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Research Article

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Receptor interactions of protoxin and activated Vip3Aa structural conformations in *Spodoptera exigua*

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Abstract

BACKGROUND: The Vip3Aa insecticidal protein, produced by *Bacillus thuringiensis*, has been effectively used in commercial Bt-crops to manage lepidopteran pests. Upon ingestion by larvae, the protoxin is processed by midgut proteases into the activated protein and binds specifically to its receptors in the midgut, leading to insect mortality. Cryo-EM resolution of the trypsin-processed Vip3Aa protein unveiled structural remodelling of the N-terminal region during the transition from protoxin to activated protein. This conformational change has been demonstrated to be crucial for toxicity against *Spodoptera exigua* larvae, a major global lepidopteran pest. In this study, we investigated the relevance of the structural remodelling for the specific binding to midgut receptors.

RESULTS: We conducted *in vitro* binding assays with radiolabelled proteins and brush border membrane vesicles (BBMV) from *S. exigua*, employing structural mutants that lock the protein in either its protoxin or its activated conformation. Our results indicate that both structural stages of the protein share binding sites in the midgut epithelium. Moreover, *in vivo* competition assays revealed that Vip3Aa is able to bind to functional receptors in *S. exigua* larvae both as protoxin and as activated protein.

CONCLUSION: Altogether, our findings point to both structural conformations contributing to receptor binding. *In vivo*, either spontaneous structural shift upon proteolytic cleavage or receptor-mediated remodelling could be occurring. However, the timing and context in which the conformational change occurs could influence membrane insertion and toxicity. Our results show the complex interplay between proteolytic processing, protein structure and receptor interactions in Vip3Aa's toxicity.
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Keywords: *Bacillus thuringiensis*; insecticidal protein; beet armyworm; biotechnological pest control; Bt-crop; biopesticide

1 INTRODUCTION

The *Spodoptera* genus, commonly known as armyworms owing to the well-known gregarious behaviour of their larvae, comprises several lepidopteran species widely recognized as major global pests.[1] These insects have become major invaders, colonizing multiple continents outside their native range.[2] Their larvae are extremely polyphagous, causing extensive damage by feeding on a wide range of crops, resulting in significant financial losses for agriculture on a global scale.[3]

Entomopathogenic bacteria can help to control these pests by providing us with a variety of insecticidal compounds. Cry proteins produced by *Bacillus thuringiensis* (Bt) are the most widely used and better studied among insecticidal proteins.[4,5] The genes coding for these proteins have been incorporated into transgenic plants (Bt-crops), offering effective pest control since the mid 90s.[6–8] However, resistance evolution to Cry proteins in some insect pests has prompted the search for alternative insecticidal proteins.[4,9–11] In this context, Vip3 proteins, also produced by Bt, have emerged as valuable candidates as a consequnce of their potency against lepidopteran insects and, most important, the fact that they do not share binding sites with Cry proteins,[12–18] and there is no reported case of cross-resistance between them.[19,20] Therefore, Vip3 proteins have become excellent partners for Cry proteins in pyramided Bt-crops, which combine different insecticidal proteins with different specificity to expand the target spectrum and prevent the appearance of insect resistance.[21–23] To date, numerous events of maize and cotton containing *vip3Aa* genes combined with *cry* genes have been approved for commercialization globally, with expectations for their adoption in many more crops in the future.[24]

Vip3 proteins are produced by Bt during its vegetative growth phase and are secreted into the media as tetrameric molecules. Each tetramer comprises four identical monomers, with each

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monomer composed by five structural domains.[25–27] The N-terminal (N-t) Domains I and II play roles in tetramer maintenance and toxicity.[28–30] Domains II–III, have been associated with binding functions,[12,29] whereas the C-terminal (C-t) Domains IV–V are carbohydrate binding motifs, and it has been proposed that they may be involved in peritrophic matrix interactions and glycosylated molecules engagement.[25,26,29,31]

Cryo Electron Microscopy (Cryo-EM) resolution of trypsin-processed Vip3Aa and Vip3Bc proteins revealed a structural remodelling of the N-t region. This enabled the distinction between two different structural conformations: the protoxin and the activated protein.[25,26] In the protoxin, the tetramer adopts a pyramidal structure in which the N-t (Domains I and II) forms a helix bundle. That region of the protein gets unfolded and remodelled into a tetrameric coiled-coil in the activated protein, which consists of a central core from which a long stalk protrudes. This coiled-coil structure is formed by Domain I and has a flexible end consisting of helix α1 (residues 23–39) (Fig. 1). The end of the stalk structure has been observed to insert into the membrane of liposomes,[25,32] in agreement with the DeepTMHMM transmembrane prediction tool[33] which indicates a putative transmembrane region in residues 12–30 of Vip3Aa (Supporting information, Fig. S1).

Regarding Vip3 proteins' mode-of-action, it is generally accepted that upon ingestion by larvae, the proteins are proteolytically cleaved between helixes α4 and α5 by midgut proteases, bind specifically to their receptors in the apical membrane of the midgut epithelium (which identity is still under debate) and get inserted into the cell membrane after remodelling into the activated conformation.[25,28,30] Because it is not clear whether the remodelling to the activated protein conformation requires binding to the membrane receptors or can take place before binding, this study aims to examine if Vip3Aa is able to bind to *Spodoptera exigua* midgut binding sites in both structural conformations. To address this question, we have used protoxin and trypsin-processed Vip3Aa proteins in *in vitro* binding assays to *S. exigua* brush border membrane vesicles (BBMV) and *in vivo* competition assays. In addition, two different structural mutants that lock the protein in either its protoxin or its activated-protein conformation have been used. On the one hand, the mutant Vip3Aa_$\Delta\alpha$1, which lacks α-helix 1, has a constitutive activated-protein conformation owing to the loss of contacts between α-helix 1 and Domain II α-helixes that stabilize the protoxin structure.[30] On the other, the mutant Vip3Aa_S-S displays a constitutive protoxin conformation as a result of the introduction of disulfide bonds cross-linking Domain I α-helixes 2 and 3 which avoids its remodelling to the coiled-coil structure that is formed in the activated conformation.[30] Both mutants were described as nontoxic against *S. exigua* larvae, indicating the importance of the helix α1 and the conformational change for the toxicity of the protein.[30] Our results show that both structural conformations of the Vip3Aa protein share binding sites in *S. exigua* midgut epithelium.

2 MATERIALS AND METHODS

2.1 Protein expression and purification

The production of Vip3Aa_Wt and structural mutants Vip3Aa_$\Delta\alpha$1 and Vip3Aa_S-S was performed as described previously with minor modifications.[26,30] Briefly, protein expression was

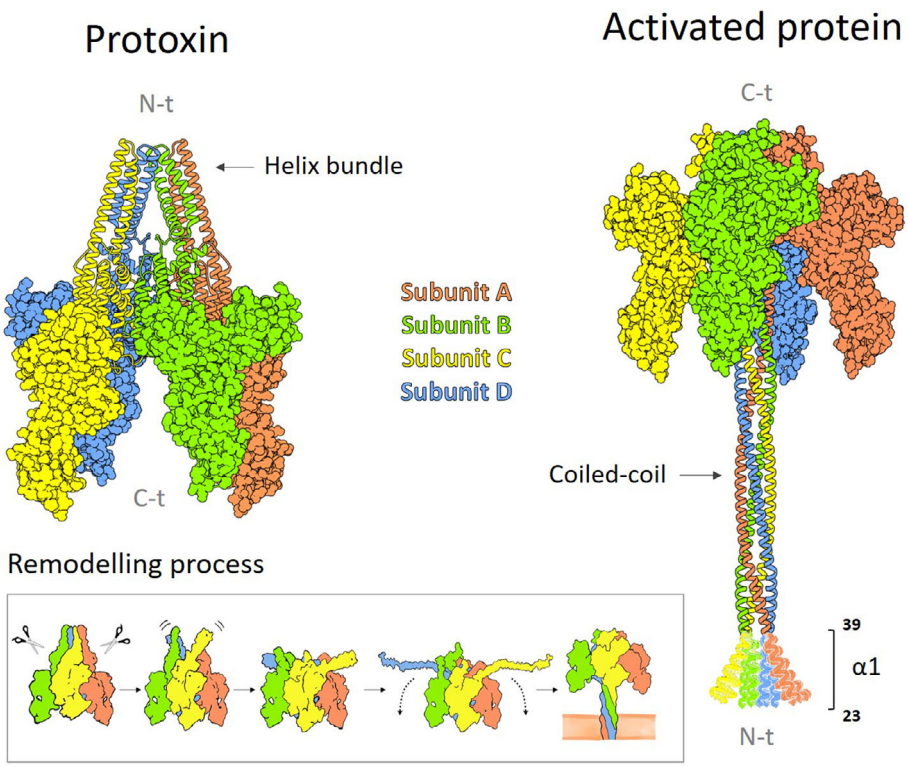

Figure 1. Vip3Aa protein structural remodelling. Vip3 proteins form tetramers in solution composed by four identical subunits. In the protoxin conformation (left panel), the N-t, composed by Domains I and II, forms a helix bundle that, after proteolytic cleavage at the primary site (residues 195–198 in the loop between Domains I and II in Vip3Aa), is remodelled into a tetrameric coiled-coil (involving Domain I) in the activated conformation (right panel), with a flexible end consisting of helix α1 (residues 23–39). The bottom panel shows the remodelling process proposed for Vip3 proteins (modified from[25]).

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conducted in *Escherichia coli* C43 (DE3) containing each of the construct vectors. Cells were grown in 700 mL Luria-Bertani broth to exponential phase [optical density at 600 nm (OD_{600}) ~ 0.6–0.8], then induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated at 25 °C overnight. Cells were collected and stored at −20 °C. For purification, thawed cells were resuspended in buffer A (50 mM Tris–HCl, 150 mM NaCl and 5 mM $MgCl_2$ pH 8.0) supplemented with 3 mg mL^{-1} lysozyme, 10 µg mL^{-1} DNase and 100 µM PMSF and incubated for 30 min at 37 °C with shaking. The lysate was then sonicated for 5 min and centrifuged at 25 000×*g* for 30 min. The clarified supernatant was loaded onto a 5-mL StrepTrap column (Cytiva, Marlborough, CA, USA) for protein purification. The protein was eluted with 5 mM D-desthiobiotin dissolved in buffer A pH 8.0. The StrepII-tag was removed by treating with 3C PreScission Protease (Cytiva) followed by dialysis in buffer A. The sample was then subjected to another step of affinity chromatography with the StrepTrap column to separate the nontagged protein from any remaining tagged molecules, and pure proteins were eluted in buffer A.

The mutant protein Vip3Aa DIP (disabled insecticidal protein) E168A was used as positive control for in *in vivo* competition assays. The protein was produced as described previously.[34,35]

The mutant protein Cry1Ab R99E was used as negative control for in *in vivo* competition assays. Heterologous expression in *E. coli*, cell lysis, protoxin solubilization, trypsin-activation and purification by anion-exchange were performed as described previously.[36]

Pure proteins were checked in 12% acrylamide SDS-PAGE gels (Fig. S2), aliquoted, flash-frozen with liquid nitrogen, and stored at −80 °C for further use.

2.2 Preparation of *S. exigua* brush border membrane vesicles

The BBMV were obtained using the differential magnesium precipitation method[37] starting with frozen midgut tissue from last instar larvae of *S. exigua*. After preparation, vesicles were divided into aliquots, rapidly frozen in liquid nitrogen, and stored at −80 °C. Before their utilization in *in vitro* binding assays, BBMV were thawed, centrifuged and resuspended in binding buffer (see Section 2.4 for buffer composition). The protein concentration in the BBMV samples was then quantified using the Bradford assay.[38]

2.3 Protein ^{125}I radiolabelling

Vip3Aa_Wt and Vip3Aa_Δα1 protoxins were radiolabelled by the chloramine T method[39] following the protocol described elsewhere.[12,34] Briefly, 25 µg of pure protein were mixed with 0.3 mCi of [^{125}I]-NaI and 1/3 (vol/vol) 18 mM chloramine T. The excess of free ^{125}I was separated from the labelled protein using a PD10 desalting column (Cytiva). The purity of the labelled proteins was checked by analyzing the eluted fractions by SDS-PAGE with 12% acrylamide gels and autoradiography (Fig. S3). Vip3Aa_Wt protein was labelled twice being the specific activity 5.07and 5.90 µCi $µg^{-1}$, respectively. The specific activity of radiolabelled Vip3Aa_Δα1 was 2.60 µCi $µg^{-1}$. Labelled proteins were stored at 4 °C and used up to 1 month after the labelling reaction.

2.4 *In vitro* binding assays with radiolabelled proteins and *S. exigua* BBMV

In vitro binding assays were performed as described previously.[34] Briefly, for all kind of assays, labelled proteins were incubated with BBMV for 1 h in 100 µL final volume of binding buffer (20 mM Tris–HCl, 1 mM $MnCl_2$, 0.1% BSA, pH 7.4). After incubation, the reaction was halted by centrifugation at 16 000×*g* for 10 min at 4 °C, and the pellet was washed with 500 µL binding buffer. Radioactivity retained in the pellet was quantified using a model 2480 WIZARD2 γ-counter (Perkin Elmer, Waltham, MA, USA) to assess bound labelled protein.

Bindability assays with a fixed amount of labelled protein (0.2 nM), increasing concentrations of BBMV and an excess of unlabelled competitor (1000-fold molar ratio) were performed for both nonprocessed and trypsin-treated samples of Vip3Aa_Wt and Vip3Aa_Δα1 labelled proteins.

Competition assays were performed by mixing a fixed amount of trypsin-processed labelled proteins (0.2 nM) with increasing concentrations of unlabelled homologous and heterologous competitors and a fixed amount of BBMV established according to the shape of their respective total binding curves in bindability assays (0.05 mg mL^{-1} for Vip3Aa_Wt and 0.1 mg mL^{-1} for Vip3Aa_Δα1 radiolabelled proteins based on BBMV concentration in which total binding is not saturated).

For measuring binding reversibility, chase-time experiments were performed by incubating a fixed amount of trypsin-processed labelled proteins (0.2 nM) with a fixed amount of BBMV (0.05 mg mL^{-1} for Vip3Aa_Wt and 0.1 mg mL^{-1} for Vip3Aa_Δα1). After an incubation time of 1 h, a 1000-fold molar excess of unlabelled competitor was added to the tubes and samples were collected after 5 min, 30 min and 1 h chase-time. The samples were centrifuged and washed as for the rest of binding assays, the radioactivity retained in the pellet corresponding to the remaining labelled protein bound after the chase time was quantified for the different time points.

Trypsin-treatment of labelled proteins and competitor samples was performed at 1% w/w, incubating at 37 °C for 1 h in all cases. The proteolytic profile of Vip3Aa_Wt, Vip3Aa_Δα1 and Vip3Aa_S-S competitor proteins after trypsin-incubation can be observed in Fig. S2. At least two technical replicates were performed for each binding assay, competition assay or chase-time experiment. Graphical representations were obtained with Prism (GraphPad, San Diego, Ca, USA). The binding parameters equilibrium dissociation constant (K_d) and binding site concentration (R_t), were estimated using LIGAND[40] for the homologous competition curves of both Vip3Aa_Wt and Vip3Aa_Δα1 radiolabelled proteins.

2.5 *Spodoptera exigua* colony rearing and *in vivo* competition assays

For surface contamination bioassays, a laboratory population of *S. exigua* was reared on semi-synthetic diet[41] under controlled conditions: 25 ± 2 °C, 70 ± 5% relative humidity (RH) and a photoperiod of 16:8 h, light:dark. *In vivo* competition bioassays were conducted using different protein: competitor molar ratios (1:0, 1:10, 1:100 and 1:1000) in buffer A, the structural mutants Vip3Aa_Δα1 and Vip3Aa_S-S were used as competitors. Negative controls included buffer (labelled as 0) and an excess of competitor (labelled as 0:1000, representing the highest competitor concentration used). A Vip3Aa DIP mutant carrying the point mutation E168A, which lacks toxicity but maintains its binding capacity, was used as a positive control of *in vivo* competition based on previous results found in *S. littoralis*.[34] The Cry1Ab R99E mutant protein, described previously as nontoxic but able to block the toxicity of its wild-type (WT) counterpart, was used as a negative control of competition.[42–44] Pure Vip3Aa at a

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constant concentration of 20 ng cm^{-2} determined from previous median lethal concentration (LC_{50}) values[30] was mixed with corresponding amounts of pure competitor proteins to create the desired ratios. Dispensing 50 μL protein preparations onto the surface of assay wells (2 cm^2 diameter) filled with semi-synthetic diet, we employed 16 wells per protein dose. Following drying of the diet surface, a single neonate larva was gently introduced into each well, and the plates were sealed. Trays were kept in a climatic chamber mimicking rearing conditions, and mortality was recorded after 7 and 10 days of incubation. Each *in vivo* competition protein combination underwent at least three biological replicates. Statistical analysis of mortality differences between 1:0 and other protein: competitor ratios was performed using a one-way ANOVA with *post hoc* Bonferroni test in Prism (GraphPad), ensuring rigorous assessment of significance with *P*-values higher than an α-value of 0.05.

3 RESULTS

3.1 *In vitro* binding assays to *S. exigua* BBMV with radiolabelled Vip3Aa proteins

In order to examine the ability of protoxin conformation and activated toxin conformation to bind to midgut receptors, radiolabelled Vip3Aa nonprocessed and trypsin-processed samples were used in binding assays to BBMV from *S. exigua*. Our results showed that Vip3Aa was able to bind in both conformations [Fig. 2(A), (B)]. However, although the protoxin showed a higher total binding (60%) [Fig. 2(A)] than the trypsin-processed protein (20% at the highest BBMV dose tested), practically most of the binding of the protoxin was nonspecific, in contrast to the binding of the trypsin-processed Vip3Aa [Fig. 2(B)].

We also performed *in vitro* binding assays with the Vip3Aa_Δα1 mutant, which shows a constitutive activated-protein conformation without the need of proteolytic cleavage. Both nonprocessed and trypsin-treated samples showed specific binding [Fig. 2(C), (D)]. Interestingly, the nonspecific binding was not dose-dependent. In addition, the trypsin-processed Vip3Aa_Δα1 showed lower nonspecific binding than the nonprocessed Vip3Aa_Δα1.

3.2 *In vitro* binding competition assays

Competition assays were performed using trypsin-treated radiolabelled Vip3Aa in combination with increasing concentrations of nonlabelled Vip3Aa (homologous competitor). The analysis of the competition curve provided a K_d of 117 ± 43 nM, with R_t of 129 ± 52 pmol mg^{-1} BBMV protein. We also used two distinct Vip3Aa structural mutants that lock the protein in either its protoxin (Vip3Aa_S-S) or its activated conformation (Vip3Aa_Δα1) as heterologous competitors. The results showed that both trypsin-processed mutant proteins were able to compete for the specific binding of trypsin-treated labelled WtT protein (Vip3Aa_Wt) [Fig. 3(A)–(C)].

Using trypsin-processed Vip3Aa_Δα1, Vip3Aa_S-S and Vip3Aa_Wt as competitors for the radiolabelled trypsin-processed Vip3Aa_Δα1 protein, we found that the three proteins were able to compete in a similar way for the specific binding of the labelled

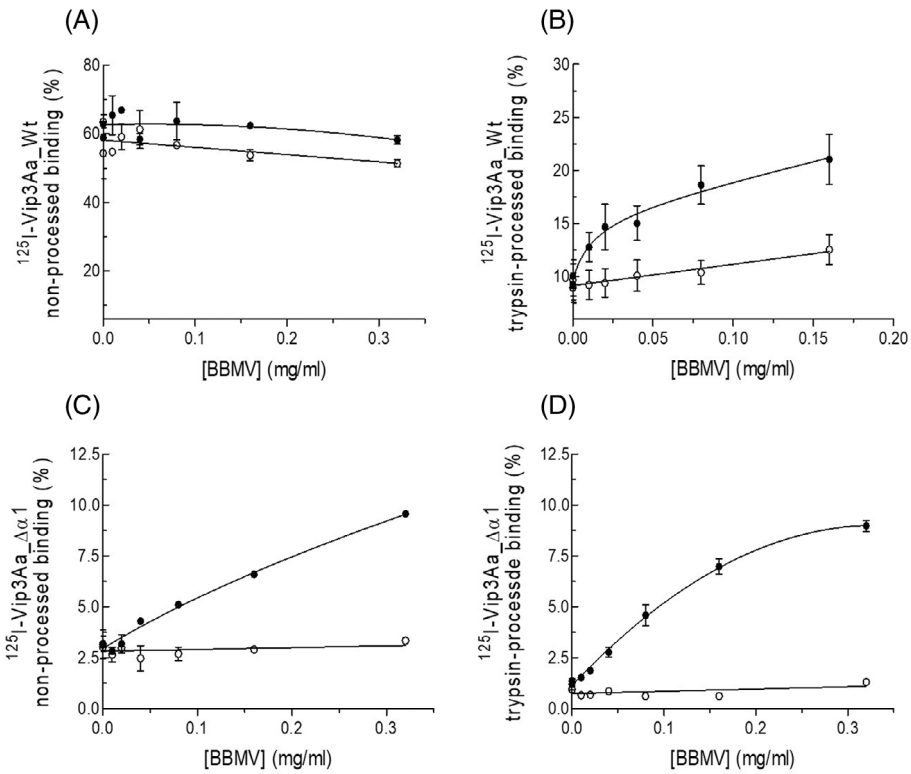

Figure 2. ^{125}I-Vip3Aa_Wt and ^{125}I-Vip3Aa_Δα1 binding assays in *Spodoptera exigua* brush border membrane vesicles (BBMV). *In vitro* binding assays were performed using nonprocessed ^{125}I-Vip3Aa_Wt (A) with only protoxin molecules, trypsin-processed ^{125}I-Vip3Aa_Wt (B) having a mixture of molecules in protoxin and activated conformations, nonprocessed ^{125}I-Vip3Aa_Δα1 (C) and trypsin-processed ^{125}I-Vip3Aa_Δα1 (D), both samples having only activated structure, in combination with increasing concentrations of *S. exigua* BBMV. Full circles and open circles represent total and nonspecific binding (determined with 1000-fold unlabelled homologous competitors), respectively. Each data point represents the mean of at least two replicates (±SEM). Wt, wild-type

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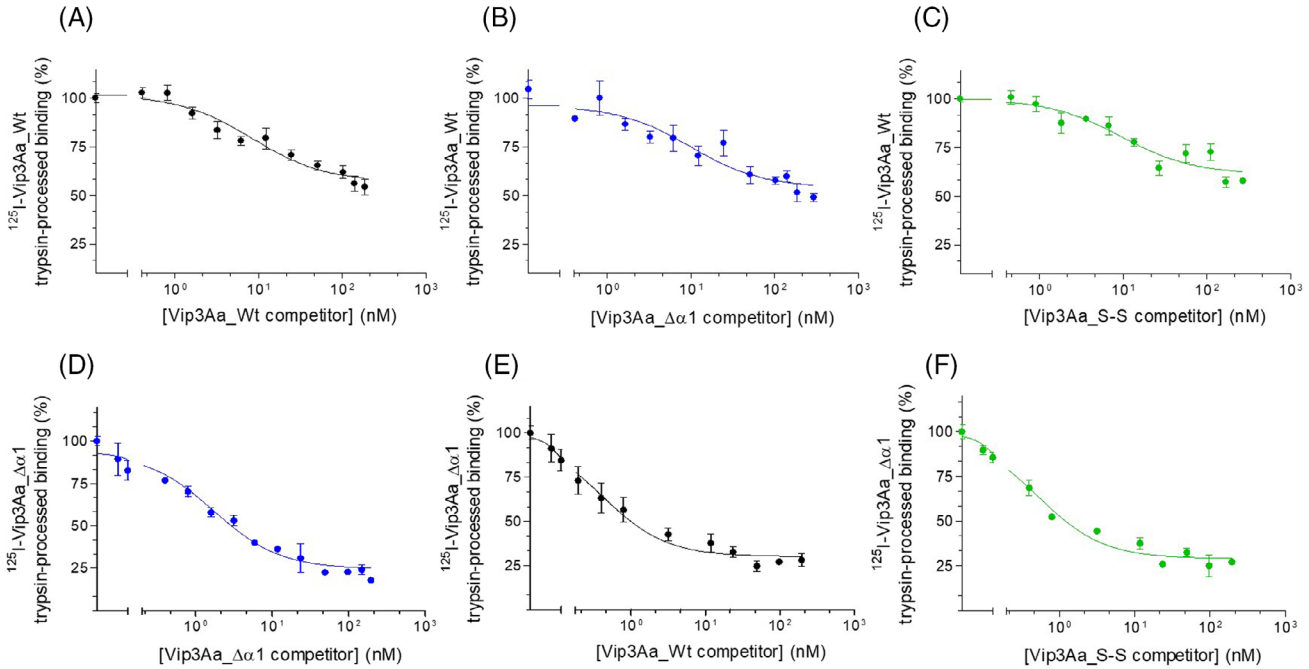

Figure 3. ^{125}I-Vip3Aa_Wt and ^{125}I-Vip3Aa_Δα1 competition binding assays in *Spodoptera exigua* brush border membrane vesicles (BBMV). Trypsin-processed labelled proteins ^{125}I-Vip3Aa_Wt (A–C) and ^{125}I-Vip3Aa_Δα1 (D–F) were used with a fixed concentration of BBMV and increasing concentrations of unlabelled competitors. Competitions in black colour refer to the use of trypsin-processed Vip3Aa_Wt as competitor, which contains a mixture of protoxin and activated-protein structural conformations. Competitions in blue refer to the use of trypsin-processed Vip3Aa_Δα1 as competitor, which displays a constitutive activated-protein structure. Competitions in green refer to the use of trypsin-processed Vip3Aa_S-S as competitor, showing a constitutive protoxin conformation. Each data point represents the mean of at least two replicates (± SEM). Wt, wild-type

protein [Fig. 3(D)–(F)] confirming that both protoxin and activated conformations bind to the same sites in the BBMV. The analysis of the homologous competition curve for trypsin-treated Vip3Aa_Δα1 provided a K_d of 22.7 ± 5.2 nM, with a R_t of 11.8 ± 2.0 pmol mg^{-1} BBMV protein.

3.3 *In vitro* binding reversibility assays

Chase-time experiments were performed to assess the binding reversibility of the Vip3Aa_Wt and Vip3Aa_Δα1 to the BBMV. Trypsin-processed Vip3Aa_Wt showed 50% irreversible binding, and the binding displacement occurred at the beginning of the chase-time [Fig. 4(A)], whereas trypsin-processed Vip3Aa_Δα1 exhibited a higher percentage of irreversible binding (80%) and the binding displacement took place later [Fig. 4(B)].

3.4 Vip3Aa *in vivo* competition assays against conformational mutants

Finally, to evaluate the functional relevance of the binding of Vip3Aa in its both structural stages, we performed *in vivo* competition assays using the conformational mutants as competitors

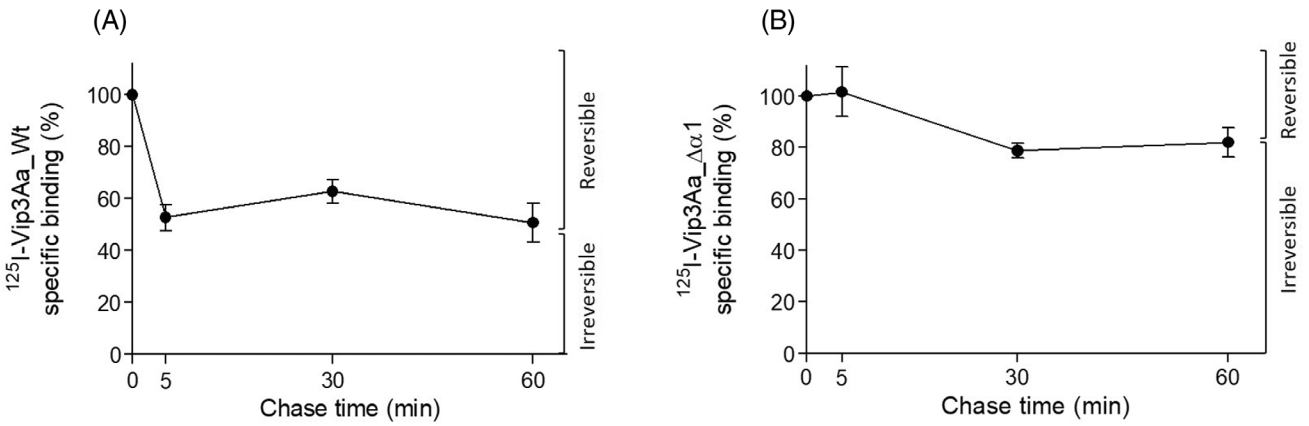

Figure 4. ^{125}I-Vip3Aa_Wt and ^{125}I-Vip3Aa_Δα1 binding reversibility assays in *Spodoptera exigua* brush border membrane vesicles (BBMV). Chase-time experiments were performed by pre-incubating trypsin-processed ^{125}I-Vip3Aa_Wt (A) (containing a mixture of both structural conformations), and trypsin-processed ^{125}I-Vip3Aa_Δα1 (B) (containing only molecules in the activated-protein conformation), with a fixed amount of *S. exigua* BBMV before adding an excess of homologous nonlabelled competitors. Samples were collected after 5, 30 and 60 min of incubation with the competitor proteins to quantify the remaining binding and evaluate binding reversibility. Each data point represents the mean of at least two replicates (± SEM). Wt, wild-type

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since none of them are toxic against *S. exigua* larvae[30] and are able to compete with Vip3Aa *in vitro*. The Vip3Aa E168A and Cry1Ab R99E mutant proteins were used as positive and negative controls of competition, respectively. The bioassays showed that in a similar way to the Vip3Aa DIP mutant used as positive control [Fig. 5(A)], both Vip3 conformational mutants, Vip3Aa_Δα1 and Vip3Aa_S-S, were able to block the Vip3Aa_Wt's toxicity [Fig. 5 (B), (C)], indicating that both conformations of the protein bind to functional receptors *in vivo*. However, as expected given the lack of shared receptors among Cry1A and Vip3A proteins, the mutant Cry1Ab R99E did not block the Vip3Aa_Wt's toxicity [Fig. 5(D)].

4 DISCUSSION

The present study focuses on examining how both the protoxin and activated protein structural conformations of the Vip3Aa insecticidal protein, bind to *S. exigua* midgut receptors. Employing an experimental approach comprising *in vitro* binding assays to BBMV and *in vivo* competition toxicity assays, our objective was to determine how the structural conformation of the protein affects its binding capacity.

The initial *in vitro* binding assays, employing radiolabelled trypsin-treated Vip3Aa_Wt, yielded results consistent with previous findings on Vip3A proteins in *Spodoptera* spp. BBMV, showing 50% of specific binding and a rapid saturation of binding [Fig. 2 (B)].[12,13,34] Based on prior Cryo-EM and Negative Staining EM (NS-EM) observations,[26,30] the trypsin-processed Vip3Aa sample contains a mixture of both protein conformations, with the proportion of molecules in the sample undergoing structural remodelling varying across replicates (50–70%). Considering these previous observations, to characterize the binding of the activated protein conformation alone, we decided to label the mutant protein Vip3Aa_Δα1 which shows a constitutive activated protein structure without the need of proteolytic processing owing to the lack of α-helix 1, which is critical for maintaining the protoxin structure.[30] Compared with the Wt protein, Vip3Aa_Δα1 had a lower nonspecific binding, which could be related with the protein conformation, but also with the lack of helix α1. In addition, comparing the nonprocessed Vip3Aa_Δα1 with the trypsin-treated Vip3Aa_Δα1, the nonprocessed protein had a higher nonspecific binding, indicating that not only the structural conformation, but also the proteolytic cleavage as well has an impact on binding specificity [Fig. 2(C), (D)].

Binding competition assays using trypsin-processed Vip3Aa_Δα1 and Vip3Aa_S-S structural mutants as competitors for the WT radiolabelled protein provided further insights into the binding mechanisms. Despite differences in their structural conformations, both mutants effectively competed with trypsin-processed Vip3Aa_Wt for its binding sites on *S. exigua* BBMV, indicating that both conformations of Vip3Aa are able to recognize the same binding sites [Fig. 3(B), (C)]. If the different conformations of Vip3Aa would bind to different binding sites, the mutant proteins could not compete completely with the Vip3Aa_Wt

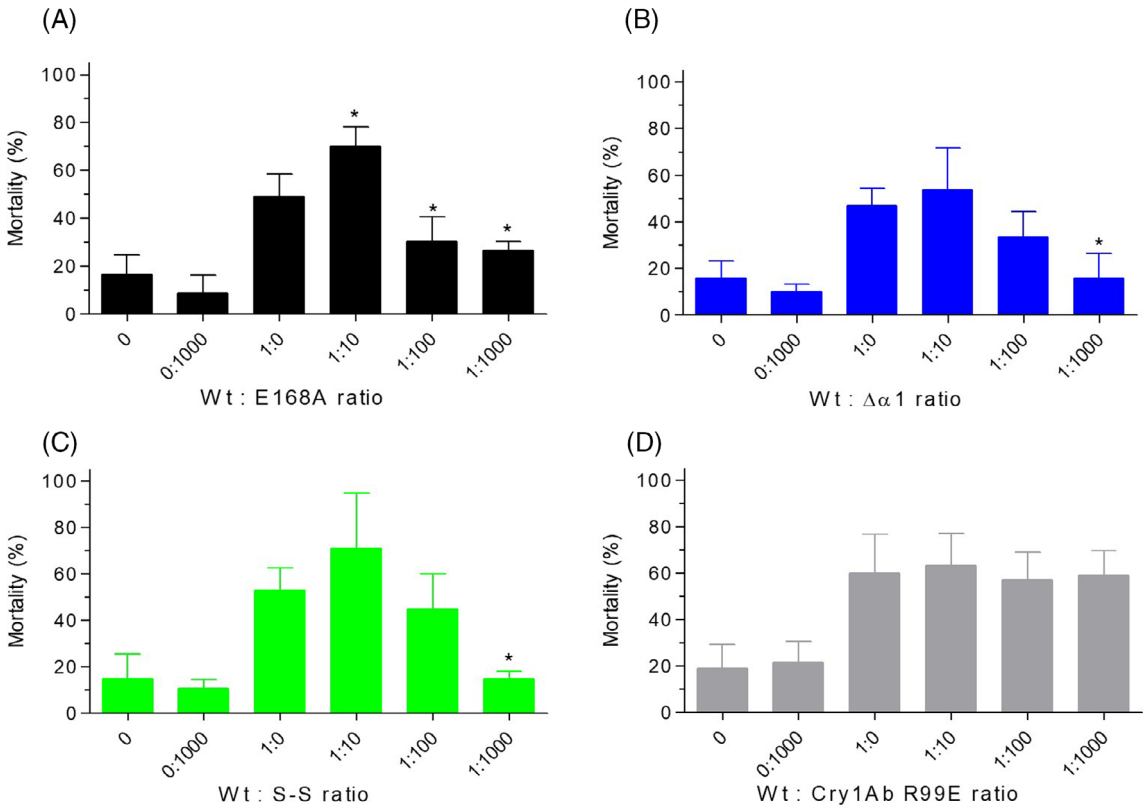

Figure 5. Vip3Aa *in vivo* competition assays against conformational mutants in *Spodoptera exigua* larvae. Bars represent the mortality recorded after 10 days for each protein: competitor molar ratio. 0, just buffer; 0:1000, just nontoxic competitor at the highest concentration tested; 1:0 just Vip3Aa_Wt at the LC_{50} dose; 1:10, 1:100 and 1:1000 ratio of Vip3Aa_Wt (at the LC_{50} dose) and nontoxic competitor. Error bars show the standard deviation (SD) among replicates. The asterisks over the bars indicate significance ($P < 0.05$) compared to the ratio 1:0 in a one way ANOVA with Bonferroni *post hoc* test. Wt, wild-type

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labelled protein and the competition curves would differ. Competition assays with the trypsin-processed Vip3Aa_Δα1 radiolabelled protein confirmed that Vip3Aa in its two conformations binds to the same binding sites *in vitro* [Fig. 3(E), (F)]. Furthermore, *in vivo* competition assays with the structural mutants confirmed the results found *in vitro*, showing that the two Vip3Aa conformations bind to functional receptors in the insect midgut because both effectively blocked the toxicity of Vip3Aa_Wt against *S. exigua* larvae [Fig. 5(B), (C)].

Interestingly, Vip3Aa_Wt protoxin is able to bind to *S. exigua* BBMV and shows higher total binding (60%) compared to the trypsin processed protein (20%). However, this binding is mainly nonspecific [Fig. 2(A)]. Therefore, despite that almost all of the protoxin's binding is nonspecific, it includes the specific and functional binding sites of the activated protein, explaining the competition observed *in vitro* for the BBMV binding sites, and the ability of the Vip3Aa_S-S mutant to block the WT's toxicity *in vivo*. The observation that both structural conformations are able to bind to the same receptors supports the proposed role of Domains II–III in receptor binding,[12,29] as this part of the protein does not undergo any dramatic structural remodelling after proteolytic processing[26] (Fig. 1).

Nonetheless, our results of binding reversibility suggest potential differences in membrane interactions possibly attributable to the remodelling into the activated structure, because the trypsin-processed Vip3Aa_Δα1 mutant (constitutive activated conformation), despite lacking of the putative transmembrane region, shows a higher level of irreversible interactions with the BBMV compared to the trypsin-processed WT protein (mixture of both structural conformations) (Fig. 4). Thus, binding irreversibility may not reflect membrane insertion, and although the primary role in receptor binding may reside in Domains II–III, the involvement of the N-t part of the activated protein in membrane engagement cannot be disregarded, as these irreversible interactions could be highly relevant for the protein's function. Differences in the binding properties of both structural stages are further supported by the analysis of binding parameters performed with the homologous competition curves of Vip3Aa_Wt and Vip3Aa_Δα1 trypsin-processed radiolabelled proteins. For the Vip3Aa_Δα1 protein, a lower K_d value was obtained, indicating a higher affinity for its binding sites in the BBMV compared to the affinity of the WT protein. Also, the higher binding affinity observed for the Vip3Aa_Δα1 mutant protein could be related with the higher percentage of irreversible interactions displayed by this mutant.

5 CONCLUSION

We have investigated how the structural conformation of Vip3Aa affects its capacity of binding to midgut receptors in *S. exigua*. Altogether, our findings point to both protoxin and activated protein structural conformations contributing to receptor binding. Therefore, either spontaneous structural shift upon proteolytic cleavage or receptor-mediated remodelling could be occurring *in vivo*. In terms of binding, protein conformation seems not to be a critical feature, because the protoxin structure, despite its high nonspecific binding observed *in vitro*, is nonetheless able to block functional binding sites *in vivo*. However, the timing and context in which the conformational change into the activated protein structure occurs, could profoundly influence membrane insertion and subsequent toxicity. Our results show the complex interplay between proteolytic processing, protein structure and receptor interactions in Vip3Aa's toxicity against *S. exigua*. Nonetheless, the higher binding affinity observed for the Vip3Aa_Δα1 mutant protein suggests that the activated conformation would be favoured *in vivo*.

AUTHOR CONTRIBUTIONS

All authors conceived the ideas and designed the experiments. ML-B performed the experimental work, analyzed the data, wrote the main draft of the manuscript and prepared the figures. All authors critically reviewed the manuscript and gave final approval for submission.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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