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Membrane binding and oligomer membrane insertion are necessary but insufficient for *Bacillus thuringiensis* Cyt1Aa toxicity

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Abstract

Bacillus thuringiensis Cyt proteins are pore-forming toxins that have insecticidal activity mainly against dipteran insects. However, certain Cyt proteins have toxicity to some insect orders, but not toxicity of Cyt1Aa against lepidopteran larvae has been found. Insect specificity has been proposed to rely in specific binding to certain lipids on the brush border membrane of midgut cells since no protein receptors have been described so far. To determine the molecular basis of Cyt1Aa insect specificity we compared different steps of Cyt1Aa mode of action in a susceptible insect as the dipteran *Aedes aegypti* and also in the non-susceptible lepidopteran *Manduca sexta*. Our data shows that the lack toxicity of Cyt1Aa to *M. sexta* larvae does not rely on protoxin processing, membrane binding interaction, and oligomerization of Cyt1Aa since these steps were similar in the two insect species analyzed.

Keywords

Cyt toxins; *Bacillus thuringiensis*; mode of action; membrane binding; oligomerization

1. Introduction

Bacillus thuringiensis (Bt) form a group of bacteria that upon sporulation produces insecticidal proteins called Cry and Cyt. Different Bt strains produce a variety of Cry or Cyt toxins that give insecticidal specificity to each Bt isolate. Cry and Cyt toxins are pore-forming toxins (PFT) that insert into the cell membrane of their hosts after undergoing structural changes making pores and killing cells by osmotic shock [2, 13, 19].

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Among the most used Bt strains for insect control is Bt subs. *israelensis* (Bti) that is highly effective against dipteran insects such as mosquitoes and black flies that are important vectors of human diseases like malaria or dengue fever. Bti produce four Cry toxins (Cry4Aa, CryBa, Cry10Aa and Cry11Aa) and two Cyt toxins (Cyt1Aa and Cyt2Ba) [2, 10]. Cry toxins produced by different Bt strains show toxicity to a number of dipteran, coleopteran and lepidopteran insects. In the case of Cry toxins insect specificity relies on specific recognition of certain larvae midgut proteins called receptors [21]. In contrast, Cyt toxins are mainly dipteran specific [2, 25, 29]. In the case of Cyt1Aa that is toxic to *Aedes aegypti* mosquito larvae it was also shown that this protein is toxic to certain coleopteran pest [15]. However, the toxicity of Cyt1Aa against lepidopteran insects is still questionable since it was reported that this toxin may be toxic to *Plutella xylostella* but a follow up study concluded that Cyt1Aa lacked toxicity to *P. xylostella*, thus this discrepancy remains to be solved [18, 24]. Interestingly, most Cyt toxins also show hemolytic activity and are toxic to certain mammalian cell lines, to cancer cells and to several bacterial species [7, 27, 28, 32]. In the case of Cyt toxins no protein receptors have been described so far and insect specificity was proposed to rely in specific binding to certain lipids on the brush border membrane of midgut cells although the molecular mechanism of Cyt1Aa insect specificity remains to be determined [12, 22].

One of the most interesting features of Cyt1Aa toxin is its capacity to synergize certain Cry toxins such as Cry4A, Cry4Ba and Cry11Aa [4, 30, 31]. This synergistic effect of Cyt1Aa on Cry4Ba or Cry11Aa depends on their binding interaction and it was proposed that Cyt1Aa is a functional Cry11Aa membrane receptor [3, 20].

The three dimensional structure of three Cyt proteins has been solved showing a single α–β domain composed of two outer layers of α-helix hairpins wrapped around a β-sheet [8, 9, 17]. The α-helices have an amphiphilic character, with the hydrophobic residues packed against the β-sheet [25]. Cyt1Aa is synthesized as a protoxin of 27 kDa that undergoes proteolytic cleavage in the amino- and carboxy-terminal ends yielding an activated toxin of 24 kDa. Cyt toxins interact with non-saturated membrane lipids, such as phosphatidylcholine, phosphatidylethanolamine and sphingomyelin [12]. The proteolytic activation of Cyt1Aa in the presence of membranes results in the formation of high molecular weight oligomers of more than 400 kDa that are proposed to be formed by ~16 Cyt monomers [6, 22]. Mutants of Cyt2Aa that affect oligomer formation have reduced hemolytic and insecticidal activity indicating that oligomerization is a crucial step for Cyt toxin action [23].

To determine the molecular basis of Cyt1Aa insect specificity we compared different steps of Cyt1Aa mode of action in a susceptible insect such as *A. aegypti* and also in the non-susceptible lepidopteran *Manduca sexta*. Our data shows that the lack of Cyt1Aa toxicity to *M. sexta* larvae does not rely on protoxin processing, membrane binding interaction, and oligomerization since these steps were similar in the two insect species analyzed.

2. Materials and Methods

2.1 Production of Cyt1Aa crystals

The Bt acrystalliferous strain 407 was transformed with pWF45 plasmid containing the cloned *cyt1Aa* gene. Toxin crystals were produced by growing the strain on HCT media plates supplemented with erythromycin (10 μg/ml) for 3 days at 30°C as previously reported [16]. Crystal production was verified by light microscopy. Cultures were recovered and washed three times with 3M NaCl /0.5 M EDTA, pH 8.0, and four times with distilled water and 1mM PMSF. Crystals were purified by discontinuous sucrose gradient as previously described [28]. Cyt1Aa containing fractions were washed and stored in 50 mM Tris, 1mM PMSF, pH 8.0.

2.2 Toxin solubilization

For the analysis of toxin solubilization at different pH's, 5 μg of Cyt1Aa crystals were centrifuged at 13200 rpm, 4°C, for 10 min of a tabletop centrifuge (Eppendorf, Hamburg, Germany), and the pellet was suspended in either 50 mM phosphate buffer at a pH of 6, 7, 8, and 12 or 50 mM carbonate buffer at pH 9, 10 and 11. DTT was added to a final concentration of 10 mM. Crystals were incubated for 1 hour at 37°C with slight shaking. Soluble protein was recovered by centrifugation for 10 min, at 13200 rpm, 4°C. Five μl of supernatant were separated in 15% SDS-PAGE gel and stained with Coomassie blue. For all other experiments, 15 μg of Cyt1Aa crystals were solubilized with 50 mM carbonate buffer pH 10.5 as described above.

2.3 Preparation of brush border membrane vesicles (BBMV)

For BBMV preparation midgut tissue of either 4th instar *A. aegypti* larvae or 3rd instar *M. sexta* larvae were dissected. Midguts and caeca were recovered, intestinal content cleared and the tissue washed and stored in cold MET buffer (300 mM Mannitol, 5 mM EGTA, 1 M Tris-HCL, pH 7.4), supplemented with 1 mM PMSF and 5 mM DTT (buffer A). *A. aegypti* midgut tissue was homogenized in 5 ml buffer A and then 4.5 ml of cold buffer A were added with 500 μl of 240 mM $MgCl_2$ and let stand on ice for 20 min. The mixture was then centrifuged at 3,000 *xg* for 15 min at 4°C. The supernatant was recovered and transferred to a fresh tube. The membrane pellet was homogenized and centrifuged twice as described above. The three supernatants were pooled and centrifuged at 100,000 *xg* for 10 min, at 4°C. The supernatant from this centrifugation was discarded and pellet collected in buffer A, aliquots made and stored at −70°C until used. For *M. sexta*, 3 g of midgut tissue were homogenized in a 1:10 ratio with cold buffer A. After that, an equal volume of 24 mM $MgCl_2$ was added and let stand on ice for 15 minutes. The supernatant was recovered by centrifugation at 2,500 *xg* for 15 min at 4°C and transferred to a fresh tube. It was again centrifuged at 30,000 *xg* for 30 min at 4°C. The pellet was suspended in 1:1 volume of cold buffer A and 24 mM $MgCl_2$. Centrifugation steps were repeated once more. The final pellet was suspended in 1 ml cold buffer A and aliquoted. BBMV were quantified by Lowry method (Bio-RAD, USA). *M. sexta* midgut juice was obtained from 3rd instar larvae by recovering food content from dissected larvae and centrifugating for 5 min at 9,300 *xg* on a tabletop centrifuge. The supernatant was stored at −20°C.

2.4 Liposome preparation

All lipids were obtained from Avanti Polar (Alabaster, Alabama, USA). For liposome preparation a total of 1.3 μmol of lipids were mixed in a 10:3:1 ratio of phosphatidilcholine, cholesterol and stearylamine. Lipids were carefully spread by rotation on the inside of crystal tube washed with chloroform, forming a lipid film by evaporation of solvent with the help of a nitrogen flow. Afterwards, the lipid film was completely dried in vacuum for 12 h on a Speed Vac SVC100 (Savant Instruments Inc, Holbrook, USA). Liposomes were hydrated with 1.3 ml of 10 mM CHES, 150 mM KCl, pH 9.0, letting them stand for 10-30 min, and removing oxygen with a nitrogen flow. The mixture was capped and briefly vortexed a couple of times to obtain multilamellar vesicles. Small unilamellar vesicles (SUV) were obtained after sonication the mixture for few min, using 1 min pulse in a Branson 1200 waterbath (Branson, USA), with 1 min rest on ice between each pulse. Liposomes were used immediately or stored at 4°C at most 2 days.

2.5 Cyt1Aa activation

Soluble Cyt1Aa protein was quantified by the Bradford method (Bio-RAD, USA) using bovine serum albumin as standard. Either proteinase K (Sigma-Aldrich) was added at 1:10 m/m ratio (proteinase K/Cyt1Aa) or *M. sexta* midgut juice at 1% or 10% v/v ratio of midgut juice/Cyt1Aa to 40 μg of solubilized Cyt1Aa. Incubation at 30°C was done for 30, 60 or 120 minutes. The reaction was inhibited by the addition of 1mM PMSF and immediately centrifuged for 10 min at 16,000 *xg*, 4°C. The supernatant was recovered and quantified and 3 μg of each digestion were loaded and run in a 15% SDS-PAGE gel and stained with Coomassie Blue. N-terminal sequencing was performed at the facilities of Instituto de Biotecnología UNAM, after SDS-PAGE 7 % and transfer onto polyvinylidene difluoride membranes.

2.6 Cyt1Aa oligomerization

For oligomerization of Cyt1Aa 400 ng of soluble Cyt1Aa protoxin were activated with proteinase K 1:10 (w/w), 10% or 1% M. sexta gastric extract in the presence or absence of 90 ul synthetic liposomes for a final volume of 100 ul, adjusted with 50 mM carbonate buffer pH 10.5. Protoxin was incubated 1 hr at 37°C, and reaction stopped with 1mM PMSF. 20 ul of reaction was heated at 65 °C and were separated on a 15% SDS-PAGE gel and transferred to a PVDF membrane (Millipore) for 16 h at 4 °C and 150 mA. The membrane was blocked for 1 h at room temperature with 5% skim milk in 0.1% Tween-20/PBS. The membrane was washed with 0.1% Tween-20/PBS. Cyt1Aa was detected by incubating 1 h with a polyclonal antibody for Cyt1Aa at 1:70,000 in 0.1% Tween-20/PBS. Washing was done as before, followed by incubation with 1:10,000 HRP-anti rabbit antibody (Sigma-Aldrich) in 0.1% Tween-20/PBS. After washing, the membrane was revealed with SuperSignal reagent (Pierce). Cyt1Aa oligomerization was also analyzed by activation of 200 ng of soluble protoxin with 40 ng proteinase K in the presence of *A. aegypti* or *M. sexta* 20 μg BBMV protein in 100 μl of 50 mM Na_2CO_3 pH 10.5 for 2 h at 37°C. Finally, 1 mM PMSF was added to stop proteolysis. Membrane fractions were separated by centrifugation 30 min at 60 000 rpm and each sample was heated 3 min at 50

°C before loading SDS-PAGE and visualized by western blot using polyclonal anti-Cyt1A antibody as described above.

2.7 Cyt1Aa labeling and detection

For biotinylation of Cyt1Aa, the proteinase K activated Cyt1Aa was dialyzed overnight at 4 °C against a borate buffered solution at pH 8.6 (0.05M boric acid, 0.05M NaOH, 0.15M NaCl) at a 1:1000 ratio of toxin/solution. Dialyzed toxin was recovered and quantified by UV absorbance on a Nanodrop 2000 equipment (Thermo Scientific, Rockford, USA). Biotinylation reagent, 20 μl, (Amersham Biosciences, Sweden) was added for each 600 μg of activated Cyt1Aa and incubated at room temperature for 1 hour. Excess biotin was removed by passing through a Sephadex G25 column equilibrated and washed with PBS by centrifugation for 2 min at 2000 rpm on a free angle rotor. The biotin was detected by immunoblotting. Briefly, samples were loaded and run in a 15% SDS-PAGE gel and transferred to PVDF membrane (Millipore, USA). The membrane was blocked for 20 min with 2% Tween-20/PBS solution and washed twice with 0.1% Tween-20/PBS. HRP-streptavidin (GE Healthcare) was added at 1:5000 in 1% Tween 20/PBS and incubated for 1 h at room temperature, washed twice as before and two times with PBS. Finally the membrane was revealed with SuperSignal reagent (Pierce, USA).

For fluorescent labeling of the Cys190 of Cyt1Aa with Alexa Fluor 350 (Life Technologies, USA), proteinase K activated Cyt1Aa was purified by ion exchange chromatography on a DEAE column (Toyopearl, Germany) washed and equilibrated with 20 mM Tris at pH 7.5. Elution of Cyt1Aa fractions were monitored by UV absorbance and Coomassie blue staining. Collected Cyt1Aa fractions were concentrated with Amicon Ultra 4 NMWL 5000 filters (Millipore, USA) and quantified by UV absorbance on a Nanodrop 2000 as before. Before labeling, activated Cyt1Aa was incubated for 5 min with 1 mM DTT. Reduced Cyt1Aa was washed with 10 volumes of degassed PBS on an Amicon Ultra-4 column. For labeling of Cyt1Aa, a 50 molar excess of Alexa-350 was added in the presence of 1mM EDTA and degassed PBS in a total reaction volume of 400 μl. Incubation was done for 2 h at 37°C, in the dark. Excess label in the sample was removed by dialyzing exhaustively with degassed PBS and then passing through a Sephadex G25 column equilibrated with degassed PBS. Labeled Cyt1Aa was eluted and labeling efficiency verified by UV absorbance at 280 nm and 364 nm on a Nanodrop 2000 equipment. Absorbance at 364 nm of labeled Cyt1Aa was corrected by determining the factor of absorbance of non-labeled Cyt1Aa at this wavelength. The following formula was used to determine labeling stoichiometry considering that activated Cyt1Aa at a concentration of 1 g/L will have an absorbance of 0.898 at 280 nm (calculated from Cyt1Aa sequence), an extinction coefficient (ε) of 19,000 for AlexaFluor 350, and a molecular weight of 24 kDa for activated Cyt1Aa.

$$\frac{Abs\,(\lambda max_{ex})}{\varepsilon_{fluorophore}} \times \frac{MW_{Cyt1Aa}}{mg/ml_{Cyt1Aa}} = \frac{moles_{fluorophore}}{moles_{Cyt1Aa}}$$

2.8 Cyt1A binding competition

For binding competition of Cyt1Aa, 10 μg of *A. aegypti* or *M. sexta* BBMV were incubated for 30 min at 37 °C with 0, 100, 500, and 1000 molar excesses of non-biotinylated Cyt1Aa. Afterwards, 5 nM of biotinylated Cyt1Aa was added to the samples and incubated for a further 30 min at 37°C. Samples were centrifuged for 1 h at 90000 rpm, 4 °C. Pellets were suspended in 15 μl of PBS, loaded on a 15% SDS-PAGE gel and transferred to a PVDF membrane. Biotinylated Cyt1Aa was detected as before.

2.9 Fluorescence quenching on BBMV

All readings were done using an AMINCO Bowman Series 2 spectrofluorometer. Four μg of AlexaFluor 350 labelled Cyt1Aa were incubated for 1 h in the presence or absence of 10 μg of BBMV derived from *A. aegypti* or *M. sexta* midguts. Afterwards, samples were centrifuged for 1 h at 90000 rpm, 4 °C. The supernatant was recovered and the pellets were suspended in 50 mM carbonate buffer, pH 10.5. Supernatant of Cyt1Aa without BBMV and pellets obtained after incubations with BBMV were analyzed. KI was added to samples at final concentration of 0, 50, 100, 250 and 500 mM. KCl was used to maintain ionic strength at 500 mM in all samples. $Na_2S_2O_3$ is added at 8 mM to the sample to avoid production iodine ions. Volumes were adjusted to 250 μl with sample buffer. Equivalent samples without labeled Cyt1Aa but with KI were used as a control. Quenching efficiency was calculated as the ratio of F/F_0, where F and F_0 are the AlexaFluor 350 fluorescence measurements in the presence or absence of KI, respectively. The Stern-Volmer constant was calculated as the slope of the linear regression of the quenching curves.

3. Results

3.1 Solubilization of Cyt1Aa at different pH's

Cyt1Aa showed a LC_{50} toxicity value of 925.5 (602.5-1824.3) ng/ml to 4th instar *A. aegypti* larvae and no toxicity to *M. sexta* neonate larvae at the highest toxin concentration tested of 2000 ng/cm^2. In comparison, previously reported toxicity of Cry1Ab toxin showed a LC_{50} value of 1 ng/cm^2 to *M. sexta* [16]. The first step in the mode of action of Cyt1Aa is its solubilization in the alkaline pH of the midgut of susceptible larvae [25]. Lepidopteran insects have alkaline pH from 8 to 10 in the different midgut regions similar to that in dipteran larvae, although the pH gradients encountered throughout the larval gut is different in both insect orders [11, 26]. To determine if Cyt1Aa solubilization could be a limiting step at certain pH's, Cyt1Aa crystals were solubilized at different pH's. Figure 1 shows that Cyt1Aa was readily solubilized from pH 9 up to pH 12 indicating that toxin solubilization should not be a limiting step in toxicity of Cyt1Aa to *M. sexta*.

3.2 Cyt1Aa activation by M. sexta midgut juice

For toxicity, Cyt1Aa is activated by midgut proteases by cleavages in both the amino- and carboxy-terminal ends [1, 25]. *In vitro*, Cyt1Aa can also be activated by trypsin or proteinase K treatment although both proteases show different sites for activation: while trypsin induced a cleavage at the N-terminal end at residue Arg25, activation with proteinase K induced cleavages at Arg30 and Val31 [1]. These differences in the processing of the

toxin have a slight effect in toxicity since Cyt1Aa trypsin activated toxin showed less efficient hemolysis activity than proteinase K activated toxin [1]. To analyze if *M. sexta* midgut proteases could affect Cyt1Aa activation and toxicity, Cyt1Aa protoxin was activated with *M. sexta* midgut juice at two different concentrations and different time points and the activated toxin was analyzed by SDS-PAGE electrophoresis. For comparison Cyt1Aa protoxin was also activated with proteinase K. Figure 2 shows that Cyt1Aa protoxin activated with midgut juice from *M. sexta* larvae yielded a band of 24 kDa. Bands of slightly smaller size (~ 23 kDa) were obtained after proteinase K treatment. Importantly, the Cyt1Aa activated toxin resists high *M. sexta* midgut juice concentrations indicating that toxin degradation could not account for the lack of toxicity of Cyt1Aa in *M. sexta* (Fig. 2). The N-terminal end sequence of Cyt1Aa after proteinase K or *M. sexta* midgut juice treatment was determined. The Cyt1Aa protein activated with *M. sexta* midgut juice showed the same sequence as the Cyt1Aa activated with proteinase K (RVEDPNIDDL) indicating that *M. sexta* midgut juice properly activated Cyt1Aa toxin.

3.3 Binding of Cyt1Aa to M. sexta and A. aegypti BBMV

To analyze if the binding of Cyt1Aa to BBMV of *M. sexta* could be a limiting step in toxicity, a qualitative binding competition assay was performed with biotin-labeled Cyt1Aa. To determine if the binding to BBMV was specific, we analyzed the binding of biotin-labeled Cyt1Aa to BBMV previously incubated with different molar excess of unlabeled Cyt1Aa. For comparison, we performed a similar binding experiment with *A. aegypti* BBMV. Figure 3 shows that Cyt1Aa labeled toxin bound to *M. sexta* BBMV in a specific manner since the binding of labeled Cyt1Aa was efficiently competed by unlabeled Cyt1Aa. Similar results were obtained in the binding experiment with *A. aegypti* BBMV, although the labeled Cyt1Aa toxin was competed with less unlabeled toxin in the case of *M. sexta* BBMV (Fig. 3).

3.4 Oligomerization of Cyt1Aa

Activation of Cyt1Aa in the presence of membrane lipids results in the formation of high molecular weight oligomers [6]. To determine if activation of Cyt1Aa with *M. sexta* midgut juice results in Cyt1Aa oligomerization, we analyzed the effect of Cyt1Aa protoxin activation with *M. sexta* midgut juice in the presence of small unilamellar vesicules (SUV) composed of phosphatidylcholine, cholesterol and stearylamine as described in Materials and Methods. Figure 4 shows that in the presence of SUV, treatment of Cyt1Aa protoxin with *M. sexta* midgut juice resulted in the formation of high molecular weight oligomers. Similar size oligomers were formed with proteinase K treatment (Fig. 4).

To determine if Cyt1Aa could form oligomers and insert into *M. sexta* BBMV we performed proteolytic activation of Cyt1Aa with proteinase K in the presence of BBMV and the membrane pellet was separated by centrifugation to analyze oligomer formation by western blot using an anti-Cyt1Aa polyclonal antibody. For comparison, a similar experiment was performed with *A. aegypti* BBMV. Figure 5 shows that Cyt1Aa could form oligomers of high molecular weight that inserted into *M. sexta* BBMV since oligomers were observed in the membrane pellet whereas monomers were detected in the supernatant. A similar result

was obtained with *A. aegypti* BBMV indicating that oligomers insert into BBMV of both the susceptible and non-susceptible insects.

3.5 Analysis of Cyt1Aa BBMV insertion by fluorescence quenching

To determine if the inserted Cyt1Aa in BBMV from the two insect species have a similar conformation we took advantage of the fact that activated Cyt1Aa contains a single cysteine residue 190 located in β7 at the carboxy-terminal end. Cys190 was labeled with fluorescent dye Alexa-350 to perform quenching experiments with KI and determine the exposure of this residue to the solvent after BBMV membrane insertion. It has been proposed that β7 inserts into the membrane in the pore formed by Cyt1Aa [9, 25]. Alexa-350 dye shows low sensitivity to changes in the polar environment but is efficiently quenched by KI [33], which is a soluble quencher. Activated Cyt1Aa was labeled with Alexa-350 as described in materials and methods and the labeling of the toxin was analyzed directly on the SDS-PAGE, visualizing the labeled protein by excitation with UV light transilluminator (data not shown). The labeled toxin was then incubated with BBMV from both insects. To determine the exposure of Cys190 to the solvent the BBMV samples were incubated with increasing concentrations of KI. As control labeled Cyt1Aa was similarly incubated in solution. The KI quencher gave a linear Stern-Volmer plot (Fig. S1). The apparent dynamic quenching constants K_{SV} derived from the slopes of these plots are presented in Table S1. The data shows that Cys190 was less exposed to the solvent after Cyt1Aa binding to BBMV from both insects since the value of Sterm Volver constant was greatly reduced in comparison to that obtained from Cyt1Aa in solution. Furthermore, the fluorescence of Cys190-Alexa350 showed a similar shift of 7 nanometers to the blue region of the Cyt1Aa bound to BBMV of both insects (Table S1). These results show a similar change in the polar environment of Cys190 when Cyt1Aa inserts into the membranes of both insect species.

4. Discussion

It has been proposed that Cyt1Aa insect specificity relies on specific recognition of certain unsaturated lipids in the membranes of gut cells of susceptible insects [12, 25]. Here we show that Cyt1Aa binds to BBMV of the non-susceptible *M. sexta* larvae in a manner that is similar to that observed in BBMV of the susceptible insect *A. aegypti*. Activation of Cyt1Aa with *M. sexta* midgut proteases resulted in an activated toxin that showed similar amino-terminal as the proteinase K treated protein and could oligomerize in the presence of synthetic lipids as well as in the presence of *M. sexta* and *A. aegypti* BBMV. Furthermore, the oligomer formed inserted into BBMV since it was obtained in membrane pellets in contrast to monomeric toxin that was always found in supernatants. Also, we performed qualitative binding assays that showed that Cyt1Aa bound to the *M. sexta* BBMV in a specific way since previous binding of non-labeled toxin inhibited the binding of the biotin-labeled toxin, suggesting that binding is specific and binding sites were saturated. Finally, fluorescence quenching assays of Cyt1Aa labeled toxin with Alexa-350 dye showed that the inserted Cyt1Aa toxin in BBMV from both insect species was protected from quenching by KI and suffered a similar shift in fluorescence maximal wavelength, suggesting a similar conformation upon insertion into membranes of both insect species. Two subtle differences were noticed in the processing of Cyt1Aa with *M. sexta* midgut juice and in the binding

competition experiments. Regarding processing of Cyt1Aa, processing with *M. sexta* midgut juice resulted in processed Cyt1Aa protein with a similar amino terminal end as the proteinase k treated protein but a slight higher molecular weight than the protein processed with protease K (Fig. 2). As mentioned previously, differences in the processing of the toxin have a slight effect in toxicity since Cyt1Aa trypsin activated toxin showed less efficient hemolysis activity than proteinase K activated toxin [1]. It remains to be determined if processing with *M. sexta* midgut juice affects Cyt1Aa toxicity although processing with *M. sexta* midgut juice triggered efficient Cyt1Aa oligomerization (Fig. 4). Regarding binding competition experiment, binding of Cyt1Aa was saturated with less excess concentration of unlabeled Cyt1Aa in *M. sexta* BBMV in comparison to *A. aegypti*. This could suggest that *M. sexta* BBMV have lower Cyt1Aa binding sites than *A. aegypti*. We confirmed 30-50 % less binding sites of Cyt1Aa in *M. sexta* BBMV in comparison to *A. aegypti* by ELISA competitive binding assays (data not shown). However, the lower amount of binding sites in *M. sexta* membranes does not explain by itself the complete lack of toxicity. Recently, it has been shown that toxicity of Cyt2Aa could be targeted to non-susceptible aphids by introducing a peptide sequence that showed binding affinity to an aminopetidase-N present in the aphid gut into certain exposed loop regions of Cyt2Aa [5]. The engineered Cyt2Aa showed enhance binding to aphid BBMV and toxicity indicating that the number of binding sites in the gut is a limiting step for Cyt2Aa toxicity to aphids [5]. Thus, it is possible that a small amount of Cyt1Aa that could reach *M. sexta* brush border membrane could be limited in the number of binding sites needed to trigger toxicity. Nevertheless, we cannot rule out other possibilities like an enhanced immune response in lepidopteran gut that could be sufficient to cope with Cyt1Aa toxicity. It is important to note that although Cyt1Aa is toxic to *A. aegypti* larvae, toxicity is low compared with other Cry mosquitocidal proteins [10]. Also, it was recently shown that in other mosquito species as *Anopheles albimanus* Cyt1Aa is not toxic but it still synergized Cry4Ba and Cry11Aa in this mosquito species [14]. It could be possible that the major role of Cyt1Aa in toxicity against dipteran larvae is its synergistic capacity to enhance Cry toxicity rather than its toxicity itself.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Cry	crystal proteins
Bt	*Bacillus thuringiensis*
PFT	pore forming toxins
Bti	*Bacillus thuringiensis* subs *israelensis*

BBMV	brush border membrane vesicles
LC_{50}	median lethal concentration
SUV	small unilamellar vesicles

References

[1]. Al-yahyaee SAS, Ellar DJ. Maximal toxicity of cloned CytA δ-endotoxin from *Bacillus thuringiensis* subsp. *israelensis* requires proteolytic processing from both the N- and C-termini. Microbiol. 1995; 141:3141–3148.

[2]. Bravo A, Likitvivatanavong S, Gill SS, Soberón M. *Bacillus thuringiensis*: a story of a successful bioinsecticide. Insect Biochem Mol Biol. 2011; 41:423–31. [PubMed: 21376122]

[3]. Cantón PE, Reyes EZ, Ruiz de Escudero I, Bravo A, Soberón M. Binding of *Bacillus thuringiensis* subsp. *israelensis* Cry4Ba to Cyt1Aa has an important role in synergism. Peptides. 2011; 32:595–600. [PubMed: 20558220]

[4]. Chang C, Yu YM, Dai SM, Law SK, Gill SS. High-level *cryIVD* and *cytA* gene expression in *Bacillus thuringiensis* does not require the 20-kilodalton protein and the coexpressed gene products are synergistic in their toxicity to mosquitoes. Appl Environ Microbiol. 1993; 59:815–821. [PubMed: 8481007]

[5]. Chougule NP, Li H, Liu S, Linz LB, Narva KE, Meade T, Bonning C. Retargeting of the *Bacillus thuringiensis* toxin Cyt2Aa against hemipteran insect pests. Proc Natl Acad Sci USA. 2013; 110:8465–8470. [PubMed: 23650347]

[6]. Chow E, Singh GJP, Gill SS. Binding and aggregation of the 24 kDa toxin of *Bacillus thuringiensis* subsp. *israelensis* to cell membranes and alteration by monoclonal antibodies and amino acid modifiers. Appl Environ Microbiol. 1989; 55:2779–2788. [PubMed: 2624459]

[7]. Cohen S, Cahan R, Ben-Dov E, Nisnevitch M, Zaritsky A, Firer MA. Specific targeting to murine myeloma cells of Cyt1aa toxin from Bacillus thuringiensis subspecies israelensis. J Biol Chem. 2007; 282:28301–28308. [PubMed: 17626007]

[8]. Cohen S, Dym O, Albeck S, Ben-Dov E, Cahan R, Firer M, Zaritsky A. High-resolution crystal structure of activated Cyt2Ba monomer from *Bacillus thuringiensis* subsp. *israelensis*. J Mol Biol. 2008; 380:820–827. [PubMed: 18571667]

[9]. Cohen S, Albeck S, Ben-Dov E, Cahan R, Firer M, Zaritsky A, Dym O. Cyt1Aa toxin: crystal structure reveals implications for its membrane perforating function. J Mol Biol. 2011; 413:804–814. [PubMed: 21959261]

[10]. Crickmore N, Bone EJ, Williams JA, Ellar DJ. Contribution of the individual components of the δ-endotoxin crystal to the mosquitocidal activity of *Bacillus thuringiensis* subsp. *israelensis*. FEMS Microbiol Lett. 1995; 131:249–254.

[11]. Dow JA. pH gradients in lepidopteran midgut. J Exp Biol. 1992; 172:355–375. [PubMed: 9874748]

[12]. Du J, Knowles BH, Li J, Ellar DJ. Biochemical characterization of *Bacillus thuringiensis* cytolytic toxins in association with phospholipid bilayer. Biochem J. 1999; 338:185–193. [PubMed: 9931315]

[13]. de Maagd RA, Bravo A, Berry C, Crickmore N, Schnepf HE. Structure, diversity and evolution of protein toxins from spore-forming entomopathogenic bacteria. Ann Rev Genet. 2003; 37:409–433. [PubMed: 14616068]

[14]. Fernández-Luna MT, Tabashnik B, Lanz-Mendoza H, Bravo A, Soberón M, Miranda-Rios J. Single-Concentration tests show synergism among *Bacillus thuringiensis* subsp. *israelensis* toxins against the malaria vector mosquito *Anopheles albimanus*. J Invertebr Pathol. 2010; 104:231–233. [PubMed: 20361977]

[15]. Federici BA, Bauer LS. Cyt1Aa protein of *Bacillus thuringiensis* is toxic to the cottonwood leaf beetle, *Chrysomela scripta*, and suppresses high levels of resistance to Cry3Aa. Appl Environ Microbiol. 1998; 64:4368–4371. [PubMed: 9797292]

[16]. Gómez I, Sánchez J, Miranda R, Bravo A, Soberón M. 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. 2002; 513:242–246. [PubMed: 11904158]

[17]. Li J, Koni PA, Ellar DJ. Structure of the mosquitocidal delta-endotoxin CytB from *Bacillus thuringiensis* sp. *kyushuensis* and implications for membrane pore formation. J Mol Biol. 1996; 257:129–152. [PubMed: 8632451]

[18]. Meyer SK, Tabashnik BE, Liu Y-B, Wirth MC, Federici BA. Cyt1A from *Bacillus thuringiensis* lacks toxicity to susceptible and resistant larvae of diamnondback moth (*Plutella xylostella*) and pink bollworm (*Pectinophora gossypiella*). Appl Environ Microbiol. 2001; 67:462–463. [PubMed: 11133481]

[19]. Parker MW, Feil SC. Pore-forming protein toxins: from structure to function. Progr Biophys Mol Biol. 2005; 88:91–142.

[20]. Pérez C, Fernandez LE, Sun J, Folch JL, Gill SS, Soberón M, Bravo A. *Bacillus thuringiensis* subsp. *israeliensis* Cyt1Aa synergizes Cry11Aa toxin by functioning as a membrane-bound receptor. Proc Natl Acad Sci USA. 2005; 102:18303–18308. [PubMed: 16339907]

[21]. Pigott CR, Ellar DJ. Role of receptors in *Bacillus thuringiensis* crystal toxin activity. Microbiol Mol Biol Rev. 2007; 71:255–281. [PubMed: 17554045]

[22]. Promdonkoy B, Ellar DJ. Membrane pore architecture of a cytolytic toxin from *Bacillus thuringiensis*. Biochem J. 2000; 350:275–282. [PubMed: 10926854]

[23]. Promdonkoy B, Rungrod A, Promdonkoy P, Pathaichindachote W, Krittanai Ch, Panyim S. Amino acid substitutions in α-A and α-C of Cyt2Aa2 alter hemolytic activity and mosquito-larvicidal specificity. J Biotechnol. 2007; 133:287–293. [PubMed: 18054404]

[24]. Sayyed A, Crickmore N, Wright DJ. Cyt1Aa from *Bacillus thuringiensis* subsp. *israelensis* is toxic to the diamondback moth, *Plutella xylostella*, and synergizes the activity of Cry1Ac towards a resistant strain. Appl Environ Microbiol. 2001; 67:5859–5861. [PubMed: 11722947]

[25]. Soberón M, López-Díaz JA, Bravo A. Cyt toxins produced by *Bacillus thuringiensis*: A protein fold conserved in several pathogenic microorganisms. Peptides. 2012; 41:87–93. [PubMed: 22691603]

[26]. Souza-Neto JA, Gusmao DS, Lemos FJ. Chitinolytic activities in the gut of *Aedes aegypti* (Diptera:Culicidae) larvae and their role in digestion of chitin rich structures. Comp Biochem Physiol A Mol Integr Physiol. 2003; 136:717–724. [PubMed: 14613799]

[27]. Teixera Corrêa RF, Ardisson-Araújo DMP, Monnerat RG, Ribeiro BM. Cytotoxicity analysis of three *Bacillus thuringiensis* subsp. *israelensis* δ-endotoxins towards insect and mammalian cells. PloS ONE. 2012:e46121. [PubMed: 23029407]

[28]. Thomas WE, Ellar DJ. *Bacillus thuringiensis* var. *israelensis* crystal δ-endotoxin: effects on insect and mammalian cells *in vitro* and *in vivo*. J Cell Sci. 1983; 60:181–197. [PubMed: 6874728]

[29]. van Frankenhuyzed K. Cros-order and cross-phylum activity of *Bacillus thuringiensis* pesticidal proteins. J Invertebr Pathol. 2013; 114:76–85. [PubMed: 23747826]

[30]. Wirth CM, Federici BA, Walton WE. Cyt1Aa from *Bacillus thuringiensis* synergizes activity of *Bacillus sphaericus* against *Aedes aegypti*. (Diptera: Culicidae). Appl Environ Microbiol. 2000; 66:1093–1097. [PubMed: 10698776]

[31]. Wu D, Johnson JJ, Federeci BA. Synergism of mosquitocidal toxicity between CytA and CryIV proteins using inclusions produced from cloned genes of *Bacillus thuringiensis* subsp. *israelensis*. Mol Microbiol. 1994; 13:965–972. [PubMed: 7854129]

[32]. Yudina TG, Konukhova AV, Revina LP, Kostina LI, Zalunin IA, Chestukhina GG. Antibacterial activity of Cry- and Cyt-proteins from *Bacillus thuringiensis* ssp. *israelensis*. Ca J Microbiol. 2003; 49:37–44.

[33]. Zavala LE, Pardo-López L, Cantón PE, Gómez I, Soberón M, Bravo A. Domains II and III of *Bacillus thuringiensis* Cry1Ab toxin remain exposed to the solvent after insertion of part of domain I into the membrane. J Biol Chem. 2011; 286:19109–19117. [PubMed: 21464133]

Highlights

1. Cyt1Aa is properly activated by *Manduca sexta* midgut proteases
2. Cyt1Aa shows specific binding to *Manduca sexta* BBMV
3. *Manduca sexta* midgut proteases facilitates Cyt1Aa oligomer formation in the presence of membrane lipids
4. A similar region containing Cys residue of Cyt1Aa inserts into *Manduca sexta* and *Aedes aegypti* midgut membranes

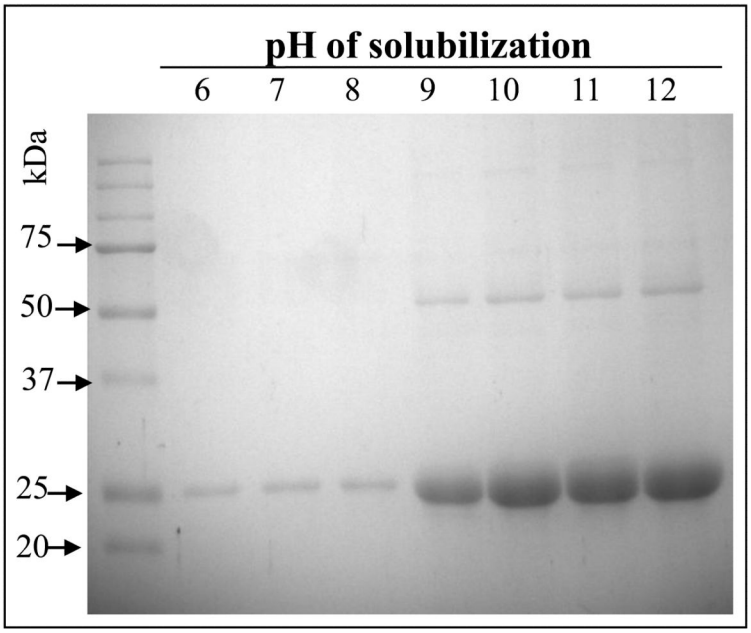

Figure 1.

Cyt1Aa protoxin is efficiently solubilized at alkaline pH's. Spore crystals suspensions were incubated at different pH's as indicated in Materials and Methods and the soluble proteins were separated by SDS-PAGE after removal of the insoluble material by centrifugation.

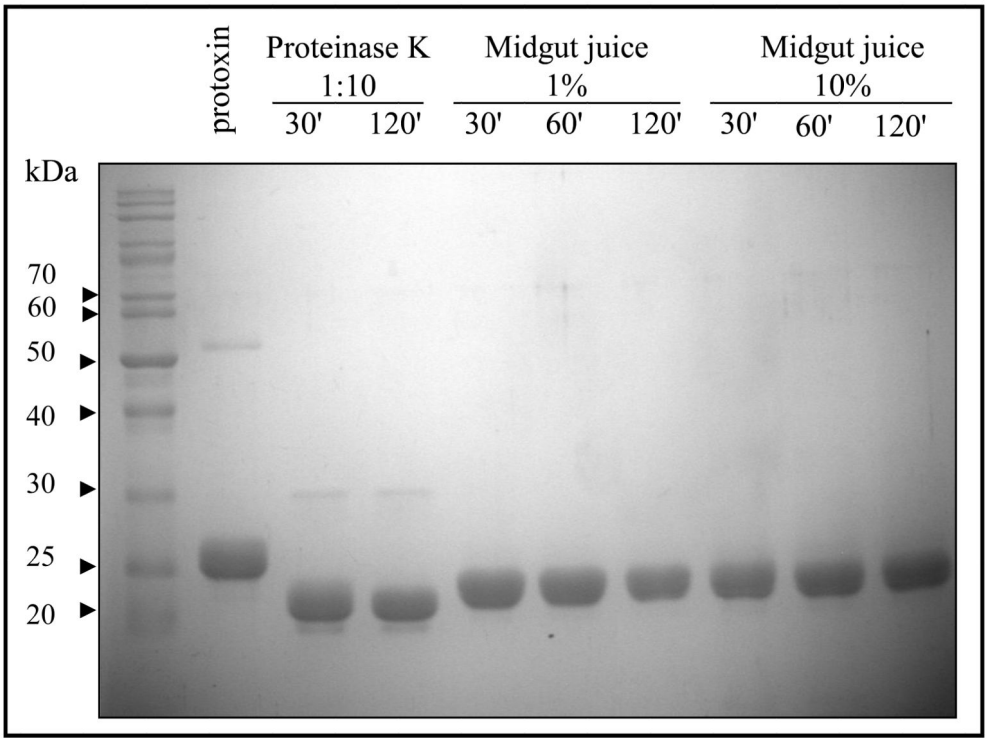

Figure 2.

Cyt1Aa protoxin is efficiently processed with *Manduca sexta* midgut juice. Cyt1Aa protoxin was treated with Proteinase K or 1% or 10 % *M. sexta* midgut juice as described in Materials and Methods and the protease treatment was stopped at different time points. Samples were separated by SDS-PAGE and stained with Coomasie blue.

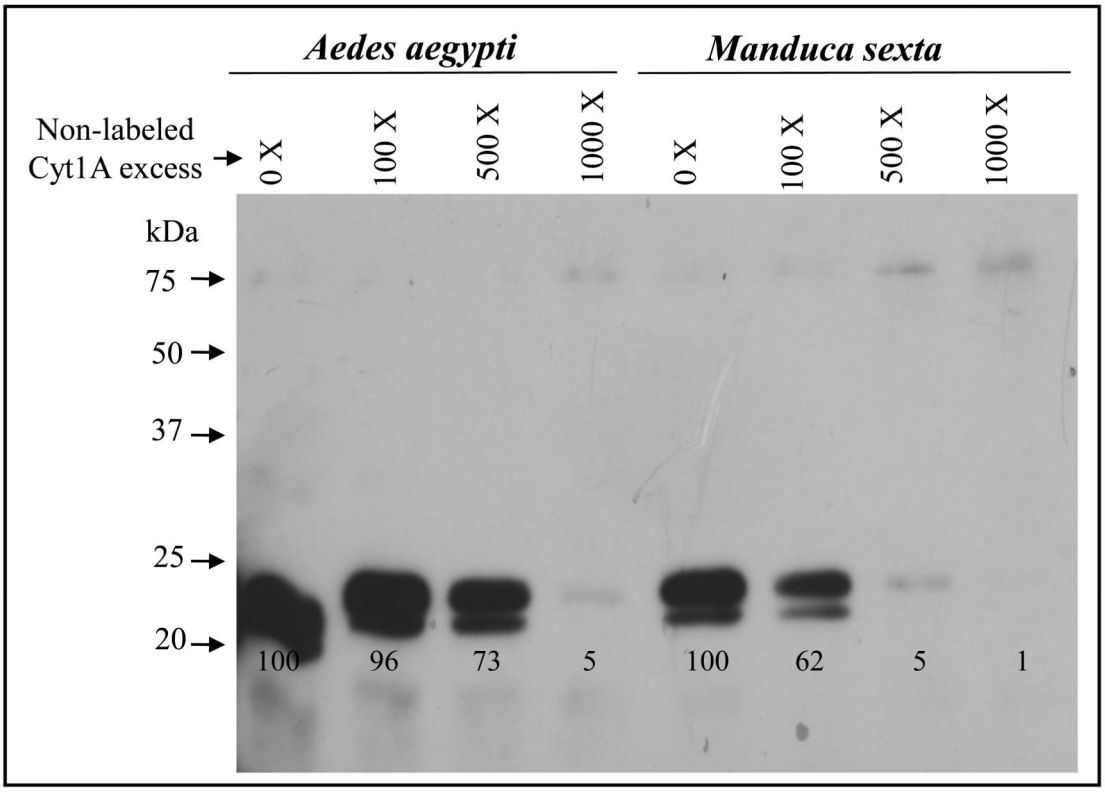

Figure 3.

Binding competition experiments shows that *Manduca sexta* BBMV have lower Cyt1Aa binding sites than *Aedes aegypti* BBMV. *M. sexta* or *A. aegypti* BBMV were incubated with different molar excess of unlabelled and biotin labeled Cyt1Aa was then bound to BBMV samples. BBMV were obtained by centrifugation and separated by SDS-PAGE. Bound biotin-Cyt1Aa was revealed with streptavidin coupled to peroxidase as indicated in Materials and Methods.

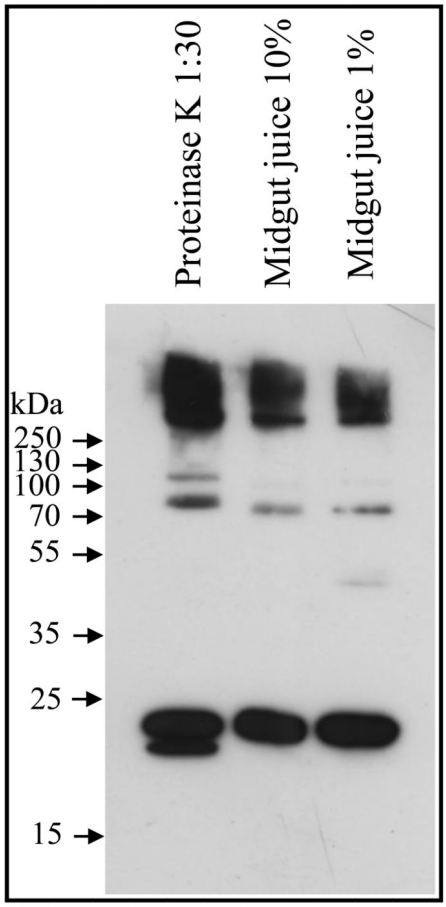

Figure 4.

Activation of Cyt1Aa protoxin by *Manduca sexta* midgut juice in the presence of synthetic membranes induces Cyt1Aa oligomerization. Cyt1Aa protoxin was treated with Proteinase K or two different *M. sexta* midgut juice concentrations in the presence of small unilaminar vesicles and the samples were loaded on SDS-PAGE and revealed by western blot with anti-Cyt1Aa antibody.

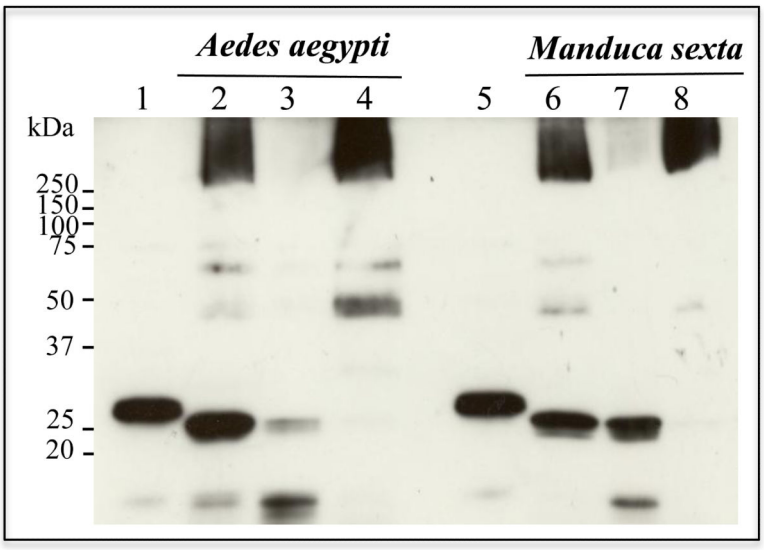

Figure 5.

Cyt1Aa oligomers insert into BBMV of *Aedes aegypti* and *Manduca sexta*. Cyt1Aa protoxin was activated with proteinase K in the presence of BBMV from both insects, finally the BBMV membranes were separated by centrifugation. The membrane pellets and the supernatants were loaded on SDS-PAGE and Cyt1Aa was revealed by western blot by anti-Cyt1Aa antibody. Lanes 2 to 4 correspond to *A. aegypti* BBMV while lanes 6 to 8 to *M. sexta* BBMV. Lanes 1 and 5 are Cyt1Aa protoxin samples, lanes 2 and 6 the correspond to the BBMV samples without separation by centrifugation, lanes 3 and 7 are the supernatants after separation of the membranes by centrifugation and lanes 4 and 8 are the BBMV membrane pellets after separation by centrifugation.