ARTÍCULO DE INVESTIGACIÓN

Preferential Protection of Domains II and III of *Bacillus thuringiensis* Cry1Aa Toxin by Brush Border Membrane Vesicles

Protección preferencial de los dominios II y III de la toxina Cry1Aa de *Bacillus thuringiensis* en Vesículas de Membrana de Borde de Cepillo

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Abstract

The surface exposed Leucine 371 on loop 2 of domain II, in Cry1Aa toxin, was mutated to Lysine to generate the trypsin-sensitive mutant, L371K. Upon trypsin digestion L371K is cleaved into approximately 37 and 26 kDa fragments. These are separable on SDS-PAGE, but remain as a single molecule of 65 kDa upon purification by liquid chromatography. The larger fragment is domain I and a portion of domain II (amino acid residues 1 to 371). The smaller 26-kDa polypeptide is the remainder of domain II and domain III (amino acids 372 to 609). When the mutant toxin was treated with high dose of *M. sexta* gut juice both fragments were degraded. However, when incubated with *M. sexta* BBMV, the 26 kDa fragment (domains II and III) was preferentially protected from gut juice proteases. As previously reported, wild type Cry1Aa toxin was also protected against degradation by gut juice proteases when incubated with *M. sexta* BBMV. On the contrary, when mouse BBMV was added to the reaction mixture neither Cry1Aa nor L371K toxins showed resistance to *M. sexta* gut juice proteases and were degraded. Since the whole Cry1Aa toxin and most of the domain II and domain III of L371K are protected from proteases in the presence of BBMV of the target insect, we suggest that the insertion of the toxin into the membrane is complex and involves all three domains.

Key words: *Bacillus thuringiensis,* site directed mutagenesis, δ -endotoxin.

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Resumen

La superficie de la toxina Cry1Aa, en el asa 2 del dominio II contiene expuesta la leucina 371, la cual fue modificada a lisina produciendo una mutante sensible a la tripsina, L371K. Esta mutante produce dos fragmentos de 37 y 26 kDa por acción de la tripsina que son separables por SDS-PAGE, pero que a la purificación por cromatografía líquida se mantienen como una sola molécula de 65 kDa. El fragmento grande contiene al dominio I y una parte del dominio II (aminoácidos 1 al 371). El polipéptido de 26 kDa contiene la parte restante del dominio II y dominio III (aminoácidos 372 al 609). Cuando la toxina mutante fue tratada con dosis altas de jugo intestinal de *Manduca sexta*, ambos fragmentos fueron degradados. Sin embargo, cuando fueron incubados en VMBC de *M. sexta*, el fragmento de 26 kDa fue protegido preferencialmente de las proteasas intestinales. Como se ha reportado, la toxina silvestre Cry1Aa también es protegida de la degradación de las proteasas cuando es incubada en VMBC de *M. sexta*. Sin embargo, cuando se adicionó VMBC de ratón a la mezcla de reacción, ni la toxina Cry1Aa ni la mutante L371K mostraron resistencia a las proteasas y fueron degradadas. Dado que la toxina completa de Cry1Aa y casi todo de los dominios II y III de L371K están protegidos de proteasas en presencia de VMBC del insecto, este estudio sugiere que la inserción de la toxina en la membrana involucra los tres dominios.

Palabras clave: *Bacillus thuringiensis*, mutagénesis sitio dirigida, δ–endotoxina

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Introduction

The mode of action of the insecticidal *Bacillus thuringiensis* (*Bt*)* δ-endotoxins, or Cry toxins, consists of solubilization of the crystals in the insect's alkaline midgut environment (Tojo and Aizawa, 1983), followed by the activation of protoxin into toxin molecule by specific midgut proteases, and binding of the toxin to midgut epithelial cell receptors (Hofmann *et al*., 1988). The binding and subsequent insertion of the toxin into the midgut membrane allows the influx of cations through either non-specific pores or ion channels, depending upon the conditions (Knowles and Ellar, 1897; English and Slatin, 1992; Schwartz *et al*., 1993).

The process of membrane insertion by the insecticidal *Bt* δ-endotoxins remains a matter of extensive research. Several theoretical assumptions were made earlier regarding the insertion of toxin into the membrane and pore formation. Two main models were proposed: the "Umbrella Model" and the "Penknife Model", both of them propose that only portions of the α-helical domain I insert in the membrane, while the remaining domains II and III were just involved in receptor recognition (Li *et al*., 1991; Knowles, 1994).

More studies on *Bt* Cry toxins have suggested that the protein could insert into the midgut membrane as a single molecule, opening the possibility for a third model for the topology of the toxin in the membrane (Aronson *et al*., 1999; Aronson, 2000; Arnold *et al*., 2001; Loseva *et al*., 2001; Alzate *et al*., 2006; Nair *et al*., 2008; Alzate *et al*., 2009). In this last model, virtually the whole toxin, when associated with BBMV, is protected from proteinase K, with the exception that (in Cry1A toxins) α-helix 1 is cleaved off of the inserted form.

In order to test the hypothesis that virtually the whole toxin inserts as a single molecule, we have constructed a trypsin-sensitive Cry1Aa mutant, in which the Leu residue at position 371 has been replaced with Lys (L371K), producing two fragments of the toxin under denaturing conditions (Figure 1). Functional studies such as toxicity, receptor binding and insertion

* Abbreviations: Bt: *Bacillus thuringiensis;* BBMV: brush border membrane vesicles.

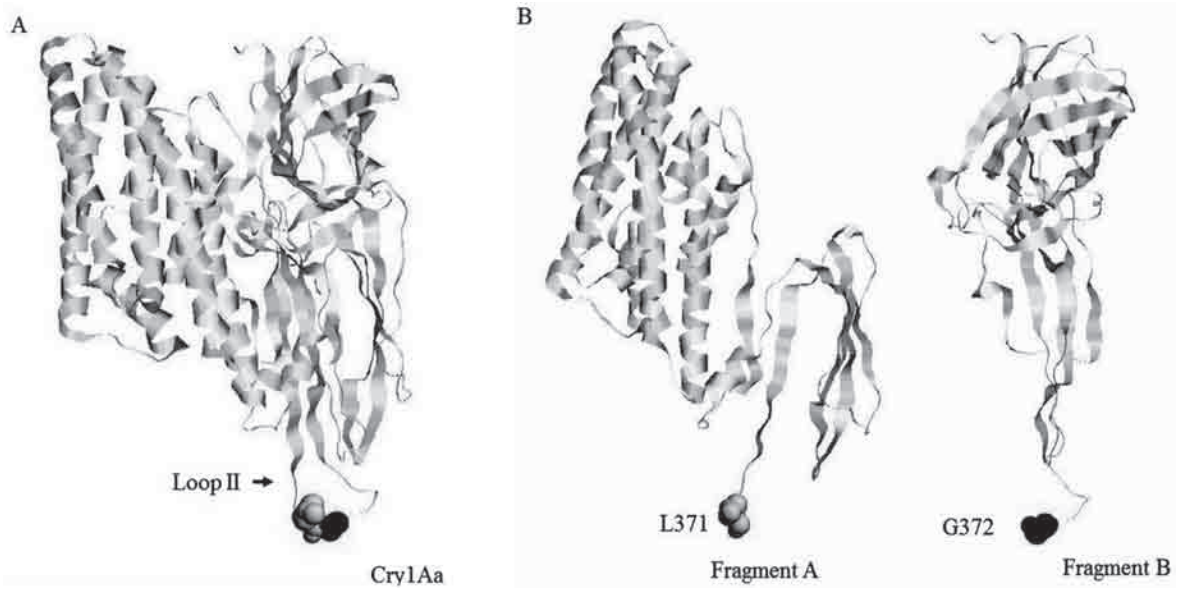

Figure 1. Structural representation of Cry1Aa with mutant toxic protein L371K. Panel A, shows the molecule with the location of the L371, in loop II. Panel B, shows the fragments that result from trypsin digestion.

with wild type and the mutant protein, have led us to propose that besides domain I, domains II and III have also the ability to penetrate the membrane in support of our previous findings (Arnold *et al*., 2001; Alzate *et al*., 2006; Nair *et al*., 2008; Alzate *et al*., 2009).

Materials and Methods

Mutant construction and screening. The construction of pOS4102 (Cry1Aa), by subcloning the Cry1Aa1 gene (Schnepf *et al*., 1985) from *Bt* HD-1 into pKK223-3 and over expression in *Escherichia coli*, has been described previously (Ge *et al*., 1989). To target the putative loop region in domain II, we sub-cloned the 354 bp *Eco*RI-*Sac*I fragment from pOS4102 into pBluescript KS^-, to obtain pBS350. Site-directed mutagenesis was performed with Muta-Gene M13 *in vitro* Mutagenesis kit (Bio-Rad) following manufacturers protocol. Mutagenic oligonucleotides were obtained from Sandoz Agro. Inc (Palo Alto, California). Potential mutant colonies were screened by the dideoxy sequencing method (Sanger *et al*., 1977), using sequenase kit (U.S Biochemical). After mutagenesis and selection, the 354 bp *Eco*RI-*Sac*I fragment was sub-cloned into expression vector pOS4102. The resulting construct was named L371K.

Toxin purification and activation. The mutant gene, *L371K*, was overexpressed in host strain MV1190. δ-endotoxin was purified from *E. coli* as described (Alzate *et al*., 2009). The purified crystal proteins were solubilized in sodium carbonate buffer (50 mM Na_2CO_3/Na_2HCO_2; 10 mM dithiothreitol, pH 9.5). Protoxin concentration was determined by Coomassie blue protein assay reagent (Pierce). The wild type (Cry1Aa) and the mutant (L371K) protoxins were digested with 3% (w/w) of trypsin at 37ºC for 4hrs. Activated toxins were analyzed as described (Alcantara *et al*., 2001). Trypsin-activated toxin was purified by liquid chromatography on a HiLoad 16/60 Superdex 200 column (Pharmacia) with a Pharmacia Äkta Explorer preparative HPLC.

Structural analyses. Thermolysin digestion was carried out with the trypsin resistant core of Cry1Aa and L371K, the procedure employed was as described (Almond and Dean, 1993). Toxins in 30 μg aliquots were incubated for 20 minutes at 50ºC, and 58ºC with 4% (w/w) of thermolysin in 50 mM Tris-HCl (pH 9.5) buffer containing 10 mM $CaCl_2$. The reaction was terminated by adding 20 mM EDTA (final concentration). Samples were analyzed by SDS-12% PAGE and stained with Coomassie brilliant blue. The relative intensities of toxin fragments at various temperatures were determined with a UVP trans-illuminator using the Labworks™ Analysis Software (UVP, Inc. Upland, CA.) and recorded as the percentage of peptide remaining against temperature.

Toxicity bioassays. *M. sexta* eggs were obtained from Dr. D. L. Dahlman (Dept. of Entomology, University of Kentucky, Lexington, Kentucky). Bioassays were performed by surface contamination of artificial diet (Bio-Serve Inc., French Town, New Jersey). The diet was poured into 24-well tissue culture plates (Falcon). 50 μL of various toxin dilutions were then applied to the surface and allowed to air-dry. Six toxin concentrations were used to calculate the median lethal concentration (LC_{50}) value with 12 *M. sexta* neonate larvae for each concentration. The average of three bioassays per toxin was used to calculate effective doses (LC_{50}) and 95% fiducial limits with the Probit analysis (Finney, 1972).

Receptor binding experiments. BBMV were prepared from fifth instar *M. sexta* larvae by the differential magnesium precipitation method as described (Wolfersberger *et al*., 1987), resuspended in binding buffer (8 mM Na_2HPO_4, 2 mM KH_2PO_4, 150 mM NaCl, pH 7.4) and stored in liquid nitrogen until further use. Iodination of toxins for competition and dissociation assays was carried out as described (Lee *et al*., 1992). The homologous competition assay was performed by competing 1nM of ^{125}I-labeled toxin with an increasing concentration of the same unlabeled toxin as described (Rajamohan *et al*., 1996). For the dissociation experiments, 50 μg of *M. sexta* BBMV was incubated with 2 nM of either ^{125}I-labeled Cry1Aa or ^{125}I-labeled L371K in 100 μl of binding buffer at room temperature. After 1hr incubation, 500-fold excess of unlabeled toxin was added to the ^{125}I-labeled toxin-BBMV suspension. The reaction was stopped at various time intervals (0 to 60 min) by spinning down the mixture. The pellets were washed twice with 300 μl of binding buffer in order to remove any unbound toxin. The iodine content of the final pellets was determined in a gamma counter (Beckman instruments). Non-specific binding was determined by adding together labeled toxin and 500 fold excess of the corresponding unlabeled toxin to the BBMV. Non-specific binding was subtracted in the final data analysis. Binding data were analyzed with Sigma Plot (Jandel Scientific Co.).

Membrane partitioning assay. *M. sexta* BBMV (45 μg) was incubated with 0.120 μg of ^{125}I-Cry1Aa or ^{125}I-L371K. The reaction volume was adjusted to 60 μl with binding buffer supplemented with 0.1% bovine serum albumin (BSA) to avoid non-specific binding. After 150 min. of incubation at 25 °C, the suspension was centrifuged and the pellets were washed with binding buffer to remove any unbound toxin. The pellets were resuspended in 30 μl of binding buffer and 10μL of *M. sexta* gut juice was added. Three vials containing the same volume of mixture and no gut juice were used as control. The suspension was incubated at 25 °C for 1hr. At the end of the incubation period 5μl of 50X protease inhibitor cocktail (Roche, Molecular Biochemicals) was added to stop the reaction. The BBMV was solubilized by adding denaturing loading gel buffer (Laemmli, 1970), and boiled for 5 min. Toxin fragments were separated from BBMV on SDS-12% PAGE and analyzed by autoradiography. As a negative control mouse BBMV was incubated with either ^{125}I-Cry1Aa or ^{125}I-L371K and subjected to same treatment as *M. sexta* BBMV. Cry1Aa and L371K toxins were also treated with *M. sexta* gut juice (10 μl) without adding any BBMV, 60 μg of BSA were added to compensate for

the BBMV peptides. Relative intensities of gut juice treated and untreated fragments were determined as described above. The data were recorded as the percentage of peptide remaining after protease digestion as compared to undigested toxin.

Results

Trypsin digestion of proteins. Solubilized crystals of both, Cry1Aa and its mutant L371K, produced 130 kDa protoxin (figure 2, lanes 1 and 3, respectively). Digestion of the mutant protoxin with trypsin produces an activated toxin that separates as a single peak, eluting at the position of a 65 kDa protein molecule, by size exclusion chromatography (figure 3). Peak fractions were analyzed by SDS-PAGE (figure 2, lanes 2 and 4, respectively). The mutant protein was cleaved into two peptides with approximate molecular weights of 37 kDa (fragment A) and 28 kDa (fragment B), in contrast to a single 65 kDa for Cry1Aa toxin (figure 2). Since the trypsin-sensitive site in L371K was created in the loop2 of domain II, we consider the 37.3 kDa peptide as domain I, plus a portion of domain II (expected molecular weight, ~37.3kDa). Domain III and the remaining of domain II correspond to the 26.2 kDa fragment (calculated molecular weight, ~26.2kDa).

Structural stability of Cry1Aa and L371K. In order to determine stability alterations in the trypsin-resistant core of L371K, it was treated with thermolysin at various temperatures and compared with the wild type toxin under the same conditions. Our data shows that fragment B of L371K was more susceptible to thermolysin than fragment A (figure 4, lanes 5, 6, and 7).

Bioassay. Trypsin-activated L371K and Cry1Aa were analyzed for toxicity against *M. sexta* neonate larvae. The mutant protein was active against the insect, although its toxicity was 7 times lower than the wild type (table 1).

Receptor binding studies. In order to study the binding ability of L371K toxin toward the membrane receptor of *M. sexta* midgut competition assays were performed. Homologous competition experiments indicated that the mutant toxin (K_{com}=14.25 ± 0.51 nM) has three-fold less initial (reversible) binding affinity than Cry1Aa toxin (K_{com} =4.37 ± 0.72 nM) (Fig. 5A). In order to determine the irreversible binding parameters of the mutation to *M. sexta* BBMV, ^{125}I-labeled wild type and mutant toxins were allowed to bind to the BBMV for 1h. In the presence of excess amounts of unlabeled toxin, 45% of the mutant toxin was chased off in the first 20 minutes and only 50% of the toxin remained bound to BBMV at 60 min. In contrast, the wild type toxin showed more irreversible association with only 17% being chased off after 60 min (figure 5B).

Evidence for toxin insertion into the BBMV. We investigated the regions of the Cry1Aa toxin that insert into the BBMV. It was observed that after incubation with excess *M. sexta* gut juice for 1h at 25 °C in the absence of BBMV, 74.2% of the Cry1Aa toxin molecules were digested (Figure 6A, lanes 1 and 2). Only 37.7% of the *M. sexta* BBMV-protected toxin molecules were degraded by the gut juice (figure 6A, lanes 3 and 4). It is seen that the digested fragment is slightly smaller than the undigested toxin. In agreement with a report showing that α-helix 1 is cleaved off during the insertion process (Aronson et al., 1999).

In the case of L371K toxin, the effect of *M. sexta* gut juice on fragments A and B was studied. In the absence of *M. sexta* BBMV both fragments showed lower band intensities (figure 6B, lane 2) than the untreated toxin (figure 6B, lane 1). There was a relatively equal degradation of fragment B (49.8%) compared with intensity on lane 1, as compared to fragment A (47.4%). In contrast to these results, after incubation with *M. sexta* BBMV and gut juice treatment, fragment B showed less degradation (17.2%) than fragment A (66.2%; figure 6B, lanes 3 and 4).

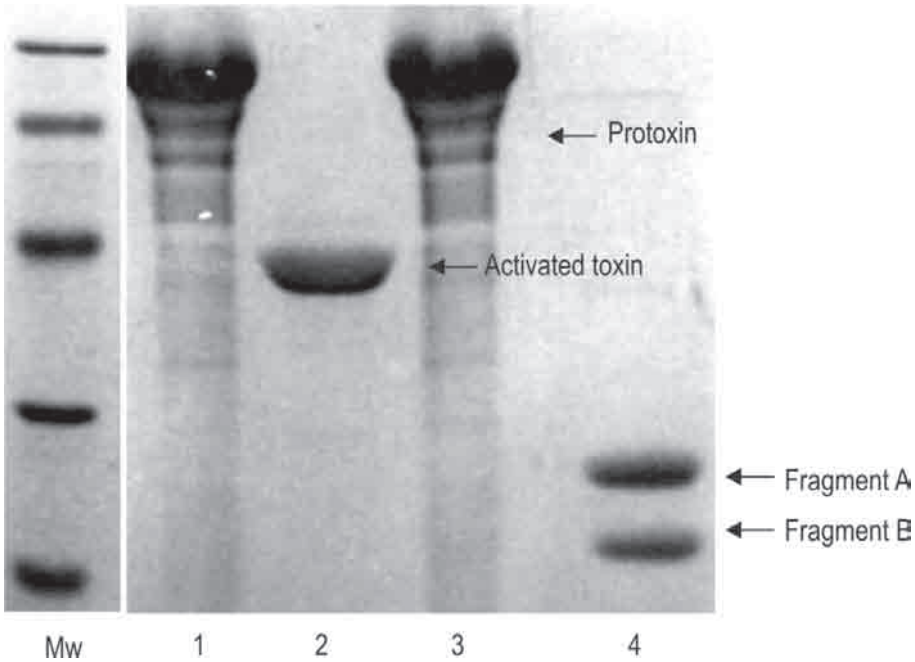

Figure 2. SDS-PAGE analysis of Cry1Aa and L371K. Lanes 1 and 3 are 130 kDa protoxin bands of Cry1Aa and L371K, respectively. Lane 2, trypsin activated Cry1Aa toxin. Lane 4 is trypsin digested fragments of L371K. Fragment A is ~37 kDa and fragment B is ~26 kDa. The left column contains the molecular weight markers.

In order to eliminate the possibility of non-specific interaction of Cry1Aa or L371K, to a lipid bilayer and thus protection from proteases, mouse BBMV was used as a control. It was observed that there was only a weak binding of either toxin to these vesicles. This may be due to the non-specific binding of toxin to the mouse BBMV. However, both Cry1Aa toxin and L371K toxin were completely degraded by gut juice proteases in the presence of mouse BBMV (figure 6, lanes 5 and 6).

Discussion

The mechanism of action of the δ-endotoxins involves binding to receptors on the microvillar membranes of the midgut, with a major portion of this interaction becoming irreversible. A direct correlation was found between toxicity and the rate of irreversible binding (Ihara *et al.*, 1993; Liang *et al*, 1995). Several indirect studies suggest that irreversible binding indicates toxin insertion into the membrane (Schnepf, 1995). More specifically it has been hypothesized that domain I is the only region involved in membrane integration and channel formation (Li *et al.*, 1991; Knowles, 1994; Gazit *et al.*, 1998; Schwartz *et al.*, 1997).

In this report we have attempted to test this hypothesis by performing membrane association studies with Cry1Aa and its mutant toxin L371K. We designed this trypsin-cleavable mutant based on previous biochemical studies that showed that Cry1A could be considered as two fragments linked by a protease susceptible loop that can be cleaved by chymotrypsin at amino acid residue 371 (Covents *et al.*, 1991). Our work shows that the digestion of L371K protoxin with trypsin produces two fragments of ~37 kDa (fragment A) and ~26 kDa (fragment B) that can be resolved on SDS-PAGE. Fragment A corresponds to domain I and a portion of domain II (residues 1 to 371), whereas fragment B contains domain III and the remaining of domain II (residues 372 to

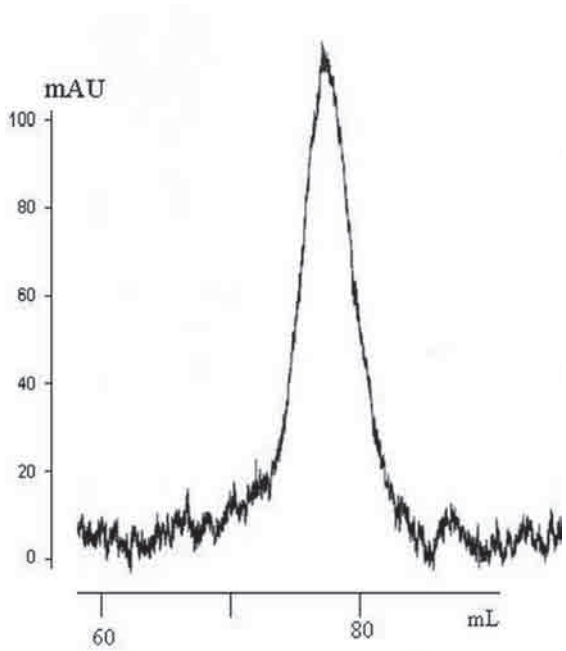

Figure 3. Chromatographic analysis of trypsin activated Cry1Aa mutant L371K toxin. Purified L371K mutant toxin elutes as a single peak, with the same molecular weight as the wild type toxin.

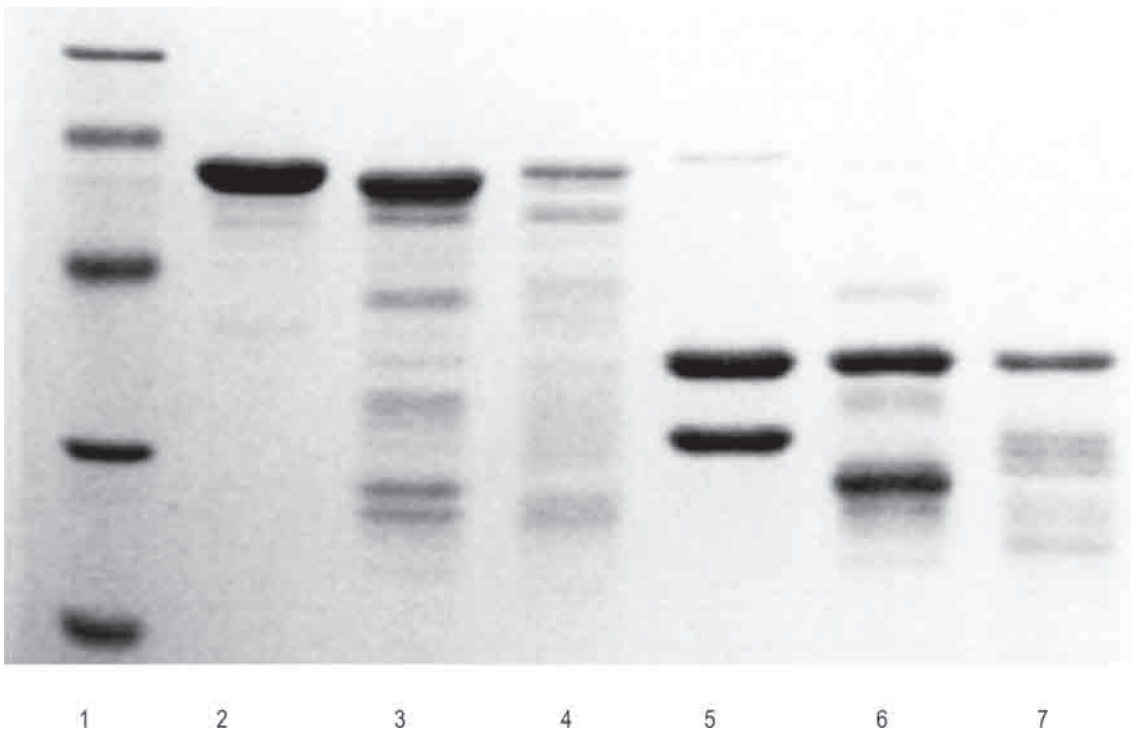

Figure. 4. Thermal stability of Cry1Aa and L371K. Lane 1, molecular weight marker. Lane2, Cry1Aa at room temperature without adding thermolysin. Lane 3, Cry1Aa at 50ºC with thermolysin. Lane 4, Cry1Aa at 58ºC with thermolysin. Lane 5, L371K at room temperature without adding thermolysin. Lanes 6 and 7, at 50 and 58ºC with thermolysin, respectively.

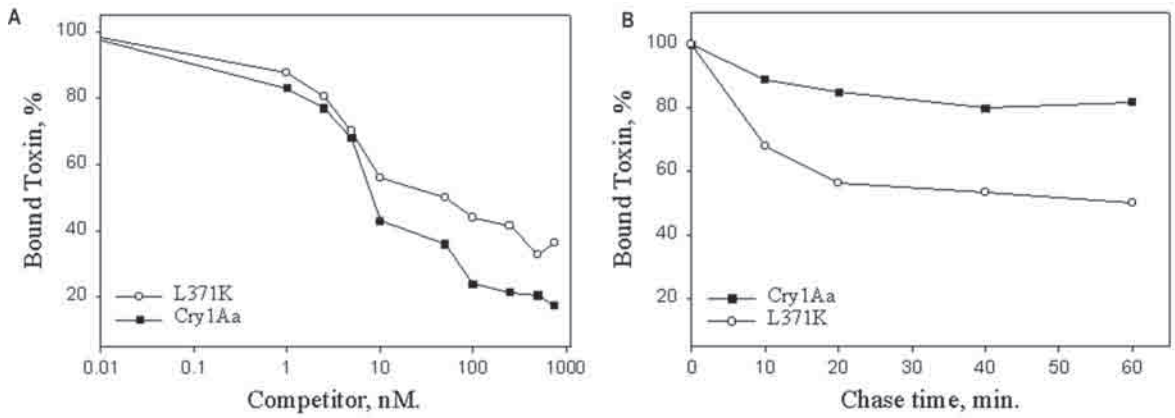

Figure 5. Binding data on M. sexta BBMV. A) Homologous competition assay of 125I-Cry1Aa and 125I-L371K. Binding is expressed as the percentage of labeled toxin bound to the BBMV after competition with increasing amount of the corresponding unlabeled toxin. B) Dissociation of the wild type and mutant toxins from M. sexta BBMV. Binding is expressed as the percentage of labeled toxin that remains associated with BBMV after adding 400 fold excess of corresponding unlabeled toxin at various time intervals.

609). In solution, the two fragments remain associated as a single 65 kDa molecule.

Thermal denaturation profile of L371K shows a greater degradation of fragment B compared to fragment A in the presence of thermolysin. This may be due to the fact that fragment A mainly has domain I, which consists of amphipathic α-helices that are close-packed keeping the hydrophobic residues away from the solvent (Li *et al.*, 1991; Grochulski *et al.*, 1995).

In Cry1A toxins, amino acid residues 365 to 371 have been shown to affect receptor binding or membrane insertion (irreversible binding), and toxicity (Rajamohan *et al.*, 1996; Lu *et al.*, 1994; Rajamohan *et al.*, 1995). The partial reduction (2 to 3 times) in the initial binding affinity of the mutant toxin L371K is likely to be caused by changes in the physicochemical properties of amino acid residue at position 371, and because of the likelihood of increased mobility of the loop due to protease cleavage. Hydrophobic amino acids at this position are required for proper binding to receptors or insertion into BBMV (Rjamohan *et al.*, 1995). The importance of this region in the Cry1A family of δ-endotoxins is further strengthen by studies showing that substituting aliphatic, hydrophilic and small side-chain residues for Phe at position 371 reduce the irreversible association and toxicity of Cry1Ab to *M. sexta* (Rajamohan *et al.*, 1995; 1996). By analogy, it can be assumed that the seven-fold reduction in toxicity for *M. sexta* larvae is mainly due to reduced binding or irreversible association (insertion) of L371K toxin.

Protection against exogenous proteases has been used to determine the topology of bacterial toxins in biological membranes (Aronson *et al.*, 1999; Aronson, 2000; Arnold *et al.*, 2001; Loseva *et al.*, 2001; Cabiaux *et al.*, 1994). Using a similar technique, protection of ^{125}I-Cry1Aa and ^{125}I-L371K toxins against *M. sexta* gut juice proteases was studied. It was observed that, after 1-hour incubation with *M. sexta* BBMV, only 37.7% of the initial concentration of Cry1Aa was digested by gut juice, while 74.2% of Cry1Aa molecules were degraded in the absence of *M. sexta* BBMV. However, there were no fragments smaller than the whole toxin, suggesting that the protein enters the membrane as a single molecule. This protection might be due to the insertion of the toxin into the membrane because a membrane-inserted configuration will make the toxin

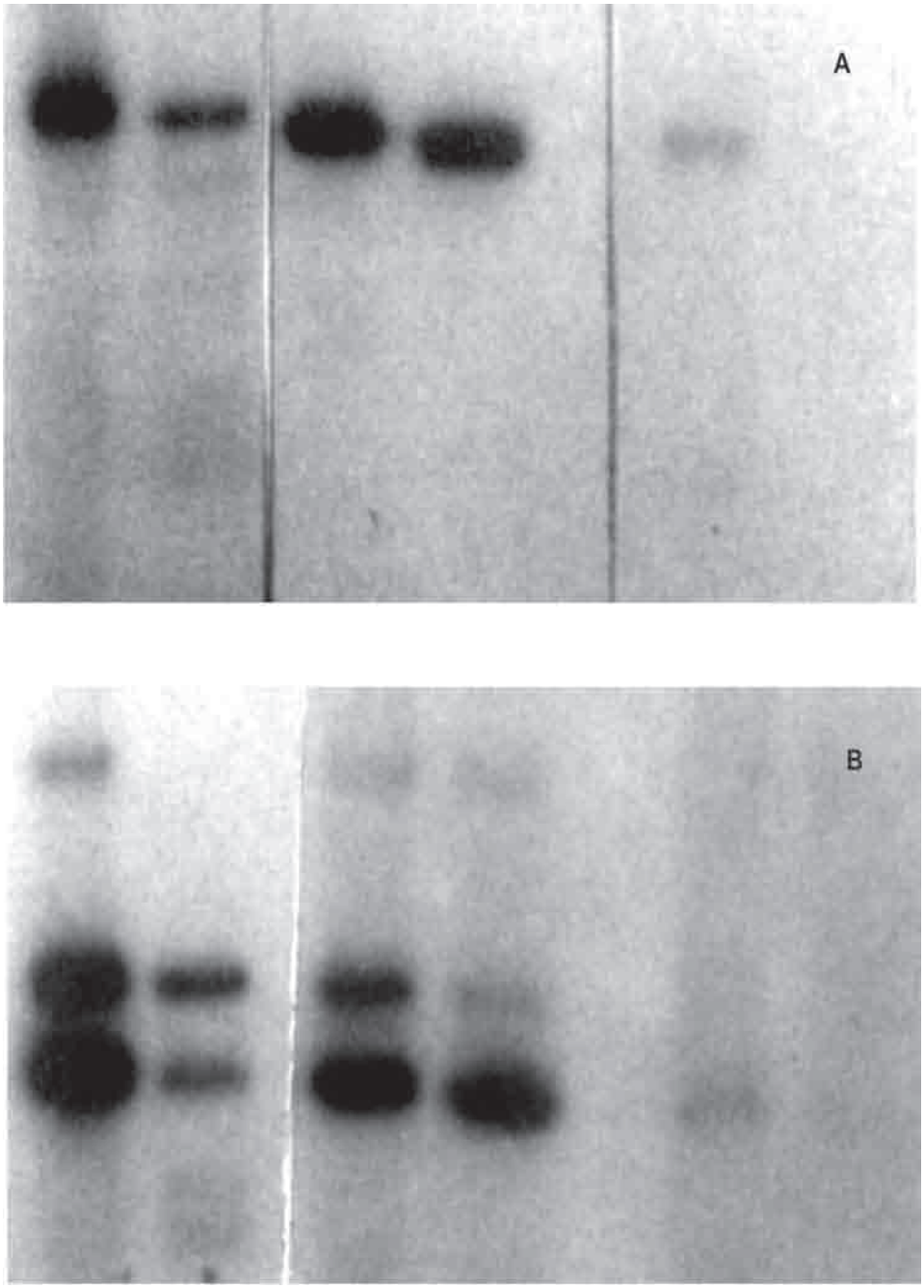

Figure 6. Autoradiogram of 125I-labeled Cry1Aa and L371K toxins incubated with M.sexta gut juice with or without BBMV. Panel A: Lane 1, 125I-Cry1Aa toxin as a control. Lane 2, Cry 1Aa toxin treated with gut juice as described in experimental procedures. Lane 3, M. sexta BBMV pellet incubated with Cry1Aa. Lane 4, M. sexta BBMV with Cry1Aa after gut juice treatment. Lane 5, mouse BBMV incubated with 125I-Cry1Aa. Lane 6, M. sexta gut juice treated mouse BBMV with Cry1Aa. Panel B: Lane 1, 125I-L371K as a control. Lane 2, L371K (fragments A and B) after M. sexta gut juice digestion. Lane 3, M. sexta BBMV incubated with L371K. Lane 4, incubation mixture of M. sexta BBMV and L371K after gut juice digestion. Lane 5, mouse BBMV incubated with 125I-L371K. Lane 6, mouse BBMV with L371K after gut juice digestion.

Table 1. Toxicity assays on M. sexta, binding parameters to purified BBMV and protection of Cry1Aa and L371K against M. sexta gut juice digestion.

Polypeptide	[a]LC_{50}(ng/cm^2)	[b]K_{com} (nM)	[c]Protein Protected No BBMV	[c]Protein Protected. BBMV
Cry1Aa	2.50 (1.7-3.6)	4.37 ±0.72	5.21	69.40
L371K	16.80 (12.5-20.8)	14.25 ±0.51	NA	NA
Fragment A	NA	NA	3.75	6.30
Fragment B	NA	NA	0.74	25.70

a: 50% lethal concentration (95% confidence limits); NA: Not applicable.
b: Data were obtained from homologous competition assay expressed as a mean of four readings.
c: Relative intensities of both wild type and mutant were compared to the untreated Cry1Aa and L371K (fragment A and B), respectively, given as % of toxin remaining after *M. sexta* gut juice digestion.

resistant to proteolytic cleavage. These observations in which proteinase K has been used to digest Cry1Ab and Cry1Ac (Aronson *et al*., 1999; Aronson, 2000; Arnold *et al*., 2001), under similar conditions as those presented here, suggest that the toxin enters the membrane as a single molecule.

Proteolytic digestion studies with mutant L371K showed differential resistance of fragment A and fragment B in the presence and absence of *M. sexta* BBMV. Both fragments were similarly degraded in solution (fragment A showed similar resistance than fragment B). The thermal stability studies, showing fragment A being more resistant to thermolysin than fragment B, are in agreement with the amphipathic α-helices of domain I being more tightly packed in the aqueous environment in the absence of the membrane. In the presence of BBMV however, fragment B showed more protection against protease digestion than fragment A, suggesting that fragment B is capable of membrane translocation by itself, without a dragging force produced by the insertion of domain I. To account for this observation we propose that at least some portions of domain II and/or domain III may become inserted into the membrane. Our hypothesis is based on studies involving the C-terminus of δ-endotoxins. It has been shown by voltage clamp and light scattering studies, that mutations of arginine residues in the conserved block 4, of domain III, causes reduction in ion-channel function of Cry1Aa toxin (Chen *et al*., 1993; Wolfersberger *et al*., 1996; Schwartz *et al*., 1997; Masson *et al*., 2002). Furthermore mutations in domain II of Cry1Ab and Cry3Aa have been shown to affect the irreversible association with the membrane (Rajamohan *et al*, 1996; 1995; Wu and Dean, 1996). Our interpretation is further supported by the earlier work indicating that the whole toxin associated with BBMV is neither attacked by proteases nor bound by monoclonal antibodies (Wolfersberger *et al*., 1986) and it is also protected from proteinase K digestion (Aronson *et al*., 1999; Aronson, 2000; Arnold *et al*., 2001). The possibility of anti-parallel β-sheets being inserted into the lipid bilayer is demonstrated by the protease protection and analytical studies on diphtheria toxin, and aerolysin (Cabiaux *et al*., 1994; Parker *et al*., 1994). The β-sheets have been suggested to serve as scaffold, so that α-helices can acquire a more favorable position to serve as the ion-channel (Hucho *et al*., 1994).

Our observation that fragment B (domains II and III) has a greater affinity to the BBMV than domain I does not agree with the Umbrella Model, which predicts that only a portion of domain I partitions into the membrane, while domains II and III do not. Two other possibilities exist for the differential protection of fragment B. A folding conformation on the surface of the membrane may make domains II and III inaccessible to proteases (the carpet model). However, it is difficult to defend the position that most of a protein, 65 kDa in size, or even fragment B of 26 kDa would lie on the surface and have no exposure to at least a clip by proteinase K or midgut juice. A second possibility is that the β-sheets of domain II and III may form oligomers after binding to the receptor, thus protecting the whole toxin from degradation. In this work we support a model for the membrane bound state of the δ-endotoxins, in which the whole toxin inserts into the membrane, in agreement with previous results (Aronson *et al.*, 1999; Aronson, 2000; Arnold *et al.*, 2001; Loseva *et al.*, 2001; Nair *et al.*, 2008; Alzate *et al.*, 2007; Alzate *et al.,* 2009) that suggests new interpretations for the mechanism of action of these important biopesticides.

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