Structural and functional role of Domain I for the insecticidal activity of the Vip3Aa protein from Bacillus thuringiensis

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Abstract
Vip3 proteins are produced by Bacillus thuringiensis and are toxic against lepidopterans, reason why the vip3Aa gene has been introduced into cotton and corn to control agricultural pests. Recently, the structure of Vip3 proteins has been determined and consists of a tetramer where each monomer is composed of five structural domains. The transition from protoxin to the trypsin-activated form involves a major conformational change of the N-terminal Domain I, which is remodelled into a tetrameric coiled-coil structure that is thought to insert into the apical membrane of the midgut cells. To better understand the relevance of this major change in Domain I for the insecticidal activity, we have generated several mutants aimed to alter the activity and remodelling capacity of this central region to understand its function. These mutants have been characterized by proteolytic processing, negative staining electron microscopy, and toxicity bioassays against Spodoptera exigua. The results show the crucial role of helix α1 for the insecticidal activity and in restraining the Domain I in the protoxin conformation, the importance of the remodelling of helices α2 and α3, the proteolytic processing that takes place between Domains I and II, and the role of the C-t Domains IV and V to sustain the conformational change necessary for toxicity.
INTRODUCTION

Vegetative insecticidal proteins (Vip3) are produced during the vegetative growth phase of the bacterium *Bacillus thuringiensis*, and are toxic against a wide range of lepidopteran pests (Chakroun et al., 2016; Estruch et al., 1996; Palma et al., 2014; Ruiz de Escudero et al., 2014). Notably, members of the Vip3A family have been used in Bt crops (crops that express one or more *B. thuringiensis* genes) in combination with the Cry insecticidal proteins to prevent the evolution of insect-resistant populations (Carrière et al., 2015; Tabashnik & Carrière, 2017), since they are toxic for insect species that have already developed resistance against the Cry proteins and do not share binding sites with them (Chakroun & Ferré, 2014; Gouffon et al., 2011; Lee et al., 2006; Quan et al., 2021; Sena et al., 2009).

The Vip3 proteins are secreted as protoxins of ~89 kDa which, once ingested by the larvae, are processed by the midgut proteases into two different fragments of 19–22 and 62–66 kDa that remain attached and conform to the activated protein (Bel et al., 2017; Chakroun & Ferré, 2014; Quan & Ferré, 2019; Zack et al., 2017). Once processed, Vip3 proteins are able to bind to specific receptors located on the brush border membrane of the midgut epithelium cells (Chakroun & Ferré, 2014; Quan et al., 2021) where they exert their toxic effect. Regarding their mode of action, it has been shown that Vip3 proteins get inserted into the membrane and induce pore formation, leading to the death of the larvae (Lee et al., 2003; Liu et al., 2011). In addition, and mainly in studies with culture cells, it has been reported that Vip3A proteins can induce apoptosis through the mitochondrial apoptotic pathway after being internalized into the cells (Hernández-Martínez et al., 2017; Hou et al., 2020; Jiang et al., 2016; Nimsanor et al., 2020).

Recently, the structure of Vip3 proteins has been determined by cryo-electron microscopy (cryo-EM), revealing that they consist of a homotetramer in which each subunit is composed of five structural domains (Byrne et al., 2021; Núñez-Ramírez et al., 2020; Zheng et al., 2020) (Figure 1). Comparison between Vip3 protoxin and trypsin-activated protein structures has demonstrated a major conformational change at the N-terminal (N-t) Domain I (Figure 1A, B). In the protoxin conformation, the N-t forms a helix bundle that is remodelled into a tetrameric coiled-coil as observed in the toxin conformation, with a flexible end that most probably is formed by helix α1 (Byrne et al., 2021; Núñez-Ramírez et al., 2020). The tip of this coiled-coil structure has been shown to get inserted into the membrane of liposomes (Byrne et al., 2021). At the C-terminus (C-t), Domains IV and V (Figure 1C) are carbohydrate-binding motifs that do not contact the N-t part of the protein or directly interact with other subunits, and are dispensable for oligomer formation (Byrne et al., 2021; Núñez-Ramírez et al., 2020; Quan & Ferré, 2019; Zheng et al., 2020). Also, it has recently been shown that they are not necessary for the specific binding of Vip3A to *Spodoptera* spp. brush border membrane vesicles (BBMV) and *Spodoptera frugiperda* Sf21 cultured cells, or for exerting toxicity against the latter (Jiang et al., 2020; Quan et al., 2021). Nevertheless, many reports have shown that they are necessary for the toxicity of Vip3A proteins in vivo (Banyuls et al., 2018, 2021; Gayen et al., 2012, 2015; Li et al., 2007; Quan & Ferré, 2019; Selvapandiyan et al., 2001).

To better understand the relevance of Domain I for the toxicity and the role of the C-t Domains IV and V in the in vivo toxicity of Vip3Aa, we have engineered a deletion mutant of these domains in addition to mutations aimed to restrict remodelling of Domain I as well as to modify it. Since activation of Vip3A proteins by the insect midgut proteases is a key step for the insecticidal activity (Chakroun et al., 2016; Hernández-Martínez et al., 2013; Lee et al., 2003), the behavior of these mutants upon proteolytic processing has been characterized. Also, we have performed negative staining EM and toxicity bioassays against the lepidopteran species *Spodoptera exigua*, to determine the effect of these mutations on the protein structure and how this affects the insecticidal activity. The results demonstrate that the integrity and the remodelling of Domain I with subsequent coiled-coil formation is essential for the in vivo insecticidal activity of Vip3Aa.

EXPERIMENTAL PROCEDURES

Mutagenesis of Vip3Aa

Vip3Aa16 (NCBI accession No. AAW65132) (residues 10–789) was cloned in LIC 1.5, as previously described (Núñez-Ramírez et al., 2020), and then was used as a template vector to prepare the mutant vectors used in this study. Specifically, the sequences of Vip3Aa_Δα1 and Vip3Aa_ΔIV-V were prepared by amplification of the vector template from residue 40 to 789 and residue 10 to 535, respectively, then cloned in LIC 1.5. The rest of the mutants were obtained using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) with the vector template to obtain the corresponding mutant vectors containing the mutant proteins. See Table S1 for the primer description used to prepare each mutant protein.

Protein expression and purification

The production of Vip3Aa WT and mutants used in this study was described previously (Núñez-Ramírez et al., 2020). Briefly, protein expression was conducted in *Escherichia coli* C43 (DE3) containing each of the
mutant vectors. Cells were grown in Luria-Bertani broth to exponential phase (OD₆₀₀ ~ 0.6), then induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), incubated at 37°C for 3 h and then centrifuged and stored at −20°C. For purification, thawed cells were re-suspended in buffer A (50 mM Tris–HCl, 0.5 M NaCl, and 50 mM MgCl₂) pH 8.0, with 1 mM phenylmethanesulfonyl fluoride and 1 mM Tris (2-carboxyethyl)phosphine hydrochloride, and sonicated for 5 min. The cell lysate was centrifuged at 25,000 g for 30 min and the clarified supernatant was loaded into a 5 ml Streptrap column (GE, Healthcare). The protein was eluted with 5 mM d-desethylbin dissolved in buffer A pH 8.0. The StreptII-tag was removed adding 3C PreScission protease (fused to an N-terminal GST-tag) to the Vip3Aa mutants at a 1:1/25 (protein:protease) molar ratio followed by dialysis. The sample was then subjected to two other steps of affinity chromatography with a Streptrap and a GST-Trap column to separate the non-tagged protein from any remaining tagged protein and protease. The non-tagged protein was further purified by gel filtration chromatography using a ProteoSEC 16/60 6–600 HR (Generon, UK). Samples containing the purest protein were concentrated to ~5 mg/ml, aliquoted, frozen with liquid nitrogen, and stored at −80°C for further use.

**In vitro proteolytic assays**

Purified proteins diluted in buffer A pH 7.5 were quantified by Bradford (1976). A fixed amount of protein (10 μg) was incubated with 10% (w/v; 1 μg) commercial trypsin or α-chymotrypsin (both from bovine pancreas, Sigma Aldrich), or 10% (w/v) midgut juice or 10% (w/w) BBMV from *S. exigua*. Midgut juice of *S. exigua* was prepared following the protocol described elsewhere (Chakroun et al., 2012), flash-frozen in liquid nitrogen and stored at −80°C. BBMV from
**RESULTS**

**Engineering of Vip3Aa mutant proteins**

To better understand the role of the structural features of Domain I in the toxicity, we have engineered several mutants targeting different regions or processes
which can be grouped into four categories: (i) mutants directed to affect the N-t part of the protein, named as Vip3Aa_Δα1, Vip3Aa_41-KVKK-44 and Vip3Aa_M34L, (ii) mutants directed to affect the proteolytic processing and conformational change of the activated protein, named as Vip3Aa_195-AVAA-198 and Vip3Aa_S-S, (iii) mutants directed to reduce the internal diameter of the tetrameric coiled-coil observed in the toxin conformation, named as Vip3Aa_L146F and Vip3Aa_L146M, and (iv) a mutant lacking the C-t Domains IV and V (Vip3Aa_ΔIV-V). A detailed description of the mutations and their expected effect on the structure and/or properties of the protein, according to the structural data, are summarized in Table 1.

In vitro proteolytic processing of the Vip3Aa WT and mutant proteins

The WT and the mutant proteins were subjected to proteolytic processing in vitro under two conditions: using commercial trypsin and using midgut juice of S. exigua larvae (SeMJ). As can be observed in Figure 2, the WT protein (~85 kDa), rendered two fragments of 20 and 65 kDa (corresponding to N-t Domain I and C-t Domains II–V, respectively) in both proteolytic conditions (Figure 2), indicating that this site was not sufficiently exposed to be cleaved by proteases. The mutant Vip3Aa_195-AVAA-198, designed to eliminate the trypsin proteolytic site KVKK located in the loop α4-α5 between Domains I and II (Figure 1C), was not digested by the commercial trypsin, as previously described (Zhang et al., 2018). However, a slight band corresponding to the 65kDa fragment was observed upon incubation with SeMJ, pointing to slight processing by the mixture of proteases in the midgut juice (Figure 2). The mutant Vip3Aa_S-S, which contained mutations D66C and N69C located in helix α2, as well as, mutations Q96C and L100C located in helix α3, (Figure 1C) was designed to cross-link helices α2-α3 probably due to different unfolding stages of the dissociated subunits under denaturing conditions affecting the electrophoretic mobility of protein–SDS interaction. MALDI-TOF analysis of the two bands indicated that they were the same protein with 99.5% of confidence. Indeed, upon processing by trypsin, both bands disappeared rendering the 65 kDa band shown in the WT and an N-t band that runs as ~16 kDa, instead of 20 kDa, due to the absence of helix α1. Using SeMJ, only the 65 kDa band was observed (Figure 2), presumably by the degradation of the ~16 kDa fragment by midgut proteases. The mutant Vip3Aa_41-KVKK-44, which includes a proteolytic site in the loop α1α2 aimed to remove helix α1 (Figure 1C), unexpectedly showed the same proteolytic pattern as the WT in both digestion conditions (Figure 2), indicating that this site was not sufficiently exposed to be cleaved by proteases.

![Figure 1C](image)

**TABLE 1** Nomenclature and description of the Vip3Aa mutants

| Category | Mutation name | Mutation description | Expected consequence of the mutation |
|----------|---------------|----------------------|-------------------------------------|
| Mutants directed to affect the N-t part of the protein and helix α1 | Vip3Aa_Δα1 | Deletion from N-t to residue 39 which removes helix α1 | To remove helix α1 |
|          | Vip3Aa_41-KVKK-44 | Mutation of residues 41-DTGG-44 for 41-KVKK-44 that incorporates a new trypsin proteolytic cleavage site after helix α1 | To insert a new trypsin cleavage site to remove helix α1 |
|          | Vip3Aa_M34L | Point mutation M34L in helix α1 | To increase the insecticidal activity of the protein (Banyuls et al., 2021) |
| Mutants directed to affect the proteolytic processing and the conformational change of the activated protein | Vip3Aa_195-AVAA-198 | Mutation of residues 195-KVKK-198 to 195-AVAA-198 | The trypsin proteolytic cleavage site is changed to avoid trypsinization (Zhang et al., 2018) |
|          | Vip3Aa_S-S | Mutation to Cys of four residues located in domain I: D66C and N69C in helix α2 and Q96C and L100C in helix α3 | To block the protoxin conformation by disulphide bridges formation through the introduced Cys residues |
| Mutants directed to reduce the inside diameter of the coiled-coil | Vip3Aa_L146F | Mutation L146F in helix α3 | To change the inside diameter of the coiled-coil |
|          | Vip3Aa_L146M | Mutation L146M in helix α3 | To change the inside diameter of the coiled-coil |
| Mutant directed to affect C-t domains | Vip3Aa_ΔDIV-V | Deletion of C-t domains IV and V | To check the role of C-t domains in maintaining the structure and function |

* See Figure 1.
by two disulphide bonds (D66C-L100C and N69C-Q96C) to avoid remodelling of both helices upon activation. This mutant showed a similar proteolytic pattern to the WT protein under reducing conditions (Figure 2). However, under non-reducing conditions the 20 kDa band runs faster in SDS-PAGE (Figure S2) indicating a more compact conformation of Domain I probably due to the cross-link between helices $\alpha_2$ and $\alpha_3$. The presence of the disulphide bonds was also confirmed by MALDI-TOF (Figure S3) and LC–MS tandem MS (Figure S4).

The three single residue mutants, the mutant Vip3Aa_M34L, having a mutation within helix $\alpha_1$ (Figure 1C), and the mutants Vip3Aa_L146F and L146M designed to reduce the internal diameter of the tetrameric coiled-coil upon activation (Figure 1D), as L146 is located in $\alpha_3$ (Figure 1B, C), all showed a similar pattern to the WT (Figure 2).

Finally, the mutant Vip3Aa_ΔIV-V, lacking the C-t Domains IV and V, showed a proteolytic pattern in both conditions composed of two bands, the 20 kDa for the Domain I and a ~38 kDa comprising Domains II and III (Figure 2).

Overall, all the mutants, except Vip3Aa_195-AVAA-198, showed proteolytic processing upon incubation with SeMJ or trypsin, although some of them had changes in the electrophoretic mobility of the protein bands compared to WT. The fact that Vip3Aa_195-AVAA-198 could not be processed by trypsin and slightly by SeMJ indicates that, in vivo, a different type of proteases may cleave the mutated binding site or there is an alternative cleavage site close to the mutated one.

Negative staining—EM visualization of protoxin and trypsin-treated Vip3Aa WT protein and its mutants

The mutations introduced in Vip3Aa_Δα1, Vip3Aa_41-KVKK-44, Vip3Aa_195-AVAA-198, Vip3Aa_S-S, and Vip3Aa_ΔIV-V (all the engineered mutants except those only changing one residue) were analysed by negative staining transmission electron microscopy to explore if they had an effect at the structural level to influence the switch from protoxin to toxin conformation (Figure 3).

As expected, the WT shows the protoxin conformation before incubation with trypsin and the toxin conformation after incubation with trypsin in a large fraction of the molecules. From our previous structural studies, we know that proteolytic processing is necessary to trigger the toxin conformation, though the percentage of molecules that appear to undergo the conformational change is variable (50%–70%). Interestingly, we observed that the mutant Vip3Aa_Δα1 adopted the toxin conformation independently of the incubation with trypsin. This result indicated that the N-t comprising helix $\alpha_1$ had a relevant role in the stabilization of the helices at Domain I. Indeed, in the protoxin conformation helix $\alpha_1$ interacts with the core of the tetramer formed by Domain II and it is also connected to the loop $\alpha_1\alpha_2$ that allows the twist of helix $\alpha_2$ (Figure 1C). Thus, its absence might release the tension generated by the twist, lowering the energetic barrier required to trigger the remodelling of helices $\alpha_2$ and $\alpha_3$. For the mutants Vip3Aa_41-KVKK-44, Vip3Aa_S-S, and Vip3Aa_ΔIV-V, the majority of the molecules showed the protoxin conformation before and after incubation with trypsin, which indicates that despite the cleavage is produced, the shift towards the toxin confirmation was not favoured. In the case of Vip3Aa_41-KVKK-44, the mutation may have reduced the flexibility of the loop $\alpha_1\alpha_2$ in the WT (41-DTGG-44) provided by two Gly, slowing down remodelling of the helices. As originally intended, mutant Vip3Aa_S-S might have cross-linked helices $\alpha_2$ and $\alpha_3$ by disulphide bonds impairing remodelling. The lack of remodelling of Vip3Aa_ΔIV-V suggests that the C-t domains IV and V may provide a frame to sustain Vip3Aa during remodelling. In the case of the mutant Vip3Aa_195-AVAA-198, just the protoxin conformation was observed as no cleavage was produced by trypsin.

Thus, our results indicate that remodelling of Domain I is driven by a fine-tuned mechanism where not only
proteolytic cleavage is necessary but also an appropriate free energy barrier to favour the conformational change.

**Insecticidal activity of Vip3Aa and its mutants against S. exigua larvae**

So far, our structural results indicate that the mutants analysed by EM were not favoured to adopt the toxin conformation in vitro upon trypsin treatment, in contrast to Vip3Aa_Δα1 that could spontaneously adopt the toxin conformation. To test the effect of the mutations on the insecticidal activity of the protein, all the mutants were bioassayed against neonate larvae of the insect species S. exigua. The WT protein showed LC₅₀ and LC₉₀ values of 19.5 (8.6–28) ng/cm² and 62.9 (47–98) ng/cm², respectively (Table 2). The mutant Vip3Aa_Δα1, lacking helix α₁, was nontoxic (Table 2 and Figure S1A); however, the mutant

| Protein             | - TRYPsin   | + TRYPsin   |
|---------------------|-------------|-------------|
| Vip3Aa_WT           | ![Image](image1) | ![Image](image2) |
| Vip3Aa_Δα1          | ![Image](image3) | ![Image](image4) |
| Vip3Aa_195-AVAA-198 | ![Image](image5) | ![Image](image6) |
| Vip3Aa_41-KVKK-44   | ![Image](image7) | ![Image](image8) |
| Vip3Aa_S-S          | ![Image](image9) | ![Image](image10) |
| Vip3Aa_ΔIV-V        | ![Image](image11) | ![Image](image12) |

**TABLE 2** Toxicity of the WT and mutant proteins against S. exigua larvae by Probit analysis

| Protein       | LC₅₀ (95% F.L.) | Relative potency (95% F.L.) at LC₅₀ | LC₉₀ (95% F.L.) | Relative potency (95% F.L.) at LC₉₀ | Slope ± SE | χ² | DF |
|---------------|-----------------|------------------------------------|-----------------|-------------------------------------|------------|----|----|
| Vip3Aa_WT     | 19.5 (8.6–28)   | –                                  | 62.9 (47–98)    | –                                   | 2.5 ± 0.4  | 45 | 28 |
| Vip3Aa_Δα1    | >315            | –                                  | >315            | –                                   | –          | –  | –  |
| Vip3Aa_41-KVKK-44 | 55.1 (26–129) | 3.8 (1.9–7.6)                     | 3082 (694–183,530) | 53.1 (5.7–498)                    | 0.7 ± 0.2  | 12 | 12 |
| Vip3Aa_M34L   | 4.5 (1.2–8.6)   | 0.23 (0.18–0.31)                  | 27.7 (17–46)    | 0.42 (0.28–0.64)                   | 1.6 ± 0.3  | 34 | 26 |
| Vip3Aa_195-AVAA-198 | 18.1 (8.8–25) | 1.4 (1–2)                         | 56.3 (42–96)    | 0.9 (0.6–1.3)                      | 2.6 ± 0.6  | 11 | 11 |
| Vip3Aa_S-S    | >315            | –                                  | >315            | –                                   | –          | –  | –  |
| Vip3Aa_L146F  | 14.9 (7.9–24)   | 1.7 (1.1–2.6)                     | 376 (155–2439)  | 6.3 (2.7–15)                       | 0.9 ± 0.1  | 24 | 14 |
| Vip3Aa_L146M  | 54.4 (17–83)    | 1.8 (1.2–2.6)                     | 202 (128–485)   | 3.9 (2–7.5)                        | 2.3 ± 0.6  | 14 | 15 |
| Vip3Aa_ΔIV-V  | >315            | –                                  | >315            | –                                   | –          | –  | –  |

*95% F.L.* indicates the fiducial limits at 95% of confidence.
*Relative potency values around 1 indicate that mutant proteins have similar toxicity compared to the WT, values higher than 1 indicate lower toxicity for the mutants, whereas relative potency values smaller than 1 indicate enhanced toxicity.
*Similar slope values indicate similar dose-responses.
*SE indicates the standard error for the calculated slope values.
*Higher χ² values indicate higher heterogeneity of the data in the Probit analysis.
*DF indicates degrees of freedom for the calculation of χ².
Vip3Aa_41-KVKK-44, designed to eliminate the same helix α1 by trypsin digestion, retained toxicity at the LC50 level with a value of 55.1 (26–129) ng/cm², whereas it essentially lost all toxicity at the LC90 level, indicating a different dose–response compared to the WT protein (Table 2 and Figure S1A). The mutant Vip3Aa_M34L showed enhanced toxicity compared to the WT protein (Table 2 and Figure S1A), with LC50 and LC90 values of 4.5 (1.2–8.6) and 27.7 (17–46) ng/cm², respectively. The mutant Vip3Aa_195-AVAA-198, in which the cleavage site was eliminated, showed toxicity values very similar to that of the WT protein (Table 2 and Figure S1B). In contrast, the mutant Vip3Aa_S-S, in which the conformational change that follows the proteolytic activation is prevented, showed a complete lack of toxicity (Table 2 and Figure S1B). That lack of toxicity could hardly be attributed to the individual mutations, as the single mutant N69C or the double mutant D66C/N69C showed toxicity values similar to the WT (LC50 values of 7.5 with FL 4.3–10.5, and 21.0 with FL 15.3–27.4, respectively) and the single mutants Q96C and L100C also showed similar toxicity as the WT (LC50 values of 9.6 with FL 6.3–12.8, and 18.4 with FL 6.4–33.3, respectively). The mutants Vip3Aa_L146F and Vip3Aa_L146M, which according to the amino acid substitutions in the atomic models likely count with a reduced internal diameter in the tetrameric coiled-coil (Figure S5), did not differ from the WT at the LC50 level, but had significantly lower toxicity at the LC90 level, with a different dose–response compared to the WT protein (Table 2 and Figure S1C). Finally, the mutant Vip3Aa_ΔIV-V, lacking the C-t Domains IV and V, also showed a complete lack of toxicity (Table 2 and Figure S1D).

Overall, these results indicate that helix α1 is essential for Vip3Aa activity, as well as remodelling of helices α2 and α3 and the presence of the C-t domains. The increased activity of Vip3Aa_M34L reinforces the role of helix α1 as a hot spot in the mode of action. Moreover, Vip3Aa_41-KVKK-44 has a decreased activity, as Vip3Aa_L146F and Vip3Aa_L146M. The fact that Vip3Aa_195-AVAA-198 showed the same insecticidal activity as the WT suggests an alternative in vivo cleavage site.

Proteolytic processing of the Vip3Aa_195-AVAA-198 mutant protein

The observation that the mutant Vip3Aa_195-AVAA-198, engineered to destroy the cleavage site, showed similar toxicity values compared to the WT despite the fact that it was not activated in vitro by commercial trypsin and only barely by SeMJ, made us search for other in vitro conditions that emulated better the in vivo conditions. Thus, we performed the in vitro digestion at higher pH, incubating with S. exigua BBMV, and replacing trypsin with α-chymotrypsin, another relatively common serine peptidase in the lepidopteran larvae midgut (Caccia et al., 2014) (Figure 4A). The mutant protein could not be processed under any of these new conditions, whereas the WT protein was completely processed by trypsin at both pH conditions and partially processed by S. exigua BBMV and α-chymotrypsin.
Since we failed to show the in vitro processing of the Vip3Aa_195-AVAA-198 mutant, we set out to demonstrate that this protein was processed at an alternative cleavage site in vivo. The in vivo digestion assay was performed by drop-feeding S. exigua larvae with either the mutant or the WT protein and then dissecting the larvae at different times. The processed proteins were detected by Western blot (Figure 4B). The results showed that both, the WT and mutant Vip3Aa_195-AVAA-198 were completely processed in vivo after 16 h of ingestion. However, the kinetics of activation for the mutant protein seemed to be slower, since after 15 min of ingestion the WT protein was completely processed whereas almost half of the mutant proteins still remained in the protoxin form. The in silico analysis of the proteolytic cleavage sites in the surroundings of 195-AVAA-198, using the ExPASy Peptide Cutter tool (Bioinformatics Resources, Swiss Institute of Bioinformatics), predicts 14 potential trypsin/chymotrypsin cleavage sites in a range from 8 to 22 amino acids upstream and downstream of the 195-AVAA-198 sequence. Most probably, in vivo, some of these sites become available for trypsin or chymotrypsin cleavage.

DISCUSSION

The recent structures of Vip3Aa and Vip3B in the protoxin and toxin conformation have revealed that remodelling events at Domain I may impact insecticidal activity. To shed light on this mechanism, we engineered several Vip3Aa mutants, in highly conserved residues of Domain I (Figure S6), and then, characterized their proteolytic and insecticidal activity, as well as their conformational flexibility trying to correlate their structural features with their activity in vivo.

Our data indicate that remodelling and toxicity are interconnected by a fine-tuned mechanism where the free energy that involves remodelling depends on the appropriate coordination of several structural features. One of these structural features corresponds to helix α1 which has proven to be essential. Its absence leads to a complete loss of toxicity, in agreement with previous reports showing that the lack of the first N-t 33 and 39 residues abolished the insecticidal activity of the protein (Bhalla et al., 2005; Selvapandiyan et al., 2001). Our data also reveal that the absence of helix α1 induces the spontaneous conformation of toxins. Thus, we can envision that helix α1 may be holding Domain I in the protoxin conformation through interactions with Domain II until the protein has been proteolytically processed in the surroundings of the membrane of the midgut cells, to favour remodelling and interaction with the membrane at the appropriate location and timing. The mutant Vip3Aa_M34L, which carries a point modification inside helix α1, shows enhanced toxicity compared to the WT protein, in agreement with previous results obtained with the Vip3Af protein and the related species Spodoptera littoralis (Banyuls et al., 2021). The fact that a point mutation inside this region increases the toxicity can be related to the amphipathic nature of helix α1, increasing its hydrophobicity to improve the interaction with the hydrophobic nature of the membrane since the hydrophobic index of Leu (0.943) is higher than Met (0.738) (Black & Mould, 1991). In amphipathic helices (AH), the hydrophobic side of the helix inserts into the interior of the bilayer, while the hydrophilic side interacts with the lipid headgroups (Drin & Antonny, 2010; Roberts et al., 2013), thus, promoting membrane curvature and membrane fission (Campelo et al., 2008; Martyna et al., 2017). AHs of many fission-inducing proteins contain at least two basic residues (Arg and/or Lys) each at the polar-non-polar interface (Zhukovsky et al., 2019). In helix α1, its sequence 23-IYGATGKIDNMIFK-39 contains two Lys, K31 and K39 which may indicate that Vip3Aa could induce membrane fission. Then, that would lead to a better insertion in the membrane to further interact with some transmembrane or intracellular compounds to lead the cell death, possibly through its internalization and activation of the apoptotic pathways as has already been described (Hernández-Martínez et al., 2017; Hou et al., 2020; Jiang et al., 2016; Nimsanor et al., 2020).

Vip3 proteins can get inserted into the membrane of liposomes through the N-t part of Domain I (Byrne et al., 2021) and have been described as pore-forming proteins (Lee et al., 2003; Liu et al., 2011). The tetrameric coiled-coil in the structure of Vip3Aa holds a divalent ion bound in an internal diameter of ~3 Å (Núñez-Ramírez et al., 2020) which size could impact pore formation, although it is much narrower compared to the pore diameters described for other pore-forming proteins (Dal Peraro & van der Goot, 2016; Meusch et al., 2014; Mueller et al., 2009). Thus, mutations at L146 to Phe and Met were aimed to reduce the diameter and possibly affect ion transportation, as these are more voluminous residues than Leu. However, just a reduction of toxicity at the LC90 level was detected, indicating that the inside diameter of the coiled-coil is not a critical feature for Vip3Aa insecticidal activity. Thus, ion binding to the tetrameric coiled-coil might help to maintain the structural stability and dynamics of the coiled-coil (Hartmann, 2017) and not serve for ion transportation, though it cannot be ruled out that the interaction of Domain I with the membrane might facilitate that ions cross the membrane through other mechanisms.

A second structural feature that is essential for the Vip3Aa activity is the remodelling of helices α2 and α3, as the mutant Vip3Aa_S-S which locked the protein in the protoxin conformation impaired the insecticidal activity. These two long helices comprise the total length of the tetrameric coiled-coil (Figure 1B) possibly serve as a scaffold for the action of helix α1 in the activity of the toxin.
A third structural feature that has been demonstrated to be relevant is the loop $\alpha 1\alpha 2$ (residues 40–47). We mutated the sequence 41-DTGG-44, which contains two Gly, for 41-KVKK-44, resulting in the reduction of the toxicity of this mutant. Although our mutation was intended to introduce a cleavage site for the release of helix $\alpha 1$, the mutation has demonstrated that stiffness in the loop between helices $\alpha 1$ and $\alpha 2$ is deleterious. Thus, flexibility in this region is important for remodelling and therefore, for the insecticidal activity of the protein.

A fourth structural feature that unexpectedly has resulted to be critical for remodelling is the C-t part of the protein, formed by Domains IV and V which are not interacting with Domain I. Their absence in Vip3Aa favoured the protoxin conformation and abrogated toxicity. This is in agreement with previous reports showing a drastic lack of toxicity of C-t truncated Vip3Aa in bioassays with several lepidopteran pests, including *S. exigua* (Banyuls et al., 2018, 2021; Gayen et al., 2012, 2015; Li et al., 2007; Quan & Ferré, 2019; Selvapandian et al., 2001). The fact that the protoxin conformation was favoured in the absence of Domains IV and V points out a role for the C-t during remodelling, most likely through the interaction with the epithelial membrane of the cells. Thus, the absence of both domains may impair Vip3Aa from targeting the membrane, affecting recognition to find an interacting surface. However, we cannot discard that the lack of these domains could affect the proteolytic activation process of the protein in vivo, and so, the absence of toxicity could be the result of different factors.

Finally, the 195-AVAA-198 cleavage site is also an essential structural feature for remodelling and toxicity. Surprisingly, we have found that alternative proteolytic processing may also take place in vivo, and this explains why the toxicity of this mutant does not differ from that of the WT, at least against *S. exigua*. Our results are in contrast to those obtained by Zhang et al. (2018), where the same mutant protein showed a significant reduction of toxicity to *S. exigua*. It is important to note that the mortality values obtained by these authors for all the Vip3Aa proteins tested were much lower than ours, suggesting important differences in the susceptibility of the *S. exigua* colonies against Vip3Aa proteins.

In conclusion, we have demonstrated here the crucial role of helix $\alpha 1$ and the remodelling which takes place in the toxin conformation at Domain I for the insecticidal activity of the Vip3Aa protein. We have proposed several structural features that are relevant for the timing in which Vip3Aa remodelling occurs to drive its biological activity: the role of helix $\alpha 1$, essential for toxicity, holding the Domain I in the protoxin conformation and most probably interacting with the membrane in the toxin conformation, the flexibility of loop $\alpha 1\alpha 2$, the remodelling of helices $\alpha 2$ and $\alpha 3$, the proteolytic processing that takes place at the loop $\alpha 4\alpha 5$ between Domains I and II and the role of the C-t Domains IV and V to sustain the conformational change.

**AUTHOR CONTRIBUTIONS**

ML-B, YB, PC, and JF designed the experiments. ML-B, FP-M, RN-R, and YB performed the experimental work. ML-B, YB, EA-P, PC, and JF discussed and interpreted the results. ML-B, PC, and JF wrote the main manuscript text. ML-B, EA-P, and PC prepared the figures. All authors reviewed the manuscript.

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**CONFLICT OF INTEREST**

The authors declare no competing interests.

**DATA AVAILABILITY STATEMENT**

The authors confirm that the data supporting the findings of this study are available within the article and supporting information.

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