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Changes in protease activity and Cry3Aa toxin binding in the Colorado potato beetle: implications for insect resistance to *Bacillus thuringiensis* toxins

Olga Loseva [1,a], Mohamed Ibrahim [1,a], Mehmet Candas [a], C. Noah Koller [b], Leah S. Bauer [b,c], Lee A. Bulla Jr. [a,*]

[a] *Center for Biotechnology and Bioinformatics and the Department of Molecular and Cell Biology, The University of Texas at Dallas, PO Box 830688, FO3.1, Richardson, TX 75083, USA*

[b] *Department of Entomology, Center for Integrated Plant Systems, Michigan State University, East Lansing, MI 48824, USA*

[c] *Forest Service, U.S. Department of Agriculture, NCRS, East Lansing, MI 48823, USA*

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Abstract

Widespread commercial use of *Bacillus thuringiensis* Cry toxins to control pest insects has increased the likelihood for development of insect resistance to this entomopathogen. In this study, we investigated protease activity profiles and toxin-binding capacities in the midgut of a strain of Colorado potato beetle (CPB) that has developed resistance to the Cry3Aa toxin of *B. thuringiensis* subsp. *tenebrionis*. Histological examination revealed that the structural integrity of the midgut tissue in the toxin-resistant (R) insect was retained whereas the same tissue was devastated by toxin action in the susceptible (S) strain. Function-based activity profiling using zymographic gels showed specific proteolytic bands present in midgut extracts and brush border membrane vesicles (BBMV) of the R strain not apparent in the S strain. Aminopeptidase activity associated with insect midgut was higher in the R strain than in the S strain. Enzymatic processing of toxin did not differ in either strain and, apparently, is not a factor in resistance. BBMV from the R strain bound ~60% less toxin than BBMV from the S strain, whereas the kinetics of toxin saturation of BBMV was 30 times less in the R strain than in the S strain. However, homologous competition inhibition binding of ^{125}I-Cry3Aa to BBMV did not reveal any differences in binding affinity (K_d~0.1 μM) between the S and R strains. The results indicate that resistance by the CPB to the Cry3Aa toxin correlates with specific alterations in protease activity in the midgut as well as with decreased toxin binding. We believe that these features reflect adaptive responses that render the insect refractory to toxin action, making this insect an ideal model to study host innate responses and adaptive changes brought on by bacterial toxin interaction.

Keywords: Colorado potato beetle; *Bacillus thuringiensis*; Cry3Aa, protease; Resistance

1. Introduction

Innate cellular defense involves nonspecific responses found in organisms ranging from plants to humans. Invertebrates, including insects, rely primarily on innate cellular responses to defend themselves against microorganisms and associated toxins (Hoffmann et al. 1996, 1999). Information relay between innate defenses and adaptive protective immunity are of considerable scientific interest and insects serve as useful model systems

Abbreviations: CPB, Colorado potato beetle; ECB, European corn borer; BBMV, brush border membrane vesicles; R, resistant strain; S, susceptible strain; APN, aminopeptidase N; PMSF, phenylmethylsulfonyl fluoride; MES, 2-[*N*-morpholino]ethanesulfonic acid; DTT, dithiothreitol; Z-Arg-Arg-MNA, *N*-carbobenzoxy-arginine-arginine-4-methoxy-β-naphthylamide; Z-Phe-Arg-MNA, *N*-carbobenzoxy-phenylalanine-arginine-4-methoxy-β-naphthylamide; LpNA, leucine p-nitroanilide; E-64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino) butane.

* Corresponding author. Center for Biotechnology and Bioinformatics, The University of Texas at Dallas, PO Box 830688, FO3.1, Richardson, TX 75083-0688, USA.

E-mail address: bulla@utdallas.edu (L.A. Bulla Jr.).

[1] These authors contributed equally to this work.

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to investigate the molecular and biochemical pathways associated with these conserved responses. For example, since the discovery of antimicrobial activities in the cecropia moth, *Hyalophora cecropia* (Steiner et al., 1981), studies of *Drosophila* and mosquitoes have revealed notable conservation of innate defense mechanisms in both insects and mammals (Hoffmann et al., 1999; Dimopoulos et al., 2001). These immune responses are triggered on the surface of epithelial cells and communication through signaling pathways promotes special physiological adaptations that somehow protect the cell against foreign invaders. Most often, these reactions sustain cellular adaptation upon prolonged exposure to microbial activity or other stress conditions and eventually lead to resistance and, in some instances, apoptosis (Hecht, 1999; Loeb et al., 2000).

Commercial formulations of *Bacillus thuringiensis*, which produces insecticidal crystalline proteins known as Cry toxins are utilized to control many agriculturally and biomedically important insects (Schnepf et al., 1998). The toxicity as well as specificity of *B. thuringiensis* Cry toxins correlates directly with binding of toxins to high-affinity receptors on the epithelial cells that line the midgut of susceptible insects. Cry toxin receptors in the midgut epithelium of certain insects, including *Manduca sexta* and *Bombyx mori* (Vadlamudi et al. 1993, 1995; Nagamatsu et al. 1998, 1999) were identified as specific cell adhesion molecules, cadherins, which represent a large family of calcium-dependent, transmembrane glycoproteins and are responsible for maintaining the integrity of cell–cell contacts in multicellular organisms (Nollet et al., 2000; Angst et al., 2001). In addition to cadherin receptors for Cry toxins, several 120-170kDa Cry toxin-binding proteins also have been identified in some lepidopteran insects (Knight et al. 1994, 1995; Sangadala et al., 1994; Valaitis et al., 1995; Gill et al., 1995; Oltean et al., 1999). These proteins, in fact, exist as multiple forms of a ubiquitous midgut protease, aminopeptidase N (APN). Although APN interacts with Cry toxins and is an important component of the insect midgut surface, APNs do not serve as functional receptors to mediate toxicity of Cry proteins. In contrast, binding of Cry toxins to the cadherin receptors in the insect midgut causes stress and, consequently, disrupts the epithelium and destroys the entire midgut tissue. Insect larvae that have ingested lethal amounts of the Cry toxin stop feeding and die. In *M. sexta*, the cadherin receptor for Cry1A toxins, BT-R_1, is specifically expressed in the midgut of the larval stage of the insect (Midboe et al., 2002). That the molecule is not present in any other stage of the insect's life cycle indicates that Cry toxin binding to developmentally important cadherins might be of evolutionary significance to the entomopathogenicity of *B. thuringiensis*. Furthermore, cadherins are implicated in resistance of insects to the insecticidal activity of Cry toxins. Resistance to the Cry1Ac toxin by *Heliothis virescens* is linked to retrotransposon-mediated disruption of a specific cadherin gene (Gahan et al., 2001), indicating that midgut epithelial cadherins are involved directly in the entomopathogenicity of *B. thuringiensis*. Of particular concern is the likelihood that insect resistance to certain Cry toxins will become prevalent (Ferre et al., 1995; Schnepf et al., 1998).

Little is known about the mechanism(s) of resistance to Cry toxins. One possible mechanism includes decrease in the toxin binding to insect midgut (Van Rie et al., 1990; Ferre et al. 1991, 1995; Sayyed et al., 2000). Others implicate proteases that interact with toxin in the insect gut. For example, proteases from a strain of tobacco budworm *H. virescens* resistant to *B. thuringiensis* subsp. *kurstaki* HD-73 were reported to degrade toxin faster than proteases from a susceptible strain (Forcada et al., 1996). Keller et al. (1996) showed that the specific activity of gut proteases increases throughout larval development. The increased activity was associated with a loss of sensitivity by late developmental stages of the larvae to Cry1C toxin, possibly, due to toxin degradation. Oppert et al. (1997) reported two resistant strains of the Indianmeal moth *Plodia interpunctella* that lacked a major gut protease involved in toxin activation. These investigations suggest that changes in the activity or composition of gut proteases are involved in altering the susceptibility by insects to Cry toxins.

In the present study, we assessed several factors that are implicated in resistance by the CPB to the Cry3Aa toxin produced by *B. thuringiensis* subsp. *tenebrionis*. We compared proteolytic activity profiles in BBMV and gut juice, aminopeptidase activity, stability of Cry3Aa toxin to BBMV and gut juice proteases and toxin binding to BBMV as well as toxin action on midgut epithelial cells in vivo. We found that resistance to Cry3Aa toxin entails decreased toxin binding and changes in the composition and activity of midgut proteolytic enzymes, especially elevated aminopeptidase activity. These factors appear to be involved in maintaining a protective state in midgut epithelial tissue, and, together, they constitute an adaptive response of the CPB to the Cry3Aa toxin.

2. Materials and methods

2.1. Insects

Colorado potato beetles *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) were collected from potato farms in seven Michigan counties during 1987 and 1988. A cohort for resistance to Cry3Aa was selected using *B. thuringiensis* subsp. *tenebrionis*-based insecticides (Whalon et al., 1993). The level of resistance at F_{35} was about 300-fold higher than the S strain (Rahardja and

Whalon, 1995). Since that time, beetle larvae have been continually reared on potted "Superior" potato at 24±2 °C with a 16:8 (light:dark) photoperiod. Then 24-h second-instar beetle larvae were placed on potato leaves dipped in M-Trak™ (a Cry3Aa-based insecticide produced by Dow Agrochemicals) for several days. Surviving larvae were transferred to untreated foliage and reared to the adult stage. At F_{54}, larvae were transferred to the foliage of NewLeaf™ Russett Burbank 2-06 (Monsanto Company, St Louis, MO). NewLeaf potatoes are transgenic potatoes that harbor the *cry3Aa* gene cloned from *B. thuringiensis* subsp. *tenebrionis*. The duration of larval exposure to the NewLeaf foliage gradually increased from 4 to 17 days to maintain high selection pressure. Cry3Aa-resistant beetles were collected from generations F_{63} through F_{72}.

2.2. Bioassays

Bioassays were done by applying 1-μl droplets of Cry3Aa purified crystals suspended in 22.5% sucrose onto 4-mm diameter potato leaf disks on agar in 24-well tissue culture plates. Individual 24-h old second-instar larvae were placed in separate wells each containing leaf disks treated with toxin at concentrations ranging from 0.32 to 400 ng/mm^2. The number of larvae tested at each concentration ranged from 12 to 20. After 24 h of exposure to the toxin-treated leaf discs, larvae were transferred in groups of 10 or less to Petri dishes (110×15 mm) containing toxin-free potato foliage. Mortality was determined at 96 and 120 h after transfer. Based on the results of five replicate assays, the 96-h LC_{50} (95% CI) for native Cry3Aa crystals in second-instar Cry3Aa-susceptible larvae was 25.31 (13.85–43.53) ng/mm^2; the 120-h LC_{50} (95% CI) was 12.65 ng/mm^2 (6.75–20.69). We were unable to determine an LC_{50} for the resistant strain. No mortality of the resistant larvae was observed after 24-h exposure to Cry3Aa crystals at a concentration of 10,000 ng/mm^2, the maximum concentration that could be applied in this bioassay procedure.

2.3. Cry3Aa purification

Cry3Aa toxin was obtained from a sporulated culture of *B. thuringiensis* subsp. *tenebrionis* grown on T3-sporulating medium (peptone/yeast extract/$MnCl_2$) at 30 °C for 3–4 days. The spore-crystal pellet was collected by centrifugation at 12,000*g* for 15 min and washed twice in 100 mM sodium phosphate buffer, pH 7.0. Crystals were solubilized in 3.3 M NaBr, 50 mM sodium phosphate buffer, pH 7.0 containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The pH of soluble Cry3Aa toxin was adjusted to pH 6.0.

2.4. Extraction of larval proteases

Extraction of gut proteases was carried out as described by Girard et al. (1998). Midguts were excised from cold anesthetized fourth-instar larvae and homogenized in cold 150 mM NaCl. The solid midgut parts were separated from soluble material by centrifugation at 16,000*g* for 20 min at 4 °C. The BBMV from third- and fourth-instar S and R larvae were prepared according to the method of Wolfersberger et al. (1987). For the first and second instars, the BBMV were prepared from whole larvae as described by MacIntosh et al. (1994). Protein concentrations in the extracts were determined by the method described by Bradford (1976) using BSA as a protein standard.

2.5. Total protease activity assays

Total proteolytic activity in larval gut juice and BBMV was measured using azocasein as substrate. Larval extracts (5 μl per assay) were incubated with 30 μl of buffer A containing 50 mM MES (2-[*N*-morpholino]-ethanesulfonic acid), pH 6.0, 5 mM dithiothreitol (DTT) and 0.1% Triton X-100 for 20 min at 37 °C before addition of 100 μl of 2% azocasein (w/v in buffer A). The reaction was allowed to proceed for 180 min at 37 °C before it was stopped with cold 10% trichloroacetic acid. Upon centrifugation at 16,000*g*, 10 μl of 10 N NaOH was added and the supernatant absorbance at 440 nm was measured with a Beckman DU 530 spectrophotometer. Rate of proteolysis of azocasein was expressed in microOD_{440} units/60 min/mg of midgut protein.

2.6. Specific protease activity assays

The activity of cysteine proteases was measured using the synthetic substrates *N*-carbobenzoxy-arginine-arginine-4-methoxy-β-naphthylamide (Z-Arg-Arg-MNA) and *N*-carbobenzoxy-phenylalanine-arginine-4-methoxy-β-naphthylamide (Z-Phe-Arg-MNA) according to Barrett (1976). The reaction mixture containing 5 μl of enzyme extract in 30 μl of buffer A was incubated for 30 min at 37 °C before adding 100 μl of 0.5 mM substrate. After incubation for 60 min at 37 °C, the reaction was stopped by adding 1.5 ml of 5 mM mersalyl–2% Tween 20–0.02 mg/ml Fast Garnet reactive solution. The resulting solution was centrifuged at 10,000*g* for 5 min and the absorbance of the supernatant was monitored at 520 nm. The rate of proteolysis of the substrates was defined as the OD_{520}/60 min/mg of midgut protein.

The activity of protease inhibitors was determined by pre-incubating the different inhibitors for 30 min at 37 °C with the enzyme extracts before addition of substrates. The following concentrations of inhibitors were used: 400 μM of *trans*-epoxysuccinyl-L-leucylamido(4-

guanidino)butane (E-64), 5 μM of pepstatin, 5 mM of PMSF and 200 μM of leupeptin.

The activity of aminopeptidases was measured using 1–2 mM leucine *p*-nitroanilide (LpNA) as substrate in a reaction mixture containing 100 mM Tris–HCl, pH 8.0 and 0.1% Triton X-100 at room temperature. The kinetics of *p*-nitroaniline release was monitored by measuring the increase in absorbance (λ_{405}) for 60 min. The reaction rate was calculated as microOD_{405} units/min/mg of midgut protein.

2.7. Assessment of Cry3Aa proteolysis in BBMV and gut juice

The purified Cry3Aa toxin in buffer A was incubated with BBMV or gut juice extracts at 1:500 ratio (w/w) for 2, 4, 6, 8 and 24 h at 37 °C. Proteolysis was stopped by boiling the samples for 10 min. Proteolytic products were separated by 10% SDS-PAGE (Laemmli, 1970) and blotted on poly(vinylidene difluoride) membrane. Western blotting was performed using anti-Cry3Aa toxin rabbit polyclonal antibodies and horseradish peroxidase-coupled goat anti-rabbit IgG antibodies. Peroxidase activity was determined using an ECL Western blotting detection kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

2.8. Effect of gut fluid from European corn borer on Cry3Aa toxin

Five microliters of gut fluid (6.2 μg/μl) from European corn borer (ECB), *Ostrinia nubilalis*, was added to 42 μl of Universal buffer with pH 3.7 or 11.1 (Koller et al., 1992). After 5 min, 3 μl of soluble S^{35}-Cry3Aa toxin (158,000 cpm) was added. This mixture was allowed to sit at room temperature for 30 min. Following incubation, 10 μl of the mixture was removed and added to 10 μl of ice-cold 10% TCA. After 10 min, the mixture was centrifuged and the supernatant was removed. The pellet was dissolved in 2 X sample buffer (Laemmli, 1970) and the proteins were separated on a 4–15% gradient SDS-PAGE gel. The gel was fixed, stained and prepared for fluorography according to Koller et al. (1992). The film was developed overnight at −80 °C.

2.9. Protease activity profiling by electrophoresis

Different forms of digestive proteases in R and S strains of CPB larvae were analyzed using 10% polyacrylamide slab gels containing 0.4% gelatin. After separation of the proteins, the gels were washed for 1 h in 2.5% Triton X-100 and then incubated overnight in buffer A at room temperature. To determine the response of proteases to class-specific irreversible inhibitors, the protein extracts were treated before electrophoresis with 400 μM E-64 or with 5 mM PMSF.

2.10. Cry3Aa radioiodination and ligand binding assays

Cry3Aa toxin was radioiodinated as described by Keeton and Bulla (1997) by the chloramine-T method. Radiolabeled toxin binding assays were performed as described by Hofmann et al. (1988). Competition binding assay was performed according to Vadlamudi et al. (1993). Samples of BBMV (10 μg) were incubated with ^{125}I-Cry3Aa toxin (1.0 nM) in the presence of increasing concentrations (0–10 μM) of unlabeled Cry3Aa toxin in a final assay volume of 100 μl of PBS, pH 7.4, containing 0.02% BSA. Binding data were analyzed by using GraphPad Prism 3 software package. All values are reported as percentages relative to the binding detected in the absence of competitor.

2.11. Microscopy

Second-instar larvae were starved for 6 h and then placed on 5-mm potato leaf disks with a 2-μl droplet of 22.5% sucrose containing 6 μg of Cry3Aa solubilized in 10 mM KOH. Larval guts were dissected 18 h after initial exposure, fixed and sectioned as described in (Bauer and Pankratz, 1992). The midgut sections were fixed overnight in cold 2.5% glutaraldehyde in 100 mM phosphate buffer, pH 7.2 and post-fixed for 1 h at room temperature in 1% OsO_4 in the 100 mM phosphate buffer. The tissues then were dehydrated through an ethanol series, treated with propylene oxide and embedded in Poly/Bed 812 (Polysciences Inc., Warrington, PA). Thin sections were examined by light microscopy using a Zeiss Axioskop 50 compound microscope (Carl Zeiss, Inc., Thornwood, NY).

3. Results

3.1. Proteolytic activity in the gut juice of CPB larvae

Total proteolytic activity in the gut juice from R and S strains of the CPB was determined using azocasein as the protein substrate. The reactions were carried out in a slightly acidic reaction mixture (pH 6.0) that correlates with the physiological pH of the insect midgut and activated by DTT (Michaud et al., 1995). Gut juice from fourth-instar larvae of both R and S strains exhibited similar hydrolytic activity against azocasein (Table 1). Incorporation of the protease inhibitors E-64 and leupeptin to the system resulted in approximately 70–75% inactivation of the proteolytic activity in both extracts whereas only about 20–25% of the protease activity in the extracts was sensitive to the serine protease inhibitor PMSF (Table 1). Pepstatin inhibited azocasein hydrolysis by about 15–20% in the extracts (Table 1), indicating

Table 1
Total proteolytic activity in gut juice of fourth-instar larvae using azocasein as substrate

Inhibitor	Concentration	OD_{440}/60min/mg		% Residual activity	
		S	R	S	R
None		1.96±0.18	2.00±0.21	100	100
PMSF	5 mM	1.54±0.12	1.52±0.11	78	76
Pepstatin	5 μM	1.64±0.13	1.60±0.13	84	80
E-64	400 μM	0.47±0.08	0.57±0.09	24	28
Leupeptin	200 μM	0.61±0.09	0.66±0.06	26	29

the presence of aspartate proteases in the midgut of the beetle.

3.2. Cysteine protease activity

To further characterize and compare proteolytic activities in larval gut juice from the R and S strains, Z-Phe-Arg-MNA was used as a specific substrate for cathepsins B and L and Z-Arg-Arg-MNA for cathepsin B. No differences in the proteolytic hydrolysis of these specific substrates were found in either the R or S strain. Hydrolysis of the substrates was almost totally inhibited by the inhibitors of cysteine proteases, E-64 and leupeptin (Table 2) (Salvesen and Nagase, 2001), indicating that cysteine proteases constitute a significant amount of the activity associated with degradation of Z-Phe-Arg-MNA. Similar results were obtained with Z-Arg-Arg-MNA (data not shown).

3.3. Aminopeptidase activity

In insects, aminopeptidases are found mainly bound to the microvillar membranes of midgut epithelial cells (Terra and Ferreira, 1994; Cristofoletti and Terra, 1999). Indeed, we detected aminopeptidase activity associated with BBMV prepared from all four larval instars of the CPB (Fig. 1). Enzymatic activity was less in the third- and fourth-instar larvae compared to the first two instars. Notably, the R strain had higher aminopeptidase activity than the S strain throughout larval development.

Table 2
Specific proteolytic activity in gut juice using Z-Phe-Arg-MNA as substrate

Inhibitor	Concentration	OD_{520}/60min/mg	
		S	R
None		32.3±0.4	33.0±0.5
PMSF	5 mM	32.1±0.2	33.2±0.5
E-64	400 μM	0.5±0.1	0.4±0.1
Leupeptin	200 μM	1.2±0.2	1.2±0.3

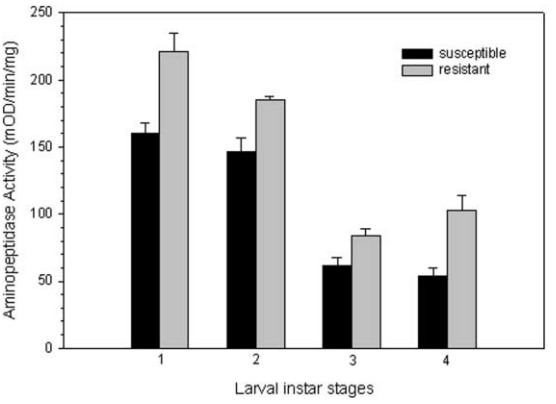

Fig. 1. Aminopeptidase activity in BBMV from susceptible and resistant CPB strains during larval development. The activity of aminopeptidases was measured with *p*-nitroaniline release from LpNA at pH 8.0. Results are expressed as microOD_{405} units/min/mg of total BBMV protein. Each column represents the mean of three assays. Vertical bars indicate standard errors of the mean.

3.4. Protease forms in midgut extracts

Electrophoretic separation of midgut proteins in gelatin-containing gels revealed multiple zones of hydrolytic activity for this substrate (Fig. 2). Both the R and the S strains possess several distinct forms of proteases in BBMV (Fig. 2A) and gut juice (Fig. 2B). Extracts from the R strain exhibited a different protease activity profile than did the S strain. Analysis of BBMV and gut juice samples from fourth-instar larvae of the R strain showed zones of gelatin hydrolysis (see arrows, lanes 3 and 4, Fig. 2A and B) on the zymograms not present in the S strain, suggesting that the Cry3Aa-resistant insects utilize different enzymes or different isoforms of the proteases. Similar observations were made for BBMV preparations from first- and second-instar larvae (data not shown). The samples were also treated with class-specific irreversible inhibitors of proteases prior to electrophoresis and analyzed for response to these inhibitors. Almost all forms of the proteases in both the S and R strains were inhibited by the cysteine proteinase inhibitor E-64 (Fig. 2B, lanes 2 and 4). These results corroborate that cysteine proteases constitute a significant part of gut

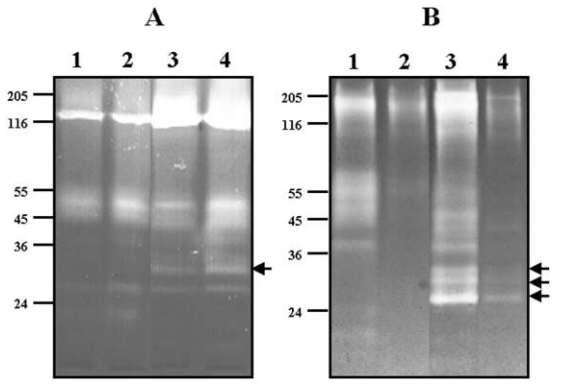

Fig. 2. Zymogram showing gelatin hydrolysis by midgut extracts from susceptible and resistant CPB strains. (A) Protease profile in BBMV (20 μg per lane) from S (lane 1, 2) and R (lane 3, 4) strain. Protein extracts were treated with PMSF (5 mM) before electrophoresis (lane 2, 4). (B) Protease profile in gut juice (15 μg per lane) from S (lane 1, 2) and R (lane 3, 4) strain. Protein extracts were treated with E-64 (400 μM) before electrophoresis (lane 2, 4). Arrows indicate additional protease forms in the R strain. The smear on the top of the gels is due to protease activity during migration. Molecular weight markers are indicated on the left.

proteolytic activity in the CPB (Michaud et al., 1995). These investigators saw a pronounced activation of proteases by PMSF in the CPB. We observed a similar phenomenon using PMSF with BBMV in the gelatin-containing gel (Fig. 2A). Increase in gelatin hydrolysis activity in PMSF-treated BBMV preparations suggests that this inhibitor affects serine proteases, which can modulate activity of other proteases.

3.5. Cry3Aa proteolysis by gut juice and BBMV

To determine whether resistance to Cry3Aa toxin is related to proteolysis of the toxin, Cry3Aa toxin degradation or modification by gut juice and BBMV was compared in the S and R strains. Cry3Aa toxin was incubated with enzyme extracts (1:500) at 37 °C for different periods of time and the samples were analyzed by Western blotting (Fig. 3). No differences were observed in BBMV and gut juice of the S and R strains, indicating that Cry3Aa toxin processing is the same in both strains (Fig. 3A and B). Apparently, toxin degradation is not a factor in the development of resistance by the CPB to Cry3Aa toxin. A 38-kDa fragment was produced upon incubation of toxin with BBMV (Fig. 3A). The fragment was resistant to any further degradation upon prolonged exposure to the gut juice extracts.

Unlike the CPB, the Cry3Aa toxin was degraded in gut fluid (pH 11.1) from the naturally resistant ECB (Fig. 3C). The major proteases of the lepidopteran insects (ECB belongs to this order) are serine proteases with alkaline pH-optimum (Schnepf et al., 1998). Significantly, the pH midgut of the CPB is mildly acidic and cysteine proteases are the common digestive enzymes in Coleoptera (Murdock et al., 1987). Evidently, E-64 and

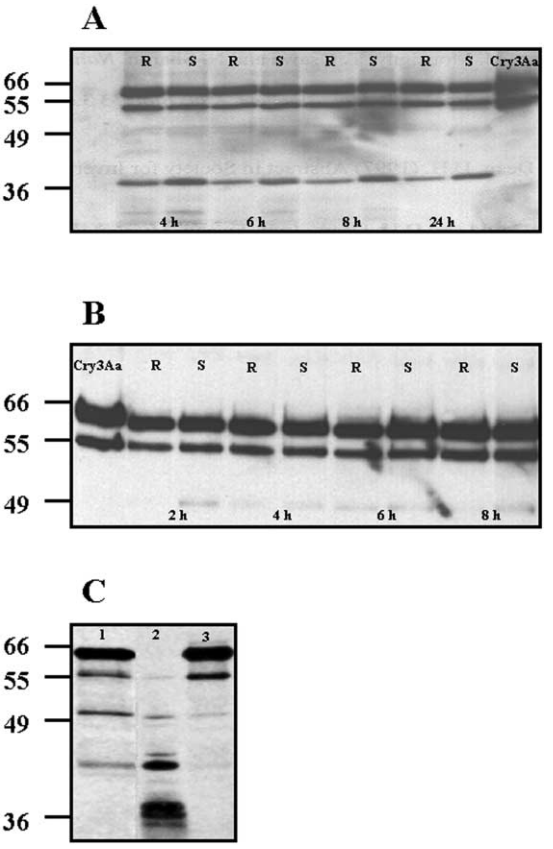

Fig. 3. Proteolytic digestion of Cry3Aa toxin by BBMV and gut juice from CBP and ECB. Cry3Aa toxin digestion by BBMV (A) and gut juice (B) from CPB at a substrate:enzyme ratio of 1:500. (C) Cry3Aa toxin digestion by gut juice from ECB: lane 1, digestion at pH 3.7; lane 2, digestion at pH 11.1; lane 3, Cry3Aa toxin at pH 11.1 without gut fluid. Molecular weight markers are indicated on the left.

leupeptine, both cysteine protease inhibitors, dramatically reduced proteolycic activity in CPB gut juice when Z-Phe-Arg-MNA was used as a substrate (Table 2).

3.6. Ligand binding analyses

To test whether there is a difference between Cry3Aa R and S strains of the CPB in binding of Cry3Aa toxin to the surface of midgut epithelial cells, saturation binding experiments were performed using ^{125}I-Cry3Aa toxin and BBMV (Fig. 4A). The results of these experiments revealed a significant difference in toxin binding to BBMV between the R and S strains (Fig. 4A). BBMV preparations from the Cry3Aa-resistant strain bound ~60% less toxin than did the Cry3Aa-susceptible strain. However, competitive binding of ^{125}I-Cry3Aa toxins to BBMV in the presence of increasing concentrations of unlabeled Cry3Aa toxins did not reveal any differences in binding affinity (K_d~0.1 μM) between the S and R strains (Fig. 4B).

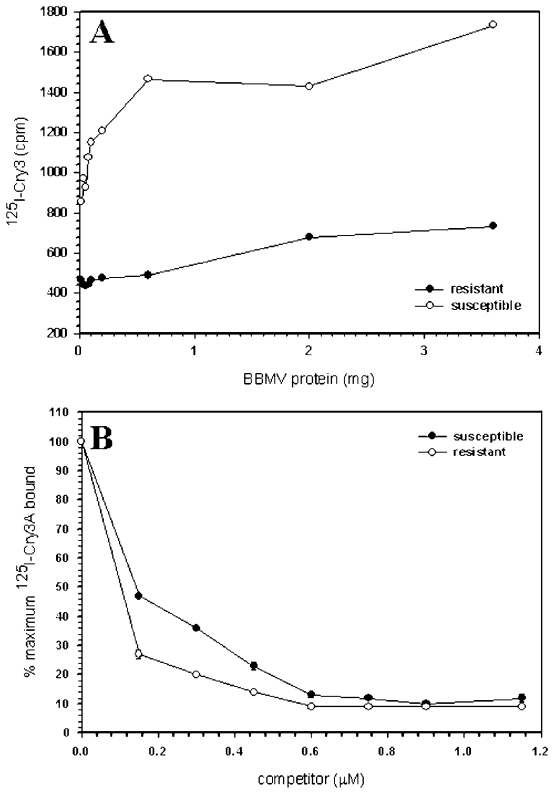

Fig. 4. Binding of ^{125}I-Cry3Aa toxin to BBMV proteins of susceptible and resistant strains of the CPB. (A) Specific binding of ^{125}I-Cry3Aa toxin to BBMV proteins. (B) Homologous competition inhibition binding of ^{125}I-Cry3Aa toxin to BBMV from S and R strains. All values are means of experiments performed in duplicate, with standard deviations indicated.

3.7. Pathological effect of Cry3Aa toxin on midgut tissue

The cytotoxic effect of Cry3Aa toxin on the midgut of the S CPB was dramatic (Fig. 5A) when compared to an unexposed midgut (Fig. 5B). Midgut epithelial cells were destroyed by the Cry3Aa toxin whereas cells in the R strain retained their structural integrity (Fig. 5C). Similar action by other Cry toxins on susceptible insects has been reported (Gill et al., 1992).

4. Discussion

B. thuringiensis synthesizes intracellular parasporal glycoprotein crystals during the sporulation cycle (Bulla et al., 1977). These glycoproteins are protoxins and exhibit insecticidal activity. The protoxin is activated proteolytically after ingestion by an insect susceptible to the toxic product (Bulla et al., 1981). The active toxin (Cry toxin) binds to a receptor on the surface of epithelial cells in the insect midgut and leads to disruption of the tissue (Höfte and Whiteley, 1989; Gill et al., 1992; Schnepf et al., 1998). Obviously, changes in the physiology and biochemistry of the insect gut can alter these processes and, consequently, diminish the effectiveness of the Cry toxin. Indeed, the CPB, which has gained tolerance to the Cry3Aa toxin of *B. thuringiensis* subsp. *tenebrionis*, displays adaptive changes that are inhibitory to toxin action. Epithelial cells in the R strain retained their structural and functional integrity (Fig. 5C) upon exposure to the toxin, whereas cells in the S strain were completely destroyed (Fig. 5A). Similar observations were made for toxin-susceptible strains of the tobacco budworm and the cottonwood leaf beetle (Bauer and Pankratz, 1992; Forcada et al., 1999). Epithelia in the R strains escaped toxin action whereas the S strains did not. Experiments with cultured midgut cells from tobacco budworm *H. virescens* revealed cell apoptosis as a result of exposure to *B. thuringiensis* toxin (Loeb et al., 2000). Regeneration of midgut cells after exposure to subletal doses of *B. thuringiensis* toxin is accompanied with increased synthesis of Lepidopteran midgut differentiation factor 1 as a marker of increased stem cell differentiation (Loeb et al., 2001). The response of cultural midgut cells to Bt toxin action is comparable to regulatory processes associated with regeneration and healing in vertebrate tissues.

Protease action on Cry toxins has been proposed as the means by which toxin activity is reduced or inhibited, thereby rendering insects resistant to *B. thuringiensis* (Oppert et al., 1997; Shao et al., 1998). No such protease action on the Cry3Aa toxin was evident in the resistant strain of the CPB. Analysis of proteolytic diges-

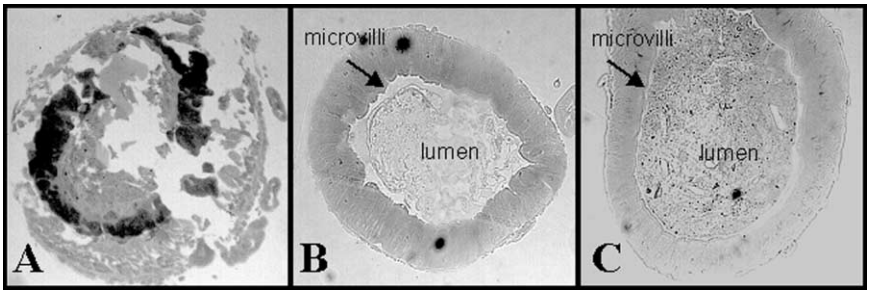

Fig. 5. Histopathology of Cry3Aa toxin action in midgut epithelial tissue. (A) Cross-section of the midgut of the S strain of the Colorado beetle fed Cry3Aa toxin. (B) Midgut of beetle not fed toxin. (C) Midgut of R strain fed Cry3Aa toxin.

tion of the Cry3Aa toxin by proteases associated with gut juice and BBMV from R and S strains of the beetle revealed no distinguishable differences between the two strains (Fig. 3A and B). Clearly, enzymatic processing or degradation of toxin does not impart Cry toxin resistance in the CPB. It is important to note that for the ECB, which is naturally resistant to the Cry3Aa toxin, there was nearly complete degradation of the toxin by gut juice at pH 11.1 (Fig. 3C). ECB gut proteases do not function at acidic pH. They are active only at alkaline pH, which is characteristic of lepidopteran midgut (Gill et al., 1992). Whether the action proteases or lack of binding the toxin by ECB BBMV (Ibrahim and Bulla, unpublished results) or combination of these renders the ECB refractory to the Cry3Aa toxin remains to be determined.

Interestingly, proteases associated with the BBMV, but not gut juice, from both S and R strains of the CPB did cleave the Cry3Aa toxin, generating a 38-kDa fragment. Evidently, there are certain proteases that act specifically on Cry toxins located exclusively on the epithelial cell membrane. Possibly, membrane components may affect the structural conformation of the toxin, exposing potential cleavage sites for protease action.

In function-based activity profiling using zymographic gels, the R strain exhibited a different overall activity profile than the S strain for proteases associated with both BBMV and gut juice extracts (Fig. 2). The differences in the profiles were both qualitative and quantitative in that the R strain exhibited distinct protease species with elevated activities. Forcada et al. (1999) also observed additional proteolytic bands in the gut extract of the resistant strain of the tobacco budworm. Apparently, changes in midgut tissue protease activity profiles correlate with Cry toxin resistance in both the tobacco budworm and the CPB. It is established that proteases are involved in innate responses in insect midgut by modulating signaling and amplification cascades that lead to the activation of specific defense mechanisms, such as pathogen recognition, melanization, coagulation and induction of antimicrobial peptides (Hoffmann et al., 1999; Barillas-Mury et al., 2000; Jiang and Kanost, 2000). Moreover, innate responses by mosquitoes to bacterial invasion of the digestive tract apparently involve induction of certain serine proteases (Dimopoulos et al., 1997; Han et al., 2000). Likewise, we believe that the appearance of new proteases in the R strain of the CPB is a result of specific gene expression responding to Cry toxin exposure.

In the present study, the R and S strains of the CPB displayed different levels of aminopeptidase activities associated with the midgut epithelial cells (Fig. 1). Aminopeptidases are associated primarily with the microvillar membrane (brush border) of midgut cells. They are the main enzymes in midgut microvillar membranes of most insects, constituting about 55% of the microvillar proteins in coleopterans (Cristofoletti and Terra 1999, 2000). Sequence analysis and substrate specificity studies have revealed that these enzymes from insects share common features with mammalian aminopeptidase N, metallopeptidase of the gluzincin superfamily (Hooper, 1994; Knight et al., 1994; Shipp and Look, 1993). Mammalian aminopeptidase N (CD13) as well as other cell surface peptidases constitute a group of ectoenzymes with a broad functional repertoire. They are implicated not only in degradation of terminal peptides and scavenging of amino acids but also in signal transduction as well, cleaving peptide mediators and modulating their activities (Shipp and Look, 1993; Reimann et al., 1999; Santos et al., 2000). Innate immune responses in mouse small intestine epithelium are modulated by aminopeptidases that act on secreted antibacterial peptides, defensins, which also are present in invertebrates (Lehane et al., 1997; Ouellette et al., 2000). In Indianmeal moth strains exhibiting different susceptibilities to Cry1A toxin (Zhu et al., 2000), increased levels of mRNA of aminopeptidase-like genes in toxin-resistant strains lends further support to our postulation that increased aminopeptidase activity in the R strain of the CPB contributes to a more effective immune state than exists in the S one.

Another factor that probably contributes to the resistance phenomenon in the CPB is decreased binding of the toxin to the receptor(s). Comparison of the binding of Cry3Aa toxin to BBMV reveals that the R strain of the CPB binds fewer toxin molecules than the S strain (Fig. 4). However, we did not observe any difference in toxin binding affinities, as delineated by identical K_d values calculated in radiolabeled toxin displacement experiments with both S and R strains (Fig. 4B). Therefore, decreased toxin binding in the R strain appears to be due to a reduced number of the toxin binding sites in the receptor molecule or to a decreased number of receptors themselves. Conversely, decreased binding also might be occasioned by changes in epithelium receptors involving specific cleavage and/or side-chain modifications. In an analogous system, a Cry3Aa-resistant strain of the cottonwood leaf beetle avoided toxin action by binding fewer and excreting more toxin molecules than did susceptible larvae (unpublished data). Results from other studies also have led to the conclusion that alterations in Cry toxin binding receptor sites correlate with resistance (Van Rie et al., 1990; Ferre et al. 1991, 1991; Ballester et al., 1999; Sayyed et al., 2000; Gahan et al., 2001; Griffitts et al., 2001). Evidently, decreased toxin binding to insect midgut epithelium is mechanistically important in increasing the insects' capacity to withstand Cry toxin action and in providing a survival advantage. In mosquito hemocyte-like cells that have been challenged by bacterial infection, decreased expression of scavenger cell surface receptors appear to contribute to the insect immune responsiveness (Dimopoulos et al., 2000). Our studies

demonstrate that resistance to the Cry3Aa toxin by the CPB correlates with reduced binding of Cry3Aa toxin to insect midgut epithelial cells along with changes in the composition as well as activity of midgut proteases. The increased activity of aminopeptidases in the R strain of the CPB indicates a higher concentration of these enzymes on the midgut and suggests that aminopeptidases do not serve as receptors for the Cry3Aa toxin in this insect as has been suggested for other Cry toxins in lepidopteran insects (Knight et al., 1994). There appears to be two levels of adaptive responses in insects that bring resistance to the lethal action of Cry toxins; the first level involves changes in the profile of midgut proteases to situate the tissue to respond to toxin stress, while the second level might resolve adaptive responses that can be genetically transmitted for survival advantage. Elucidating the mechanism(s) of how midgut epithelium cells in resistant insects avoid toxin binding will be one of the priorities in understanding insect resistance. The innate responses and adaptive changes in the insect midgut epithelium and their functional involvement in rendering immunity to *B. thuringiensis* toxins is fundamentally important to explaining resistance.

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