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Identification of novel Cry1Ac binding proteins in midgut membranes from *Heliothis virescens* using proteomic analyses

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Abstract

Proteins such as aminopeptidases and alkaline phosphatases, both glycosyl-phosphatidyl-inositol (GPI) anchored proteins, were previously identified as Cry1Ac binding proteins in the *Heliothis virescens* midgut. To identify additional toxin binding proteins, brush border membrane vesicles from *H. virescens* larvae were treated with phosphatidyl inositol phospholipase C, and released proteins were resolved by two-dimensional electrophoresis. Protein spots selected by their ability to bind Cry1Ac were identified by MALDI-TOF mass spectrometry coupled to peptide mass fingerprinting (PMF) and database searching. As in previous studies, *H. virescens* alkaline phosphatase was identified as a Cry1Ac binding protein. V-ATP synthase subunit A and actin were identified as novel Cry1Ac binding proteins in *H. virescens*. Additional toxin-binding proteins were predicted based on MS/MS fragmentation and de novo sequencing, providing amino acid sequences that were used in database searches to identify a phosphatase and a putative protein of the cadherin superfamily as additional Cry1Ac binding proteins.

Keywords: *Bacillus thuringiensis*; 2D electrophoresis; *Heliothis virescens*; Cry1Ac; Alkaline phosphatase; Proteomics; Peptide mass fingerprinting

1. Introduction

Larvae of the lepidopteran *Heliothis virescens* (tobacco budworm) are controlled by genetically engineered cotton expressing the *Bacillus thuringiensis* (Bt) Cry1Ac toxin (Betz et al., 2000). Cry1Ac was engineered into Bt cotton due to its high potency against *H. virescens* and other Heliothine species. According to a recent model (Bravo et al., 2004), ingestion of Cry1Ac by a susceptible larva initiates a cascade of events culminating in insect mortality. The Cry1Ac protein is processed from a protoxin form by midgut proteases to a 60-kDa toxin, translocates through the peritrophic matrix to the brush border membrane, and undergoes a final proteolytic cleavage step after interaction with a cadherin receptor. Processed toxin then oligomerizes into tetramers and binds to a second receptor, such as aminopeptidase-N (APN) or alkaline phosphatase (ALP]), that is GPI-anchored to the cell membrane and localized in specific cell surface microdomains called lipid rafts (Zhuang et al., 2002; Jurat-Fuentes and Adang, 2004). Binding to this second receptor leads to insertion of the toxin tetramers, forming pores in the epithelial cell membrane that cause cell death by osmotic shock and eventual insect mortality. Additionally, it has also been proposed that binding of toxin monomers to cadherin may lead to the activation of an intracellular cascade resulting in apoptosis (Zhang et al., 2005, 2006).

H. virescens larvae have three groups (A, B and C) of Cry1 binding sites (Van Rie et al., 1989; Jurat-Fuentes and

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Adang, 2001). Cry1Ac toxin binds to all three sites, sharing site A with Cry1Aa, Cry1Ab, Cry1Fa, and Cry1Ja, and site B with Cry1Ab. The cadherin-like protein HevCaLP (Jurat-Fuentes et al., 2004) and the 170- and 130-kDa APNs (Luo et al., 1997; Oltean et al., 1999) are considered components of site A. The HevCaLP component of site A is especially critical, since genetic knock-out of the *BtR4* gene encoding this protein is associated with high levels of Bt resistance and lack of Cry1Aa binding in the YHD2 and KCBhyb strains of *H. virescens* (Gahan et al., 2001; Jurat-Fuentes et al., 2004). However, higher levels of resistance were correlated with reducing binding not only to site A, but also to sites B and C (Jurat-Fuentes et al., 2002; Jurat-Fuentes and Adang, 2004), suggesting that all binding sites have a role in toxicity. The B group of binding sites has been associated with a 130-kDa protein (Jurat-Fuentes and Adang, 2001), while the C binding sites include HvALP, a membrane-bound form of alkaline phosphatase (Jurat-Fuentes and Adang, 2004) and other proteins of less than 100-kDa in size. The recognition of multiple sites by Cry1Ac correlates positively with its high toxicity towards *H. virescens*.

Proteomic approaches based on two-dimensional (2D) gel electrophoresis and mass spectrometry have been used to discover novel Bt toxin binding proteins and elucidate changes in midgut proteins associated with Bt resistance. McNall and Adang (McNall and Adang, 2003) identified a membrane-bound form of alkaline phosphatase (mALP) and actin as novel Cry1Ac toxin-binding proteins in the brush border midgut membrane proteome of *Manduca sexta* larvae. Candas et al. (Candas et al., 2003) used differential-in-gel electrophoretic (DIGE) analysis to compare Bt susceptible and resistant larvae of *Plodia interpunctella*. These authors detected increased levels of midgut enzymes associated with oxidative metabolism and altered migration of an F_1F_o-ATPase in resistant larvae when compared to susceptible proteins on 2D gels. Candas et al. also detected reduced levels of alkaline chymotrypsin in the resistant *P. interpunctella* larvae associated with reduced capacity for protoxin activation.

The goal of this study was to identify additional Cry1Ac binding proteins in *H. virescens* BBMV using 2D gel electrophoresis and peptide mass fingerprinting (PMF). Based on results from previous proteomic analyses (McNall and Adang, 2003) and the ability of Cry1Ac to bind multiple sites on *H. virescens* BBMV (Banks et al., 2001), we hypothesized that novel binding proteins would be discovered. Since some GPI-anchored proteins bind toxin, and toxin localizes in membrane regions enriched in GPI-proteins, most of our investigation targeted GPI-protein released by phosphatidyl inositol-specific phospholipase C (PIPLC) treatment of the BBMV. Cry1Ac binding proteins were detected by probing blots of BBMV proteins separated by 2D electrophoresis with biotinylated toxin. Biotinylated Cry1Ac bound to several protein spots in 2D ligand blots, including protein spots detected by antisera against mALP. Cry1Ac-binding proteins were identified by peptide mass fingerprinting and de novo sequencing.

2. Materials and methods

2.1. Bacterial strains and toxin purification

Bt strain HD-73 producing Cry1Ac was obtained from the *Bacillus* Genetic Stock Collection (Columbus, Ohio). Cry1Ac toxin was prepared from crystals of strain HD-73 as previously described (Luo et al., 1999). Purified toxin appeared as a single band after separation by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-10% PAGE, data not shown). Cry1Ac (0.5 mg) was biotinylated by incubating a 1:30 molar ratio of toxin: EZ-Link^{TM} sulfo-NHS-LC-Biotin (Pierce) for 30 min at room temperature. Uncoupled biotin was removed by extensive dialysis at 4 °C in 20 mM Na_2CO_3 pH 9.6, 200 mM NaCl. Toxin preparations were quantified using the Bradford protein assay (Bradford 1976) with BSA as standard and stored at −80 °C until needed.

2.2. Insect rearing, midgut dissection and BBMV preparation

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

BBMV were prepared by the differential magnesium precipitation method (Wolfersberger et al., 1987), with minor modifications. Briefly, midguts were homogenized in SET buffer containing a protease inhibitor cocktail (Complete, Roche). After addition of one volume of 24 mM $MgCl_2$ 250 mM sucrose, samples were incubated on ice for 15 min before centrifugation at $2500 \times g$ for 15 min. The supernatant was collected and centrifuged at $27{,}000 \times g$ for 30 min. The resulting pellet was suspended in half the original volume of SET buffer and the centrifugation cycle repeated. The final BBMV pellet was resuspended in ice-cold PBS buffer (135 mM NaCl, 2 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.5) containing a protease inhibitor cocktail (Complete, Roche). Protein concentration was quantified as for toxin samples, and BBMV were stored at −80 °C until needed.

Aminopeptidase specific activity using leucine-ρ-nitroanilide as substrate served as an enzymatic marker for brush border membrane enrichment (Terra and Ferreira, 1994). Typical activity enrichment in the BBMV preparations was five-fold relative to the initial midgut homogenate.

2.3. Solubilization of GPI-anchored proteins

Glycosylphosphatidylinositol (GPI) anchors of BBMV proteins were digested according to (Luo et al., 1997). BBMV proteins (1 mg) in PBS buffer were incubated with 1 unit of phosphatidylinositol-specific phospholipase

C (PIPLC, Sigma) for 4 h at 37 °C with continuous mixing. After centrifugation at 15,700 × *g* for 10 min, solubilized proteins were recovered in the supernatant. To quantify percentage release of a GPI-anchored enzyme by PIPLC digestion, we assayed aminopeptidase activity using leucine-*ρ*-nitroanilide as substrate. In these assays about 29% of the aminopeptidase activity was present in the supernatant from PIPLC-treated vesicles (data not shown). Solubilized protein samples were quantified (Bradford, 1976) and used immediately for 2D sample preparation to minimize the risk of degradation.

2.4. Sample preparation and 2D electrophoresis

Proteins to be analyzed by 2D gel electrophoresis (BBMV and PIPLC-released proteins) were extracted and precipitated using the 2D Clean-Up Kit (GE Biosciences). Precipitated proteins were dissolved in solubilization buffer (5 M urea [Plus-One; GE Biosciences], 2 M thiourea [Sigma], 2% CHAPS [Plus-One, GE Biosciences] and Complete™ protease inhibitors cocktail). Proteins were forced into solution by sonication and vortexing. Samples were then centrifuged at 15,700 × *g* for 20 min to pellet insoluble proteins and cell debris. Solubilized proteins were quantified using the 2D Quant Kit (GE Biosciences).

For 2D electrophoresis, Immobiline DryStrips (GE Biosciences) were rehydrated overnight with solubilized protein (10 to 30 μg for silver staining, 30 μg for western blots) in rehydration buffer (solubilization buffer plus 0.002% bromophenol blue, 0.018 M dithiothreitol [DTT], and 0.5% ampholytes [GE Biosciences]). Strips of three different lengths (7, 11 and 18 cm) and two pH ranges (pH 3-10 NL or pH 4-7) were used. Following rehydration, the strips were subjected to isoelectric focusing using a Multiphor II unit following manufacturer's recommendations (GE Biosciences). Temperature was maintained at 20 °C throughout focusing. Focused strips were equilibrated for 15 min in equilibration buffer (6 M urea [Plus-One; GE Biosciences], 2% SDS, 30% glycerol, 0.05 M Tris [pH 8.8], 0.002% bromophenol blue) containing 1% DTT followed by a second equilibration for 15 min in equilibration buffer plus 4% iodoacetamide. For second dimension separation we used the Ettan Dalt*six* system (GE Biosciences). Equilibrated strips were overlaid on SDS-8% PAGE gels and electrophoresis run at 5 W per gel for 30 min, and then current was increased to a maximum of 15 W per gel until the dye front reached the bottom of the gel. Separated proteins were either silver stained or transferred to polyvinylidene difluoride Q (PVDF) membrane filters (Millipore).

2.5. Ligand and Western blotting

After 1D and 2D electrophoretic separation, BBMV proteins were transferred overnight to PVDF filters at 20 V constant voltage. Filters were blocked for 1 h in PBST (135 mM NaCl, 2 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.5, 0.1% Tween-20) plus 3% BSA. After blocking, all filter incubations and washes were in PBST plus 0.1% BSA.

For toxin blots, blocked filters were incubated either with biotinylated Cry1Ac (5 nM) alone, or combined with 300-fold unlabeled Cry1Ac, 1 h at room temperature. After washing, filters were incubated 1 h with a 1:50,000 dilution of streptavidin-horseradish peroxidase (HRP) conjugate (Sigma). Binding proteins were visualized using enhanced chemiluminescence (SuperSignal West Pico, Pierce) following the manufacturer's instructions.

For detection of mALP, blocked filters were probed with a 1:25,000 dilution of rabbit antisera against *Bombyx mori* mALP (a gift from Dr. Masanobu Itoh, Kyoto Institute of Technology, Japan). Goat anti-rabbit sera conjugated with either HRP or alkaline phosphatase (both from Sigma) were used as secondary antibodies. Blots probed with alkaline phosphatase conjugated antibodies were developed using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). No endogenous alkaline phosphatase activity was detected when probing blots of BBMV proteins with streptavidin-AP or exposing directly to NBT-BCIP. Blots probed with HRP conjugate were developed as above. All blots were repeated in triplicate to ensure reproducibility of results.

2.6. Mass spectrometry

BBMV proteins (200 μg) or BBMV proteins solubilized by PIPLC digestion (100 μg) were loaded onto an 18 cm, pH 4-7 Immobiline DryStrip (GE Biosciences) and separated by 2D gel electrophoresis. The gel was then stained with SyproRuby (Invitrogen, Molecular Probes) or Deep Purple (GE Biosciences) following instructions from the manufacturer and imaged using a Typhoon Scanner (GE Biosciences) with emission and excitation wavelengths at 610 and 532 nm, respectively. Quantitative analysis of spots on the gel was by Decyder software (Amersham Biosciences). Spots of interest were excised and digested with trypsin using an Ettan Spot Handling workstation (GE Biosciences). Selected gel plugs (1.4 mm^3) were treated with 140 ng of sequencing grade trypsin (Promega) in 20 mM ammonium bicarbonate at 37 °C for 2 h. Peptides were extracted by incubating the gel plugs with 50% acetonitrile, 0.1% trifluoroacetic acid for 20 min. The extract was saved and the procedure repeated on the same gel plug. The pooled extracts were then dried using a speedvac concentrator. Dried peptides were dissolved in 5 μl of 50% acetonitrile, 0.1% trifluoroacetic acid. An aliquot (1/10th of the sample volume) was spotted on a matrix assisted laser/desorption ionization (MALDI) plate using 50% saturated alpha-cyano-4-hydroxy-cinnamic acid as a matrix. MALDI-time of flight (ToF) mass spectrometry was carried out using a 4700 Proteomics Analyzer (ABI) to obtain peptide mass fingerprints. Spectra were acquired in positive reflection mode, from 900–3500 m/z, altering the laser intensity for optimal resolution. Spectral

calibration was done using trypsin autolysis peaks of m/z 1045.556 and 2211.096.

A total of seven peptides from PMFs of distinct protein spots were selected based on their MALDI-TOF spectral intensity for MS/MS fractionation. *De novo* sequencing was conducted on the resulting spectra using the *de novo* Explorer software package (Applied Biosystems) and by personal inspection.

2.7. MS data analysis

Multivariate analysis was used to confirm that similar protein spots picked from independent gels represented the same protein. Mass spectral data were analyzed in Excel using statistiXL, with masses rounded to 1 decimal place and isotopic cluster area expressed as a proportion of the total isotopic cluster area of each mass spectrum. Isotopic cluster areas for masses differing by 0.1 Da were pooled. Mass spectra were clustered using Bray and Curtis hierarchical clustering by nearest neighbor, and patterns visualized using principle components analysis. Classification was performed using linear discriminate analysis. Mass spectra from identified protein spots were used as a measure of the validity of the clustering/classification algorithm. The spots of interest from both datasets clustered together using all three multivariate analysis algorithms, strongly suggesting that these spots represented the same protein (data not shown).

PMF searches to identify proteins were performed using two databases (NCBI and Swiss-Prot) to increase the likelihood of protein identification. NCBInr searches of Metazoa were with ProFound (http://prowl.rockefeller.edu), while searches on the Swiss-Prot/TrEMBL Metazoa database were with Aldente (Tuloup et al., 2003) (http://www.expasy.org/tools/aldente). Tryptic autolysis peaks were deleted from the mass list prior to submission to the database. Mass accuracy of 0.1 Da, partial and complete methionine oxidation and cysteine carbamidomethylation, and one or no missed trypsin cleavages were factors considered in the searches. The quality of the search results was interpreted considering the observed and expected pI and molecular weight, the specific percentage coverage, the stringency of the search conditions, and the probability of obtaining the same result in a random match (Z-score for ProFound, p Value for Aldente). The percentage of coverage indicates the ratio of the portion of protein sequence covered by matched peptides to the whole length of protein sequence. The Z-score calculated by the ProFound database search engine provides an estimation of the probability that the match is not random. Z-score values of 2.32 or higher indicate the search results are at the 99% percentile, or that there are about 1% of random matches that could yield higher Z scores than this search. The p Value calculated as probability estimation by the Aldente search engine represents the probability of finding, for a given PMF, a protein match with the same score value in a random protein database. Protein scores with lower p Values or higher Z-scores represent better matches. When PMFs matched to predicted or non-arthropod proteins with high probability, we performed BLAST searches on the Arthropoda subset of NCBInr using the matched sequence as query to identify potential homologues.

Amino acid sequences obtained from de novo sequencing were used to search the metazoan subset of Swiss-Prot using SPIDER (http://bif.csd.uwo.ca/spider/) and of NCBInr using the MS BLAST (http://dove.embl-heidelberg.de/Blast2/msblast.html) software. The SPIDER program is based on an algorithm to match sequence tags with errors to database sequences to identify peptides from de novo generated sequences (Han et al., 2005), while the MS BLAST program is designed to identify proteins whose sequence does not exist in the databases (Shevchenko et al., 2001). Due to the possibility the specific target sequence was not in the databases, we used non-gapped homology matches as query type with SPIDER, with a mass tolerance of 0.3 Da. The hit scores provided by the search engines were used to interpret the quality of the match.

3. Results

3.1. Detection of Heliothis virescens BBMV proteins separated by 1D and 2D gel electrophoresis

The protein complexity of the midgut brush border membrane of *H. virescens* larvae was compared by 1D and 2D gel electrophoresis. BBMV proteins separated by 1D electrophoresis (Fig. 1A) were detected as a pattern of discrete bands spanning from 170- to less than 20-kDa in size against a continuous background of less abundant proteins. When BBMV proteins were separated by isoelectric focusing with a pH 3-10 (non linear) range, more than 200 protein spots with a size range similar to 1D separations were detected (Fig. 1B), the exception being the absence of proteins larger than 120-kDa on 2D gels. Some proteins were localized in a chain of spots migrating at the same molecular size, representing either distinct proteins, or, more likely, a series of post-translational modifications of the same protein. Most of the resolved protein spots had pI values between pH 4 and 7, with only a few protein spots localized in more acidic or basic pH ranges. Accordingly, we used strips with a narrower pH range (pH 4-7) to increase resolution of the protein spots on gels (Fig. 1C).

3.2. Detection of Cry1Ac binding proteins and HvALP on blots of BBMV proteins

Cry1Ac-binding proteins in BBMV were detected by probing blots of 1D and 2D gels with biotinylated toxin. This approach provides a direct method for detecting Cry1Ac toxin-binding proteins, with the caveat that BBMV proteins on the blot are denatured. On the 1D blot (Fig. 2A), Cry1Ac bound to proteins of 170-, 120-, 100-, 110-, 65-, and

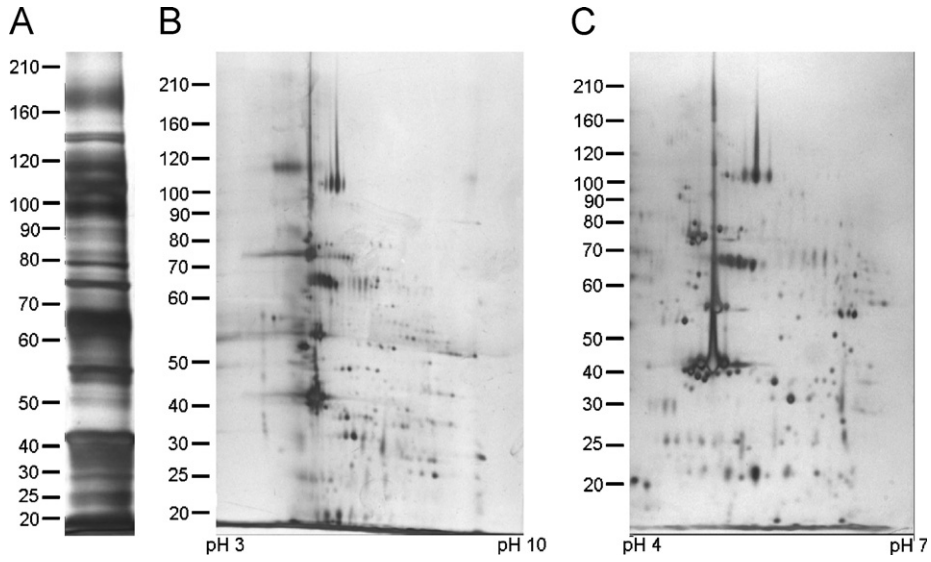

Fig. 1. 1D and 2D polyacrylamide gel separations of *H. virescens* BBMV proteins. Positions of molecular size markers (kDa) are indicated on the side of each gel and pH range using for the isoelectric focusing is indicated at the bottom.

45-kDa among a background of other minor toxin binding bands. On the 2D blot, Cry1Ac toxin mostly bound to two chains of protein spots at 65- and 45-kDa and individual spots of 180-, 100-, 70-, and 55-kDa (Fig. 2B). As noted in the silver stained gels, the most apparent difference between blots of 1D and 2D gels was the decreased detection of proteins larger than 120-kDa in size in the 2D blots.

Based on the molecular sizes and pI values reported for insect midgut mALP (Okada et al., 1989; Takesue et al., 1989; McNall and Adang, 2003), we predicted that the Cry1Ac-binding spots at 65-kDa were forms of HvALP. To test this possibility, 2D blots of BBMV proteins were probed with anti-mALP sera (Azuma and Eguchi, 1989). As previously reported (Jurat-Fuentes and Adang, 2004), this antisera detected a single protein band of about 65-kDa for HvALP in 1D blots (Fig. 2C). In comparison, in 2D blots a chain of protein spots of 65-kDa was detected (Fig. 2D). These HvALP spots co-localized with Cry1Ac-binding spots in ligand blots. This observation was confirmed by probing a 2D blot of BBMV proteins first with biotinylated Cry1Ac, developing the blot with enhanced chemiluminescence, and then reprobing the same blot with anti-ALP serum and developing the blot using colorimetric substrates (data not shown).

We hypothesized the toxin-binding protein spots at 45-kDa to be forms of actin based on previous reports of actin from *M. sexta* BBMV binding to Cry1Ac toxin (McNall and Adang, 2003). Antisera against actin detected protein bands of 55- and 45-kDa in a 1D blot of BBMV proteins (Fig. 2E). On the 2D blots of the same BBMV proteins, actin was detected as two protein spots of the same size as the 1D bands. Both of these spots were also observed in the Cry1Ac ligand blots.

3.3. Identification of Cry1Ac binding proteins by peptide mass fingerprinting (PMF)

Based on their Cry1Ac-binding attributes, we selected four protein spots from the BBMV proteome for protein identification using PMF analyses (Fig. 2G). These spots were picked, digested with trypsin and the resulting peptides analyzed by MALDI-TOF MS. ProFound and Aldente software packages were used to search the NCBInr and Swiss-Prot metazoan databases, respectively. The results from these searches are shown in Table 1.

Cry1Ac-binding protein spot 1 matched to H^+ATPase (V-ATPase) subunit A from *M. sexta* with a high Z probability score (Z score 2.34 indicates >99.9% probability of an accurate match) and low *p* Value. The expected pI for this matched protein agreed with the position observed for spot 1 in the 2D gels, yet the expected molecular size (68 kDa) was slightly smaller than the observed size of spot 1 (about 72 kDa). This discrepancy is probably due to interspecific variations between V-ATPase subunit A homologues from *M. sexta* and *H. virescens*.

Based on previous reports (McNall and Adang, 2003), Cry1Ac binding (Fig. 2B), and recognition by anti-mALP (Fig. 2D) or anti-actin sera (Fig. 2F), we anticipated spots 2 and 3 to match to alkaline phosphatase and spot 4 to match actin proteins. Spots 2 and 3 matched to the membrane-bound alkaline phosphatase from *B. mori* with very high probability (*Z*-score 2.32) using Profound, yet no significant matches were obtained with the Aldente program. To confirm this identification and to validate subsequent MS/MS analyses, we selected a common peptide (1128.58 Da) from the PMF of spots 2 and 3 and performed MS/MS and *de novo* sequencing. Searches with both the SPIDER and MS BLAST programs using the

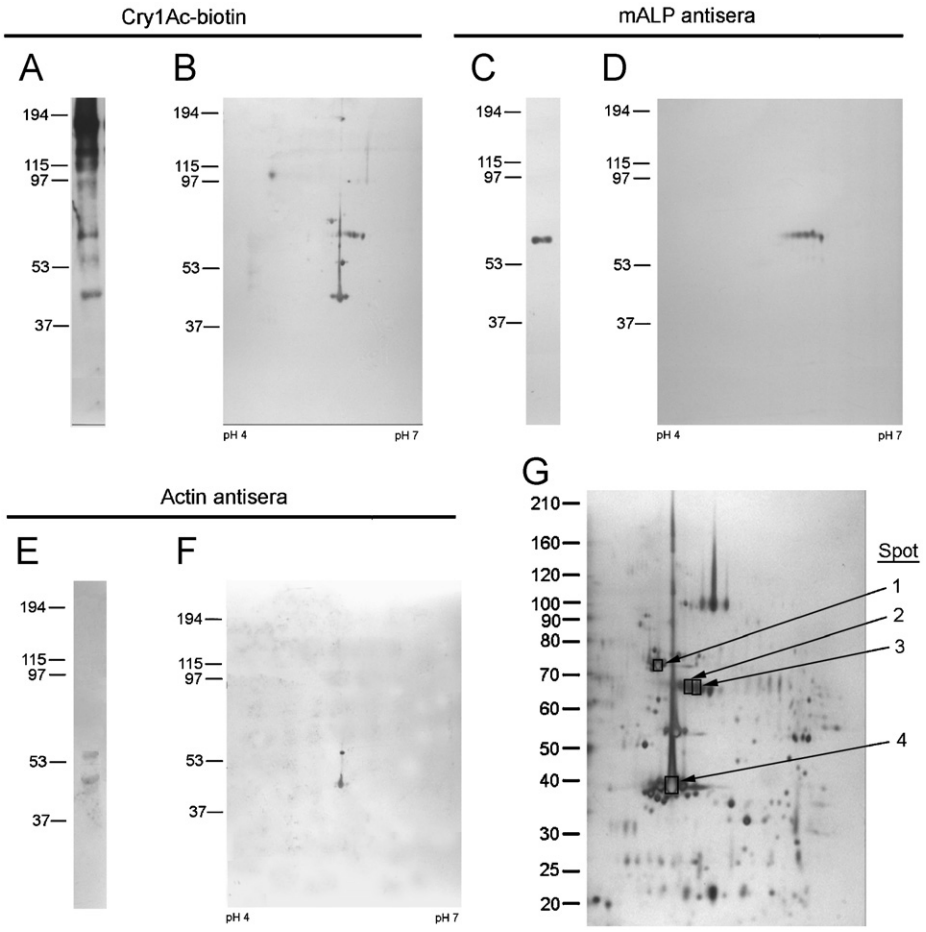

Fig. 2. Detection of Cry1Ac binding proteins (A and B), HvALP (C and D) and actin (E and F) in blots of 1D (A, C and E) and 2D (B, D and F) resolved *H. virescens* BBMV proteins. Filters were probed with 5 nM of biotinylated Cry1Ac (A and B), antisera against *B. mori* membrane ALP (C and D), or sera against chicken actin (E and F). Panel G is a representative silver stained 2D gel showing the position of the protein spots selected for PMF analysis.

generated sequence (GFFLFVENR) as query returned matches to the ^{347}GFFLFVEGG^{355} peptide from the membrane-bound form of alkaline phosphatase from *B. mori* (accession number 15128500), which is also present in the soluble form of alkaline phosphatase from the same organism (^{271}GFFLFVEGG^{277}, accession number 74764705). Furthermore, additional alkaline phosphatases from diverse organisms also contained the GFFLFVE peptide, suggesting that this specific fragment is highly conserved between alkaline phosphatases.

The PMF of spot 4 matched to a predicted actin protein from *Anopheles gambiae* (accession number 31210881) with very high probability (*Z*-score 2.39). BLAST searches of the NCBInr Arthropoda database with this *A. gambiae* protein returned a high probability match (probability of random hit 0.0) to *H. virescens* actin (accession number 14010639). To confirm this identification, an additional database search using the Aldente program was performed, obtaining high probability matches to the protein identified by Profound, and to midgut actin from *B. mori* (*p* Value 4.3e-14, accession number 113216) and *H. virescens* (*p* Value 5.2e-11, accession number 14010639).

3.4. Analyses of GPI-anchored subproteome

Identification of protein spots using the whole BBMV proteome, as described above, proved to be difficult for less abundant proteins. Since both HvALP and APNs are GPI-anchored BBMV proteins and bind Cry1Ac (Luo et al., 1997; Banks et al., 2001; Jurat-Fuentes and Adang, 2004), we investigated the sub-proteome of GPI-anchored proteins released from BBMV by PIPLC digestion. Numerous protein spots ranging in size from 120- to 20-kDa were observed in the PIPLC-released protein sample when analyzed by 2D electrophoresis (Fig. 3A). Several individual spots and the chain of protein spots at 42-kDa previously identified as actin were only observed in the BBMV pellet after PIPLC treatment (Fig. 3B). This result was expected, as actin is a cytosolic protein that is not solubilized by PIPLC digestion. Interestingly, some of the PIPLC-solubilized protein spots also were solubilized by incubation with buffer alone (Fig. 3C), suggesting the presence of endogenous PIPLC activity or proteins loosely attached to the brush border membrane.

Cry1Ac binding proteins in the PIPLC-released fraction were detected by probing blots of 2D gels with biotinylated

Table 1
Results from peptide mass fingerprint database searches using the Profound and Aldente programs and deduced amino acid sequences for proteins identified using tandem MS/MS and de novo sequencing

Spot #	Observed mass (kDa)	Predicted mass (kDa)	Accession number	Species	Top ranking match	Analysis	% coverage[a]	Z-score[b]	pValue[c]	Deduced sequence
1	72	68	11062	*M. sexta*	H (+) ATPase subunit A	MS (PMF)	53	2.34	1.3e–17	
2	65	59	15128500	*B. mori*	Membrane-bound ALP	MS (PMF)	10	1.19		GFFLFVENR
			15128500	*B. mori*	Membrane-bound ALP	MS/MS sequencing				
3	65	59	15128500	*B. mori*	Membrane-bound ALP	MS (PMF)	8	2.32		
4	42	42.8	31210881	*A. gamhiae*	Actin	MS (PMF)	42	2.39	6.2e-15	
		42	113216	*B mori*	Actin		39	—	4.3e-14	
5	100	101.8	1345364	*H. sapiens*	Desmocollin	MS (PMF)	6	2.43	6.8e-4	
6	100	96.8	17506361	*C. elegans*	Putative Protein 1J65	MS (PMF)	12	1.45	—	
		101 8	13435364	*H. sapiens*	Desmocollin		11	—	2.4e-3	
7	100	101 8	13435364	*H. sapiens*	Desmocollin	MS (PMF)	6	2.43	—	
		101	54035735	*M. musculus*	Afadin Af-6		9	—	1e-2	
8	68	66.7	7495764	*C. elegans*	Hypothetical ser/thr Kinase	MS (PMF)	17	1.29	—	
		63	14916956	*H. sapiens*	ATP-dependent Clp protease		27	—	6.2e-5	
		59	66523631	*A. mellifera*	Similarto CG16771-PA isoforrn 1	MS/MS sequencing				THVATPSP-LYAHSASR
		59	15128500	*B. mori*	Membrane-bound ALP					
9	65	64.9	4589606	*H. sapiens*	KIAA0981 protein	MS (PMF)	7	0.51	—	
		63	14916956	*H. sapiens*	ATP-dependent Clp protease		23	—	7e-6	
10	68	64.9	4589606	*H. sapiens*	KIAA0981 protein	MS (PMF)	7	1.57	—	
		63	14916956	*H. sapiens*	ATP-dependent Clp protease		15	—	1.8e-3	
11	66	64.9	4589606	*H. sapiens*	KIAA0981 protein	MS (PMF)	7	0.36	—	
			14916956	*H. sapiens*	ATP-dependent Clp protease		25	—	9.9e-6	
12	68	64.9	4589606	*H. sapiens*	KIAA0981 protein	MS (PMF)	5	0.22	—	
		63	14916956	*H. sapiens*	ATP-dependent Clp protease		18	—	2e-3	
13	68	64.9	4589606	*H. sapiens*	KIAA0981 protein	MS (PMF)	5	0.93		
		63	14916956	*H. sapiens*	ATP-dependent Clp protease		13	—	1.8e-3	

For the Profound searches of the NCBlnr meiazoan database, the *Z*-score for the best match is shown. pValues correspond to the best matches obtained with Aldente searches of the Swiss-Prot metazoan database.

[a]Percentage coverage is defined as the ratio of the length of the protein sequence covered by the matched peptides to the whole protein sequence.

[b]*Z*-score is a probability indicator of the quality of the result match in the Profound software and it is the defined as the distance to the population mean in a unit of standard deviation The following is a list of *Z*-score and corresponding percentile: 1.282 = 90.0, 1 645 = 95.0, 2.362 = 99 0

[c]pValue is a probability indicator of the quality of the match in the Aldente program, and it is defined as the probability that a protein score would assume a value greater that or equal to the observed value strictly by chance.

Cry1Ac (Fig. 4A). When 300-fold excess unlabelled Cry1Ac was included in the 2D ligand blots, no binding of biotinylated Cry1Ac was detected (Fig. 4B), demonstrating specificity of toxin binding. In agreement with Fig. 2A, Cry1Ac bound to a chain of protein spots migrating at 65-kDa (Fig. 4A) that was detected by anti-mALP sera (Fig. 4C). Cry1Ac also bound to two additional chains of protein spots in the 65- to 70-kDa size range. One of these chains had a more basic pI than the mALP spots, while the other chain resolved to a more acidic region of the strip. In contrast to the protein patterns observed in the whole BBMV proteome, clusters of Cry1Ac-binding protein spots of about 115- and 97-kDa in size were detected in the PIPLC-released BBMV proteome. Confirmation of the Cry1Ac-binding ability of all these spots was obtained by detecting matching spot patterns in blots probed with Cry1Ac and antisera against the toxin instead of biotinylated toxin (data not shown). These proteins were similar in mass to previously described Cry1Ac-binding APNs (Gill et al., 1995; Oltean et al., 1999; Banks et al., 2001). However, antisera against the 120-kDa APN from *M. sexta*, which cross-reacts with *H. virescens* APNs (Luo et al., 1997), did not detect any protein spots on 2D gel blots of PIPLC-released BBMV proteins (data not shown).

3.5. Identification of Cry1Ac-binding proteins from the PIPLC-released subproteome

We identified Cry1Ac-binding proteins released from BBMV by PIPLC using PMF analyses, and in some cases

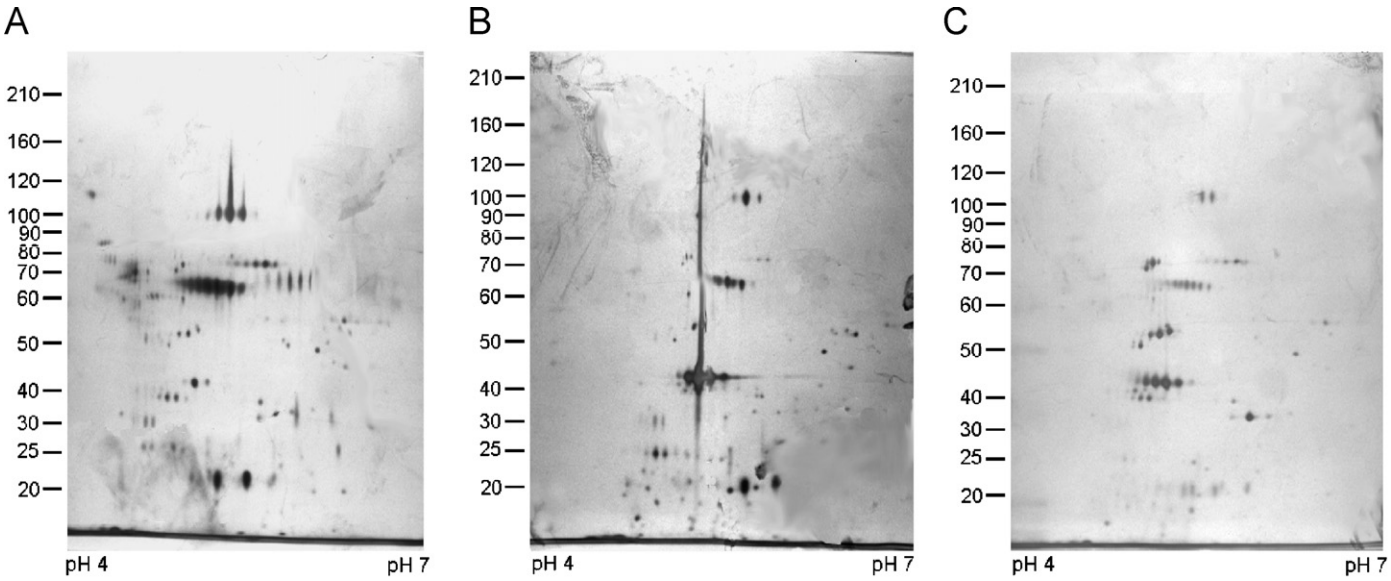

Fig. 3. Solubilization of *H. virescens* BBMV proteins by PIPLC digestion. BBMV proteins solubilized (A) or unsolubilized (B) after PIPLC treatment were separated by 2D electrophoresis. Solubilized BBMV proteins after incubation in buffer without PIPLC (C) are also included for comparison. Positions of molecular size markers (kDa) are indicated on the side of each gel.

MS/MS-based *de novo* sequencing. Only proteins spots that were detected as Cry1Ac-binding proteins using both antisera against the toxin and biotinylated Cry1Ac were selected for identification. Unfortunately, some of the Cry1Ac-binding protein spots present in chains at 115-kDa and in the 65–70-kDa were not stained by SyproRuby or Deep Purple stains, and these proteins were excluded from our PMF analysis. The nine protein spots that were detected and selected for identification are shown in Fig. 5. The results from the database searches are presented in Table 1.

Database searches with peptides derived from spots 5, 6 and 7 returned matches of distinct probability to human desmocollin (accession number 13435364) and mouse afadin (accession number 5852977) in the Swiss-Prot database. Desmocollins are proteins that belong to the cadherin superfamily and are involved in cell-cell adhesion and stratification (Mechanic et al., 1991). Similarly, afadin participates in cell-cell adhesion junctions in polarized epithelia, together with the cadherin-catenin system (Ikeda et al., 1999). Searches of the NCBInr database with the same PMFs using the Profound program, returned high probability matches to human desmocollin for spots 5 and 7 (*Z*-score 2.43), while spot 6 matched a predicted protein from *Caenorhabditis elegans*. We were unsuccessful in finding homologues of this predicted protein in the NCBInr Arthropoda database. Additional low-probability matches to alternative proteins of the cadherin superfamily were observed when searching both the NCBInr and the Swiss-Prot (subset Metazoa) databases. To validate these putative identifications, we performed MS/MS and *de novo* sequencing on 4 selected peptides common to all spots. Unfortunately, the PMF peaks matched by the top ranking hits in the PMF database searches could not be used for MS/MS fractionation due to low spectral intensity. NCBInr database searches with de novo generated sequences from the 4 selected peptides (954.507 Da: YNLE MLR; 1644.8 Da: EGFLSANSPY[404]R; 1843.99 Da: LS[422] TSLPTVLHTP; 1949.91 Da: [492]EPYNLAFDSFVR) using the MS BLAST program did not return significant match scores. Additional Swiss-Prot database searches with the SPIDER program returned matches to a cell division control protein from yeast (accession number 1351679), a phosphoinositide kinase from mouse (accession number 6685475) and hemocyanin from octopus (accession number 6685487). However, the molecular mass of all these proteins was notably larger than the mass observed for spots 5, 6, or 7 in our 2D gels. Database queries with alternative programs (Phenyx platform, http://www.phenyx-ms.com) did not return any matches.

Multivariate analysis of the PMF spectra from spots 8 to 13, which had molecular sizes between 65- to 70-kDa and more alkaline pI than spots 2 and 3, were used to confirm that they represented the same protein (data not shown). In both Swiss-Prot and NCBInr metazoan database searches with these PMF patterns, we obtained moderate to low probability matches to human proteins and a protein from *C. elegans* (Table 1). Despite the low probability scores for the matches, noticeably all three matched proteins contained phosphatase domains. To obtain further information on the identity of these spots, we performed *de novo* sequencing on peptide 1694.86 Da, which, although it did not match any of the peptides matched in the PMF searches, was common to all spots. In searches of the NCBInr database using the BLAST program, the *de novo* generated sequence from this peak (THVATPSPLYAHSASR) returned a statistically

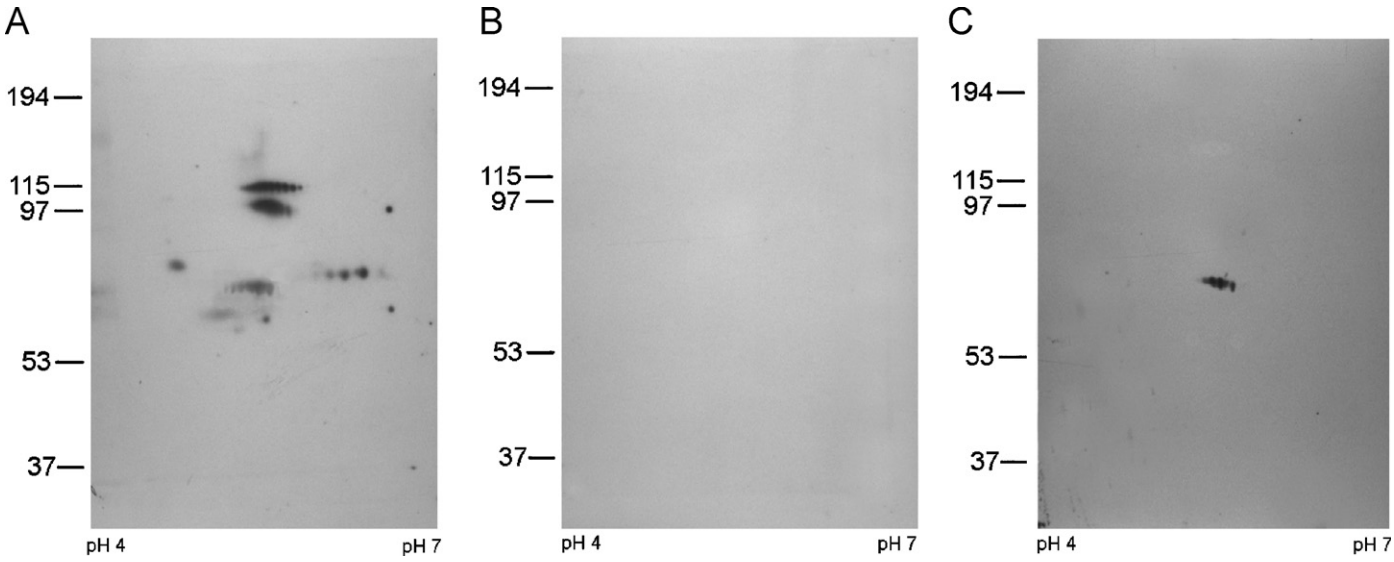

Fig. 4. Ligand (A and B) and immunoblots (C) of PIPLC-solubilized *H. virescens* BBMV proteins. PIPLC-solubilized proteins were resolved by 2D electrophoresis and transferred to PVDF filters, and were probed with 5 nM of biotinylated Cry1Ac alone (A) or in the presence of 300-fold excess unlabeled Cry1Ac (B), or with antisera against *B. mori* mALP (C). Positions of molecular size markers are indicated on the side of each gel.

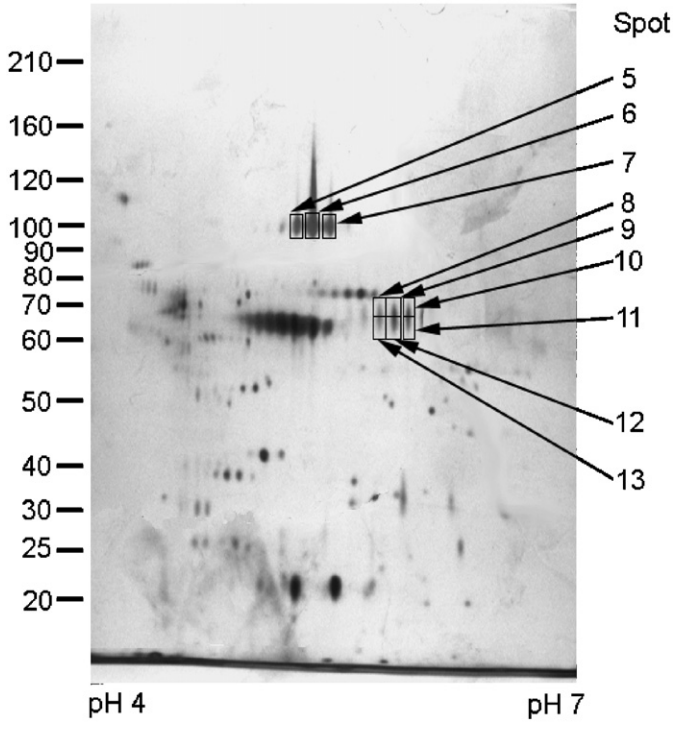

Fig. 5. Representative silver stained gel of protein spots selected for PMF analysis from the PIPLC-solubilized BBMV subproteome. Based on their Cry1Ac-binding properties, nine protein spots were selected for mass spectrometric analyses. Molecular weights of markers (kDa), pI and spot pattern were used to correlate spots on ligand and western blots with those on a Deep Purple-stained gel. Selected spots were numbered 5 through 13 to consider the four spots previously picked from the whole BBMV proteome.

significant match having 81% amino acid identify to the ^{166}TH-ATPAPLAHSANR^{180} peptide of an alkaline phosphatase isozyme from honey bee (accession number 394009). This identification was confirmed when searching the SwissProt database using the SPIDER software.

We used the LALIGN program (http://www.ch.embnet.org/software/LALIGN_form.html) to perform local sequence alignments between the de novo-generated sequence and alkaline phosphatases from *B. mori*. The alignments identified peptides in both the soluble (^{117}THASPAGAYAHTADR^{131}) and the membrane bound (^{192}THASPAGTFAKVANR^{206}) forms as having 40% sequence identity with the de novo-generated peptide.

4. Discussion

We described the identification of Cry1Ac-binding proteins in the BBMV proteome and the GPI-anchored brush border membrane sub-proteome of *H. virescens* using 2D electrophoresis and mass spectrometry. Through this study, several previously reported and novel Cry1Ac-binding proteins were detected and identified. In several cases, the limited number of lepidopteran sequences in databases available for PMF searches resulted in low coverage and probability scores or no significant matches. Database searches with *de novo* generated sequences for selected peptides allowed us to overcome some of these limitations and identify protein homologues for the selected proteins. Following this combined PMF and *de novo* sequencing approach we were able to identify V-ATPase subunit A, actin, membrane-bound alkaline phosphatase, a novel alkaline phosphatase, and a desmocollin-like protein as Cry1Ac binding proteins.

To our knowledge, this is the first direct reported evidence of interactions between Cry1Ac and vacuolar ATPase subunits. We detected and identified the A subunit of the midgut V-ATPase as an intracellular

Cry1Ac-binding protein. The V-ATPase enzyme is localized to the apical membrane of goblet cells and represents the primary energy source for secretion and absorption by serving as an H^+/K^+ electrogenic transporter across the insect midgut epithelium (Wieczorek et al., 1999). The different V-ATPase subunits have specific roles in the assembly, regulation and catalytic activity of the ATPase, which is reflected in the low degree of sequence conservation among distinct subunits. Subunit A is part of the peripheral catalytic V_1 complex of vacuolar ATPase and is essential for assembly and catalytic function (reviewed in Wieczorek et al., 2000). Previous 2D-DIGE studies on Bt susceptible and resistant larvae of *P. interpunctella* showed increased levels of V-ATPase in resistant insects (Candas et al., 2003). It was suggested that this increase would result in an elevated energetic midgut state that facilitates resistance.

Even though actin was previously detected as a Cry1Ac-binding protein in *M. sexta* BBMV (McNall and Adang, 2003), this is the first report of this protein as Cry1Ac-binding protein in the BBMV proteome from *H. virescens*. It is difficult to explain how Cry1Ac would interact with a cytosolic subunit of the V-ATPase or with the actin cytoskeleton. However, recent reports suggest the possibility that Cry toxins may interact with intracellular proteins during key steps of their mode of action (Griffitts et al., 2003). There is also reported evidence for the hypothetical activation of intracellular apoptotic pathways by Cry1Ac toxin after binding to cadherin (Zhang et al., 2005). In *D. melanogaster*, the actin cytoskeleton interacts with the cytosolic domain of cadherin proteins, controlling cellular division and cell-cell interactions in epithelia (Woods et al., 1997; Shimada et al., 2001). Similar interactions between V-ATPase subunits and actin filaments regulate reversible dissociation of the enzyme complex under starvation or molting conditions (Vitavska et al., 2003, 2005). Although speculative, it is possible that toxin binding to cadherin and insertion on the cell membrane may facilitate interactions between toxin and cytosolic proteins. It is also likely that Cry1Ac binding to V-ATPase interferes with the H^+/K^+ transport and hence destabilizes both the ionic and pH balance as observed in the toxin mode of action (Knowles 1994). Interestingly, Cry1Ac also inhibits the mammalian (NA,K)-ATPase (English and Cantley, 1986) and other phosphatases (English and Readdy, 1989; Sangadala et al., 1994). Accordingly, increased levels of V-ATPase in resistant insects, as previously detected (Candas et al., 2003), would help to overcome the toxic effect of Cry1Ac binding on V-ATPase function.

As we attempted identification of additional protein spots by PMF database searching, we encountered a problem with insufficient material for complete mass spectra. Increasing protein loads to overcome this problem resulted in overlapping spots and no significant matches in PMF searches. Since Cry1Ac-binding alkaline phosphatases (ALPs) and N-aminopeptidases (APNs) in *H. virescens* are GPI-anchored proteins (Luo et al., 1997; Oltean et al., 1999; Jurat-Fuentes and Adang, 2004), we decided to concentrate our efforts on the GPI-anchored BBMV proteome released by PIPLC digestion. Solubilization of GPI-anchored proteins eliminated some of the most prominent protein chain spots, facilitating spot picking to obtain PMFs. A caveat to this approach is that not all GPI-anchored proteins are susceptible to PIPLC cleavage (Luo et al., 1997), and that non-GPI anchored Cry1Ac receptors, like the cadherin HevCaLP, may be excluded from the analysis. Additionally, the assay was not restrictive for some non-GPI anchored proteins that were solubilized by incubation in buffer alone. For example, it was unexpected to observe the presence of V-ATPase subunits and a potential cadherin-like protein in the PIPLC-solubilized BBMV sample. Detection of these non-GPI anchored proteins in the PIPLC-solubilized subproteome suggests that some proteins are loosely attached to the BBMV surface or the existence of PIPLC activity associated with these vesicles.

According to the model of (Bravo et al., 2004), GPI-anchored proteins that localize to lipid rafts, such as APNs and ALPs (Zhuang et al., 2002; Jurat-Fuentes and Adang, 2004), bind toxin oligomers and promote toxin insertion into the membrane. There is direct evidence that Cry1Ac binds ALPs in blots (McNall and Adang, 2003) and in native state (English and Readdy, 1989; Sangadala et al., 1994) and that they can be functional Cry toxin receptors (Fernandez et al., 2006). In *B. mori*, membrane-bound (mALP) and soluble (sALP) forms of alkaline phosphatase have been described (Okada et al., 1989). When representative spots that bound Cry1Ac and mALP antisera were picked for PMF analysis, high probability matches to the membrane-bound form of *B. mori* ALP in the databases were obtained. This identification was confirmed by de novo sequencing of the peptide GFFLFVE, which is part of the alkaline phosphatase fingerprint in the PRINTS data bank of conserved protein family fingerprints (http://umber.sbs.man.ac.uk/dbbrowser/sprint/). According to the Swiss-Prot sequence annotation, this peptide is present in the mature form of the protein and is predicted to contain a metal ligand binding site important for enzymatic activity, which may explain the observed high level of conservation for this sequence. Based on recognition by mALP antisera and Cry1Ac binding, these ALP spots are synonymous with HvALP (Jurat-Fuentes and Adang, 2004). Interestingly, even though sera against sALP does not recognize mALP forms in *B. mori* (Azuma et al., 1991), both mALP and sALP antisera recognized the same HvALP protein spots in 2D blots of *H. virescens* BBMV (data not shown). This observation suggests the existence of regions in HvALP that are conserved in both sALP and mALP forms from *B. mori*. The detected chain-like distribution of HvALP spots suggest that all the individual spots within the HvALP chain probably represent post-translational modifications of the same protein.

The Cry1Ac spots observed in the most basic region of the PIPLC-digested subproteome gels were identified as a

novel form of alkaline phosphatase based on *de novo* sequencing and database searches. Searches using the PMF from these spots returned matches to proteins containing kinase and phosphatase activity regions. Accordingly, homology searches with a *de novo* generated peptide (THVATPSPLYAHSASR) from one of the peptides common to all these spots returned high identity matches to an alkaline phosphatase from honey bee . It is uncertain whether this phosphatase is soluble or membrane bound. Analysis of the honey bee phosphatase by the DGPI program identified a potential GPI anchor attachment site near the C-terminus, but did not predict the signal peptide required of a GPI-anchored protein. Peptide sequences with lower identity to this peptide were also found in both soluble and membrane-bound forms of ALP from *B. mori*. In agreement with this low sequence identity and in contrast to the HvALP spots, this second group of ALP spots did not cross-react with mALP or sALP antisera (Jurat-Fuentes, unpublished data), suggesting that they represent a novel phosphatase. Preliminary lectin blotting suggests that these protein spots are glycosylated (Jurat-Fuentes, unpublished data), a modification characteristic of membrane proteins. A vacuolar-type ATPase activity distinct from the goblet cell V-ATPase was previously detected in BBMV from *B. mori* (Minami et al., 1991) and *Galleria mellonella* (Bandani et al., 2001). Further research is needed to accurately identify and characterize this putative phosphatase and to establish its role as Cry1Ac binding protein and potential receptor.

Because aminopeptidases (APNs) are abundant Cry1Ac-binding proteins in *H. virescens* BBMV, we expected to detect these GPI-anchored proteins in our 2D Cry1Ac ligand blots. Based on reported sizes for APNs in this insect (Gill et al., 1995; Luo et al., 1997; Oltean et al., 1999; Banks et al., 2001), we predicted the 100-kDa spots detected by Cry1Ac to be forms of APN. However, these spots were not recognized by sera against APN and their PMF spectra matched to human desmocollin, a protein of the cadherin superfamily. There are a number of explanations for this lack of APN spots in our Cry1Ac ligand blots. Our current hypothesis is that the sample preparation method used for 2D analysis selects against certain proteins, including APNs (McNall and Adang, 2003). Theoretically, PIPLC digestion would facilitate solubilization of GPI-anchored proteins, including APNs. However, previous reports and the low percentage of APN activity released from the BBMV by PIPLC digestion (about 30%, data not shown), demonstrate that at least some Cry1Ac-binding APNs are resistant to PIPLC digestion (Luo et al., 1997). It is possible that some of the Cry1Ac-binding protein spots not detected in our fluorescent staining are forms of APN. However, none of these spots were recognized by cross-reactive sera against APN (Jurat-Fuentes, unpublished observation).

Desmocollins are a group of cadherin-like proteins that share low homology with classical cadherins and protocadherins (Nollet et al., 2000) and are involved in controlling rearrangements of the actin cytoskeleton at desmosomal junctions, especially in the context of cytokinesis and cell polarization (reviewed in (Ishii and Green, 2001)). We were unable to find insect homologues for the specific matched desmocollin. Sequence alignment revealed that the matched desmocollin shared only 13% identity with the *H. virescens* HevCaLP cadherin Cry1Ac receptor (accession number 15149240), suggesting that the protein spots may represent an alternative novel cadherin-like protein. In support of this hypothesis, these spots were not detected by antisera against HevCaLP (Jurat-Fuentes, unpublished data). Even though the *H. virescens* HevCaLP cadherin is a putative Cry1Ac receptor (Gahan et al., 2001; Jurat-Fuentes et al., 2004), it is seldom detected in 1D Cry1Ac ligand blots of BBMV proteins, and only binding to HevCaLP peptides expressed in *Escherichia coli* has been reported (Xie et al., 2005). Similarly, no cadherin protein was detected in the BBMV proteome from *M. sexta* (McNall and Adang, 2003). Considering that the HevCaLP cadherin is naturally processed into fragments of size similar to some of the 100-kDa Cry1Ac-binding spots (Jurat-Fuentes et al., 2004), we considered the possibility that they may represent truncated HevCaLP peptides. However, we were unable to confirm the identification of these protein spots using de novo sequencing, and only low identity matches were obtained. Because of the lack of high identity sequence tag matches, further research is needed to accurately identify these Cry1Ac-binding spots.

The present study demonstrates the value of two-dimensional electrophoresis, peptide mass fingerprinting, and *de novo* sequencing for identification of Bt toxin-binding proteins in larval gut samples. Whether the identified proteins are functional Cry1Ac receptors or "null receptors", which account for toxin binding that does not result in mortality, is yet to be established. Notably, our Cry1Ac binding results with HvALP confirm and support the hypothetical role of this protein in Cry1Ac toxicity and resistance ((Jurat-Fuentes and Adang, 2004), Jurat-Fuentes and Adang, unpublished data). Using bioinformatics and current available databases containing protein fingerprints and amino acid sequences, proteomics is a very useful tool in protein identification (Lester and Hubbard, 2002). However, the caveat of this technique is the quality of the PMF matches obtained when the sequences of the specific organism under study are not abundant in the databases. Increasing numbers of relevant sequenced genomes in the databases should improve the quantity of the significant PMF search matches. In this sense, proteomics could be very helpful for the identification of new targets to improve insecticide effectivity or to combat the problem of insecticide resistance. Further studies on the novel Cry1Ac binding proteins relating to their potential function as toxin receptors should prove useful in further resolving the Cry1Ac intoxication process.

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