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Full Length Article

Bacillus thuringiensis Vip3Aa and Vip3Ad Chimeric Proteins Improve their Insecticidal Activity against Lepidopteran Insects

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Abstract

Vegetative insecticidal proteins (Vips) are potential alternatives for *Bacillus thuringiensis* endotoxins. Vip3Aa39 showed high toxicity against lepidopterans, whereas Vip3Ad displayed no toxicity. In this study, sequence alignment analysis indicated that Vip3Aa39 and Vip3Ad share 85% homology with one another. Amino acid fragment interchange between Vip3Aa39 and Vip3Ad was carried out by the overlapping extension PCR method, and 6 chimeric genes were obtained successfully. These chimeric genes were transformed and expressed in *Escherichia coli* strain BL21. SDS-PAGE analysis confirmed the expression of 88 kDa proteins. Insect bioassays were conducted with *Helicoverpa armigera* and *Plutella xylostella*, and after the N-terminal amino acid fragment (1-60) of Vip3Aa39 was replaced by that of Vip3Ad protein, the recombinant protein Vip3AdAa1 exhibited high toxicity against both insects. Vip3AdAa2 (amino acids 1-118 replaced by that of Vip3Ad) displayed no significant difference in toxicity compared with Vip3Aa39. Vip3AaAd1 showed no toxicity against *H. armigera* and *P. xylostella*. The chimeric proteins Vip3AaAdAa1, Vip3AaAdAa2 and Vip3AaAd2 displayed differences in insecticidal spectra against the two insects. This study also evaluated the hydrolysis of chimeric proteins with trypsin. Five chimeric proteins were cleaved into 62 kDa fragments, except for Vip3AaAd1. These new Bt proteins constructed by protein engineering can effectively enrich insecticidal protein resources. This study also provides a reference for in-depth understanding of the key activity areas of Vip3A insecticidal proteins. © 2020 Friends Science Publishers

Keywords: *Bacillus thuringiensis*; Chimeric; Toxicity; Vip3Aa39; Vip3Ad

Introduction

Vegetative insecticidal proteins (Vips) are produced during the vegetative growth phase of *Bacillus thuringiensis*. As the second generation of biological insecticides, Vips have no homology in the evolution of amino acid sequences and no competition in insecticidal targets with Cry proteins. These can prevent or delay insect resistance and expand the insecticidal spectrum by using alone or in combination with Cry proteins (Ben Hamadou-Charfi *et al.*, 2013; Bergamasco *et al.*, 2013; Wang *et al.*, 2018), which have become new strategy for pest control. To date, Vip toxins have been classified into four subfamilies according to their sequence homology. Vip1 and Vip2 are binary toxins with specific insecticidal activity against Coleoptera and Hemipteran insect pests. Most studies on Vip toxins have focused on Vip3 (Song *et al.*, 2016; Ferre and Escriche 2017), mainly involving 98 *vip3A*, 5 *vip3B*, and 4 *vip3Ca* genes (Crickmore *et al.*, 2018). Among the Vip3 proteins investigated, Vip3A proteins are the most abundant and account for 91.5% of known Vip3 proteins (Baranek *et al.*, 2015; Lone *et al.*, 2018). Although the sequence differences between Vip3A proteins are less than 22%, their insecticidal activities and insecticidal spectrum are significantly different. The Vip3Aa protein shows strong insecticidal activities against lepidopterans such as *Agrotis ipsilon*, *Spodoptera exigua*, *Earias vitella*, and *Helicoverpa armigera* (Wu *et al.*, 2004; Yu *et al.*, 2011; Pradhan *et al.*, 2016). Vip3Ad has no activity against many lepidopteran pests (Ruiz de Escudero *et al.*, 2014; Boukedi *et al.*, 2018). However, the molecular structures of Vip3Aa and Vip3Ad have not been revealed (Song *et al.*, 2016; Ferre and Escriche, 2017).

Protein engineering has been used to elucidate the role of some amino acid residues in the structural stability of Cry toxins and to generate improved Bt toxicity (de Maagd *et al.*, 2000). De Maagd *et al.* (1999) constructed a chimeric protein (1Ab-1Ab-1C) of Cry1Ab by replacing with domain III of Cry1C, and the insecticidal activity of 1Ab-1Ab-1C was enhanced approximately 10-fold. However, recombinant protein (AhAhAb) formed from Cry1Ab and Cry1Ah had no insecticidal activity against *H. armigera* and showed a significant reduction in activity against *P. xylostella*. The chimeric protein (AaAaAb) constructed from Cry3Aa and Cry1Ab gained toxicity to the western corn

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rootworm (Walters *et al.*, 2010). Vip3AcAa is a chimeric protein composed of a 600 amino acid fragment at the N-terminus from Vip3Ac1 and a 189 amino acid fragment at the C-terminus from Vip3Aa1. Vip3AcAa not only exhibited a higher activity against the fall armyworm but also gained a novel activity against the European corn borer (Fang *et al.*, 2007). It also has broad-spectrum insecticidal activity against lepidopteran pests (Chen *et al.*, 2017). Transferring Vip3AcAa+Cry1Ac into cotton can effectively delay the resistance of *H. armigera* to Cry protein (Chen *et al.*, 2018). Sellami *et al.* (2018) constructed the Vip3Aa16-Cry1Ac protein, which enhanced insecticidal activity to *Ephestia kuehniella*.

H. armigera and *P. xylostella* are two important lepidopteran agricultural pests in China. In recent years, the drug resistant of *them* has caused great harm. In this study, 6 chimeric proteins were constructed from Vip3Aa39 and Vip3Ad. Insecticidal activities of chimeric proteins against the two insect pests were determined. Trypsin sensitivity of recombinant proteins was assayed. Major objectives of this study were to provide: (1) genetic materials for the construction of engineered bacteria and transgenic plants, and (2) a reference for in-depth understanding of the key activity areas of Vip3A insecticidal proteins and further revealing the evolution governing Vip3A insecticidal specificity.

Materials and Methods

Strains and Plasmids

E. coli JM109 and BL21 were used as host strains for cloning and expression. The pET-21-b (+) plasmid was used as the expression vector, and maintained in our laboratary. The vip3Ad gene (GenBank accession number: KP346519) comes from *B. thuringiensis* BJG810, which was isolated from the soil of Guangxi. The *vip3Aa39* gene from *B. thuringiensis* (GenBank accession number: HM117631) was maintained in the Biochemistry and Molecular Biology Laboratory of Northeast Agricultural University (China).

Construction of Chimeric Genes

This experiment adopted overlapping extension polymerase chain reaction (PCR) to construct chimeric proteins, comparing the amino acid sequences of Vip3Aa39 and Vip3Ad, selecting conserved regions, designing 8 pairs of overlapping primers (JB1F/JBIR, JB2F/JB2R, JB3F/JB3R, JB4F/JB4R, JB5F/JB5R, JB6F/JB6R, JB7F/JB7R, and JB8F/JB8R) and 2 pairs of full length primers (39F/39R and DF/DR), and adding *Bam*HI and *Sal*I enzyme cutting points to the full-length primer, according to Table 1. The fragments were changed with reference to the overlapping extension PCR method, and chimeric gene fragments were constructed by using Vip3Aa39 and Vip3Ad genes as templates. Fragments were amplified with a high-fidelity enzyme, products were recovered and then overlap fragments were amplified with the full-length primer to create the chimeric gene containing the gene fragments of Vip3Aa39 and Vip3Ad (Thornton 2016). The recombinant gene was ligated into the pET-21b plasmid at 4°C overnight after double enzyme digestion. The recombinant plasmid was transferred to BL21 competent cells, and positive clones were picked and sent to the Bioengineering Company of Shanghai for gene sequencing, using the NCBI database and the DNAMAN software to align the results.

The *vip3AdAa* chimeric gene was formed by splicing the N-terminal nucleotide sequence provided by Vip3Ad and the C-terminal nucleotide sequence provided by Vip3Aa39. Based on the construction of the chimeric gene *vip3AdAa1*, using the constructed plasmid pET-*vip3Ad* as a template, PCR amplification was conducted with the DF/JB1R primers to obtain the *vip3Ad* (1-207) sequence fragment, named *vDN1*. Continuing to use the pET-vip3Aa39 plasmid as a template, PCR amplification was performed with the primers JB1F/39R to obtain the sequence fragment of *vip3Aa39* (181-2370), named *v9C1*. Using the products *vDN1* and *v9C1*, purified from a gel, from the previous two steps of PCR as a template, fusion amplification was carried out with the primers DF/39R, obtaining the gene *vip3AdAa1*, composed of *vip3Ad* (1-207) and *vip3Aa39* (208-2370). Similarly, the *vip3AdAa2* gene was obtained, composed of *vip3Ad* (1-354) and *vip3Aa39* (355-2370).

Meanwhile, the *vip3AaAd* chimeric gene was formed by combining the N-terminal nucleotide sequence from *vip3Aa39* with the C-terminal nucleotide sequence from *vip3Ad*. Similar to the *vip3AdAa* constructs, *vip3AaAd1* is composed of *vip3Aa39* (1-2199) and *vip3Ad* (2200-2361), and *vip3AaAd2* consists of *vip3Aa39* (1-2316) and *vip3Ad* (2317-2361).

The chimeric gene *vip3AaAdAa* was developed by combining the beginning and end nucleotide sequences provided by *vip3Aa39* and the middle nucleotide sequence provided by *vip3Ad*. The chimeric gene *vip3AaAdAa1* was formed by fusion of *vip3Aa39* (1-810), *vip3Ad* (811-1035) and *vip3Aa39* (1036-2370). The *vip3AaAdAa2* gene was developed by fusion of *vip3Aa39* (1-1365), *vip3Ad* (1366-1898), and vip3*Aa39* (1899-2370). The full lengths of *vip3AdAa*, *vip3AaAd* and *vip3AaAdAa* are 2370, 2361, and 2370 bp, respectively. The corresponding templates, primers and amplified sequences are shown in Table 2.

Protein Expression and Extraction

The *E. coli* BL21 recombinant strains containing the wild-type *vip3Aa39* gene, *vip3Ad* and the chimeric genes were inoculated into 5 mL of LB liquid medium and incubated overnight. Then, 1 mL of bacteria was inoculated into 100 mL of LB liquid medium. The cells were cultured at 37°C and 220 r/min until the OD_{600} was approximately 0.5. Isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM, and the expression of protein was

Table 1: PCR primers used for amplification

Primers	Sequences (5'-3')	Description (position)
JB1F	ATTTCTGGTAAATTGGATGGGGTAAAT	181-207
JB1R	ATTTACCCCATCCAATTTACCAGAAAT	
JB2F	CCTAAAATTACATCTATGTTAAGTGA	355-380
JB2R	TCACTTAACATAGATGTAATTTTAGG	
JB3F	GTTTATAATTTCTTAATTGTATTAACA	811-837
JB3R	TGTTAATACAATTAAGAAATTATAAAC	
JB4F	AAGATGATTGTGGAAGCTAAACCA	1036-1059
JB4R	TGGTTTAGCTTCCACAATCATCTTTG	
JB5F	AAAGTAGAATCAAGTGAAGCGGAGTATA	1366-1393
JB5R	TATACTCCGCTTCACTTGATTCTACTTT	
JB6F	TAAACGTTTTACTACAGGAACTGATT	1899-1924
JB6R	AATCAGTTCCTGTAGTAAAACGTTTA	
JB7F	CGGAGATGCTAATGTAAGGATT	2178-2199
JB7R	AATCCTTACATTAGCATCTCCG	
JB8F	TAGAGCTTTCTCAAGGG	2300-2316
JB8R	CCCTTGAGAAAGCTCTA	
39F	CGCGGATCCGATGAATATGAATAATACTAAATTAAACG	1-28
39R	ACGCGTCGACTTACTTAATTGAGACATCGTTAAACT	2345-2370
DF	CGCGGATCCGATGAACATGAATAATGCTAAATTAAAT	1-27
DR	ACGCGTCGACTTATTTAATAGAGAAATCATAAAAT	2335-2361

Note. F means forward and R means reverse, and the underlined sequences are the restriction enzyme sites engineered for cloning of both fragments

Table 2: Template and primers used for the construction of chimeric gene

Name	Template	Primer pair	Amplified fragment	Product size (bp)
vAdN1	pET-vip3Ad	DF/JB1R	*vip3Ad* (bases 1 to 207)	207
vAaC1	pET-vip3Aa39	JB1F/39R	*vip3Aa39* (bases 181 to 2370)	2189
Vip3AdAa1	*vAdN1+vAaC1*	DF/39R	*vip3Ad* (bases 1 to 180) and *vip3Aa39* (bases 181 to 2370)	2370
vAdN2	pET-vip3Ad	DF/JB2R	*vip3Ad* (bases 1 to 380)	380
vAaC2	pET-vip3Aa39	JB2F/39R	*vip3Aa39* (bases 355 to 2370)	2015
Vip3AdAa2	*vAdN2+vAaC2*	DF/39R	*vip3Ad* (bases 1 to 354) and *vip3Aa39* (bases 355 to 2370)	2370
vAaN1	pET-vip3Aa39	39F/JB7R	*vip3Aa39* (bases 1 to 2199)	2199
vAdC1	pET-vip3Ad	JB7F/DR	*vip3Ad* (bases 2179 to 2361)	182
Vip3AaAd1	*vAaN1+vAdC1*	39F/DR	*vip3Aa39* (bases 1 to 2199) and *vip3Ad* (bases 2200 to 2361)	2361
vAaN2	pET-vip3Aa39	39F/JB8R	*vip3Aa39* (bases 1 to 2316)	2316
vAdC2	pET-vip3Ad	JB8F/DR	*vip3Ad* (bases 2300 to 2361)	61
Vip3AaAd2	*vAaN2+vAdC2*	39F/DR	*vip3Aa39* (bases 1 to 2316) and *vip3Ad* (bases 2317 to 2361)	2361
vAaN3	pET-vip3Aa39	39F/JB3R	*vip3Aa39* (bases 1 to 837)	837
vAdM1	pET-vip3Ad	JB3F/JB4R	*vip3Ad* (bases 811 to 1059)	248
vAaAd1	*vAaN3+vAdM1*	39F/JB4R	*vip3Aa39* (bases 1 to 810) and *vip3Ad* (bases 811 to 1059)	1059
vAaC3	pET-vip3Aa39	JB4F/39R	*vip3Aa39* (bases 1036 to 2370)	1334
vip3AaAdAa1	*vAaAd1+vAaC3*	39F/39R	*vip3Ad* (bases 811 to 1035) insert to the same position of *vip3Aa39*	2370
vAaN4	pET-vip3Aa39	39F/JB5R	*vip3Aa39* (bases 1 to 1393)	1393
vAdM2	pET-vip3Ad	JB5F/JB6R	*vip3Ad* (bases 1366 to 1924)	558
vAaAd2	*vAaN4+vAdM2*	39F/JB6R	*vip3Aa39* (bases 1 to 1365) and *vip3Ad (bases* 1366 to 1924)	1924
vAaC4	pET-vip3Aa39	JB6F/39R	*vip3Aa39* (bases 1900 to 2370)	470
vip3AaAdAa2	*vAaAd2+vAaC4*	39F/39R	*vip3Ad* (bases 1366 to 1899) insert to the same position of *vip3Aa39*	2370

induced at 16°C and 160 r/min for 12 h. The cells were collected by centrifugation, resuspended in 20 mM Tris-HCl (pH 8.0) buffer, sonicated for 10 min (ultrasound power: 80%; ultrasound frequency: pulse on 3 s, pulse off 3 s) and centrifuged at 4°C and 12 000 r/min for 15 min. The supernatants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Recombinant proteins were quantified (Bradford 1976).

Bioassays

The tested insects were supplied by Jiyuan Henan Baiyun Industrial Co., Ltd (China) and were reared in a culture room under controlled conditions of temperature of 27°C, relative humidity of 65% and a photoperiod of 15 h light and 9 h dark. Bioassays were performed using crude proteins that were diluted into six gradient concentrations in 20 mM Tris-HCl buffer (pH 8.0). The tested insects had a mortality rate of less than 100% within four concentrations of proteins, Vip3Aa39 and Vip3Ad, as well as a control, empty plasmid was used as the negative control.

For *P. xylostella*, fresh leaves of kohlrabi (*Brassica oleracea* L. gongylodes group) were cut into small circular pieces (2×2 cm^2), cleaned and soaked in protein solution, removed and dried. A circular filter paper piece was placed into the bottom of the culture dish to maintain moisture. The dried circular kohlrabi leaf pieces were put into the culture dish, and then first instar larvae were gently attached to the circular pieces with a soft writing brush. Bioassays were replicated three times using 30 newly hatched larvae per concentration.

For *H. armigera*, 2.0 mL of each concentration of the protein solution to be tested was added to 20 g of feed and stirred, cooled down at room temperature to evaporate excess water and sub packaging in a sterilized 24-well cell culture plate (50 μL/well). The newly hatched larvae were reared in wells (one larva per well) on a diet for each dose along with the control set, and each treatment was replicated three times. Mortality in each case was recorded up to 3 days for *P. xylostella* and 7 days for *H. armigera*.

The Proteins Treated by Trypsin

Vip3Aa39, Vip3Ad and the chimeric proteins in 20 mM PBS buffer (pH 8.0) were quantified and adjusted to 300 μg/mL. To determine the digestion time for the chimeric proteins, Vip3Aa39 and Vip3Ad proteins were treated with trypsin (0.1mg/mL), according to a ratio of trypsin to quantified protein of 1:10 and were added into a 1.5 mL EP tube. After being mixed quickly and evenly, the mixture was placed into a 37°C water bath pot to be incubated for different times (0 s, 15 s, 30 s, 1 min, 2 min, 5 min, 10 min, 20 min), and then the loading buffer was added to stop the reaction, with degeneration in boiling water for ten min. Then, proteolysis products were separated by SDS-PAGE. The chimeric proteins, after ultrasonic crushing treatment, was trypsinized in PBS buffer (pH 8.0). The Vip3Aa39 and Vip3Ad proteins were treated as positive control. All experimental results were from three separate trials.

Statistics and Analysis

Results were statistically analyzed with three replications. Probit analysis was conducted using SPSS 13.0 software to determine the fifty percent lethal concentration (LC_{50}) with 95% confidence limits in each case (Cai *et al.,* 2006; Hernandez-Martinez *et al.,* 2013). LC_{50} values were considered significantly different when fiducial limits did not overlap.

Results

The Acquisition of Chimeric Gene

The electrophoresis results showed that the molecular weight of 22 PCR amplification fragments was consistent with the expectation. The recombinant plasmid was transferred to BL21 competent cells, and positive clones were picked and sent for sequence testing. Using the NCBI database and the DNAMAN tools to align the gene sequences as well as deduced amino acid sequences, we verified that the base fragments were inserted correctly. Six chimeric genes were constructed successfully (Fig. 1). The Vip3Aa39 gene is 2370 bp in length and encodes a polypeptide of 790 amino acids. Chimeric genes (*vip3AdAa1*, *vip3AdAa2*, *vip3AaAdAa1* and *vip3AaAdAa2*) with the C-terminus of *vip3Aa39* are the same as vip3Aa39 in length. Whereas chimeric genes (*vip3AaAd1* and *vip3AaAd2*) with the C-terminus of *vip3Ad* are 2361 bp in length and encode a polypeptide of 787 amino acids which are the same as *vip3Ad*.

Expression of the Chimeric Proteins in *E. coli*

The expression levels of all the target proteins were quantified by SDS–PAGE (Fig. 2). All the proteins had an apparent molecular mass of ~88 kDa, which was basically consistent with the theoretical calculation. Preliminary results indicated that 6 chimeric proteins, together with Vip3Aa39 and Vip3Ad proteins, were expressed in E. *coli* BL21, mainly in 20 mM Tris-HCl supernatant.

Insecticidal Activity

Insecticidal activity of these proteins was assessed toward *H. armigera* and *P. xylostella* (Table 3). The results demonstrated that Vip3Aa39 showed toxicity with LC_{50} values of 114.174 $\mu g \cdot mL^{-1}$ and 141.83 $\mu g \cdot mL^{-1}$ against *H. armigera* and *P. xylostella*, respectively, whereas Vip3Ad and Vip3AaAd1 had no activity. For *H. armigera*, Vip3AdAa1, Vip3AaAd3 and Vip3AaAdAa2 were more active than Vip3Aa39, with LC_{50} values of 26.908, 74.277 and 24.298 $\mu g \cdot mL^{-1}$, respectively. The toxicity of Vip3AdAa2 and Vip3AaAdAa2 showed no significant difference with that of Vip3Aa39. Bioassays with *P. xylostella* showed that Vip3AdAa1 had very high toxicity (1.94-fold), and Vip3AaAdAa1 and Vip3AaAdAa2 were significantly less active than Vip3Aa39 (0.53-0.48-fold). The toxicity of Vip3AdAa2 was not significantly different from that of Vip3Aa39.

Proteolysis of Chimeric Proteins by Trypsin

Vip3Aa39 and Vip3Ad proteins were processed with trypsin at different times and analyzed by SDS-PAGE as shown in Fig. 3. With increasing trypsin digestion time, the intensity of protein (88 kDa) decreased gradually, whereas the core polypeptide of 62 kDa appeared gradually. The 88 kDa band degraded fully when Vip3Aa39 protein was activated by incubation with trypsin 2 to 5 min, whereas that of Vip3Ad degraded in 10 to 20 min (Fig. 3).

Based on the results of the trypsin experiment on Vip3Aa39 and Vip3Ad, the sensitivity of trypsin to chimeric proteins was analyzed by treatment with trypsin for 10 min. The chimeric proteins could all be activated by trypsin, except for Vip3AaAd1, and produced an approximate 62 kDa fragment, which is also known as the Vip3A toxic core. The core band of 62 kDa was not found for Vip3AaAd1, even after trypsinization for 20 min (Fig. 4).

Discussion

Vegetative insecticidal proteins (Vips), a new class of insecticidal proteins that are secreted during the period of vegetative growth of *B. thuringiensis*, have high insecticidal activity against lepidopteran pests (Estruch *et al.,* 1996;

Table 3: Comparative toxicity analysis of Vip3Aa39 and its chimeric proteins

Protein	Toxicity			
	H. armigera		*P. xylostella*	
	LC_{50} (μg•mL^{-1})	Toxicity fold	LC_{50} (μg•mL^{-1})	Toxicity fold
Vip3Aa39	114.174(97.5-133.707)[a]	1	141.83(119.96-161.46)	1
Vip3Ad	NA	-	NA	-
Vip3AdAa1	26.908(22.313-31.446)	4.2	73.171(63.08-82.389)	1.94
Vip3AdAa2	105.928(96.011-116.012)	1.08	163.177(145.284-180.219)	0.87
Vip3AaAd1	NA	-	NA	-
Vip3AaAd2	74.277(63.208-84.296)	1.53	>500	-
Vip3AaAdAa1	24.298(21.377-27.14)	4.70	270.04(241.4-297.7)	0.53
Vip3AaAdAa2	121.761(111.828-132.485)	0.94	295.132(276.463-315.673)	0.48

NA: No activity; [a] Fiducial limit (95%) are given in parenthesis

Mesrati *et al.*, 2005; Doss 2009). However, we still have little information about the structure and function of each part of the protein (Boukedi *et al.*, 2018; Lone *et al.*, 2018). The C-terminal fragment is less homologous, while the N-terminal fragment of Vip3 is highly conserved (Estruch *et al.*, 1996; Bel *et al.*, 2017). When 39 N-terminal amino acids of the Vip3Aa protein were deleted, the mutant protein showed a significant effect on susceptible pests such as *S. litura* and *C. partellus*. Adding more amino acids to its C-terminus resulted in decreased stability of Vip3 (Sellami *et al.*, 2016). The S9N mutant of Vip3Aa11 showed improved activity against *H. armigera*, but the R115H mutant showed significantly decreased activity (Liu *et al.*, 2017). The construction of chimeric proteins is an effective way to enhance the toxicity of insecticidal proteins (Zaheer Abbas 2013). Vip3Aa39 showed toxicity with LC_{50} values of 114.174 μg·mL^{-1} and 141.83 μg·mL^{-1} against *H. armigera* and *P. xylostella*, respectively. However, Vip3Ad had no activities on the two pests. To evaluate the roles of several regions of the Vip3A proteins in their toxicity and host specificity and to explore the possibility for toxin improvement by artificial gene recombination, we constructed 6 chimeric genes.

One study suggested that 198 amino acids at the N-terminus of Vip3A can ensure correct folding. *Vip3AdAa1* is a chimeric protein consisting of the N-terminal 60 amino acid residues of Vip3Ad and the C-terminal 730 amino acid residues of Vip3Aa39. When comparing the amino acid sequence of Vip3AdAa1 to Vip3Aa39, there are only three amino acid differences between these (T6A, N45D, E60D), and Vip3AdAa1 showed enhanced toxicity compared to the Vip3Aa39 protein against *H. armigera* and *P. xylostella*. It was hypothesized that the three amino acid sites facilitated the improvement of their insecticidal activities. However, 118 amino acid fragments at the N-terminus of Vip3Aa39 were replaced by the corresponding amino acid fragments of Vip3Ad (Vip3AdAa2). Although this toxin differs from Vip3Aa39 in only eight amino acids (8 amino acid difference: T6A, N45D, E60D, E88Q, I92V, A108T, T112S, H115K), Vip3AdAa2 had no significant difference in insecticidal activity on the two insects compared to Vip3Aa39. It is thus possible that five amino acids retain their insecticidal function changes caused by the three amino acids. Further studies are needed to understand the reasons.

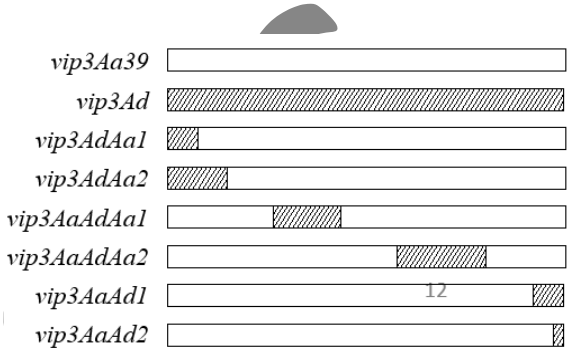

Fig. 1: The construct of chimeric gene

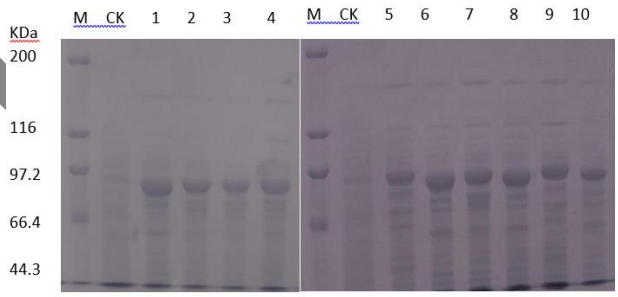

Fig. 2: Expression of chimeric and their parental genes in *E. coli* BL21. M: Protein marker; CK: pET21b empty vector; lane 1: Vip3AdAa1; lane 2: Vip3AdAa2; lane 3: Vip3Aa39; lane 4: Vip3Ad; lane 5: Vip3AaAd1; lane 6: Vip3AaAd2; lane 7: Vip3AaAdAa1; lane 8: Vip3AaAdAa2; lane 9: Vip3Ad; lane 10: Vip3Aa39

Vip3A proteins exhibited significant differences in toxicity to the same insect species (Lone *et al.*, 2016), which was further confirmed according to our results. For *H. armigera*, Vip3AaAd2 (1.53-fold) and Vip3AaAdAa1 (4.70-fold) were significantly more active than Vip3Aa39, whereas Vip3AaAdAa2 showed no significant differences in activity. Bioassays with *P. xylostella* showed that Vip3AaAd2, Vip3AaAdAa1 and Vip3AaAdAa2 were significantly less active than Vip3Aa39. Differences between the sequences of the various members of the Vip3A family vary widely but occur mostly in the C-terminal region. The C-terminal region was proposed to be involved in target specificity (Wu *et al.*, 2007; Gayen *et al.*,, 2012). The high divergence of the C-terminal among Vip3A toxins might result in the discrepant insecticidal activities among

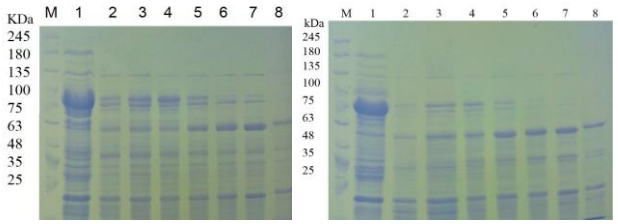

Fig. 3: Proteolytic activation of Vip3Aa and Vip3Ad by commercial trypsin. Proteins were incubated at 30ºC for different times. A: Vip3Ad; B: Vip3Aa39. Lane M: Molecular weight markers; Lane 1: un-induced protein; lane 2: protein induced after 15s; lane 3: protein induced after 30s; lane 4: protein induced after 1 min; lane 5: protein induced after 2 min; lane 6: protein induced after 5 min; lane 7: protein induced after 10 min; lane 8: protein induced after 20 min

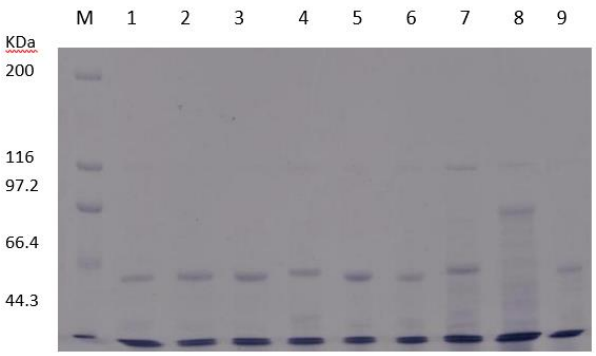

Fig. 4: SDS-PAGE analysis of proteins by trypsin treatment for 10 min; lane M: Protein marker; lane 1: Vip3AdAa1; lane 2: Vip3AdAa2; lane 3*:* Vip3Aa39; lane 4: Vip3Ad; lane 5,6: Vip3AaAdAa1; lane 7: Vip3AaAdAa2; lane 8: Vip3AaAd1; lane 9: Vip3AaAd2

those proteins due to their binding properties (Pan *et al.*, 2017). Vip3AaAd1 was constructed by replacing an amino acid fragment of Vip3Aa39 (734 to 790) with the corresponding amino acid fragment of Vip3Ad (734 to 787). For the Vip3AaAd2 protein, a different amino acid fragment of Vip3Aa39 (773 to 790) was replaced with the corresponding amino acid fragment of Vip3Ad (773 to 787). Unlike Vip3AaAd2, Vip3AaAd1 showed no toxicity against *H. armigera* and *P. xylostella*, which also indicated that the amino acid fragment (734-772) was related to the loss of insecticidal activity of the Vip3Ad protein.

Previous studies in some lepidopteran insects have reported that Vip3A proteins must be activated by proteolysis and then the active form binds to specific receptors on the midgut brush border membrane which eventually leads to pore formation and cell lysis (Chakroun *et al.*, 2012, Sellami *et al.*, 2015). The hydrolysis of trypsin on chimeric proteins is necessary for their toxicity to target insects. This study evaluated the hydrolysis of chimeric proteins with trypsin. Five chimeric proteins were degraded to 62 kDa, except for Vip3AaAd1, which confirmed that trypsin is involved in the activation of chimeric proteins. This result further indicated that the Vip3AaAd1 protein was inactive because it did not digest the active core fragment. This study also demonstrates that using exchange of variable regions across classes of Vip proteins can result in novel bioactivity.

Conclusion

We performed the functional analysis of 6 new chimeric Vip3A toxin proteins. These findings showed that recombinant proteins (Vip3AdAa1, Vip3AaAd2 and Vip3AaAdAa1) might be useful for controlling *H. armigera*. Vip3AdAa1 also exhibited an important role in controlling *P. xylostella.* These toxins could be potential materials for genetically modified plants or as bio-insecticides. The study also revealed that the C-terminal amino acid fragment plays an important role in insecticidal activity in the two insects.

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