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Biological activities of the vegetative insecticidal protein Vip3Aa against beet armyworm (*Spodoptera exigua*)

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ABSTRACT: Vegetative insecticidal proteins (Vips) are produced by different bacterial species including *Bacillus cereus* and *Bacillus thuringiensis* during vegetative stage. They have broad spectrum toxicity against various pests including Lepidopteran. This study aimed to investigate the biological activity of Vip3Aa protein against beet armyworm (*Spodoptera exigua*). The 88.5-kDa recombinant Vip3Aa protein was produced in *E. coli* as a soluble protoxin. The purified Vip3Aa protoxin was processed by either trypsin or midgut proteases to yield a 62-kDa active toxin. At day 7 post feeding to the *S. exigua* 2nd instar lavae, the LC_{50} values of the Vip3Aa protoxin, the trypsin-activated Vip3Aa toxin, and the midgut proteases-activated Vip3Aa toxin were 556, 277, and 43 ng/cm^2, respectively. The midgut epithelial cells of the *S. exigua* 3rd instar larvae fed with Vip3Aa protoxin for 1 and 2 days were observed under scanning electron microscope (SEM). The epithelial cells were found swollen, misshaped, and lysed.

KEYWORDS: *Bacillus thuringiensis*, larvicidal activity, Vip3Aa, *Spodoptera exigua*, Beet armyworm

INTRODUCTION

Bacillus thuringiensis (*Bt*) is a Gram-positive soil bacterium that can produce a wide range of toxic proteins called Bt toxins. These toxins are toxic to the larvae of various insect orders. The main group of Bt toxins is δ-endotoxin known as Crystal (Cry) and Cytolytic (Cyt) toxins that are produced during sporulation stage. The δ-endotoxin is the first generation of insecticides from the *Bt*. It has been used as biopesticides in commercial transgenic insect-resistant crops for the development of green agriculture [1]. However, there have been some problems relating to the use of δ-endotoxin to date, such as insect resistance and narrow host range [2, 3]. The second generation Bt toxins, i.e., vegetative insecticidal proteins (Vips), were classified into four classes: Vip1, Vip2 (binary toxic proteins), Vip3 (single-chain toxic proteins), and Vip4 (recently identified) [4]. Most of the Bt toxins are produced as protoxin and become toxic after proteolysis. Vips are produced as protoxins during vegetative stage and secreted outside the cell. The Vip1 and the Vip2 protoxins showed toxicity against the Coleopteran pest, *Sitophilus zeamais* [5]. Among the Vip protoxins, the Vip3 has an insecticidal activity against a wide variety of Lepidopteran species. The molecular weight of Vip3Aa protoxin is approximately 88.5 kDa [6]. When the Vip3Aa is processed, an active protein of approximately 62 kDa will be obtained [7]. The N-terminal half of Vip3Aa is highly conserved, while the C-terminal region is highly variable [8, 9]. The C-terminus of Vip3Aa has been proposed for being related to target specificity [9]. The Bt toxins in the epithelial cell of insect midgut can kill the insect [10]. The first generation Bt toxins (Cry and Cyt) could form pores in the insect midgut leading to cell disruption [11]; but the mode of action of Vip3 is still unclear. The toxicities of Vip3Aa16 [12] and Vip3Aa58 [13] were reported against *Spodoptera frugiperda*, *S. exigua*, and *S. litura* [14] with the LC_{50} values in the range of 35–290 ng/cm^2.

In this study, *vip3Aa35* gene of *Bacillus thuringiensis* was cloned and expressed in the *E. coli* BL21 (DE3) pLysS and then purified. Insecticidal activities of the Vip3Aa35 protein were determined in beet armyworm (*S. exigua*). The midgut pathology of the *S. exigua* treated with the Vip3Aa35 protein was also revealed under SEM.

MATERIALS AND METHODS

Bacterial strain and plasmid

The *E. coli* BL21(DE3) pLysS was used throughout the experiment. The recombinant plasmid pET28b-*vip3Aa* containing the full-length *vip3A35* gene was obtained as described previously [15]. This plasmid encodes the Vip3Aa with 6xHistidine tagged at its N-terminus (6xHis-Vip3Aa).

Protein preparation

The 1–3% of *E. coli* BL21(DE3) pLysS harboring recombinant plasmid pET28b-*vip3Aa* culture was transferred into a 200 ml new LB broth medium containing 34 μg/ml chloramphenicol and 50 μg/ml kanamycin. The cell cultures were grown at 37 °C until the OD_{600} of the culture reached 0.4–0.6. The isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the final

concentration of 0.4 mM, and the culture was incubated at 25 °C for 16–18 h. The cells were harvested, re-suspended in 1X PBS buffer pH 7.4, and then lysed by ultra-sonication. The solution was centrifuged at 12 000 *g* for 10 min at 4 °C. The recombinant soluble proteins in the supernatant were harvested and purified by affinity chromatography using Ni^{2+} bead (Thermo Fisher Scientific, MA, USA). The column was equilibrated with 25 mM imidazole in PBS buffer. The protein sample (supernatant) was loaded into the column, and 25 mM imidazole in PBS buffer was used as the wash buffer. The Vip3Aa protein was eluted by PBS pH 7.4 containing 100–250 mM imidazole. All fractions: the supernatant, the flow through, the wash, and the eluted were analyzed by 12% SDS-PAGE and visualized by InstantBlue™ (Expedeon, CA, USA). The purified Vip3Aa protein was concentrated, and the imidazole was removed using PD10 desalting column. The Vip3Aa protein band was excised from the gel and identified by mass spectrometry (LC/MS) at the Proteomics Service, Faculty of Medical Technology, Mahidol University. The purified Vip3Aa protein was stored at 4 °C. To activate the protoxin, the purified Vip3Aa protein was treated with either 1% (w/w) trypsin or 1% (w/w) *S. exigua*'s midgut proteases at 37 °C for 2 h. The 1 mM PMSF was added to inhibit the activity of trypsin. The 1X protease inhibitor cocktail (consisting of AEBSF, aprotinin, bestatin, E-64, leupeptin, and pepstatin A) was added to inhibit the activity of the midgut proteases.

Biological activity assays

The 2nd instar larvae of *S. exigua* were fed with artificial insect diet overlaid with eight concentrations of Vip3Aa protoxin (50, 100, 250, 500, 1000, 1500, 2000, and 2500 ng/cm^2) and five concentrations each of trypsin-activated Vip3Aa toxin and midgut proteases-activated Vip3Aa toxin (125, 250, 500, 1000, and 2000 ng/cm^2). The 1X PBS buffer pH 7.4 was used as a negative control. Thirty larvae were used for each toxin concentration. All larvae were kept at room temperature and mortality was recorded on day 5 and day 7 post feedings. The LC_{50} and the LC_{95} values were calculated by the Probit analysis program and the IBM SPSS Statistics 20 (IBM Analytics, USA). The experiments were done in three replicates.

Histopathological assay of *S. exigua* midgut by SEM

The 3rd instar larvae of *S. exigua* were used in this experiment. The larvae were fed with artificial diet overlaid with Vip3Aa protoxin at 2 μg/cm^2. The 1X PBS buffer pH 7.4 was used as a negative control. At 24 h and 48 h post feeding, the larvae were removed, placed in 70% ethanol, and washed twice in distilled water. Larval midguts were pulled out and fixed in a cold 3% glutaraldehyde in 1X PBS buffer pH 7.4

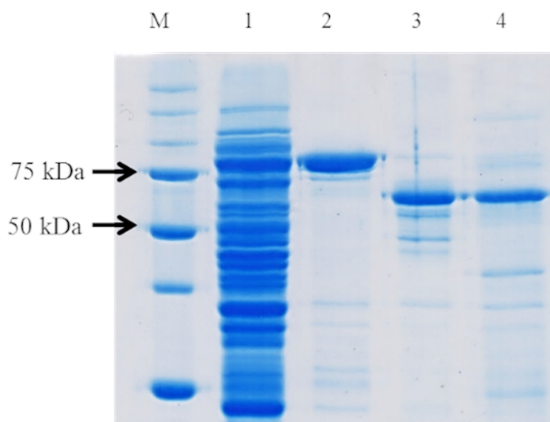

Fig. 1 Recombinant Vip3Aa expression as protein marker, lane M; soluble protoxins, lane 1; purified Vip3Aa protoxin, lane 2; 62 kDa trypsin-activated Vip3Aa toxin, lane 3; and 62 kDa *S. exigua*'s midgut proteases-activated Vip3Aa toxin, lane 4.

for 1 h at 4 °C. Midguts were opened by scalpel and immediately fixed in a cold 3% glutaraldehyde for 3 h at 4 °C. After fixation, the samples were rinsed 4 times with a cold 1X PBS buffer pH 7.4 at 4 °C, 15 min each. The fixed samples were dehydrated by rinsing in a series of ethanol concentrations (50%, 60% 70%, 80%, 90%, and 100%) for 15 min each. They were kept in the 100% ethanol until critical point dried. Then, the samples were mounted in stubs, coated with gold-palladium following the standard technique, and observed under a FEI Quanta 450 SME (Thermo Fisher Scientific, MA, USA).

RESULTS

Protein expression, purification, and activation

The Vip3Aa protoxin was produced in *E. coli* BL21(DE3) pLysS as a soluble protein with a molecular weight of 88.5 kDa (Fig. 1, lane 1). The protoxin was successfully purified by affinity chromatography using Ni^{2+} bead with 100–250 mM imidazole elution (Fig. 1, lane 2). The purified 88.5 kDa Vip3Aa protoxin was activated by either trypsin or *S. exigua*'s midgut proteases to obtain a major band at 62 kDa (Fig. 1, lanes 3,4). The purified protein was confirmed to be Vip3Aa by LC/MS spectroscopy (data not shown).

Biological activity assays

The mortality of *S. exigua* larvae was recorded at day 5 and day 7 post feeding with Vip3Aa protoxin and activated Vip3Aa toxins. The mortality percentage was increased at higher concentrations of the Vip3Aa protoxin. With similar concentrations of Vip3Aa protoxin, the percentage of mortality on day 7 post feeding was higher than the day 5 post feeding (Fig. 2A). In addition, the percentage of mortality of the midgut

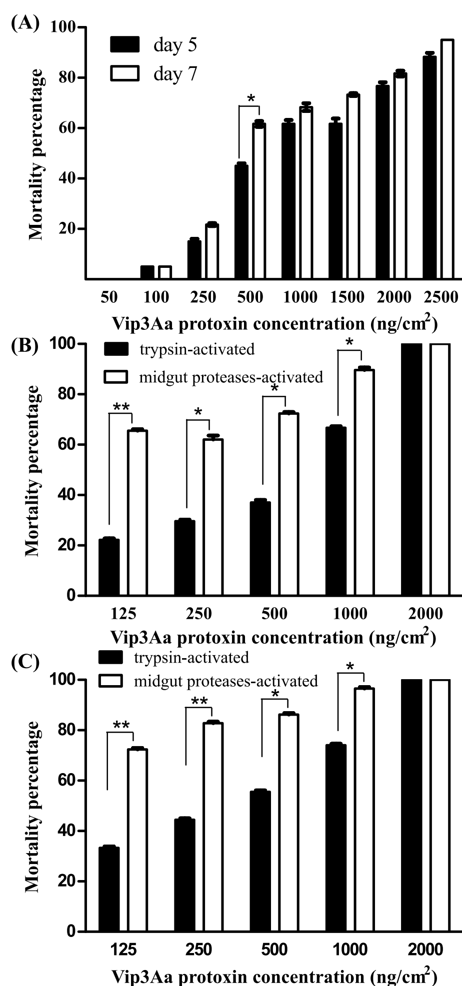

Fig. 2 Mortality percentages of *S. exigua* after treated with Vip3Aa: (A), Vip3Aa protoxin treatment at day 5 and day 7; (B), trypsin- and midgut proteases-activated Vip3Aa toxins at day 5; and (C), trypsin- and midgut proteases-activated Vip3Aa toxins at day 7 (*p-value $\leqslant 0.05$, **p-value $\leqslant 0.001$).

Table 1 LC_{50} and LC_{95} of the Vip3Aa protoxin, the trypsin-activated Vip3Aa toxin, and the midgut proteases-activated Vip3Aa toxin.

		$LC_{50} \pm$ SEM (ng/cm^2)	$LC_{95} \pm$ SEM (ng/cm^2)
Vip3A protoxin	5 days	763 ± 0.16	5434 ± 0.16
	7 days	556 ± 0.16	3686 ± 0.16
Trypsin-activated Vip3Aa toxin	5 days	436 ± 1.67	4239 ± 1.67
	7 days	277 ± 1.45	3768 ± 1.45
Midgut proteases-activated Vip3Aa toxin	5 days	92.64 ± 1.24	1948.41 ± 1.24
	7 days	43.37 ± 1.26	870.80 ± 1.26

proteases-activated Vip3Aa toxin is higher than the trypsin-activated Vip3Aa toxin for both the day 5 and the day 7 post feedings (Fig. 2B,C). The LC_{50} and the LC_{95} values of the Vip3Aa protoxin, the trypsin-activated Vip3Aa toxin, and the midgut proteases-activated Vip3Aa toxin against the *S. exigua* 2nd instar larvae were determined as shown in Table 1 with 95% confidence limits. The activated toxins were more toxic than the protoxin. In addition, the midgut proteases-activated Vip3Aa toxin was more toxic than the trypsin-activated Vip3Aa toxin. No mortality was observed for the larvae fed with the diet overlaid with the negative control PBS buffer. These results clearly demonstrated that the recombinant Vip3Aa produced in *E. coli* was active against *S. exigua*.

Histopathological assay of *S. exigua* midgut by SEM

The midguts of the *S. exigua* 3rd instar larvae fed with the Vip3Aa protoxin for 24 and 48 h were visualized under SEM. Results showed that the epithelial cells of the midgut larvae treated with Vip3Aa protoxin had changed. While the epithelial cells of the control larvae showed densely packed microvilli on the surface (Fig. 3A-C), the epithelial cells of the larvae treated with Vip3Aa toxin for 24 h had lost some of the surface microvilli (Fig. 3D-F) and lost all of them after receiving Vip3Aa toxin for 48 h (Fig. 3G-I). Moreover, the epithelial cells of the larvae fed with Vip3Aa toxin were swollen, misshaped, and lysed.

DISCUSSION

The larvicidal activities of Vip3Aa protoxin and activated toxins were examined in the beet armyworm, *S. exigua*, and their LC_{50} and LC_{95} are summarized in Table 1. The results showed that the toxicity of the Vip3Aa35 against the insect larvae used in this experiment was similar to the other Vip3Aa toxins, Vip3Aa16 and Vip3Aa58 [12–14]. The amino acid sequence identity and similarity of the Vip3Aa35 compared with the Vip3Aa16 [12] and the Vip3Aa58 [13, 14] were 98.8% and 99.1%, respectively. The differences of amino acids among these three Vip3Aa's were found in 10 residues (Fig. 4). These variations might affect their toxicity. There was a report showing that when tested against neonate larvae of *S. frugiperda*, the LC_{50} of the crude extract Vip3Aa protoxin and the trypsin-activated Vip3Aa toxin were 620 and 150 ng/cm^2, respectively [16]. The results were similar to ours even though different insect species were used. The toxicity of Vip3Aa against the *S. frugiperda* was slightly different from the *S. exigua* in this study. This might be due to the susceptibility variation in different insect species. The toxicities of the crude lysate and the trypsin-activated Vip3Aa16 to the 1st instar larvae of *S. frugiperda* and *S. exigua* were previously investigated [12]. The LC_{50} values of crude Vip3Aa against *S. frugiperda* and *S. exigua* were 340 and 2600 ng/cm^2,

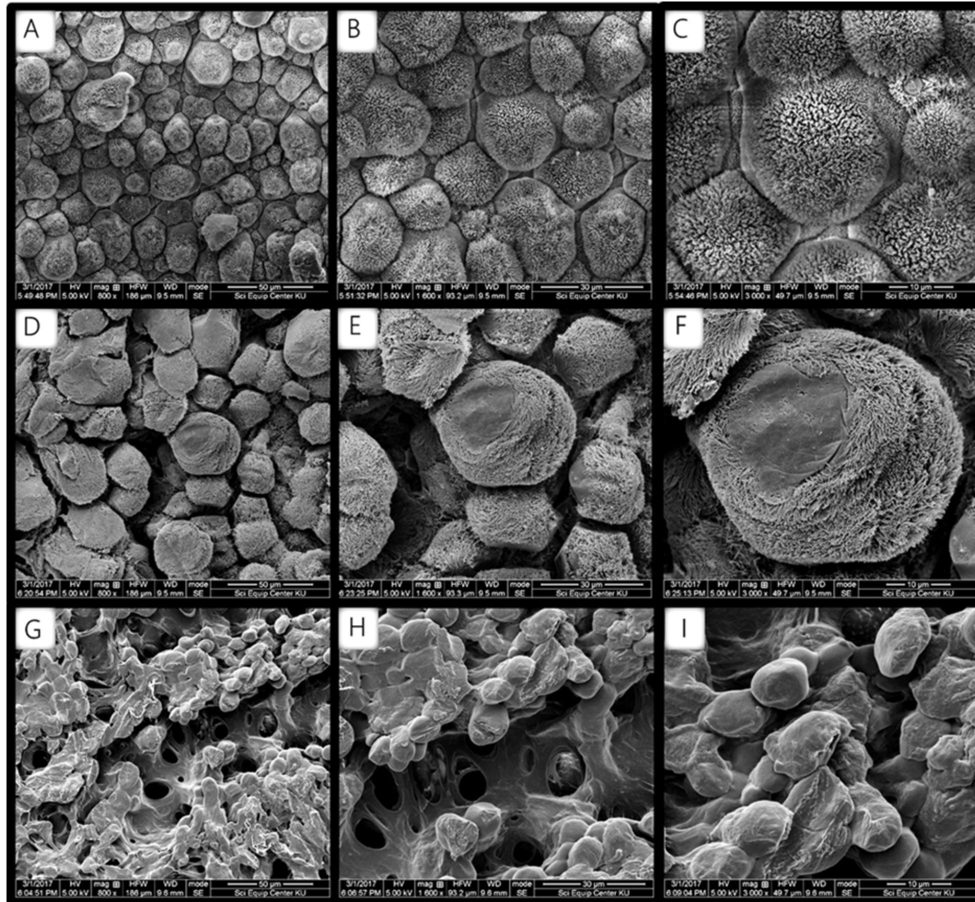

Fig. 3 Scanning electron micrographs of midgut epithelial cells of *S. exigua* 3rd instar larvae: (A, B, C), fed with PBS buffer at 800X, 1600X, and 3000X, respectively; (D, E, F), fed with 2 µg Vip3Aa protoxin for 1 day at 800X, 1600X, and 3000X, respectively; and (G, H, I), fed with 2 µg Vip3Aa toxin for 2 days at 800X, 1600X, and 3000X, respectively.

respectively, whereas the LC_{50} of trypsin-activated Vip3Aa against *S. frugiperda* and *S. exigua* were 41 and 35 ng/cm^2, respectively. These results suggested that the gut proteases in *S. exigua* were not as effective as those in *S. frugiperda* in activating the Vip3Aa protoxin. However, the comparable LC_{50} values of the activated toxin against both insect species indicated that both insects were susceptible to Vip3Aa. In our experiments, LC_{50} values at day 7 post feeding of the purified Vip3Aa35 protoxin, the trypsin-activated Vip3Aa toxin, and the midgut proteases-activated Vip3Aa toxin were 556, 277, and 43 ng/cm^2, respectively. The LC_{50} ratio of Vip3Aa protoxin over the trypsin-activated Vip3Aa toxin was 1.4 indicating that the toxicity of these two types of toxins were not much different. These results corresponded to a study on proteolytic activation of Vip3Aa protoxin showing that the activated toxin and the protoxin did not make a big difference in the insect toxicity [12, 17]. However, the toxicity of the midgut proteases-activated Vip3Aa was much higher than the protoxin and the trypsin-activated toxin. The midgut juice composes of many types of proteases that have different properties and different digestion sites in comparison to trypsin. The activation by midgut juice proteases gave higher toxicity than that of the trypsin indicating that different activated products were produced. The larvicidal activity of Vip3Aa was varied depending on the growth stage of insects. In 2016, Song et al [14] studied on neonate and 1st to 3rd instar larvae of *S. litura* fed with Vip3Aa protoxin. At day 3 post feeding, the LC_{50} values of Vip3Aa protoxin for the neonatal larvae, the 1st instar larvae, the 2nd instar larvae, and the 3rd instar larvae were 2.609, 28.778, 70.460, and 200.627 ng/cm^2, respectively. The results showed that the younger larvae were more sensitive to the toxin than the older ones.

The results of the midgut epithelial cells of *S. exigua* 3rd instar larvae observed under SEM showed that the Vip3Aa protein affected the epithelial cells by causing cell swelling and lysis. The results were similar to those observed in other insect species treated with many *B. thuringiensis* toxins. In another study [18],

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Vip3Aa35   MNKNNTKLSTRALPSFIDYFNGIYGFATGIKDIMNMIFKTDTGGDLTLDEILKNQQLLND   60
Vip3Aa58   MNKNNTKLSTRALPSFIDYFNGIYGFATGIKDIMNMIFKTDTGGDLTLDEILKNQQLLND   60
Vip3Aa16   MNKNNTKLSTRALPSFIDYFNGIYGFATGIKDIMNMIFKTDTGGDLTLDEILKNQQLLND   60
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Vip3Aa35   ISGKLDGVNGSLNDLIAQGNLNTELSKEILKIANEQNQVLNDVNNKLDAINTMLRVYLPK   120
Vip3Aa58   ISGKLDGVNGSLNDLIAQGNLNTELSKEILKIANEQNQVLNDVNNKLDAINTMLRVYLPK   120
Vip3Aa16   ISGKLDGVNGSLNDLIAQGNLNTELSKEILKIANEQNQVLNDVNNKLDAINTMLRVYLPK   120
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Vip3Aa35   ITSMLSDVMKQNYALSLQIEYLSKQLQEISDKLDIINVNVLINSTLTEITPAYQRIKYVN   180
Vip3Aa58   ITSMLSDVMKQNYALSLQIEYLSKQLQEISDKLDIINVNVLINSTLTEITPAYQRIKYVN   180
Vip3Aa16   LTSMLSDVMKQNYALSLQIEYLSKQLQEISDKLDIINVNVLINSTLTEITPAYQRIKYVN   180
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Vip3Aa35   EKFEELTFATETSSKVKKDGSPADILDELTELTELAKSVTKNDVDGFEFYLNTFHDVMVG   240
Vip3Aa58   EKFEELTFATETSSKVKKDGSPADILDELTELTELAKSVTKNDVDGFEFYLNTFHDVMVG   240
Vip3Aa16   EKFEELTFATETSSKVKKDGSPADILDELTELTELAKSVTKNDVDGFEFYLNTFHDVMVG   240
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Vip3Aa35   NNLFGRSALKTASELITKENVKTSGSEVGNVYNFLIVLTALQAKAFLTLTTCRKLLGLAD   300
Vip3Aa58   NNLFGRSALKTASELITKENVKTSGSEVGNVYNFLIVLTALQAKAFLTLTTCRKLLGLAD   300
Vip3Aa16   NNLFGRSALKTASELITKENVKTSGSEVGNVYNFLIVLTALQAKAFLTLTTCRKLLGLAD   300
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Vip3Aa35   IDYTSIMNEHLNKEKEEFRVNILPTLSNTFSNPNYAKVKGSDEDAKMIVEAKPGHALVGF   360
Vip3Aa58   IDYTSIMNEHLNKEKEEFRVNILPTLSNTFSNPNYAKVKGSDEDAKMIVEAKPGHALIGF   360
Vip3Aa16   IDYTSIMNEHLNKEKEEFRVNILPTLSNTFSNPNYAKVKGSDEDAKMIVEAKPGHALIGF   360
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Vip3Aa35   EISNDSITVLKVYEAKLKQNYQVDKDSLSEVIYGDMDKLLCPDQSEQIYYTNNIVFPNEY   420
Vip3Aa58   EISNDSITVLKVYEAKLKQNYQVDKDSLSEVIYGDMDKLLCPDQSEQIYYTNNIVFPNEY   420
Vip3Aa16   EISNDSITVLKVYEAKLKQNYQVDKDSLSEVIYGDMDKLLCPDQSEQIYYTNNIVFPNEY   420
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Vip3Aa35   VITKIDFTKKMKTLRYEVTANFYDSSTGEIDLNKKKVESSEAEYRTLSANDDGVYMPLGV   480
Vip3Aa58   VITKIDFTKKMKTLRYEVTANFYDSSTGEIDLNKKKVESSEAEYRTLSANDDGVYMPLGV   480
Vip3Aa16   VITKIDFTKKMKTLRYEVTANFYDSSTGEIDLNKKKVESSEAEYRTLSANDDGVYMPLGV   480
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Vip3Aa35   ISETFLTPINGFGLQADENSRLITLTCKSYLRELLLATDLSNKETKLIVPPSGFIKNIVE   540
Vip3Aa58   ISETFLTPINGFGLQADENSRLITLTCKSYLRELLLATDLSNKETKLIVPPSGFISNIVE   540
Vip3Aa16   ISETFLTPINGFGLQADENSRLITLTCKSYLRELLLATDLSNKETKLIVPPSGFISNIVE   540
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Vip3Aa35   NGSIEEDNLEPWKANNKNAYVDHTGGVNGTKALYVHKDGGISQFIGDKLKPKTEYVIQYT   600
Vip3Aa58   NGSIEEDNLEPWKANNKNAYVDHTGGVNGTKALYVHKDGGISQFIGDKLKPKTEYVIQYT   600
Vip3Aa16   NGSIEEDNLEPWKANNKNAYVDHTGGVNGTKALYVHKDGGISQFIGDKLKPKTEYVIQYT   600
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Vip3Aa35   VKGKPSIHLKDENTGYIHYEDTNNNLEDYQTITKRFTTGTDLKGVYLILKSQNGDEAWGD   660
Vip3Aa58   VKGKPSIHLKDENTGYIHYEDTNNNLEDYQTINKRFTTGTDLKGVYLILKSQNGDEAWGD   660
Vip3Aa16   VKGKPSIHLKDENTGYIHYEDTNNNLEDYQTINKRFTTGTDLKGVYLILKSQNGDEAWGD   660
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Vip3Aa35   NFIILEISPSEKLLSPELINTNNWTSTGSTNISGNTLTLYQGGRGILKQNLQLDSFSTYR   720
Vip3Aa58   NFIILEISPSEKLLSPELINTNNWTSTGSTNISGNTLTLYQGGRGILKQNLQLDSFSTYR   720
Vip3Aa16   NFIILEISPSEKLLSPELINTNNWTSTGSTNISGNTLTLYQGGRGILKQNLQLDSFSTYR   720
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Vip3Aa35   VYFSVSGDANVRIRNSREVLFEKRYMSGAKDVSEIFTTKLGKDNFYIELSQGNNLNGGPI   780
Vip3Aa58   VYFSVSGDANVRIRNSREVLFEKRYMSGAKDVSEMFTTKFEKDNFYIELSQGNNLYGGPI   780
Vip3Aa16   VYFSVSGDANVRIRNSREVLFEKRYMSGAKDVSEMFTTKFEKDNFYIELSQGNNLYGGPI   780
           **********************************:****: ************** ****

Vip3Aa35   VKFSDVSIK 789
Vip3Aa58   VHFYDVSIK 789
Vip3Aa16   VHFYDVSIK 789
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Fig. 4 Amino acid sequence alignments of Vip3Aa35 (this study), Vip3Aa16 [12], and Vip3Aa58 [13, 14]. The red box showed the residues that were different.

the midgut of the *Heliothis virescens* 4th instar larvae treated with Cry1Ac toxin was severely damaged, and many activities were clearly observed such as cell swelling, reduction of microvilli, and cell ruptures. The histopathological effects of Vip3Aa toxin against the *S. litura* 3rd instar larvae was investigated [14], and the examination of the larvae's midgut cross-sections showed a wide damage of the midgut epithelial cells. The histopathological changes included vacuolization of the cytoplasm, cellular swelling, and brush border membrane destruction. Many reports demonstrated that Vip3Aa protein caused extensive damages to the midgut epithelial cells: cell swelling, leaking of cellular materials out to the gut lumen, and eventual cell lysis [19–23]. *In vitro* cytotoxicity assay against Sf9 cell line revealed that, upon internalization, Vip3Aa caused cell division disruption and could induce cell death via apoptosis [24]. Our *in vivo* results showed that Vip3Aa could induce cell swelling, misshaping, and lysis. Taken together, we conclude that the ingested Vip3Aa protein is processed by the larval gut proteases. The processed protein then binds to a specific receptor

on the epithelial cell membrane and internalizes into the cytosol. The Vip3Aa protein could disturb midgut epithelial cells leading to the larvae's death.

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