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Original Research Article

A strategy to enhance the insecticidal potency of Vip3Aa by introducing additional cleavage sites to increase its proteolytic activation efficiency

Kun Jiang[a,#,*], Zhe Chen[a,#], Yiting Shi[b,c], Yuanrong Zang[a], Chengbin Shang[b], Xi Huang[b], Jiahe Zang[b], Zhudong Bai[b], Xuyao Jiao[a], Jun Cai[d], Xiang Gao[a,*]

[a] State Key Laboratory of Microbial Technology, Shandong University, Qingdao 266237, China
[b] School of Life Sciences, Shandong University, Qingdao 266237, China
[c] Taishan College, Shandong University, Jinan 250100, China
[d] Department of Microbiology, College of Life Sciences, Nankai University, Tianjin 300071, China

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ABSTRACT

Microbially derived, protein-based biopesticides have become a vital element in pest management strategies. Vip3 family proteins from *Bacillus thuringiensis* have distinct characteristics from known insecticidal Cry toxins and show efficient insecticidal activity against several detrimental lepidopteran pests. They are considered to be a promising toxic candidate for the management of various detrimental pests. In this study, we found that in addition to the preliminary digestion sites lysine, there are multiple cleavage activation sites in the linker region between domain I (DI) and DII of Vip3Aa. We further demonstrated that by adding more cleavage sites between DI and DII of Vip3Aa, its proteolysis efficiency by midgut proteases can be significantly increased, and correspondingly enhance its insecticidal activity against *Spodoptera frugiperda* and *Helicoverpa armigera* larvae. Our study promotes the understanding of the insecticidal mechanism of Vip3 proteins and illustrates an easily implementable strategy to increase the insecticidal potency of Vip3Aa. This facilitates their potential future development and efficient application for sustainable agriculture.

1. Introduction

Microbial insecticidal proteins are important supplements and potential substitutes for synthetic pesticides because of their highly specific insecticidal effect and are environmentally friendly, etc. [1–5]. *Bacillus thuringiensis* (Bt) is the most extensively used microbial insecticide worldwide, due to the successful application of Cry insecticidal proteins [6,7]. Cry proteins are potent endotoxins, which are mainly produced by Bt during sporulation. The structure and molecular mechanism of action for Cry proteins is well documented, and they are widely used for the biological control of insects both in transgenic crops and formulated sprays [6,8-11]. However, many pests are not sensitive to Cry proteins, and with their long-term use, many pests have evolved resistance to Cry proteins [3,4,7,12]. Therefore, the development of new appropriate insecticidal protein resources is imperative.

Unlike Cry proteins, vegetative insecticidal proteins (Vip3) are secreted by Bt during its vegetative growth phase [13,14]. Vip3 proteins have different characteristics and mechanisms of action from Cry proteins, and have high insecticidal activity, especially against *Lepidoptera*. They are considered to be a new generation of insecticidal proteins that can be utilized in practice after Cry proteins [2,13,15-18]. In response to the increasing pest resistance to Cry proteins, Vip3 proteins have been used in genetically engineered crops in combination with Cry proteins, which have exhibited a highly synergistic insecticidal effect and promising application potential for the management of resistance and crop protection [2,15].

Vip3 consists of about 789 amino acid residues. Since their discovery in 1996 [14], more than 132 Vip3 proteins have been identified based on their structural homology [19,20]. It is generally recognized that Vip3A changes from the inactive protoxin to activated toxin after digestion by trypsin or midgut proteases [20–23]. The ~88 kDa Vip3A protein could be cleaved into two fragments of around 20 and 66 kDa that remain strongly associated, which is essential for its toxicity [24–28]. Recently, the crystal and cryo-EM structures of Vip3 have been successfully reported [29–32]. The Vip3 protoxin consists of five domains and is assembled into a highly stable tetrameric dimer of dimers. After domain I (corresponding to the N-terminal 198 amino acids) and domain II are cleaved by trypsin, the domain I undergoes a

* Corresponding authors.
E-mail addresses: kunjiang@sdu.edu.cn (K. Jiang), xgao@email.sdu.edu.cn (X. Gao).
# These authors contributed equally to this work.

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huge conformational change, and is remodeled into an extended four-helix coiled coil, which is proposed to be required for the pore formation of Vip3.

Several studies have reported improvements to the Vip3A's insecticidal activity or insecticidal spectrum. One such improvement was a mutation of Met^{34} to Lys^{34} which increased the toxicity of Vip3Af to *Spodoptera littoralis* [33]. Mutations $Vip3Aa^{S543N}$, $Vip3Aa^{I544L}$, and $Vip3Aa^{S686R}$ increased the toxicity of Vip3Aa to *S. exigua*, while the toxicity of Vip3Aa to *Helicoverpa armigera* and *Agrotis ipsilon* was decreased, respectively [34]. The $Vip3Aa^{S543N/I544L/S686R}$ mutation exhibited increased toxicity against *S. frugiperda* and *H. armigera;* while the $Vip3Aa^{S543N/I544L/E627A}$ mutation exhibited increased toxicity against *S. frugiperda* and decreased toxicity against *Mythimna separata* compared with that of the wild-type Vip3Aa protein [35]. Vip3AcAa, a chimeric protein, constructed through combining the N-terminal of Vip3Ac1 and C-terminal Vip3Aa1, gained toxicity against *Ostrinia nubilalis* and showed enhanced toxicity against *S. frugiperda* [36]. A chimera with the N-terminal domain (domain I) from Vip3Aa and the remainder of the protein derived from Vip3Ca also showed enhanced toxicity against *S. frugiperda* compared to the parental Vip3Ca [37]. These studies were primarily based on the sequence differences between different Vip3A proteins to exchange various sites or fragments of Vip3A, and mainly increased the toxicity of Vip3A to a specific pest. However, there are no modification methods based on the activation mechanism of Vip3A and few to generally improve the insecticidal activity of Vip3A.

In this study, we explored the activation mechanism of Vip3Aa and identified multiple protease activation sites in the loop region between DI and DII of Vip3Aa. Additionally, we further demonstrated that increasing the proteolysis efficiency of the loop region between DI and DII of Vip3Aa promoted its insecticidal potency. We propose a practical strategy to increase the insecticidal activity of Vip3Aa, which will potentially enhance the development of Vip3Aa into a more efficient bio-insecticide.

2. Materials and methods

2.1. Bacterial strains and insects

E. coli B21 (DE3) was cultured at 37 ˚C in lysogeny broth (LB) or agar for plasmid construction and protein purification. Bt 9816C was cultured at 30 ˚C in LB and agitated at 200 rpm. *S. frugiperda* and *H. armigera* larvae were used for the bioassays as described below.

2.2. Plasmid construction

For the recombinant protein expression in *E. coli*, the *Vip3Aa11* gene was cloned into the pET28a expression vector with an N-terminal 6 × His-SUMO (small ubiquitin-like motif) tag using the Gibson assembly strategy [38,39]. All point mutations of *Vip3Aa* were generated as full-length *Vip3Aa*. All plasmids were verified by DNA sequencing and listed in Table S1. All the primers used are listed in Table S2.

2.3. Protein expression and purification

E. coli B21 (DE3) harboring the plasmids expressing the different Vip3Aa constructs were grown in autoinduction terrific broth medium at 25 ˚C for 48 h. Bacterial cells were collected by centrifugation, and the pellet was resuspended in lysis buffer (20 mM Tris–HCl (pH 8.0) and 300 mM NaCl). Bacterial cells were lysed by a high-pressure cell crusher (Union-Biotech, Shanghai, China), the supernatant was collected after centrifugation at 18,000 × g for 45 min, run through Ni-NTA beads (Qiagen, Valencia, CA), and washed with lysis buffer. The SUMO tag was removed with in-house generated His-tagged ULP1(ubiquitin-like-specific protease 1) protease at room temperature for 3 h and proteins were eluted in lysis buffer. The proteins were further purified through an anion-exchange column (Hitrap Q, GE Healthcare) and gel filtration chromatography (Superdex 200 Increase 10/300 GL, GE Healthcare). Peak fractions were determined and used for subsequent functional studies. The concentration of protein was quantified by a NanoPhotometer N60 (Implen, Germany).

2.4. Midgut juice (MJ) or trypsin proteolysis assay

The fourth instar larvae of *S. frugiperda* or *H. armigera* were anesthetized on ice for 10 min and dissected to collect the midguts. The peritrophic matrix containing the food bolus was removed, and the midgut tissues were mixed and centrifuged at 4 ˚C for 10 min at 16,000 × g. The supernatant was separated into small aliquots, immediately frozen in liquid nitrogen, and stored at −80 ˚C.

The Vip3Aa11 protein (15 μg) was incubated with MJ at a ratio of 1:16 (MJ:Vip3Aa, wt:wt) in a final volume of 40 μL and incubated at 27 ˚C for 12 h, unless otherwise stated. For the trypsin proteolysis assay, Vip3Aa protein (15 μg) was incubated with trypsin at a ratio of 1:50 (trypsin:Vip3Aa, wt:wt), at 4 ˚C for 6 h, unless otherwise stated. Prior to electrophoresis, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) protease inhibitor was used to halt the reaction, followed by SDS-PAGE loading buffer, and the samples were boiled for 5 min and analyzed on a 15% SDS-PAGE.

2.5. Edman degradation sequence analysis

Protein sequencing by Edman degradation was performed as previously described [22]. Briefly, the ~66 kDa protein fragment produced by MJ-treated $Vip3Aa^{5M}$ was separated by a 12% SDS-PAGE and electrotransfered onto a polyvinylidene fluoride (PVDF) membrane (Millipore). The PVDF membrane was stained with Ponceau S for 1 min and decolorized with 50% methanol. The ~66 kDa protein fragment was excised and submitted for amino acid sequencing using an SHIMADZU automated peptide sequencer (PPSQ-33A, Japan). The original data generated by PPSQ-33A were analyzed by the PPSQ-30 DataProcessing software.

2.6. Bioassay

Bioassays were assessed using a surface contamination method with *S. frugiperda* or *H. armigera* larvae, which were maintained in a rearing chamber at 27 ˚C, with 50% relative humidity [23]. The artificial diet was prepared and poured in a 24-well cell culture plate. Different concentrations of Vip3Aa proteins (~50 μL) were added to the diet, as indicated in the figure legends. Lysis buffer was used as a blank control. The diet was dried completely at room temperature for about 1 h, and *S. frugiperda* or *H. armigera* larvae were added to each well of the diet (n = 24 total; 1 per well) and allowed to feed for at least seven days. The mortality rate after 7 days was measured using three independent replications. The larvae remaining in the initial instar stage were recorded as functional mortality. GraphPad Prism v.8.0 (GraphPad, USA) was used to calculate the lethal concentration (LC_{50}) values.

2.7. Statistical analysis

Data are shown as the arithmetic mean ± standard deviation (SD). All statistical data were calculated using GraphPad Prism v.8.0 (GraphPad). For the comparison of the means of two groups, unpaired two-sided *t*-tests were used. For the comparison between multiple groups with a control group, one-way analysis of variance (ANOVA) was used. $P < 0.05$ was considered to be statistically significant.

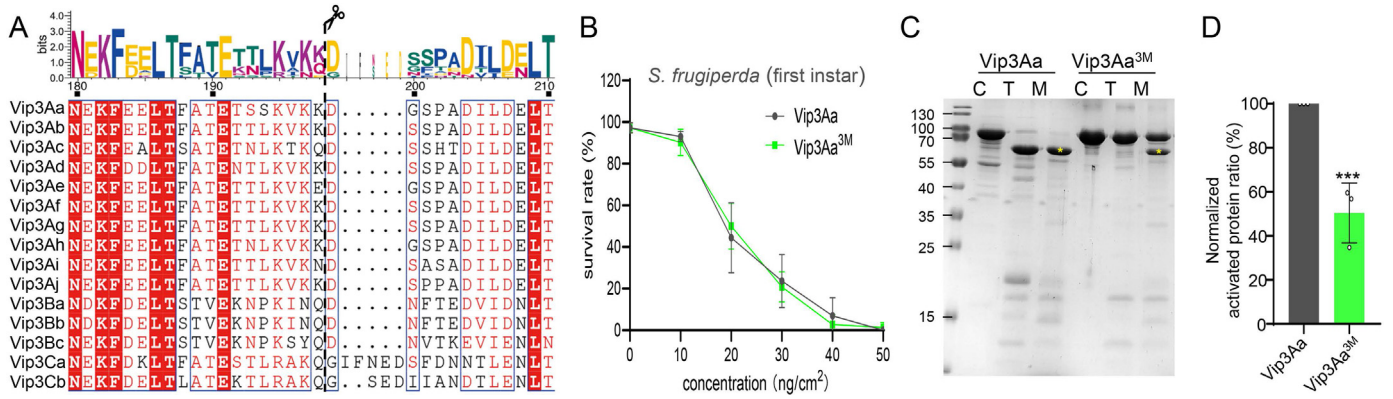


Fig. 1. Lysine is not the only proteolytic cleavage site of Vip3Aa.

(A) Sequence alignment of the selected amino acids between domain I (DI) and domain II (DII) of the different subclasses of Vip3 family proteins. The scissor cartoon indicates the protease cleavage site. The Weblogo was generated using WebLogo 3: Public Beta.

(B) Insecticidal activity of Vip3Aa and Vip3Aa^{3M} to *S. frugiperda* larvae (first instar) at concentrations of 10 ng/cm^2, 20 ng/cm^2, 30 ng/cm^2, 40 ng/cm^2, and 50 ng/cm^2 (n = 24).

(C) SDS-PAGE analysis of Vip3Aa and Vip3Aa^{3M} after treatment with trypsin or *S. frugiperda* midgut juice (MJ). The proteins were treated trypsin (1:50, trypsin:Vip3Aa, wt:wt) or MJ (1:16, MJ:Vip3Aa, wt:wt), and the interaction halted with the protease inhibitor AEBSF. "C": proteins untreated as a control; "T": proteins treated with trypsin; "M": proteins treated with MJ; * indicates the ~66 kDa bands produced through proteolysis by MJ. The images are representative at least three independent experiments.

(D) Intensity ratios of the ~66 kDa bands (*) produced by MJ treatment compared to that of the untreated control (C) samples of each indicated protein. Because Vip3Aa was cleaved completely, the ratio of the ~66 kDa band generated by Vip3Aa is normalized as 1. Data are expressed as the mean ± standard deviation (SD) of three independent experiments. Statistical analysis was performed using unpaired two-sided *t*-tests: ***, $P < 0.001$.

3. Results

3.1. Trypsin was not the only protease that cleaved and activated vip3Aa in the insect midgut

The Vip3 proteins are produced as inactive protoxins. Proteolytic cleavage between DI and DII is an essential process to activate Vip3, which produced two associated bands as components of the activated protein [24,25,32,40]. However, the mechanism of Vip3 activation through proteolysis is still not fully understood. Sequence alignment showed that the amino acid residues of the loop region between DI and DII of the Vip3 proteins were relatively less conserved than the rest of the DI and DII sequences (Fig. 1A and SFig 1). The structural analysis showed that this loop region was apparently flexible, which is supported by the lack of electron density or high B-factor of the loop region in the reported Vip3 structures (SFig 2).

A previous study found that Vip3Aa^{3M} (195KVKKD199 to 195AVAAD199) could not be cleaved by trypsin, and its insecticidal toxicity was reduced relative to that of Vip3Aa wild-type, which indicates that these three lysine residues (K195,197, and 198) are the primary trypsin cleavage sites of Vip3Aa [22]. However, we found that the toxicity of Vip3Aa^{3M} against *S. frugiperda* larvae was similar to that of the wild-type Vip3Aa (Fig. 1B and Table 1). Further proteolysis assays and SDS-PAGE analysis showed that Vip3Aa^{3M} could not be cleaved by trypsin; however, it could be cleaved by the midgut juice (MJ) of *S. frugiperda* larvae (Fig. 1C and 1D), although with reduced efficiency compared to that of Vip3Aa. These results indicate that trypsin is not the only protease able to cleave and activate Vip3Aa in *S. frugiperda* midgut.

Table 1
Mortality analysis of *Spodoptera frugiperda* larvae (first instar) as induced by Vip3Aa and the indicated mutant proteins.

Protein	LC50 (ng/cm^2)	95% Fiducial limit (ng/cm^2)-Lower	95% Fiducial limit (ng/cm^2)-Upper
Vip3Aa	19.56	17.42	21.71
Vip3Aa^{3M}	19.90	18.22	21.51
Vip3Aa^{4M}	20.99	19.81	22.07
Vip3Aa^{5M}	24.85	22.50	27.15
Vip3Aa^{7M}	34.83	32.23	37.38
Vip3Aa^{10M}	219.0	195.2	243.4
Vip3Aa^{13M}	No[a]	N/A	N/A

3.2. Vip3Aa has multiple proteolytic cleavage sites in the loop region between DI and DII for its activation

To further investigate whether there are other midgut protease cleavage sites between DI and DII, we initially constructed Vip3Aa^{4M} (195KVKKD199 to 195AVAAA199) and Vip3Aa^{5M} (195KVKKD199 to 195AGAAA199) mutants around the triple lysines of Vip3Aa and found that they were also cleaved by MJ (Fig. 2A, B). However, the Vip3Aa^{5M} mutant showed reduced cleavage efficiency and ~0.5 × lower insecticidal activity compared to Vip3Aa (Fig. 2A–2C and Table 1). To characterize the additional cleavage sites, the ~66 kDa protein fragment produced by MJ-treated Vip3Aa^{5M} was subjected to Edman degradation analysis. The N-terminal sequence of the tested fragment was AGAAAG, which matched the sequence of the 195AGAAAG200 of the Vip3Aa^{5M} protein sequence (SFig 3). This result indicated that MJ could still cleave Vip3Aa^{5M} between the Ser194 and Ala195. Therefore, we further constructed Vip3Aa^{7M} (SFig 4), adding SS193/194AA to the Vip3Aa^{5M}. Proteolysis assays and bioassays showed that the insecticidal activity of Vip3Aa^{7M} was about 2 × less than that of Vip3Aa which is consistent with the reduced cleavage efficiency by MJ (Fig. 2A–C and Table 1).

In addition, further limited proteolysis assays showed that digestion with protease V8 and MJ yielded similar digestion product profiles (Fig. 2D and SFig 5). The cleavage sites of the V8 protease are aspartic acid (D) and glutamate (E) [41], and there are multiple conserved D and E residues around the loop region of DI and DII (Fig. 1A). Therefore, we further constructed Vip3Aa^{10M} by adding E184/185/191A to Vip3Aa^{7M} and Vip3Aa^{13M} and adding D204/207A-E208A to Vip3Aa^{10M} (SFig 4). Proteolysis assays showed that the cleavage efficiency of Vip3Aa^{10M} and Vip3Aa^{13M} by MJ were both remarkably reduced, especially Vip3Aa^{13M}, which as scarcely cleaved by MJ (Fig. 2E and 2F). Correspondingly, bioassays showed Vip3Aa^{10M} and Vip3Aa^{13M} have clearer phenotypes with 11 × less and no toxicity, respectively, than that of Vip3Aa (Fig. 2G and Table 1). These results indicate that, in addition to lysines 195, 197,

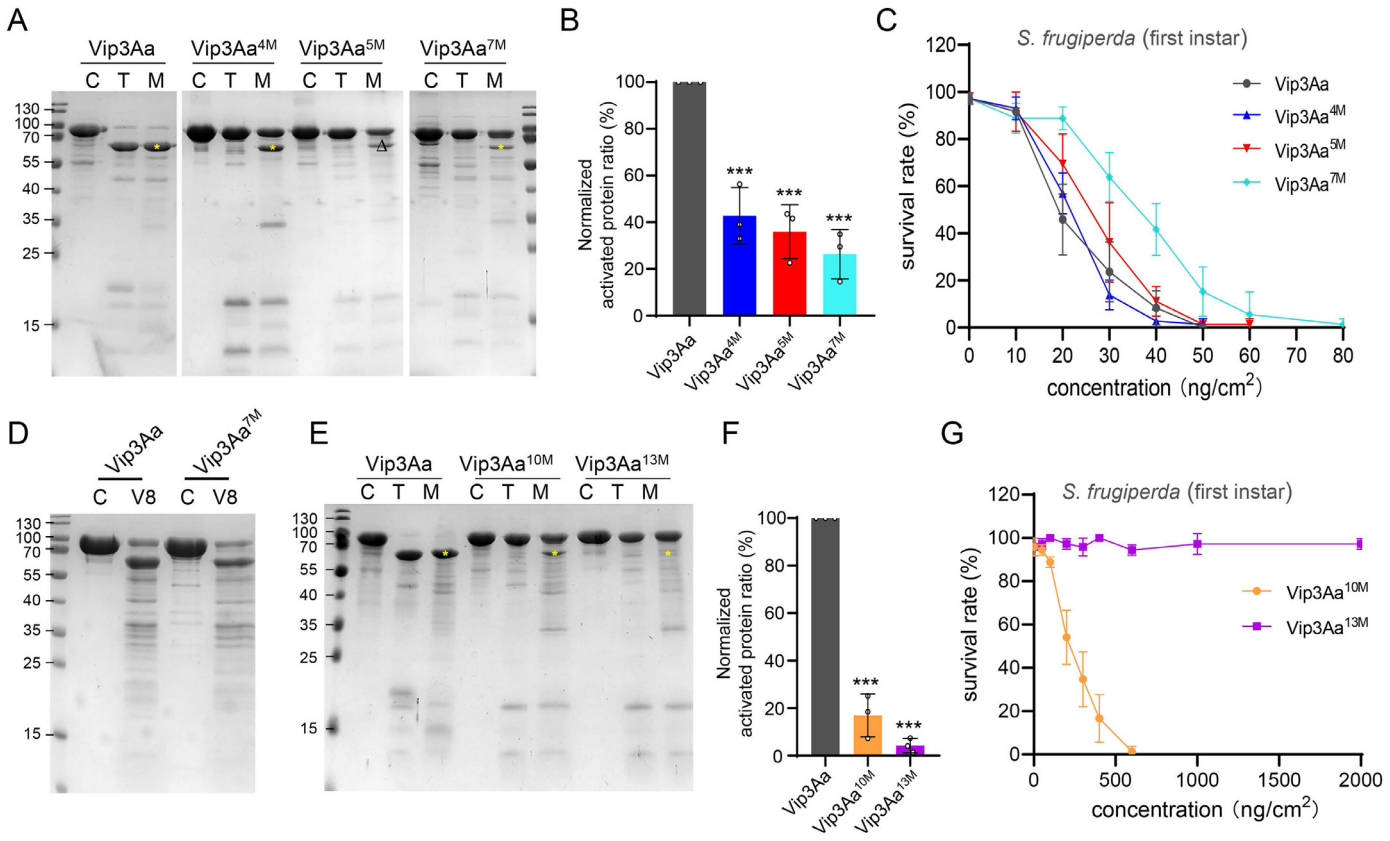


Fig. 2. Vip3Aa has multiple proteolytic cleavage activation sites

(A and E) SDS-PAGE analysis of Vip3Aa and the indicated Vip3Aa mutants after treatment with trypsin or *S. frugiperda* MJ. The proteins were treated with trypsin (1:50, trypsin:Vip3Aa, wt:wt) or MJ (1:16, MJ:Vip3Aa, wt:wt), and the reaction was halted with the protease inhibitor AEBSF. "C": proteins untreated as a control; "T": proteins treated with trypsin; "M": proteins treated with MJ; * indicates the ~66 kDa bands produced through proteolysis by MJ; Δ indicates the bands analyzed by Edman degradation. The images represent at least three independent experiments.

(B and F) Intensity ratios of the ~66 kDa bands (*) produced by MJ treatment compared to that of the untreated control (C) sample of each indicated protein of (A) and (E). Because Vip3Aa is cleaved completely, the ratio of the ~66 kDa band generated by Vip3Aa is normalized as 1. Data are expressed as the mean ± standard deviation (SD) from three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Duncan's multiple range test (MRT): ***, $P < 0.001$.

(C) Insecticidal activity of Vip3Aa and the indicated Vip3Aa mutants against *S. frugiperda* larvae (first instar) at concentrations of 10 ng/cm^2, 20 ng/cm^2, 30 ng/cm^2, 40 ng/cm^2, 50 ng/cm^2, 60 ng/cm^2, and 80 ng/cm^2 ($n = 24$).

(D) SDS-PAGE analysis of the Vip3Aa and Vip3Aa^{7M} after treatment with V8 protease. The proteins were treated with V8 protease at 4 °C for 30 min at a ratio of 1:200 (V8:Vip3Aa, wt:wt). The reaction was halted with the protease inhibitor AEBSF.

(G) Insecticidal activity of Vip3Aa, Vip3Aa^{10M}, and Vip3Aa^{13M} against *S. frugiperda* larvae (first instar) at concentrations of 50 ng/cm^2, 100 ng/cm^2, 200 ng/cm^2, 300 ng/cm^2, 400 ng/cm^2, 600 ng/cm^2, 1 μg/cm^2, and 2 μg/cm^2 ($n = 24$).

In (C) and (G), data are expressed as the mean ± SD from three independent experiments.

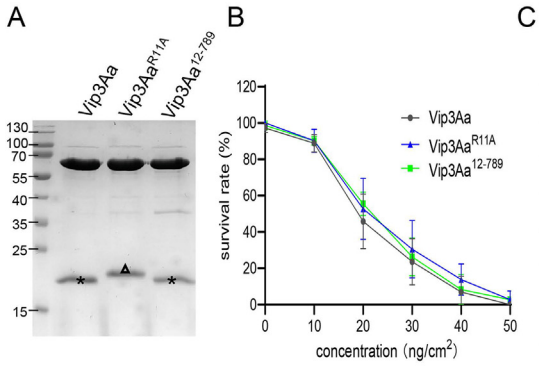
and 198, several other protease cutting sites, including aspartic acid, glutamic acid, and serine, are recognized as cleavage sites to activate Vip3Aa by the MJ of *S. frugiperda* larvae between DI and DII.

3.3. Adding additional cleavage sites between DI and DII increases the proteolysis efficiency of vip3Aa

By investigating the detailed cleavage process between DI and DII of Vip3Aa, we found that the toxicity of Vip3Aa was positively correlated with its cleavability by MJ. Therefore, we hypothesized that by adding additional cleavage sites in the loop region between DI and DII, a more efficient activation could be achieved to increase the insecticidal toxicity of Vip3Aa.

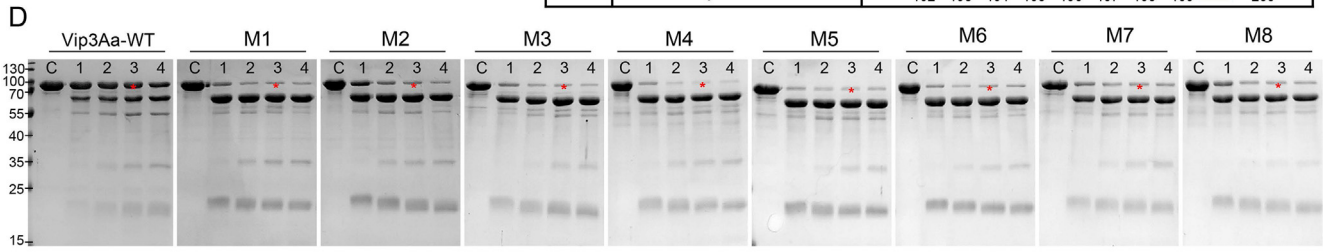
Through sequence alignment, we found that the sequence identity of DI is relatively high conserved across the Vip3 protein family, excluding the first ~10 amino acids of Vip3 proteins (SFig 1), which are speculated to be a potential signaling sequence [26,37]. We constructed the Vip3Aa^{R11A} mutant and Vip3Aa$^{12–789}$ truncation variants to evaluate whether the first eleven amino acids were essential for Vip3 functioning. Through proteolysis assays and bioassays, we demonstrated that the first 11 amino acids until the conserved arginine residue (R11) of the Vip3Aa DI could be removed by trypsin without affecting the stability and toxicity of Vip3Aa (Fig. 3A and 3B), and further suggested that the exposed R-A residues of Vip3Aa could be efficiently cleaved by trypsin or MJ. This is consistent with previous findings that R-A pair performs better among the cleavage sites of trypsin, because of the preferable interaction between arginine with the aspartate located at the bottom of the substrate-binding pocket of trypsin and less steric resistance caused by alanine [42].

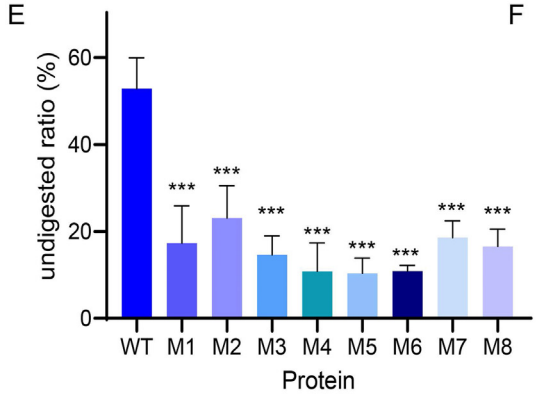
To test whether the introduction of additional cleavage sites between DI and DII increased the hydrolysis efficiency of Vip3Aa by MJ, we first inserted R-A residues between T192 and G200 of Vip3Aa (Fig. 3C). After treatment with MJ from *S. frugiperda* in time course experiments, we found that these mutants could be more efficiently cleaved than Vip3Aa, especially when R-A is inserted between K195 and K198 of Vip3Aa (Fig. 3D, E). In addition, we further examined whether replacing the inefficient cleavage sites between DI and DII with R-A increased the cleavage efficiency of Vip3Aa by MJ. Consistent with this assump-



C

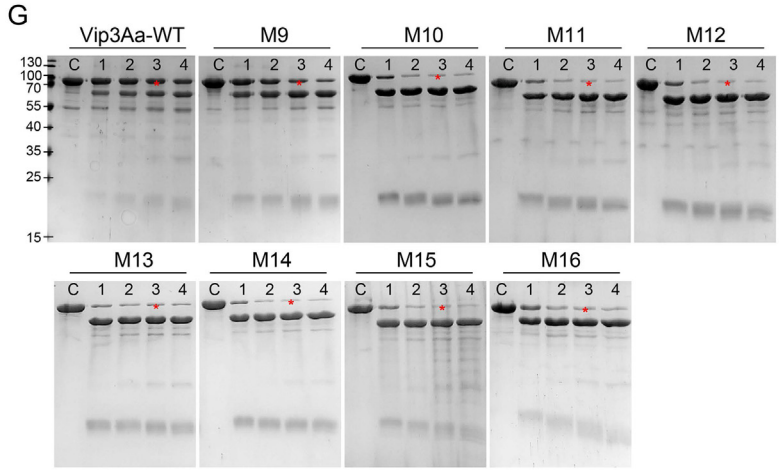
No.	Name	Corresponding amino acid residue mutation sites
WT	Vip3Aa-192_200	$T_{192}S_{193}S_{194}K_{195}V_{196}K_{197}K_{198}D_{199}G_{200}$
M1	Vip3A^{193RA}	T_{192} **RA** $S_{193}S_{194}K_{195}V_{196}K_{197}K_{198}D_{199}G_{200}$
M2	Vip3A^{194RA}	$T_{192}S_{193}$ **RA** $S_{194}K_{195}V_{196}K_{197}K_{198}D_{199}G_{200}$
M3	Vip3A^{195RA}	$T_{192}S_{193}S_{194}$ **RA** $K_{195}V_{196}K_{197}K_{198}D_{199}G_{200}$
M4	Vip3A^{196RA}	$T_{192}S_{193}S_{194}K_{195}$ **RA** $V_{196}K_{197}K_{198}D_{199}G_{200}$
M5	Vip3A^{197RA}	$T_{192}S_{193}S_{194}K_{195}V_{196}$ **RA** $K_{197}K_{198}D_{199}G_{200}$
M6	Vip3A^{198RA}	$T_{192}S_{193}S_{194}K_{195}V_{196}K_{197}$ **RA** $K_{198}D_{199}G_{200}$
M7	Vip3A^{199RA}	$T_{192}S_{193}S_{194}K_{195}V_{196}K_{197}K_{198}$ **RA** $D_{199}G_{200}$
M8	Vip3A^{200RA}	$T_{192}S_{193}S_{194}K_{195}V_{196}K_{197}K_{198}D_{199}$ **RA** G_{200}





F

No.	Name	Corresponding amino acid residue mutation sites
M9	Vip3A^{SS193RA}	T_{192} $R_{193}A_{194}$ $K_{195}V_{196}K_{197}K_{198}D_{199}G_{200}$
M10	Vip3A$^{SS193RA/193RA}$	T_{192} **RA** $R_{193}A_{194}K_{195}V_{196}K_{197}K_{198}D_{199}G_{200}$
M11	Vip3A$^{SS193RA/195RA}$	$T_{192}R_{193}A_{194}$ **RA** $K_{195}V_{196}K_{197}K_{198}D_{199}G_{200}$
M12	Vip3A$^{SS193RA/196RA}$	$T_{192}R_{193}A_{194}K_{195}$ **RA** $V_{196}K_{197}K_{198}D_{199}G_{200}$
M13	Vip3A$^{SS193RA/197RA}$	$T_{192}R_{193}A_{194}K_{195}V_{196}$ **RA** $K_{197}K_{198}D_{199}G_{200}$
M14	Vip3A$^{SS193RA/198RA}$	$T_{192}R_{193}A_{194}K_{195}V_{196}K_{197}$ **RA** $K_{198}D_{199}G_{200}$
M15	Vip3A$^{SS193RA/199RA}$	$T_{192}R_{193}A_{194}K_{195}V_{196}K_{197}K_{198}$ **RA** $D_{199}G_{200}$
M16	Vip3A$^{SS193RA/V196DE}$	$T_{192}R_{193}A_{194}K_{195}$ **DE** $K_{197}K_{198}D_{199}G_{200}$



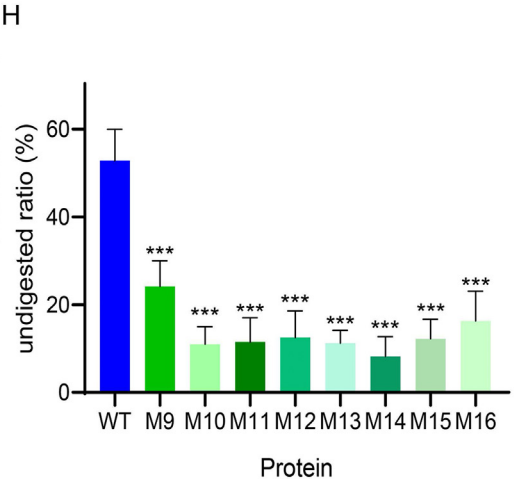


Fig. 3. Adding additional cleavage sites between DI and DII can increase the proteolysis efficiency of Vip3Aa.
(A) SDS-PAGE analysis of Vip3Aa, Vip3Aa^{R11A}, and Vip3Aa$^{12–789}$ after treatment with trypsin (1:50, trypsin:Vip3Aa, wt:wt) for 6 h. * indicates that the DI's bands produced by Vip3Aa and Vip3Aa$^{12–789}$;Δ indicates the DI's bands produced by Vip3Aa^{R11A}.
(B) Insecticidal activity of Vip3Aa, Vip3Aa^{R11A}, and Vip3Aa$^{12–789}$ against *S. frugiperda* larvae (first instar) at concentrations of 10 ng/cm^2, 20 ng/cm^2, 30 ng/cm^2, 40 ng/cm^2, and 50 ng/cm^2 (n = 24).
(C and F) The corresponding amino acid residue mutation sites between T192 and G200 of Vip3Aa. The inserted R-A residues are highlighted in red.
(D and G) SDS-PAGE analysis of the Vip3Aa and the indicated Vip3Aa mutants after exposure to *S. frugiperda* MJ. The proteins were treated with MJ at a ratio of 1:16 (MJ:Vip3, wt:wt). The reaction was halted by the addition of the protease inhibitor AEBSF after 1, 2, 3, or 4 h. The images represent at least three independent experiments.
(E and H) Histograms showing the ratio of the intensity of undigested protein (*) compared to that of the control sample (C) of each indicated protein in (D) and (G), after 3 h of MJ treatment. Data are expressed as the mean ± standard deviation (SD) from three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Duncan's multiple range test (MRT): ***, $P < 0.001$.

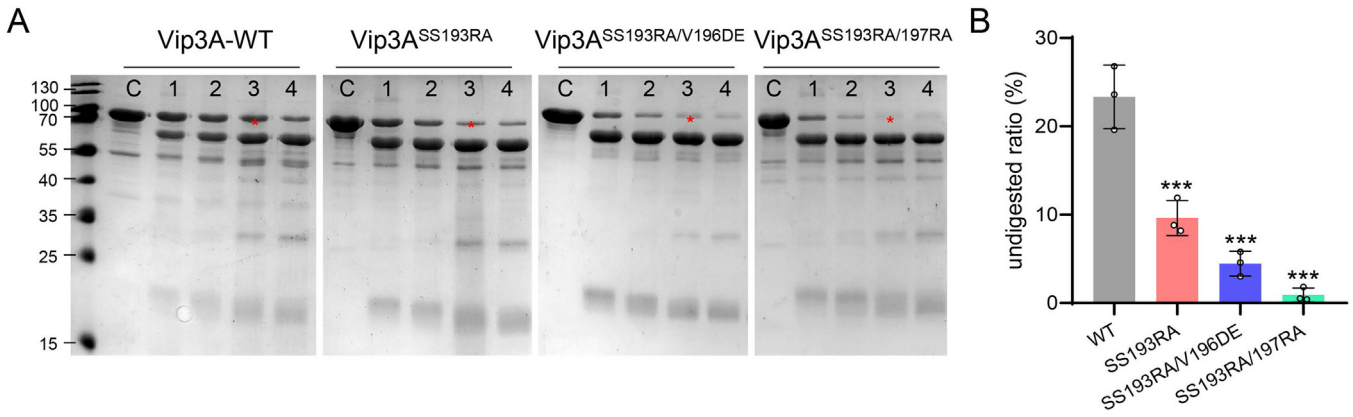


Fig. 4. Increasing the proteolysis efficiency of Vip3Aa enhances its insecticidal potency.
(A) SDS-PAGE analysis of the Vip3Aa and the indicated Vip3Aa mutants after exposure to *H. armigera* MJ. The proteins were treated with MJ at a ratio of 1: 16 (MJ:Vip3, wt:wt). The reaction was halted by the addition of the protease inhibitor AEBSF after 1, 2, 3, or 4 h. The images represent at least three independent experiments.
(B) Histograms showing the ratio of the intensity of undigested protein (*) compared to that of the control (C) sample of each indicated protein of (A), after 3 h of MJ treatment. Data are expressed as the mean ± standard deviation (SD) from three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Duncan's multiple range test (MRT): ***, $P < 0.001$.

tion, the efficiency of Vip3Aa hydrolyzed by MJ could be significantly increased by replacing S193–S194 with R-A (Vip3A^{SS193RA}) (Fig. 3F–H). Furthermore, introducing this mutation into the Vip3Aa mutants in Fig. 3C increased the efficiency of the mutants being cleaved by MJ to varying degrees (Fig. 3F–3H).

In addition, the D, E residues of Vip3Aa could also be efficiently cleaved by MJ of *S. frugiperda*. We, therefore, constructed Vip3A$^{SS193RA/V196DE}$, adding the V196/DE (V196 replaced by DE) mutation to Vip3A^{SS193RA} (Fig. 3F). Proteolysis assays showed that the cleavage efficiency of Vip3A$^{SS193RA/V196DE}$ by MJ increased (Fig. 3G and 3H). Together, these results indicated that introducing additional cleavage sites in the loop region between DI and DII increased the proteolysis efficiency of Vip3Aa by the MJ of *S. frugiperda*.

3.4. Increasing the proteolysis efficiency of vip3Aa promotes its insecticidal potency

Having demonstrated that introducing additional cleavage sites into Vip3Aa increased its proteolysis efficiency by the MJ of *S. frugiperda*. We further examined whether this modification could also increase their cleavage efficiency by MJ from another insect larval. After treatment with MJ from *H. armigera*, proteolysis assays showed that the three representative mutations Vip3A^{SS193RA}, Vip3A$^{SS193RA/V196DE}$, and Vip3A$^{SS193RA/197RA}$ of Vip3Aa could be cleaved more efficiently than Vip3Aa (Fig. 4A, B), suggesting that this rule also applies to the MJ from other insect larvae for cleavage of the Vip3Aa mutations.

Increasing the insecticidal activity of Vip3 enhances its efficiency as a biopesticide. Therefore, we further tested whether these three Vip3Aa mutations increased the insecticidal activity of Vip3Aa. After feeding them to the second instar larvae of *S. frugiperda* and *H. armigera*, bioassays showed that the toxicity of Vip3A^{SS193RA}, Vip3A$^{SS193RA/V196DE}$, and Vip3A$^{SS193RA/197RA}$ to *S. frugiperda* and *H. armigera* were increased compared with the Vip3Aa wild-type (Table 2). Consistent with the increase in their cleavage efficiency by MJ, the insecticidal activity of Vip3A$^{SS193RA/197RA}$ increased most obviously. These results further indicated that promoting the proteolysis efficiency of Vip3Aa increased its insecticidal potency.

4. Discussion

It has been reported that in agriculture alone, pests will reduce the worldwide agricultural yield by 10%–16% before harvest and will consume a similar amount after harvest [43]. Synthetic chemical pesticides are a double-edged sword due to their indiscriminate harm. Although they control pests, they also cause profound damage to human health and the ecosystem. With the advantages of their highly efficient and specific insecticidal effects, biodegradability, and environmental safety, microbially derived, protein-based biopesticides have become important and sustainable insect pest management tools [1–5].

As an important entomopathogenic bacterium, Bt has been used worldwide due to its ability to produce insecticidal crystal proteins, known as Cry toxins, through transgenic crops, or traditional spray approaches. However, Cry toxins are also face the challenges of limited insecticidal spectrum and the evolution of pest resistance [3,4,7]. Among the 16 kinds of bacterial insecticidal proteins [19,20], the Vip3 family proteins have been theorized to be particularly useful insecticidal proteins due to their characteristics being distinct from Cry toxins, including their efficient and broad-spectrum insecticidal activity against lepidopteran pests [2,13,15,16,44]. Therefore, Vip3s represent a "new generation" of insecticidal toxins after Cry toxins, and have been utilized together with Cry proteins in commercial transgenic crops in response to the increasing pest resistance to Cry proteins [2,15]. Intensive studies have been performed to investigate the functioning mechanism of Vip3 [2,15], and some studies have tried to improve its insecticidal activity or spectrum [33–37]. However, due to Vip3's complicated structural composition and its complex biological processes inside the host insects' midgut, few general pathways have been reported to enhance the insecticidal activity of Vip3 based on its mechanism of action.

Proteolytic activation is regarded as a critical step in triggering the toxicity of Vip3 proteins [2,15]. Previous studies have shown that three lysine residues located between the amino acids 195–200 are the primary cleavage sites of the Vip3Aa protein [22]. Whether this cleavage site is the only proteolysis activation site was undetermined. In this study, we illustrated that in addition to lysine, there are several cleavage activation sites in the linker region between DI and DII of Vip3Aa, including arginine, aspartic acid, glutamic acid, and serine. These multiple cleavage activation sites of Vip3 might be evolutionary adaption of Bt in response to long-term interactions with variable protease compositions in the midgut of different insect hosts, thereby increasing the insecticidal spectrum of Bt.

Additionally, although practical field resistance to Vip3A proteins has not been reported, some studies have provided strong evidences of an early warning of field-evolved resistance to Vip3Aa in several pests [45,46]. Different proteolytic efficiencies are thought to be associated

Table 2
Mortality analysis of *Spodoptera frugiperda* larvae and *Helicoverpa armigera* larvae (second instar) as induced by Vip3Aa and the indicated mutant proteins.

	S. frugiperda (second instar)			*H. armigera* (second instar)		
Protein	LC50 (ng/cm$^{2)}$	95% Fiducial limit-Lower	95% Fiducial limit-Upper	LC50 (ng/cm^2)	95% Fiducial limit-Lower	95% Fiducial limit-Upper
Vip3Aa	56.76	52.84	60.69	270.1	246.5	294.0
Vip3Aa^{SS193RA}	47.43	43.62	51.14	193.4	173.8	213.4
Vip3Aa$^{SS193RA/V196DE}$	38.16	34.70	41.66	164.8	148.2	181.8
Vip3Aa$^{SS193RA/197RA}$	32.94	30.46	35.50	148.2	133.0	163.7

with the distinct insecticidal toxicities of Vip3 or Cry proteins [22,47-50]. The specific mechanism in the field-evolved or laboratory-induced resistance to Vip3Aa has not been reported; however, similar to the evolution of resistance to Cry proteins, lowering the proteolytic activity was proposed as a driver of pest insect resistance to Vip3Aa [7]. Here, we observed that by adding more cleavage sites near the preliminary digestion sites of Vip3Aa increased its proteolytic efficiency by MJ, and thus enhanced its insecticidal activity. This finding provides a novel strategy to manage biopesticide resistance in insect pests due to reduced protease activity in the midgut. Furthermore, increasing the proteolytic efficiency is a practical method to increase the insecticidal activity of Vip3. However, the reasonable use of this strategy in practical applications requires further research.

Furthermore, the cryo-ET data in previous reports showed that although Vip3 was almost completely cleaved by trypsin, a subset of the activated protein conformation could be classified in the cryo-EM structure of the protoxin. In addition, nearly 30% of Vip3Aa16 and 16% of Vip3Bc1 activated protein still maintained the same configuration corresponding to the uncleaved protoxin [31,32], suggesting that cleavage of the protease may be necessary for Vip3 activation but there are other factors involved in its activation. Consistent with the previous cryo-electron microscopy findings, we found that the cleavage efficiency of the Vip3Aa mutants by MJ was substantially increased by adding more efficient cleavage sites. However, the insecticidal activity of these mutants did not increase proportionally, further suggesting that there are other important factors contributing to the insecticidal activity of Vip3A besides the cleavage of the protease. The identification of other factors involved in the physiological processes between protease cleavage, allosteric activation, and insecticidal activity of Vip3A in the insect gut may also play vital roles in improving its insecticidal activity, which requires further investigation.

5. Conclusion

In conclusion, we investigated the activation process of Vip3Aa and found that there were multiple protease activation sites between DI and DII. We demonstrated that increasing the proteolysis efficiency by introducing extra cleavage sites between DI and DII was a viable strategy to enhance the insecticidal activity of Vip3Aa. Because all members of the Vip3 family are highly conserved and have similar mechanisms of action, these results, and unique insights can likely be applied to other members of this protein family. This study facilitates the understanding of the insecticidal mechanism of Vip3 proteins and promotes their future application.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Patent application on improving the insecticidal activity of Vip3A by increasing the digestion efficiency on its loop region between domain I and domain II has been filed by Shandong University.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.engmic.2023.100083.

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