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Role of toxin activation on binding and pore formation activity of the *Bacillus thuringiensis* Cry3 toxins in membranes of *Leptinotarsa decemlineata* (Say)

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Abstract

Binding and pore formation constitute key steps in the mode of action of *Bacillus thuringiensis* δ-endotoxins.

In this work, we present a comparative analysis of toxin-binding capacities of proteolytically processed Cry3A, Cry3B and Cry3C toxins to brush border membranes (BBMV) of the Colorado potato beetle *Leptinotarsa decemlineata* (CPB), a major potato coleopteran-insect pest. Competition experiments showed that the three Cry3 proteolytically activated toxins share a common binding site. Also heterologous competition experiments showed that Cry3Aa and Cry3Ca toxins have an extra binding site that is not shared with Cry3Ba toxin. The pore formation activity of the three different Cry3 toxins is analysed. High pore-formation activities were observed in Cry3 toxins obtained by proteolytical activation with CPB BBMV in contrast to toxins activated with either trypsin or chymotrypsin proteases. The pore-formation activity correlated with the formation of soluble oligomeric structures. Our data support that, similarly to the Cry1A toxins, the Cry3 oligomer is formed after receptor binding and before membrane insertion, forming a pre-pore structure that is insertion-competent.

Keywords: *Bacillus thuringiensis*; Protoxin activation; Mode of action

1. Introduction

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say) is a major potato insect pest in Europe, North and Central America, which to date has developed resistance to a wide variety of synthetic chemical insecticides [1]. Alternative approaches for effective control of this pest rely on the use of new insecticidal molecules with a totally different mode of action in order to circumvent the problem of resistance developed to conventional insecticides. Bio-insecticides based on *Bacillus thuringiensis* (Bt), which are safe for users and the environment, meet the requirements to offer a good alternative to chemical insecticides. Bt-based products have been increasingly used over the past decades for many different insect pests.

The gram-positive bacterium *B. thuringiensis* produces parasporal crystalline inclusions that contain proteins (δ-endotoxins) that are toxic to a broad range of insect species and other invertebrates such as nematodes, mites and protozoans [2]. The detailed molecular mechanism that mediates the insecticidal activity of these toxins is still being elucidated but basically it has been described as a multi-step process, which initiates upon ingestion of the parasporal inclusions by the susceptible larvae. The specific pH and proteases in the insect midgut favour solubilization and proteolytic cleavage of the inclusions. The activated toxins bind to highly specific receptors on the insect midgut brush border membrane (BBMV) [3,4]. Following binding, it is proposed that at least part of the toxin inserts into the membrane resulting in pore formation [5,6]. It has been proposed that an oligomer formation is a necessary step for pore formation. Supporting this hypothesis, a tetrameric oligomer of Cry1Ac was observed in synthetic membranes [7] and the formation of a stable oligomeric structure of Cry1A toxins by incubation of

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Cry proteins with BBMV of target insects has been described [8–10]. The oligomeric structure leads finally to the formation of lytic pores [8] followed by cell lysis, and insect death.

The δ-endotoxins from Bt comprise a group of over 116 Cry proteins that have been classified into 40 subgroups based on amino acid identity [11]. The *cry3* class genes, which occur in *B. thuringiensis* subsp. *tenebrionis*, *san diego*, *morrisoni*, *tolworthi*, and *galleriae*, encode for the Cry3A, Cry3B and Cry3C groups of proteins, which exhibit coleopteran-specific activity [12–15]. The CPB has been reported to be susceptible to the three Cry3 toxins [16–18].

Cry3A interaction with CPB midgut cell membranes has been extensively studied and a number of reports concerning its toxic effect and particular aspects of its mode of action have been described [19–23]. However, until now there are no studies describing the pore formation activity of Cry3 toxins in membranes of this insect.

It is generally accepted that protease activity influences Cry toxin target specificity. The effect of toxin proteolytic cleavage by different insect midgut proteases correlates with the toxicity of some Bt toxins against different insect pests [24,25]. The Cry protoxin activation is a complex process, since besides the proteolysis at the N- and C-termini, intramolecular processing within domains I and II have been reported for several toxins including Cry3 toxins [20,24,26,27]. Cleavage within domain I has been correlated with toxin activation [8,21,26,28]. On the other hand, the lack of a major gut protease could result in insect resistance to Cry toxins [27] and the fast degradation of some Cry toxins was associated with low sensitivity for those Cry toxins [29].

In this work, we present a comparative analysis of toxin-binding capacities of proteolytically processed Cry3 toxins to BBMV isolated from midgut cells from CPB. Also the pore formation activity of the three different Cry3 toxins is presented. Our results show that as in Cry1A lepidopteran-specific toxins, the pore formation activity of Cry3 toxins also depends on the formation of an oligomeric pre-pore structure after protoxin interaction with the microvilli membranes.

2. Materials and methods

2.1. Insects

L. decemlineata eggs were kindly provided by Dr. Leo Koopman from the University of Wageningen (The Netherlands). Larvae and adults were reared on potato leaves at 25 ± 1 °C and a photoperiod of 16:8 (light/dark).

2.2. Crystal purification

Cry3Aa, Cry3Ba and Cry3Ca crystals were produced in Bt strains BTS1, BTS00125L and BTS02109P, respectively. Crystal inclusions were purified from spores and cell debris by centrifugation in discontinuous 67%, 72%, 79%, 84%, and 90% (w/v) sucrose gradients in 50 mM Tris–HCl, pH 7.5, as described by Thomas and Ellar [30]. The crystal band was removed and washed three times in 50 mM Tris–HCl pH 7.5. Purity of the crystal preparation was monitored by phase contrast microscopy and analysed by 10% SDS-PAGE as described by Laemmli [31]. Protein concentration was measured by the protein-dye method of Bradford [32], using bovine serum albumin (BSA) (New England Bio-Labs, Beverly, MA) as a standard.

2.3. Protein solubilization and processing of B. thuringiensis toxins

Crystal proteins were solubilized in extraction buffer (50 mM Na_2CO_3, pH 10.2) at 37 °C for 12 h. The pH of the supernatant was adjusted to 8.5 with 50 mM Tris–HCl pH 8, and protoxins were digested with trypsin (1:5 w/w, enzyme/substrate ratio) or with chymotrypsin (2:1 w/w, enzyme/substrate ratio) for 12 h at 37 °C. The digestion was stopped by addition of 0.1 mM PMSF final concentration. Alternatively, Cry3 protoxins were activated with intrinsic proteases present in the BBMV preparations obtained in the absence of protease inhibitors. Cry protoxins (100 nM) were incubated 30 min with 10 μg BBMV, reaction was stopped with 0.1 mM PMSF and centrifuged to obtain the activated Cry3 toxins in the soluble fraction (20 min at 12,000 × *g*). Processed toxins were loaded in 10% SDS-PAGE gels to check the extent of digestion and the amount of protein obtained was determined according to Bradford [32].

2.4. Preparation of brush border membrane vesicles

BBMVs were prepared from last instar *L. decemlineata* larvae according to the method of Wolfersberger et al. [33], as modified by Reuveni and Dunn [34]. Larvae were dissected in storage buffer (300 mM mannitol, 20 mM 2-mercaptoethanol, 5 mM EGTA, 1 mM EDTA, 0.1 mM PMSF, 150 μg/ml pepstatin A, 100 μg/ml leupeptin, 1 μg/ml soybean trypsin inhibitor, 10 mM HEPES 2.4 μg/ml neomycin sulfate pH 7.5) and the insect midguts obtained were immediately frozen and stored at − 80 °C until use. For BBMV preparation, frozen midguts were mechanically homogenized in homogenization buffer (200 mM mannitol, 10 mM ascorbic acid, 5 mM EDTA, 0.03% w/v PMSF, 1% mM PVPP, 0.2 mM leupeptin, 2 mM DTT, 10 mM HEPES pH 7.4) for 10 s. One volume of 24 mM $MgCl_2$ was added and the mixture was 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.

2.5. Binding assays on isolated BBMVS

Processed toxins were biotinylated using biotinyl-*N*-hydroxysuccinimide ester (RPN28, Amersham) according

to the manufacturer indications. Biotinylated chymotrypsinized toxins (20 nM Cry3Aa, 20 nM Cry3Ba, 10 nM Cry3Ca) or biotinylated trypsinized toxins (5 nM Cry3Aa, 20 nM Cry3Ba and 5 nM Cry3Ca) were incubated with 10 μg of BBMVs in PBS buffer, pH 7.6, for 1 h in the presence or absence of 250–500-fold excess of unlabelled chymotrypsinized or trypsinized toxins, respectively. Subsequently, unbound toxin was removed by centrifugation (10 min at 14,000 × *g*) and BBMV were washed twice with the same buffer (500 μl); BBMV were suspended in 20 μl of PBS, and an equal volume of Laemmli sample loading buffer 2 × was added. Samples were boiled for 5 min, loaded in a SDS-PAGE gel, and electrotransferred to a nitrocellulose membrane. The biotinylated proteins that were bound to the blotted protein vesicles were visualized by incubating with streptavidin-peroxidase conjugate (1:4000 dilution) for 1 h, followed by addition of Supersignal West Pico Chemiluminescent substrate (Pierce), as described by the manufacturers.

2.6. Fluorescence measurements

Membrane potential was monitored with the fluorescent positively charged dye, 3,3'-dipropylthiodicarbocyanine (Dis-C_3-(5)), at 620–670 nm wavelengths in an Aminco Bowman Luminescence Spectrometer (Urbana IL, USA) as in Ref. [5]. Hyperpolarization causes dye internalization into the BBMV and a decrease in fluorescence; depolarization causes the opposite effect. BBMV (10 μg) previously loaded with 150 mM KCl were suspended in 900 μl of 150 mM *N*-methyl-D-glucamine chloride (MeGluCl), 10 mM MES pH 6 buffer. After equilibration of the dye (2 min), 50 nM Cry3 toxin was added. Changes in membrane potential were monitored by successive additions of KCl (3, 6, 12, 24 and 50 mM) to the BBMV suspension. Analyses of the slope (*m*) of Δ*F* [%] vs. K^+ equilibrium potential (E_K) (mV) curve are reported in this work. E_K was calculated with the Nernst equation. Membrane potential determinations were done three times.

Calcein leakage experiments were performed as described [35]. Calcein containing vesicles were prepared by sonication (three times for 30 s) of the BBMV (300 μg protein) in calcein 80 mM (Molecular Probes, Eugene, OR) dissolved in 150 mM KCl, Chaps 10 mM pH 9. Non-entrapped calcein was removed by gel filtration on sephadex G-50 (1 × 30 cm column) with the elution buffer containing 150 mM KCl, CHES 10 mM, pH 9. Ten micrograms of BBMV were added to 900 ml 150 mM KCl, CHES 10 mM, pH 9. After mixing BBMV and toxin (50 nM), the release of calcein produced an increase in fluorescence due to the dequenching of the dye into the external medium. Calcein fluorescence was excited at 490 nm (10 nm slit) and monitored at 520 nm with an Aminco Bowman Luminescence Spectrometer. Maximal leakage at the end of each experiment was assessed by lysis with 0.1% Triton X-100 (final concentration). All experiments were performed in triplicate at room temperature.

2.7. Bioassays

Toxicity assays were performed on neonate larvae of *L. decemlineata* exposing 12 larvae to 3.5 cm^2 potato leaf disks previously dipped for 1 min in different trypsinized Cry3Ba toxin solutions diluted in PBS, 0.1% BSA, 0.2% Triton AG-98. The toxin concentrations assayed ranged from 0.35 to 35 μg/ml, and two replicates per dose and a control of disk leaf without toxin were included. After 24 h, the leaf disks were replaced by fresh leaves without toxin. Mortality was recorded 5 days after the initial treatment.

3. Results

3.1. Proteolytic activation of Cry3 protoxins

Previous work on CPB demonstrated that chymotrypsin-activated Cry3Aa showed specific and saturable binding to BBMV [19,20]. In this work, we want to compare binding and pore formation activities of the three Cry3 toxins described as being active against this insect pest. The Cry3A, Cry3B and Cry3C protoxins were activated with trypsin or chymotrypsin. Fig. 1 shows SDS-PAGE analysis of the activated toxins. The trypsin treatment of the Cry3A and Cry3B protoxins produced a 55 kDa polypeptide, whereas the same treatment with Cry3C protoxin produced a 53 kDa fragment. In contrast, the treatment of Cry3A protoxin with chymotrypsin resulted in the production of a

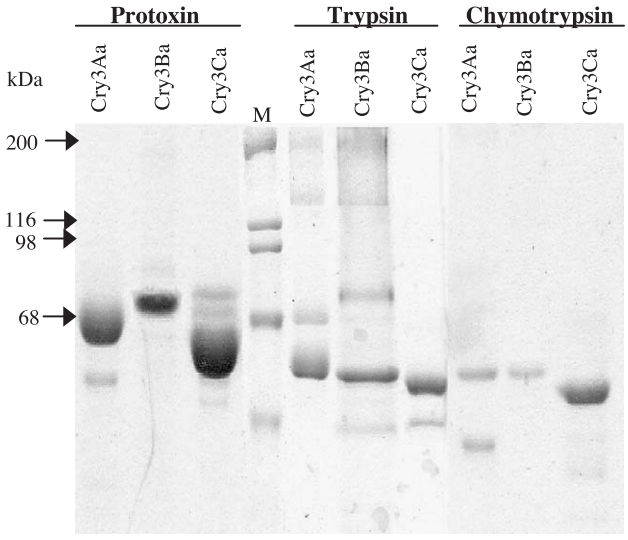

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the protease resistant fragment obtained after digestion of solubilized Cry3Aa, Cry3Ba and Cry3Ca protoxins with trypsin or with chymotrypsin.

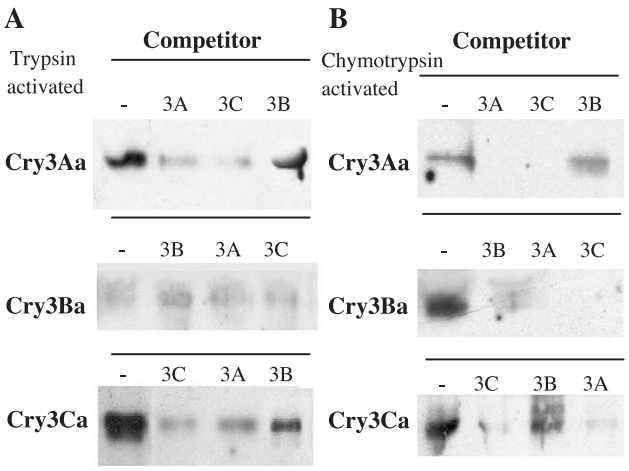

Fig. 2. Homologous and heterologous competition binding assays on BBMV isolated from *L. decemlineata* larvae. Biotinylated trypsin-activated Cry3 toxins (Panel A), or biotinylated chymotrypsin-activated Cry3 toxins (Panel B) were incubated with the BBMV in the absence or in the presence of 250–500-fold excess of unlabelled toxin. After 1 h of incubation, unbound toxins were removed and vesicles containing bound toxins were loaded onto a SDS-PAGE and blotted to a nitrocellulose membrane. Labelled proteins were visualized by means of streptavidin-peroxidase conjugate.

49 kDa polypeptide. Previously, Carrol et al. [20] demonstrated that the chymotrypsin-treated Cry3A protein remains soluble under different pH conditions tested and retains insecticidal activity against CPB. The Cry3Ba treated with chymotrypsin produced a 55 kDa polypeptide. Finally, the treatment of Cry3Ca protoxin with chymotrypsin produced a 53 kDa toxin.

3.2. Binding analysis of Cry3-activated toxins with different proteases

Trypsin and chymotrypsin-activated Cry3 toxins were labelled with biotin and homologous and heterologous competition binding assays were performed on BBMV of CPB with the three labelled toxins. The homologous competition of Cry3 toxins activated with trypsin showed that both Cry3Aa and Cry3Ca are able to bind to CPB-BBMV and this interaction is specific since it was competed with 250-fold of unlabelled trypsin-activated Cry3A or Cry3C toxins, respectively (Fig. 2A). In contrast, the trypsin-activated Cry3Ba toxin did not bind to the BBMV. Insecticidal activity of trypsin-activated Cry3Ba toxin demonstrated that trypsin activation severely affects the toxicity in CPB larvae since no mortality was observed with 35 μg/ml. The heterologous competition of trypsin-activated Cry3Aa with Cry3Ca toxins and vice versa showed that both toxins are able to compete binding of the other toxin, indicating that both toxins may be sharing a common binding site (Fig. 2A). A similar result was observed with chymotrypsinized Cry3Aa and Cry3Ca toxins, the homologous competition assays showed that the interaction of both toxins was specific, and heterologous competition assays showed that they share a common binding site (Fig. 2B). In contrast the chymotrypsinized Cry3Ba showed specific binding as proven by homologous competition assays but was unable to compete for the binding of Cry3Aa or Cry3Ca (Fig. 2B). Surprisingly, unlabelled chymotrypsinized Cry3Aa and Cry3Ca were able to compete for the binding of labelled chymotrypsinized Cry3Ba. These data suggest that Cry3A and Cry3C may have two different binding sites and only one is shared with Cry3Ba toxin (Fig. 2B).

3.3. In vitro pore formation activity of Cry3 toxins activated with different proteases

To analyse the effect of different protease treatments on the activation of Cry3 protoxins, in vitro K^+ permeability assays were performed as previously reported [5]. Fig. 3A

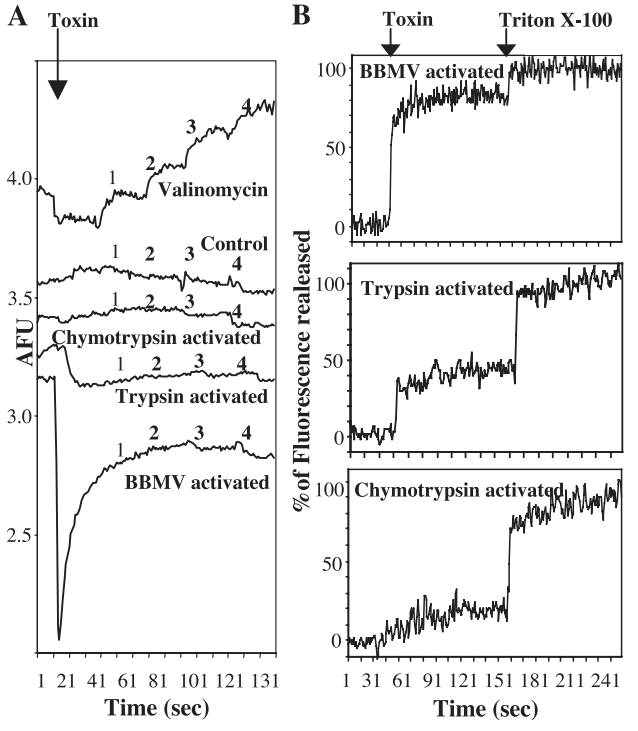

Fig. 3. In vitro pore formation activity of Cry3 toxins. Representative traces of Cry3A toxin are presented. (Panel A) Effect of Cry3A toxins on the membrane potential in BBMV isolated from *L. decemlineata* larvae. Membrane potential of BBMV was recorded as described in Materials and methods by using the Dis-C_3-(5) fluorescent dye. A downward deflection indicates a membrane potential hyperpolarization. The arrow on top of the traces corresponds to the time of toxin addition. AFU = arbitrary fluorescence units. Final K^+ concentrations were (mM): 1 = 3; 2 = 6; 3 = 12 and 4 = 24. In the control, buffer was added instead of toxin. (Panel B) Analysis of Cry3A toxin induced calcein leakage from BBMV. BBMV containing entrapped calcein were prepared as described in Materials and methods. Time course of calcein leakage was recorded, the time of toxin addition is indicated by the arrow. At the end of each run, Triton X-100 was added to determine the maximal extent of leakage.

shows that trypsin- and chymotrypsin-activated Cry3 protoxins had no effect on the membrane potential of CPB BBMV. In the Cry1A toxins, high pore formation activities correlated with the formation of a tetrameric oligomer structure after proteolytic activation in the presence of receptor or by the BBMV. Therefore, the Cry3 toxins were proteolytically activated by CPB BBMV. The Cry3 protoxins were incubated for 30 min with BBMV isolated in the absence of protease inhibitors, the proteases associated to the membrane were able to activate the protoxins and also produced higher molecular size bands in the SDS-PAGE (Fig. 4A) that could be revealed with a polyclonal antibody against Cry3 (Fig. 4B). These bands represent oligomeric structures of Cry3 toxins similar as the one reported for Cry1A toxins [8]. The addition of 50 nM of the activated toxins with the membrane-associated proteases present in the BBMV preparation produced a fast hyperpolarization followed by a clear depolarization of the membrane, when compared to the control in which the same amount of buffer was added (Fig. 3A). After each KCl addition, no response was observed suggesting that the K^+ gradient may be dissipated due to the toxin action.

An additional pore formation assay was performed to corroborate the pore formation activity of Cry3 toxins. BBMV loaded with calcein were treated with the three different activated Cry3A, Cry3B and Cry3C preparations and the release of calcein was determined by the increase in fluorescence after toxin addition. Fig. 3B and Table 1 show that protoxins activated with the proteases associated to the BBMV had higher pore formation activity than the trypsin- or chymotrypsin-activated toxins.

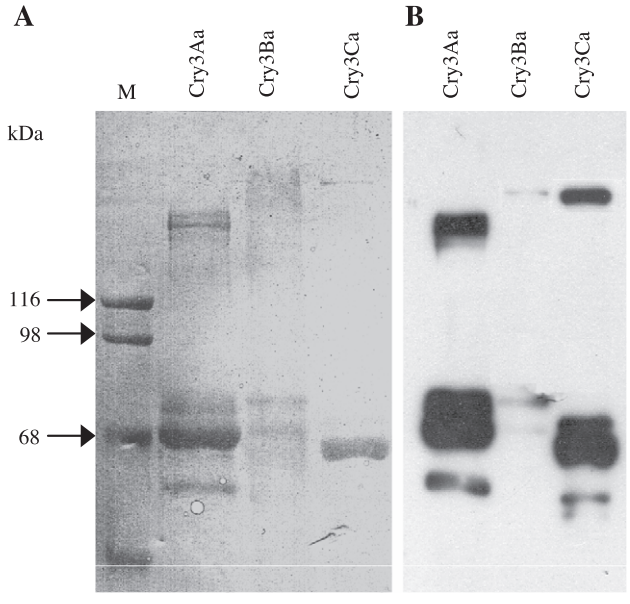

Fig. 4. SDS-PAGE electrophoresis and Western blots of Cry3 toxins activated with BBMV that were isolated without protease inhibitors. (Panel A) SDS-PAGE electrophoresis (7.5% acrylamide). (Panel B) Western blotting of the same protoxin-activated samples. Proteins were revealed with anti-Cry3A polyclonal.

Table 1
Percentage of maximal calcein leakage of BBMV induced by Cry3 toxins

Cry toxin	Activation procedure		
	Trypsin (%)	Chymotrypsin (%)	BBMV (%)
Cry3A	25.9	6.2	77.7
Cry3B	12.8	3.5	78.1
Cry3C	35.1	6.0	59.2

4. Discussion

In this work, we analysed the effect of different proteolytic activation procedures on binding and pore formation activity of Cry3Aa, Cry3Ba and Cry3Ca toxins. Previously, Carroll et al. [20] reported the activation of solubilized Cry3A protoxin with several proteases and demonstrated that two polypeptides of 55 and 49 kDa were produced in vivo in CPB, similar to the polypeptide products obtained after chymotrypsin and trypsin treatment of the protoxin. However, the main digestive proteases of Coleoptera are cysteine and aspartic proteases, whereas those of Lepidoptera and Diptera are serine proteases [36].

Here we show that the activation of Cry3 protoxins with different proteases (trypsin vs. chymotrypsin) could affect toxin activity, since trypsin-activated Cry3Ba was unable to bind to BBMV, in contrast to Cry3A and Cry3C, and this was correlated with the loss of Cry3Ba toxin activity in vivo. The loss of binding of trypsin-activated Cry3Ba toxin could be due to structural changes related to proteolysis or to processing of important binding epitopes in the toxin. In the case of the Cry3A toxin, loops I and III of domain 2 have been implicated in the toxin-receptor recognition [21,22]. On the other side, the activity of midgut proteases could be modulated by changes related to larval development, periods of starvation, changes in diet and responses to inhibitors or toxic compounds. Therefore, changes in protease expression could lead to differential processing of Cry protoxin and affect the toxin activity. In a recent paper from Loseva et al. [23] it was reported that resistance of CPB to Cry3A toxin correlates with the presence of different forms of digestive proteases in the resistant strain as well as with a decreased binding of the toxin.

Binding assays and heterologous competition experiments showed that Cry3A, Cry3B and Cry3C toxins share a common binding site. Cry3Aa and Cry3Ca were able to compete for the binding of labelled chymotrypsinized Cry3Ba but Cry3Ba did not compete for the binding of the other two toxins. These data suggest that Cry3A and Cry3C may have two different binding sites and only one is shared with Cry3Ba toxin (Fig. 2B).

This is the first report of pore formation activity of coleopteran-active toxins at nanomolar concentrations in

BBMV isolated from CPB. Pore formation was not observed in trypsin- or chymotrypsin-activated toxins and was only observed when Cry3 protoxins were proteolytically activated with BBMV containing functional proteases and receptors (Fig. 3 and Table 1). For several pore-forming toxins, it has been shown that association with their receptors is a crucial step in order to induce proteolytical processing by a membrane-associated protease, triggering oligomerization and insertion into membrane [37,38]. For example, aerolysin binds to a GPI-anchored receptor promoting cleavage by Furin and the formation of a functional pre-pore oligomeric structure that inserts into the membrane [39].

Previous observations in our laboratory showed that trypsin-activated Cry1A protoxins resulted in toxin preparations with low pore formation activity in vitro despite the fact that they retained toxicity [8,26], suggesting an incomplete activation. We showed that Cry1Ab toxin binding to the cadherin-like receptor promotes a complete proteolytic activation of the toxin and the formation of a tetrameric pre-pore that is insertion-competent leading to the formation of functional ionic pores in vitro [8]. For Cry1A toxins, it has been shown that the toxin is organized as a tetramer in the membrane inserted state [7] data that correlates with the proposed structure of the oligomer in solution [8]. We show here that interaction with CPB-BBMV promotes efficient proteolytic activation of the three Cry3 coleopteran-specific toxins that also leads to an oligomeric structure formation that correlates with pore formation activity, as in Cry1A toxins [8]. However, the final structure of the Cry3 toxin in the membrane and the characterization of the proteolytic processing on the Cry3 oligomeric structure still remain to be determined.

Our data supports that similar to the Cry1A toxins, the Cry3 oligomer is formed after receptor binding and before membrane insertion, forming a pre-pore structure that is insertion-competent. Since Cry1 and Cry3 toxins share low sequence similarity, our results suggest that the formation of the tetrameric pre-pore intermediate may be a general mechanism in membrane insertion of the whole three-domain Cry toxin family.

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References

[1] V. Sanchis, J. Chaufaux, D. Lereclus, Utilisations de *Bacillus thuringiensis* en protection des cultures et résistance des insects, Cah. Agric. 4 (1995) 405–416.

[2] E. Schnepf, N. Crickmore, J. Van Rie, D. Lereclus, J. Baum, J. Feitelson, D.R. Zeigler, D.H. Dean, *Bacillus thuringiensis* and its pesticidal crystal proteins, Microbiol. Mol. Biol. Rev. 62 (1998) 775–806.

[3] C. Hofmann, H. Vanderbruggen, H. Höfte, J. van Rie, S. Jansens, H. Van Mallaert, Specificity of *Bacillus thuringiensis* δ-endotoxins is correlated with the presence of high affinity binding sites in the brush border membranes of target insect midguts, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 7844–7848.

[4] A. Bravo, S. Jansens, M. Peferoen, Immunocytochemical localization of *Bacillus thuringiensis* insecticidal crystal proteins in intoxicated insects, J. Invertebr. Pathol. 60 (1992) 237–246.

[5] A. Lorence, A. Darszon, C. Díaz, A. Liébano, R. Quintero, A. Bravo, δ-Endotoxins induce cation channels in *Spodoptera frugiperda* brush border membranes in suspension and in planar lipid bilayers, FEBS Lett. 360 (1995) 217–222.

[6] J.L. Schwartz, R. Laprade, in: A. Charles, A. Delecluse, C. Nielson-Leroux (Eds.), Entomopathogenic Bacteria: from Laboratory to Field Application, Kluwer Academic Publishing, Norwell, MA, 2000, p. 1.

[7] V. Vie, N. Vau Mau, P. Pomarde, C. Dance, J.L. Schwartz, R. Laprade, R. Frutos, C. Rang, L. Masson, F. Heitz, C. Le Grimellec, Lipid-induced pore formation of the *Bacillus thuringiensis* Cry1Aa insecticidal toxin, J. Membr. Biol. 180 (2001) 195–203.

[8] 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.

[9] A. Aronson, Ch. Geng, L. Wu, Aggregation of *Bacillus thuringiensis* Cry1A toxins upon binding to target insect larval midgut vesicles, Appl. Environ. Microbiol. 65 (1999) 2503–2507.

[10] J.N. Tigue, J. Jacoby, D.J. Ellar, The alpha-helix 4 residue, Asn135, is involved in the oligomerization of Cry1Ac1 and Cry1Ab5 *Bacillus thuringiensis* toxins, Appl. Envioron. Microbiol. 67 (2001) 5715–5720.

[11] N. Crickmore, D.R. Zeigler, J. Feitelson, E. Schnepf, J. Van Rie, D. Lereclus, J. Baum, D.H. Dean, Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins, Microbiol. Mol. Biol. Rev. 62 (1998) 807–813.

[12] A. Krieg, A.M. Huger, G.A. Langenbruch, W. Schenetter, *Bacillus thuringiensis* var. *tenebrionis*: ein neuer, gegenüber Larven von Coleopteren wirksamer Pathotype, Z. Angew. Entomol. 96 (1983) 500–508.

[13] C. Hernstadt, G.C. Soares, E.R. Wilcox, D.L. Edwards, A new strain of *Bacillus thuringiensis* with activity against coleopteran insects, Bio/Technology 4 (1986) 305–308.

[14] C. Hernstadt, T.E. Gilroy, D.A. Solesk, B.D. Bennet, F.H. Gautner, Nucleotide sequence and deduced amino acid sequence of a coleopteran active delta-endotoxin gene from *Bacillus thuringiensis* subsp. *san diego*, Gene 57 (1987) 81–116.

[15] A. Sick, F. Gaerter, A. Wong, Nucleotide sequence of a coleopteran-active toxin gene from a new isolate of *Bacillus thuringiensis* subsp. *tolworthi*, Nucleic Acids Res. 18 (1990) 1305.

[16] W.P. Donovan, M.J. Rupar, K.A.C. Slaney, T. Malvar, M.C. Gowran-Burke, T.B.C. Johnson, Characterization of two genes encoding *Bacillus thuringiensis* insecticidal crystal proteins toxic to coleopteran species, Appl. Environ. Microbiol. 58 (1992) 3921–3927.

[17] B. Lambert, W. Theunis, K.R. Ageda, K. Van Audenhove, K.C. Dewell, S. Janssens, K.J. Seurin, M. Peferoen, Nucleotide sequence of gene cry III D encoding a novel coleopteran-active crystal protein from strain BT109p of *Bacillus thuringiensis* subsp. *kurstaki*, Gene 110 (1992) 131–132.

[18] Y.Z. Haffani, C. Cloutier, F.J. Bezile, *Bacillus thuringiensis* Cry3Ca1 protein is toxic to the Colorado potato beetle *Leptinotarsa decemlineata* (Say), Biotechnol. Prog. 17 (1996) 211–216.

[19] 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.

[20] 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.
[21] S.J. Wu, D.H. Dean, Functional significance of loops in the receptor binding domain of *Bacillus thuringiensis* CryIIIA δ-endotoxin, J. Mol. Biol. 255 (1996) 628–640.
[22] S.J. Wu, C.N. Koller, D.L. Miller, L.S. Bauer, D.H. Dean, Enhanced toxicity of *Bacillus thuringiensis* Cry3A δ-endotoxin in coleopterans by mutagenesis in a receptor binding loop, FEBS Lett. 473 (2000) 227–232.
[23] 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 (2001) 567–577.
[24] D.J. Lightwood, D.J. Ellar, P. Jarrett, Role of proteolysis in determining potency of *Bacillus thuringiensis* Cry1Ac delta-endotoxin, Appl. Environ. Microbiol. 66 (2000) 5174–5181.
[25] M.Z. Haider, B.H. Knowles, D.J. Ellar, Specificity of *Bacillus thuringiensis* var. *colmeri* insecticidal δ-endotoxin is determined by differential proteolytic processing of the protoxin by larval gut proteases, Eur. J. Biochem. 156 (1986) 531–540.
[26] R. Miranda, F.Z. Zamudio, A. Bravo, Processing of Cry1Ab delta-endotoxin from *Bacillus thuringiensis* by *Manduca sexta* and *Spodoptera frugiperda* midgut proteases: role in protoxin activation and toxin inactivation, Insect Biochem. Mol. Biol. 31 (2001) 1155–1163.
[27] B. Oppert, K.J. Kramer, R.W. Beaman, D.E. Johnson, W.H. McGaughey, Proteinase-mediated insect resistance to *Bacillus thuringiensis* toxins, J. Biol. Chem. 272 (1997) 23473–23476.
[28] 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.
[29] M. Keller, B. Sneh, N. Strizhov, E. Prudovsky, A. Regev, C. Koncz, J. Schell, A. Zilberstein, Digestion of δ-endotoxin by gut proteases may explain reduced sensitivity of advanced instar larvae of *Spodoptera littoralis* to CryIC, Insect Biochem. Mol. Biol. 26 (1996) 365–373.
[30] W.E. Thomas, D.J. Ellar, *Bacillus thuringiensis* var. *israelensis* crystal delta-endotoxin: effects on insect and mammalian cells in vitro and in vivo, J. Cell. Sci. 60 (1983) 181–197.
[31] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature (Lond.) 227 (1970) 680–685.
[32] 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.
[33] M. Wolfersberger, 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 from the larval midgut of the cabbage butterfly (*Pieris brassicae*), Comp. Biochem. Physiol. 86A (1987) 301–308.
[34] M. Reuveni, P.E. Dunn, Differential inhibition by *Bacillus thuringiensis* delta endotoxin of leucine and aspartic uptake into BBMV from midgut of *Manduca Sexta*, Biochem. Biophys. Res. Commun. 181 (1991) 1089–1093.
[35] R.R.C. New, Characterization of liposomes, in: R.R.C. New (Ed.), Practical Approach Series, IRL Press, Oxford, 1994, p. 105.
[36] 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.
[37] C.M. Rosenberger, J.H. Brumell, B.B. Finlay, Microbial pathogenesis: lipid rafts as pathogen portals, Curr. Biol. 10 (2000) 823–825.
[38] V. Cabiaux, Ch. Wolff, J.M. Ruysschaert, Interaction with a lipid membrane: a key step in bacterial toxins virulence, Int. J. Biol. Macromol. 21 (1997) 285–298.
[39] L. Abrami, F.G. van der Goot, Plasma membrane microdomains act as concentration platforms to facilitate intoxication by aerolysin, J. Cell Biol. 147 (1999) 175–184.