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A membrane associated metalloprotease cleaves Cry3Aa *Bacillus thuringiensis* toxin reducing pore formation in Colorado potato beetle brush border membrane vesicles

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Abstract

Insect proteases are implicated in *Bacillus thuringiensis* insecticidal proteins mode of action determining toxin specificity and sensitivity. Few data are available on the involvement of proteases in the later steps of toxicity such as protease interaction with toxin–receptor complexes and the pore formation process. In this study, a Colorado potato beetle (CPB) midgut membrane metalloprotease was found to be involved in the proteolytic processing of Cry3Aa. Interaction of Cry3Aa with BBMV membrane proteases resulted in a distinct pattern of proteolysis. Cleavage was demonstrated to occur in protease accessible regions of domain III and was specifically inhibited by the metalloprotease inhibitors 1,10-phenanthroline and acetohydroxamic acid. Proteolytic inhibition by a peptide representing a segment of proteolysis in domain III and the metalloprotease inhibitor acetohydroxamic acid correlated with increased pore formation, evidencing that Cry3Aa is a specific target of a CPB membrane metalloprotease that degrades potentially active toxin.

Keywords: *Bacillus thuringiensis*; Colorado potato beetle; Insecticidal toxin; Protease; Cry3Aa proteolysis; Pore formation

1. Introduction

Bacillus thuringiensis (*Bt*) insecticidal proteins mode of action has been described as a step-wise mechanism that initiates with ingestion, followed by solubilization and protease activation in the lumen of the insect midgut, and culminates in the vicinity of the midgut membrane with the postactivation events of toxin–receptor interaction, oligomerization and pore formation. To exert their action, toxins need to be previously activated by insect midgut proteases that must play a major role in toxicity [1].

Lepidopteran-active toxins are obtained via proteolytic processing which has been reported to proceed by removal of both N- and C-terminal peptides. The 73 kDa coleopteran-specific Cry3Aa toxin has been considered as a naturally truncated version of the lepidopteran toxins [2]. Nevertheless, extensive N-terminal processing of this toxin resulted in a more toxic form than the native toxin [3]. In *Leptinotarsa decemlineata*, Colorado potato beetle (CPB), although midgut proteases are mainly cysteine and aspartate proteinases [4] a chymotrypsin-like serine-protease has been proposed to activate Cry3Aa toxin to a 49-kDa form which can be detected *in vivo* [5] and is able to bind to CPB brush border membrane vesicles (BBMV) [6].

The activity or composition of gut proteases influences susceptibility by insects to Cry toxins and may provide the basis for the specificity of *Bt* in different insect species and resistance development [7]. In CPB, Loseva et al. [8] reported that resistance to Cry3Aa toxin correlates with the presence of different forms of digestive proteases in a resistant strain that also displayed decreased binding of the toxin.

Excessive toxin degradation has been implicated in insensivity to *Bt* toxins. Strong proteolytic activity of the midgut juice contributed to further degradation of activated toxin in *Heliothis armigera* [9] and in *Choristoneura fumiferana* [10] rendering both insects less susceptible to *Bt* toxins than *Bombyx mori* where activated toxin remains unprocessed. Resistance to

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Bt toxins developed by *Heliothis virescens* in a resistant strain has been related to slower Cry1Ac protoxin activation and faster processing of the active toxin than in the susceptible strain [11].

While there is reasonable understanding of the effect of soluble midgut digestive proteases in toxin activation, little is known about the role of membrane protease processing of *Bt* toxins in relation to toxin–receptor complex and pore formation events of the *Bt* mode of action. It has been reported that the dipteran active Cry11Aa toxin undergoes a specific pattern of proteolysis when incubated with *Culex quinquefasciatus* BBMV [12] and that proteases associated with CPB BBMV, but not gut juice, cleave the Cry3Aa toxin generating a 38-kDa fragment [8]. In both cases the functional significance of this processing remains unclear. We have previously demonstrated that Cry3Aa toxin pre-incubated with CPB BBMV in the absence of midgut juice proteases form pores more efficiently that the native toxin [13], suggesting that membrane molecules could be involved in the mechanism of activation of this toxin for effective membrane insertion, as it had been proposed for the lepidopteran-specific toxin Cry1Ab [14]. In contrast, Kirouac et al. [15] recently reported that Cry1Aa toxin, once activated by midgut juice proteases, is completely functional and does not require further BBMV proteolysis.

In the present study, we show that Cry3Aa toxin is specifically cleaved by BBMV associated metalloproteases, which drastically reduce the pore forming activity of this protein. Elimination of *Bt* toxins via degradation by midgut membrane proteases may account for the observed decrease in pore formation and has to be considered as an additional mechanism that impairs the Cry toxins entomopathogenic action. Membrane associated proteases could therefore be involved either in toxin activation or in its further degradation, both at the same time or neither.

2. Materials and methods

2.1. Insects

A laboratory colony of *L. decemlineata* (Colorado potato beetle) founded from eggs taken from the field was used. Larvae and adults were reared on potato leaves at 25 °C and with a photoperiod of 16:8 (light/dark).

2.2. Preparation of brush border membrane vesicles (BBMV)

BBMV were prepared from last instar *L. decemlineata* larvae according to the method of Wolfersberger et al. [16], as modified by Reuveni and Dunn [17]. Larvae were dissected in storage buffer (300 mM mannitol, 20 mM 2-mercaptoethanol, 5 mM EGTA, 1 mM EDTA, 10 mM HEPES, 2.4 μg/ml neomycin sulphate, pH 7.5) and the insect midguts obtained were immediately frozen and stored at −80 °C until use. Frozen midguts were mechanically homogenized in homogenization buffer (200 mM mannitol, 10 mM ascorbic acid, 5 mM EDTA, 2 mM DTT, 10 mM HEPES, pH 7.4) for 10 s. One volume of 24 mM $MgCl_2$ was added and the mixture incubated for 10 min. Following centrifugation of the mixture (10 min, 6000×*g* at 4 °C), the supernatant was further centrifuged (30 min, 30,000×*g* at 4 °C) and the final pellet suspended in 200 mM mannitol, 1 mM DTT, 1 mM HEPES–Tris, pH 7.4, frozen and stored at −80 °C until use. The protein concentration of BBMV was measured by Bradford's procedure [18] using bovine serum albumin (BSA) as a standard.

2.3. Crystal purification

Cry3Aa crystals were produced in *Bt* strain BTS1. Crystal inclusions were separated from spores and cell debris by centrifugation in discontinuous 67%, 72%, 79%, and 84% (w/v) sucrose gradients in 50 mM Tris–HCl, pH 7.5, as described by Thomas and Ellar [19]. Crystal proteins were solubilized in 50 mM Na_2CO_3, pH 10.5, at 37 °C for 12 h. Purity of the crystal preparation was monitored by phase contrast microscopy and analyzed by 10% SDS-PAGE. Protein concentration was measured by the protein-dye method of Bradford [18], using BSA as a standard.

2.4. Incubation of Cry3Aa with CPB BBMV

Cry3Aa toxin (2 μM) was incubated with 20 μg BBMV in a final volume of 30 μl PBS (8 mM Na_2HPO_4, 2 mM KH_2PO_4, 150 mM NaCl, pH 7.4), for 10 min at room temperature, and centrifuged 20 min at 12,000×*g*. The pellet and/or supernatant were loaded in 10% SDS-PAGE gels and the resolved proteins were transferred onto a nitrocellulose membrane (Millipore) and immunoblotted against anti-Cry3Aa polyclonal antibody. The secondary antibody was alkaline phosphatase-conjugated anti-rabbit antibody (Sigma). The immunoreactive proteins were visualized using the ECL detection system Immobilon Western (Millipore).

In Cry3Aa cleavage inhibition assays, incubation was performed in the presence or absence of protease inhibitors and peptides designed to inhibit proteolysis. The concentration and type of inhibitor used in each experiment is described in the figure legends.

2.5. Calcein release experiments

Calcein containing CPB BBMV were prepared as described previously [13]. Calcein leakage experiments were performed with 75 μg of calcein loaded BBMV added to 900 μl of 150 mM KCl, CHES 10 mM, pH 9.0, and 20 μl of the supernatant of Cry3Aa pre-incubated with BBMV, in the presence or absence of 150 μM pep-99 peptide or 2 μM of the proteolytic cleavage inhibitor aceto-hydroxamic acid (final concentration). Maximal leakage at the end of each experiment was assessed by lysis with Triton X-100 (0.1% final concentration).

The increase in fluorescence due to the release of calcein in the solution was monitored at room temperature with constant agitation in a VARIAN Cary Eclipse Fluorescence Spectrophotometer equipped with a thermostatically controlled cell holder, with excitation and emission wavelengths of 490 and 520 nm, respectively.

2.6. Cry3Aa labelling

Solubilized Cry3Aa toxin (200 μg) was precipitated using the Plus One 2-D Clean-Up Kit (Amersham) and re-suspended in 50 μl of buffer containing 30 mM Tris–HCl, pH 8.5, 7 M urea, 2 M thiourea and 4% (w/v) Chaps. The protein concentration was determined by the RC-DC Protein Assay (Bio-Rad). Minimal labelling of Cry3Aa with Cy DIGE fluorophores was performed using succinimidyl ester of propyl-Cy3 and methyl-Cy5 fluorophores following Amersham Biosciences instructions.

2.7. Two-dimensional electrophoresis

The first dimension, isoelectric focusing (IEF), was performed in 3 mm× 24 cm immobiline™ Dry Strips (Amersham Biosciences) with a NL pH 3–10 gradient. The strips were rehydrated with a solution containing 7 M urea, 2 M thiourea, 2% CHAPS, 20 mM DTT, 1.2% (v:v) Destreak (Amersham) and 0.5% Pharmalyte 3–10 NL. Following overnight rehydration, Cy3- and Cy5-labelled protein samples (100 μg) were loaded onto the strips using a cup loading technique. IEF was performed in a voltage step-gradient (300 V for 3 h; 1000 V for 6 h; 8000 V for 3 h; 8000 V 4 h; 500 V 10 h), using Ettam IPGPhor isoelectric focusing system (Amersham Biosciences). Prior to the second dimension (SDS-PAGE), the strips were equilibrated for 15 min in a solution containing 50 mM Tris–HCl buffer, pH 8.6, 6 M urea, 30% glycerol, 2% SDS 1% DTT followed by a 15-min incubation with 50 mM Tris–HCl buffer, pH 8.6, 6 M urea, 30% glycerol, 2% SDS and 2.5% iodoacetamide. After equilibration,

the strips were applied to 10% (w/v) polyacrylamide-SDS gels in low-fluorescence glass plates. Electrophoresis was performed at 20 °C in an Ettan DALTsix electrophoresis unit and the gels were run overnight at constant current at 1 W per gel. Cy3- and Cy5-labelled polypeptides were detected in gels using a Typhoon 9400TM scanner (Amersham Biosciences).

For N-terminal sequencing, after 2-D electrophoresis the gel was electroblotted onto a PVDF membrane and protein spots excised for Edman degradation.

3. Results

3.1. Intramolecular proteolytic cleavage of Cry3Aa catalyzed by CPB BBMV

In a previous work we demonstrated that Cry3Aa incubation with CPB BBMV resulted in the formation of a high molecular weight oligomeric structure [13]. Concomitantly, we observed that the toxin was readily proteolyzed by proteases associated to BBMV yielding fragments which can be separated from the membranes by centrifugation (Fig. 1, panel A). Cry3Aa was mainly processed into three bands, with an apparent molecular mass of 42, 30 and 26 kDa on SDS-PAGE (Fig. 1, panel A lane 2). In some supernatants where the lower molecular mass fragments of 30 and 26 kDa appear very faint relatively to the 42-kDa fragment, a 38-kDa fragment was also observed (Fig. 4, Panel A, first lane). No changes in the proteolytic profile were observed increasing the incubation time up to 2 h within the pH range from 7.4 to 11.0 (data not shown).

To analyze the polypeptide profile of Cry3Aa cleaved by BBMV proteases, the toxin was fluorescent-labelled and the proteolysis fragments separated by two-dimensional electrophoresis. Cy5-Cry3Aa labelled toxin was incubated with CPB BBMV and the resulting soluble fraction containing the cleavage fragments was mixed with Cy3-Cry3Aa labelled non-cleaved control toxin. Proteins in the mixture were separated by two-dimensional electrophoresis (Fig. 1, panel B). Green fluorescent spots corresponded to Cy3-Cry3Aa non-cleaved control toxin, red spots to fragments generated by BBMV proteolytic cleavage of Cy5-Cry3Aa toxin and yellow spots to Cry3Aa fragments that were present in both Cy3-Cry3Aa non-cleaved toxin and proteolyzed Cy5-Cry3Aa toxin. To characterize the proteolytic fragments, red spots were selected and N-terminal sequenced. Two sequences were unambiguously determined, SLDGA and DKV, with serine-578 and aspartic acid-631 of the deduced Cry3Aa amino acid sequence at the N-terminus, respectively, both located in domain III loops facing the same surface of interaction with the membrane protease (Fig. 2). The SLDGA sequence and its flanking regions in the Cry3Aa sequence were selected to design a 12-mer peptide, pep-99 (Ac-FTLSLDGAP-FNQ-NH_2), to be used as competitor in Cry3Aa cleavage assays with CPB BBMV. As it is shown in Fig. 3, pep-99 prevented Cry3Aa proteolysis in a dose-dependent manner. This result indicates that this sequence might be a specific target of a CPB BBMV protease.

3.2. Inhibitors of metalloproteases prevent Cry3Aa cleavage by CPB BBMV

To characterize the membrane proteases involved in Cry3Aa proteolytic cleavage, the effect of a variety of protease inhibitors was analyzed (Fig. 4, panel A). The most effective inhibition was observed with metalloprotease inhibitors, whereas other types had minimal or no inhibitory effect. This suggests that a metalloprotease associated to CPB BBMV is responsible of Cry3Aa cleavage. The sensitivity of Cry3Aa proteolysis to metalloprotease inhibitors was mainly due to 1,10-phenanthroline (Fig. 4, panel B) which was shown to be dose-dependent (Fig. 4, panel

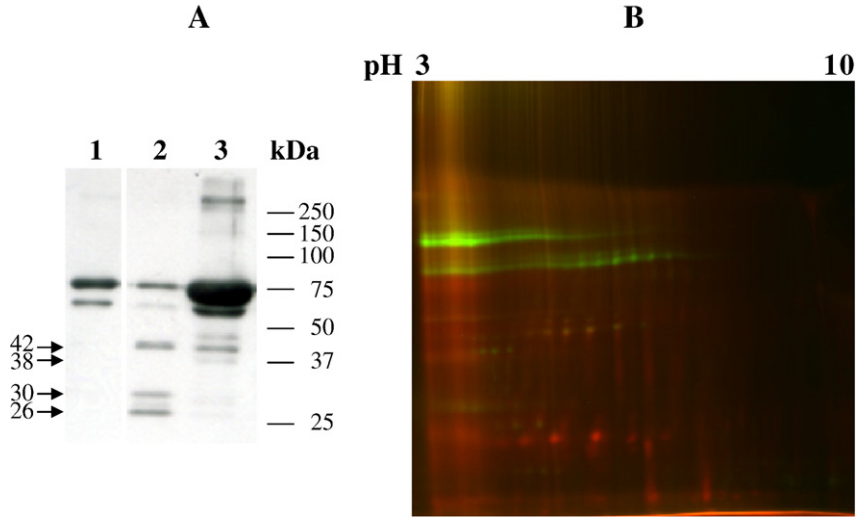

Fig. 1. Polypeptide profile of Cry3Aa cleaved by CPB BBMV proteases. Panel A, western blot of Cry3Aa toxin incubated with CPB BBMV. Lane 1, Cry3Aa protoxin; lanes 2 and 3, supernatant and pellet of Cry3Aa incubated with CPB BBMV. Panel B, fluorescent scanning of the two-dimensional electrophoretic separation of fragments in a mixture of 50 μg of Cy3-Cry3Aa labelled non-cleaved control toxin (green spots) and 50 μg of Cy5-Cry3Aa labelled toxin proteolytically cleaved by incubation with CPB BBMV (red spots). Yellow spots correspond to the superimposed image of a green and a red spot. The pH range of isoelectric focusing is indicated at the top. *Note*. The white space between lanes 1 and 2 denotes that the lanes were not contiguous in the gel.

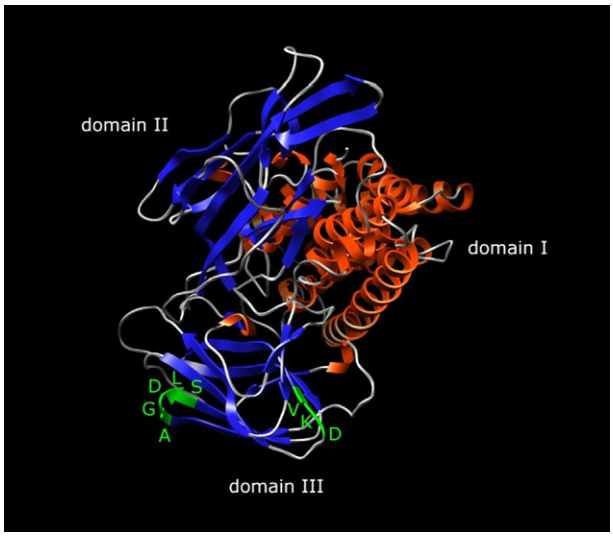

Fig. 2. Schematic ribbon presentation of the Cry3Aa toxin three domain organization. Molecular graphical model of Cry3Aa toxin was produced with Chimera [30]. Highlighted in green are the segments corresponding to N-terminal sequences of two cleavage fragments in domain III obtained by CPB BBMV proteolysis.

C). Another metalloprotease inhibitor, acetohydroxamic acid, also significantly inhibited Cry3Aa proteolysis (Fig. 4, panel B).

3.3. Functional significance of BBMV proteolysis of Cry3Aa

Previous work on CPB demonstrated that Cry3Aa protein incubated with CPB BBMV had higher pore formation activity than the protoxin itself [13]. Therefore to assess the effect of Cry3Aa proteolysis on the ability of this toxin to form pores *in vitro,* calcein leakage experiments were carried out using the soluble fraction of Cry3Aa pre-incubated with CPB BBMV, where proteolytic fragments are mainly recovered. Supernatants obtained after the pre-incubation in the presence or absence of inhibitors of the metalloprotease inhibitors 1,10-phenanthroline and acetohydroxamic acid, or the competitor peptide pep-99 were added to calcein loaded BBMV and the release of calcein was monitored (Fig. 5). Unfortunately, 1,10-phenanthroline could not be used in calcein release experiments because it interfered with calcein fluorescence emission. Cry3Aa pore formation activity in the presence of the metalloprotease inhibitor acetohydroxamic acid or the competitor peptide pep-99 was significantly higher than that of Cry3Aa. These data are in accordance with the existence of a degradative membrane associated proteolytic activity that if inhibited results in a higher effective Cry3Aa concentration that efficiently forms membrane pores.

Comparison of curves labelled 3A and 3A (BBMV pre-incubated) in Fig. 5 also shows that interaction with BBMV is essential to activate the toxin and, as previously reported [13], this can be achieved only if toxin is BBMV pre-incubated. The sequence of changes associated to the pre-incubation is not well understood. We varied BBMV and toxin concentration, incubation time and buffer composition and pH (data not shown), and we observed that in the experimental conditions stated in Materials and methods the ratio toxin:BBMV in a fixed volume is essential to effectively activate the toxin.

4. Discussion

Colorado potato beetle has become the most significant pest on potato and other Solanacea, which includes economically important crop plants such as tomato and eggplant. *Bt* based bioinsecticides, safe for users and the environment, represent a viable alternative for the biological control of this pest, which to date has developed resistance to multiple synthetic chemical insecticides. Managing the pest successfully requires identification of midgut protease activities involved in toxin activation and/or degradation and membrane molecules that can act as receptors that promote membrane insertion leading to cell toxicity.

We observed that it was not possible to incubate Cry3Aa with CPB BBMV without proteolytic cleavage, as it has been also described for the dipteran specific Cry11Aa toxin [12]. This may be a relevant process in the insecticidal action that diminishes toxin effectiveness. Our observation agrees with the Cry3Aa proteolytic processing by CPB BBMV previously reported [8] in which a major 38-kDa band resistant to any further degradation was obtained, although we also detected a 42-kDa band and in some experiments, proteolytic fragments lower than 38 kDa. Replicated experiments evidenced that the relative intensity of the Cry3Aa proteolytic bands depended on the BBMV and Cry3Aa toxin preparations, and that the proteolytic profile obtained did not change upon prolonged BBMV incubation (data not shown), except for the 38-kDa band that seems to be only detected when is not completely further processed to the 30- and 26-kDa bands. Proteolysis bands resolved in two-dimensional electrophoresis revealed a reproducible more complex membrane proteolytic pattern (Fig. 1, panel B). The specific proteolytic profile obtained upon incubation with CPB BBMV allowed us to identify by N-terminal analysis two cleavage sequences, SLDGA and DKV. These protease-sensitive sequences are located in regions accessible to proteases in Cry3Aa domain III (Fig. 2) [20]. The N-terminal DKV polypeptide contains a block of 10 amino acids (DKVYIDKYEFIPVN) at the extreme C-terminal of Cry3Aa found to be almost identical in other Cry toxins, including

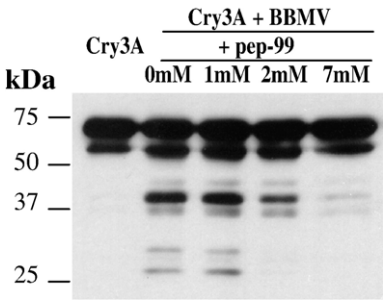

Fig. 3. Effect on Cry3Aa proteolysis by CPB BBMV of synthetic peptide pep-99. Western blot of the supernatants obtained after incubation of Cry3Aa toxin with CPB BBMV in the absence or presence of increasing concentrations of pep-99 (Ac-FTLSLDGAPFNQ-NH_2).

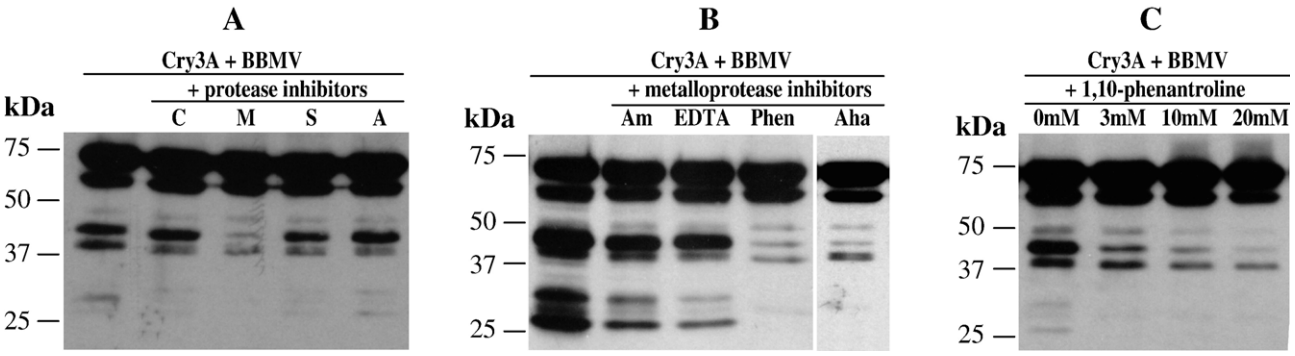

Fig. 4. Effect of different protease inhibitors on Cry3Aa proteolytic cleavage catalyzed by CPB BBMV. All panels show western blots of the supernatants obtained after incubation of Cry3Aa with CPB BBMV in the absence or presence of protease inhibitors. Panel A, four different cocktails of class-specific protease inhibitors: C, Cysteinprotease inhibitors (1 mM PMSF, 10 μM E-64, 100 μM leupeptin); M, Metalloprotease inhibitors (0.1 mM amastatin, 10 mM EDTA, 10 mM 1–10, phenanthroline); S, Serinprotease inhibitors (1 mM PMSF, 100 μM leupeptin, 0.1 mM TPCK, 0.1 mM TLCK, 5 mM Pefabloc, 0.1 mg/ml soybean trypsin inhibitor); A, Aspartylprotease inhibitor (50 μM pepstatin A). Panel B, four different metalloprotease inhibitors, (0.1 mM amastatin, 10 mM EDTA, 10 mM 1–10, phenanthroline, 50 mM acetohydroxamic acid); and Panel C, increasing concentrations of the metalloprotease inhibitor 1,10-phenanthroline. Lines at the left of each blot represent molecular weight markers corresponding to 75, 50, 37 and 25 kDa. *Note*. Last lane, Panel B (Aha) is separated from the rest because is not adjacent to the others in the gel.

three specific for Lepidoptera and two active against Diptera [3,21]. This region that is essential for toxicity in *Manduca sexta* [22], may also be in CPB, therefore the C-terminal processing observed in Cry3Aa would result in a loss of activity *in vivo* against CPB.

The N-terminal SLDGA polypeptide is located in a segment on and adjacent to β-18 within domain III outer sheet of Cry3Aa. In this region, Cry1Ac displays a six-residue insertion unique to this toxin [23] which is important to GalNAc-mediated receptor binding [24]. To investigate the specificity of this site of cleavage, we designed a 12-mer peptide with an amino acid sequence containing the SLDGA region (pep-99) that was confirmed to effectively inhibit proteolysis (Fig. 3). Given the multiple steps in the mechanism of action of these pore-forming toxins, membrane permeabilization experiments constitute a very useful approach to assess toxicity. The rate of BBMV calcein release in the presence of this peptide was increased, showing that Cry3Aa membrane associated proteolysis leads to reduced pore formation. These results indicate that Cry3Aa domain III is specifically recognized and cleaved by membrane proteases, which eliminate potentially active toxin via degradation. In *H. armigera*, it has been suggested that further degradation of activated toxin may occur *in vivo,* because bioassays in which HD-1 protoxin was combined with serine protease inhibitors showed an increase of mortality from 17% to 70% [9].

To analyze the effect of Cry3Aa proteolysis on the *in vitro* pore formation activity of this toxin, protease inhibitors that effectively blocked toxin proteolytic processing were also assayed in pore formation experiments. Significant proteolysis

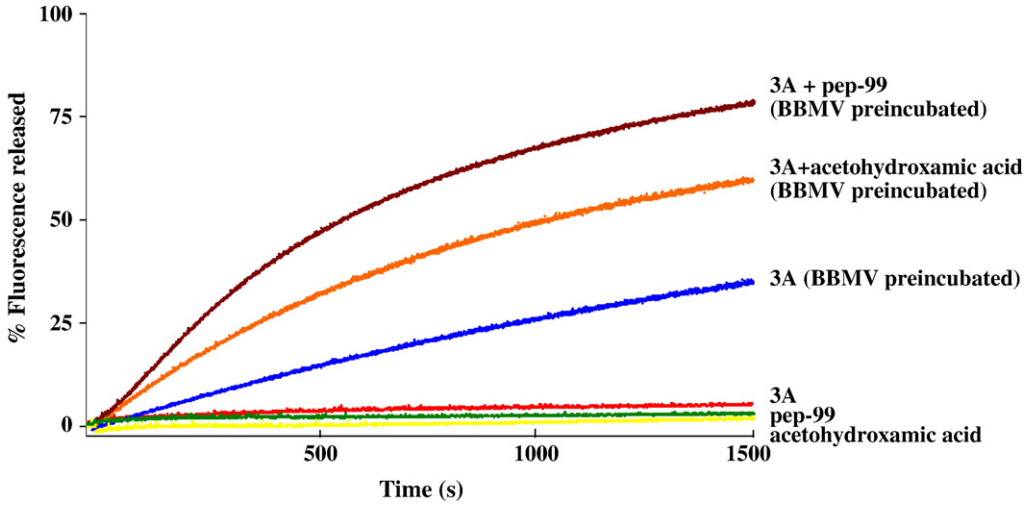

Fig. 5. Analysis of Cry3Aa toxin induced calcein leakage from CPB BBMV in the absence and presence of the cleavage inhibitors pep-99 and acetohydroxamic acid. Experiments were performed at least in triplicate as described in Materials and methods and representative traces are shown. Pep-99 peptide (150 μM), acetohydroxamic acid (2 μM), and Cry3Aa (44 nM) not pre-incubated with BBMV were independently added to calcein loaded vesicles, as controls. Release of calcein was monitored for 1500 s, excitation and emission wavelengths of 490 and 520 nm, respectively. Maximum fluorescence intensity obtained by addition of Triton X-100 corresponded to 100% of released calcein.

inhibition was detected with the metalloprotease inhibitors 1,10-phenanthroline and acetohydroxamic acid. Given that 1,10-phenanthroline interfered with calcein fluorescence emission, acetohydroxamic acid was assayed in pore formation experiments. As observed when pep-99 was used to prevent Cry3Aa cleavage, in the presence of acetohydroxamic acid the rate of Cry3Aa pore formation was increased, confirming the degradative action of CPB membrane metalloproteases on this toxin. Carroll et al. [5] reported that chymotrypsinized Cry3A was as toxic as the native toxin and proposed this was due to the fact that all Cry3A segments remained associated in solution and the insecticidal activity was not affected by the nicking of the polypeptide backbone. Our results also support a role for all Cry3A constituent parts since pore-forming activity significantly increased when proteolysis was prevented. We have previously reported that toxin preincubation with CPB BBMV more effectively increased Cry3A pore-forming activity than proteolytic processing by enzymes such as trypsin and chymotrypsin [13]. In the present study we give further evidence to the relevance of the interaction of the toxin with the midgut membranes to become activated. Western blot analysis reveals that the most active toxin in terms of pore formation (Cry3Aa BBMV preincubated in the presence of pep-99 and acetohydroxamic acid) resembles that of the non activated Cry3Aa protoxin in that both of them lack the proteolytic fragments. Whether toxin interaction with the membrane metalloprotease, independently of its catalytic activity, may account for the activation effect observed upon BBMV contact is still unknown, but it cannot be ruled out in view of its dramatic impact on pore-forming activity despite Cry3Aa proteolysis by CPB BBMV appeared to affect only a small fraction of the protoxin.

Metalloproteases are the most diverse of the four main types of proteases, with more than 50 families identified to date [25]. Although some of the most widely studied metalloproteases have been digestive enzymes such as carboxypeptidases A and B and thermolysin, a considerable advance in the understanding of structure and function of membrane metalloendopeptidases has been made within the last years. These proteases are implicated not only in protein degradation but also are important in many aspects of biology, ranging from cell proliferation, differentiation and remodelling of the extracellular matrix (ECM) to vascularization, cell migration and tumor progression. Most of these processes require a delicate balance between the functions of matrix metalloproteases (MMPs) or metalloprotease-disintegrins (ADAMs) and natural tissue inhibitors of metalloproteases (TIMPs) [26]. To our knowledge only one insect metalloprotease has been reported to process *Bt* toxins, a thermolysin-like enzyme activity detected in *C. quinquefasciatus* BBMV, which was responsible for Cry11Aa cleavage [12]. In *Culex*, as well as it has been described in CPB with Cry3Aa [5,6,27], it is difficult to demonstrate binding to BBMV receptors most probably due to the rapid proteolysis of the toxin. The relevance of the Cry11Aa proteolysis with regard to pore formation and toxicity in *C. quinquefasciatus* has not been evaluated. Whether or not the inverse relationship between membrane proteolytic processing and pore formation that we describe here applies for other *Bt* toxins would need further research. In the case of Cry1A toxins that do not seem to undergo BBMV proteolytic processing [28], it has been reported that protease inhibitors did not alter pore formation [15].

It has been proposed that aminopeptidases do not serve as receptors for the Cry3Aa toxin [8] as it has been reported for other Cry toxins in lepidopteran insects [29]. Remarkably, the aminopeptidase N, as Cry1Ac receptor is independent of its metalloprotease enzymatic activity, but the Cry1Ac region of interaction with this receptor matched the SLDGA sequence of Cry3Aa domain III that undergoes cleavage. In CPB, the metalloprotease that cleaves Cry3Aa could be the counterpart of the aminopeptidase-N identified in Lepidoptera. If this were the case, interaction of the toxin with the metalloprotease could render an activated form of the toxin able to trigger subsequent steps leading to toxicity. The overall sensitivity of an insect to *Bt* toxins may reflect the balance between the activation of the toxin to an insertional-competent form and its degradative processing by midgut insect proteases.

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References

[1] A. Bravo, S.S. Gill, M. Soberón, *Bacillus thuringiensis* mechanisms and use, in: L.I. Gilbert, K. Iatrou, S.S. Gill (Eds.), Comprehensive Molecular Insect Science, vol. 6, Elsevier B.V., Amsterdam, 2005, pp. 175–206.

[2] H. Höfte, J. Seurinck, A. Van Houtven, M. Vaeck, Nucleotide sequence of a gene encoding an insecticidal protein of *Bacillus thuringiensis* var. tenebrionis toxic against Coleoptera, Nucleic Acids Res. 15 (1987) 7183.

[3] J. Carroll, J. Li, D.J. Ellar, Proteolytic processing of a coleopteran-specific δ-endotoxin produced by *Bacillus thuringiensis* var. *tenebrionis*, Biochem. J. 261 (1989) 99–105.

[4] D. Michaud, N. Bernier-Vornais, S. Overney, S. Yelle, Constitutive expression of digestive cysteine proteinase forms during development of the Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae), Insect Biochem. Mol. Biol. 25 (1995) 1041–1048.

[5] J. Carroll, D. Convents, J. Van Damm, A. Boets, J. Van Rie, D.J. Ellar, Intramolecular proteolytic cleavage of *Bacillus thuringiensis* Cry3A δ-endotoxin may facilitate its coleopteran toxicity, J. Invertebr. Pathol. 70 (1997) 41–49.

[6] A.C. Martínez-Ramírez, M.D. Real, Proteolytic processing of *Bacillus thuringiensis* CryIIIA toxin and specific binding to the brush-border

membrane vesicles of *Leptinotarsa decemlineata* (Colorado potato beetle), Pestic. Biochem. Physiol. 54 (1996) 115–122.
[7] B. Oppert, Protease interactions with *Bacillus thuringiensis* insecticidal toxins, Arch. Insect Biochem. Physiol. 42 (1999) 1–12.
[8] O. Loseva, M. Ibrahim, M. Candas, N.C. Koller, L.S. Bauer, L.A. Bulla Jr., Changes in protease activity and Cry3A toxin binding in the Colorado potato beetle: implications for insect resistance to *Bacillus thuringiensis* toxins, Insect Biochem. Mol. Biol. 32 (2002) 567–577.
[9] Z. Shao, Y. Cui, X. Liu, H. Yi, J. Ji, Z. Yu, Processing of delta-endotoxin of *Bacillus thuringiensis* subsp. kurstaki HD-1 in *Heliothis armigera* midgut juice and the effects of protease inhibitors, J. Invertebr. Pathol. 72 (1998) 73–81.
[10] A.S.D. Pang, J.L. Gringorten, Degradation of *Bacillus thuringiensis* δ-endotoxin in host insect gut juice, FEMS Microbiol. Lett. 167 (1998) 281–285.
[11] C. Forcada, E. Alcácer, M.D. Garcerá, R. Martínez, Differences in the midgut proteolytic activity of two *Heliothis virescens* strains, one susceptible and one resistant to *Bacillus thuringiensis* toxins, Arch. Insect Biochem. Physiol. 31 (1996) 257–272.
[12] S. Dai, S.S. Gill, In vitro and in vivo proteolysis of the *Bacillus thuringiensis* subsp. *israeliensis* CryIVD protein by *Culex quinquefasciatus* larval midgut proteases, Insect Biochem. Mol. Biol. 23 (1993) 273–283.
[13] C. Rausell, I. García-Robles, J. Sánchez, C. Muñoz-Garay, A.C. Martínez-Ramírez, M.D. Real, A. Bravo, Role of toxin activation on binding and pore formation activity of the *Bacillus thuringiensis* Cry3 toxins in membranes of *Leptinotarsa decemlineata* (Say), Biochim. Biophys. Acta 1660 (2004) 99–105.
[14] I. Gómez, J. Sánchez, R. Miranda, A. Bravo, M. Soberón, Cadherin-like receptor binding facilitates proteolytic cleavage of helix α-1 in domain I and oligomer pre-pore formation of *Bacillus thuringiensis* Cry1Ab toxin, FEBS Lett. 513 (2002) 242–246.
[15] M. Kirouac, V. Vachon, D. Quievy, J.L. Schwartz, R. Laprade, Protease inhibitors fail to prevent pore formation by the activated *Bacillus thuringiensis* toxin Cry1Aa in insect brush border membrane vesicles, Appl. Environ. Microbiol. 72 (2006) 506–515.
[16] M. Wolfesberger, P. Lüthy, A. Maurer, F. Parenti, V. Sacchi, B. Giordana, G.M. Hanozet, Preparation and partial characterization of amino acid transporting brush border membrane vesicles form the larval midgut of the cabbage butterfly (*Pieris brassicae*), Comp. Biochem. Physiol. 86A (1987) 301–308.
[17] M. Reuveni, P.E. Dunn, Differential inhibition by *Bacillus thuringiensis* delta endotoxin of leucine and aspartic uptake in BBMV from midgut of *Manduca sexta*, Biochem. Biophys. Res. Commun. 181 (1991) 1089–1093.
[18] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[19] W.E. Thomas, D.J. Ellar, *Bacillus thuringiensis* var. *israeliensis* crystal delta-endotoxin: effects on insect and mammalian cells in vitro and in vivo, J. Cell. Sci. 60 (1983) 181–197.
[20] J. Li, J. Carroll, D.J. Ellar, Crystal structure of insecticidal δ-endotoxin from *Bacillus thuringiensis* at 2.5 Å resolution, Nature 353 (1991) 815–821.
[21] W. Chungjatupornchai, H. Höfte, J. Seurinck, C. Angsuthanasombat, M. Vaeck, Common features of *Bacillus thuringiensis* toxins specific for Diptera and Lepidoptera, Eur. J. Biochem. 173 (1988) 9–16.
[22] H.E. Schnepf, H.R. Whiteley, Delineation of a toxin-encoding segment of a *Bacillus thuringiensis* crystal protein gene, J. Biol. Chem. 260 (1985) 6273–6280.
[23] J. Li, D.J. Deryshire, B. Promdonkoy, D.J. Ellar, Structural implications for the transformation of the *Bacillus thuringiensis* δ-endotoxins from water-soluble to membrane-inserted forms, Biochem. Soc. Transac. 29 (2001) 571–577.
[24] S.L. Burton, D.J. Ellar, J. Li, D.J. Derbyshire, N-acetylgalactosamine on the putative insect receptor aminopeptidase N is recognised by a site on the domain III lectin-like fold of a *Bacillus thuringiensis* insecticidal toxin, J. Mol. Biol. 287 (1999) 1011–1022.
[25] N.D. Rawlings, A.J. Barrett, Evolutionary families of metallopeptidases, Methods Enzymol. 248 (1995) 183–228.
[26] C. Chang, Z. Werb, The many faces of metalloproteases: cell growth, invasion, angiogenesis and metastasis, Trends Cell Biol. 11 (2001) S37–S43.
[27] C. Herrnstadt, G.C. Soares, E.R. Wilcox, D.L. Wilcox, A new strain of *Bacillus thuringiensis* with activity against coleopteran insects, Bio/Technology 4 (1986) 305–308.
[28] N.J. Tigue, J. Jacoby, D.J. Ellar, The α- helix 4 residue, Asn135, is involved in the oligomerization of Cry1Ac and Cry1Ab5 *Bacillus thuringiensis* toxins, Appl. Environ. Microbiol. 67 (2001) 5715–5720.
[30] E.F. Pettersen, T.D. Goddard, C.C. Huang, G.S. Couch, D.M. Greenblatt, E.C. Meng, T.E. Ferrin, UCSF chimera—A visualization system for exploratory research and analysis, J. Comput. Chem. 25 (13) (2004) 1605–1612.