

# A proteomic approach to study Cry1Ac binding proteins and their alterations in resistant *Heliothis virescens* larvae

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## Abstract

Binding of the *Bacillus thuringiensis* Cry1Ac toxin to specific receptors in the midgut brush border membrane is required for toxicity. Alteration of these receptors is the most reported mechanism of resistance. We used a proteomic approach to identify Cry1Ac binding proteins from intestinal brush border membrane (BBM) prepared from *Heliothis virescens* larvae. Cry1Ac binding BBM proteins were detected in 2D blots and identified using peptide mass fingerprinting (PMF) or *de novo* sequencing. Among other proteins, the membrane bound alkaline phosphatase (HvALP), and a novel phosphatase, were identified as Cry1Ac binding proteins. Reduction of HvALP expression levels correlated directly with resistance to Cry1Ac in the YHD2-B strain of *H. virescens*. To study additional proteomic alterations in resistant *H. virescens* larvae, we used two-dimensional differential in-gel electrophoresis (2D-DIGE) to compare three independent resistant strains with a susceptible strain. Our results validate the use of proteomic approaches to identify toxin binding proteins and proteome alterations in resistant insects.

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## 1. Introduction

Acreage devoted to transgenic crops expressing Cry toxins from *Bacillus thuringiensis* (Bt crops) has steadily increased since the introduction of this technology for efficient and environmentally-safe insect pest control (James, 2005). Increased adoption levels represent higher selection pressure for the evolution of insect resistance to these crops. Even though there are no reported episodes of resistance after 10 years of use, results from laboratory selection of target pests demonstrates that the potential for resistance evolution exists (Ferre and Van Rie, 2002). Both development of sensitive resistance detection methods as well as design of effective resistance management strategies rely on detailed knowledge on the Cry intoxication process

and alterations in this process that result in insect resistance.

The molecular details of the Cry toxin mode-of-action remain controversial. Toxin crystals need to be solubilized and activated by midgut proteases to an active toxin core. Activated toxin passes through the peritrophic matrix and interacts with specific receptors on the brush border cell membrane, determining toxin specificity (Hofmann et al., 1988). According to the model proposed by Bravo et al (Bravo et al., 2004), initial binding of toxin monomers to a cadherin protein results in further toxin processing and oligomerization. Toxin oligomers display high affinity binding to carbohydrate residues on certain glycosylphosphatidylinositol (GPI)-anchored proteins, such as *N*-aminopeptidase (APN) or alkaline phosphatase (ALP). This second binding step results in a conformational change in the toxin oligomer that leads to insertion in the membrane, formation of a pore and cell death due to osmotic shock (Pardo-Lopez et al., 2006). An alternative model (Zhang et al., 2006), suggests

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that toxin binding to cadherin activates intracellular apoptotic pathways that result in cell death.

Because Cry toxin mode of action is a multi-step process, resistance to Cry toxins can theoretically develop by alterations in any of the steps. However, in most cases of strains selected in the laboratory, resistance correlates with alterations of toxin binding molecules in the brush border membrane (Ferre and Van Rie, 2002). In *Heliothis virescens* (tobacco budworm), alterations in the cadherin protein HvCaLP (Gahan et al., 2001; Jurat-Fuentes et al., 2004), and the membrane-bound alkaline phosphatase HvALP (Jurat-Fuentes and Adang, 2004) have been reported to be involved in high levels of resistance to Cry1Ac. More recently, alterations in midgut serine-protease activities have been proposed to contribute to resistance in strains of *H. virescens* (Karumbaiah et al., 2007).

Our research goals are focused on studying the mechanisms of resistance in *H. virescens* against Cry toxins with two goals in mind: (1) identification of key elements in the mode of action that when altered result in resistance, (2) identification of potential resistance markers. To attain these goals, we used proteomics as a high throughput approach that allows us to identify toxin binding proteins and screen for alterations of these proteins in brush border membrane preparations from resistant larvae.

## 2. Proteomic identification of phosphatases as Cry1Ac binding proteins

Proteomics involves the study of the identity and function of all the proteins from a cell, tissue or organism at a given time. This group of proteins, or proteome, is highly dynamic and its composition reflects the physiological state of the specific sample studied. Proteomic approaches have been previously used to identify novel Cry toxin binding proteins (McNall and Adang, 2003) or compare proteomes from Cry-susceptible and -resistant insects (Candas et al., 2003) or cell lines (Liu et al., 2004). In these studies, the increased resolving power of 2D electrophoresis allowed for identification of proteins that were not successfully resolved using traditional SDS-PAGE electrophoresis.

Because successful identification of Cry toxin receptors is crucial to characterize alterations resulting in resistance, our first objective was to identify Cry1Ac binding proteins in the brush border membrane (BBM) proteome of susceptible *H. virescens* larvae. In ligand blots of BBM proteins separated using two-dimensional (2D) gel electrophoresis and probed with Cry1Ac, we detected several toxin binding proteins (Fig. 1). These proteins were identified by peptide mass fingerprinting or by *de novo* sequencing of peptides (Krishnamoorthy et al., 2007). Using this approach, we confirmed our previous identification of the membrane-bound alkaline phosphatase HvALP as a Cry1Ac binding protein (Jurat-Fuentes and Adang, 2004). Interestingly, an additional phosphatase in BBM protein samples (Fig. 1) was identified as a Cry1Ac binding protein (Krishnamoorthy et al., 2007). Protein spots corresponding to

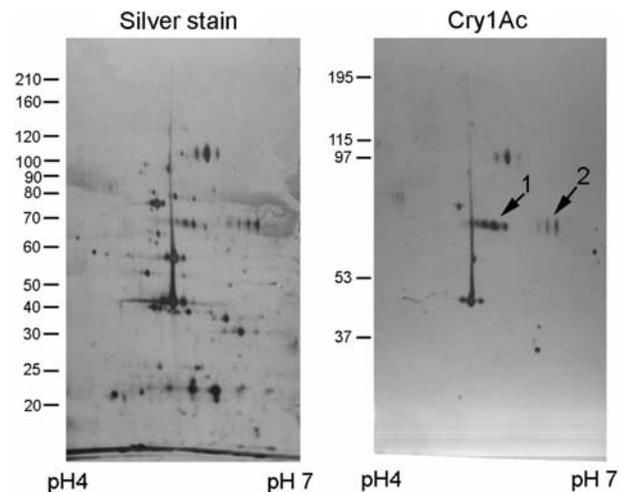


Fig. 1. Detection and identification of Cry1Ac binding proteins in BBM proteins from *H. virescens* larvae separated using 2D electrophoresis. BBM proteins (30  $\mu$ g) were processed and separated as previously described (Krishnamoorthy et al., 2007). Resolved proteins were silver stained or transferred to PVDF filters and probed with Cry1Ac as indicated. Proteins binding Cry1Ac were detected using polyclonal antisera to Cry1Ac. Arrows indicate localization of HvALP spots (1) or the novel phosphatase spots (2).

this phosphatase were not recognized by the antisera used to detect HvALP (Fig. 2).

Previous reports suggested interactions between Cry1Ac and phosphatases under native conditions. Cry1Ac inhibited alkaline phosphatase activity in midgut epithelial membrane samples from *H. virescens* (English and Readdy, 1989) and *M. sexta* (Sangadala et al., 1994). Because neither *N*-aminopeptidase (APN) nor bovine alkaline phosphatase activities were affected by incubation with the same toxin, inhibition of phosphatase activity by Cry1Ac seems specific for insect alkaline phosphatase, suggesting an important role for this enzyme during intoxication.

Using lectin blots and glycosidase digestions we previously determined that the protein band identified as HvALP in SDS-PAGE contained a terminal *N*-acetylgalactosamine (GalNAc) residue that was recognized by Cry1Ac (Jurat-Fuentes and Adang, 2004). 2D electrophoresis allowed us to study more specifically which protein spots contained the specific GalNAc residues. In lectin blots of BBM proteins separated by 2D electrophoresis, soybean lectin (SBA), concanavalin A (ConA), and the lectin from *Wistaria floribunda* (WFL) bound to the HvALP spots (Fig. 2). Both SBA and WFL recognize terminal GalNAc, while ConA binds to the trimannosidic core characteristic of N-linked glycans (Debray et al., 1981). These results confirm our previous observations using lectin blots of SDS-gels (Jurat-Fuentes and Adang, 2004), and demonstrate the presence of an N-linked glycan on HvALP containing a terminal GalNAc residue. In comparison, and even though weak binding of SBA and ConA was detected, only WFL bound strongly to the novel phosphatase spots

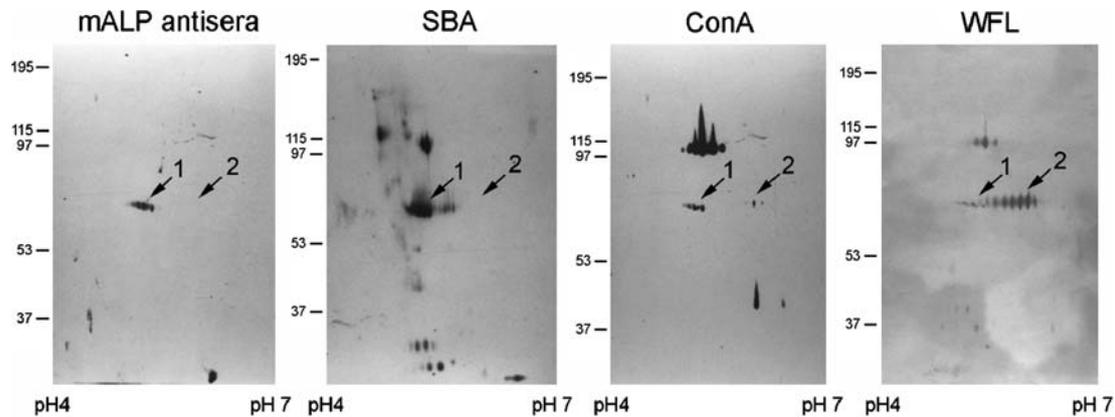


Fig. 2. Detection of HvALP spots (mALP antisera), or proteins binding soybean agglutinin (SBA), concanavalin A (ConA) or the lectin from *W. floribunda* (WFL). HvALP spots were detected using antisera to the membrane-bound form of alkaline phosphatase from *B. mori*. Methods for lectin blots were as previously described (Jurat-Fuentes and Adang, 2004). Arrows indicate the positions of HvALP (1) or the novel Cry1Ac binding phosphatase (2).

(Fig. 2), suggesting the presence of extensive O-linked glycosylation on this protein. Glycosylation of the novel phosphatase spots is evidence for the membrane localization of this protein. Interestingly, Cry1Ac binding was much more intense to HvALP spots than to the novel phosphatase (Fig. 1). Although further research is necessary to determine specific toxin binding affinities, this observation may suggest that Cry1Ac may have higher affinity for the terminal GalNAc residue in the N-linked glycan than the O-linked sugar residues in the novel phosphatase. High affinity Cry1Ac toxin oligomer binding to terminal GalNAc residues has been proposed as a crucial step in the intoxication process (Pardo-Lopez et al., 2006).

### 3. HvALP levels and resistance to Cry1Ac

We previously detected reduced expression of HvALP in larvae from the Cry1Ac-resistant YHD2-B strain of *H. virescens* (Jurat-Fuentes and Adang, 2004). Detection of HvALP protein spots on 2D gels allowed us to test

for alterations of HvALP in the resistant proteome. When comparing 2D blots of BBM proteins from susceptible (YDK) and Cry1Ac-resistant (YHD2-B) larvae, the levels of HvALP were clearly decreased in the resistant proteome (Fig. 3).

Because Cry1Ac resistance in the YHD2-B strain is recessive, larvae from the F1 generation of backcrosses between YDK and YHD2-B moths are susceptible to Cry1Ac (Jurat-Fuentes et al., 2002). When we detected HvALP levels in the F1 larval proteome, the amounts of HvALP were similar to the levels detected for the susceptible parental sample (Fig. 3). This reduction in HvALP levels correlates with reduced alkaline phosphatase activity in BBM vesicles (Jurat-Fuentes and Adang, 2004). These results are evidence of a correlation between Cry1Ac susceptibility and HvALP expression levels. According to this correlation, reduction of HvALP expression holds promise as candidate for the development of an efficient Cry1Ac resistance marker. We are currently testing this possibility and the genetic linkage between reduced HvALP expression and Cry1Ac resistance in our laboratories.

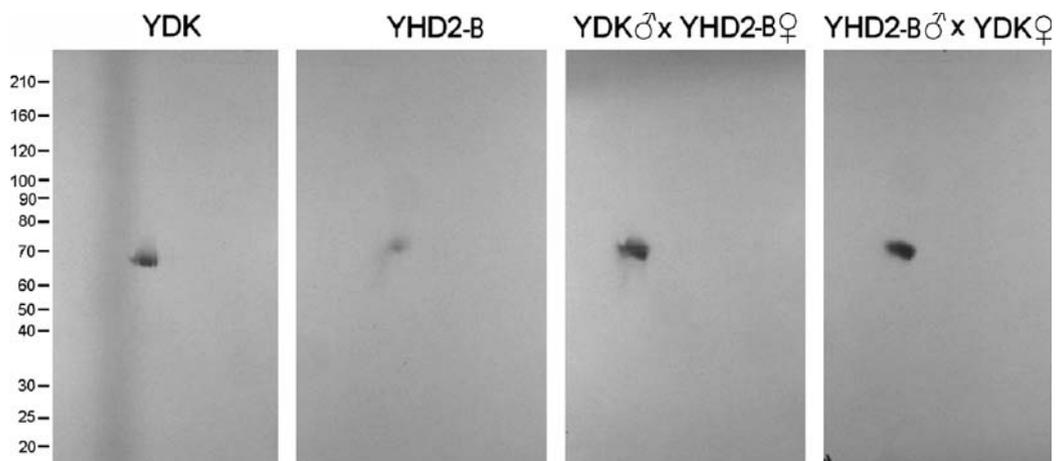


Fig. 3. Detection of HvALP protein spots in 2D blots of BBM proteins from susceptible (YDK), Cry1Ac-resistant (YHD2-B), or F1 larvae from crosses of susceptible and resistant moths as indicated. HvALP spots were detected with mALP antisera as described in Fig. 2.

#### 4. Proteomics of Bt resistance

Identification of HvALP and other Cry1Ac binding proteins allow us to study potential alterations of these proteins that correlate with resistance. To further characterize proteome alterations that correlate with resistance to Cry toxins, we compared BBM proteomes of one susceptible (YDK) and three resistant (YHD2-B, CXC, and KCBhyb) *H. virescens* strains using 2D differential in gel electrophoresis (2D-DIGE). In this approach (Fig. 4), proteins from susceptible and resistant larvae are labeled with distinct CyDye fluorophores (GE Healthcare) and resolved in the same 2D gel. An internal control sample consisting of a mixture of equal amounts of protein from all samples, thus containing all the proteins of each sample under study, is labeled with a third CyDye. Using this methodology we compared three independent BBM protein preparations from each strain, with samples labeled with two different dyes to eliminate the possibility of a labeling effect on the analysis. After protein separation, gels were imaged for the specific CyDyes and the scanned images compared using the DeCyder analysis software (GE Healthcare). This program detects and quantifies all protein spots in the imaged gel and matches detected spots to the internal reference gel. Because spot matching to the reference image is generated for each gel, high accuracy comparisons can be established between samples within the same gel and between different gels. In our analysis, we detected more than 780 proteins, of which 86 protein

spots had differential expression of at least 2-fold between the strains. We are currently using peptide mass fingerprinting and *de novo* sequencing (Krishnamoorthy et al., 2007) to identify these proteins. Because we are using three resistant strains with very distinct resistance and cross-resistance phenotypes (Jurat-Fuentes and Adang, 2006), we expect to identify specific proteins whose expression correlates with specific resistance phenotypes.

#### 5. Identification of additional Cry1Ac binding proteins and future prospects

Our results clearly demonstrate that HvALP is a Cry1Ac binding protein and putative receptor. Alteration of HvALP expression levels correlates with Cry1Ac resistance in the YHD2-B strain of *H. virescens* and suggests the potential utility of this enzyme as a resistance marker. We are currently testing this possibility using alternative resistant strains as well as different insect/toxin combinations.

Proteomic analysis of Cry1Ac binding proteins in the BBM proteome allowed for identification of novel toxin binding proteins, including actin and the A subunit of the vacuolar ATPase (Krishnamoorthy et al., 2007). Both of these proteins are localized to the cytosolic surface of the cell membrane, a region that traditionally has not been implicated in Cry1Ac intoxication. However, Cry1Ac binding to actin has been previously detected in 2D ligand blots using the BBM proteome from *Manduca sexta* larvae (McNall and Adang, 2003), and binding of Cry1Ac to cytosolic subunits of the mammalian Na–K ATPase has also been reported (English and Cantley, 1986). Interestingly, actin is one of the main components of the BBM proteome, reflective of the important role of this family of proteins in microvillae formation and organization (Dallai et al., 1998). Actin interacts through a number of phosphorylation signaling pathways with the cytosolic domain of cadherin proteins to activate intracellular pathways in response to extracellular signals (Lilien and Balsamo, 2005). Detection of Cry1Ac binding to both ATPase and actin suggests the possibility of potentially relevant interactions between part of the Cry1Ac toxin and intracellular proteins. These interactions would help explain toxin internalization as observed in *Caenorhabditis elegans* (Griffitts et al., 2001). Because protein denaturation may affect Cry1Ac toxin binding (Daniel et al., 2002), further research on the post binding steps in Cry intoxication is necessary to elucidate the potential role of these interactions *in vivo*.

Availability of independently-selected Cry1Ac and Cry2Aa resistant strains of *H. virescens* with distinct resistance phenotypes allowed us to study the alterations in the BBM proteome that result in resistance and cross-resistance to Cry toxins. However, because intracellular signaling pathways may be directly involved in Cry1Ac intoxication (Zhang et al., 2006), our analysis is not limited to toxin binding proteins. Using differential in-gel electrophoretic (DIGE) analysis we expect to detect and identify

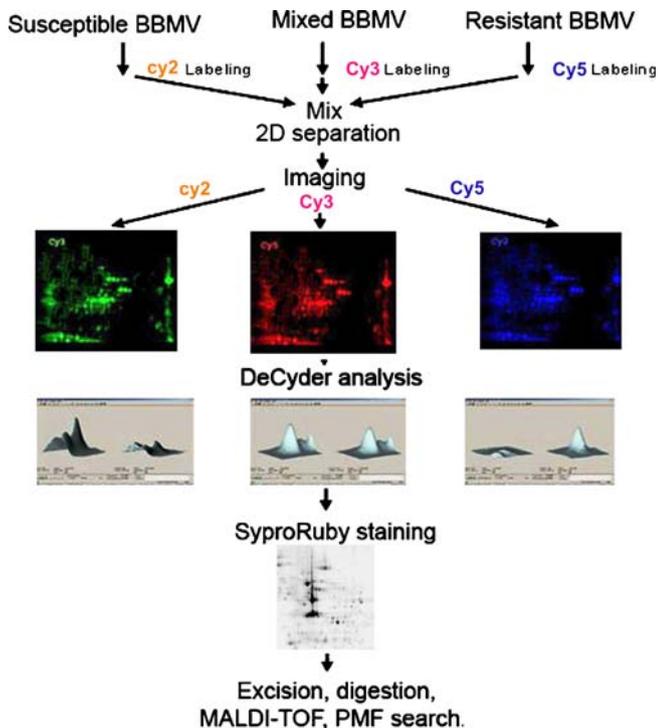


Fig. 4. Differential in-gel electrophoresis (DIGE) strategy used to compare BBM proteins from larvae of susceptible and three distinct Cry1Ac-resistant strains of *H. virescens*.

alterations in the BBM proteome in the resistant larvae that directly correlate with specific resistance phenotypes. The results from this analysis should allow for identification of proteins implicated in Cry1Ac and Cry2A intoxication as well as candidate biomarkers for resistance detection. Considering that second generation Bt cotton produces both these toxins for increased pest control, results from our 2D-DIGE analysis would be directly relevant to evaluate potential cross-resistance risks.

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