of NCBI using ProFound, a web-interface program (prowl.rockefeller.edu). Several potential identifications were made (Table 1.1). The PMF of spot 1, corresponding to a GPI-anchored Cry1Ac binding protein, had low probability matches to APN proteins from a number of Lepidoptera, all of which had been initially identified as *B. thuringiensis* toxin binding molecules. High probability matches to actin were produced by the PMF of spots 8 and 9, both corresponding to toxin binding but not GPI-anchored proteins. Very low probability matches were obtained by searches with the PMF of three spots (2, 3, and 4). Surprisingly, the PMF data from spot 4 did not result in a clear identification whereas spots 5, 6, and 7 (picked from portions of a neighboring long spot) gave high probability matches to *B. mori* alkaline phosphatase. For all presumptive identifications, only two proteins were from *M. sexta*, although there were matches to other Lepidoptera (*B. mori*, *Heliothis virescens*, *Helicoverpa punctigera*) as well as a few Diptera (*Anopheles gambiae, Sarcophaga peregrine, Drosophila* sp.).

FindMod was used to determine if post-translational modifications might contribute to the low probability matches reflected by low Z-scores for spot 1 (0.03 for *M. sexta* aminopeptidase), and to determine if *M. sexta* actin is an appropriate match for spot 9 (Table 1.2). We compared the PMF of spot 1 with *M. sexta* APN and the PMF of spot 9 with both *A. gambiae* actin and *M. sexta* actin. Of the ten peptides entered from spot 1, two matched directly,

two others matched with potential modifications confirmed by the rules, and three others matched with potential modifications not confirmed by the rules. There was one peptide, not identified in the potential modification searches, which matched with single amino acid substitutions. Only two peptides did not match under any of these circumstances.

For spot 9, the PMF consisted of 33 peptides. When FindMod was used to compare the PMF of this spot against *A. gambiae* actin (the best match identified by ProFound), a third of the peptides were an exact match, a small number matched with potential modifications confirmed by the rules, and several more matched with potential post-translational modifications that were not confirmed by the rules. A third of the peptides (not identified in potential modification results) matched with single amino acid substitutions. Only three peptides did not match under any of these circumstances. A comparison of the PMF from spot 9 and *M. sexta* actin revealed similar findings. There were an almost equal number of exact matches and peptides matching with rule-confirmed potential modifications. Very few peptides matched with potential modifications not confirmed by the rules. A third of the peptides (not identified in the potential modification results) matched with single amino acid substitutions. Six peptides didn't match under any of these circumstances.

3.5. Aminopeptidase Blots

To verify the identifications predicted by PMF, Western blots of 1D and 2D separated BBMV were probed with antibody against MsAPN1 (Luo et al., 1999). For 1D blots, a 120 kDa band predominated (Figure 1.5A) with two minor bands seen at 100 and 105 kDa. For the 2D blot (Figure 1.5B), a spot at 120 kDa (arrow) was seen although at much weaker levels than the

1D blot and was in the same position as the spot that had been identified as APN by mass spectrometry. Other minor spots were also detected.

3.6. Alkaline Phosphatase Blots

An antibody that recognizes the membrane form of *B. mori* alkaline phosphatase identified spots that correspond to spots 5, 6, & 7 which mass spectroscopy identified as alkaline phosphatase. In 1D blots, a strong wide band was detected at 65 kDa (Figure 1.6A) and may consist of a doublet. In 2D blots, two slightly overlapping chains of spots (65 kDa and 62 kDa) were recognized by the antibody (Figure 1.6B).

3.7. Actin Blots

Spots 8 and 9 were confirmed as actin by probing blots with an antibody against actin. In both 1D and 2D blots a strong signal was detected at 42 kDa (Figure 1.7A and 1.7B). This protein exists in several isoforms as evidenced by the chain of spots detected by anti-actin on the 2D blot (Figure 1.7B).

4. Discussion

4.1. Identification of M. sexta BBMV proteins

Two subsets of *M. sexta* BBMV proteins were investigated in this study: *B. thuringiensis* Cry1Ac binding proteins and GPI-anchored proteins. The approach used was a combination of mass spectrometry and blot analyses of proteins separated by 2DE. Through these techniques, alkaline phosphatase and actin were identified as novel Cry1Ac binding proteins.

Alkaline phosphatase was identified on 2D blots as a Cry1Ac binding protein (Figure 2B). Identification of this protein was made by PMF and confirmed by anti-alkaline phosphatase antibody. English and Readdy (1989) first identified a 72 kDa alkaline phosphatase as a Cry1Ac binding protein in *H. virescens*. A relationship between alkaline phosphatase activity and Cry1Ac was also established for *M. sexta*. Ligand blots of BBMV probed with ^{125}I-Cry1Ac produced a strong band at 120 kDa (APN) and a minor band at 65 kDa (Garczynski et al., 1991; Sangadala et al., 1994). An immunopurified preparation of BBMV protein containing both of these proteins was treated with Cry1Ac and resulted in a decrease in alkaline phosphatase activity (Sangadala et al., 1994). In consideration of our results, it is likely that the 65 kDa protein observed by Sangadala et al. (1994) was alkaline phosphatase. In the present study, anti-alkaline phosphatase antibody recognized two overlapping chains of proteins on 2DE blots at 65 and 62 kDa. These chains most likely represent the soluble and membrane forms of this protein. In *B. mori* midgut, the soluble form of alkaline phosphatase is 61 kDa and the membrane form is 58 kDa (Azuma et al., 1991). Taken together, the results of the present and previous study suggest that alkaline phosphatase may be involved in Cry1Ac mode-of-action in *M. sexta*.

Actin was identified as a Cry1Ac binding protein on 2D blots. Identification was made by PMF and confirmed with a Western blot. In a previous 2DE study of *M. sexta* cytoskeleton, Bonfanti et al. (1992) reported a chain of spots at 43 kDa with a pI of approximately 5.7 that was immunoreactive with an actin antibody. Our findings agree with their results. BBMV preparations not only isolate the microvilli from midgut columnar cells, but contain very small amounts of 'contaminating' material from mitochondria, microsomes, basolateral membranes, and cytoskeletal elements (Cioffi and Wolfersberger, 1983; Capella et al., 1997). Since actin is organized into a cytoskeletal structure similar to vertebrate intestinal brush border (Bonfanti et

al., 1992), it is unlikely that this protein is serving as a receptor for *B. thuringiensis* toxin. Actin generally forms ordered arrays with other proteins and serves to support the apical surface of the brush border in the midgut (Mooseker, 1985). Due to the localization of actin in the cell, contacts between *B. thuringiensis* toxin and actin would occur after toxin penetrates the epithelial barrier of the midgut. This interaction could occur when toxin inserts into the membrane or once the integrity of the cells is weakened due to the lytic process.

Actin interactions with *B. thuringiensis* toxin may result in the disruption of its normal function in the cytoskeleton. This structural network is not static, but the synthesis and turnover of the cytoskeletal components is regulated by the metabolic state of the tissue (Mooseker, 1985). In *M. sexta* skeletal muscle, a reduction in myosin and actin expression correlated to the time of cell death (Schwartz et al., 1993). Since actin expression levels vary and this variability is related to the physiological state of the cell, occupation of actin by *B. thuringiensis* toxins may contribute to the weakening of the integrity of midgut epithelia. This speculation is supported by the fact that associations between actin and septate junctions are critical to maintenance of epithelial barriers in invertebrates (Bonfanti et al., 1992). In *Drosophila melanogaster*, cadherin is indirectly linked to the actin cytoskeleton (Pacquelet et al., 2003). Although no functional data has been published for a lepidopteran cadherin, some functional information can be inferred for Bt-R1 based on sequence homology to well-characterized cadherins. Bt-R1 contains several cell-adhesion and integrin binding sites (Candas et al., 2002), implicating a role for this protein in cell interactions and suggesting that lepidopteran cadherins may function similar to those in *Drosophila*. Until studies analyzing the functions of Bt-R1 are conducted, however, the roles for this cadherin remain speculation. Interestingly, the proteomic study of *P. interpunctella* found a decrease in peroxinectin, a cell adhesion protein, in resistant animals (Candas et al., 2003).

Therefore, it is reasonable that disruption of the cellular adhesion-cytoskeletal substructure is part of the intoxication process for Cry1 toxins.

GPI-anchored proteins were isolated from BBMV using PIPLC to cleave the anchors and TX-114 phase separation. This approach has been used to depict this sub-proteome in *Arabidopsis thaliana* (Sherrier et al., 1999) and was a simple way to isolate a sub-set of midgut brush border proteins. The 120 kDa APN was detected on anti-CRD blots of GPI-anchored proteins separated by both 1DE and 2DE (Figures 1.3A and 1.3B). In this study, both Western blots and PMF identified proteins of 65 kDa as alkaline phosphatase. This is known to be a GPI-anchored protein (Takesue et al., 1989; Garczynski and Adang, 1995). Interestingly, GPI-anchored proteins were not detected at 65 kDa on Cry1Ac blots. It is possible that this protein precipitated during separation by 2DE and is the vertical band detected at 55-65 kDa in the 2D anti-CRD blot, however this is speculation at this time. Additionally, spots 3 and 4 were identified as GPI-anchored proteins from anti-CRD blots, yet searches with PMF data suggest that these proteins may be peptidases or (part of) membrane transporters (Table 1.1). Both these classes of proteins are transmembrane proteins, not GPI-anchored proteins. This discrepancy may be the result of picking overlapping spots for mass spectrometry analysis, or more likely that the Z-scores are quite low and these identifications are incorrect.

A comparison of Cry1Ac binding proteins detected on blots of 2D gels versus the more conventional 1D gels showed that the greatest difference was in the intensity of the detection of recognized proteins. For example, the detection of a 120 kDa protein, corresponding to MsAPN1, was very strong on 1D Cry1Ac blots (Figure 1.2A). In contrast, detection of APN was relatively weak on 2D blots (Figure 1.2B). This difference may be due to the inherent bias for certain proteins in 2DE. Membrane proteins, for example, are harder to resolve by this technique

(reviewed in Santoni et al., 2000). Additionally, larger proteins (>100 kDa) are much harder to resolve by 2DE using IPG strips. This phenomenon can be partially overcome, however, by active rehydration where overnight strip rehydration occurs under a low voltage (30V) and the entry of high molecular weight proteins (≥100 kDa) is facilitated (Görg et al., 1998). This technique requires that the isoelectric focusing step is conducted using and IPGphor instrument (Amersham) that was not available for these studies.

The challenge of resolving high molecular weight proteins is reflected in our 2D gel that shows few proteins larger than 100 kDa (Figure 1.1B). Correspondingly, we did not detect Cry1Ac binding to the 210 kDa cadherin in 2D separations. Cry1Ac binding to this protein was also not detected in the 1D blot. Although it is most likely that cadherin-Cry1Ac binding is not observed because cadherin is not entering the gel, particularly in the case of 2DE, the absence of binding may also be related to the treatment of BBMV proteins prior to electrophoresis. This treatment may alter the availability of the binding epitopes of *B. thuringiensis* toxin-binding proteins. Using 1D separated *M. sexta* BBMV, another study described the differences seen in ligand blots subjected to either native or denaturing conditions. The authors determined denaturation of proteins increases detection by Cry1 toxins (Daniel et al., 2002). This explanation seems unlikely in the present study since the second dimensional separation step of 2DE is identical to the same denaturing condition as 1DE. More likely any difference in epitope availability is due to the isoelectric focusing step of 2DE.

4.2. Use of mass spectrometry data to identify proteins from a species with a limited sequence data set

Identification of lepidopteran proteins by 2DE and PMF proved challenging and required further computer analyses to corroborate appropriate identifications. PMF data used in ProFound searches resulted in presumptive identifications with low Z-scores to *M. sexta* proteins or high Z-score matches to proteins that were not from *M. sexta* (Table 1.1). A theoretical digest of MsAPN1 was conducted using PeptideCutter (us.expasy.org/tools/peptidecutter/) and revealed 77 potential trypsin digestion sites (data not shown). The experimental digest of the APN spot only resulted in 10 peptides. A likely explanation for this is that higher molecular weight proteins, especially those at relatively low concentrations, generate less robust mass spectra. Not only is less protein available for digestion, but the concentration of recovered peptides will be low as well. Therefore the PMF will be derived from the most abundant peptides. This fact can contribute to low Z-scores as the number of peptides available to be matched to the theoretical digest is small.

To determine what consequence the number of input peptides had on Z-score, a second ProFound search was conducted using only those peptides for spots 1 and 8 (2 and 6 respectively) that had produced exact matches to APN and actin respectively (data not shown). For spot 8, no change in either the resulting matches or their Z-scores was seen. For spot 1, this type of search resulted in *M. sexta* APN being the best match with a higher Z-score (0.43) than a search with all peptides of the PMF (0.03). This is still a poor match by the definition of Z-score.

There are approximately 500 protein entries for *M. sexta* in NCBI and 80,000 entries in the ‘Other Metazoa’ subset of NCBI. This means that the number of potential non-matching

proteins is much larger than the number of potential matching peptides and the estimated Z-score will be affected. It is also necessary, therefore, to examine the PMF data with other tools to adequately evaluate the candidate matches produced by ProFound.

As mentioned previously, the database used in the search can affect the Z-score and the number of matching peptides. Additionally, sequences deposited in NCBI consist of protein sequence alone and have none of the post-translational modifications found *in vivo* or those that can arise during protein isolation and separation. When a spot from 2D separated BBMV is digested and the peptides are analyzed by mass spectrometry, the resulting spectra will represent peptides with post-translational modifications. By searching against peptides without these modifications, mass mismatches can occur which will lower the probability of correct identifications. FindMod accounts for these mismatches by predicting potential sites of post-translational modifications, adding the appropriate mass of the modification, and then comparing the mass-corrected peptides against the experimentally derived peptide masses (Wilkins et al., 1999). This program was used on two spots (1 and 9) to match a large proportion of the input peptides providing added confidence to the identifications produced by ProFound searches (Table 1.2).

Candas et al. (2003) also reported challenges obtaining identifications using PMF data from *P. interpunctella* proteins, however, they did not report Z-scores for candidate matches. They suggested that un-matched proteins were due to mass variations in the tryptic peptides for homologous proteins in different organisms. While that may be the case, it is also apparent that mass differences can also be due to post-translational modifications since not all *Manduca* peptides matched those we obtained from the same protein isolated from BBMV.

4.3. Conclusions

The identification of alkaline phosphatase and actin as Cry1Ac binding proteins is only the first step in elucidating their roles in the mode-of-action of *B. thuringiensis* toxins. Future experiments with purified or expressed proteins will be necessary to establish detailed binding characteristics and potential mediation of cell toxicity. The separation and identification of these two proteins, as well as other *M. sexta* BBMV proteins proved to be a challenging task. Although 2DE and mass spectrometry techniques have become more accessible and user-friendly, limitations are still encountered when working with organisms with fewer sequenced proteins than widely studied organisms or those with sequenced genomes. Additional insect protein sequences, especially Lepidoptera, are needed to yield high confidence identifications from database searches with PMF data. Until then, the use of additional analyses, such as those described here, may continue to be necessary to maximize the power of 2DE and mass spectrometry.

Acknowledgements

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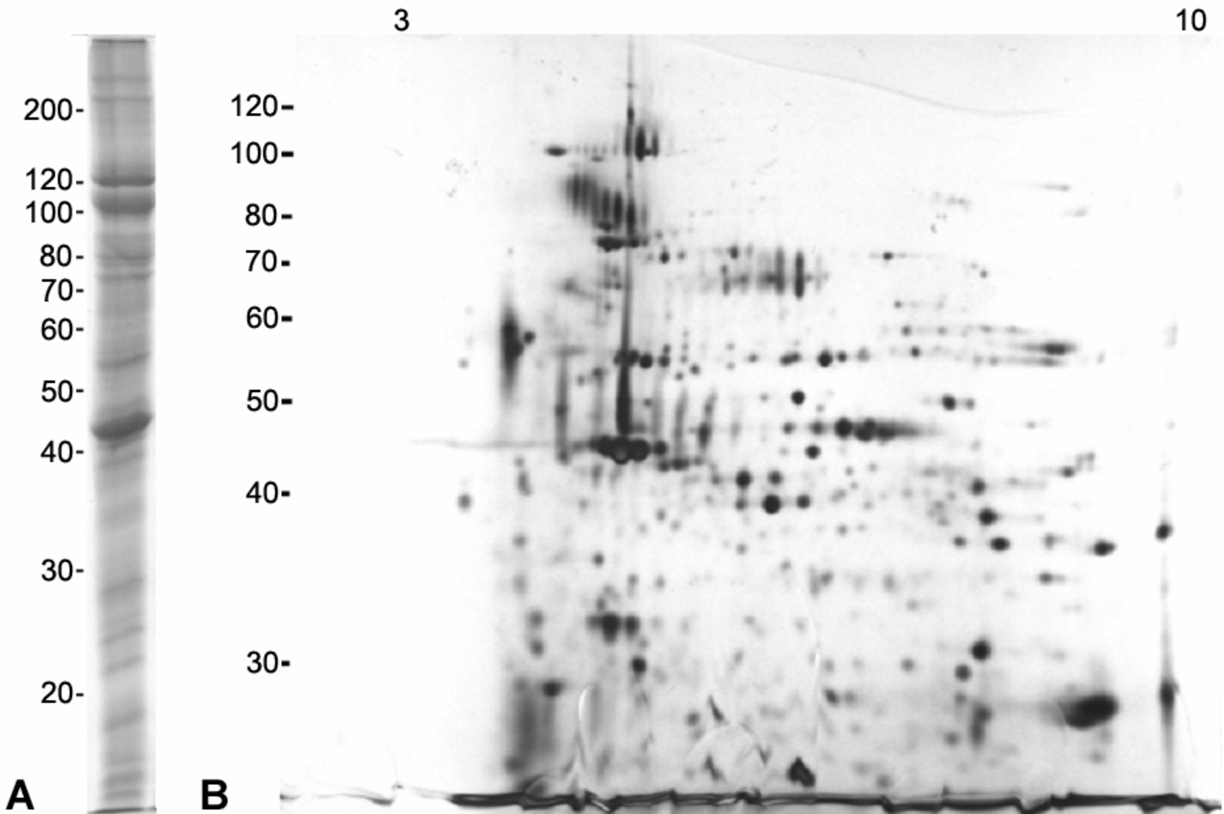

Figure 1.1. Silver stained gels of electrophoretic separations of *M. sexta* BBMV proteins. A. 10 μg of BBMV protein separated by 10% SDS-PAGE. B. 45 μg of BBMV protein separated by 2DE using a 13 cm pH 3-10 non-linear Immobiline DryStrip (Amersham) for the first dimension and a 10% SDS-gel as the second dimension. Masses of molecular weight markers (kDa) on the left of each gel and pH range of isoelectric focusing at the top are indicated.

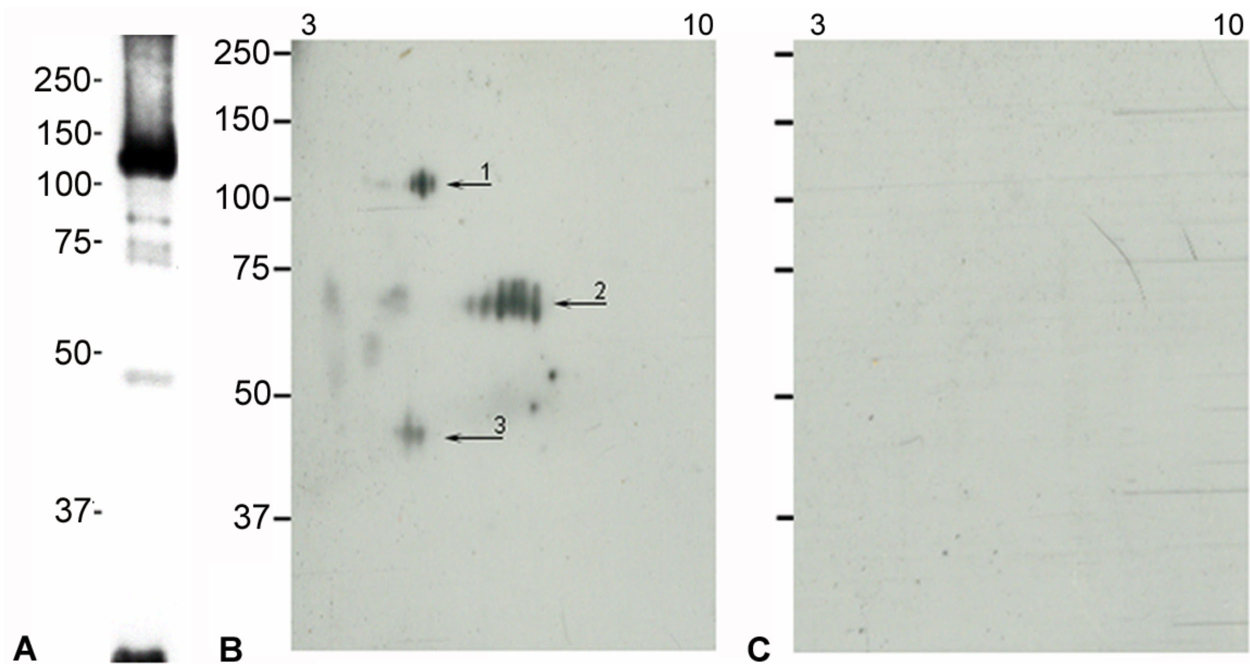

Figure 1.2. Cry1Ac ligand blot of 1D and 2D separated *M. sexta* BBMV proteins. 5 nM of biotinylated Cry1Ac and a monoclonal antibody against biotin were used to probe blots of 10 µg of BBMV protein separated by (A) 10% SDS-PAGE and (B), (C) 2DE using a 7 cm pH 3-10NL Immobiline DryStrip (Amersham) for the first dimension and a 10% SDS-gel as the second dimension. The addition of 500 nM of unlabeled Cry1Ac was used as a competitor in (C). Masses of molecular weight markers (kDa) are indicated at the left of each gel. See text for significance of arrows.

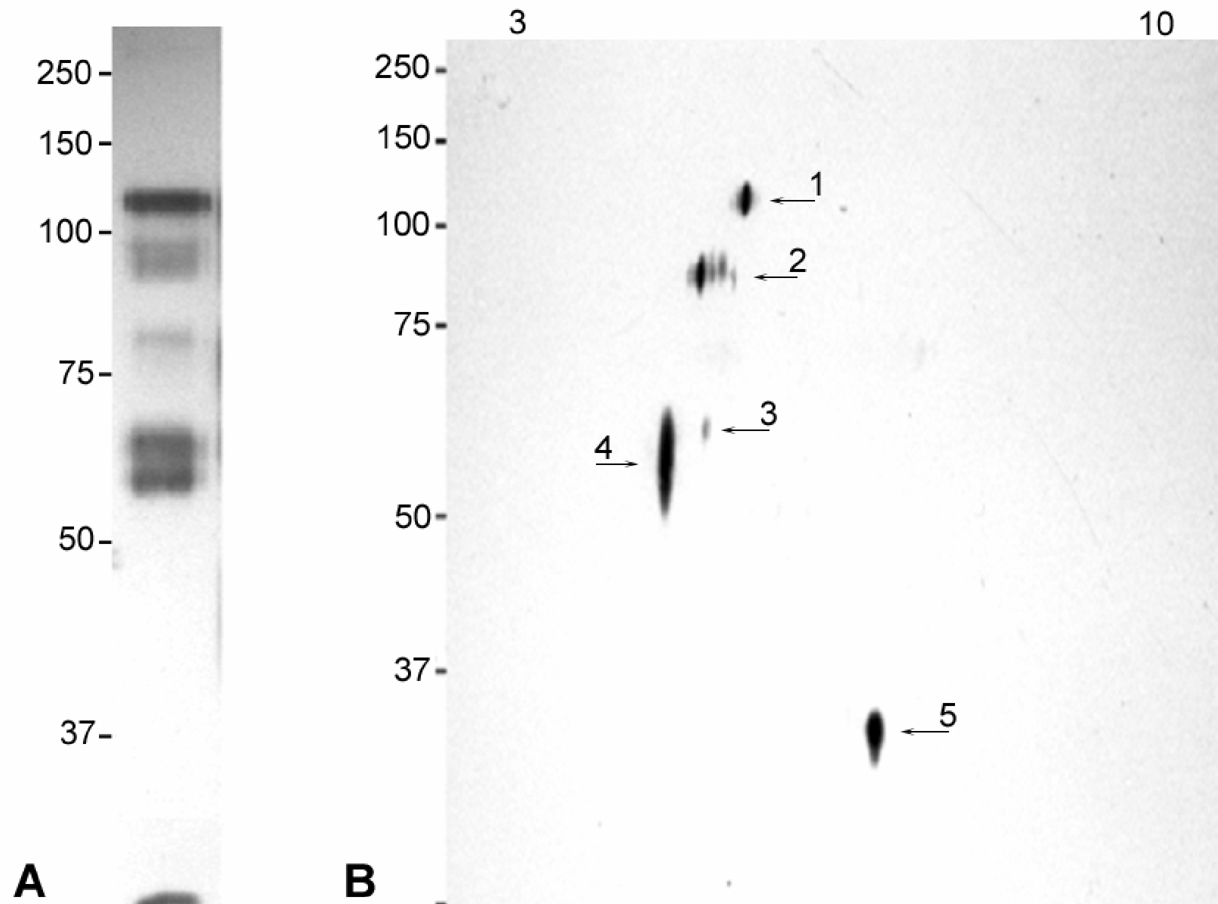

Figure 1.3. GPI-anchored proteins in *M. sexta* BBMV. A rabbit anti-CRD antibody and a goat-anti-rabbit antibody were used to probe blots of (A) 10 µg of isolated GPI-anchored proteins separated by 10% SDS-PAGE and (B) 10 µg of protein separated by 2DE using a 7 cm pH 3-10NL Immobiline DryStrip (Amersham) for the first dimension and a 10% SDS-gel as the second dimension. Masses of molecular weight markers (kDa) are indicated at the left of each gel. See text for significance of arrows.

Table 1.1. Peptide mass fingerprint results from ProFound using methionine oxidation as a protein modification.

Spot Number	Type of Match[a]	Accession Number	Species	Protein Identified	Predicted Mass(kDa)/pI	Z-Score[b]
1	TB	6425002	*H. virescens*	Cry1A toxin receptor A	112.20 / 5.7	0.07
	GPI	6358530	*M. sexta*	Aminopeptidase N	111.88 / 5.8	0.03
		2499901	*M. sexta*	Membrane alanyl aminopeptidase precursor (aminopeptidase N-like protein) (Cry1Ac receptor)	111.28 / 6.0	0.02
2	GPI	21296061	*A. gambiae*	agCP1751 (ABC transporters, ATPase component)[c]	84.98 / 5.8	0.38
		13359138	*B. mori*	Neutral endopeptidase 24.11	87.75 / 5.8	0.03
		21295000	*A. gambiae*	agCP10681 (peptide transporter)[c]	78.59 / 5.1	
3	GPI	21296061	*A. gambiae*	agCP1751 (ABC transporter, ATPase component)[c]	84.98 / 5.8	0.22
		21288397	*A. gambiae*	agCP9476	83.81 / 5.0	0.06
		21297926	*A. gambiae*	agCP14130 (peptidase family M49)[c]	84.17 / 5.2	0.02
4	TB	21294833	*A. gambiae*	agCP7205	61.24 / 6.4	0.12
		423944	*Drosophila*	Octamer-dependent transcription factor	55.39 / 6.0	0.04
		21299921	*A. gambiae*	agCP9061 (sodium channel)[c]	59.24 / 5.8	
5	TB	14917025	*B. mori*	Membrane-bound alkaline phosphatase	59.22 / 5.9	2.43
6	TB	15128500	*B. mori*	Membrane-bound alkaline phosphatase	59.13 / 5.8	2.43
7	TB	14917025	*B. mori*	Membrane-bound alkaline phosphatase	59.22 / 5.9	2.43
8	TB	224305	*Pecten sp.*	Actin	41.55 / 5.3	1.33
		113281	*P. ochraceus*	Actin, muscle	41.93 / 5.3	1.30
9	TB	21297650	*A. gambiae*	agCP8921 (actin)[c]	42.38 / 5.5	2.43

[a]Spots chosen for mass spectrometry analysis were in the same position on a SyproRuby stained 2D gel as positive spots on Cry1Ac (Toxin Binding and/or anti-CRD (GPI) blots.
[b]Z-score is an indicator of the quality of the search result. The following is a list of Z-score and corresponding percentile: 1.282 = 90.0, 1.645 = 95.0, 2.326 = 99.0, 3.090 = 99.9. A detailed description of Z-score can be found at: http://129.85.19.192/profound/help.html
[c] If searches returned *Anopheles gambiae* sequences as a top match, these sequences were used to search NCBInr using BLAST since they are not yet annotated as to possible function. A description of homology to conserved domains is reported in those cases where a potential identification could be made.

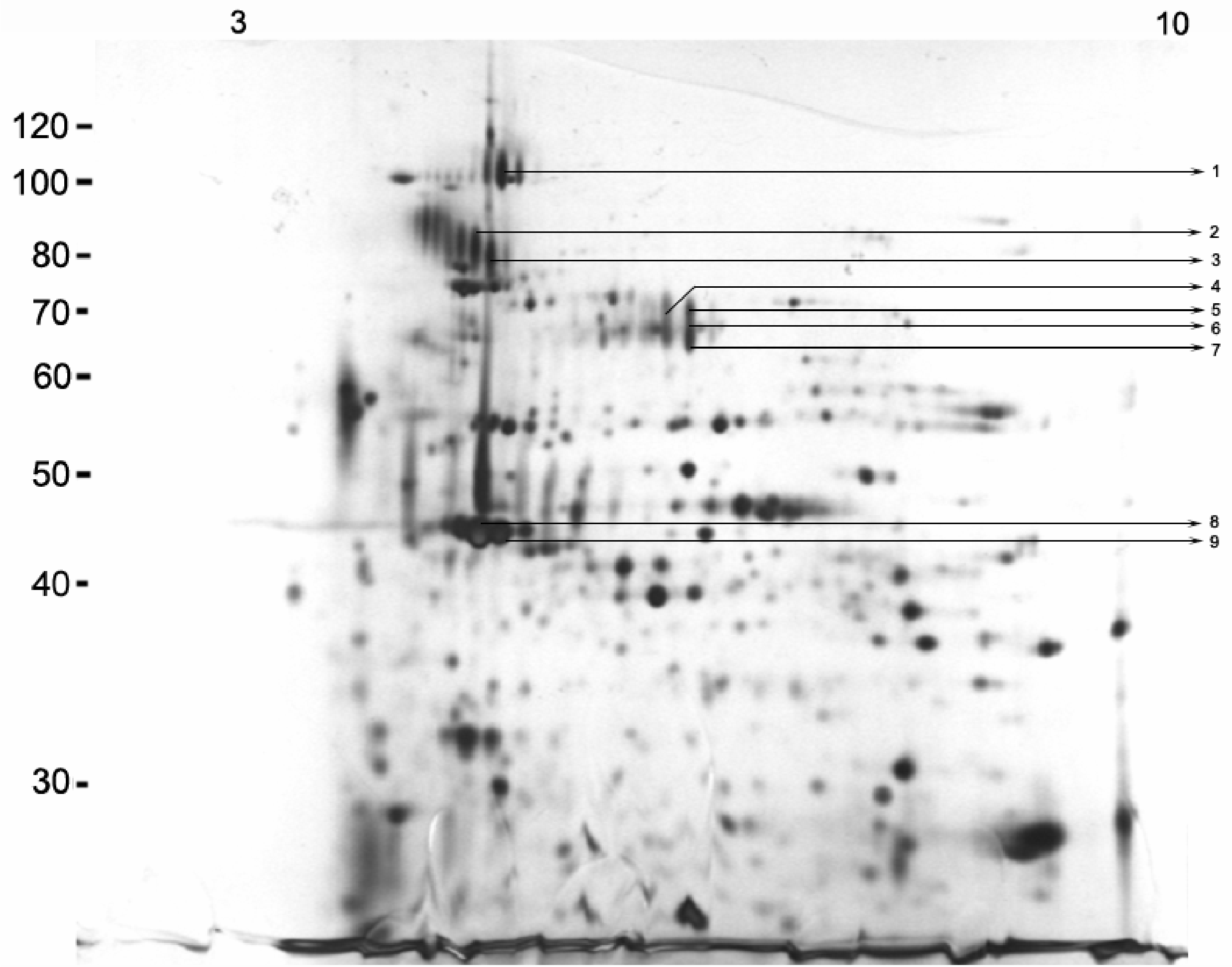

Figure 1.4. Representation of spots analyzed by peptide mass fingerprinting. Molecular sizes of the standards, approximate pI, and spot pattern were used to correlate spots detected on Cry1Ac and anti-CRD blots with spots on a SyproRuby stained gel. Nine spots were chosen for analysis. Spots 5, 6, and 7 were excised from the top, middle, and bottom region of the same long spot.

Table 1.2. Results of number of matching peptides between PMF data and known proteins using FindMod[a].

Spot Number	Peptides in PMF	Protein	Accession Number	Exact Match Peptides	Peptides Matched with Potential Post-translational Modifications[b]		Matched Peptides with Substitutions[c]
					Confirmed by rules	Not conforming to rules	
1	10	*M. sexta* APN	6358530	2	2	3	1
9	33	*A. gambiae* cytoplasmic actin	21297650	11	3	6	10
		M. sexta cytoplasmic actin	28070937	8	7	3	9

[a]FindMod (http:// us.expasy.org/tools/findmod/) predicts sites of post-translational modifications for 22 types of modifications, then compares modified peptides to experimentally derived peptides.
[b]Predictions are based on a set of rules which are defined using the information in PROSITE, by examining the annotations in Swiss-Prot, and examining the literature. Matches either conform to the rules or are detected by a mass difference, but can't be confirmed by the rules.
[c]Single potential amino acid substitutions detected by mass difference.

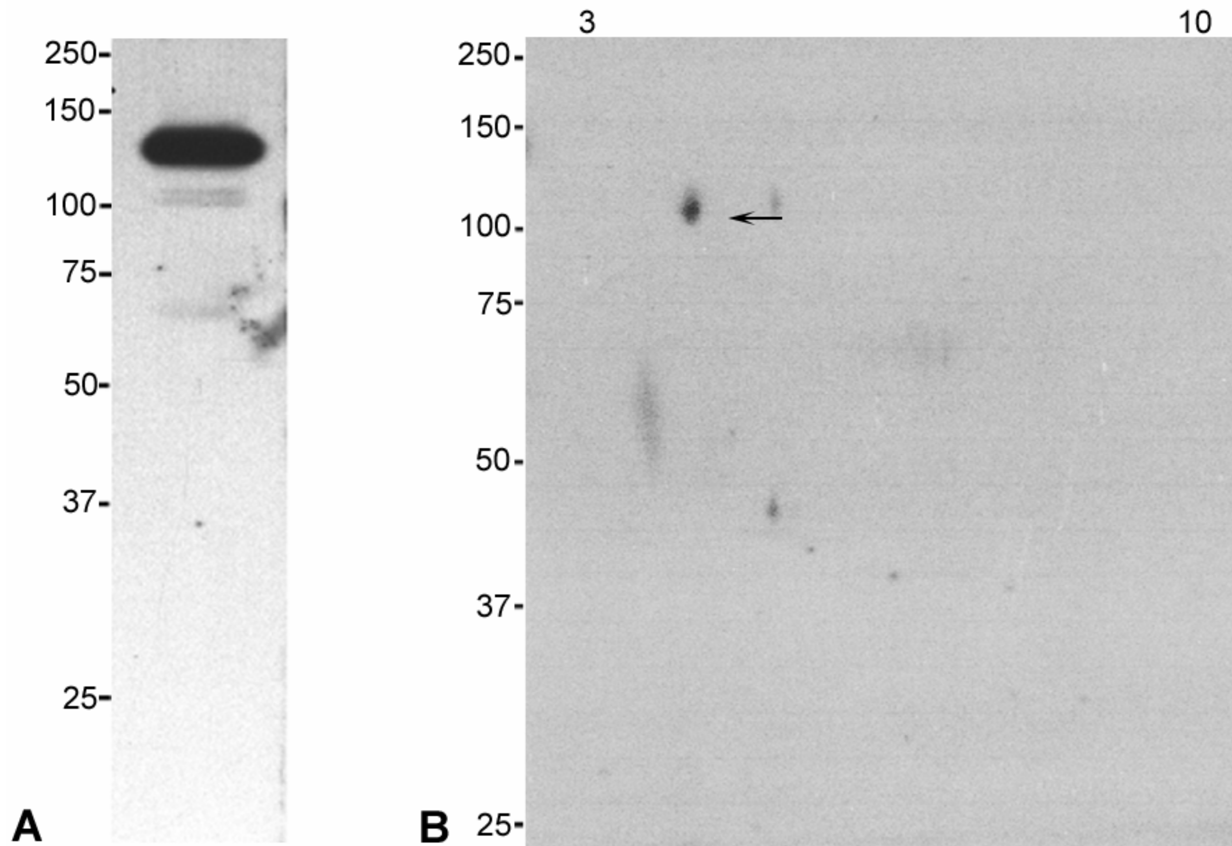

Figure 1.5. Aminopeptidase blots of 1D and 2D separated *M. sexta* proteins. A rabbit antibody against MsAPN1 and a goat anti-rabbit antibody were used to probe blots of (A) 10 µg of BBMV protein separated by 10% SDS-PAGE and (B) 10 µg of BBMV protein separated by 2DE using a 7 cm pH 3-10NL Immobiline DryStrip (Amersham) for the first dimension and a 10% SDS-gel as the second dimension. Masses of molecular weight markers (kDa) are indicated at the left of each gel. The arrow marks the 120 kDa protein.

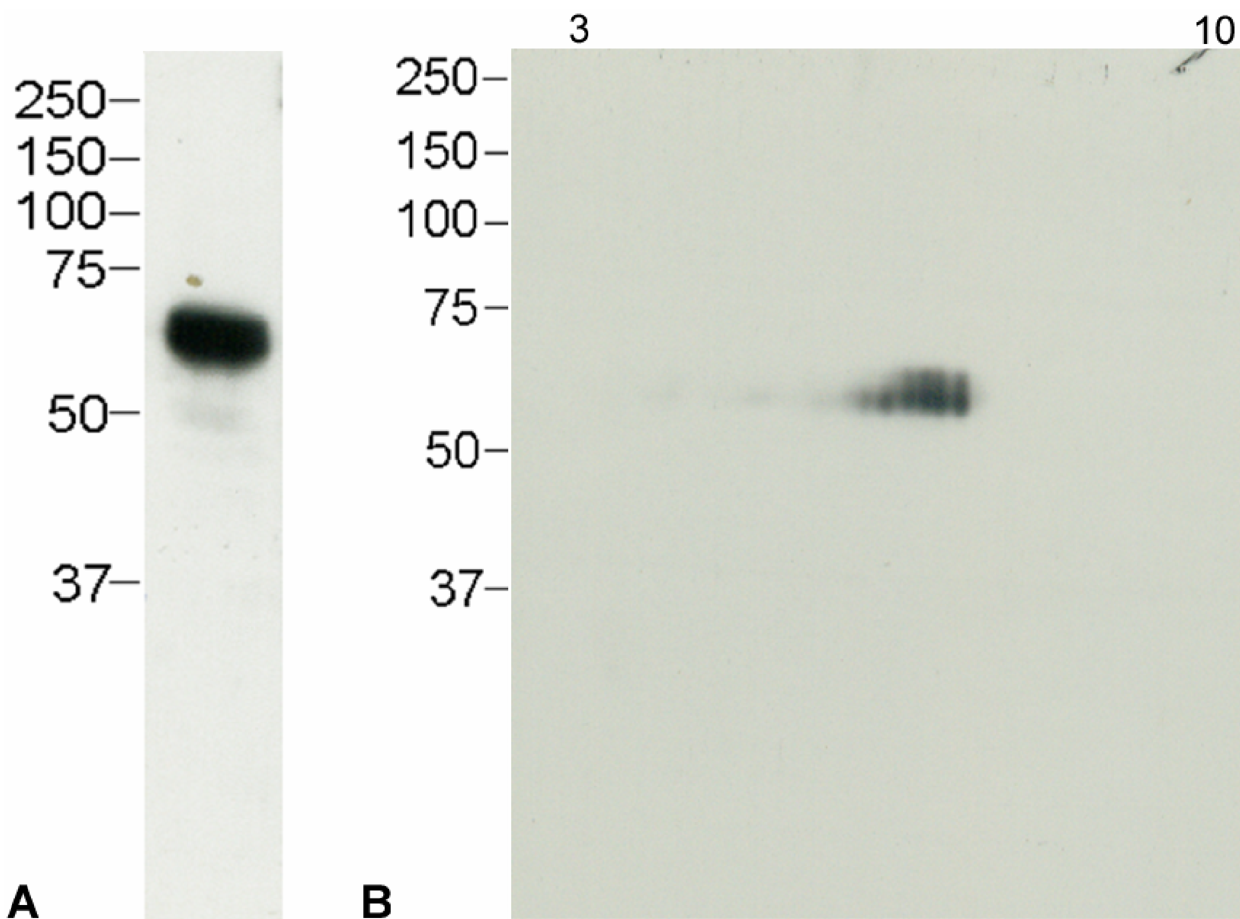

Figure 1.6. Membrane alkaline phosphatase blots of 1D and 2D separated *M. sexta* proteins. A rabbit antibody against *B. mori* membrane alkaline phosphatase and a goat anti-rabbit antibody were used to probe blots of (A) 10 μg of BBMV protein separated by 10% SDS-PAGE and (B) 10 μg of BBMV protein separated by 2DE using a 7 cm pH3-10NL Immobiline DryStrip (Amersham) for the first dimension and a 10% SDS-gel as the second dimension. Masses of molecular weight markers (kDa) are indicated at the left of each gel.

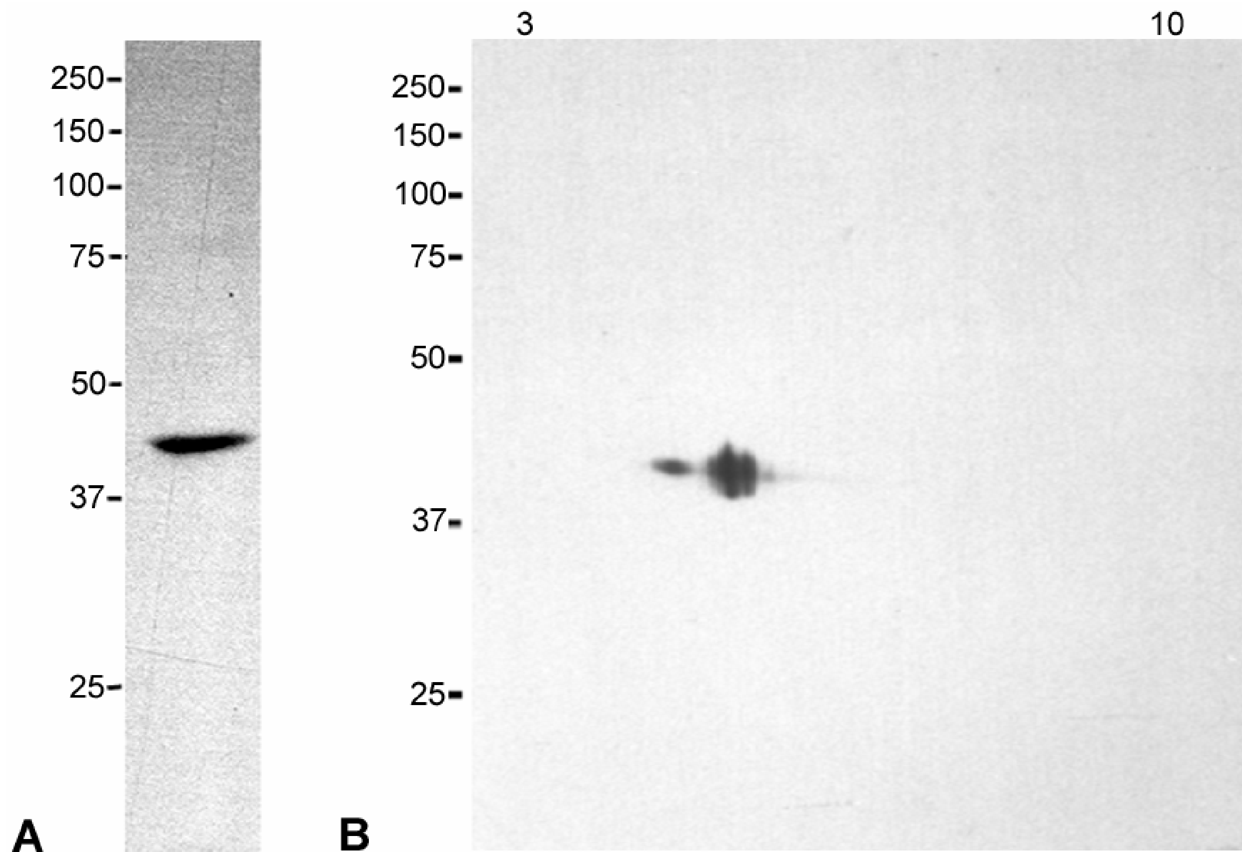

Figure 1.7. Actin blots of 1D and 2D separated *M. sexta* proteins. A rabbit antibody against chicken muscle actin and a goat anti-rabbit antibody were used to probe blots of (A) 10 μg of BBMV protein separated by 10% SDS-PAGE and (B) 10 μg of BBMV protein separated by 2DE using a 7 cm pH 3-10NL Immobiline DryStrip (Amersham) for the first dimension and a 10% SDS-gel as the second dimension. Masses of molecular weight markers (kDa) are indicated at the left of each gel.

CHAPTER 3

COMPARISON OF MIDGUT PROTEINS FROM *BACILLUS THURINGIENSIS* CRY1AC SUSCEPTIBLE AND RESISTANT POPULATIONS OF *PLUTELLA XYLOSTELLA*.[1]

[1] McNall, R. J. and M. J. Adang. 2004. To be submitted to *Insect Biochem. Mol.Biol.*

Abstract

The diamondback moth, *Plutella xylostella*, is the only insect known to have field populations resistant to *Bacillus thuringiensis* (*Bt*) toxins. Previous studies have demonstrated a reduction in Cry1A toxin binding sites using *in vitro* binding assays. However, a Cry1Ac binding protein, a 120 kDa aminopeptidase N, is present at similar levels in susceptible and resistant insects. The identity of molecules responsible for resistance is still unknown. In this study, brush border membrane vesicles were prepared from whole insects and dissected midguts of diet-adapted *Bt* susceptible and resistant *Plutella xylostella*. The protein composition of these vesicles was compared using fluorescent dyes and two-dimensional electrophoresis. The identification of some altered proteins was accomplished through peptide mass fingerprinting and sequencing by MALDI-ToF ToF. Although vesicles prepared from whole insects contained a larger number of proteins, more changes between susceptible and resistant insects were detected using dissected midgut preparations. In vesicles prepared from dissected midguts, actin was identified by peptide mass fingerprinting as a down regulated protein in resistant *P. xylostella*. Glucosinolate sulphatase was identified through fingerprinting and *de novo* sequencing as an up regulated protein in resistant insects in both types of vesicle preparations. A western blot comparison of diet-adapted strains with cabbage-reared strains revealed that GSS protein levels are increased in resistant animals from both food sources. Alkaline phosphatase activity followed a similar pattern as that seen for glucosinolate sulphatase. These findings suggest that *P. xylostella* resistance to *Bt* is multifaceted.

Keywords: *Bacillus thuringiensis***,** resistance, 2D-DIGE, *Plutella xylostella*

Abbreviations: 2D-DIGE – two dimensional difference gel electrophoresis, BBMV – brush border membrane vesicles, *Bt* – *Bacillus thuringiensis*, GSS – glucosinolate sulphatase, MALDI-ToF – matrix assisted laser desorption/ionization time of flight, MS/MS – tandem mass spectrometry, PMF – peptide mass fingerprinting.

1. Introduction

The soil bacterium *Bacillus thuringiensis* (*Bt*) produces inclusion bodies that have insecticidal activity. When a susceptible insect ingests these crystalline proteins, the inclusion body is solubilized releasing protoxin. This form is cleaved by midgut proteases resulting in a toxic core protein. Once toxins bind to target molecules at the brush border of the midgut epithelium, the insect dies as pores are formed (reviewed in Schnepf *et al.*, 1998). *Bt* toxins are highly specific to their target pests, which is a welcome alternative to other commercial insecticides that are less specific and environmentally unfriendly.

Bt proteins were first incorporated into sprayable formulations, however genes encoding these proteins have been engineered into a variety of crops which provide constitutive protection against insect feeding damage (Betz *et al.*, 2000). The selection pressure exerted by constant exposure to *Bt* toxins, however, has made resistance development a threat to the efficacy of *Bt* crops. Research that addresses the physiological changes in resistant insects will facilitate the long-term efficacy of *Bt* sprays and transgenic crops.

Bt resistant field populations of *Plutella xylostella*, the diamondback moth, have been found in areas of extended usage of spray formulations (Ferre & Van Rie, 2002). The first field resistant strains of *P. xylostella* were detected in Hawaii, but additional populations have been found in other parts of the United States as well as other countries (Ferre and Van Rie, 2002). In

most resistant populations, alterations were found in the binding of toxins to brush border membrane vesicles (BBMV). Solution based binding assays with BBMV from different strains of resistant *P. xylostella* have correlated loss of toxicity to reduced toxin binding (reviewed in Ferre and Van Rie, 2002). Studies with a *Bt* resistant strain from Hawaii, NO-QA, demonstrated reduced binding of Cry1Aa, Cry1Ab, Cry1Ac, and Cry1F toxins compared to the susceptible strain LAB-P (Tabashnik *et al.*, 1994; Tabashnik *et al.*, 1997). These results suggest that resistance to all four toxins is due to an alteration in a shared binding site, resulting in multiple toxin cross resistance.

A linkage analysis in the strain NO-QA revealed a correlation between an autosomal recessive gene and resistance to Cry1Ac (Heckel *et al.*, 1999). Recent AFLP analyses found no correlation of toxin resistance with cadherin (David Heckel, personal communication), a midgut protein identified as a toxin binding molecule in a number of insects (Adang *et al.*, 2002). With another resistant strain, PHI, isozyme polymorphism linkage analysis demonstrated that resistance strongly correlated to two mannose-6-phosphate isomerase isozymes (Herrero *et al.*, 2001).

Although binding of toxin to BBMV from LAB-P and NO-QA strains was reduced, surface plasmon resonance experiments conducted on sonicated BBMV indicated only a slight reduction in the number of binding sites in resistant insects (Masson *et al.*, 1995). In Western blot analyses with BBMV from these same strains, a 120 kDa protein bound labeled toxin in both sets of insects (Luo *et al.*, 1997). This candidate Cry1Ac receptor was identified as an aminopeptidase N (APN) and was immunoaffinity purified from both susceptible and resistant *P. xylostella* in equal amounts (Luo *et al.*, 1997). In different *Bt*- susceptible and –resistant strains, two proteins (probably APN) of 116 and 110 kDa with similar binding intensities were detected

in Cry1Ac ligand blots (Kumaraswami *et al.*, 2001). The results from ligand blotting and surface plasmon resonance experiments indicate that proteins capable of binding toxin are present in resistant *P. xylostella* even though BBMV binding assays demonstrate low levels of toxin binding. This suggests that there are other molecules responsible for resistance.

Proteomic analyses of complex biological samples have become a favored approach to identify proteins implicated in biological processes or different disease states. Two recent studies have used two-dimensional electrophoresis (2DE) to examine the role of midgut proteins in *Bt* toxin mode-of-action. In *Manduca sexta*, ligand blots of BBMV proteins separated by 2DE demonstrated Cry1Ac toxin binding to alkaline phosphatase and actin, in addition to the previously identified toxin binding protein MsAPN1 (McNall and Adang, 2003). Candas et al. (2003) compared BBMV proteins from *Bt* susceptible and resistant *Plodia interpunctella* using differential fluorescence dyes in conjunction with 2DE. BBMV from resistant strains have increased levels of proteins associated with oxidative metabolism suggesting an involvement in *Bt* resistance. Additionally, there was a decrease in chymotrypsin protein and its corresponding activity in resistant *P. interpunctella*. This may reflect a difference in toxin processing (Candas *et al.*, 2003). These studies demonstrated the power of proteomics in examining the mode of action as well as complex physiological changes in *Bt* resistant insects.

In this study, a proteomic approach was used to analyze BBMV proteins from diet-adapted susceptible and resistant *P. xylostella* (Tabashnik *et al.*, 2000). Diet-adapted insects are a preferred alternative to animals reared on plants due to the relative simplicity of colony maintenance and bioassay. Unlike the adaptation of a susceptible strain (Geneva 88) to diet (Shelton *et al.*, 1991), diet-adaptation of a *Bt* resistant strain (NO-QA) was challenging. The survival of NO-QA larvae was poor on artificial diet, so NO-QA and Geneva 88 were crossed.

Once crossed, the new NO-QAGE strain was maintained by inbreeding and reared on artificial diet. Periodic toxin selections were conducted with Cry1Ac (Tabashnik *et al.*, 2000). These insects are resistant to *Bt* at the same levels as their cabbage-reared resistant parent (Tabashnik *et al.*, 2000). Two-dimensional differential gel electrophoresis (2D-DIGE) was used to examine changes in midgut proteins between the two diet-adapted strains of insects by comparing two types of vesicle preparations: whole-larvae and dissected midgut. Identification of altered proteins was accomplished by mass spectrometry. The potential role of these identified proteins in *Bt* mode-of-action is discussed.

2. Materials and Methods.

2.1. Insects and brush border membrane vesicle (BBMV) preparation

P. xylostella eggs (Geneva 88 and NO-QAGE) were obtained from Benzon Research (Carlisle, PA) and reared on artificial diet (Southland; Lake Village, AR) to first day, fourth-instar larvae at 26°C with a 12:12 (L:D) photoperiod. Larvae were either frozen at -80°C or midguts were isolated. BBMV were prepared from either whole animals or isolated midguts using the $CaCl_2$ precipitation method (English and Readdy, 1989), except that protease inhibitors (Complete, Roche) were used in the grinding buffer instead of PMSF. For whole animal preparations, the following modifications were made. First, phenylthiourea was added to the grinding buffer at 0.1 mg/ml to inhibit the phenoloxidase system of the larvae. Second, the supernatant of the 4,000 x *g* centrifugation was filtered through a layer of cheesecloth to remove exoskeletal material (MacIntosh *et al.*, 1994) and the 4,000 x *g* centrifugation step was repeated. For all preparations, final pellets were resuspended in deionized water and protein concentration determined by the method of Bradford (1976) using BSA as a standard.

P. xylostella larvae (LAB-P and NO-QA) were reared on cabbage in 1996 as described (Tabashnik *et al.*, 1994). Fourth-instar larvae were collected and frozen at -80°C until used in this study. BBMV were prepared from whole animals as described above.

Resuspended BBMV were subjected to protein precipitation using the Plus-One 2-D Clean-Up kit (Amersham Biosciences) as described by the manufacturer. This step was necessary to obtain high quality 2D gels. Recovered precipitated proteins were resuspended in solubilization buffer (5M urea, 2M thiourea, 2% CHAPS, 2% SB3-10 (Sigma), 10 mM Tris (pH 8.3) and protease inhibitors (Complete, Roche)) by using a sonicating water bath and vortexing. The protein solution was then centrifuged at 30,000 x *g* for 30 minutes yielding a supernatant containing the solubilized BBMV proteins. Protein concentrations were determined using the Plus-One 2-D Quant Kit (Amersham Biosciences).

2.2 One-dimensional electrophoresis and Western blots

BBMV proteins were separated by 10% SDS-PAGE and either stained with BioSafe Coomassie Blue (Pierce) or transferred to PVDF membranes(Towbin *et al.*, 1979). To confirm the identification of altered proteins, western blots were conducted. All steps were conducted at room temperature. For western blots, PVDF membranes were blocked with 5% BSA in 1x TBS/0.1% Tween-20 (TBST) for 1 hr then incubated with anti- *P. xylostella* glucosinolate sulphatase (GSS) antibody (gift from Dr. Heiko Vogel, Max Planck Institute) diluted to 1:300,000 in TBST for 1 hr. Blots were washed with three changes of TBST for 15 minutes each. Washed blots were incubated with goat anti-rabbit antibody diluted to 1:20,000 in TBST for 1 hr. After another set of washes, blots were developed with ECL chemiluminescence

substrate (Amersham Biosciences) and exposed to film. Western blots were conducted on three different BBMV preparations.

The intensity of GSS detected bands was quantified by spot densitometry using the densitometry program associated with the AlphaImager (Alpha Innotech Corporation, San Leandro, CA). The program assigns an integrated density value to a selected region, and the sum of the signal intensities of all selected regions is defined as 100%.

2.3. Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE)

2D-DIGE was performed at the Proteomics Resource Facility at the University of Georgia. Protein extract (50 μg) of Geneva 88 and NO-QAGE proteins were each minimally labeled with either Cy3 or Cy5 fluorescent dye (Amersham Biosciences) as per the manufacturer's directions. The labeled samples were pooled and brought up to 300 μl with rehydration solution (solubilization buffer with the addition of bromophenol blue, 18 mM DTT, and 0.5% ampholines). This solution was used to rehydrate a 18 cm Immobiline DryStrip (Amersham Biosciences) overnight at room temperature. Isoelectric focusing (IEF) was conducted with an IPGphor (Amersham Biosciences) as per the manufacturer's suggestions for a duration of 32000 Vhr. Focused strips were equilibrated with buffer (6M urea, 2% SDS, 30% glycerol, 50 mM Tris (pH 8.8), bromophenol blue) containing 1% DTT followed by 4% iodoacetamide for 15 minutes each. Equilibrated strips were overlaid on an 8-20% SDS-PAGE and run at 20 mA until the dye front reached the bottom of the gel.

Upon completion of electrophoresis, the gels were fixed in 30% ethanol, 7.5% acetic acid overnight at room temperature. Gels were imaged with the Typhoon 9400 (Amersham Biosciences), optimizing the photomultiplier tubes for each laser to achieve the broadest

dynamic range. Wavelengths for the filters/lasers were set at: 532 nm/580 nm for Cy3 and 633 nm /670 nm for Cy5. Decyder software (Amersham Biosciences) was used to analyze differences between images. Spots were considered ‘different’ if there was a 2.0-fold difference in the spot-volume between the two samples. In figure 2, altered spots are circled in blue if more abundant in the resistant strain and red if more abundant in the susceptible strain.

Three replicates were conducted for each pair of strains using three different BBMV preparations. At least one replicate was done reversing the cy dye labeling. The ‘fold change’ was calculated from all spots within a given charge strain from all three replicates.

2.4. Peptide Mass Fingerprinting (PMF)

Gels were stained with Sypro Ruby (Molecular Probes), destained in 10% methanol, 6% acetic acid for 30 minutes at room temperature. Stained gels were imaged and matched to cy dye images using Decyder software. Pick lists for mass spectrometry were created based on the Sypro image. Mass spectrometry was performed at the Proteomics Resource Facility at the University of Georgia on spots of interest as described by McNall and Adang (2003).

Correlative searching strategies with peptide mass fingerprint (PMF) data were used to search the Metazoan subset of NCBI using ProFound (http://prowl.rockefeller.edu/) with a confidence level of 0.1 Da.

2.5. MS/MS Sequencing

Further fragmentation of selected peptides was conducted to elucidate protein identifications. Based on the intensity of the spectra produced from initial MALDI-ToF, two peptides were selected for further analysis. *De novo* sequencing was conducted on the resulting

spectra using the *De Novo* Explorer software package (Applied Biosystems) and by personal inspection. All resulting sequences were used to search the 'other metazoa' subset of NCBI using PepFrag (prowl.rockefeller.edu/prowl/pepfragch.html) and MS Blast (dove.embl-heidelberg.de/Blast2/msblast.html).

2.6. Alkaline Phosphatase Activity Assay

Enzyme activities were measured for whole-animal preparations of BBMV (before solubilization) from cabbage-reared and diet-adapted animals as previously described (Garczynski & Adang, 1995). Enzyme assays were done in triplicate for three different BBMV preparations for each sample. Mean activities of the four BBMV (Geneva 88, NO-QAGE, LAB-P, and NO-QA) were compared using ANOVA followed by Tukey's test ($\alpha = 0.05$). Mean activities of diet-reared strains were compared to cabbage-reared strains using a t-test. A t-test was also used to compare mean activities of susceptible versus resistant strains. All data conformed to normality and equal variance assumptions.

3. Results

3.1. One-dimensional comparison of BBMV

A comparison of BBMV preparations from whole animals and dissected midguts by one-dimensional SDS-PAGE did not reveal differences between susceptible and resistant insects (Fig. 2.1). However, differences were apparent between the two types of preparation. Proteins at 250 ,130, 32, and 17 kDa were more abundant in whole-animal BBMV preparations while proteins at 90, 53, 28, and 14 kDa were more abundant in dissected midgut preparations.

3.2. Two dimensional difference gel electrophoresis (2D-DIGE)

A more detailed comparison of whole-animal and dissected midgut BBMV preparation and *Bt* susceptibility and resistance was conducted using 2D-DIGE. Whole-animal preparations showed a large number of spots (approximately 600), covering the pI spectrum, with molecular sizes ranging from 150 kDa to 10 kDa (Fig. 2.2 A, B). Numerous charge trains were seen which typically represent one protein in slightly different charge states often due to post translational modifications. However, based on the parameters in Decyder, few significant differences were detected between protein spots from susceptible and resistant animals. All differences were quantitative as there were no unique spots detected. Of these, there were no decreased spots, but 12 increased spots in the resistant strain. These increases were in a charge train at 80 kDa, a pair of spots at 70 kDa, a single spot at 23 kDa, and another isolated spot at 12 kDa. Images of dissected gut BBMV preparations had fewer spots (approximately 400), also covering the pI range, but with molecular sizes ranging from 110 kDa to 10 kDa (Fig. 2.2 C, D). In contrast to the whole-animal preparations, many differences were observed between susceptible and resistant animals. In the resistant sample, there were 45 increased spots and 58 decreased relative to the susceptible sample, representing approximately 25% of the total spots detected. One of these spots (group 2, arrows) was at the same molecular size and pI as that seen in the whole-animal preparations.

Although many statistically significant changes in protein spots were detected, four trains of altered spots were chosen for mass spectrometry analysis (Fig. 2.2 D, 1-4) based on their abundance. Selecting spots within a charge train allows replicated PMF data for a given protein and at least two spots were analyzed for each selected spot train. An additional spot that was unchanged between susceptible and resistant samples was analyzed (Fig. 2.2 D, spot 5).

3.3. PMF Analysis

MALDI-ToF analyses of selected spots resulted in PMFs that generally produced poor matches to proteins in the Metazoa subset of NCBI. Table 2.1 shows the results of these database searches. Group 1 spots matched to several different proteins with poor Z-scores and coverages. Spots from group 2 and 3 tentatively matched to intermediate filament sequences and alpha-amylase respectively. These matches were with Z-scores near or above the 90th percentile. Spots chosen from group 4 were identified as actin with good coverage and high Z-scores. Group 5 spots (the unchanged group) matched to several different protein sequences. None of these matches had high Z-scores but several did have good percent coverages. The best of these matches was to SPARC (secreted protein acidic and rich in cysteine) which is a glycoprotein involved in cell-matrix interactions.

3.4. De Novo Sequencing

To confirm the potential match of spots from group 2 with intermediate filament, two peptides from the PMF (1326.7 and 1426.7) were fragmented into partial peptide fragments by MALDI ToF-ToF. Sequences were generated for each peptide through de novo sequencing. Database searches with these peptides produced matches to glucosinolate sulphatase (GSS) from *P. xylostella* (Table 2.2). One peptide (1326.7) was homologous to ^{45}SVLTPNLDVLTR^{56} with a discrepancy of an inverse of the 'PN' amino acid pair. The other peptide (1426.7) was identical to ^{107}LISQYLQDAGYR^{118}.

Because the original identification by PMF (Table 2.1) did not match the *de novo* sequencing result, we repeated the PMF search using a larger molecular size window. Initially, a mass window of 65-90 kDa was selected based on the apparent sizes of the protein spots (80

kDa). The lower limit of the size window was changed to 55 kDa to encompass the predicted size of the GSS protein (61 kDa). This PMF search resulted in the identification of GSS with a high Z-score and good coverage (Table 2.3).

3.5. Glucosinolate Sulphatase Western Blot

Since these strains were adapted to diet at different times, the differences in the up-regulation of GSS could be related to the selection process and not to *Bt* resistance. To test this hypothesis, a related pair of cabbage-reared susceptible and resistant (LAB-P and NO-QA) *P. xylostella* was used in a western blot as a comparison to the diet-adapted strains.

Western blots of 1D-separated BBMV probed with anti-GSS antibody revealed that more GSS was present in BBMV from resistant larvae regardless of the food source (Fig. 2.3A), although the difference was less pronounced in the cabbage-reared insects as measured by densitometry (data not shown). Additionally, levels of GSS were higher in diet-adapted animals versus cabbage-fed animals (Fig. 2.3A). To examine whether or not the protein loads and general band pattern of all four samples were consistent, blots were probed with an antibody against MsAPN1 since previous studies had shown aminopeptidase levels are similar between susceptible and resistant *P. xylostella* (Luo *et al.*, 1997). A doublet at 120 kDa was present at equal intensities was detected in all samples (Fig. 2.3B). Therefore, the increases in GSS levels were positively correlated with resistance and diet-adaptation, and not due to an alteration in overall BBMV protein composition.

3.6. Alkaline Phosphatase Activity

Alkaline phosphatase is routinely used as a midgut marker. As another measure of the consistency of BBMV proteins in cabbage-reared and diet-adapted animals, the alkaline phosphatase activity of the BBMV preparations were compared (Table 2.4). Alkaline phosphatase specific activity was higher in the diet-adapted strains than in the cabbage-reared strains ($p=0.005$) and resistant animals had higher enzyme activities than susceptible animals ($p=0.034$). The four strain types (Geneva 88, NO-QAGE, LAB-P, and NO-QA) were statistically different than each other ($p<0.009$) with exception of Geneva 88 and NO-QA ($p=0.681$).

4. Discussion

In this study, BBMV proteins from *Bt* susceptible and resistant *P. xylostella* were analyzed by 2D-DIGE. Only a subset of the entire BBMV protein composition was apparent in 2D gels, however, comparisons between BBMV preparations can still be made. In this study, a comparison of proteins from whole-animal and dissected midgut preparations showed that more differences were detected between susceptible and resistant animals when dissected midguts were used. Whole-animal BBMV preparations, therefore, may not be an accurate representation of *P. xylostella* midgut brush border membrane proteins. The use of dissected midgut BBMV revealed both up- and down-regulated proteins were detected in resistant animals compared to susceptible animals. In this preliminary study, three of these altered proteins were identified by mass spectrometry: actin, glucosinolate sulphatase (GSS) and tentatively alpha-amylase.

An earlier report described a method of BBMV preparation for *P. xylostella* using whole animals rather than dissected midguts (MacIntosh *et al.*, 1994). Eschriche et al. (1995) tested

these BBMV in vesicle binding assays with Cry1Ac toxin. Calculated binding constants revealed no significant differences between whole-animal or dissected gut preparations (Escriche *et al.*, 1995). Our preparations analyzed by SDS-PAGE and 2DE clearly show differences in proteins between the two types of BBMV preparations. Perhaps more importantly, dissected midgut BBMV revealed a number of altered proteins that were undetected in whole-animal preparations (Figure 2.2). Even though one group of altered proteins was detected in the whole-animal preparations, more spots were visible on gels from these preparations when compared to dissected midgut BBMV. A simple explanation for both these observations lies in the source of the material used. By using whole animals, the resulting BBMV will consist of membrane vesicles from guts as well as other tissues. Not only is this likely to be the cause of the more robust protein pattern, but proteins from other tissues will mask differences between susceptible and resistant animals. It is therefore more informative to use dissected midgut BBMV preparations when trying to elucidate protein differences by 2DE.

Although the levels of many proteins were altered in resistant *P. xylostella*, we focused on four chains of proteins for identification by mass spectrometry. Initial searches with peptide mass fingerprint (PMF) data resulted in the identification of actin and tentative identification of alpha-amylase. A third identification, intermediate filament, was subsequently shown to be incorrect. *De novo* sequencing, MS/MS fragmentation, of two peptides from this spot demonstrated that it was GSS. Since MS/MS sequencing identified GSS, a second PMF search was done. This protein has a predicted size of 61 kDa but an apparent size of 80 kDa on gels. By using wider molecular mass parameters in PMF searches, GSS was positively identified.

This example clearly illustrates several challenges of searching protein databases with mass spectrometry data. First, if matches are made to organisms other than the one being

analyzed, identifications based on moderate Z-scores (<1) and limited percent coverage are at best, tentative identifications. Second, databases consist of protein sequence alone; a search with peptides that have post-translational modifications will not match peptides from proteins in the database based on size alone. As discussed by McNall and Adang (2003), there are web-based programs, such as FindMod (us.expasy.org/tools/findmod/), which can be used to facilitate and confirm identifications of proteins with post-translational modifications. In fact, FindMod re-confirmed the GSS identification made through analyses with mass spectrometry data. The use of these tools, complementary techniques such as western blotting or N-terminal sequencing, along with peptide sequencing through mass spectrometry can also improve reliability of identifications.

In an attempt to discover if altered midgut proteins are capable of binding to Bt toxins, Cry1Ac ligand blotting was done with BBMV prepared from dissected midguts (data not shown). Surprisingly, no binding proteins were detected in 2D separated BBMV proteins from either Bt-susceptible or –resistant insects. Because membrane proteins are typically hard to resolve by 2DE (Gygi *et al*., 2000), this may be why the 120 kDa APN was undetected. The treatment of the BBMV, protein precipitation and solubilization, may affect binding epitopes present in the 2DE resolved proteins thus rendering them incapable of binding toxin. Alternately, toxin binding proteins may simply be eliminated during these treatments. Regardless, the failure of ligand blotting makes direct links between altered proteins and Bt resistance harder to establish.

GSS is an enzyme used by *P. xylostella* to protect itself against the accumulation of toxic compounds from Brassicaceae (Ratzka *et al*., 2002). When these plants are damaged due to herbivory, a myrosinase processes glucosinolates into isothiocyanates, nitriles, and thiocyanates

which are all toxic to the insect (Li *et al.*, 2000). *P. xylostella* counters this process by using GSS to convert glucosinolates into non-toxic compounds and sulfate which inhibits myrosinase activity in the plant. However, it was not known if the up regulation of GSS in resistant *P. xylostella* was due to *Bt* resistance or a result of diet adaptation.

The *Bt* -resistant NO-QAGE strain was derived from crosses between Geneva 88, a susceptible diet-adapted strain, and NO-QA, a resistant cabbage-reared strain (Tabashnik *et al.*, 2000). A reasonable explanation for the elevated levels of GSS in the NO-QAGE resistant insects is that Geneva 88 had some basal level of the enzyme, and increased GSS levels were introduced from the NO-QA genetic background during the crosses. Perhaps the NO-QAGE animals may have higher GSS levels because regulation of GSS is linked to resistance in the NO-QA strain. Alternatively GSS activity may reflect dietary selection. NO-QAGE larvae are more recently removed from cabbage compared to Geneva 88. Perhaps insects that lacked the requirement of GSS for survival on cabbage, reduced the amount of GSS produced and used their energy sources in other ways. Since Geneva 88 was adapted to diet many years before NO-QAGE (Shelton *et al.*, 1991; Tabashnik *et al.*, 2000), these animals may have less GSS than their resistant counterpart. To examine if the difference in protein amounts is similar in cabbage-reared animals, frozen stocks of LAB-P and NO-QA (a pair of susceptible and resistant cabbage-reared strains) were used. Only whole-animal BBMV could be prepared, which is an adequate model in this case as GSS was found at increased levels in both whole-animal and diet-adapted BBMV preparations in 2D-DIGE experiments (arrows in Fig 2.2).

Contrary to expectations, BBMV from cabbage-reared animals had lower levels of GSS protein than the diet-adapted strains, and the difference in GSS levels between susceptible and resistant BBMV was the same. What the specific role of increased levels of GSS is in *Bt*

resistance is unknown, but several scenarios are possible. Previous studies have shown that the amount of glucosinolates in crucifers had no effect on *P. xylostella* herbivory (Li *et al.*, 2000), so it seems unlikely that the up regulation of GSS in diet-reared animals relates to food source. Additionally, artificial diet presumably has lower levels of glucosinolates than crucifers making it unlikely that higher levels of GSS in diet-adapted strains are a response to the food source. A more likely explanation for the high GSS levels in diet-adapted *P. xylostella* is that the insect challenged by *Bt* toxins is stressed. In this case, GSS up regulation would be a general protective response rather than a specific resistance mechanism to *Bt*.

Related to this is a second possibility in which the up-regulation of GSS is part of a stress response. *Bt* resistance in *P. interpunctella* is correlated with increased oxidative metabolism and enhancement of cellular stress responses (Candas *et al.*, 2003). In *P. xylostella*, both *Bt* resistant and diet-adapted insects have elevated GSS levels. Both phenomena are suggestive of an adaptive stress response. In *Drosophila*, the link between stress response and life-span is well-established. Selection of laboratory strains for delayed senescence also had increased tolerance to starvation, heat, and oxidative damage (Service, 1987; Rose *et al.*, 1992). In 1998, Lin et al. identified a mutation of the methuselah gene of *Drosophila* that caused a 35% increase in life span as well as enhanced global resistance to various stressors such as starvation, elevated temperatures, and paraquat, a herbicide (Lin *et al.*, 1998). The methuselah gene encodes a protein with predicted homology to a G-coupled protein receptor indicating that signal transduction pathways may play a role in successfully overcoming stress (Lin *et al.*, 1998). The GSS pathway in *P. xylostella* may be part of a similar stress response pathway.

The third potential role for overproduction of GSS is that the product of its reaction (desulfo-glucosinolates) interferes with the toxic action of Bt. Previous studies have shown that

the presence of GalNAc can inhibit toxin binding and that mutations in domain III affect binding to aminopeptidases (Jenkins *et al.*, 1999; Burton *et al.*, 1999. Domain III, as well as domain II, share structural homology to various lectins (Grochulski *et al.*, 1995). Desulfo-glucosinolates, which contain a glucose moiety, may bind to toxin preventing interactions with functional receptors in the insect midgut. The increase in GSS levels lead to an increase in these compounds which would occupy more toxin molecules. Further experiments will need to be conducted to definitively state that increased GSS protein is a mechanism for Bt resistance

The finding that the pattern of alkaline phosphatase activity among the four strains tested mimics the pattern of GSS protein expression suggests a commonality perhaps through a broad stress response. Additionally, diet-adapted animals may have shifted their production of alkaline phosphatase in order to properly digest the artificial food source. Lehane et al. (1995) demonstrated that phytophagous insects have the potential to alter digestive enzymes in response to different diets. This may explain why diet-adapted animals had higher levels of alkaline phosphatase activity than their cabbage-reared counterparts. We do not know if the pattern of alkaline phosphatase activity observed in this study is due to stress, a necessary digestive response to the diet, or a combination of both. It is also important to note that the alkaline phosphatase activities were determined for whole-animal BBMV preparations. To verify that these differences are real, the same experiments as well as western blotting should be done on BBMV prepared from dissected midguts. It would be, therefore, premature to assign the difference in alkaline phosphatase activities are true differences.

Actin was identified as a less abundant protein in *Bt* resistant P. *xylostella*. Lionetto et al. (2002) demonstrated that actin levels in the apical brush border of eel intestine were reduced in response to hypotonic stress. This type of response may be what occurs in the midgut brush

border of *Bt* resistant *P. xylostella* or this response may be more directly related to the mode-of-action of the toxin. Recent work by McNall and Adang (2003) demonstrated that in *M. sexta*, actin binds Cry1Ac toxin. They suggested that this interaction takes place downstream of initial binding at the midgut epithelium resulting in disruption of normal actin function and contributing to the deconstruction of midgut cells (McNall and Adang, 2003). Thus, decreased levels of actin would minimize interactions between toxin and actin thereby increasing the insect's resistance to the pathogenic effects of *Bt* toxins. With lower levels of actin, however, comes the cost of losing such an important structural protein. Groeters et al (1994) reported that NO-QA had reduced fecundity, egg hatch, and survival. Another study, however, demonstrated that a resistant *P. xylostella* colony from Florida had no fitness costs (Tang *et al.*, 1997). Although the simplest explanation for the findings of Groeters is that the fitness costs observed are due to *Bt* resistance, the authors did acknowledge that the differences could be due to genetic drift or inbreeding in the resistant colony (Groeters *et al.*, 1994). The role of actin in the various fitness measures is unknown.

The proteins identified in this preliminary study indicate the complexity of the resistance response to *Bt* toxin. The use of proteomics approaches, particularly comparative ones, will be invaluable to elucidating protein changes in *Bt* resistant animals that are not detected in functional assays. By developing a complete picture of protein alterations in these animals' midguts, strategies for combating field resistance to *Bt* can be developed. The lack of availability of sequenced genomes for lepidopteran pests makes identification of altered proteins challenging. However, as these data becomes available, the utility of proteomic and other high-throughput experiments will become invaluable in the elucidation of the complete mode-of-

action of *Bt* toxins. This knowledge will help maintain their utility as an important pest management tool.

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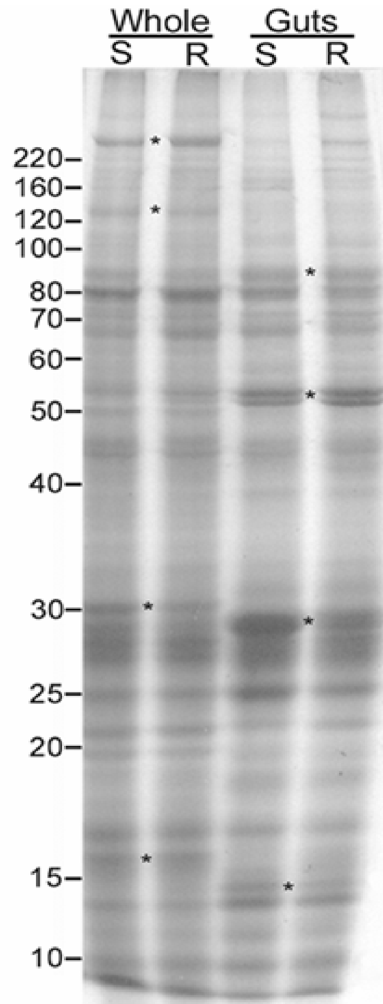

Figure 2.1. Coomassie Blue stained 10% SDS-PAGE of 20 µg of *P. xylostella* BBMV proteins. 'Whole', whole animal preparation; 'Guts', dissected gut preparation; 'S', susceptible; 'R', resistant. Asterisks indicate bands more prevalent in the marked lanes versus the unmarked lanes. Masses (kDa) of molecular weight markers (Benchmark, Invitrogen) are indicated on the left of the gel.

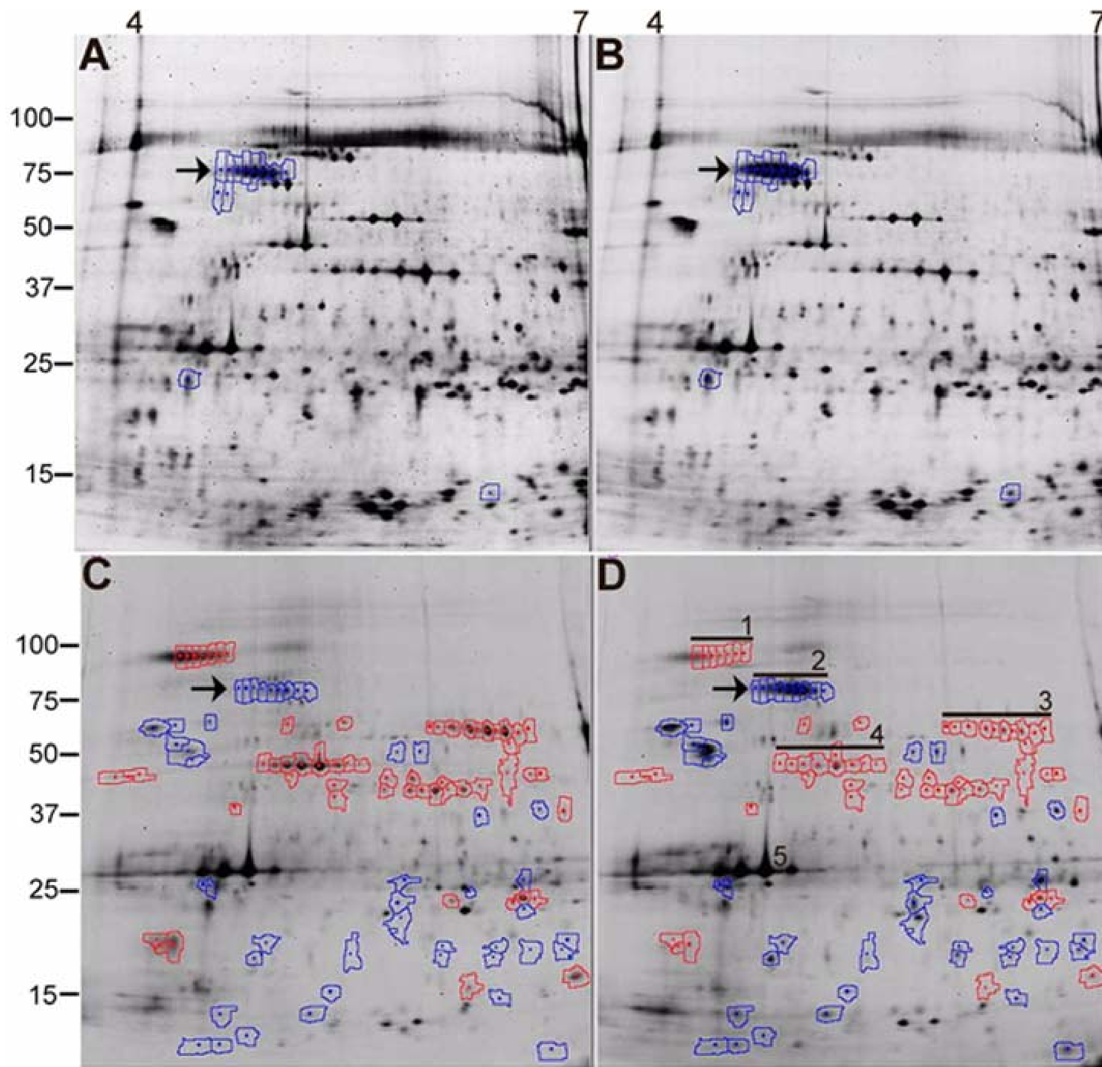

Figure 2.2. Cydye images of 2D-separated *P. xylostella* BBMV proteins (50 μg). A, Susceptible whole; B, Resistant whole; C, Susceptible guts; D, Resistant guts. Spots in blue are at higher levels in resistant samples, while spots in red are higher in susceptible. (See methods for details of quantitative comparisons). Numbers indicate groups from which spots were excised for mass spectrometry analysis. Arrows indicate the same altered charge train (GSS) in all four panels. Masses (kDa) of molecular weight markers (Precision unstained, BioRad) are on the left of the figure and pH range of isoelectric focusing are at the top.

Table 2.1. Peptide mass fingerprint results from searches of the 'Other Metazoa' subset of NCBI using ProFound.

Group[a]	Spot	Z-Score[b]	Percent Coverage	Species	Identification	Predicted Mass(kDa)/pI	Fold Change[c]
1	a	0.47	2	*Anthocidaris crassispina*	Outer arm dynein intermediate chain 1	91.96 / 4.4	-2.96
		0.39	4	*Anopheles gambiae*	ENSANGP00000016987	81.56 / 4.6	
	b	0.54	5	*A. gambiae*	ENSANGP00000016570 (calpain) [d]	89.51 / 4.8	
		0.20	2	*A. crassispina*	Outer arm dynein intermediate chain	91.96 / 4.4	
2	a	0.59	12	*Ommastrephes sloani*	Intermediate filament protein	70.15 / 5.3	4.48
		0.36	10	*H. medicinalis*	Intermediate filament gliarin	71.32 / 5.4	
	b	1.22	10	*O. sloani*	Intermediate filament protein	70.15 / 5.3	
		0.64	9	*Arenicola marina*	Fibrillar collagen	68.35 / 5.2	
3	a	1.54	7	*Drosophila rufa*	Alpha-amylase	40.86 / 6.3	-4.61
		1.06	5	*Coptotermes formosanus*	Endo-b-1,4-glucanase	49.13 / 6.2	
	b	0.46	7	*A. gambiae*	ENSANGP00000003806 (phosphomannomutase) [d]	54.70 / 5.9	
		0.34	7	*D. rufa*	Alpha-amylase	40.36 / 6.3	
		0.07	8	*Schistosoma mansoni*	Probable protein disulfide isomerase	54.93 / 6.3	
4	a	2.43	36	*A. gambiae*	agCP8921 (actin)[d]	42.81 / 5.5	-4.46
	b	2.43	38	*Limulus polyphemus*	Actin	42.22 / 5.3	
5	a	0.74	15	*Artemia franciscana*	SPARC	34.90 / 5.3	
		0.57	8	*D. ananassae*	Alpha-amylase	33.60 / 5.4	
		0.53	17	*A. gambiae*	ENSANGP00000013448	33.58 / 5.1	

[a]Group refers to the location of the charge train indicated in figure 2, panel D.
[b]Z-score is an indicator of the quality of the search result. The following is a list of Z-score and corresponding percentile: 1.282 = 90.0, 1.645 = 95.0, 2.326 = 99.0, 3.090 = 99.9. A detailed description of Z-score can be found at: http://129.85.19.192/profound/help.html
[c] Fold change represents the average of the change in all protein spots in a group for resistant BBMV vs. susceptible BBMV.
[d] If searches returned *Anopheles gambiae* sequences as a top match, these sequences were used to search NCBInr using BLAST since they are not yet annotated as to possible function. A description of homology to known proteins is reported in those cases where a potential identification could be made.

Table 2.2. Results of BLAST search using *de novo* sequences derived from MALDI-ToF ToF.

Group	Spot	Peptide Fragment	*De novo* sequence	Database Sequence Matched	Protein Identified (organism)
2	a	1326.7	SVLT**NP**LDVLTR	^{45}SVLT**PN**LDVLTR^{56}	Glucosinolate Sulphatase (*P. xylostella*)
		1426.7	LLSQYLQDAGY	^{107}LISQYLQDAGYR^{118}	Glucosinolate Sulphatase (*P. xylostella*)

Table 2.3. Peptide mass fingerprint searches using a wider molecular size window.

Group	Spot	Z-Score[a]	Coverage	Accession Number	Species	Identification	Predicted Mass(kDa)/pI
2	a	2.31	21	22450123	*P. xylostella*	Glucosinolate sulphatase	60.8 / 5.1
	b	2.30	27	22450123	*P. xylostella*	Glucosinolate sulphatase	60.8 / 5.1

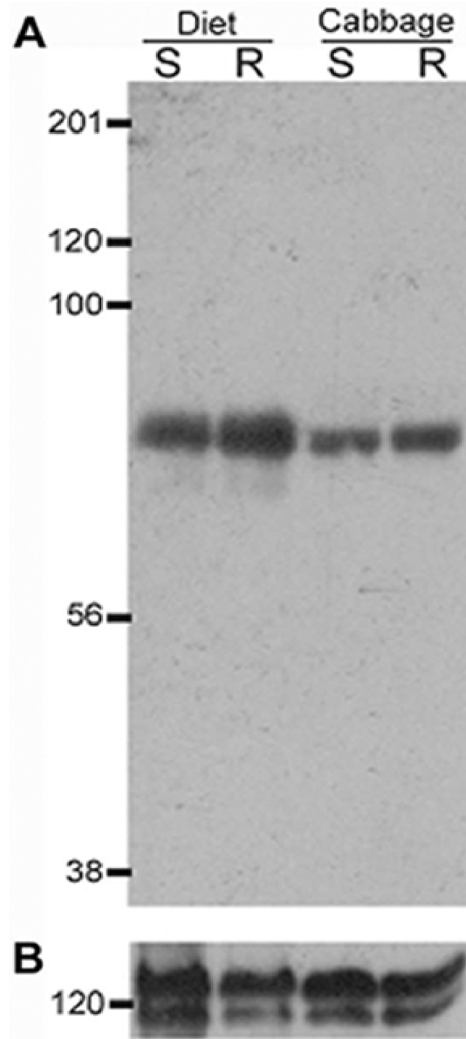

Figure 2.3. GSS western blot of cabbage-reared and diet-adapted *P. xylostella* BBMV proteins. 15 µg of BBMV protein were separated by 10% SDS-PAGE. Diet adapted: S, Geneva 88; R, NO-QAGE. Cabbage reared: S, LAB-P; R, NO-QA. A. GSS protein was detected with a rabbit antibody against *P. xylostella* GSS and a goat anti-rabbit antibody. B. Aminopeptidase protein was detected with a rabbit antibody MsAPN1 and a goat anti-rabbit antibody. Masses (kDa) of molecular weight markers (Broad prestained, BioRad) are indicated on the left of the blot.

Table 2. 4. *P. xylostella* BBMV Alkaline Phosphatase Activity.

		Activity[1] (± S.D.)[2]
Diet-Adapted		
	Geneva 88 (S)[3]	20.9 ± 2.1 a[4]
	NO-QAGE (R)	27.9 ± 0.3 b
Cabbage-Reared		
	LAB-P (S)	13.2 ± 1.0 c
	NO-QA (R)	19.4 ± 2.4 a

[1]Expressed as μmol/min/mg.
[2]S.D. - standard deviation.
[3]S denotes susceptible to Cry1Ac, R denotes resistant to Cry1Ac.
[4]Means followed by the same letter are not significantly different. (Tukey's test, α=0.05)

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSION

The research in this dissertation describes the development of proteomic techniques for analyzing insect midgut proteins. These studies are among the first 2DE analyses of insect midguts and are certainly the first that describe the further steps (including bioinformatics analyses and blotting studies) that are required to confirm mass spectrometry identifications. The use of this combination of techniques resulted in the identification of two new Cry1Ac binding proteins in *M. sexta* (actin and alkaline phosphatase) that may expand the understanding the mode-of-action of Bt toxins. The use of 2D-DIGE to compare BBMV proteins from Bt susceptible and resistant *P. xylostella* revealed that BBMV prepared from whole animals do not reveal as many differences as those prepared from dissected midguts. This finding demonstrates that whole animal BBMV may not be a proper model for studying Bt action and resistance mechanisms. Additionally, comparative proteomics revealed many alterations in protein expression levels in Bt resistant *P. xylostella* and two of these proteins were successfully identified (actin and glucosinolate sulphatase (GSS)). These proteins, as well as alkaline phosphatase, are new proteins that are implicated in Bt resistance mechanisms in *P. xylostella*. Perhaps most importantly, this work has demonstrated the value of proteomics as a method for determining broad responses to Bt toxins and for generating new avenues of study.

The evidence for actin being implicated as an important protein in Bt action was discussed in Chapters 2 and 3 of this dissertation. In *M. sexta*, Cry1Ac ligand blots of 2DE separated BBMV demonstrated that actin is a toxin binding protein. In *P. xylostella*, the BBMV from resistant larvae had lower levels of actin than their susceptible counterparts. These findings suggest that actin plays a role in the pathogenic effects of Bt toxin possibly through apoptosis. A role for apoptosis in Bt pathogenicity was observed by Loeb *et al*. (2000). It was demonstrated that Bt toxin effected an increase in apoptosis of *H. virescens* cultured midgut cells (Loeb et al.,

2000). In *M. sexta* skeletal muscle, a reduction in myosin and actin expression correlated to the time of cell death (Schwartz et al., 1993). Future studies directed at measuring the changes in expression levels of genes specifically implicated in apoptotic pathways (such as the caspase cascade (Thornberry, 1997)) will need to be measured to determine how important apoptosis may be in Bt action and whether it is part of the cause or just an effect of toxicity.

As discussed in Chapter 3, increased alkaline phosphatase activity and GSS protein levels were detected in Bt resistant *P. xylostella*. These findings may be simply correlative, or indicative of changes in cellular defense mechanisms. A 2D-DIGE experiment examining differences in levels of specific proteins (spots) between Bt-susceptible and –resistant BBMV from *P. interpunctella* revealed differences implicating increased oxidative metabolism and glutathione requirements in resistant animals (Candas et al., 2003). For example, resistant animals had increased levels of GSH transferase, cytochrome oxidase subunit I, and NADH dehydrogenase subunit V. Those researchers suggest that resistance is biphasic. First, acute responses related to strengthening midgut epithelia and enhancing cellular responses provide immediate protection. Second, adaptive changes and enhanced stress responses render the insect immune from Bt action. Perhaps in *P. xylostella* the increases in GSS and alkaline phosphatase function in some aspects of these responses. Increased GSS levels may play a role in a general immune, or stress, response, while increased levels of alkaline phosphatase activity may be developed as a specific response to Bt toxins. However, this remains speculation as none of the proteins identified in either the current or *P. interpunctella* 2D-DIGE study have been shown to be directly responsible for Bt-resistance.

An approach that should help elucidate proteins responsible for the evolution of Bt resistance is to use incremental doses of toxin to measure changes in gene expression and

corresponding protein levels. Selection pressure by sub-lethal doses of toxin may trigger alterations in gene activity that result in reduced or enhanced levels the corresponding protein. These types of changes may reveal broader responses to Bt toxins as well as implicate important proteins in Bt mode-of-action. This type of approach was recently used in *S. exigua*. Larvae were challenged with sub-lethal doses of toxins and cDNA libraries were generated (R. de Maagd et al., 2003). Microarrays were used to quantitate differences between treated and untreated samples and trends were analyzed. One of four APN genes was no longer expressed in challenged which correlates with a reduction in toxin binding (R. de Maagd et al., 2003). Additional down-regulated genes were mostly lipases involved in fatty acid metabolism (R. de Maagd et al., 2003). The researchers suggest that this finding may be related to lower energy requirements of resistant animals for protecting intact epithelial cells. Unfortunately most of the altered genes had sequences that did not match genes with known activities. This is a problem also encountered in the proteomic experiments in this dissertation. Relatively few Lepidopteran gene and protein sequences have been deposited in data bases making identification of altered genes or proteins difficult. Until the genomes for these insects are sequenced, annotated, and functions assigned to the genes, the use of these approaches will continue to be limited. The use of other models, such as mosquitoes, *Drosophila*, and *C. elegans* may be more useful for surveying and identifying proteins important in Bt mode-of-action and resistance.

An example of what can be learned by using an organism with a fully sequenced and well annotated genome comes from a series of papers from the Aroian group (Griffitts et al., 2003; Marroquin et al., 2000; Wei et al., 2003). *C. elegans* mutants were identified that confer resistance to Cry5B. Several of these genes were identified as glycosyltransferases the orthologs of which have been implicated in the production of glycosphingolipids in *Drosophila* (Griffitts et

al., 2003). A recent microarray study comparing *C. elegans* cRNA from nematodes treated with Cry5B (toxic) and Cry5A (nontoxic) found that after 8 hrs, 1200 genes were induced and 500 were reduced (Huffman and Aroian, 2003). When these nematodes were fed cadmium at a dose that kills nematodes at similar levels to Bt, only 600 genes were induced and 250 genes were reduced demonstrating the specificity of the response to Bt. One of the genes upregulated by Cry5B was identified as map kinase kinase (Huffman and Aroian, 2003). In *Drosophila*, this protein has been shown to play a role in innate immunity to bacterial pathogens. This type of study again indicates that resistance mechanisms are diverse and seem to involve a variety of signaling and protective pathways.

The selection of Bt resistant insect colonies was begun before field resistance had been detected (Ferre and Van Rie, 2002). These colonies serve as models that can be used to study the evolution of resistance and thereby devise strategies that will prolong the efficacy of Bt crops and sprays. Additionally, understanding resistance mechanisms should provide insights into the pathogenic action of Bt toxins. Although laboratory selected colonies of *P. interpunctella* and *H. virescens* have been in existence for more than a decade, relatively little is understood about Bt action in resistant larvae beyond toxin binding at the midgut epithelial surface.

Progress has been made, however, towards understanding the evolution of resistance mechanisms. In *P. interpunctella*, a deficiency in midgut proteases in Bt resistant animals has been well established (Oppert et al., 1997; Oppert et al., 1996; Oppert et al., 1994). This change affects appropriate processing of Bt toxin, thereby preventing contacts with binding molecules that mediate pathogenicity. In two other insects, however, changes in binding proteins have been revealed. Cadherin disruptions have been identified in Bt resistant populations of both *H. virescens* (Gahan et al., 2001) and *P. gossypiella* (Morin et al., 2003). The current understanding

is that these disruptions cause cadherin to be in a soluble form rather than anchored to the membrane, thus not in an appropriate position to mediate pore formation and cell death. A recent study demonstrated that in Bt resistant *H. virescens*, reduced levels of alkaline phosphatase correlated strongly to a reduction in binding and increased resistance (Jurat-Fuentes and Adang, submitted).

It is noteworthy that the mechanism(s) of Bt resistance differs between populations from the field and populations derived from laboratory selection experiments. A recent AFLP study has found no link between cadherin and Bt resistance in *P. xylostella* (David Heckel, personal communication). Additionally, a Cry1Ac binding APN is present at similar levels in both susceptible and resistant larvae (Luo et al., 1997). These results indicate that either there are other binding molecules that mediate toxicity or that Bt resistance is due to alteration in other steps in the mode-of-action. Although there has been one report of altered proteolytic processing of Bt in *P. xylostella*, this was a relatively small difference that contributes only slightly to Bt resistance (Liu et al., 2000). Overall it appears that the mechanism for Bt resistance in this insect lies downstream of binding events.

Although there is an ongoing debate as whether lipid rafts exist *in vivo* or are artifacts of detergent treatments (reviewed in Zajchowski and Robbins, 2002), several findings suggest that lipid rafts play a critical role in mediating effective Bt toxin action. Zhuang *et al*. (2002) that Cry1A toxins associate with these rafts containing APN and can form pores only when lipid rafts are intact. Additional experiments have demonstrated that cadherin mediates the interactions between APN and toxin (Zhuang et al., 2003). These interactions rely on the presence of lipid rafts which provide fluidity in plasma membranes. A recent review describes the role lipid rafts play in regulating signaling events and the means by which pathogens exploit these rafts to try to

evade host defenses (Manes et al., 2003). Glycolipid-toxin interactions have been identified (Garczynski and Adang, 2000) and diminished levels of glycolipids have been detected in Bt-resistant *P. xylostella* (Kumaraswami et al., 2001). It is possible that interactions with, or disruptions of, lipid rafts by Bt toxins can trigger apoptotic pathways or interfere with normal signaling pathways. In fact, rearrangement of the plasma membrane is required for removal of apoptotic cells by phagocytes (Williamson and Schlegel, 2002).

The research in this dissertation describes the development of proteomics as an approach to understanding insect responses to Bt toxins. The power of this technique lies in the ability to survey a large number of proteins at once, however the limitation lies in the ability to identify proteins of interest. It is important to remember that lepidopteran sequences are a limited dataset in NCBI and Swiss-Prot, and therefore mass spectrometry identifications need to be reported cautiously. Although the 2D-DIGE study of *P. xylostella* is certainly preliminary in that only a few proteins were examined and identified, it is almost equally certain that the analysis of more proteins would have been problematic resulting in few certain identifications. The fact that there are only 132 protein sequences for *P. xylostella* in NCBI is a serious limitation. When compared to other organisms that have been successfully analyzed by 2DE (e.g. 63,000 proteins described for *S. cerevisiae*), it is not surprising that protein identifications were challenging.

Although *M. sexta* also has a small representation in NCBI (~500 sequences) I was able to determine two novel Cry1Ac binding proteins in *M. sexta*: actin and alkaline phosphatase. The availability of antibodies to these proteins, as well as to GSS in *P. xylostella*, made confirming identifications with western blotting possible. Experiments designed to confirm identifications (western blots for proteomics and RT-PCR for microarrays) will continue to be a necessary step until ESTs and complete genome sequences are available for Lepidoptera.

Despite these limitations, however, the proteomic research presented in this dissertation has uncovered several new directions for future experimentation as has the microarray work in *S. exigua* and *C. elegans*. Hopefully these findings will help broaden research efforts from the focus on Bt binding proteins and toward the elucidation of intracellular toxin effects and interactions. A better understanding of both Bt mode-of-action and resistance mechanisms will help prolong the long-term usage of Bt crops and sprays for insect control.

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