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Article

Structural domains of the *Bacillus thuringiensis* Vip3Af protein unraveled by tryptic digestion of alanine mutants

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Abstract: Vip3 proteins are increasingly used in insect control in transgenic crops. To shed light on the structure of these proteins, we used the approach of trypsin fragmentation of mutants altering the conformation of the Vip3Af protein. From an alanine scanning on Vip3Af, we selected mutants with an altered proteolytic pattern. Based on the protease digestion patterns, their effect on oligomer formation, and theoretical cleavage sites, we generated a map of the Vip3Af protein with five domains, which match some of the domains proposed independently by two *in silico* models. Domain I ranges from aa12-198, domain II from aa199-313, domain III from aa314-526, domain IV from aa527-668 and domain V from aa669-788. The effect of some of the mutations on the ability to form a tetrameric molecule revealed that domains I-III are required for tetramerization, while domain V is not. The involvement of domain IV in the tetramer formation is not clear. Some mutations distributed from near the end of domain I up to the end of domain II affect the stability of the first three domains of the protein and negatively impact oligomerization upon trypsin treatment. Because of the high sequence similarity among Vip3 proteins, we propose that our domain map can be extended to many other members of the Vip3 family of proteins.

Keywords: Bt toxins; insecticidal proteins; trypsin cleavage; tetrameric proteins; domain map

Key Contribution: Five structural domains have been defined in the Vip3 proteins based on tryptic patterns of Ala-mutants. Domain V is not necessary for maintaining the tetrameric form of the protein.

1. Introduction

Vip3A proteins are produced during the vegetative phase of growth of *Bacillus thuringiensis* and are of practical interest because of their insecticidal activity against Lepidoptera [1]. Because Vip3A proteins share no sequence and structural homology with *B. thuringiensis* Cry proteins, they are considered an excellent complement of Cry proteins in crop protection and resistance management. Some commercial Bt-crops (crops protected from insect attack by expressing insecticidal proteins from *B. thuringiensis*) combine Cry and Vip3 proteins and this strategy of pyramiding proteins with different modes of action is expected to continue in the future [2].

Despite the increasing interest in Vip3 proteins, their mode of action is not completely understood and their 3D structure still remains unknown. Recently, a number of studies have provided valuable information towards the structure of these proteins. Multiple alignment of Vip3 proteins has shown that they contain from 786 to 803 amino acids (corresponding to a molecular weight of around 89 kDa), with a highly conserved N-terminal part (up to residue 334 in Vip3Aa1) and a highly variable C-terminal region [1]. Proteolytical activation in the midgut of insects

eliminates a small part of the N-terminus, which in the case of Vip3Ab and Vip3Af proteins takes place at residue R11/12 [3, 4], and in the case of Vip3Bc1 at R20 [3], followed by cleavage of the protein at the primary cleavage site, which in Vip3Aa and Vip3Af is K198/D199 [4, 5]. Then, two peptides are generated, of about 19 and 65 kDa, which remain strongly attached [3, 6, 7]. More recently, it has been shown that Vip3 proteins are found in solution as homo-tetramers, both as protoxins and after activation by proteases [3, 7, 8, 9].

To date, a high resolution 3D structure of a Vip3 tetrameric protein is lacking, though low resolution images have been obtained [8, 10]. In an attempt to propose a 3D structure for Vip3 proteins, Vip3Af1 and Vip3Aa16 have been subjected to *in silico* modelling and several domains have been proposed. For Vip3Af, five structural domains were proposed [4], with domain 1 spanning from the N-terminus to residue 188, domain 2 from residue 189 to 272, domain 3 from 273 to 542, domain 4 from 543 to 715, and domain 5 from 716 to the end. For Vip3Aa16, three domains were proposed, though domain 1 was further subdivided into three domains [11]: subdomain 1.1 spanned from the N-terminus to residue 313, subdomain 1.2.1 from 314 to 441, subdomain 1.2.2 from 442 to 532, domain 2 from 533 to 667, and domain 3 from 668 to the end. Given the high sequence similarity between the two proteins (92.7%), the discrepancy between them regarding the predicted regions spanned by the domains likely reflects inaccuracies of the modelling programs used.

With the aim to shed light on the putative functional and structural domains of Vip3 proteins, we have made use of selected Vip3Af Ala-mutants with altered proteolytic patterns [4], with the rationale that proteolytic fragments may unravel structural and functional domains. The results, based on the protease digestion patterns, oligomer formation, and theoretical tryptic sites, have allowed us to propose a map of the Vip3Af protein with five domains. The information thus generated will contribute to the better understanding of the structure of Vip3 proteins and may be useful in the understanding of the mode of action of this family of proteins.

2. Results

2.1 Effect of Residue Substitution on the Proteolytic Cleavage of Vip3Af

Changes in protein conformation may expose potential cleavage sites, otherwise buried inside the protein, and when exposed to proteases give rise to altered patterns of fragments compared with that of the wild type protein. Proteolytic patterns may thus unravel structural domains in the Vip3Af protein.

We confirmed the altered proteolytic patterns obtained previously with Ala-mutants [4]. To better define the major fragments generated by the action of trypsin on each of the mutants, we used an irreversible trypsin inhibitor to terminate the reaction and avoid further processing during SDS denaturation before gel loading [5, 7]. Figure. 1 shows the SDS-PAGE separation of the tryptic fragments from six selected mutants (T167A, F229A, E483A, W552A, G689A, and I699A). Regarding the major fragments, Vip3Af(WT) and mutant T167A showed the 65 and 19 kDa bands (pattern “a”), as a result of the cleavage at the primary cleavage site after residue K198. The other patterns either lacked the 65 kDa band or this did not represent the major band. Patterns “b”, “c” and “e” contained the 19 kDa band, indicating that they altered the C-terminal part of the protein but not the N-terminal part. Patterns “d” and “f” did not contain the 19 kDa band either, indicating that the conformational change had a larger effect on the overall structure of the protein. Pattern “b” and “c” share the 35 and 19 kDa bands; in addition, the former showed strong bands of 38 and 10 kDa, whereas the latter showed main bands of 53, 17 (a doublet) and 15 kDa. Patterns “d” and “f” lack large fragments (larger than 30 kDa) and instead they share a main band of 27 kDa; in addition, pattern “d” has a strong band of 17 kDa and pattern “f” a strong band of <10 kDa. Finally, pattern “e” is the same as pattern “c” but still maintaining the band of 65 kDa, as if mutant G689A (the only representative of pattern “e”) was relatively stable compared with those mutants with pattern “c”.

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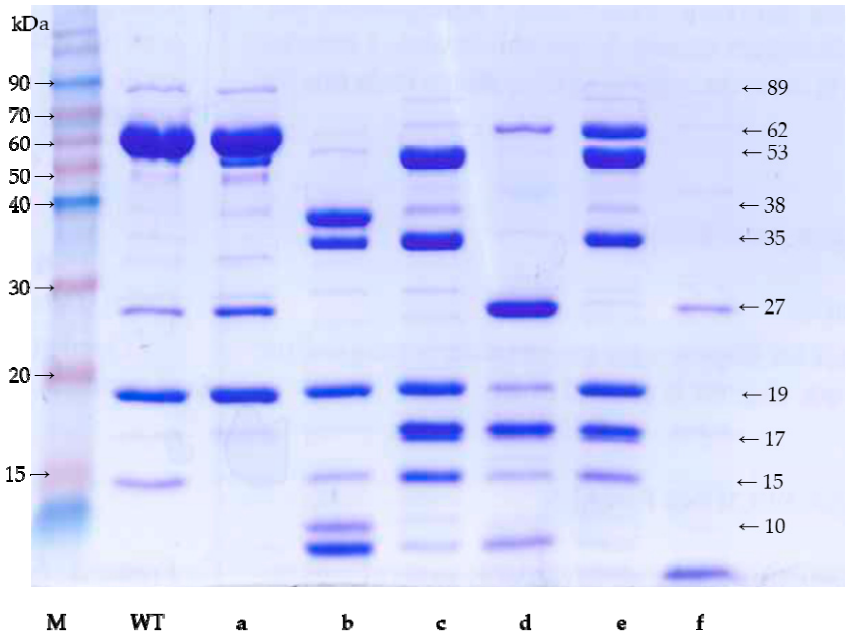

Figure 1. Trypsin digestion of Vip3Af(WT) and some of the selected mutants (patterns "a" to "f") after SDS-PAGE. The proteins were treated with 5% trypsin (w/w) at 30 ℃ for 24 h, and the reaction was then stopped with addition of irreversible trypsin protease inhibitor (1 mM AEBSF at room temperature for 10 min). M: molecular weight markers. Patterns "a" to "f" were obtained from mutants T167A, W552A, I699A, F229A, G689A, and E483A, respectively.

2.2 Insecticidal Activity of the Ala-Mutants after Trypsin Treatment

The mutants selected in this study had been shown to have decreased insecticidal activity when tested as protoxins [4]. Here we tested their activity after *in vitro* treatment with trypsin (Table 1). The results were similar to the ones reported previously for the protoxin form, confirming that these mutations have a strong deleterious effect on the insecticidal activity of the protein. It is worth to mention the differences in insecticidal activity observed among mutants with the same proteolytic pattern, for example, between P171A and F229A (both pattern "d"), and among I699A, Y719A and G727A (all pattern "c"). This might be explained by the residue substitution itself or by differences in their stability to proteases. Mutant G689A, which gives pattern "e" after trypsin treatment, is the most toxic one among those tested. As mentioned above, this mutant has a pattern "c" with contribution of the 65 kDa fragment, reflecting its higher stability compared with mutants giving pattern "c".

Table 1. Toxicity of trypsin-treated Vip3Af and some mutants (1 μg/cm²) against *S. frugiperda*.

Toxins	Tryptic pattern	Mortality (%)	FM (%)[1]
WT	a	100	100
T167A	a	13±10	19±13
E168A	a	0	3.1±3.1
P171A	d	16.7±3.3	40.0±6.7
F229A	d	6.3±6.3	6.3±6.3
E483A	f	3.3±3.3	16.7±3.3
W552A	b	0	0
G689A	e	42±21	48±15
I699A	c	0	0
Y719A	c	26.7±6.7	46.7±6.7
G727A	c	0	0

[1] Functional mortality: mortality plus 1-instar larvae.

2.3 Effect of Residue Substitution on Vip3Af Oligomerization

Residue substitutions may affect the capacity of the Vip3Af protein to form the tetramer [7], the form that Vip3 proteins adopt in solution [3, 7, 8, 9, 10]. We used gel filtration chromatography to determine the oligomerization state of the Ala-mutants, both as protoxins and after trypsin treatment. First, we tested the wild type Vip3Af (from now on: Vip3Af(WT)) and determined the possible effect of pH on oligomerization. Fig. 2A shows that, at the pH range tested (pH 7, 9 and 11), there was no effect on the tetramerization of the Vip3Af(WT) protoxin. The chromatograms showed the main peak at 24.0 min, corresponding to a molecular weight of approximately 370 kDa (a tetramer of the 89 kDa protoxin should theoretically be of 356 kDa). The trypsin-treated Vip3Af(WT) also showed just one peak at 24 min (Fig. 2A). SDS-PAGE analysis of the peak showed the 19 and 65 kDa bands, confirming that trypsin treatment did not induce separation of the two fragments [3, 5, 6, 7, 9]. Mutants T167A and E168A (both pattern "a") showed chromatograms that did not differ from that of the wild type (data not shown).

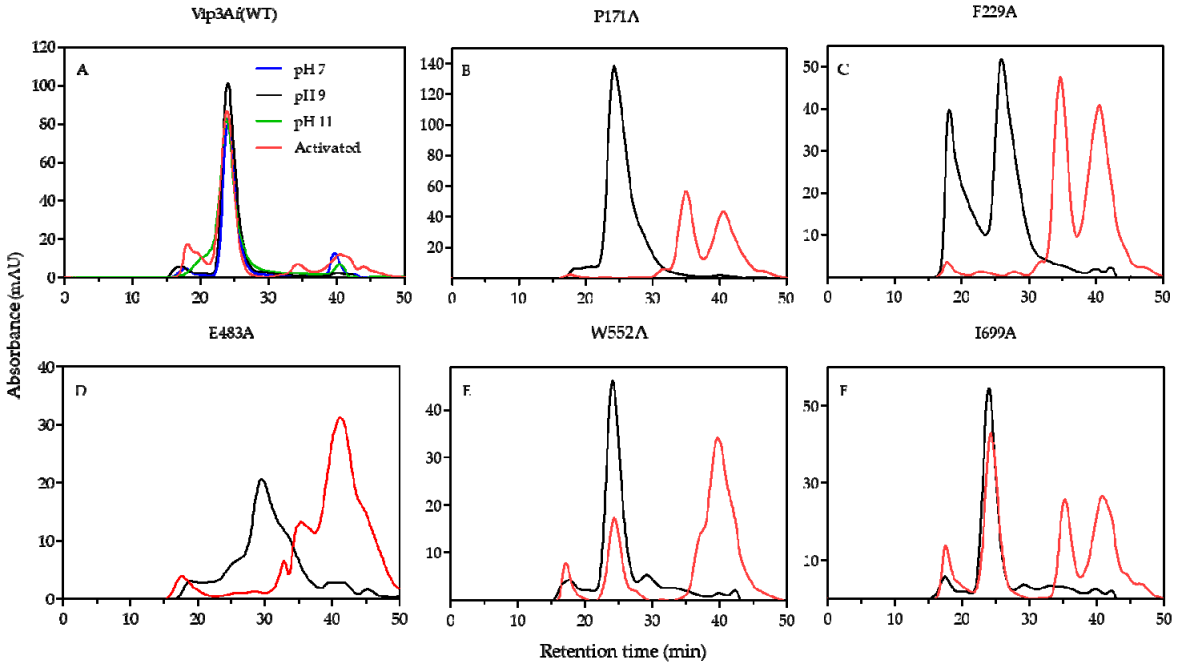

Figure 2. Gel filtration chromatography of Vip3Af(WT) and selected Ala-mutants. Tris buffer (50 mM Tris, 150 mM NaCl, pH 9.0) was used in all cases (black line: protoxin; red line: trypsin-treated). For the Vip3Af(WT), elution was also performed in phosphate buffer (50 mM phosphate, 150 mM NaCl, pH 7.0)(blue line) and carbonate buffer (50 mM Na_2CO_3, 150 mM NaCl, pH 11.0)(green line).

With mutant P171A (pattern "d") the protoxin eluted at 24 min, revealing a tetrameric form; however, just small fragments (eluting at 34.8 min) were observed after trypsinization (Fig. 2B). SDS-PAGE of the peak at 34.8 min showed a 27 kDa strong band (Fig. S1). This chromatography profile was also observed for the other mutants with pattern "d", except for F229A. The chromatogram of F229A (pattern "d") showed strong peaks at 18 and 26 min (Fig. 2C), the former coinciding with the exclusion volume of the column and corresponding to protein aggregates. The peak at 26 min indicated a molecular weight of approximately 230 kDa, which would best fit a dimeric form of the protein; this mutant, upon trypsin treatment, only showed small fragments eluting at 34.8 min. SDS-PAGE of the fraction at 34.8 min revealed a main fragment of 27 kDa (Fig. S2).

Neither the protoxin nor the trypsin-treated mutant E483A (pattern "f") showed any tetramer in solution (Fig. 2D); the main peak of the protoxin eluted at 29.5 min, (corresponding to an approximate estimated molecular weight of 122 kDa (best fitting a monomer), whereas the

trypsinized protein eluted at around 35 min, corresponding to the 27 kDa fragment and smaller fragment (<10 kDa) as revealed by SDS-PAGE (Fig. S1).

Mutant W552A (pattern "b") formed a tetramer both as protoxin and after trypsin treatment (Fig. 2E); however, upon trypsin treatment, the amount of tetramer was reduced and a large peak corresponding to small fragments appeared. Analysis of the fraction at 24 min by SDS-PAGE showed the presence of the 38, 35 and 19 kDa fragments (Fig. S2).

Mutant I699A (pattern "c") also tetramerized as protoxin and after trypsin treatment (Fig. 2F); however, after trypsinization it also showed fragments eluting at 35.2 min. This chromatography profile was also observed for the other mutants with pattern "c" and "e". The SDS-PAGE analysis of the fraction at 24 min revealed, among other minor bands, the 53, 35 and 19 kDa fragments, whereas the fraction at 35.2 min revealed a fragment of 17 kDa (Fig. S1).

From the chromatographic analysis we can conclude that the 27 and 17 kDa fragments, once cleaved by trypsin, are released from the structure and no longer form part of the oligomer. However, the tetrameric structure of Vip3Af can still be maintained in the presence of fragments 38+35+19 kDa (such as in mutant W552A, pattern "b") or fragments 53+35+19 kDa (such as mutants with pattern "c"). Residues F229 and E483 must have a key role in the oligomerization of the Vip3Af protoxin, since their exchange for alanine prevents tetramer formation even before trypsin treatment.

2.4 Identification of 17, 27 and 38 kDa Tryptic Fragments by Peptide Fingerprinting

Identification of the tryptic fragments was performed after separation of the fragments by 2D gel electrophoresis and/or size filtration chromatography followed by SDS-PAGE. The results of the peptide fingerprint were matched with those of the tryptic sites in the primary sequence of Vip3Af, and the estimated size of the fragment was taken into account to define the fragment limits. By 2D gel electrophoresis we could separate and analyze the 17 and 27 kDa spots from trypsin-treated F229A mutant. The results of the peptide fingerprint, along with the tryptic sites in the sequence of Vip3Af, indicated that the 27 kDa fragment corresponded to residues from 523/526 to probably the C-terminus of the protein. The same type of analysis with the spot of 17 kDa indicated that it corresponded to residues from 523/526 to 661/663. The 17 kDa fragment from the trypsin-treated I699A mutant was analyzed from the chromatographic fraction B15 (peak 34.8 min) of this mutant. The results indicated the same match as the 17 kDa fragment from mutant F229A. The identity of the 38 kDa fragment was determined from the chromatography fraction A11 (24.2 min) from trypsin-treated W552A; the peptide fingerprint indicated that the fragment corresponded to residues from 313/315 to 523/526.

3. Discussion

Banyuls et al. [4] defined six proteolytic patterns of mutants with strongly impaired insecticidal activity. With minor modifications in the methodology, we have confirmed and refined such patterns with the aim of revealing the major fragments generated by trypsin and then identify their position in the primary structure of the protein. The only difference observed with the previous proteolytic patterns is that, using the irreversible trypsin inhibitor to stop the reaction, we obtained a strong band of 35 kDa in patterns "b" and "c", which was not observed previously. We also detected bands smaller than 19 kDa by stopping the electrophoresis before they ran out of the gel. Altogether we ended up with fragments of 53, 38, 35, 27, 19, 17 and <10 kDa, most of them shared by various patterns. We hypothesized that the limits of these fragments may correlate with the structural domains of the wild type protein.

In a previous study, Banyuls et al. [4] identified the tryptic fragments of 62 (here referred to as 65), 55 (here referred to as 53), 27, and 20 (here referred to as 19) kDa. Our peptide fingerprint results of fragments of 17, 27 and 38 kDa, taking into account the tryptic sites in the sequence of Vip3Af, allowed us to define their position in the sequence of the protein. Putting all this information together, we propose a map of the tryptic fragments such as the one shown in Fig. 3, which defines five domains. In this map, domain I spans the region covered by the 19 kDa fragment (from residues

12 to 198); domain II spans the region from the primary cleavage site to the N-terminus of the 38 kDa fragment (from residues 200 to 313/315); domain III spans from the N-terminus of the 38 kDa fragment up to the N-terminus of fragments of 17 and 27 kDa (from residues E314-E316 to 523/526); domain IV spans the 17 kDa fragment (from residue 524/527 to residue 661/668) and basically consists of the carbohydrate-binding motif common to all Vip3 proteins with the exception of Vip3Ba [1]; and domain V spans from the end of the 17 kDa fragment (and also the end of the 38 and 53 kDa fragments) to the C-terminus of the protein (from residue 662/669 to 788). Compared with the proposed domains by in silico modelling, the domain I proposed by us is in good agreement with domain 1 proposed by Banyuls et al. [4] for Vip3Af (from 1 to 188), though there is no further correlation between both models for the other domains. However, the boundaries between domain II and III, III and IV, and IV and V in our proposed map show good correspondence with the domains proposed by Sellami et al. [11] for Vip3Aa (at residues 313, 532, and 667, respectively). The agreement between the domain limits proposed by us with some of those defined by *in silico* modelling supports the predictive value of the tryptic fragments approach to unravel structural domains of the Vip3A proteins.

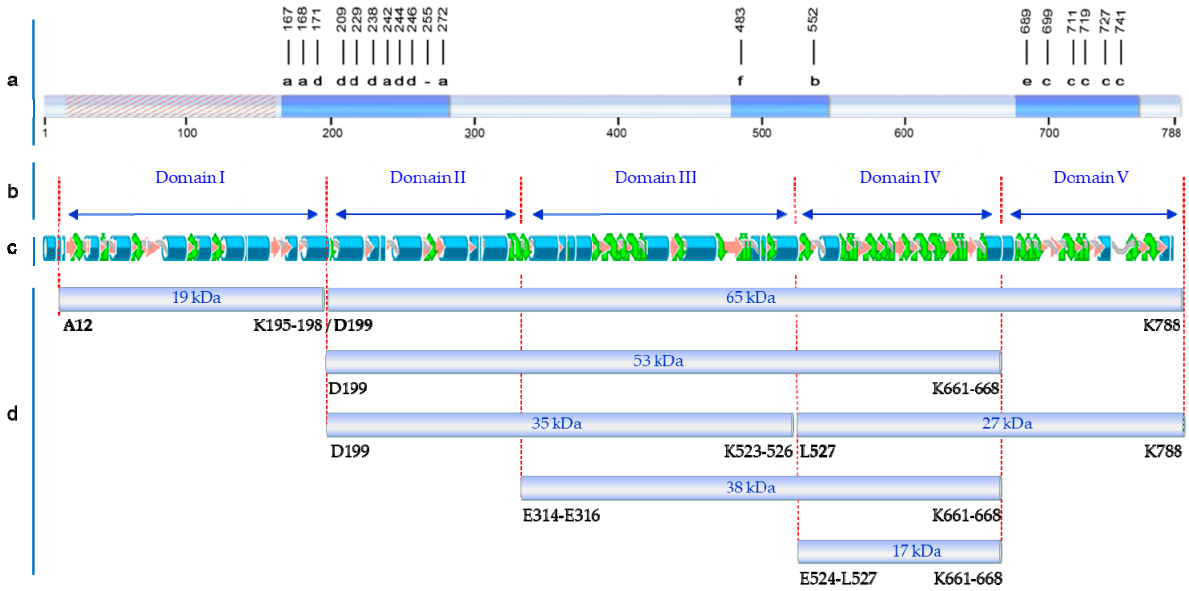

Figure 3. Schematic representation of the Vip3Af protein. (a) Distribution of critical residues affecting the insecticidal activity (dashed region: non-analyzed) after Banyuls et al. [4]; (b) proposed structural domains as defined by the tryptic fragments; (c) secondary structure of Vip3Af after Banyuls et al. [4]; (d) main fragments after trypsin treatment of the Vip3Af1 protein and their Ala-mutants (amino acid residues identified by Edman's degradation are shown in bold).

An interesting observation from patterns "b" and "c" is that the 65 kDa fragment is spliced in two alternative ways, giving rise to two strong bands that otherwise could not come from the same original fragment. In the case of pattern "b", these bands correspond to fragments of 35 and 38 kDa. In the case of mutants with pattern "c", the strongest bands are those corresponding to fragments of 17, 35 and 53 kDa. Obviously, there must be an alternative splicing of the 65 kDa band, approximately at a ratio 1:1. One possible explanation for this is that the tetrameric structure adopted by the protoxin does not have a symmetry of a regular tetramer, but a symmetry of two dimers. This could explain why half of the monomers would have exposed tryptic sites which would not be exposed in the other half.

The results from gel filtration chromatography of the Ala-mutants shed light on the structural role of the proposed domains. Mutants belonging to patterns "b", "c" and "e" are found forming a tetramer both as protoxins and also after trypsin treatment. Since trypsin digests the 27 kDa

fragment (the only one containing domain V), we can conclude that domain V is not necessary to maintain the oligomeric structure. All these mutants, after trypsin treatment, have in common fragments of 19 kDa (domain I) and 35 kDa (domains II and III), plus another larger fragment (either of 38 or 53 kDa) which includes domain IV. Despite the fact that the 17 kDa fragment (which corresponds to domain IV) elutes separately from the tetramer in the chromatography of mutants with patterns "c" and "e", the tetramer contains domain IV in the structure, as part of the 53 kDa fragment. Therefore, according to the results, domains I-III are required to form the tetrameric structure, the need for domain IV is not clear, and domain V is not necessary.

The requirement of domain I to form the tetramer, along with domain exchange studies between the 19 kDa fragment and the rest of the protein with Vip3Ab and Vip3Bb [3] support the functional role of this domain and rules out the initial assumption that the 19 kDa fragment was non-essential in the insecticidal activity of Vip3 proteins and that only the 65 kDa fragment was the active core. It has been reported that deletion of this domain in Vip3Aa completely abolished toxicity and produced a 62 kDa protein highly sensitive to trypsin degradation [12]. However, some studies have shown that domain I can withstand short N-terminal deletions without affecting the insecticidal activity [13, 14]. In contradiction to the above results, [15] reported an active Vip3Aa protein without domain I.

From the distribution of Ala-mutants with decreased insecticidal activity in the primary structure of Vip3Af (Fig. 3), we can observe that they gather into two clusters, except for mutant E483A (the only representative of pattern "f") and mutant W552A (the only representative of pattern "b"). The first cluster contains all mutants with either pattern "a" or "d". Mutations altering the structure and giving pattern "d" are concentrated at the end of domain I and the first part of domain II. This region of the protein, around the primary cleavage site, must have an important role in maintaining the 19 and 65 kDa fragments together, which might be essential to preserve the overall structure of the tetrameric protein. The second cluster is in domain V and contains all the mutants with either pattern "c" or "e". These mutants destabilize domain V, which is further digested by trypsin with the result of fragment 27 kDa being converted to the 17 kDa fragment.

4. Conclusions

Using the approach of trypsin fragmentation of mutants altering the conformation of the Vip3Af protein, we have defined five domains in the structure of Vip3Af which match some of the domains proposed independently by two *in silico* models. The effect of some of the mutations on the ability to form a tetrameric molecule reveals that domains I-III are required for tetramerization, while domain V is not. The involvement of domain IV in the tetramer formation is not clear. The overlapping fragments in the proteolytic patterns suggest a tetramer with distinct disposition of the monomers, in such a way that the tryptic sites exposed in two molecules are different to those exposed in the other two. Residues around the primary cleavage site are important to maintain the structure of the protein, since trypsin processing in mutants of pattern "d" digests most of the protein and destroys the tetrameric form. Mutants in domain V belonging to pattern "c" destabilize this domain, though they do not affect the tetrameric structure after trypsin processing. Because of the high sequence similarity among Vip3 proteins, we think that our domain map proposal may be valid to many members of the Vip3 family of proteins. The information provided here contributes to the better knowledge of the structure of Vip3 proteins and may be useful in the better understanding of their mode of action.

5. Materials and Methods

5.1 Protein Source, Expression and Purification

The source of the 788 amino acid protein Vip3Af1(WT) (NCBI accession No. CAI43275 and that of its mutant proteins has been described in Banyuls et al. [4]. The mutant proteins, all with decreased insecticidal activity, differed from Vip3Af(WT), and from each other, by a single amino acid residue which had been changed to an alanine residue. Expression and purification of

Vip3Af(WT) and the mutant proteins was carried out as described before [4], using 1 ml HisTrap FF columns (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Vip3Af proteins were eluted with phosphate buffer (50 mM phosphate, 300 mM NaCl, pH 7.4) containing 150 mM imidazole, and 1 ml fractions were collected in tubes containing 50 µl of 0.1 M EDTA. Fractions with high protein concentration (determined photometrically at 280 nm) were pooled and dialyzed overnight at 4°C against TNE buffer (20 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, pH 8.6). The purity of the preparation (10 µl) was checked by SDS-PAGE and the protein concentration was determined by the Bradford's method. After dialysis, the proteins were stored at — 20°C until used.

5.2 Trypsin Treatment and SDS-PAGE Analysis of the Tryptic Fragments

The purified Vip3Af protoxins were subjected to proteolytic activation with commercial trypsin (trypsin from bovine pancreas, SIGMA T8003, Sigma-Aldrich, St. Louis, MO, USA). A mixture of protein:trypsin (5:100, w/w), in TNE buffer, was incubated at 30°C for 24 h. Aliquots (10 µl) of the trypsinized proteins were subjected to 12% SDS-PAGE. Prior to electrophoresis, the samples were made 1 mM with AEBSF protease inhibitor (ThermoFisher, Waltham, MA, USA), let stand for 10 min at room temperature, and then heated at 100°C for 5 min with loading buffer (0.2 M Tris-HCl pH 6.8, 1 M sucrose, 5 mM EDTA, 0.1% bromophenol blue, 2.5% SDS, and 5% β-mercaptoethanol) (2:1, sample:loading buffer). The trypsin-treated samples to be used for chromatography and bioassays were stored at — 20°C for less than one week.

5.3 Insect Rearing and Bioassays

Insect rearing and bioassays was carried out on a semi-synthetic diet in a rearing chamber maintained at 25 ± 2°C, 70 ± 5% RH and 16:8 h L:D. Surface contamination assays were performed with 50 µl of protein sample on 2 cm^2 diameter well plates. The concentration of Vip3Af protein was 1 $\mu g/cm^2$, concentration at which the Vip3Af(WT) kills 100% of the larvae. Tris buffer (20 mM Tris-HCl, 150 mM NaCl, pH 8.6) was used as a blank control. Once the surface was dry, a neonate *S. frugiperda* larvae was gently placed into the well and then sealed. The number of dead and 1-instar larvae were recorded after 7 days. The mean mortality and functional mortality (dead larvae plus 1-instar larvae) was determined from two replicates of 96 insects each.

5.4 Gel Filtration Chromatography

Gel filtration chromatography was performed with an ÄKTA explorer 100 chromatography system in a Superdex 200 10/300 GL column (GE Healthcare Life Sciences, Uppsala, Sweden) at a flow rate of 0.5 mL/min of Tris buffer (50 mM Tris-HCl, 150 mM NaCl, pH 9.0), unless otherwise indicated. To estimate the molecular weight of the peaks, the column was calibrated with the following mix of standards: 4 mg/ml ovalbumin (44 kDa), 3 mg/ml conalbumin (75 kDa), 4 mg/ml aldolase (158 kDa), 0.3 mg/ml ferritin (440 kDa), 5 mg/ml thyroglobulin (6690 kDa), and Blue Dextran 200 (exclusion limit), dissolved in water.

5.5 Identification of Tryptic Fragments

Major bands (27 and 17 kDa) from trypsin-treated F229A mutant were identified after separation in a 2D-gel. The 17 kDa band from trypsin-treated I699A mutant was first separated by chromatography in the Superdex 200 column and then by SDS-PAGE. The 38 kDa band from trypsin-treated W552A mutant was first isolated by Superdex 200 chromatography and then by SDS-PAGE.

For the peptide mass fingerprinting, protein bands were directly cut out from the gel and digested with trypsin. The peptide mass and sequence was determined by liquid chromatography and tandem mass spectrometry (LC-MS/MS) in a nanoESI qQTOF (5600 TripleTOF, ABSCIEX) The mass transitions were scanned first from 350–1250 m/z and then followed by a second scan from 100–1500 m/z. The peptides sequence identified were compared to the Vip3Af1(WT) protein

sequence to match the region corresponding to each SDS-PAGE proteolytic band. Expected molecular weights were calculated using the online SIB Compute pI/Mw tool 38.

Supplementary Materials: The following are available online, Figure S1: SDS-PAGE analysis of chromatographic peaks of trypsin-treated Vip3Af Ala-mutants after gel filtration chromatography in Superdex 200, Figure S2: SDS-PAGE analysis of chromatographic peaks of trypsin-treated W552A and F229A mutants after gel filtration chromatography in Superdex 200.

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Conflicts of Interest: The authors declare no competing financial interests.

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