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 LC50 values in the range of 35–290 ng/cm².

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 vip3Aa35 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 OD600 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 Ni²⁺ 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/cm²) and five concentrations each of trypsin-activated Vip3Aa toxin and midgut proteases-activated Vip3Aa toxin (125, 250, 500, 1000, and 2000 ng/cm²). 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 LC₅₀ and the LC₉₅ 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/cm². 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 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 Ni²⁺ 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...
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 ⩽ 0.05, **p*-value ⩽ 0.001).

<table>
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<tr>
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<th>LC$_{50}$ ± SEM (ng/cm$^2$)</th>
<th>LC$_{95}$ ± SEM (ng/cm$^2$)</th>
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<tr>
<td>Vip3A protoxin</td>
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<tr>
<td>5 days</td>
<td>763 ± 0.16</td>
<td>5434 ± 0.16</td>
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<td>7 days</td>
<td>556 ± 0.16</td>
<td>3686 ± 0.16</td>
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<tr>
<td>Trypsin-activated Vip3Aa</td>
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<tr>
<td>5 days</td>
<td>436 ± 1.67</td>
<td>4239 ± 1.67</td>
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<tr>
<td>7 days</td>
<td>277 ± 1.45</td>
<td>3768 ± 1.45</td>
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<tr>
<td>Midgut proteases-activated Vip3Aa toxin</td>
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<tr>
<td>5 days</td>
<td>92.64 ± 1.24</td>
<td>1948.41 ± 1.24</td>
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<tr>
<td>7 days</td>
<td>43.37 ± 1.26</td>
<td>870.80 ± 1.26</td>
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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$^2$. 

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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],
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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REFERENCES