# รายงานวิจัยฉบับสมบูรณ์

# การหาโปรตีนตัวรับสำหรับโปรตีนฆ่าแมลง (Vip3A) ที่ผลิตในช่วงก่อนการสร้างสปอร์ของ *Bacillus thuringiensis* จากหนอนกระทู้หอม *Spodoptera exigua*

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย และ
ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ
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**Abstract**

Vegetative insecticidal proteins (Vip3A) produced by *Bacillus thuringiensis* (Bt) is highly toxic to some Lepidopteran insect larvae that are serious agricultural pests. We have identified and isolated a new Vip3A toxin from local strain of *B. thuringiensis*. The toxin is highly produced and secreted into the culture medium and shows high toxicity against *Spodoptera exigua* and *Spodoptera litura* that are important insect pests in Thailand. In this project we have tried to identify Vip3Aa receptors from susceptible larvae *S. exigua*, a major pest for several vegetable and fruit crops in Thailand. The *vip3Aa* gene was cloned in *E. coli* to be expressed as a 6xHis-tag fusion protein. This protein was used in ligand blot to fishing out its receptor from brush broader membrane extracted from midguts of *S. exigua* larvae. An interesting spot was identified from 2-dimensional gel electrophoresis couple with immunoblot detection. Molecular weight of this spot is about 170 kDa with pI approximately 7. Unfortunately, the identity of this spot has not been resolved since it could not be distinguished from other proteins in the surrounding area. The putative receptor binding motif in Vip3A has been predicted from its amino acid sequence. This region is located at the C-terminal part consisting of amino acid around positions 530-690. This region should adopt 3D structure similar to a carbohydrate binding module (CBM) of other protein that can bind to a sugar residue of a glycoprotein that should acts as a Vip3A receptor. Amino acid substitutions in this region such as W552L, D616A and W684L affected the toxin activity suggested that these changes may interfere their binding to a receptor on the gut cell membrane. Feeding the *S. litura* larvae with a mixture of the full-length Vip3A and its C-terminal fragment resulted in a reduction of larvicidal activity indicated that the C-terminal part containing a CBM completes for the receptor binding thus rendering the toxin activity. Different Vip3A proteins such as Vip3Aa, Vip3Ad and Vip3Af show a highly conserved amino acid sequences at the N-terminal part but highly variable in the C-terminal part thus these toxins exhibit different specificity to different insects. These data indicated that the receptor binding motif of Vip3A is located in the C-terminal region. In addition, the Tyr-776 play a crucial role in maintaining the toxin stability upon long term storage.

**Key words:** *Bacillus thuringiensis*, insecticidal protein, receptor, *Spodoptera exigua*, *Spodoptera litura*, Vip3A

## บทคัดย่อ

โปรตีน Vip3A ที่ผลิตจากแบคทีเรียบีที (*Bacillus thuringiensis*) สามารถออกฤทธิ์ต่อหนอนแมลงศัตรูพืชจำพวกหนอนผีเสื้อได้ดี เราได้ทำการค้นพบโปรตีน Vip3A จากแบคทีเรียบีทีสายพันธุ์ท้องถิ่นที่แยกได้ในประเทศไทย โดยพบว่าโปรตีนตัวนี้สร้างและหลั่งออกมาปริมาณมากละลายอยู่ในอาหารเลี้ยงเชื้อและสามารถออกฤทธิ์ได้ดีต่อหนอนกระทู้หอมและหนอนกระทู้ผักซึ่งเป็นแมลงศัตรูพืชที่สำคัญของประเทศไทย เราได้ทำการโคลนยีนเพื่อสร้างโปรตีนนี้ใน *E. coli* โดยสามารถผลิตและแยกบริสุทธิ์ได้ปริมาณสูงในรูปที่ต่อเชื่อมกับ 6x-His tag เมื่อทำการวิเคราะห์หาโปรตีนตัวรับจำเพาะของ Vip3A จากเยื่อหุ้มเซลล์ของหนอนกระทู้หอมโดยใช้เทคนิค ligand blot จากโปรตีนของเยื่อหุ้มเซลล์ทางเดินอาหารของหนอนกระทู้หอมโดยใช้เทคนิค 2D-gel พบสัญญาณการจับของ Vip3A กับโปรตีนที่มีขนาดประมาณ 170 kDa และมีค่า pI ประมาณ 7 แต่ยังไม่สามารถระบุชนิดของโปรตีนนี้ได้เนื่องจากยังไม่สามารถแยกจุดของโปรตีนนี้ออกจากโปรตีนอื่นที่อยู่บริเวณเดียวกันและมีจำนวนมากได้ จากการวิเคราะห์ลำดับกรดอะมิโนพบว่าโปรตีน Vip3A น่าจะใช้กรดอะมิโนประมาณตำแหน่งที่ 530-690 ที่อยู่ส่วนปลาย C-terminal จับกับตัวรับเนื่องจากส่วนนี้มีลักษณะคล้ายกับ carbohydrate binding module (CBM) ของโปรตีนอื่นที่ทราบโครงสร้างและหน้าที่แล้ว เราจึงได้ทำการแทนที่กรดอะมิโนในบริเวณนี้ และพบว่าโปรตีนกลาย W552L, D616A และ W684L ไม่สามารถฆ่าหนอนได้ ซึ่งอาจเกิดจากการที่โปรตีนกลายไม่สามารถเข้าจับกับตัวรับจำเพาะบนเยื่อหุ้มเซลล์ของหนอน เมื่อให้หนอนกระทู้ผักกินโปรตีนที่เป็นส่วนผสมระหว่าง full-length Vip3A และ ชิ้นส่วน CBM ของ Vip3A ที่มีขนาด 22 kDa พบว่าชิ้นส่วนนี้สามารถยับยั้งการออกฤทธิ์ของ Vip3A ซึ่งชี้ให้เห็นว่าชิ้นส่วน CBM สามารถเข้าแย่งจับกับตัวรับจำเพาะทำให้ Vip3A เข้าจับได้น้อยลง เมื่อทำการสำรวจ Vip3A จากบีทีสายพันธุ์ท้องถิ่นอื่นๆที่คัดแยกได้ในประเทศไทย พบว่ามีลำดับกรดอะมิโนด้านปลาย N-terminal ที่คล้ายกันมาก แต่มีกรดอะมิโนด้าน C-terminal แตกต่างกัน และมีความสามารถในการฆ่าหนอนแมลงแตกต่างกัน เช่น Vip3Aa, Vip3Ad และ Vip3Af ดังนั้นจึงเป็นไปได้ว่ากรดอะมิโนที่อยู่ด้าน C-terminal มีบทบาทสำคัญต่อการออกฤทธิ์ที่จำเพาะต่อแมลงแต่ละชนิด นอกจากนี้เรายังพบว่ากรดอะมิโน Tyrosine ที่ตำแหน่ง 776 มีความสำคัญต่อการรักษาโครงสร้างและการออกฤทธิ์ของโปรตีน Vip3A โดยเฉพาะเมื่อผ่านการเก็บรักษาเป็นเวลานาน



**คำสำคัญ** แบคทีเรียบีที โปรตีนฆ่าหนอนแมลง ตัวรับจำเพาะ หนอนกระทู้หอม หนอนกระทู้ผัก โปรตีน Vip3A

## Executive Summary

Vegetative insecticidal proteins (Vip) are a group of proteins recently discovered as secreted proteins from some strains of *Bacillus thuringiensis* (Bt). The protein is produced during vegetative phase of growth and secreted to the culture medium. Different Bt strains produce different Vip proteins and toxic to different target insects. Vip proteins identified so far could be divided into 4 major groups, Vip1, Vip2, Vip3 and Vip4. The most abundance of all is Vip3 which is toxic to a wide range of Lepidopteran larvae, major insect pests that cause serious damage to most economic crops in Thailand. The mechanism of action of Vip3 is not understood but the susceptible larvae stop feeding within a short time after treated with the toxin and eventually die a few days later. Vip3 proteins are highly toxic to insect larvae and have $LD_{50}$ comparable to that of crystal proteins (Cry toxins) from *B. thuringiensis*. In addition, Vip proteins are active against the Cry toxin-resistant insects. It is expected that proteins from both groups employ different mechanisms to kill the insects. There is a high potential to develop this toxin as an environmental friendly bio-insecticide to substitute chemical insecticides. However, there is a precaution that repeated application of this toxin could accelerate resistant development in the target insects. Since resistant development usually involves changes in the receptor on insect cell membrane, it is therefore necessary to identify a functional receptor for Vip3A protein and investigate its binding mechanism.

We have successfully cloned and produced Vip3A from Bt Thai isolate. The toxin has been purified to high purity and exhibited high toxicity against *S. axigua* and *S. litura* larvae. Ligand blots have been performed to identify a Vip3A receptor from *S. exigua* larval gut cells using both conventional SDS-PAGE and 2D-gel electrophoresis. A protein spot at approximately 170 kDa with pI about 7 was detected to bind to Vip3A. However, the identity of this protein spot has not been resolved since it is not well separated from the surrounding proteins. Additional techniques are required to purify this spot for further analysis.

The full-length Vip3Aa consists of 789 amino acids with molecular weight ~88 kDa. *In vitro* digestion of Vip3Aa by trypsin or insect gut juice proteases yields two fragments about 22 kDa and 66 kDa from its N- and C-termini, respectively. The protease cleavage site was identified after K198. Both fragments are held together and could not be separated under normal physiological condition. In order to investigate the role of the 66

kDa fragment, the gene encoding the C-terminal fragment corresponding to amino acids D199-K789 (assigned as Vip3Aa-D199) was cloned and expressed in *Escherichia coli*. The production level of the Vip3Aa-D199 was significantly decreased comparing to that of the full-length protein. Bioassays revealed that the Vip3Aa-D199 was not toxic to *Spodoptera litura* larvae when fed artificial diet overlaid with the protein up to 12,500 ng/cm$^2$ whereas the full-length Vip3Aa showed high toxicity with $LC_{50}$ about 200 ng/cm$^2$. Feeding the larvae with mixtures of the full-length Vip3Aa and Vip3Aa-D199 at different ratios (200:200, 200:1,000 and 200:5,000 ng/cm$^2$) revealed inhibition effect of the truncated toxin on the toxicity of the full-length toxin. Results showed that $LT_{50}$ and $LT_{95}$ of larvae fed the mixture toxins were longer than those fed the full-length toxin alone. The C-terminal fragment might compete for the membrane binding thus rendering activity of the full-length toxin. It is possible that the receptor binding motif of Vip3Aa resides in its C-terminal part.

To identify amino acids playing important role during intoxication, single amino acid substitutions were employed at ten positions using site-directed mutagenesis technique (P551A, W552L, E594A, Q598A, D616A, N623A, T631A, F636I, W684L and W691L). These residues are located in a putative carbohydrate binding module that could play essential role during receptor binding. All mutant proteins were effectively produced in *E. coli* and specifically reacted to anti-Vip3A antibody. Bioassays against *S. exigua* larvae revealed that most of the mutants retain their larvicidal activity comparable to that of the wild type except W552L, D616A and W684L which were unable to kill the larvae. These three residues might be important for interaction to a sugar moiety of a glycoprotein on the gut cell membrane that acts as a specific receptor for Vip3A.

It has been proposed that Vip3A proteins produced by different Bt strains show variable toxicity and specificity to different insects. Twenty Bt isolates collected from various locations in Thailand were screened for a new Vip3A by PCR using primers specific to *vip3* gene. Most of Bt isolates were found to carry *vip3Aa* gene. Only Bt isolates number 22 and 107 were found to carry *vip3Af* and *vip3Ad*, respectively. GenBank accession number for *vip3Ad* from Bt#107 and *vip3Af* from Bt#22 are KX595193 and KX595192. Both genes were cloned into pET28b to obtained 6xHis-tag fusion proteins. The fusion proteins were highly produced in *E. coli*. Insect bioassays revealed that Vip3Ad from Bt#107 was not toxic to *S. exigua* and *S. litura* larvae. Vip3Af from Bt#22 caused mortality to 20% in *S. exigua* and 60% in *S. litura* when feeding both insects with the toxin at 2 μg/cm$^2$. Amino acid sequence alignment showed variable in the C-terminal part which

might responsible for differences in specificity or receptor recognition to different insects. Although the newly discovered Vip3Ad and Vip3Af were not as good as the benchmark protein (Vip3Aa) when tested against *S. exigua* and *S. litura*, both toxins might have higher activity against other insects.

## 1. Introduction

*Bacillus thuringiensis* is a Gram positive soil bacterium, which has the insecticidal activity against many agricultural important pests (Schnepf *et al*., 1998). This bacterium produces intracellular crystal toxins (Cry toxins) at the sporulation phase and produces soluble vegetative insecticidal proteins (Vip) at the vegetative phase (Estruch *et al.,* 1996, Estruch and Yu, 2001, Selvapandiyan *et al*., 2001, Warren, 1997, Yu *et al*., 1997). To date there are 3 groups of Vip have been identified. Vip1 and Vip2 act as a binary toxin and highly specific to Coleopteran larvae (Nishimatsu & Jackson, 1998). Another group of toxins that can act independently is Vip3. Proteins in this group show broad spectrum of insecticidal activity against Lepidoptera and has acute bioactivity towards the black cutworm (BCW; *Agrotis ipsilon*), fall armyworm (FAW; *Spodoptera frugiperda*), beet armyworm (*Spodoptera exigua*) , tobacco budworm (*Heliothis virescens*), western bean cutworm (*Striacosta albicosta*) and corn earworm (*Helicoverpa zea*) (Abdelkefi-Mersati *et al.,* 2005a; Chen *et al*., 2003; Estruch *et al*., 1996; Estruch and C.-G., 2001; Shi *et al.*, 2004; Warren *et al.,* 1997; Yu *et al*., 1997). The difference in insecticidal activity of Vip depends on their sequence variation as Fang *et al*., found the activity of both Vip3Aa1 and Vip3Ac1 are not identical although their activities are limited to lepidopterans (Fang *et al*., 2007). Vip3A was first purified from *Bacillus thuringiensis* strain AB88 isolated from sour milk (Estruch *et al.,* 1996). Previous studies have shown that Vip3A has a higher insecticidal activity than some Cry1A proteins in insects (Estruch *et al.,* 1996, MacIntosh *et al.,* 1990). The *vip3A* gene encodes an 88.5-kDa protein, which has no homology with the known proteins (Estruch *et al.,* 1996). This protein can be secreted into the medium in *Bacillus thuringiensis* cultures without N-terminal processing (Estruch *et al.,* 1996). The secretion levels depend on strains of *Bacillus thuringiensis* (Loguercio *et al.,* 2002). Previous studies have indicated that Vip3A proteins have no similarity to the Cry toxins in *Bacillus thuringiensis* strain AB88 (Estruch *et al.,* 1996) and Vip3 and Cry toxins do not share the same receptors in the midgut of their insect hosts (Abdelkefi-Mersati *et al.,* 2009; Lee *et al.,* 2003). Specific binding of Vip3A to the midgut BBMV has been documented (Sena *et al*., 2009). Thus, Vip3A has become an important insecticidal toxin for development of new insecticidal products for control of agriculturally important pests and management of resistance development to Cry toxins in many important insects (MacIntosh *et al.,* 1990; Matten, 2007; Matten *et al*., 2008; Müller-Cohn *et al.,* 1996; Tabashnik *et al*., 2000).

The mode of action of Vip3A toxin involves a complex cascade, but is not well understood (Lee *et al.,* 2003; Yu *et al.,* 1997). This protein is soluble across a broad range of pH values from at least 5.0 to 10.0 (Lee *et al.,* 2003). In the larval midgut, Vip3A is activated by a proteolytic process to remove the 199-amino acid N-terminal fragment. Yu *et al*., reported that Vip3A toxin is activated by larval gut fluid, resulting in four products of 62, 45, 33, and 22 kDa in molecular weight (Yu *et al.,* 1997). Lee *et al.,* showed that the unprocessed VIP3A is incapable of forming a pore in the midgut BBMV *in vitro* (Lee *et al.,* 2003). After proteolytic activation, the toxin binds to the brush border membrane in larvae is started (Lee *et al*., 2003, Schnepf *et al.,* 1998). *In vivo* immunolocalization studies has indicated that the binding of Vip3A is restricted to the midgut cells in susceptible insects and the binding appears to be specific to the microvilli of columnar cells, and no binding occurs to goblet cells. A comparative binding analysis of Vip3A in the susceptible *A. ipsilon* and nonsusceptible *O. nubilalis* showed that Vip3A bound to the midgut tissue of the susceptible but not a non-susceptible insect (Yu *et al.,* 1997). Hence, the specific binding of the toxin to the midgut epithelium cells of susceptible insect is a key factor for determination of the insect host range for Vip3A (Chapman, 1985, Yu *et al.,* 1997). Candidate Vip3A binding proteins have been indicated in several insects. For example, Vip3A was found to bind to the BBMV proteins of 80 and 100-kDa in molecular weight in Linnaeus (Lee *et al*., 2003), whereas Vip3Aa16 was found to bind to a putative receptor of about 65 kDa in Linnaeus (Abdelkefi-Mesrati *et al*., 2009). Thus, the binding proteins for this toxin may vary from an insect to another (Abdelkefi-Mesrati *et al*., 2011). The binding of the toxin to the midgut cells leads to morphological alteration and eventually cell death.

Reported pathological symptoms of Vip3A toxicity include cytoplasm vacuolization, brush border membrane destruction, vesicle formation in the apical region of cells toward the midgut lumen, goblet cells damage, epithelium columnar cells destruction (distended and bulbours) in the midgut of Vip3A fed larvae, material and debris leaked into the lumen leading to the larval death (Abdelkefi-Mersati *et al.,* 2011, Yu *et al.,* 1997). The midgut cell lysis associated with cholesterol oxidase is suggested to be a mechanism of toxicity (Yu *et al.,* 1997). Estruch and Yu proposed that apoptotic pathway may occur after Vip3A had bound to its respective midgut receptors in susceptible insects (Estruch and Yu, 2001). Toxin activation is not only one determining factor for toxicity since gut juice from nonsusceptible insects (such as gut juice from *Ostrinia nubilalis*) can process Vip3A protoxin as well (Lee *et al.,* 2003; Yu *et al.,* 1997). Lee *et al.,* found that the

activated fragment of Vip3A, a 62-kDa protein, can be found from the gut juice of both susceptible and nonsusceptible lepidopterans (Lee *et al.,* 2003). In addition, Yu *et al*., found that the activated fragment of Vip3A from non-susceptible insect (*Ostrinia nubilalis*) was toxic to susceptible insects (Yu *et al.,* 1997). In contrast, the binding affinity to the midgut epithelium cells is considered to be a major factor determining its insect specificity. Voltage clamping assays with dissected midgut from the susceptible insect, *M. sexta*, showed pore formation by Vip3A treated with lepidopteran gut juice. Lee *et al.,* indicated that pore formation is the inherent property of Vip3A since they found both activated Vip3A by lepidopteran gut juice and by trypsin can lead to formation of ion channels in planar lipid bilayers. The authors suggested that these ion channels were voltage independent and highly cation selective, exhibiting their own conductance state and cation specificity (Lee *et al.,* 2003).

So far, the Vip3A receptor had not been clearly identified. Thus, we would like to investigate a receptor for Vip3A in the larval midgut of *Spodoptera exigua* which is a serious pest of various vegetable and other crops in Thailand, Asia, North America, parts of Africa, Oriental and Indo-Australian regions (Hardee and Herzog, 1997). Information obtained from this work can be used to pave the way for further improvement of this toxin as an effective bio-insecticide and to combat resistant development in insects.

## 2. Objectives

2.1 Identify a specific receptor of Vip3Aa from midguts of *Spodoptera exigua* larvae

2.2 Identify a receptor binding motif in Vip3Aa its and amino acid positions responsible for receptor binding

2.3 Investigate amino acid positions playing important role for protein stability, specificity and larvicidal activity

## 3. Materials & Methods

### 3.1 Bacterial cells, plasmids, oligonucleotide primers and insect larvae

*Escherichia coli* K12 JM109 and DH5α were used as cloning host cells and *E. coli* BL21(DE3)pLysS was used as an expression host to produce recombinant proteins. Plasmid pGEM-Teasy and pJET pJET1_2 Blunt were used for gene cloning and pET28b was used as expression vector to produce 6xHis-tag recombinant proteins.

Oliginucleotide primers for *vip3A* screening and cloning were shown used in Table 1 and primers for site-directed mutagenesis were in Table 2. The laboratory reared *Spodopter exigua* and *Spodoptera litura* larvae were obtained from the Biotec NPV Pilot Plant.

**Table 1.** Primer sequences used for screening and cloning of *vip3* gene.

| **Primer name** | **DNA sequences (5′ to 3′)** |
|---|---|
| Vip3 screening Fw | TGCCACTGGTATCAARGA |
| Vip3 screening Rv | TCCTCCTGTATGATCTACATATGCATTYTTRTTRTT |
| Vip3A full-length Fw | CCGCGGCCGCGGAGGATTAACATATGAACAAGAATAATACTAA ATTAA |
| Vip3Aa full-length Rv | CTCGAGTTACTTAATAGAGACATCGGA |
| Vip3Ad full-length Rv | CTCGAGTTATTTAATAGAGAAATCATAAAAATGTA |
| Vip3Af full-length Rv | CTCGAGTTATTTAATAGAAACGTTTTCAAAT |

**Table 2.** The mutagenic primers for generation of Vip3Ag2 mutant toxins

Recognition sites introduced in the primers for restriction endonuclease analysis are underlined. Mutated nucleotides are shown in bold; fw and rv represent forward and reverse primers, respectively.

| **Primer** | **Sequence** | **Enzyme** |
|---|---|---|
| P551Afw | 5’-GAAAACTTAGAG**GC**CTGGAAAGCAAATAAC-3’ | *Stu*I |
| P551Arv | 5’-ATTTGCTTTCCA**GG**CCTCTAAGTTTTCTCC-3’ | |
| E594Afw | 5’-TCGAAAACAG**C**ATATGTAATTCAATATATT-3’ | *Nde*I |
| E594Arv | 5’-TTGAATTACATAT**G**CTGTTTTCGATTTCAA-3’ | |
| Q598Afw | 5’-GAATATGTAATT**GC**ATATATTGTAAAGGGA-3’ | - |
| Q598Arv | 5’-TTACAATATAT**GC**AATTACATATTCTGTTT-3’ | |
| D616Afw | 5’-GAAAAAAATGGTG**CA**TGCATTTATGAAGAT-3’ | *Sph*I |
| D616Arv | 5’-TTCATAAATGCA**TG**CACCATTTTTTTCATC-3’ | |
| N623Afw | 5’-TATGAAGATAC**GGCC**AATGGTTTAGAAGAT-3’ | *Eae*I |
| N623Arv | 5’-TCTAAACCATT**GGCC**GTATCTTCATAAATG-3’ | |
| T631Afw | 5’-GAAGATTTTCA**GGCC**GTTACTAAAAGTTTT-3’ | *Hae*III |
| T631Arv | 5’-TTTAGTAACGG**CC**TGAAAATCTTCTAATAA-3’ | |
| F636Ifw | 5’-CCATTACTAAA**TCGA**TTATTACAGGAACGG-3’ | *Cla*I |
| F636Irv | 5’-TCCTGTAATAA**TCGA**TTTAGTAACGGTTTG-3’ | |
| W684Lfw | 5’-TAAATTCAGATGC**ACTAGT**TGGATCTCAGG-3’ | *Spe*I |
| W684Lrv | 5’-TGAGATCCAA**CTAGT**GCATCTGAATTTATC-3’ | |
| W691Lfw | 5’-TCTCAGGGAAC**ACTAGT**TTCAGGAAATTCA-3’ | *Spe*I |
| W691Lrv | 5’-AATTTCCTGAA**CTAGT**GTTCCCTGAGATC-3’ | |

### 3.2 Plasmid construction

The full length *vip190* (or *vip3Aa35*) gene from *B. thuringiensis* strain M190 (Genbank accession no. GU733921) was amplified by PCR using primers for *vip3A* full length gene as shown in Table 1. The PCR products were cloned into pGEM-Teasy vector and transformed into *E. coli* JM109. DNA sequence of the inserted gene was verified by automated DNA sequencing (Macrogen, Korea). The *vip3A* gene in pGEM-Teasy was subcloned into pET28b between *Nde*I and *Bam*HI sites to be expressed as a 6xHis-Vip3A fusion protein in BL21(DE3)pLysS

To constructed a plasmid for producing the C-terminal fragment of Vip3Aa, the 1.7 kb fragment of *vip3Aa* gene encoding amino acids D199-K789 was amplified by PCR using pET28-Vip3Aa as a template couple with forward primer: 5'-GCC ATA TGG ATG GCT CTC CTG CAG ATA TTC-3' and reverse primer: 5'-GCG GAT CCT TAC TTA ATA GAG ACA TCG GAA-3'. The underlined sequences indicate recognition sites, *Nde*I for the forward primer and *Bam*HI for the reversed primer. The PCR product was digested with *Nde*I and *Bam*HI and cloned into pET-28b between *Nde*I and *Bam*HI sites (Figure 1). This recombinant plasmid encodes a truncated fragment of Vip3Aa (D199-K789) with a polyhistidine tag at its N-terminus to facilitate protein purification. The protein was named “Vip3Aa-D199”.

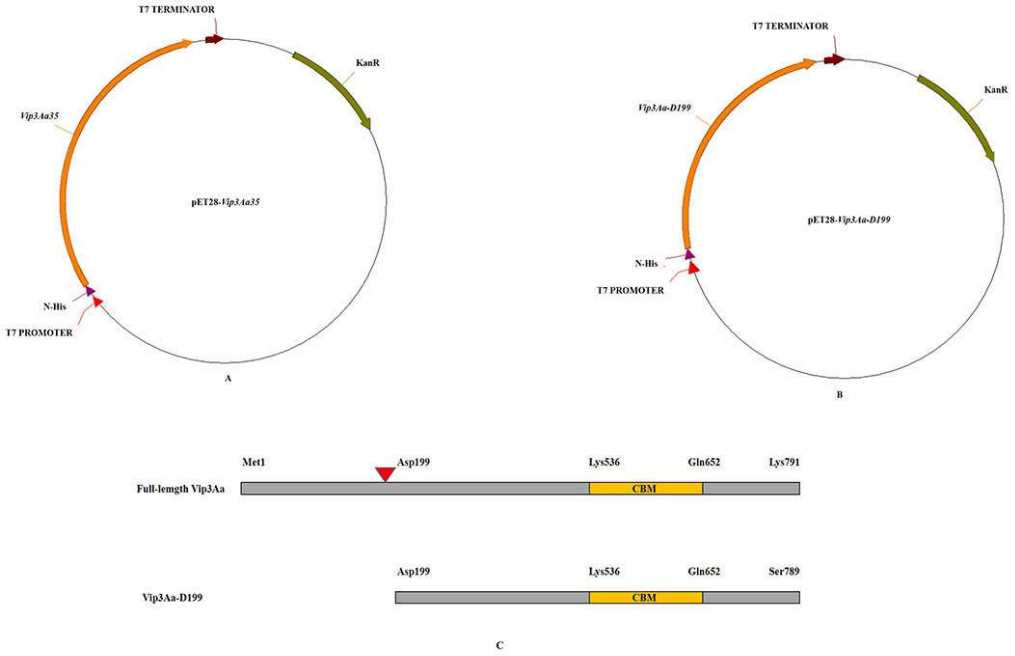


**Figure 1.** Recombinant plasmids of pET-28b containing vip3Aa gene

The 2.4 kb fragment of the full-length *vip3Aa35* gene (A) and 1.7 kb fragment of *vip3Aa-D199* gene (B) obtained from *B. thuringiensis* strain M190 was inserted into the *Nde*I-*Bam*HI sites of pET-28b expression vector under control of *T7* promoter.

### 3.3 Screening of *vip3* genes

Total genomic DNA from several *B. thuringiensis* isolates were extracted and purified using QIAGEN genomic extraction kit. The genomic DNA was used as a template for PCR screening using a pair of Vip3 screening primers as shown in Table 1. Samples that showed positive results were selected for another PCR using specific primers for Vip3Aa, Vip3Ad and Vip3Af (Table 1). The PCR products were cloned into pJET1_2 Blunt and transformed in to *E. coli* JM109. The recombinant plasmid was extracted and the DNA sequence of the entire insert gene was determined by automated DNA sequencer (Macrogen, Korea).

### 3.4 Protein preparation

*E. coli* cells producing Vip3A proteins were collected by centrifugation at 10,000xg, 4°C for 5 minutes. The cell pellet was resuspended in PBS pH 7.4. Vip3A protein was released from the cell by ultrasonication. After centrifugation at 10,000xg, 4°C for 10 minutes, soluble Vip3A protein in the supernatant was purified by affinity chromatography using HisTrap® FF column or HiTrap Chelating HP column following the manufacturer instruction. The 6xHis-Vip3A proteins were eluted using PBS pH 7.4 containing 100-250 mM immidazole. After that, the eluted protein was concentrated using Amicon® Ultra-15 centrifugal filter devices MWCO 30 kDa. Imidazole remained in concentrated protein was removed by gel filtration chromatography using HiTrap desalting column. The purified proteins were analyzed by SDS-PAGE and the identity of Vip3A was verified by immunoblotting using rabbit anti-Vip3A polyclonal antibody as a primary antibody.

### 3.5 Brush broader membrane vesicle (BBMV) preparation

BBMVs from 3$^{rd}$-instar larvae of *S. exigua* were prepared by using differential magnesium precipitation method as described by Wolfersberger *et al.* (Wolfersberger *et al.*, 1987) with some modifications. First, the anterior and posterior of third instar larvae from *S. exigua* were excised and their guts were dissected longitudinally. The luminal content enveloped in the peritrophic membrane was removed. Then, guts were pulled out of the dissected bodies and were homogenized in ice-cold GET buffer containing 1x protease inhibitor cocktails. An equal volume of ice-cold 24 mM $MgCl_2$ was added to the gut homogenate and the mixture was incubated on ice for 15 min. After centrifugation at

2500 x g (Sorvall ® Biofuge Primo R), 4°C for 15 min had done, the supernatant was transferred to the ultracentrifuge tube. The pellet was suspended in ice-cold GET buffer containing 1x protease inhibitor cocktails with 1 volume of the gut homogenate. Then, 24 mM $MgCl_2$ was added in the suspension and the protocol mentioned above was repeated. All supernatant fractions from $MgCl_2$ treatment were pooled and centrifuged at 50,000 x g for 45 min using swinging bucket rotor (TH-641, Sorvall ultra pro 80). The final BBMVs pellet was resuspended in GET buffer containing 1x protease inhibitor cocktails and was stored at -80°C before used in the ligand blot analysis and 2D-Gel Electrophoresis. The protein concentration of BBMVs was determined by Bradford method (Bradford, 1976) as mentioned previously.

### 3.6 Vip3A receptor identification

**Ligand blot from SDS-PAGE**

Various amounts of BBMV proteins from *S. exigua* larvae were loaded on SDS-polyacrylamide gel. The protein electrophoresis was operated at constant voltage (120 Volts) until the tracking dye reached the bottom of the gel. Proteins separated on SDS-PAGE were transferred to nitrocellulose membranes using a Bio-Rad Mini Trans-blot apparatus (BIO-RAD) at constant current (189 mA), 4°C for 2 hours. The membrane was blocked with blocking solution (5% skim milk in 1x PBS buffer pH 7.4), at 4°C for overnight. The 20 µg/ml of Vip3Aa in blocking solution was overlaid on the membrane for 1 hour at room temperature. The membrane was washed with 1x PBS buffer pH 7.4 for 3 times, 10 min per each time. The bound Vip3Aa was detected by probing with rabbit anti-Vip3A polyclonal antibody (1:5000), gently shaking at room temperature for 2 hours. The unbound proteins were removed by washing with 0.1% Tween-20 in 1x PBS buffer pH 7.4 for 2 times, 10 min per each time followed by washing with 1x PBS buffer pH 7.4 for 2 times, 5 min per each time. Then, the membrane was incubated with goat anti-rabbit IgG alkaline phosphatase-conjugated antibody (1:8000) with gently shaking at room temperature for 1 hour. After that, the membrane was washed with 0.1% Tween-20 in 1x PBS pH 7.4 for 2 times, 10 min per each time followed by washing with 1x PBS buffer pH 7.4 for 2 times, 5 min per each time. The immunoreactive signal on the membrane was detected by using the reaction mixture consists of 1.5% NBT in DMF (200 µl), 1% BCIP in DMF (200 µl), and 20 ml of carbonate buffer pH 9.8.

**Ligand blot from 2D-gel electrophoresis**

The immobilized pH gradient gel strip size 7 cm, pH 3-10 NL (GE healthcare Immobiline ™ Drystrip) was used for the first dimensional gel electrophoresis. The 65-180 µg of solubilized BBMV proteins were mixed with 2 µl of immobilized pH gradient (IPG) buffer and 0.75 µl of 1% bromophenol blue. Then, lysis CT buffer was added until the protein mixture reached the final volume 130 µl. Before loaded protein sample 125 µl into ceramics strip holder (Amersham Biosciences), the protein mixture was centrifuged at 12,000 rpm, 4°C for 5 min. The strip cover fluid (mineral oil) 500 µl was laid on top of the gel strip and then the strip holder was closed with the cap. The isoelectric focusing was conducted for 12,100 Volt-Hours after 12 hours passive in-gel rehydration. The experiment was done at 20°C, 50 µA/ strip by using Ettan IPGPhor II (Amersham Biosciences). After isoelectric focusing had completed, the gel strip was placed into a freshly prepared solution of 25 mg DTT in 2.5 ml SDS-PAGE equilibration buffer (2% SDS, 50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 0.002% bromophenol blue). The solution was gently shaken for 15 min, and then the DTT solution was replaced with a freshly prepared solution of 60 mg IAA in 2.5 ml SDS-PAGE equilibration buffer in order to prevent the re-formation of reduced disulfide bond.

The second dimensional gel electrophoresis was performed with a Tris-glycine buffer system using Amersham Biosciences Minive complete (GE Healthcare). The 10-12.5 % SDS-polyacrylamide gels were used for separating proteins by their molecular weight. After the gel strip had equilibrated, the gel strip was gently inserted into the top of SDS-polyacrylamide gel. A prestained protein ladder (Thermo scientific) was placed on the anode end of the gel strip. The protein electrophoresis was operated at constant voltage (130 Volts), until the tracking dye reached the bottom of the gel. The spot of separated proteins was envisioned by staining the gel in the staining solution using Colloidal Coomassie Blue G-250 (CBG) for overnight with gently shaking. The gel was subsequently destained in the deionized water (Milli Q) for overnight or until the background was clearly removed.

After the 2D-gel electrophoresis had completed, the ligand blot was performed in order to detect the binding between putative receptor protein and activated Vip3Aa. Proteins from the 2D-gel were transferred to 0.45 µm nitrocellulose membranes at constant

current (190 mA), 4°C for 2 hours using a Bio-Rad Mini Trans-blot apparatus (BIO-RAD). The internal positive control was 30 ng activated Vip3Aa dotted at the right bottom of each blotted nitrocellulose membrane. The membrane was blocked with blocking solution (5% skim milk in 1x PBS buffer pH 7.4) at 4°C for overnight. After that, the 20 µg/ml of activated Vip3Aa in blocking solution was overlaid at room temperature, for 1 hour. The membrane was subsequently washed with 1x PBS buffer pH 7.4 for 3 times, 10 min per each time. The bound activated Vip3Aa was detected by probing with rabbit anti-Vip3A polyclonal antibody (1:5000), gently shaking at room temperature for 2 hours. The unbound proteins were removed by washing with 0.1% Tween-20 in 1x PBS buffer pH 7.4 for 2 times, 5 min per each time followed by washing with 1x PBS buffer pH 7.4 for 2 times, 2 min 30 sec per each time. Then, the membrane was incubated with goat anti-rabbit HRP-conjugated polyclonal antibody (1:10,000), gently shaking at room temperature for 1 hour and 30 min. After that, the membrane was washed with 0.1% Tween-20 in 1x PBS pH 7.4 for 2 times, 5 min per each time followed by washing with 1x PBS buffer pH 7.4 for 2 times, 2 min 30 sec per each time. The immunoreactive signal on the membrane was detected by enhanced chemiluminescent detection system using supersignal west Pico chemiluminescent substrate, which is composed of supersignal west Pico Luminol/ enhancer solution and supersignal west Pico stable peroxide solution (Thermo Scientific). The Luminol/ enhancer solution 500 µl was mixed with the stable peroxide solution 500 µl. Then, this developer solution was spread all over the nitrocellulose membrane. The enhanced chemiluminescent reaction was incubated at room temperature, for 5 min in the dark room. Then, the nitrocellulose membrane was placed in the cassette for X-ray film exposure. The X-ray film (Kodak) was exposed in the dark room until the dark spots of signal could be visualized by naked eyes. The X-ray film was subsequently developed by using Kodak Medical X-ray Processor.

### 3.7 Dot blot analysis and sensitivity test

The 0.45 µm nitrocellulose membrane was equilibrated in 1x PBS pH 7.4 for 15 min prior to inserting into the Bio-Dot ™ apparatus (BIO-RAD). Then 225 µg of *S. exigua*'s BBMVs suspension was dotted on the membrane at room temperature using the Bio-Dot ™ apparatus. For the sensitivity test, 8-1000 ng of purified 6xHis-Vip3Aa was dotted on the same membrane. Two negative control dots were GET buffer and Tris-NaCl buffer pH 8.0 without protein. Membranes were blocked with blocking solution (5% skim

milk in 1x PBS buffer pH 7.4), at 4°C for overnight. After that, the 20 µg/ml of purified 6xHis-Vip3Aa in blocking solution was overlaid for 1 hour at room temperature. After the membrane had overlaid with Vip3Aa, it was subsequently washed with 1x PBS pH 7.4 for 3 times, 10 min per each time. The bound 6xHis-Vip3Aa was detected by probing with anti-polyhistidine monoclonal antibody (1:2000), gently shaking at room temperature for 2 hours. The unbound proteins were removed by washing with 1x PBS pH 7.4 for 3 times, 10 min per each time. The immunoreactive signal on the membrane was detected by using the reaction mixture consists of 12 mg 4-chloro-1-naphthol, methanol 4 ml, TBS buffer pH 8.0 ( 50 mM Tris-HCl pH 8.0, 150 mM NaCl) 20 ml, and 0.015% $H_2O_2$.

### 3.8 Larvicidal activity assay against *S. exigua* and *S. litura*

The surface treatment bioassays were carried out using second instar larvae of *S. exigua* and *S. litura* obtained from the Nuclear Polyhedrosis Virus (NPV) Production Pilot Plant, National Center for Genetic Engineering and Biotechnology, Thailand. Various concentrations (1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, and 3.91 ng/cm$^2$) of the full-length or trypsin activated Vip3Aa were applied over the surface of an artificial insect diet that was poured into polystyrene 24-well plate. The negative control wells were overlaid with 50 mM Tris-NaCl buffer pH 8.0. After the toxin was absorbed on the surface of the artificial diet, a 2$^{nd}$- instar larvae was placed in each well. At least 8 larvae were used for each protein concentration and the larvae were kept at room temperature with 12/12 hr light/dark cycle and 60% relative humidity. Mortality was recorded daily for 7 days. Three independent experiments were performed and the toxin concentrations that kill 50% and 95% of the tested larvae ($LD_{50}$ and $LD_{95}$) and time required to kill 50% and 95% of tested larvae ($LT_{50}$, and $LT_{95}$) were calculated using Probit analysis (Finney, 1971).

To evaluate the effect of the C-terminal fragment, the full-length Vip3Aa was mixed with the truncated protein at 1:1, 1:5, and 1:25 (w/w) and fed to 2$^{nd}$ instar *S. litura* larvae as described above. The full-length Vip3Aa at 200 ng/cm$^2$ was used as a positive control whereas 50 mM Tris-NaCl pH 8.0 buffer was used as a negative control. The experiment was performed at room temperature in 24 wells/treatment (1 larvae/well). The accumulative mortality was recorded daily for 7 days. $LT_{50}$ and $LT_{95}$ were calculated from three independent experiments using Probit analysis (Finney, 1971).

### 3.9 Site-directed mutagenesis

Ten Vip3A mutants (P551A, W552L, E594A, Q598A, D616A, F636I, T631A, N623A, W684L, and W691L) were generated based on Stratagene's QuikChange™ Site-Directed Mutagenesis (Strategenes). The recombinant plasmid pET28-Vip3A (wild type) was used as a template for single substitution together with appropriate primers shown in Table 2. The recombinant plasmids obtained for each mutant were distinguished from the template by restriction endonuclease digestion with the appropriate enzyme (Table 2). DNA sequences of the full-length gene from all mutants were verified by automated DNA sequencing.

## 4. Results & Discussion

### 4.1 Protein quality and larvicidal activity of Vip3A

*Escherichia coli* BL21 (DE3) pLysS was used as a host strain for the expression of 6xHis-Vip3Aa protein. After the cells had been induced by 1 mM IPTG for 5 hours, the culture equivalent to O.D.600 of 1 was harvested and analyzed on SDS-PAGE. Results showed that the 6xHis-Vip3Aa was expressed as the 88-kDa protein after the cell culture was induced. It was released from host cells after breaking the cells by ultrasonication. The 6xHis-Vip3Aa was detected only in the supernatant fraction after sonication whereas none of the corresponding protein band was observed in the insoluble fraction. This result indicated that the protein was produced as a soluble protein.

Western blot analysis using rabbit anti-Vip3A polyclonal primary antibody and goat anti-rabbit IgG alkaline phosphatase-conjugated secondary antibody was performed in order to verify if the 88 kDa-protein band was Vip3Aa. Figure 2A and Figure 2B (lane 1-3) showed the corresponding protein band of 6xHis-Vip3Aa after the cell culture was induced with 1 mM IPTG at various times whereas no band was detected in lane 4, which was the non-induced culture (Figure 2B).

The 6xHis-Vip3Aa was purified by Immobilized Metal ion Affinity Chromatography (IMAC) using HiTrap Chelating HP column (GE healthcare). In this experiment, $Ni^{2+}$ was used as the chelated ion. The column was pre-equilibrated with Tris-NaCl buffer pH 8.0 (50 mM Tris pH 8.0 and 200 mM NaCl). Soluble proteins received after sonication was loaded into the column by using flow rate 1 ml/min. The purified fraction of 6xHis-Vip3Aa was eluted from the column with flow rate 5 ml/min

by using Tris-NaCl buffer pH 8.0 with 100 mM and 250 mM imidazole as shown Figure 3A, Lane 7 and Lane 8, respectively. In addition, western blot analysis of purified proteins from each fraction also indicated that the 88 kDa-protein eluted from the column was 6xHis-Vip3Aa (Figure 13B). After the protein had been purified and concentrated via Amicon ® Ultra Centrifugal Filter, imidazole was removed from the concentrated protein by gel filtration chromatography using HP desalting column (GE Healthcare).

The purified 6xHis-Vip3Aa was digested with 2% trypsin (%W/W) (Trypsin TPCK treated from bovine pancreas, Sigma) in order to produce the active fragment of Vip3Aa. The 10 mM PMSF was used as the protease inhibitor for stopping the digestive reaction. The protoxin 6xHis-Vip3Aa (88 kDa) was completely activated by trypsin to yield two fragments approximately 62 kDa and 22 kDa as shown in Figure 4.

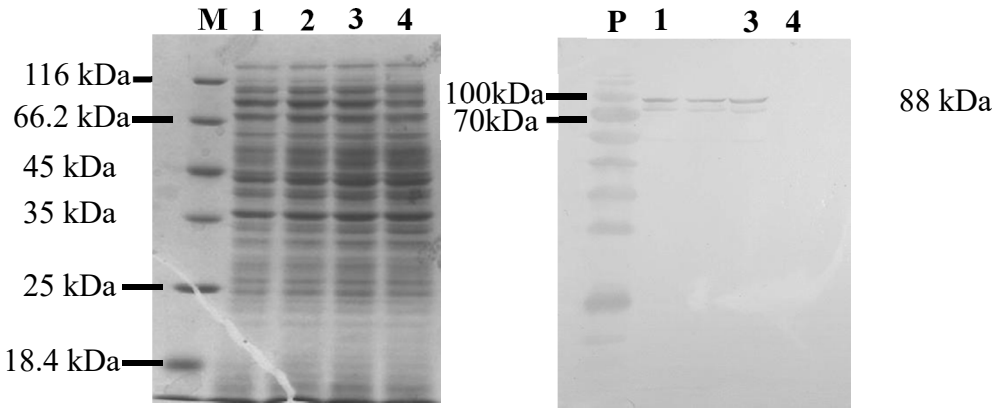


**Figure 2. Production of Vip3Aa in *E. coli* after induction with 1 mM IPTG at various times**

The expression profile of 6xHis-Vip3Aa was analyzed on 12% SDS-polyacrylamide gel. The cell culture was grown at 37°C in LB broth for 2.5, 3, and 3.5 hours and then, the expression of 6xHis-Vip3Aa was induced by adding 1 mM IPTG. The culture equivalent to $O.D._{600}$ of 1 was harvested and loaded on each well of the gel. The gel was stained with Coomassie R250 (right panel). Western blot was performed to detect 6xHis-Vip3Aa as shown in the left panel. The 6xHis-Vip3Aa was detected as a 88-kDa protein band.

M : Protein molecular weight marker

P : PageRuler ™ Plus Prestained Protein Ladder

Lane 1 : Total proteins in *E. coli* cell induced with IPTG after culture for 2.5 hours

Lane 2 : Total proteins in *E. coli* cell induced with IPTG after culture for 3.0 hours

Lane 3 : Total proteins in *E. coli* cell induced with IPTG after culture for 3.5 hours

Lane 4 : Total proteins in non-induced cell culture

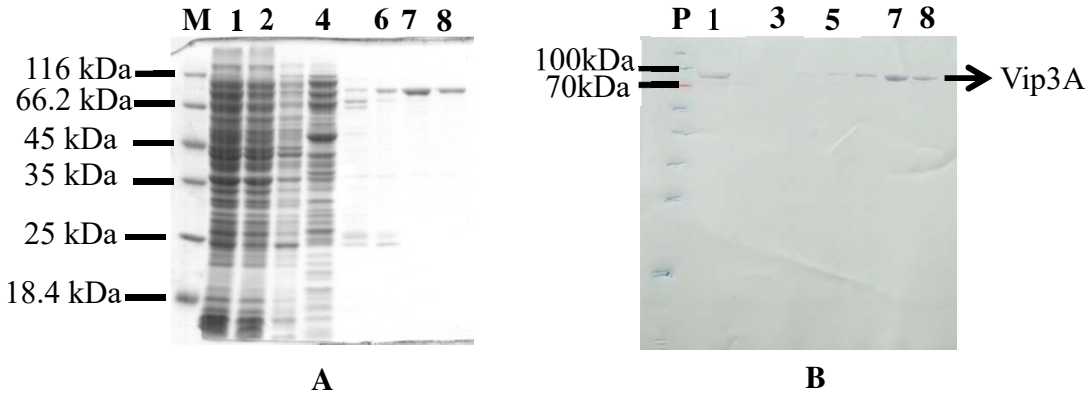


**Figure 3.** Purification of 6xHis-Vip3Aa from *E. coli*

The 6xHis-Vip3Aa was purified by Immobilized Metal ion Affinity Chromatography (IMAC). Protein fractions were eluted from the column using buffers containing 100 mM and 250 mM imidazole and loaded on SDS-polyacrylamide gel. The gel was stained with Coomassie blue R250 (Figure 13A). Western blot analysis to detect the Vip3A protein was shown in Figure 13B.

| | |
|---|---|
| M | Protein molecular weight marker |
| P | PageRuler ™ Plus Prestained Protein Ladder |
| Lane 1 | Supernatant from E. coli cell lysate after sonication |
| Lane 2 | Flow through fraction after soluble proteins from E. coli cell lysate containing 6xHis-Vip3Aa had flowed into the column |
| Lane 3 | Washed fraction by using tris-NaCl buffer pH 8.0 |
| Lane 4 | Washed fraction by using tris-NaCl buffer pH 8.0 with 40 mM imidazole |
| Lane 5 | Washed fraction by using tris-NaCl buffer pH 8.0 with 60 mM imidazole |
| Lane 6 | Washed fraction by using tris-NaCl buffer pH 8.0 with 80 mM imidazole |
| Lane 7 | Eluted fraction by using tris-NaCl buffer pH 8.0 with 100 mM imidazole |
| Lane 8 | Eluted fraction by using tris-NaCl buffer pH 8.0 with 250 mM imidazole |

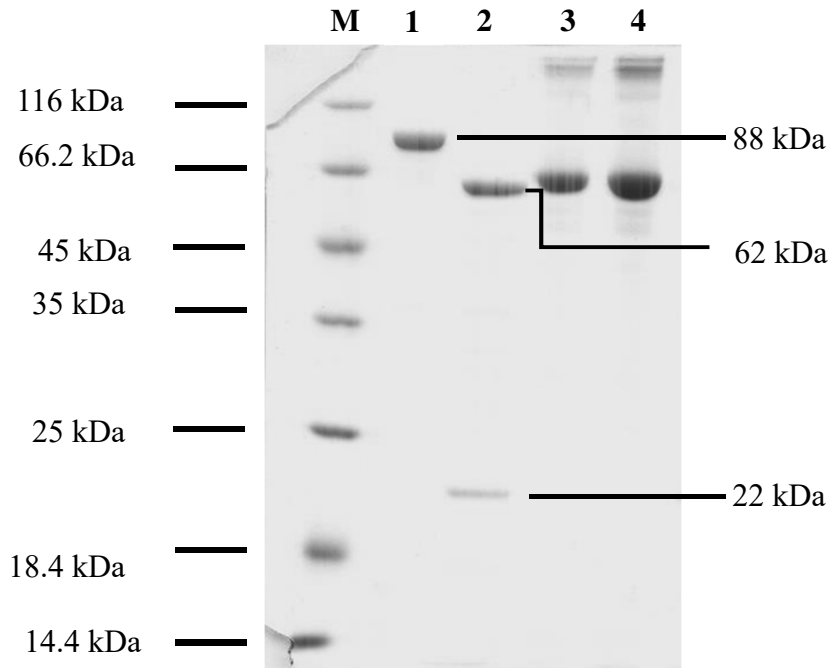


**Figure 4.** Protein profile of the trypsin activated Vip3Aa

The 6xHis-Vip3Aa was activated with 2% trypsin (%W/W) at 37°C for 2 hours. The tryptic digestion was stopped by adding 10 mM PMSF as the protease inhibitor. The activated Vip3Aa was analyzed on 12% SDS-polyacrylamide gel.

M Protein molecular weight marker

Lane 1 Purified 6xHis-Vip3Aa

Lane 2 Activated fragment of Vip3Aa after tryptic digestion

Lane 3-4 Standard BSA 1µg and 2µg, respectively

Larvicidal activity assay was performed to ensure that both of purified 6xHis-Vip3Aa (protoxin) and activated Vip3Aa still contained their own toxicity and did not lose their toxicity during the process of protein purification, concentration, desalting, and tryptic digestion. Various amounts (from 2 to 0.125 µg/cm$^2$) of both protoxin (6xHis-Vip3Aa) and activated Vip3Aa were used for testing against 2$^{nd}$ instar larvae of *S. exigua* in order to determine $LD_{50}$ values of each toxin. Experiments were performed at room temperature in 4 replicates. The accumulate mortality was recorded daily for 7 days. Then, protein amounts that kill 50% and 95% of larvae ($LD_{50}$ and $LD_{95}$) and time required to kill 50% of larvae ($LT_{50}$) were calculated by using Probit analysis (Finney, 1971).

| **Sample** | **$LD_{50}$ (ng/cm$^2$)** | **$LD_{95}$ (ng/cm$^2$)** |
|---|---|---|
| 6xHis-Vip3Aa | 500 (343 – 729) | 1,248 (824 – 3,819) |
| Activated Vip3Aa | 275 (121 – 458) | 1,598 (813 – 14,478) |

**Table 3.** $LD_{50}$ and $LD_{95}$ values of the protoxin (6xHis-Vip3Aa) and activated Vip3Aa

Both 6xHis-Vip3Aa (protoxin) and activated Vip3Aa were tested against 2$^{nd}$ instar larvae of *S. exigua*. Various amounts of both protoxin (6xHis-Vip3Aa) and activated Vip3Aa were used (2, 1, 0.5, 0.25, 0.125 µg/cm$^2$). Larvicidal activity assays were performed at room temperature in 4 replicates. The accumulated mortality was recorded daily for 7 days. $LD_{50}$ and $LD_{95}$ were calculated from three independent experiments using Probit analysis (Finney, 1971). Fiducial limits at 95% confident were shown in parentheses.

| Sample | Accumulate mortality (%) | | | | | | | $LT_{50}$ (days) |
|---|---|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 | |
| 6xHis-Vip3Aa | 0 | 38 | 88 | 100 | 100 | 100 | 100 | 2.2 (1.6 – 2.6) |
| Activated Vip3Aa | 0 | 25 | 88 | 100 | 100 | 100 | 100 | 2.3 (1.8 – 2.8) |
| Negative control | 0 | 0 | 0 | 0 | 0 | 0 | 0 | - |

**Table 4.** $LT_{50}$ of 6xHis-Vip3Aa and activated Vip3Aa

Both 6xHis-Vip3Aa (protoxin) and activated Vip3Aa were tested against 2nd instar larvae of *S. exigua*. Protoxin and activated toxin at 2 µg/cm$^2$ were applied over the surface of an artificial diet. The 2nd instar larvae of *S. exigua* were placed in each well (2 larvae/ well) and kept at room temperature. The accumulated mortality was recorded daily for 7 days. $LT_{50}$ was calculated using Probit analysis (Finney, 1971). Fiducial limits at 95% confident were shown in parentheses.

### 4.2 Identification of Vip3A receptor on *S. exigua* larval gut cells

It has been postulated that Vip3A binds to a specific receptor on the membrane of epithelium cells lining the gut lumen of susceptible insect larvae in a similar fashion to other insecticidal proteins. Therefore, it is possible to fishing out the Vip3A receptor using ligand blot technique. Several insecticidal proteins such as Cry proteins from *B. thuringiensis* and a binary toxin from *L. sphaericus* employ glycoproteins on the cell membrane of susceptible insect as a landing platform by binding to its carbohydrate moiety. Amino acid sequence analysis revealed that Vip3Aa contains a putative carbohydrate binding domain that usually recognize a sugar molecule. Thus, the proposed interaction between Vip3Aa and a sugar moiety in a glycoprotein present on the gut cell membrane should be detected using ligand blot.

### 4.2.1 Ligand blot using BBMV extracted from the whole gut

Previous data indicated that Vip3A bind to a specific receptor present on the epithelium cell membrane in larval guts and this receptor is different from that binds to Cry toxins (Lee et al., 2003 & 2006). To identify the binding between Vip3A and its receptor on the gut cell membrane, BBMVs were prepared from the whole gut of *S. exigua* larvae as described in materials & methods section. The whole larval gut was chosen in this experiment since the gut was too small and difficult to do dissection to isolate only the gut cell out of other part of the larvae. The whole guts were homogenized using a plastic pestle. Different amounts of whole guts were loaded in 12% SDS-polyacrylamide gel in order to observe the appropriate amounts of loaded whole gut proteins that suitable for the analysis on SDS-PAGE. The quality of the homogenized whole guts was shown in Figure 5. Results indicated that using 3 guts gave sufficient amount of proteins required for performing ligand blot experiment (Figure 5).

To do a ligand bloting, BBMVs equivalent to three whole guts were loaded in 10% SDS-polyacrylamide gel. Then, these proteins were transferred to nitrocellulose membrane by western blot. The bound Vip3Aa to its binding proteins was detected by probing with rabbit anti-Vip3A polyclonal primary antibody and goat anti-rabbit IgG alkaline phosphatase-conjugated secondary antibody, respectively.

The internal controls of ligand blotting experiment were 6xHis-Vip3Aa and activated Vip3Aa that were spotted directly on the nitrocellulose membrane. Four nitrocellulose membranes were used. In order to investigate the background signal which was occurred from the binding between those two antibodies and *S. exigua* proteins, the negative control (membrane C) without Vip3Aa overlaid, but probed with both of rabbit anti-Vip3A polyclonal antibody (primary antibody) and goat anti-rabbit IgG alkaline phosphatase-conjugated antibody (secondary antibody) was performed. Another negative control (membrane D), which was overlaid with 20 µg/ml of activated Vip3Aa, but did not probed with rabbit-anti Vip3A polyclonal antibody (primary antibody) was done in order to investigate the background signal, which was occurred from the binding between goat anti-rabbit IgG alkaline-phosphatase-conjugated antibody and larval gut proteins.

According to results shown in Figure 6, the negative control membrane (membrane D) showed none of the binding signal, including non-specific binding signal. The binding signal occurred between *S. exigua*'s gut proteins and Vip3Aa could be observed as shown in Figure 6A and Figure 6B. However, the non-specific binding signal

could be detected in the other negative control membrane (membrane C), which was not overlaid with Vip3Aa (Figure 6C). In addition, when compared the binding signal in Figure 6A and Figure 6B, the binding signal from isolated BBMV proteins (Figure 6A, lane 1 and Figure 6B, lane1) could be observed as a discrete band at much clearer than that of the whole gut proteins (lane 2). Thus, the BBMVs were used for all of the remaining experiments.

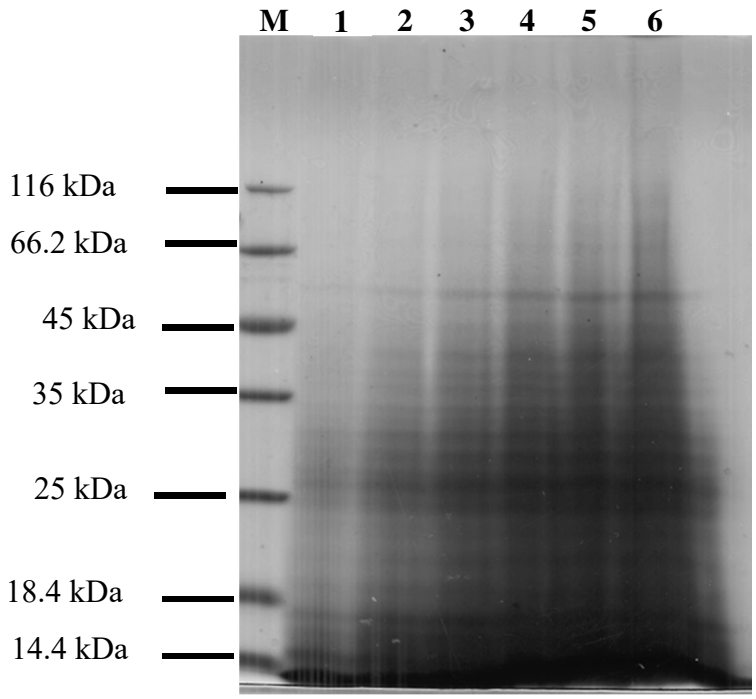


**Figure 5. Protein profile from the whole gut extract of *S. exigua* larvae**

Whole guts were pulled out of the dissected bodies and were washed in GET buffer in order to eliminate the food boluses. The 5x sample buffer containing SDS was added in the suspension prior to homogenizing. The total of 24 guts were homogenized thoroughly and loaded on each well of 12% SDS-polyacrylamide gel.

| | |
|---|---|
| M | Protein molecular weight marker |
| Lanes 1-6 | Proteins from homogenized sample equivalent to 0.5, 1, 1.5, 2, 2.5 and 3 guts, respectively |

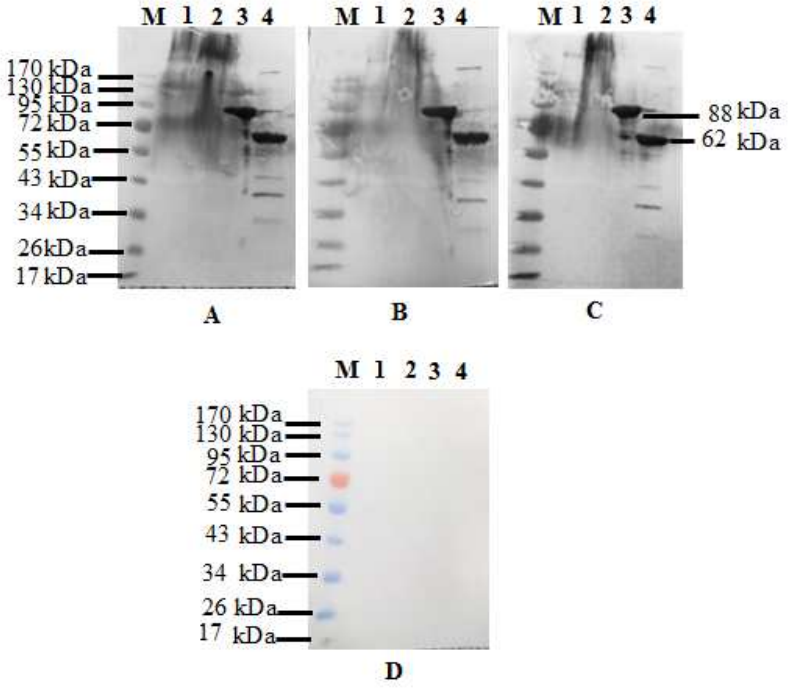


**Figure 6. Ligand blot results using the whole gut**

25 µg of BBMV proteins (lane 1) and proteins from 3 whole guts (lane 2) were loaded in 10% SDS-polyacrylamide gel. The internal controls were loaded with 1 µg of either 6xHis-Vip3Aa (lane 3) or trypsin activated Vip3Aa (lane 4). The 20 µg/ml of Vip3Aa in blocking solution was overlaid for 1 hour at room temperature. The total of four nitrocellulose membranes were used. Two of them were membranes of interest; a membrane overlaid with 6xHis-Vip3Aa (Figure 6A) and a membrane overlaid with activated Vip3Aa (Figure 6B). The other two membranes were negative controls; a membrane without Vip3Aa overlay (Figure 6C) and a membrane overlaid with activated Vip3Aa, but did not probe with primary antibody (Figure 6D). Lane M is a prestained protein ladder.

### 4.2.2 Ligand blot results from BBMV extracted from isolated guts

BBMV extracted from the whole gut gave high background and unable to identify a specific band that bind to Vip3A. Therefore, the isolated guts were used in subsequence experiments. The quality of the extracted BBMVs was analyzed on 12% SDS-polyacrylamide gel (Figure 7). Results indicated that the protein characteristic profile from *S. exigua* BBMVs composes of 3 major protein bands. The two major protein bands were the bands with molecular weight of approximately 116-120 kDa and one major protein band was the band with molecular weight of approximately 43 kDa. The major protein bands observed at approximately 116-120 kDa could be aminopeptidase N which is a common membrane protein found in epithelium gut cells of all insects. A smaller protein at about 43 kDa would be a phosphatase which is also commonly found in gut cells of most insects.

BBMV proteins were separated using SDS-PAGE and transferred to nitrocellulose membrane by western blot. The binding signal between Vip3Aa and its binding protein was detected by probing with rabbit anti-Vip3A polyclonal primary antibody and goat anti-rabbit IgG alkaline phosphatase-conjugated secondary antibody, respectively. The internal controls of ligand blotting experiment were immobilized purified 6xHis-Vip3Aa and immobilized activated Vip3Aa. In order to investigate the background signal, which might be occurred from the binding between those two antibodies and BBMV proteins, the negative control (membrane C) without Vip3Aa overlay, but probed with rabbit anti-Vip3A polyclonal primary antibody and goat anti-rabbit IgG alkaline phosphatase-conjugated secondary antibody was performed.

The binding signal could be observed as shown in Figure 8A and Figure 8B. However, the non-specific binding signal could be detected in the negative control membrane (membrane C) (Figure 8C). Non-specific signals observed in Figure 6 and Figure 8 indicated that non-specific binding was occurred between the rabbit anti-Vip3A polyclonal antibody and *S. exigua*'s gut proteins. Thus, another type of antibody (monoclonal antibody) was used for the following experiments.

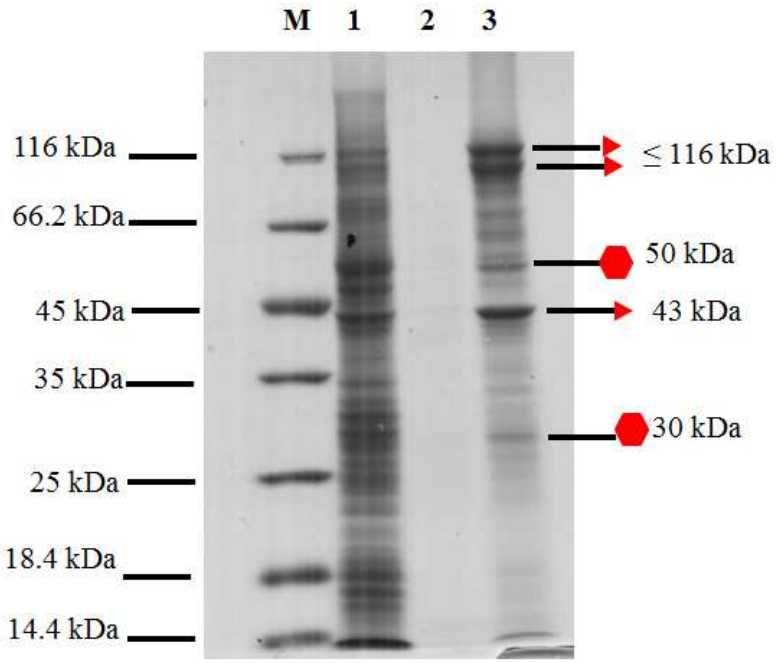


**Figure 7. Protein profile of BBMV isolated from *S. exigua* guts**

BBMVs from 3rd instar larvae of *S. exigua* were prepared by using differential magnesium precipitation method as described by Wolfersberger *et al.* (Wolfersberger *et al.*, 1987). After the centrifugation at 2500 x g, 4°C for 15 min, the supernatant was transferred to the ultracentrifuge tube for ultracentrifugation at 50,000 x g for 45 min. The final BBMVs pellet was resuspended in GET buffer containing 1x protease inhibitor cocktails. 20 µl of sample were loaded in each well.

M Protein molecular weight marker

Lane 1 Insoluble materials obtained after centrifugation at 2500 x g

Lane 2 Supernatant obtained after ultracentrifugation at 50,000 x g, 4°C for 45 min

Lane 3 BBMV proteins from *S. exigua* larval guts

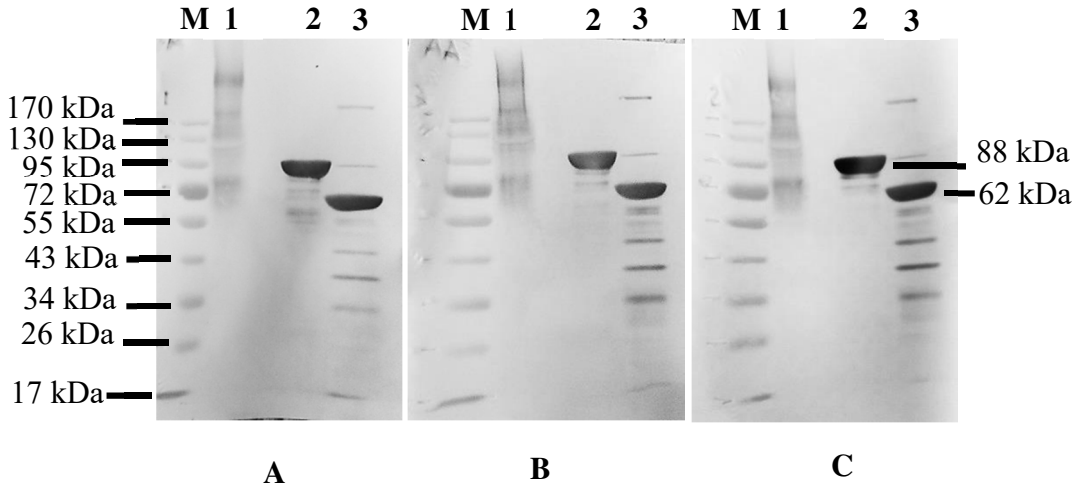


**Figure 8. Ligand blot results of BBMV extracted from isolated guts**

BBMV proteins from isolated guts were separated on SDS-PAGE (lane 1) and transferred to nitrocellulose membrane using western blotting. The internal controls were loaded with purified 6xHis-Vip3Aa (lane 2) and activated Vip3Aa (lane 3). The 20 µg/ml of Vip3Aa in blocking solution was overlaid for 1 hour at room temperature. Membrane **A was** overlaid with 6xHis-Vip3Aa. Membrane B was overlaid with activated Vip3Aa. Membrane C is a negative control without Vip3Aa overlay. M is a Prestained protein ladder**.**

Since results from the above experiments gave non-specific binding signal which could be arise from interaction between the primary antibody (rabbit anti-Vip3A polyclonal antibody) and the proteins from larval gut cells, another type of antibody (monoclonal antibody) was used for ligand blot analysis.

BBMV proteins were separated on SDS-PAGE and were transferred to nitrocellulose membrane by western blot. Binding of Vip3Aa to its binding proteins from the larval guts was detected by probing with anti-polyhistidine monoclonal antibody (HRP-conjugated). The internal controls of ligand blotting experiment were immobilized purified 6xHis-Vip3Aa and immobilized activated Vip3Aa. Three nitrocellulose membranes were used.

In order to investigate the background signal which might be occurred from the binding between this antibody and proteins from *S. exigua*'s BBMVs, the negative control (membrane B), which was overlaid with activated Vip3Aa (without His-tag) was performed. Results from Figure 9B showed that the observed protein band after probed the membrane with anti-polyhistidine monoclonal antibody (HRP conjugated) was the band corresponding to 6xHis-Vip3Aa (lane 2) whereas the protein band corresponding to the activated Vip3Aa (lane 3) could not be detected. However, no binding signal was detected in lane 1 which was loaded with BBMV proteins (Figure 9A and 9B). It is possible that the protoxin may not be able to bind to its receptor. Alternatively, the binding might occur but the detection sensitivity may not be high enough to be detected. The other negative control (membrane C), which was overlaid with 6xHis-Vip3Aa, but did not probe with anti-polyhistidine monoclonal antibody was performed and no signal was detected (Figure 9C).

According to Figure 9C, which was the membrane with immobilized *S. exigua*'s BBMV proteins that overlaid with 6xHis-Vip3Aa (protoxin), but did not probe with anti-polyhistidine monoclonal antibody, the result showed none of the protein band corresponding to 6xHis-Vip3Aa (protoxin) in lane 2. In contrast, the signal of protein band corresponding to 6xHis-Vip3Aa (protoxin) could be detected in lane 2 of the membrane of interest (Figure 9A). These results indicated that the detection system in this experiment was worked. Considering these results together (Figure 9A and Figure 9C), it could be implied that the 6x-His-Vip3Aa cannot bind to *S. exigua*'s BBMV proteins since there was no signal occurred in lane 1 of membrane A. However, other experiments are required to verify this possibility. Additionally, since one of the disadvantages of SDS-PAGE-ligand blotting technique is the limited volume of protein sample that could be loaded in each well

of SDS-polyacrylamide gel. Thus, dot blot analysis was done in order to confirm the binding results obtained from ligand blot analysis.

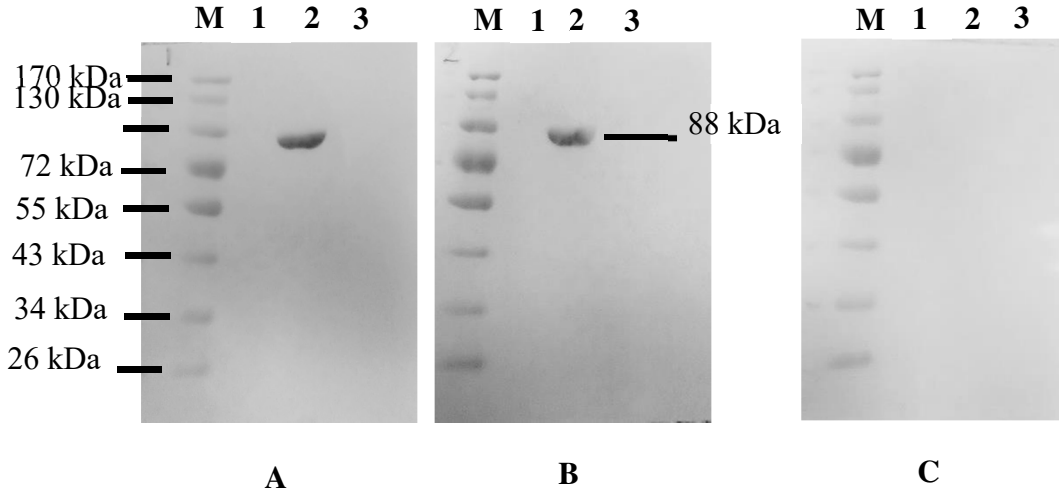


**Figure 9. Ligand blot results using anti-polyhistidine monoclonal antibody**

BBMV proteins were separated on SDS-PAGE (lane 1) and transferred to nitrocellulose membrane. The internal controls were immobilized purified 6xHis-Vip3Aa (lane 2) and immobilized activated Vip3Aa (lane 3). The 20 µg/ml of Vip3Aa in blocking solution was overlaid for 1 hour at room temperature. Membrane A was overlaid with 6xHis-Vip3Aa. Membrane B was overlaid with activated Vip3Aa. The negative control, membrane C was overlaid with 6xHis-Vip3Aa but was not probe with anti-polyhistidine monoclonal antibody. M is a prestained protein molecular ladder

Dot blot is one of the useful methods for studying protein-protein interactions without denaturing proteins, so this method was the good option for observing the binding between *S. exigua*'s BBMV proteins and 6xHis-Vip3Aa (protoxin) in non-denaturing condition. In addition, a large amount of protein sample could be applied on the nitrocellulose membrane enabling detection of low sensitivity signal.

Results from previous experiment demonstrated that the anti-polyhistidine monoclonal antibody was highly specific to polyhistidine-tagged protein. Thus, the 6xHis-Vip3Aa was used in dot blot analysis. First of all, the 225 µg of BBMVs from *S. exigua* were immobilized on nitrocellulose membrane by using Bio-Dot ™ apparatus (BIO-RAD). During the immobilization of BBMV proteins on nitrocellulose membrane, proteins are typically still retained their native conformation. Various amounts of 6xHis-Vip3Aa from 8-1000 ng were used as the internal control of dot blot analysis. The sensitivity test was performed to assess the ability of anti-polyhistidine monoclonal antibody to bind to different amounts of 6xHis-Vip3Aa. The result of sensitivity detection was summarized in Table 5. The lowest amount of the 6xHis-Vip3Aa that could be detected by this technique was 8 ng.

Results from dot blot analysis were shown in Figure 10. In order to investigate the background signal which might be occurred from the binding between this antibody and proteins from *S. exigua*'s BBMVs, the negative control (Figure 10B), which was not overlaid with 6xHis-Vip3Aa, was performed. The result from the negative control membrane at Row C; Figure 10B showed no binding signal. This result corresponded to results from the previous blotting (Figure 9B) and also corresponded to the fact that the protein containing polyhistidine does not exist in *S. exigua*. However, dot blot analysis revealed that no binding signal between *S. exigua*'s BBMV proteins and 6xHis-Vip3Aa occurred even though the maximum amount of immobilized *S. exigua*'s BBMVs was used (225 µg) (Figure 10A; Row C, Dot C1). This result corresponded to that obtained in Figure 9. Results from both experiments suggested that only the activated Vip3Aa could bind to its receptor whereas the 6xHis-Vip3Aa protoxin could not. Thus, only the activated Vip3Aa was used in subsequence experiments. In this work, the Two-Dimensional Polyacrylamide Gel Electrophoresis (2-D PAGE) and ligand blot analysis were chosen for studying the binding between *S. exigua*'s BBMV proteins and activated Vip3Aa.

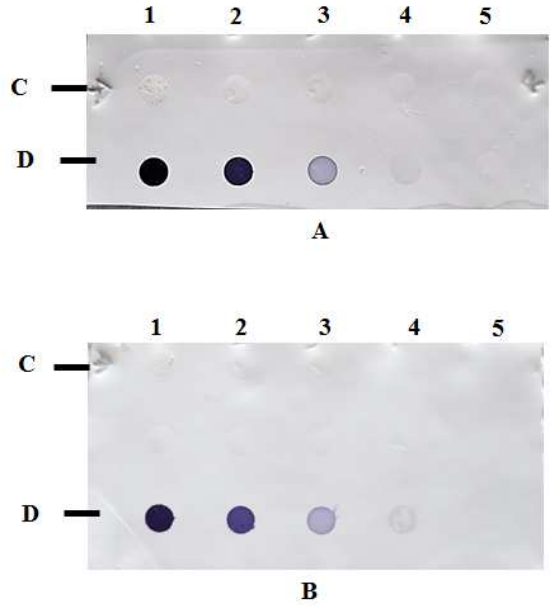


**Figure 10. Dot blot analysis of *S. exigua*'s BBMV proteins and 6xHis-Vip3Aa, using anti-polyhistidine monoclonal antibody [HIS-1] (HRP)**

The 225 µg of *S. exigua*'s BBMV proteins were dotted on the nitrocellulose membrane at room temperature by using Bio-Dot ™ apparatus (BIO-RAD). For the sensitivity test (Row D), 8-1000 ng of purified 6xHis-Vip3Aa was dotted on the nitrocellulose membrane. The internal negative control for BBMVs dot and purified 6xHis-Vip3Aa dot were GET buffer and tris-NaCl buffer pH 8.0, respectively. The 20 µg/ml of purified 6xHis-Vip3Aa in blocking solution was overlaid for 1 hour at room temperature. Two nitrocellulose membranes were used; a membrane overlaid with 6xHis-Vip3Aa (Figure 10A) and a negative control; a membrane without 6xHis-Vip3Aa overlay (Figure 10B)

Row C : dotted BBMV proteins from *S. exigua*

Lane C5 : dotted GET buffer

Row D : dotted purified 6xHis-Vip3Aa with various amounts

Lane D1 : dotted purified 6xHis-Vip3Aa 1000 ng

Lane D2 : dotted purified 6xHis-Vip3Aa 200 ng

Lane D3 : dotted purified 6xHis-Vip3Aa 40 ng

Lane D4 : dotted purified 6xHis-Vip3Aa 8 ng

Lane D5 : dotted tris-NaCl buffer pH 8.0

| Amount of 6xHis-Vip3Aa (ng) | The sensitivity signals |
|---|---|
| 1000 | ++++ |
| 200 | +++ |
| 40 | ++ |
| 8 | + |

**Table 5. The sensitivity test of purified 6xHis-Vip3Aa at various protein amounts**

8-1000 ng of purified 6xHis-Vip3Aa was dotted on the nitrocellulose membrane. The negative control for BBMVs dot and purified 6xHis-Vip3Aa dot were GET buffer and tris-NaCl buffer pH 8.0, respectively. The 20 µg/ml of purified 6xHis-Vip3Aa in blocking solution was overlaid for 1 hour at room temperature and the bound 6xHis-Vip3Aa was detected by probing with anti-polyhistidine monoclonal antibody (1:2000). The sensitivity signals were estimated by naked eyes. The ++++ signal represented the highest intensity signal whereas the + represented the lowest intensity signal.

### 4.2.3 Identification of Vip3A binding protein using 2D-gel electrophoresis

Several attempts have been made to separate the Vip3A binding protein from *S. exigua* larval gut cell membrane using SDS-PAGE. Unfortunately, those attempts failed to eliminate the background and unable to identify a specific binding signal. It might be possible to increase protein separation efficiency by using 2D-gel electrophoresis prior to the ligand blotting. Extraction of the membrane proteins from BBMV is also required to improve the resolution of proteins on the 2D-gel.

2D-Gel Electrophoresis is a technique used to separate proteins based on their isoelectric point at the first dimensional gel strip and separation based on their molecular weight at the second dimensional SDS-polyacrylamide gel. This technique is capable of extremely high resolution with proteins differing by a single charge being fractionated into separate bands. Isoelectric focusing can resolve proteins that differ in pI value by as little as 0.01.

Before performing 2D- gel electrophoresis, the *S. exigua*'s BBMV proteins were solubilized with lysis CT (CHAPS-Triton X-100) buffer. In this experiment, urea and thiourea were added in the reaction as the chaotropic agents and DTT was added in the reaction as a reducing agent. Triton X-100 and CHAPS in the reaction acted as a detergent to solubilized the proteins out of the lipid membrane. The protein profile after solubilization was analyzed on SDS-PAGE (Figure 11). After BBMV proteins had been solubilized with lysis CT buffer, some extra protein bands could be visualized and some remaining protein bands were appeared more intense when compared to those before solubilization. The extra and prominent protein bands appeared after solubilization with lysis CT buffer were the proteins with molecular weight of approximately 170 kDa, 110 kDa, and 105 kDa whereas the protein band at approximately 43 kDa was more intense after solubilization. In addition, the extra intense protein smeared at the top of the gel could be clearly observed.

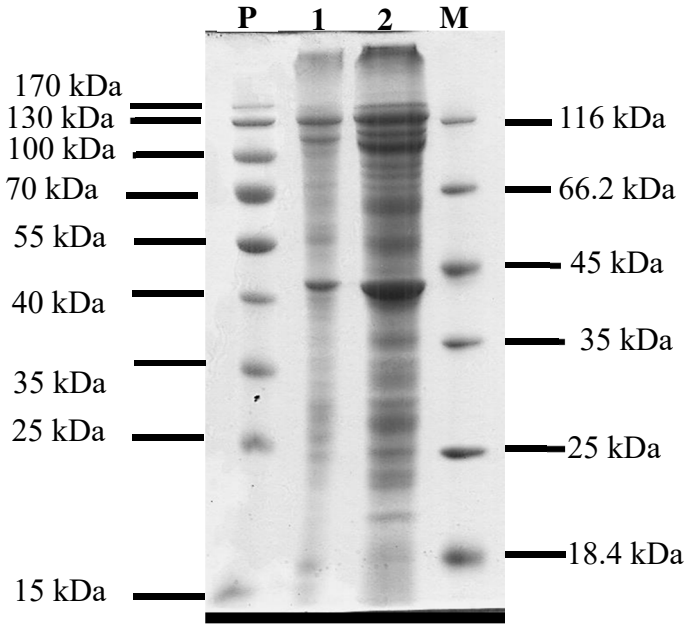


**Figure 11. Protein profile of solubilized BBMVs from *S. exigua* larvae**

*S. exigua* BBMVs were solubilized in CT buffer (CHAPS-Triton X-100) and loaded on 12% SDS-polyacrylamide gel.

P PageRuler ™ Plus Prestained Protein Ladder

M Protein molecular weight marker

Lane 1 BBMV proteins from *S. exigua* (before solubilization)

Lane 2 Solubilized BBMV proteins from *S. exigua*

The 150 µg of solubilized BBMV proteins were used for 2-D gel. In this experiment, the immobilized pH gradient gel strip size 7 cm, pH 3-10 NL (GE healthcare Immobiline™ Drystrip) was used for the first dimensional gel electrophoresis. The isoelectric focusing was conducted for 12,100 Volt-Hours after 12 hours passive in-gel rehydration. After the gel strip had equilibrated with 25 mM DTT following with 60 mM IAA, it was gently inserted into the top of 12.5% SDS-polyacrylamide gel (10 cm x 10.5 cm). The second dimensional gel electrophoresis was performed with a Tris-glycine buffer system using Amersham Biosciences Minive complete (GE Healthcare).

After 2-D PAGE had been done, the ligand blot was performed in order to detect the binding between BBMV proteins and activated Vip3Aa. The negative control in this experiment was the membrane containing immobilized BBMV proteins without Vip3Aa overlay. The bound activated Vip3Aa was detected by probing with rabbit anti-Vip3A polyclonal antibody and goat anti-rabbit HRP-conjugated polyclonal antibody, respectively. The immunoreactive signal on the membrane was detected by enhanced chemiluminescent detection system. The X-ray films (Kodak) were exposed for 3 min in the dark room and were developed by using Kodak Medical X-ray Processor. The exposed X-ray film was shown in Figure 12.

According to Figure 12C, after the exposed X-ray film from negative control (Figure 12B) had superimposed to the studied X-ray film (Figure 12A), the extra protein spots could be seen as shown in the circle area whereas the background of non-specific binding could be seen in the square area. The extra protein spots with molecular weight of approximately 40 and 100 kDa were ceased at high pI (pI more than 7.0) whereas the other protein spot with molecular weight of approximately 60 kDa was ceased at low pI (pI less than 7.0). The result from Figure 12C revealed that most of the extra protein spots occurred at the upper part of the gel. None of the interacted protein was observed at the lower part of the gel (below 35 kDa). These results gave some information about the Vip3Aa binding proteins from *S. exigua* gut cells which might involve with the medium to large molecular weight proteins.

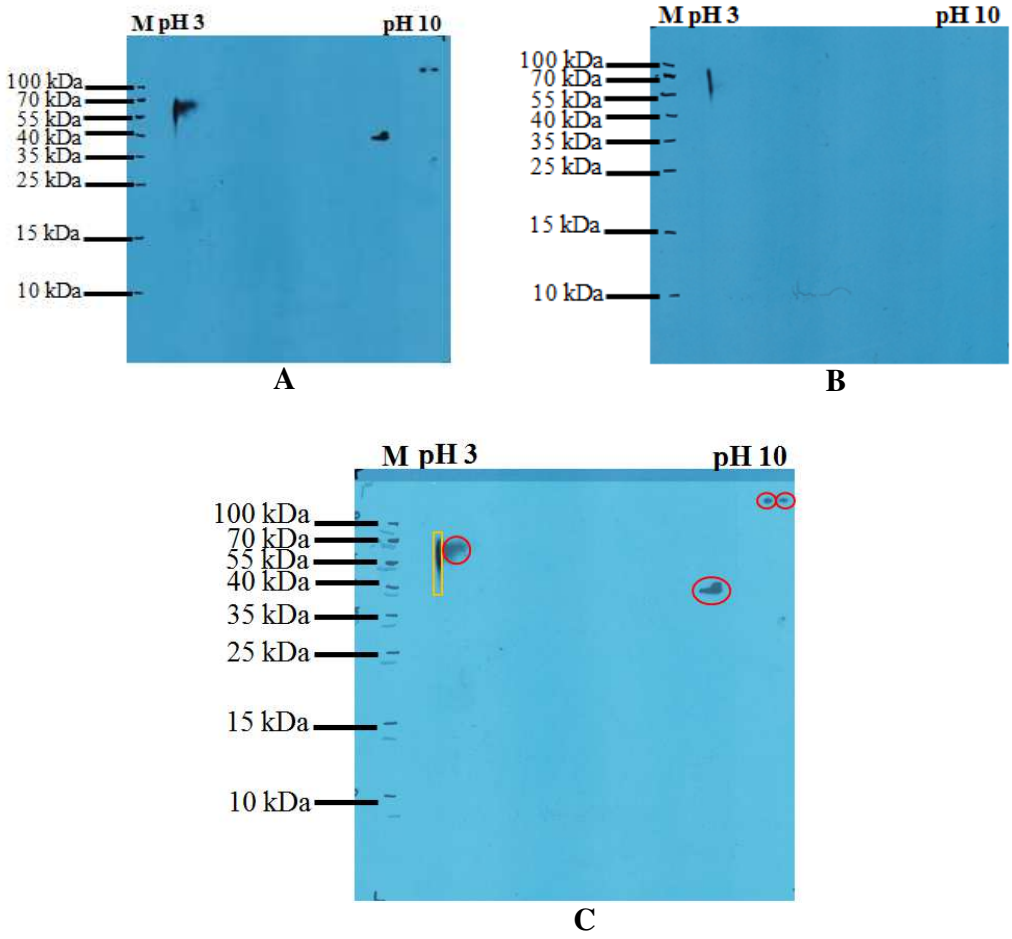


**Figure 12. Protein spots from 2-D gel and ligand blotting**

The 150 µg of solubilized BBMV proteins were applied on the immobilized pH gradient gel strip, pH 3-10 NL follow by SDS-PAGE. Proteins separated from 2D-gel were transferred to nitrocellulose membrane and the immunoreactive signal on the membrane was detected by enhanced chemiluminescent detection system. Two nitrocellulose membranes were used for film exposure; the first membrane was overlaid with activated Vip3Aa (Figure 12A) and the second membrane is a negative control without Vip3Aa overlay (Figure 12B). Images from both films were superimposed as shown in Figure 12C to identify the specific signal that appeared only in membrane A. Circle indicates extra spots observed only in membrane A and rectangular indicates non-specific signal found in both membrane. M represents PageRuler ™ Prestained Protein Ladder.

In order to confirm if the results obtained from the first 2D-gel experiment is reproducible, another 2D-gel was performed using 83 µg of solubilized BBMV proteins to apply on the gel. After completing 2D-gel and all proteins were transferred to nitrocellulose membrane, 40 ng of activated Vip3Aa was applied directly at the lower right part of each blotted nitrocellulose membrane as an internal control spot. The Coomassie blue G (CBG) stain was simultaneously performed with another 2D-gel that was run using identical sample and running conditions to those used for blotting.

Figure 13C shows superimposed images of the detection membrane (Figure 13A) to the negative membrane (Figure 13B). Extra protein spots could be seen as shown in the circle area whereas the background of non-specific binding could be seen in the square area. The extra spots with molecular weight of approximately 170 kDa (S1 and S2 spots) were ceased at low pI (pI less than 7.0). The spot of internal positive control (40 ng of activated Vip3Aa) was clearly seen at the right bottom of each membrane. The result from Figure 13C revealed that most of the extra spots occurred at the upper part of the gel similar to those found in the first 2D-gel experiment. None of the interacted protein was observed at the bottom of the gel (below 35 kDa). However, positions of these extra spots were not the same as those observed in the first 2D-gel. To locate the position of these spots on the CBG stained gel, image from the blotting membrane was superimposed to the CBG-stained gel (Figure 14), the extra spot S2 could be visualized as shown in the circle area whereas the S1 protein spot could not be detected in the CBG stained gel.

It was clearly observed that most of the signals obtained from 2D-gel and blotting were concentrated at low pH area and moved at very short distant in the second dimension gel. It could be possible that the membrane proteins used in these experiments were not well solubilized or remained clumping or associated as a large complex. Therefore, repetitive sonication of the solubilized BBMV proteins was performed for 40 minutes in order to increase the protein solubility and protein resolution at the isoelectric focusing step of the first dimensional gel electrophoresis. The protein concentration of solubilized BBMVs was quantified again by using Bradford method. The amount of total protein loaded on the gel was also reduced to 67 µg to improve the protein resolution.

Results from Figure 15C showed the extra spots in the circle area (S1 and S2 spot) whereas the background of non-specific binding could be seen in the square area. The obvious extra protein spot was the spot with molecular weight of approximately 170 kDa (S1), which was ceased at low pI (pI less than 7.0). However, the S2 spot could not be

completely differentiated from the negative control since the large intensity of background signal in the negative control appeared over the same area. Results from this experiment revealed that most of the extra spots occurred at the upper part of the gel. None of the interacted protein was observed at the bottom of the gel (below 35 kDa). These results were consistent to the results of the previous experiment (Figures 12 & 13). When superimposed signal from figure 26 to the CBG stained 2D-gel as shown in Figure 16, the extra protein spot S2 could be visualized as shown in the circle area whereas the S1 protein spot could not be detected by CBG staining. Considering all of these results together, it could be implied that the Vip3Aa binding proteins from *S. exigua*'s BBMVs are large molecular weight proteins.

Nevertheless, this experiment had to be repeated again in order to confirm the reproducibility since the protein spot of the internal positive control (40 ng of activated Vip3Aa) was not clearly seen at the right bottom of the studied X-ray film whereas it could be clearly detected on the negative control film. In addition, since the high molecular weight protein could be the Vip3Aa binding protein, a better protein separation around this region was required. In this experiment, the high molecular weight proteins, which have molecular weight greater than 100 kDa were not well separated. Considering these results from figure 12, 13 & 15 which had the problem about the resolution of high molecular weight proteins, the longer running time for the second dimensional gel electrophoresis is required. In addition, 10% SDS-PAGE would be used in the second dimensional gel electrophoresis in order to increase protein separation and transfer efficiency of high molecular weight proteins to nitrocellulose membrane. Thus, the clear binding signal between the high molecular weight proteins from *S. exigua* gut cell membrane to activated Vip3Aa could be achieved.

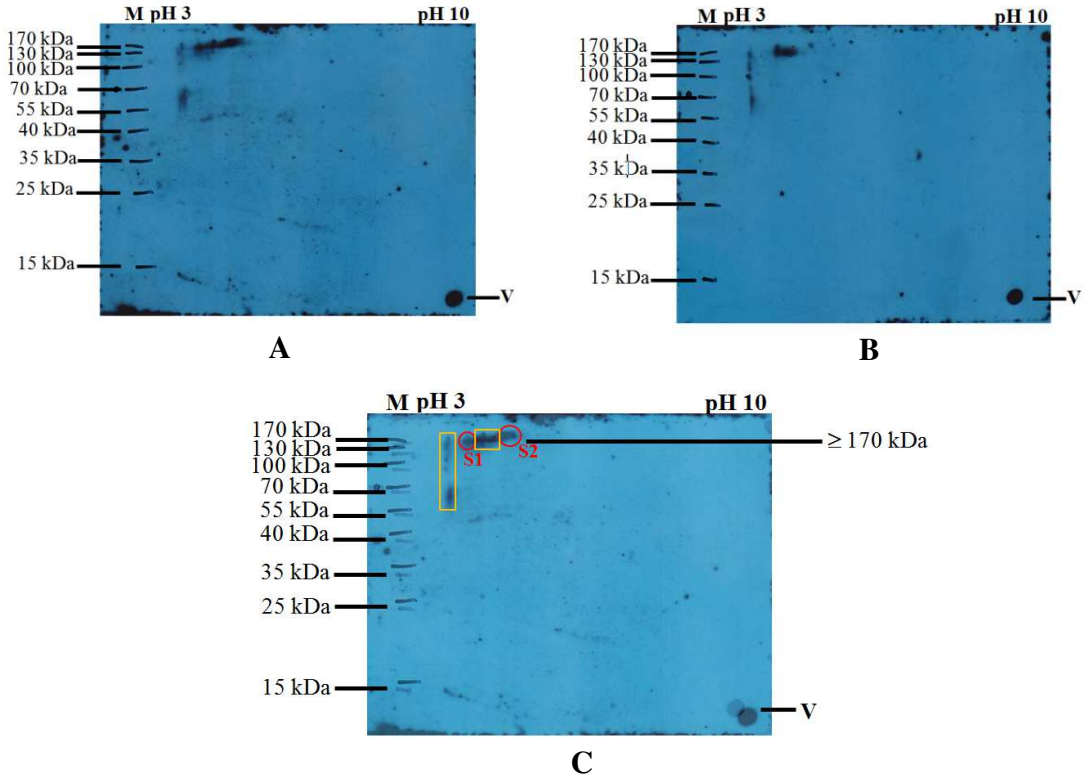


**Figure 13. Protein spots from 2D-gel and ligand blotting with Vip3A as an internal control**

The 83 µg of solubilized BBMV proteins were applied on the immobilized pH gradient gel strip, pH 3-10 NL. The internal positive control was 40 ng of dotted activated Vip3Aa located at the right bottom of each blotted nitrocellulose membrane. The immunoreactive signal on the membrane was detected by enhanced chemiluminescent detection system. Two nitrocellulose membranes were used for detection; the first membrane was overlaid with activated Vip3Aa (Figure 13A) and the second membrane use as a negative control without Vip3Aa overlay (Figure 13B). Images from both membranes were superimposed as shown in Figure 13C to identify the specific signal. Circle indicates the extra spots found only in the detection membrane and rectangular indicates the immunoreactive signal from non-specific binding. M represents PageRuler ™ Prestained Protein Ladder. V represents 40 ng of activated Vip3Aa applied directly on the nitrocellulose membrane as an internal control.

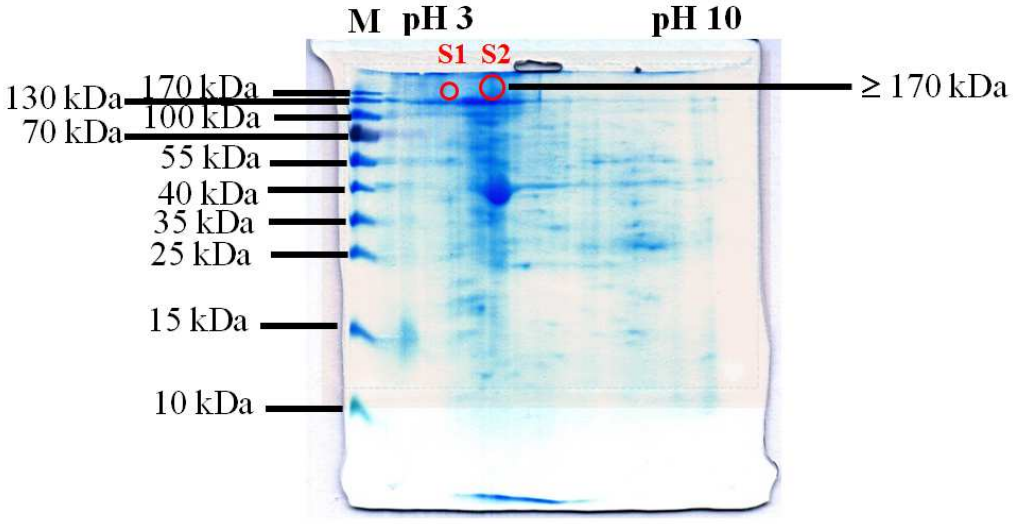


**Figure 14. Identification of a potential Vip3Aa-binding protein *S. exigua* gut cells**

Image from figure 24C was superimposed to the CBG stained gel. Positions of potential Vip3A-binding proteins are shown in circles (S1 and S2). M represents PageRuler ™ Prestained Protein Ladder.

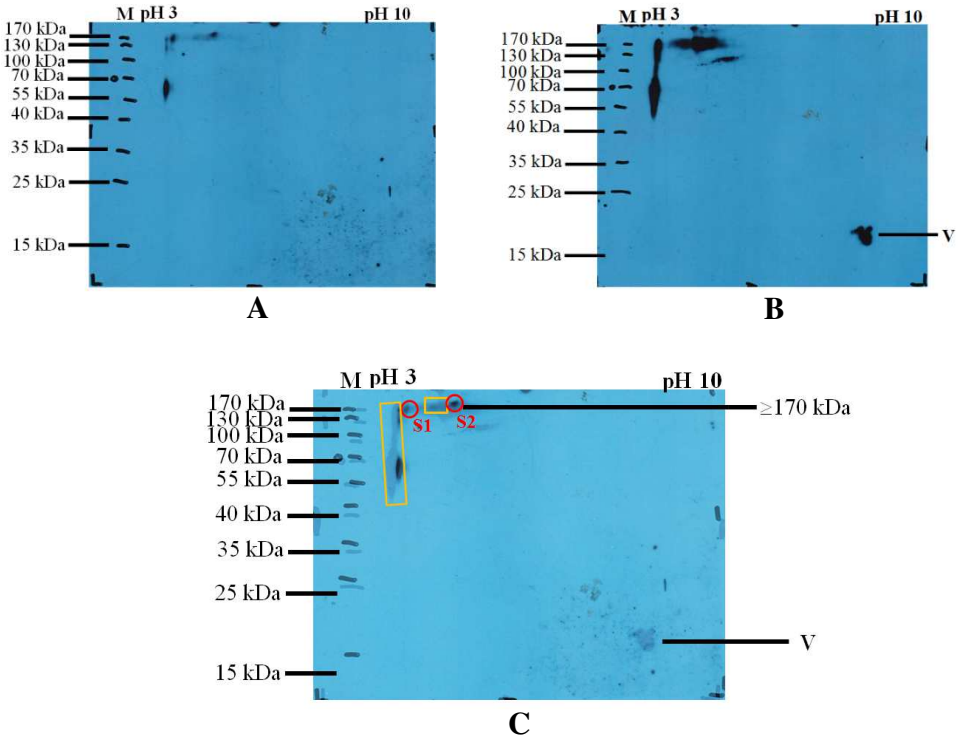


**Figure 15. Identification of Vip3A-binding protein from S. exigua gut cell membrane using 2D-gel and immunoblotting**

The 67 µg of the completely solubilized BBMV proteins were applied on the immobilized pH gradient gel strip, pH 3-10 NL. The internal positive control was 40 ng of dotted activated Vip3Aa located at the right bottom of each blotted nitrocellulose membrane. The immunoreactive signal on the membrane was detected by enhanced chemiluminescent detection system. Two nitrocellulose membranes were used for detection; the first membrane was overlaid with activated Vip3Aa (Figure 15A) and the second membrane was a negative control without Vip3Aa overlay (Figure 15B). Images from both membranes were superimposed as shown in Figure 15C. Circle indicates the extra spots on the detected membrane and rectangular indicates the non-specific binding signal. M represents PageRuler ™ Prestained Protein Ladder. V represents 40 ng of activated Vip3Aa applied directly on the nitrocellulose membrane.

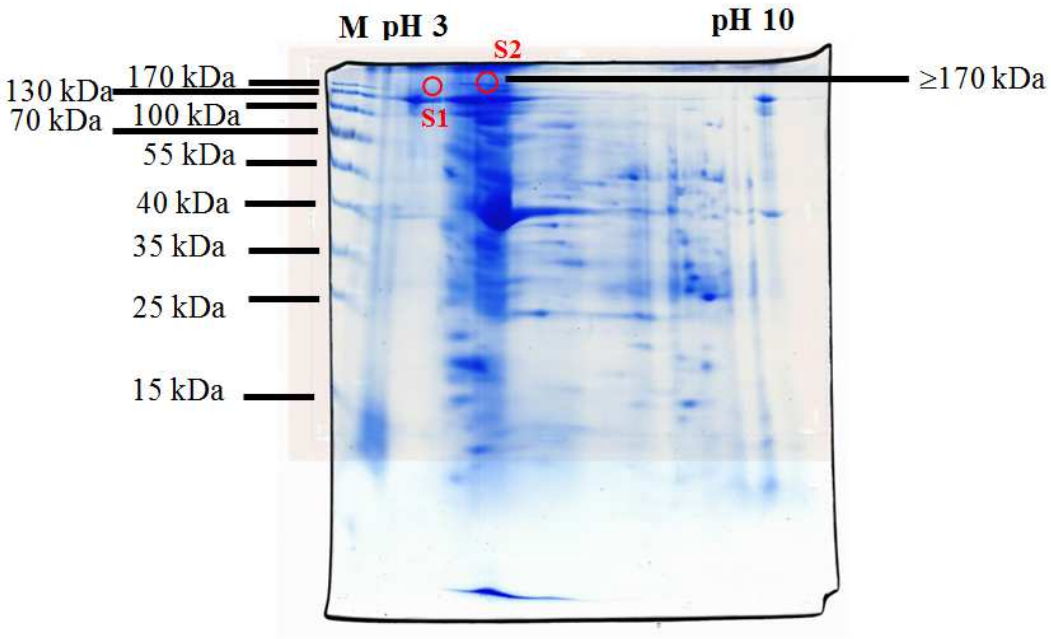


**Figure 16. Identification of Vip3Aa-binding spots from *S.exigua* gut cell membrane on CBG stained gel**

Image from figure 15C was superimposed to the CBG stained gel. Positions of potential Vip3A-binding proteins are shown in circles (S1 and S2). M represents PageRuler™ Prestained Protein Ladder.

In order to improve the resolution of the high molecular weight proteins on 2D-gel, concentration of polyacrylamide was reduced to 10% in the second dimension gel and the running time was increased to 100 minutes. In addition, the amount of total BBMV proteins was increased to 180 µg to improve the signal intensity. Results in figure 17 revealed extra spots as shown in the circle area whereas the background of non-specific binding could be seen in the square area. Figure 17C showed extra protein spots at S2, S4, S5 that were welled separated and other 3 spots located in the same area (A3). The S2 protein spot was the spot with molecular weight greater than 170 kDa, which was ceased at low pI (pI less than 7.0) whereas other two spots, which were S4 and S5, were the protein spots with molecular weight greater than or equal to 170 kDa, which were ceased at high pI (pI greater than 7.0). The extra protein spots in area A3 were proteins with molecular weight greater than or equal to 170 kDa, which were ceased at high pI (pI greater than 7.0). The result from Figure 17C revealed that most of the extra protein spots occurred at the upper part of the gel. None of the interacted protein was observed at the bottom of the gel (below 35 kDa). These results were consistent to the results of previous experiments (figures 12, 13 & 15). After superimposed the images from figure 17 to the CBG-stained 2-D polyacrylamide gel as shown in Figure 18, the extra protein spot S2 could be visualized as shown in the circle area whereas the other spots (S4, S5, and 3 spots in area A3) could not be detected by CBG staining. Although the S2 spot could be observed via CBG staining, the clear and distinct spot of S2 protein still could not be achieved from the CBG-stained-2-D polyacrylamide gel (Figure 18). Considering all of these results together, it could be implied that the Vip3Aa binding proteins from *S. exigua*'s BBMVs could be a large protein with high molecular weight ≥170 kDa and pI more than 7.

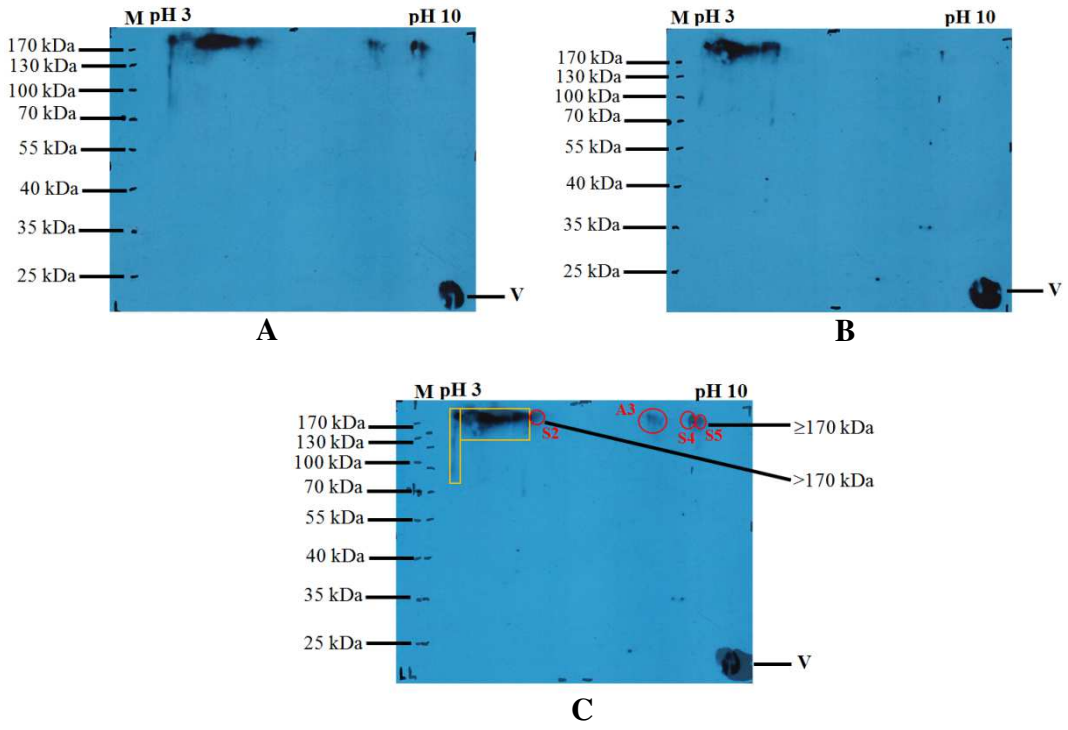


**Figure 17. Protein spots from 2D-gel and ligand blotting using 10% SDS-PAGE and longer running time**

The 180 µg of solubilized BBMV proteins were applied on the immobilized pH gradient gel strip, pH 3-10 NL. The internal positive control was 30 ng of dotted activated Vip3Aa located at the right bottom of each blotted nitrocellulose membrane. The immunoreactive signal on the membrane was detected by enhanced chemiluminescent detection system. Two nitrocellulose membranes were used for detection; the first membrane was overlaid with activated Vip3Aa (Figure 17A) and the second membrane was a negative control without Vip3Aa overlay (Figure 17B). Images from both membranes were superimposed as shown in Figure 17C. Circle indicates the extra spots on the detected membrane and rectangular indicates the non-specific binding signal. M represents PageRuler™ Prestained Protein Ladder. V represents 30 ng of activated Vip3Aa applied directly on the nitrocellulose membrane.

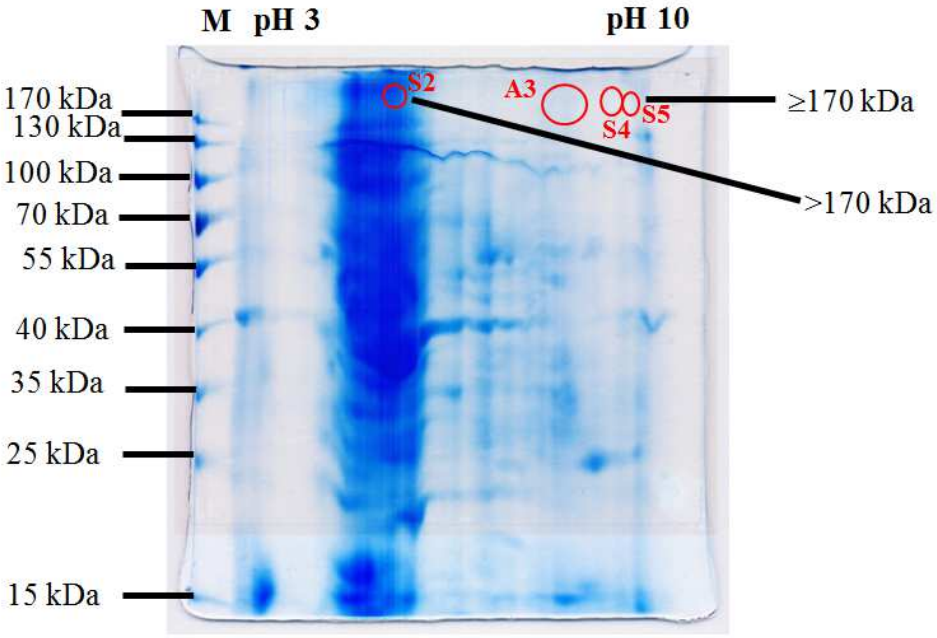


**Figure 18. Identification of Vip3Aa-binding protein spots from *S. exigua* larval gut cells on CBG-stained 2D-gel**

Image from figure 17C was superimposed to the CBG stained gel. Positions of potential Vip3A-binding proteins are shown in circles (S2, S4, S5 and A3). M represents PageRuler™ Prestained Protein Ladder.

### 4.3 Receptor binding module of Vip3A

The analysis of Vip3A protein sequences revealed the presence of a carbohydrate-binding motif (CBM) in all Vip3A proteins (Chakroun et al., 2016). The CBM spans from position 536 to a position near amino acid 652. Previous studies revealed that CBMs from many proteins bind to their ligands with high affinity and increase the efficiency of the catalytic components by targeting the enzymes to its substrate through interactions mediated by the carbohydrate-binding domain (Bae et al., 2008; Coutinho & Henrissat, 1999). Thus, CBM region presented in Vip3Aa might play a role in lepidopteran's receptor binding.

To date, a specific receptor for Vip3Aa has not been identified and the 3D structural of Vip3A has not been elucidated. To date, there is no experimental evidence to show the receptor binding motif in Vip3A. In this work, we have studied the effect of the C-terminal fragment of Vip3Aa consisting of amino acids D199-K789 (Vip3Aa-D199) on the larvicidal activity of its full-length protein. The full-length Vip3Aa and Vip3Aa-D199 were expressed in *E. coli* and their toxicity was tested against *S. litura* larvae. It was found that the larvicidal activity of the full-length toxin is inhibited by its C-terminal fragment. This could be due to the competitive binding to the same receptor on the insect gut cell membrane. Data obtained from this work suggests that the receptor binding region of Vip3Aa resides in its C-terminal region.

The full length and truncated vip3Aa genes were cloned in *E. coli* to be expressed as polyhistidine tag fusion proteins. Therefore, both proteins contain extra 20 amino acids at their N-terminus (Figure 19). It has been demonstrated that these extra amino acids did not affect larvicidal activity of the toxin (Kunthic et al., 2017). Result in Figure 20 showed that the full-length Vip3Aa was produced as an 88 kDa protein (lane 1) whereas the C-terminal fragment was observed as a major band approximately 62 kDa (lane 2). Both proteins were produced in soluble form when the cells were grown at 18-25°C. Proteins become accumulated as inclusion bodies when the induced culture was grown at 37°C. Expression level of the C-terminal fragment was significantly lower than that of the full-length protein. Similar results were found when 39 and 81 amino acids at the N-terminus were deleted from Vip3A (Chen et al., 2003b; Selvapandiyan et al., 2001). This suggests that amino acids in the N-terminal part are required for folding and stability of Vip3A.

MGSSHHHHHHSSGLVPRGSH

MNKNNTKLSTRALPSFIDYFNGIYGFATGIKDIMNMIFKTDTGGDLTLDEILKNQQLLND 60

ISGKLDGVNGSLNDLIAQGNLNTELSKEILKIANEQNQVLNDVNNKLDAINTMLRVYLPK 120

ITSMLSDVMKQNYALSLQIEYLSKQLQEISDKLDIINVNVLINSTLTEITPAYQRIKYVN 180

EKFEELTFATETSSKVKK**DGSPADILDELTELTELAKSVTKNDVDGFEFYLNTFHDVMVG** 240

**NNLFGRSALKTASELITKENVKTSGSEVGNVYNFLIVLTALQAKAFLTLTTCRKLLGLAD** 300

**IDYTSIMNEHLNKEKEEFRVNILPTLSNTFSNPNYAKVKGSDEDAKMIVEAKPGHALVGF** 360

**EISNDSITVLKVYEAKLKQNYQVDKDSLSEVIYGDMDKLLCPDQSEQIYYTNNIVFPNEY** 420

**VITKIDFTKKMKTLRYEVTANFYDSSTGEIDLNKKKVESSEAEYRTLSANDDGVYMPLGV** 480

**ISETFLTPINGFGLQADENSRLITLTCKSYLRELLLATDLSNKETKLIVPPSGFIKNIVE** 540

**NGSIEEDNLEPWKANNKNAYVDHTGGVNGTKALYVHKDGGISQFIGDKLKPKTEYVIQYT** 600

**VKGKPSIHLKDENTGYIHYEDTNNNLEDYQTITKRFTTGTDLKGVYLILKSQNGDEAWGD** 660

**NFIILEISPSEKLLSPELINTNNWTSTGSTNISGNTLTLYQGGRGILKQNLQLDSFSTYR** 720

**VYFSVSGDANVRIRNSREVLFEKRYMSGAKDVSEIFTTKLGKDNFYIELSQGNNLNGGPI** 780

**VKFSDVSIK** 789

**Figure 19.** Amino acid sequence of Vip3Aa (GenBank accession # GU733921)

The full-length Vip3Aa consists of 789 amino acids was produced in *E. coli* as a polyhistidine-tag fusion protein. Therefore 20 extra amino acids were added at its N-terminal end (underlined). The truncated fragment (Vip3Aa-D199) containing amino acid positions D199-K789 (**bold letters**) was also produced in *E. coli* with a polyhistidine-tag attached to its N-terminal end. The predicted carbohydrate binding motif (CBM) are highlighted. The protease cleavage site is indicated by an arrow.

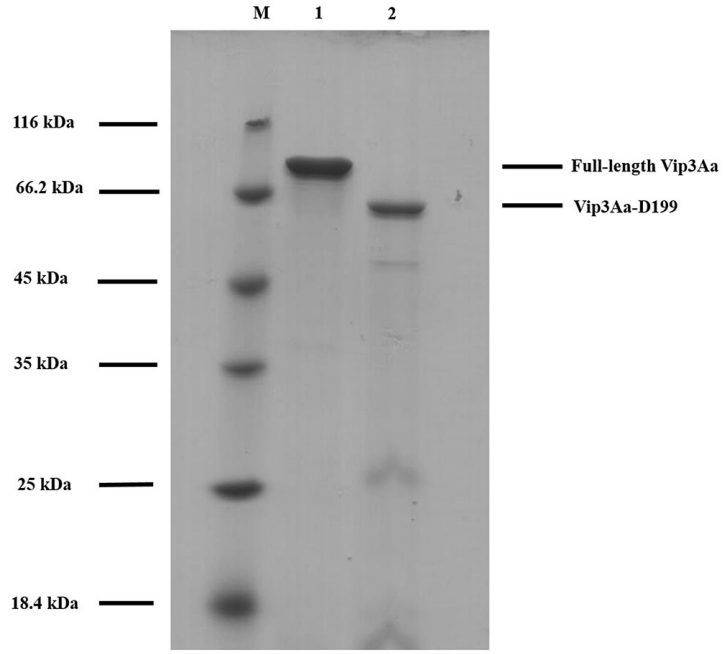


**Figure 20.** Protein profile of the full-length Vip3Aa and its C-terminal fragment (Vip3Aa-D199)

Full-length Vip3Aa and its truncated fragment were purified by Immobilized Metal ion Affinity Chromatography (IMAC). The fraction of full-length Vip3Aa (88 kDa) and its truncated fragment (62 kDa) was eluted from the column by using 250 mM and 100 mM imidazole as the eluent, respectively. Imidazole remained in concentrated protein was removed by gel filtration chromatography. The desalted full-length Vip3Aa and its N-terminally truncated fragment protein were analysed on 12% SDS-polyacrylamide gel. M: molecular weight markers; lane 1: the full-length Vip3Aa; lane 2: the truncated fragment of Vip3Aa (Vip3Aa-D199).

Mortality of the larvae fed with artificial diet overlaid with the toxin at various concentrations was recorded daily for 7 days. The full-length Vip3Aa exhibited high larvicidal activity against *S. litura* with $LD_{50}$ = 201 (138-301) ng/cm$^2$ and $LD_{95}$ = 1,097 (624-3,181) ng/cm$^2$ (fiducial limits at 95% confident are shown in parentheses). The $LT_{50}$ of the full-length Vip3Aa against *S. litura* larvae when fed the toxin at 200 ng/cm$^2$ = 2.3 (1.2-3.2) days and $LT_{95}$ = 4.7 (3.4-16.1) days. The fiducial limits at 95% confident are shown in parentheses. Exposure of *S. litura* larvae to Tris-NaCl buffer (negative control) did not cause mortality. No mortality was observed when *S. litura* larvae were fed with the C-terminal fragment (Vip3Aa-D199) up to 12,500 ng/cm$^2$. Therefore, this fragment is considered as an inactive protein.

To determine the effect of the truncated fragment of Vip3Aa on larvicidal activity of the full-length toxin, various mixtures of Vip3Aa and Vip3Aa-D199 were prepared and fed to 2$^{nd}$-instar *S. litura* larvae. Results in Table 6 showed that none of the larvae was killed when fed with Vip3Aa-D199 alone up to 5,000 ng/cm$^2$. Interestingly, the truncated fragment showed toxicity inhibition effect when fed together with the full-length toxin. The $LT_{50}$ and $LT_{95}$ when fed the larvae with the full-length toxin alone at 200 ng/cm$^2$ were only 2.3 and 4.7 days whereas these increased to 3.9 and 11.3 days when the larvae were fed with the mixture of Vip3Aa:Vip3Aa-D199 at 200:5,000 ng/cm$^2$ (1:25 w/w). Furthermore, the full-length Vip3Aa could kill the larvae more than 95% within 3 days after challenged the toxin. The C-terminal fragment effectively reduced mortality and the result was more prominent in the case of the mixture between the full-length and truncated toxins at 1:25 (w/w). Only 50% of the larvae were killed after 3 days and the mortality reached a plateau at 66.7% from days 4-7. Addition of Vip3Aa-D199 at lower ratios (1:1 and 1:5 w/w) showed less toxicity inhibition effect. The $LT_{50}$ for both mixtures increased about 1 day comparing to those fed with the full-length toxin alone and the mortality for both mixtures reached a plateau at about 80% from days 4-7.

Different growth rates of larvae were observed between those challenged with Vip3Aa alone and mixtures of Vip3Aa plus Vip3Aa-D199 (Figure 21). The larvae stopped feeding within a few hours after challenged with Vip3Aa. No more growth was detectable in this group and most of the larvae died within 2-3 days. In contrast, larvae that received the truncated toxin alone (Vip3Aa-D199 at 5,000 ng/cm$^2$) grew normally similar to those in negative control group that received Tris-NaCl buffer. The growth rate of larvae fed with the mixture of Vip3Aa and Vip3Aa-D199 was not significantly

different from the negative control group after challenged the toxin for 1 day. However, the growth rate of larvae in these groups was slower from day 2 onward comparing to that of the negative control.

| **Vip3Aa:Vip3Aa-D199** | | **Toxicity** | |
|---|---|---|---|
| **(ng/cm$^2$)** | **Ratio (w/w)** | **$LT_{50}$ (Days)** | **$LT_{95}$ (Days)** |
| 200 : 0 | - | 2.3 (1.2-3.2) | 4.7 (3.4-16.1) |
| 200 : 200 | 1 : 1 | 3.2 (2.0-4.4) | 8.7 (5.8-34.7) |
| 200 : 1,000 | 1 : 5 | 3.1 (2.2-4.0) | 6.9 (5.2-15.3) |
| 200 : 5,000 | 1 : 25 | 3.9 (3.3-4.5) | 11.3 (8.6-18.2) |
| 0 : 5,000 | - | NT | NT |

**Table 6.** Time required to kill 50% ($LT_{50}$) and 95% ($LT_{95}$) of the larvae fed with the mixtures between the full-length Vip3Aa and its C-terminal fragment (Vip3Aa-D199)

Mixture of Vip3Aa and Vip3Aa-D199 at 1:1, 1:5 and 1:25 (w/w) was overlaid on the surface of an artificial diet. The second instar *S. litura* larvae was placed in each well (1 larvae/ well) and kept at room temperature. The accumulated mortality was recorded daily for 7 days after feeding the mixture toxins. $LT_{50}$ and $LT_{95}$ were calculated from three independent experiments using Probit analysis (Finney, 1971). Fiducial limits at 95% confident are shown in parentheses. (NT: not toxic)

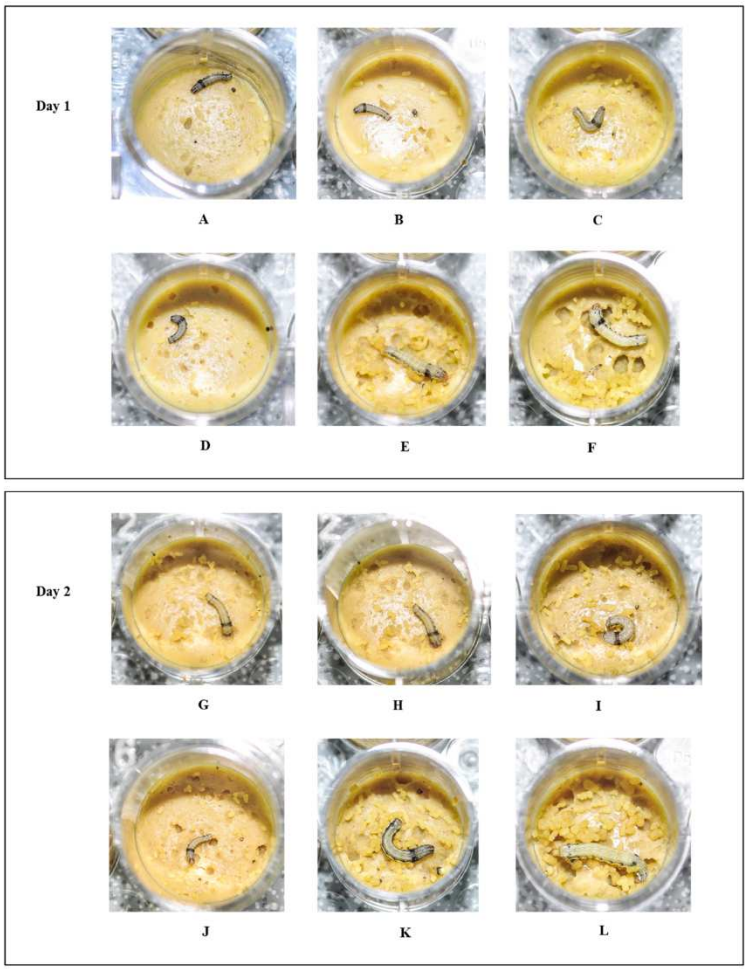


**Figure 21.** Photographs showing different growth rate of *S. litura* larvae after feeding artificial diet overlaid with the toxin mixture between the full-length Vip3Aa and its C-terminal fragment (Vip3Aa-D199) at 200:200 ng/cm$^2$ (1:1 w/w), 200:1000 ng/cm$^2$ (1:5 w/w) and 200:5,000 ng/cm$^2$ (1:25 w/w).

The full-length Vip3Aa was mixed with the truncated protein (Vip3Aa-D199) at 1:1, 1:5, 1:25 (w/w) and overlaid on the surface of the artificial diet and fed to second instar *S. litura* larvae. The growth rate of larvae in each group was monitored and recorded by taking photographs after the larvae were challenged with the toxin for 1 and 2 days. Larvae fed with the mixture toxin at 1:1 (w/w) are shown in figure A (at day 1) and G (at day 2) whereas the larvae fed with the mixture toxin at 1:5 (w/w) are shown in figure B (at day 1) and H (at day 2). Figure C and I show larvae that was fed with the mixture toxin at 1:25 (w/w) at day 1 and day 2, respectively. Larvae fed with the full-length Vip3Aa alone (200 ng/cm$^2$) are shown in figures D (at day 1) and J (at day 2). Figures E and K represent larvae fed the truncated fragment alone (5,000 ng/cm$^2$) at day 1 and day 2, respectively. Larvae from the negative control group (without toxin) are shown in figures F and L for day 1 and day 2, respectively.

The full-length Vip3Aa was produced in *E. coli* as an 88-kDa protein with polyhistidine-tagged at the N-terminus in order to facilitate protein purification by affinity chromatography. The purified Vip3Aa showed high toxicity to *S. litura* larvae with $LD_{50}$ about 200 ng/cm$^2$ comparable to those reported previously (Kunthic et al., 2017; Chen et al., 2003b). However, the truncated fragment (Vip3Aa-D199) was not toxic to *S. litura* larvae although high concentration of the truncated toxin was applied up to 12,500 ng/cm$^2$. This result is in agreement to those observed previously which indicated that the deletion of 81 amino acids from the N-terminus of Vip3A resulted in the total loss of toxicity against *S. litura* larvae (Chen et al., 2003b). Naturally, Vip3A is produced by B. thuringiensis and secreted into the culture media without N-terminal processing (Estruch et al., 1996; Li et al., 2007). The N-terminus part might be required for the correct folding or maintaining the protein structure. In this work, we found that the production level of Vip3Aa-D199 is lower than that of its full-length protein (Figure 20). These results suggests that some of the 198 amino acids at the N-terminal part play a role during protein folding or might have a crucial effect in protecting Vip3A from degradation by the host cell endogenous proteases.

There was a report demonstrating that deletion at the N-terminal part of Vip3Aa14 up to 33 amino acids did not alter protein production level and its toxicity against *S. litura* larvae (Chen et al., 2003b). Interestingly, deletion of 39 amino acids at the N-terminus significantly decreased Vip3A's toxicity (Selvapandiyan et al., 2001). The deletion of 81 amino acids from the N-terminus of Vip3A resulted in the total loss of toxicity against *S. litura* larvae (Chen et al., 2003b). Thus, the finding of a total loss in toxicity of the Vip3Aa-D199 in our work in which 198 amino acids were deleted from the N-terminus was not unexpected.

The molecular mechanism in which Vip3A toxin employed to kill the insect cells is not clearly understood and difficult to predict since the lack of the toxin structure information. Furthermore, the functional receptor for Vip3A on the target insect cell membrane has not been identified. Amino acid sequence analysis revealed that all Vip3 proteins share homology to a carbohydrate binding module, CBM_4-9 superfamily that recognizes sugar molecules similar to that found in mannanase A (Bae et al., 2008). This CBM-like region is located around amino acid positions 530-650 which are at the C-terminal part of all Vip3 proteins. It might be possible that amino acids in this region interact to a specific receptor on the insect gut cell membrane.

Several bacterial toxins including *B. thuringiensis* crystal proteins bind to a sugar moiety of some GPI-anchor glycoproteins to initiate their activity against susceptible insects (Schnepf et al., 1998; Fernandez et al., 2006; Knight et al., 2004; Valaitis et al., 2001). The predicted CBM region is present in the full-length Vip3Aa as well as in the truncated fragment (Vip3Aa-D199). Therefore both proteins could bind to the same receptor on the larval gut cell membrane as a competitive fashion. In absent of the truncated fragment, the full-length toxin could bind to the receptor more efficiently and exhibits its full toxicity. Addition of the Vip3Aa-D199 might compete for the receptor binding of the Vip3A thus reducing its toxicity. Alternatively, the toxicity inhibition effect might occur from inter-molecular interaction or binding between the truncated fragment and the full-length Vip3Aa. This interaction may lead to protein accumulation or forming a protein complex thus reducing the amount of the Vip3Aa to bind to its receptor on the cell membrane. The toxicity inhibition effect was more obvious up on increasing the ratio of Vip3Aa-D199. The larvicidal activity of Vip3Aa might be completely abolished if the mixture composed of Vip3Aa:Vip3Aa-D199 at 1:100 (w/w) or higher. However, we were unable to prepare the truncated toxin at that high concentration (e.g. Vip3Aa:Vip3Aa-D199 at 200:20,000 ng/cm$^2$).

### 4.4 Amino acids affecting receptor binding of Vip3A

Recently, we have successfully identified Vip3Ag2 from *B. thuringiensis* local isolate no. M191 (GenBank # FJ556803). The toxin is highly active against many lepidoteran larvae such as beet armyworm (*Spodoptera exigua*) and common cutworm (*Spodoptera litula*) which are important pests for major economic crops in Thailand. The toxin is produced and secreted at high level up to 800 mg/liter in rich media. There is a potential to develop this protein as an effective bio-insecticide and our group is currently pursuing this goal. Although Vip3A proteins are highly toxic to insect larvae including those resistant to Cry toxins, their specific receptors on the target insects are remained unidentified. Amino acid sequences of all Vip3A proteins are highly conserved and share no homology to any protein with known 3D structure in the database. However, a putative carbohydrate binding module (CBM) has been identified in all Vip3A proteins. This part could bind to a sugar moiety of a glycoprotein that acts as a receptor on the insect gut cell membrane similar to other insecticidal proteins such as *B. thuringiensis* Cry4B (Aroonkasorn et al., 2015)

Vip3Ag2 consists of 787 amino acids with molecular mass about 88 kDa (GenBank # ACL97352). A putative CBM is identified between amino acid positions 526-694. Homology modeling of this region using a CBM from *Thermoanaerobacterium polysaccharolyticum* ManA (PDB # 2ZEW; Bae et al., 2008), as a template suggests specific amino acids that could make interaction to a sugar molecule in glycoproteins. Amino acid replacements at ten selected positions in a putative CBM of Vip3Ag2 were employed to verify if these residues are required for its activity and play a key role during receptor binding.

The plasmid pET-Vip3Ag2 was constructed by inserting Vip3Ag2 gene from *B. thuringiensis* M191 into pET28b between *Nde*I and *Bam*HI sites. To produce Vip3Ag2 protein, the plasmid pET-Vip3Ag2 was transformed into *E. coli* BL21(DE3)pLysS. After induction with IPTG, the protein was detected in the cell supernatant suggesting that the protein was produced as a soluble protein. All ten mutant proteins were also produced as soluble proteins similar to the wild type (Figure 22). Western blot and immuno-detection using polyclonal antibody against Vip3Ag2 demonstrated that all mutants could react to the antibody (Figure 22). It should be noted that some mutants e.g. W552L, N623A and W684L were produced at lower level than the wild type. Other mutants were produced at similar level to the wild type. It remains to be confirmed if these mutants are sensitive to proteolytic digestion by the host cell proteases.

Bioassay results against *S. exigua* larvae were shown in Table 7. Seven mutants (P551A, E594A, Q598A, N623A, T631A, F636I and W691L) exhibited comparable larvicidal activity to the wild type. Three mutants (W552L, D616A and W684L) were unable to kill the larvae. All mutants are predicted to be located in the CBM region and should play a part during receptor binding. Our results suggested that single amino acid substitution at P551, E594, Q598A, N623, T631, F636 and W691 did not affect receptor binding. However, different positions might contribute differently and could be compensated by amino acids at other positions. Receptor binding is generally involved interactions of many residues. Therefore double and triple mutations are required to elucidate the receptor binding region of Vip3Ag2. Larvicidal activity was completely abolished when amino acid replacements were made at positions W552, D616 and W684. This result indicates that amino acids in these positions are essential for the toxin action. They could play a key role during toxin binding to the sugar moiety of the glycoprotein in the larval gut cell membrane. By analogy to *T. polysaccharolyticum* ManA (Bae et al.,

2008), W552, D616 and W684 in Vip3Ag2 might function similar to W20, Q81 and W125 in ManA in which they make direct interaction to a sugar residue. Nevertheless, structural alteration caused by the replacement in these positions should not be rule out for the loss of larvicidal activity. We are in the process to investigate the overall conformation of the wild type toxin and its mutants. Comparative binding between the wild type toxin and its mutants to BBMV from *S. exigua* larvae are also in progress.

**Table 7.** Larvicidal activity of the wild-type toxin and its mutants against *S. exigua* larvae.

Fifty µl of supernatant from cell lysate of *E. coli* expressing Vip3Ag2 was fed to $2^{nd}$-instar larvae and mortality was recorded after incubation at room temperature for seven days.

| Sample | % Mortality |
|---|---|
| Wild type | 100 |
| P551A | 100 |
| W552L | 0 |
| P551A | 100 |
| E594A | 100 |
| Q598A | 100 |
| D616A | 0 |
| N623A | 100 |
| T631A | 100 |
| F636I | 100 |
| W684L | 0 |
| W691L | 100 |

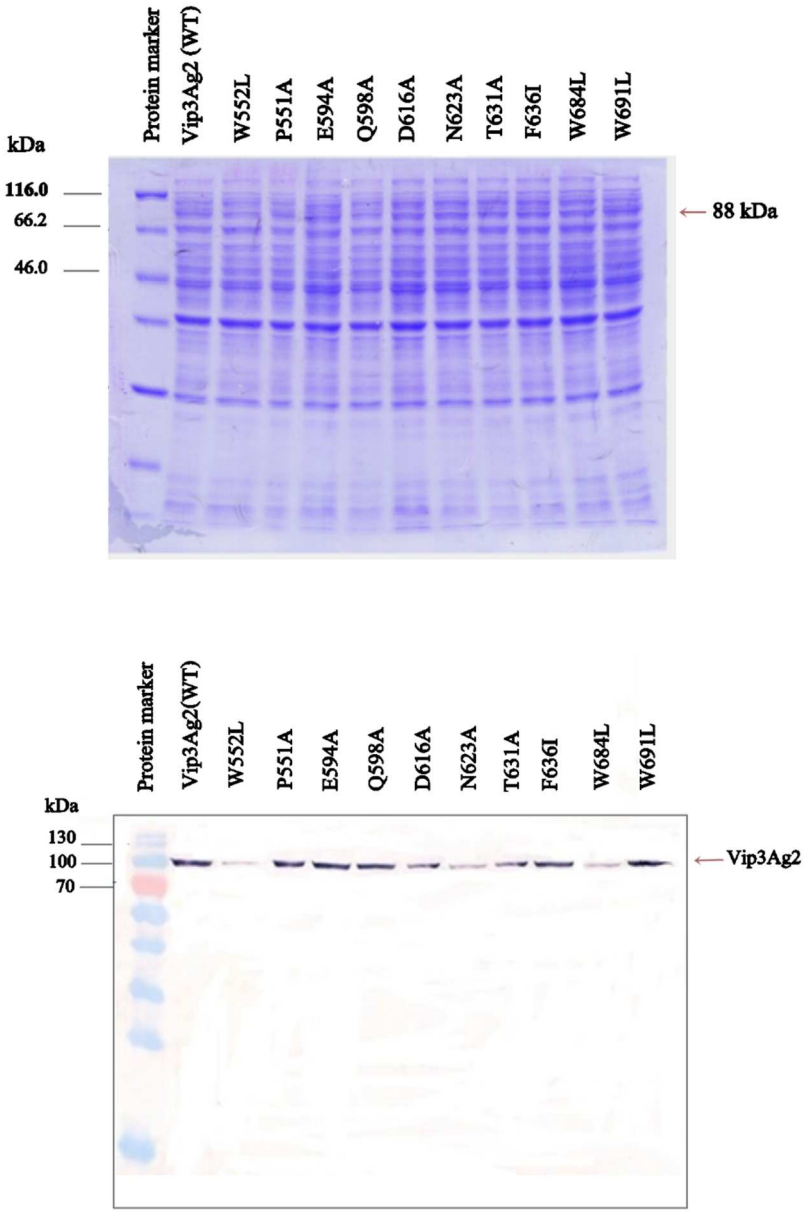


**Figure 22.** Production of Vip3Ag2

*E. coli* cells expressing the wild type Vip3Ag2 and its mutants were collected and lysed by ultrasonication. Soluble proteins from the cell lysate were collected after centrifugation and subjected to SDS-PAGE. Upper panel is a coomassie blue stained polyacrylamide gel and the lower panel is a western blot membrane detected by anti-Vip3Ag2 polyclonal antibody.

### 4.5 Comparison of amino acid sequences from different *B. thuringiensis* strains

Vip3 proteins are the most commonly found in several *B. thuringiensis* strains. More than 100 different *vip3* genes have been identified ranging from *vip3Aa*, *vip3Ab*, v*ip3Ac*,…to *vip3Ca* (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/vip.html). Different *B. thuringiensis* strains produce different Vip3 toxins and show specific activity against different insects [8]. All Vip3 proteins share high homology to each other but show no homology to any known protein. Therefore it is difficult to predict the toxin 3D structure and its molecular mechanism employed to kill the insect. Previous reports found that Vip3 can kill insects that are resistant to Cry toxins (Jackson et al., 2007). This suggests that Vip3 recognizes different receptor to that of the Cry toxin and uses different mechanism to kill the insect cells. This finding leads to application of both Cry & Vip3 in the field to prevent insect resistant development (Zhu et al., 2006; Chen et al., 2010).

Thailand is rich in biodiversity and should have a large pool of *B. thuringiensis* strains producing novel Vip3 proteins that have high activity against major insect pests. This work aimed to screen for new *vip3* genes from *B. thuringiensis* local isolates. The identified genes were cloned and expressed in *Escherichia coli*. The proteins were purified and their toxicity against insect larvae was demonstrated.

Preliminary data from Biocontrol Research Laboratory, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency indicates that about ¼ of *B. thuringiensis* local isolates collected from various locations in Thailand are able to produce Vip3 protein. In this work, 20 isolates were selected from a total of 1,000 isolates to screen for new *vip3* genes. PCR screening from the genomic DNA of these isolates using a pair of Vip3 screening primers (Table 1) revealed that all isolate carry *vip3* gene (Figure 23). Since the primers were designed from the highly conserved region for all *vip3* genes that give the same PCR product size at 1.6 kb (Hernandez-Rodriguez et al., 2009). It is not possible to identify the subtype of those *vip3* genes.

It has been shown that all *vip3* genes are highly conserved at the 5′-end and highly variable at the 3′-end. Therefore it is possible to distinguish these genes by PCR using different primers at the 3′-end. Second PCR reactions were performed using genomic DNA from those 20 isolates as templates with the universal forward primer designed for all *vip3* genes and reversed primers specific for different subtype of *vip3* genes (Table 1). This reaction could amplify the full-length *vip3* gene about 2.4 kb. Most

of the samples tested gave a positive result when using primer for *vip3Aa*. This suggests that most *B. thuringiensis* isolates contain *vip3Aa* gene in agreement with previous reports. Only isolates no.22 and no.107 gave PCR product when using primers specific to *vip3Af* and *vip3Ad*, respectively. PCR products from both reactions were cloned into a PCR cloning vector, pJET1_2 Blunt and transformed into *E. coli* JM109. DNA sequencing analysis revealed that isolate no.22 contains a 2,367-bp fragment that showed 99.7% identity to *vip3Af1* gene (GenBank #AJ872070). This fragment could be translated to a 788-amino acid protein that has 99.7% identity to the full-length Vip3Af protein. Isolate no.107 carried a 2,361-bp fragment that is very similar to *vip3Ad2* gene (99.9% identity, GenBank #AJ872071). Amino acid sequence of this fragment shows 99.6% identity to the full-length Vip3Ad2 protein. DNA and amino acid sequences of the new *vip3Af* (isolate no.22) and *vip3Ad* (isolates no.107) were deposited in GenBank under accession number **KX595192** and **KX595193**, respectively. These new Vip3Ad and Vip3Af show some variation from the prototype Vip3Aa mostly at the C-terminal part as shown in Figure 24.

The new *vip3Af* from isolate no.22 and *vip3Ad* from isolates no.107 were subcloned into pET28b in order to be expressed as 6xHis-tag fusion proteins. Both constructs were highly expressed in *E. coli* BL21(DE3)pLysS as soluble proteins. Both proteins could be purified using Ni-NTA affinity column (HisTrap FF column). The final yield of both proteins was comparable to that of the prototype toxin, Vip3Aa. It should be noted that both proteins could be eluted from the column when using buffer containing 100-250 mM imidazole although at 250 mM immidazole gave higher purity (Figure 25).

To access larvicidal activity, both proteins were fed to *S. exigua* and *S. litura* larvae. These two species are major insect pests that infest many important crops in Thailand e.g. grape, shallot, cabbage, kale, asparagus and other vegetables and fruits. Vip3Ad showed very low activity to both larvae even when used at very high concentration up to 40 μg/cm$^2$ (Table 8). The prototype toxin, Vip3Aa, was highly active against both larvae and could give 100% mortality when using the toxin more than 2 μg/cm$^2$. Vip3Af was active against *S. litura* more than *S. exigua*. Larvicidal activity of Vip3Af against *S. litura* was highly fluctuate and could give mortality up to 93.75% in one experiment and decreased to 43.75% in another experiment. Different lots of insect larvae may somehow contribute to this variation. Although our Vip3Ad and Vip3Af exhibited lower toxicity to *S. exigua* and *S. litura* comparing with that of the prototype

Vip3Aa, both toxins might have high activity against other insects. There was a report demonstrating that Vip3Af exhibited higher toxicity than Vip3Aa when tested against *Spodoptera frugiperda* (Hernandez-Martinez et al., 2013). Vip3Af also showed comparable toxicity to Vip3Aa when tested with some insects such as *Helicoverpa armigera*, *Mamestra brassicae*, *Spodoptera littoralis* and *Lobesia botrana* larvae (Ruiz et al., 2014). Vip3Ad was also showed some activity against *H. armigera* larvae (Ruiz et al., 2014). It is remain to be investigated if our new Vip3Ad and Vip3Af are toxic to those insects.

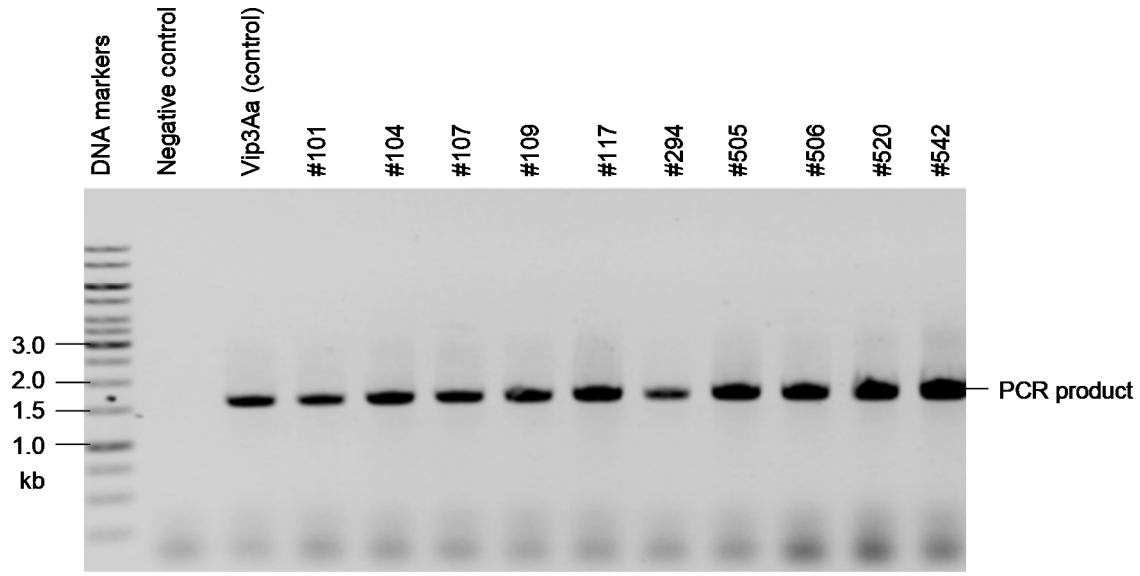


**Figure 23.** PCR products from reaction using genomic DNA from different *B. thuringiensis* isolates as templates with Vip3 screening primers. Lanes labeled with #101–#542 represent *B. thuringiensis* isolate number 101–542.

```
Vip3Ad      MNKNNTKLNARALPSFIDYFNGIYGFATGIKDIMNMIFKTDTGSNLTLDEILKNQQLLNE   60
Vip3Aa      MNKNNTKLSTRALPSFIDYFNGIYGFATGIKDIMNMIFKTDTGGDLTLDEILKNQQLLND   60
Vip3Af      MNKNNTKLNARALPSFIDYFNGIYGFATGIKDIMNMIFKTDTGGNLTLDEILKNQQLLNE   60
            ********.:**********************************.:**************:

Vip3Ad      ISGKLDGVNGSLNDLIAQGNLNTELAKQILKVANEQNQVLNDVNNKLDAINSMLKIYLPK   120
Vip3Aa      ISGKLDGVNGSLNDLIAQGNLNTELSKEILKIANEQNQVLNDVNNKLDAINTMLRVYLPK   120
Vip3Af      ISGKLDGVNGSLNDLIAQGNLNTELSKEILKIANEQNQVLNDVNNKLDAINTMLHIYLPK   120
            ************************:*:***:******************:**::****

Vip3Ad      ITSMLSDVMKQNYVLSLQIEYLSKQLQEISDKLDIINVNVLINSTLTEITPAYQRMKYVN   180
Vip3Aa      ITSMLSDVMKQNYALSLQIEYLSKQLQEISDKLDIINVNVLINSTLTEITPAYQRIKYVN   180
Vip3Af      ITSMLSDVMKQNYALSLQIEYLSKQLQEISDKLDIINVNVLINSTLTEITPAYQRIKYVN   180
            *************.*****************************************:****

Vip3Ad      EKFEELTFATETTLKVKKDSPPADILDELTELTELAKSVTKNDVDGFEFYLNTFHDVMVG   240
Vip3Aa      EKFEELTFATETSSKVKKDGSPADILDELTELTELAKSVTKNDVDGFEFYLNTFHDVMVG   240
Vip3Af      EKFEELTFATETTLKVKKDSSPADILDELTELTELAKSVTKNDVDGFEFYLNTFHDVMVG   240
            ************: *****. ************************************

Vip3Ad      NNLFGRSALKTASELIAKENVKTSGSEVGNVYNFLIVLTALQAKAFLTLTTCRKLLGLAD   300
Vip3Aa      NNLFGRSALKTASELITKENVKTSGSEVGNVYNFLIVLTALQAKAFLTLTTCRKLLGLAD   300
Vip3Af      NNLFGRSALKTASELIAKENVKTSGSEVGNVYNFLIVLTALQAKAFLTLTTCRKLLGLAD   300
            ****************:*********************************************

Vip3Ad      IDYTSIMNEHLNKEKEEFRVNILPTLSNTFSNPNYAKAKGSNEDTKMIVEAKPGYVLVGF   360
Vip3Aa      IDYTSIMNEHLNKEKEEFRVNILPTLSNTFSNPNYAKVKGSDEDAKMIVEAKPGHALVGF   360
Vip3Af      IDYTSIMNEHLNKEKEEFRVNILPTLSNTFSNPNYAKVKGSDEDAKMIVEAKPGHALVGF   360
            ************************************.***:**:*********:.****

Vip3Ad      EMSNDSITVLKAYQAKLKKDYQIDKDSLSEIIYSDTDKLLCPDQSEQIYYTKNIAFPNEY   420
Vip3Aa      EISNDSITVLKVYEAKLKQNYQVDKDSLSEVIYGDMDKLLCPDQSEQIYYTNNIVFPNEY   420
Vip3Af      EMSNDSITVLKVYEAKLKQNYQVDKDSLSEVIYGDTDKLLCPDQSEQIYYTNNIVFPNEY   420
            *:**********.*:****::**:*******:**.* ***************:**.*****

Vip3Ad      VITKIAFTKKMNSLRYEATANFYDSSTGDIDLNKTKVESSEAEYSMLKASDDEVYMPLGL   480
Vip3Aa      VITKIDFTKKMKTLRYEVTANFYDSSTGEIDLNKKKVESSEAEYRTLSANDDGVYMPLGV   480
Vip3Af      VITKIDFTKKMKTLRYEVTANFYDSSTGEIDLNKKKVESSEAEYRTLSANDDGVYMPLGV   480
            ***** *****::****.**********:*****.*********  *.*.** ******:

Vip3Ad      ISETFLNPINGFRLAVDENSRLVTLTCRSYLRETLLATDLNNKETKLIVPPNVFISNIVE   540
Vip3Aa      ISETFLTPINGFGLQADENSRLITLTCKSYLRELLLATDLSNKETKLIVPPSGFIKNIVE   540
Vip3Af      ISETFLTPINGFGLQADENSRLITLTCKSYLRELLLATDLSNKETKLIVPPSGFISNIVE   540
            ******.***** * .******:****:***** ******.**********. **.****

Vip3Ad      NGNIEMDTLEPWKANNENANVDYSGGVNGTRALYVHKDGEFSHFIGDKLKSKTEYLIRYI   600
Vip3Aa      NGSIEEDNLEPWKANNKNAYVDHTGGVNGTKALYVHKDGGISQFIGDKLKPKTEYVIQYT   600
Vip3Af      NGSIEEDNLEPWKANNKNAYVDHTGGVNGTKALYVHKDGGFSQFIGDKLKPKTEYVIQYT   600
            **.** *.********:** **::******:******** :*:******* ****:*:*

Vip3Ad      VKGKASIFLKDEKNENYIYEDTNNNLEDYQTITKRFTTGTDSTGVYLIFNSQNGDEAWGD   660
Vip3Aa      VKGKPSIHLKDENTGYIHYEDTNNNLEDYQTITKRFTTGTDLKGVYLILKSQNGDEAWGD   660
Vip3Af      VKGKPSIHLKDENTGYIHYEDTNNNLKDYQTITKRFTTGTDLKGVYLILKSQNGDEAWGD   660
            **** **.****:.    ********:************** .*****::**********

Vip3Ad      NFIILEISPCEKLLSPELIKTDKWNSTGSTYISDDRLTLYRGGRGILKQNLQLDGFSTYR   720
Vip3Aa      NFIILEISPSEKLLSPELINTNNWTSTGSTNISGNTLTLYQGGRGILKQNLQLDSFSTYR   720
Vip3Af      KFTILEIKPAEDLLSPELINPNSWITTPGASISGNKLFINLGTNGTFRQSLSLNSYSTYS   720
            :* ****.*.*.*******: :.* :* .: ** : * :  * .* ::*.*.*:.:***

Vip3Ad      VNFSVDGDANVRIRNSREVLLEKRYLN--RKGVSEMFTTKFDKDNFYVELSQGDNLGTS-   777
Vip3Aa      VYFSVSGDANVRIRNSREVLFEKRY-MSGAKDVSEIFTTKLGKDNFYIELSQGNNLNGGP   779
Vip3Af      ISFTASGPFNVTVRNSREVLFERSNLMSSTSHISGTFKTESNNTGLYVELSRRS--GGGG   778
            : *:..*  ** :*******:*:       . :*  *.*:  :  :*:***: .    .

Vip3Ad      -VHFYDFSIK     786
Vip3Aa      IVKFSDVSIK     789
Vip3Af      HISFENVSIK     788
             : * :.***
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**Figure 24.** Alignment of Vip3Aa, Vip3Ad and Vip3Af

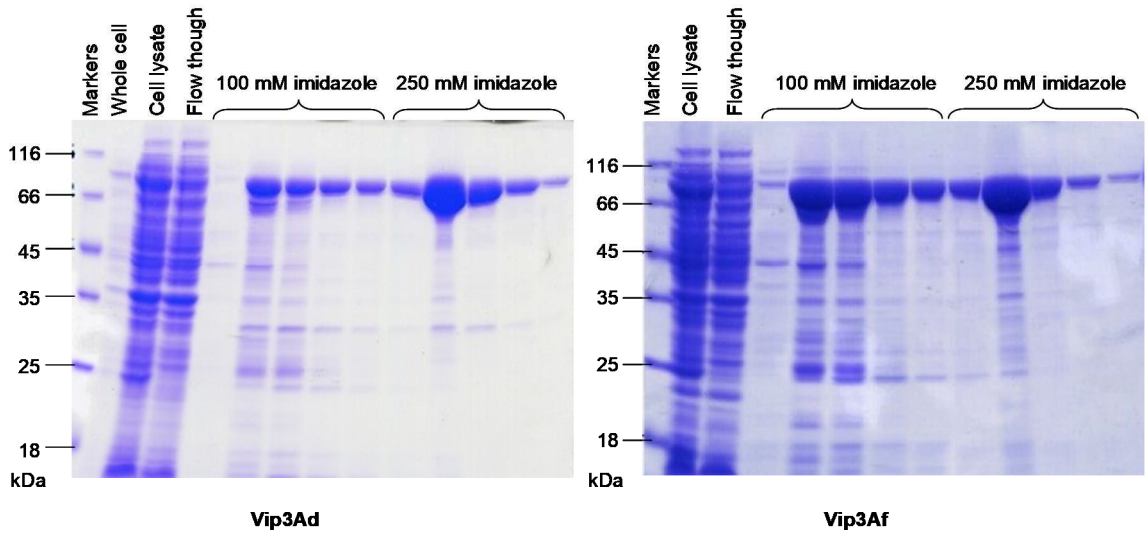



**Figure 25.** Coomassie blue stain of SDS-Polyacrylamide gel of the purified proteins from HisTrap FF column. Proteins were eluted in fraction containing 100-250 mM imidazole.

**Table 8.** Larvicidal activity of purified proteins against *S. exigua* and *S. litura* larvae

The purified protein was applied on the surface of artificial diet in each well. Two 2$^{nd}$-instar larvae were placed in each well and a total of 16 larvae were used for each sample. Three independent experiments were performed and mortality was recorded after 7 days.

| **Sample** | **% mortality (average ± SD)** | |
|---|---|---|
| | *S. exigua* | *S. litura* |
| 6xHis-Vip3Aa (2 µg/cm$^2$) | 83.33 ± 18.04 | 91.67 ± 9.55 |
| 6xHis-Vip3Ad (40 µg/cm$^2$) | 8.33 ± 7.22 | 2.08 ± 3.61 |
| 6xHis-Vip3Af (2 µg/cm$^2$) | 20.83 ± 3.61 | 60.42 ± 28.87 |
| PBS pH 7.4 (negative control) | 0 | 0 |

### 4.6 Amino acids affecting stability & activity of Vip3A

Initially, Vip3A from strain M190 in our *B. thuringiensis* collection was discovered to be effective for control of *S. exigua*. However, its application is limited by the protein's short half-life at high temperature. Therefore, we screened for *B. thuringiensis* strains that could produce Vip3A in large amounts and with higher thermostability, and identified Bt isolate 294 as expressing a protein with the desired qualities. Interestingly, both protein sequences are very similar, differing in only 9 residues, 6 of which cluster at the C terminus. Therefore, we further determined the amino acids crucial for higher stability of Vip3A produced from Bt294 through mutagenesis and evaluated larvicidal activity of mutants against *S. exigua* after high temperature storage.

It was initially observed that the culture media from *Bt* M190 contained a large amount of Vip3A protein that could be visualized by SDS-PAGE, migrating as a band around 88 kDa in size (Figure 26). The *vip3A* gene was cloned from *Bt*M190 to obtain its nucleotide sequence (GenBank accession no. GU733921), and the protein was designated as Vip3Aa35 in accordance with the Bt Toxin Nomenclature (Crickmore et al., 2016). Vip3Aa35 was the most abundant protein observed in culture media and its concentration was ~ 200 mg/l, high enough for direct application for plant protection. This culture media was also highly toxic against *S. exigua* larvae ($LC_{50}$ about 250 ng/cm$^2$), showing potential for use as a bioinsecticide. However, we noticed a protein band at ~ 62 kDa, presumably processed Vip3A, that was always detected accompanying the full-length protein (Fig. 1). The abundance of the 62-kDa band varied from batch to batch of *Bt* culture, and increased over time if the culture media had not been kept at -20 °C. This suggests that Vip3A is continuously processed by proteases from *Bt* to yield the 62-kDa fragment, which is approximately the same size with the active form of Vip3A.

To explore the application of Vip3Aa35 under Thai environmental conditions, culture media from Bt*M190* was stored at 37 °C to study the effect of high temperature on protein toxicity since such temperatures are unavoidable either during storage or application in the field. It was found that after 1 month of 37 °C storage, most of the 88-kDa protein was processed into 62 kDa and the culture media completely lost larvicidal activity (Figure 26). Therefore, although the high production level and toxicity of Vip3Aa35 render it a highly desirable bioinsecticide for control of lepidopteran pests, its application is limited by its low stability at high temperature.

With the low stability of Vip3Aa at high temperature limiting its application, 500 *Bt* isolates collected from various locations in Thailand were therefore screened for the presence of highly expressed, strongly toxic and thermostable Vip3A. Firstly, we looked for the presence of a protein band at 88 kDa, which were confirmed to be Vip3A by western blot analysis. From these, 78 isolates (~15%) produced Vip3A at high levels in culture media, with most of them (76 out of 78) being toxic to *S. exigua* larvae. Culture media from these strains were then tested for thermostability upon heat treatment. *Bt*M190 lost its biological activity after 30 min of heat treatment at 55 and 60 °C, but 37 strains retained larvicidal activity after the 55 °C incubation and 8 strains (*Bt* isolates 64, 81, 98, 117, 294, 501, 506, and 583) were still active after the 60 °C incubation. By SDS-PAGE analysis, all 8 strains produced Vip3A at high levels with moderate amounts of 62-kDa fragment and caused 100% larvae mortality at a protein concentration of 2 μg/cm$^2$ like *Bt* M190 (Figure 27). The 8 strains were further evaluated for stability after long storage by assessing the occurrence of processed protein and toxicity over a period of 3 months at 37 °C. Only *Bt*294 and *Bt*506 retained larvicidal activity after 2 months of storage, and only *Bt*294 was still active against *S. exigua* larvae after 3 months.

SDS-PAGE analysis revealed that the longer the storage period, the more processed Vip3A was detected (Figure 27). Noticeably, although almost all full-length Vip3A produced from *Bt*294 was processed into the 62-kDa fragment within 2 months of 37 °C storage, *Bt*294 still retained larvicidal activity. On the other hand, loss of toxicity of Vip3Aa35 from *Bt*M190 appeared to associate with occurrence of the 62-kDa processed protein, in which partially processed Vip3Aa35 resulted in slight toxicity loss (1-month storage) and almost completely processed Vip3Aa35 resulted in total loss of toxicity (2-month storage) (Figure 27A and 27B). The 62-kDa protein fragments of both Vip3A in culture media were analyzed by liquid chromatography mass-tandem spectrometry (LC-MS/MS), and were both found to be comprised of amino acid 199-789 (data not shown), consistent with the proteolytically processed 62-kDa active core produced from larvae gut juice (Chakroun et al., 2016). This suggests that both proteins were processed at the same position. However, Vip3Aa35 from *Bt*M190 loses its toxicity whereas Vip3A from *Bt*294 retains toxicity.

Due to the substantially higher stability of *Bt*294-derived Vip3A, the *vip3A* was cloned from *Bt*294 and analyzed. The protein was designated as Vip3Aa64 according to Bt toxin Nomenclature (Crickmore et al., 2016). The amino acid sequence of Vip3Aa64 is

identical to many previously described Vip3A, including Vip3A6, 7, 10, 11, 12, 15, 21, 33, 34, 35, 37, 43, 44, 55, and 58. Like Vip3Aa35, Vip3Aa64 is 789 amino acids in length, and both proteins share a highly similar sequence with only 9 amino acid differences. These amino acids are at positions 358, 536, 633, 755, 760, 761, 776, 782, and 784 (Figure 28A). Purified Vip3Aa64 exhibited about 8-fold higher toxicity than Vip3Aa35. Moreover, Vip3Aa64 also had higher stability after 37 °C storage compared to Vip3Aa35 (Figure 29), consistent with the results seen for native proteins produced from *Bt* cells. This implies that an intrinsic property of Vip3A proteins significantly influences their thermostability.

Vip3Aa35 and Vip3Aa64 have different amino acids at 9 positions, with 6 of them clustering at the C terminus of the protein. First, we wanted to determine whether amino acids located in the N terminus (positions 358, 536, and 633) or C terminus (positions 755, 760, 761, 776, 782, and 784) of Vip3A proteins are involved in protein stability. Thus, chimeric proteins were constructed where the N terminus (amino acids 1–705) and C terminus (amino acid 706–789) of the two proteins were exchanged (Figure 28B). While both recombinant proteins (N)3Aa64/3Aa35(C) and (N)3Aa35/3Aa64(C) showed similar toxicity against *S. exigua* larvae, only (N)3Aa35/3Aa64(C) exhibited high stability after 37 °C storage (Figure 29), suggesting that amino acids clustered at the C terminus of the protein were responsible for the higher stability of Vip3Aa64.

To identify residues important for Vip3Aa64 stability, site-directed mutagenesis was performed to individually replace residues in Vip3Aa35 with the corresponding amino acids from Vip3Aa64. Only the mutant Vip3Aa35:G761E completely lost larvicidal activity, whereas the others retained toxicity to the larvae, albeit with slightly increased $LC_{50}$ compared to Vip3Aa35 and Vip3Aa64. Most notably, the single mutant Vip3Aa35:N776Y acquired increased stability after 37 °C storage, illustrating that Tyr776 by itself contributed significantly to the higher thermostability of Vip3Aa35. To further confirm the significance of this residue, Tyr776 in Vip3Aa64 was substituted with the Asn of Vip3Aa35. As expected, Vip3Aa64:Y776N lost protein stability, exhibiting a Vip3Aa35-like rapid loss of larvicidal activity (Figure 30), thus validating the importance of this Tyr residue.

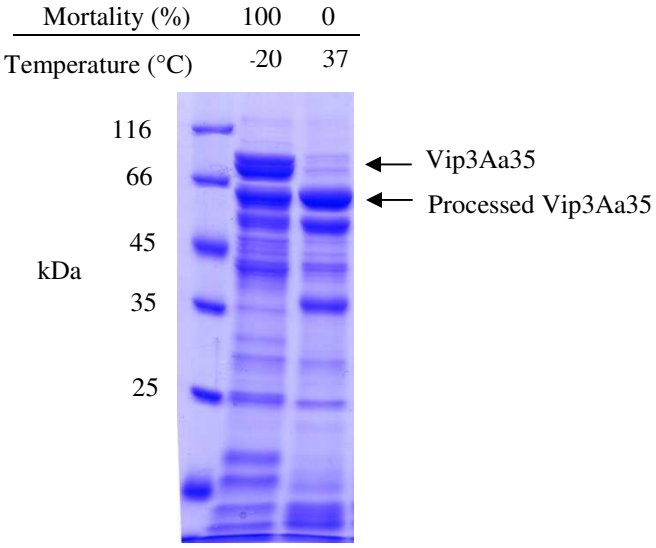
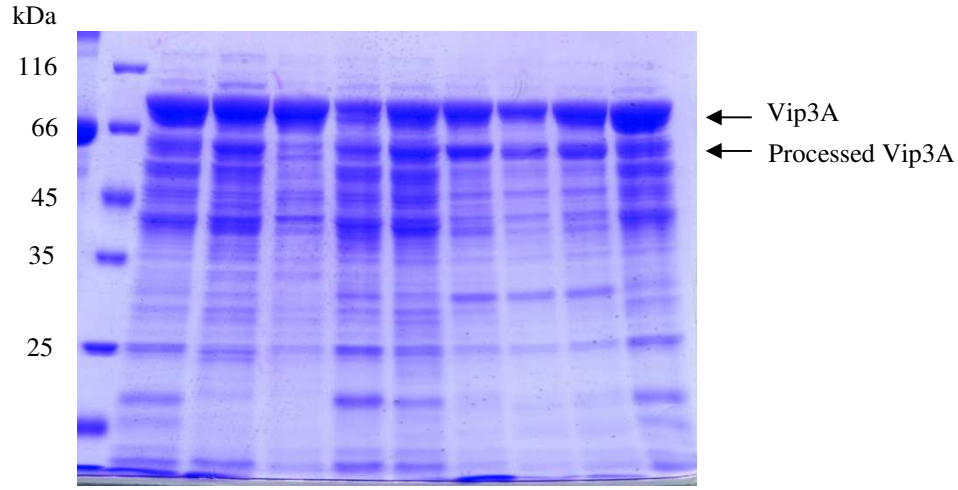



**Figure 26.** Vip3Aa35 stability in culture media after 1 month of 37 °C storage. The same batch of culture media containing Vip3Aa35 was separately kept at -20 °C and 37 °C for 1 month before SDS-PAGE analysis and larvicidal activity assay. The protein markers are shown on the left. Larvicidal activity against *S. exigua* is presented at the top. Vip3Aa35 was tested at a concentration of 2 μg/cm$^2$ for the -20 °C samples, and the same volumes of culture media were used for the 37 °C samples.

**A**.




**B**.

**Figure 27.** Vip3A production and larvicidal activity in culture media upon 37 °C storage for selected *Bt* strains. A) Prior to 37 °C storage, 15 µl of culture media were analyzed by SDS-PAGE. The protein marker lane is shown on the left. *Bt* strain numbers are indicated, with their corresponding larvicidal activity against *S. exigua* (2 µg/cm$^2$) after 37 °C storage over a period of 3 months. M represents the storage period in months. B) SDS-PAGE analysis of culture media from *Bt*M190 and *Bt*294 after storage at 37 °C. Protein markers are on the left.

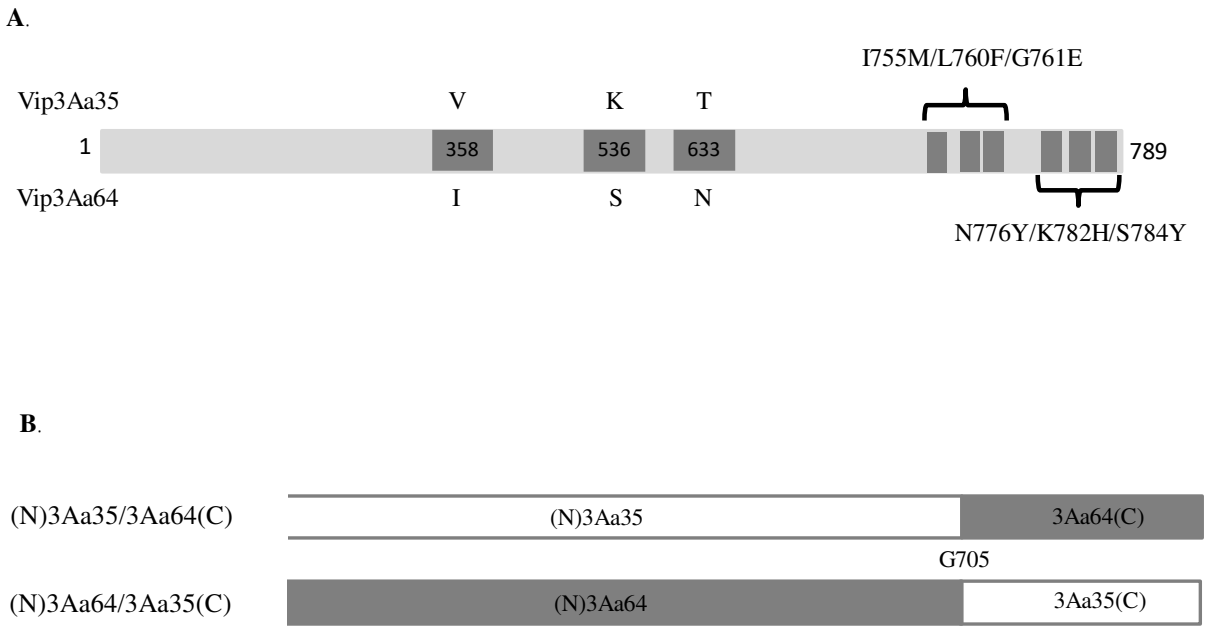



**Figure 28.** Diagrams of Vip3Aa35, Vip3Aa64, and their chimeras. A) The 9 differing amino acids between Vip3Aa35 and Vip3Aa64 are shown. For the C-terminal amino acids, the Vip3Aa35 residues are indicated in front of the position number, while the Vip3Aa64 residues are shown after the number. B) Illustration of gene fragment swapping between Vip3Aa35 and Vip3Aa64 based on restriction digest at position G705. N denotes the protein region covering amino acids 1–705 and C denotes the protein region covering amino acids 706–789.

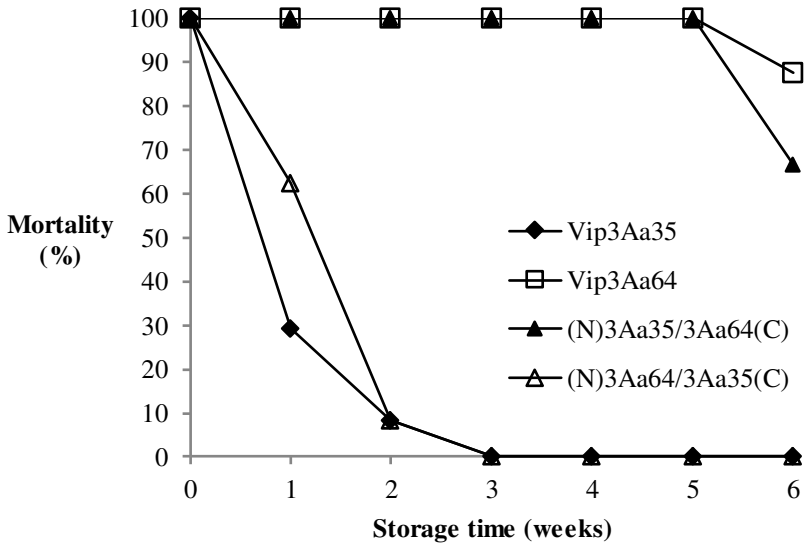



**Figure 29.** Activity of purified wild-type and chimeric Vip3A upon storage at 37 °C. Proteins were kept at 37 °C for 6 weeks and larvicidal activities against *S. exigua* were tested every week at a concentration of 2 μg/cm$^2$ to evaluate protein stability.

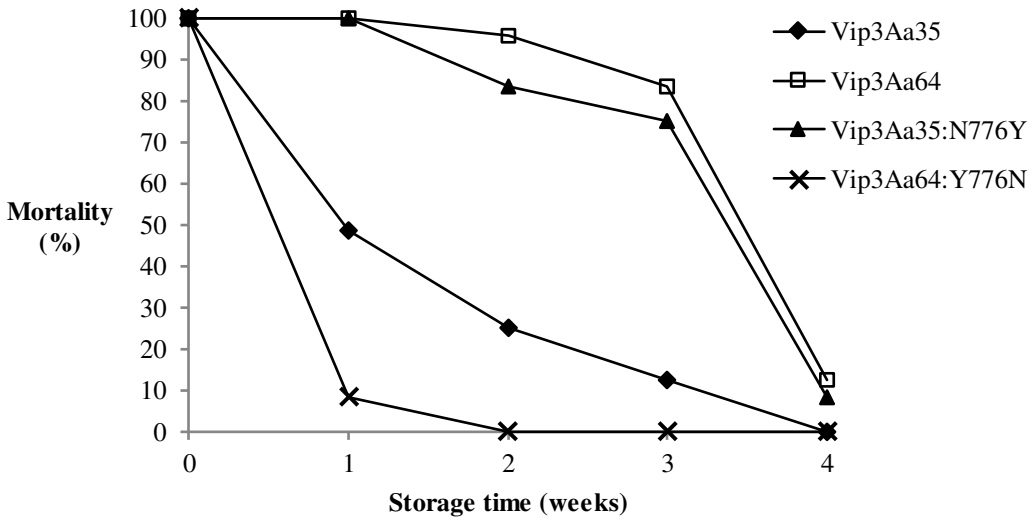



**Figure 30.** Activity of purified wild-type and mutant Vip3Aa35 and Vip3Aa64 upon storage at 37 °C. Proteins were kept at 37 °C for 4 weeks and larvicidal activities against *S. exigua* were tested every week at a concentration of 2 μg/cm$^2$ to evaluate protein stability.

**Table 9.** Larvicidal activity of Vip190 mutants after storage at 37˚C storage.

| Protein | $LC_{50}$ (ng/cm$^2$)[a] | Mortality (%)[b] | | | | |
|---|---|---|---|---|---|---|
| | | Week 0 | Week 2 | Week 4 | Week6 | Week 7 |
| Vip190 | 250 (206-298) | 100 | 41.6 | 4.2 | -[c] | - |
| Vip294 | 32 (11-74) | 100 | 100 | 100 | 100 | 83.3 |
| Vip190:I755M | 720 (210-1,910) | 93.8 | 8.3 | - | - | - |
| Vip190:L760F | 650 (550-770) | 95.8 | 4.2 | - | - | - |
| Vip190:G761E | >2000 | - | - | - | - | - |
| Vip190:N776Y | 110 (95-130) | 100 | 100 | 100 | 83.3 | 45.8 |
| Vip190:K782H | 265 (120-1,220) | 98.6 | 79.2 | 8.3 | - | - |
| Vip190:S784Y | 588 (310-1,320) | 95.8 | 12.5 | - | - | - |

[a] 95% confidence limits are shown in parenthesis from 3 replicates .

[b] Tested protein concentration for stability was 2 μg/cm$^2$.

[c] Not determined (no dead).

**Output from the project**

Detail of the following outputs are attached in appendices.

1. Identify a membrane protein about 170 kDa and pI about 7 from *S. exigua* larvae gut cells that could act as a Vip3A receptor.
2. Identify the C-terminal region in Vip3A that play important role during membrane binding and found that W552, D616 and W684 might play important role in the receptor binding. The manuscript in this topic is in preparation and is expected to submit to Journal of Invertebrate Pathology.
3. The C-terminal part of Vip3A could inhibit larvicidal activity of the full-length Vip3A when feeding as a mixture to *S. litura* larvae. This fragment might complete for the receptor binding. The manuscript in this topic is submitted to Biocontrol Science and Technology. This manuscript is under revision.
4. Different Vip3A show variable amino acid sequences at their C-terminal part resulted in selecting toxicity to different insects. This observation could be found in many Vip3A such as Vip3Aa, Vip3Ad and Vip3Af. This data have been published in Veridian e-Journal of Science and Technology

**References**

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# Appendices

1. Manuscript submitted to Biocontrol Science and Technology (under revision)
2. Manuscript published in Veridian Journal of Science and Technology (Nimsanor S., Chimpalee S., Rungrod A., Trakulnaleamsai C., Jongsareejit, B. and Promdonkoy B. (2016) Cloning and characterization of vegetative insecticidal protein (Vip3A) from Thai isolated *Bacillus thuringiensis*. Veridian 3(5); 228-236.)
3. Proceeding of the 11$^{th}$ International Symposium of the Protein Society of Thailand, 3-6 August 2016
4. DNA & amino acid sequences deposited in GenBank (Vip190, Vip294, Vip3Ad, Vip3Af)


# Larvicidal activity of *Bacillus thuringiensis* Vip3Aa is inhibited by its C-terminal fragment

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# Larvicidal activity of *Bacillus thuringiensis* Vip3Aa is inhibited by its C-terminal fragment

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**Running title:** Truncated Vip3Aa reduces toxicity of its full-length toxin

# Larvicidal activity of *Bacillus thuringiensis* Vip3Aa is inhibited by its C-terminal fragment


## Abstract

Vip3Aa is a vegetative insecticidal protein produced from *Bacillus thuringiensis* and is highly toxic to Lepidopteran larvae. The full-length Vip3Aa consists of 789 amino acids with molecular weight ~88 kDa. *In vitro* digestion of Vip3Aa by trypsin or insect gut juice proteases yields two fragments about 22 kDa and 66 kDa from its N- and C-termini, respectively. The protease cleavage site was identified after K198. Both fragments are held together and could not be separated under normal physiological condition. In order to investigate the role of the 66 kDa fragment, the gene encoding the C-terminal fragment corresponding to amino acids D199-K789 (assigned as Vip3Aa-D199) was cloned and expressed in *Escherichia coli*. The production level of the Vip3Aa-D199 was significantly decreased comparing to that of the full-length protein. Bioassays revealed that the Vip3Aa-D199 was not toxic to *Spodoptera litura* larvae when fed artificial diet overlaid with the protein up to 12,500 ng/cm$^2$ whereas the full-length Vip3Aa showed high toxicity with $LC_{50}$ about 200 ng/cm$^2$. Feeding the larvae with mixtures of the full-length Vip3Aa and Vip3Aa-D199 at different ratios (200:200, 200:1,000 and 200:5,000 ng/cm$^2$) revealed inhibition effect of the truncated toxin on the toxicity of the full-length toxin. Results showed that $LT_{50}$ and $LT_{95}$ of larvae fed the mixture toxins were longer than those fed the full-length toxin alone. The C-terminal fragment might compete for the membrane binding thus rendering activity of the full-length toxin. It is possible that the receptor binding motif of Vip3Aa resides in its C-terminal part.


**Keywords:** *Bacillus thuringiensis*, inhibition effect, larvicidal activity, *Spodoptera litura*, truncated protein, Vip3A

## Introduction

Vip3Aa is a vegetative insecticidal protein produced by some strains of *Bacillus thuringiensis* as a soluble protein during vegetative growth (Estruch et al., 1996; Chakroun et al., 2016). This protein is toxic against Lepidopteran larvae which are important insect pests for major crops worldwide. Vip3Aa is produced as a protoxin about 88-kDa and secreted into the culture medium without N-terminal processing (Li et al., 2007; Milne et al., 2008). After ingestion by susceptible larvae, the protoxin is activated by gut proteases to yield an active toxin that is able to bind to a specific receptor on the gut cell membrane and induces cell death (Abdelkefi-Mesrati et al., 2011a; Liu et al., 2011). The mode of action of Vip3A is not fully understood since its 3D structure has not been elucidated and the lack of information about a specific receptor for this toxin on the target insects.

*In vitro* activation using trypsin and insect gut juice proteases generated two major fragments approximately 21-22 kDa N-terminal fragment and 62-66 kDa C-terminal part (Kunthic et al., 2017; Selvapandiyan et al., 2001; Yu et al., 1997). The cleavage site was identified to be at the carboxyl terminal of Lys-198. When the C-terminal 66-kDa fragment was cloned and expressed in *E. coli*, no toxicity was observed against insect species previously found to be susceptible, suggesting that the 22-kDa N-terminal portion is necessary for toxicity or folding (Estruch et al., 1996; Chen et al., 2003b; Li et al., 2007). Previous studies indicated that deletion of 33 amino acids from the N-terminus of Vip3A expressed in *E. coli* did not alter its toxicity against *Spodoptera litura* larvae (Bhalla et al., 2005) whereas deletion of 39 and 81 amino acids from the N-terminus of Vip3A resulted in the total loss of toxicity against *S. litura* larvae (Chen et al., 2003b; Selvapandiyan et al., 2001). In addition, many evidences indicated that Vip3A sequence variation at C-terminus is the critical region responsible for its toxicity and host range (Fang et al., 2007).

After proteolytic activation had occurred, the binding to the brush border membrane of the larvae is started (Lee et al., 2003; Yu et al., 1997). The binding ability of Vip3A proteins to gut cells supposed to be a major factor of insect host range and insect specificity determination. Vip3A proteins did not share the same binding sites to Cry proteins. They have different putative receptors depending on different types of insects (Abdelkefi-Mesrati et al., 2011b). The progressive degeneration of the epithelial layer, gut paralysis, and complete lysis of gut epithelium cells due to the osmotic imbalance were occurred, leading to the death of susceptible larvae (Abdelkefi-Mesrati et al., 2011a; Yu et al., 1997).

The analysis of Vip3A protein sequences revealed the presence of a carbohydrate-binding motif (CBM) in all Vip3A proteins (Chakroun et al., 2016). The CBM spans from position 536 to a position near amino acid 652. Previous studies revealed that CBMs from many proteins bind to their ligands with high affinity and increase the efficiency of the catalytic components by targeting the enzymes to its substrate through interactions mediated by the carbohydrate-binding domain (Bae et al., 2008; Coutinho & Henrissat, 1999). Thus, CBM region presented in Vip3Aa might play a role in lepidopteran's receptor binding.

To date, a specific receptor for Vip3Aa has not been identified and the 3D structural of Vip3A has not been elucidated. To date, there is no experimental evidence to show the receptor binding motif in Vip3A. In this work, we have studied the effect of the C-terminal fragment of Vip3Aa consisting of amino acids D199-K789 (Vip3Aa-D199) on the larvicidal activity of its full-length protein. The full-length Vip3Aa and Vip3Aa-D199 were expressed in *E. coli* and their toxicity was tested against *S. litura* larvae. It was found that the larvicidal activity of the full-length toxin is inhibited by its C-terminal fragment. This could be due to the competitive binding to the same receptor on the insect gut cell membrane. Data obtained from this work suggests that the receptor binding region of Vip3Aa resides in its C-terminal region.

## Materials and Methods

### *Plasmid Construction*

The recombinant plasmid pET-Vip3Aa carrying the full length *vip3Aa* gene was as described previously (Kunthic et al., 2017). To constructed a plasmid for producing the C-terminal fragment of Vip3Aa, the 1.7 kb fragment of *vip3Aa* gene encoding amino acids D199-K789 was amplified by PCR using pET28-Vip3Aa as a template couple with forward primer: 5'-GCC ATA TGG ATG GCT CTC CTG CAG ATA TTC-3' and reverse primer: 5'-GCG GAT CCT TAC TTA ATA GAG ACA TCG GAA-3'. The underlined sequences indicate recognition sites, *Nde*I for the forward primer and *Bam*HI for the reversed primer. The PCR product was digested with *Nde*I and *Bam*HI and cloned into pET-28b between *Nde*I and *Bam*HI sites (**Figure 1**). This recombinant plasmid encodes a truncated fragment of Vip3Aa (D199-K789) with a polyhistidine tag at its N-terminus to facilitate protein purification. The protein was named "Vip3Aa-D199".

### *Protein production and purification*

*E. coli* BL21 (DE3) pLysS was used as the host strain for the expression of the recombinant truncated fragment and the full-length Vip3Aa under the control of T7 promoter. *E. coli* cells harboring pET28-Vip3Aa and pET28-Vip3Aa-D199 were grown in Luria-Bertani medium (LB broth) containing chloramphenicol 34 µg/ml and kanamycin 50 µg/ml and were incubated at 37°C with 200-250 rpm shaking for overnight. 0.7 ml of the overnight culture was inoculated in 700 ml of LB broth containing antibiotics as mentioned above and incubated at 37°C with 200-250 rpm shaking until the $OD_{600}$ reached 0.6-0.7 then the protein expression was induced by adding 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). The induced culture was grown for an additional 6 hours at 25°C with 200-250 rpm shaking.

*E. coli* cells were harvested by centrifugation at 10,000 g for 5 min at 4°C and the cell pellet was resuspended in 1xPBS pH 7.4. Proteins were released from *E. coli* cells by ultrasonication. Soluble and insoluble proteins were separated by centrifugation at 12,000 g at 4°C for 10 min. The truncated fragment (Vip3Aa-D199) and the full-length Vip3Aa were purified by Immobilized Metal ion Affinity Chromatography (IMAC) using HiTrap Chelating HP column (5 ml matrix volume, GE healthcare). In this experiment, $Ni^{2+}$ was used as a chelated ion. The truncated fragment of Vip3Aa were eluted from the column by 20 mM Tris pH 8.0, 300 mM NaCl supplemented with 100 mM imidazole whereas the full-length Vip3Aa was eluted from the column by 50 mM Tris pH 8.0, 200 mM NaCl supplemented with 250 mM imidazole. After that, the eluted protein was concentrated using Amicon® Ultra-15 centrifugal filter devices MWCO 30 kDa. Imidazole remained in concentrated protein was removed by gel filtration chromatography using HiTrap desalting column (GE healthcare). The column was pre-equilibrated with 20 mM Tris pH 8.0, 300 mM NaCl for the truncated fragment and pre-equilibrated with 50 mM Tris pH 8.0, 200 mM NaCl for the full-length Vip3Aa. Then, the protein was eluted from the column with flow rate 5 ml/min by using the equilibration buffer mentioned above and was stored at 4°C until required. The desalted protein was analysed on 12% SDS-PAGE. The protein was quantified by scanning the density of a protein band on the Coomassie blue strained polyacrylamide gel using Gene Tools software from Syngene version 4.03.02.0 and BSA was used as a standard.

***Larvicidal activity assay against Spodoptera litura***

The surface treatment bioassays were carried out using second instar larvae of *S. litura* obtained from the Nuclear Polyhedrosis Virus (NPV) Production Pilot Plant, National Center for Genetic Engineering and Biotechnology, Thailand. The concentration of the full-length Vip3Aa that kill 50% of larvae ($LD_{50}$) were determined. Various amounts (1000, 500,

250, 125, 62.5, 31.25, 15.63, 7.81, and 3.91 ng/cm$^2$) of the full-length Vip3Aa were applied over the surface of an artificial *S. litura* diet that was poured into polystyrene 24-well plate (Corning incorporated). The negative control wells were overlaid with 50 mM Tris-NaCl buffer pH 8.0. After the toxin was absorbed on the surface of the artificial diet, a second instar larvae of *S. litura* was placed in each well. A total of 12 larvae were used for each protein concentration and the larvae were kept at room temperature with 12/12 hr light/dark cycle and 60% relative humidity. Mortality was recorded daily for 7 days. Three independent experiments were performed and the toxin concentrations that kill 50% and 95% of the tested larvae ($LD_{50}$ and $LD_{95}$a) and time required to kill 50% and 95% of tested larvae ($LT_{50}$, and $LT_{95}$) were calculated using Probit analysis (Finney, 1971).

To evaluate the effect of the C-terminal fragment, the full-length Vip3Aa was mixed with the truncated protein at 1:1, 1:5, and 1:25 (w/w) as shown in Table 1 and fed to second instar *S. litura* larvae as described above. The full-length Vip3Aa at 200 ng/cm$^2$ was used as the positive control whereas 50 mM Tris-NaCl pH 8.0 buffer was used as the negative control. The experiment was performed at room temperature in 24 wells/treatment (1 larvae/well). The accumulative mortality was recorded daily for 7 days. $LT_{50}$ and $LT_{95}$ were calculated from three independent experiments using Probit analysis (Finney, 1971).

## Results

### *Production level of the full-length and truncated fragment of Vip3Aa*

The full length and truncated *vip3Aa* genes were cloned in *E. coli* to be expressed as polyhistidine tag fusion proteins. Therefore, both proteins contain extra 20 amino acids at their N-terminus (Supplement 1). It has been demonstrated that these extra amino acids did not affect larvicidal activity of the toxin (Kunthic et al., 2017). Result in **Figure 2** showed that the full-length Vip3Aa was produced as an 88 kDa protein (**lane 1**) whereas the C-

terminal fragment was observed as a major band approximately 62 kDa (**lane 2**). Both proteins were produced in soluble form when the cells were grown at 18-25°C. Proteins become accumulated as inclusion bodies when the induced culture was grown at 37°C. Expression level of the C-terminal fragment was significantly lower than that of the full-length protein. Similar results were found when 39 and 81 amino acids at the N-terminus were deleted from Vip3A (Chen et al., 2003b; Selvapandiyan et al., 2001). This suggests that amino acids in the N-terminal part are required for folding and stability of Vip3A.

***Larvicidal activity against Spodoptera litura***

Mortality of the larvae fed with artificial diet overlaid with the toxin at various concentrations was recorded daily for 7 days. The full-length Vip3Aa exhibited high larvicidal activity against *S. litura* with $LD_{50}$ = 201 (138-301) ng/cm$^2$ and $LD_{95}$ = 1,097 (624-3,181) ng/cm$^2$ (fiducial limits at 95% confident are shown in parentheses). The $LT_{50}$ of the full-length Vip3Aa against *S. litura* larvae when fed the toxin at 200 ng/cm$^2$ = 2.3 (1.2-3.2) days and $LT_{95}$ = 4.7 (3.4-16.1) days. The fiducial limits at 95% confident are shown in parentheses. Exposure of *S. litura* larvae to Tris-NaCl buffer (negative control) did not cause mortality. No mortality was observed when *S. litura* larvae were fed with the C-terminal fragment (Vip3Aa-D199) up to 12,500 ng/cm$^2$. Therefore, this fragment is considered as an inactive protein.

***Toxicity inhibition effect of the truncated fragment of Vip3Aa***

To determine the effect of the truncated fragment of Vip3Aa on larvicidal activity of the full-length toxin, various mixtures of Vip3Aa and Vip3Aa-D199 were prepared and fed to 2$^{nd}$-instar *S. litura* larvae. Results in **Table 1** showed that none of the larvae was dead when fed with Vip3Aa-D199 alone up to 5,000 ng/cm$^2$. Interestingly, the truncated fragment

showed toxicity inhibition effect when fed together with the full-length toxin. The $LT_{50}$ and $LT_{95}$ when fed the larvae with the full-length toxin alone at 200 ng/cm$^2$ were only 2.3 and 4.7 days whereas these increased to 3.9 and 11.3 days when the larvae were fed with the mixture of Vip3Aa:Vip3Aa-D199 at 200:5,000 ng/cm$^2$ (1:25 w/w). Furthermore, the full-length Vip3Aa could kill the larvae more than 95% within 3 days after challenged the toxin (Supplementary 2). The C-terminal fragment effectively reduced mortality and the result was more prominent in the case of the mixture between the full-length and truncated toxins at 1:25 (w/w). Only 50% of the larvae were killed after 3 days and the mortality reached a plateau at 66.7% from days 4-7. Addition of Vip3Aa-D199 at lower ratios (1:1 and 1:5 w/w) showed less toxicity inhibition effect. The $LT_{50}$ for both mixtures increased about 1 day comparing to those fed with the full-length toxin alone and the mortality for both mixtures reached a plateau at about 80% from days 4-7.

Different growth rates of larvae were observed between those challenged with Vip3Aa alone and mixtures of Vip3Aa plus Vip3Aa-D199 (Supplementary 3). The larvae stopped feeding within a few hours after challenged with Vip3Aa. No more growth was detectable in this group and most of the larvae died within 2-3 days. In contrast, larvae that received the truncated toxin alone (Vip3Aa-D199 at 5,000 ng/cm$^2$) grew normally similar to those in negative control group that received Tris-NaCl buffer. The growth rate of larvae fed with the mixture of Vip3Aa and Vip3Aa-D199 was not significantly different from the negative control group after challenged the toxin for 1 day. However, the growth rate of larvae in these groups was slower from day 2 onward comparing to that of the negative control.

## Discussion

The full-length Vip3Aa was produced in *E. coli* as an 88-kDa protein with polyhistidine-tagged at the N-terminus in order to facilitate protein purification by affinity chromatography. The purified Vip3Aa showed high toxicity to *S. litura* larvae with $LD_{50}$ about 200 ng/cm$^2$ comparable to those reported previously (Kunthic et al., 2017; Chen et al., 2003b). However, the truncated fragment (Vip3Aa-D199) was not toxic to *S. litura* larvae although high concentration of the truncated toxin was applied up to 12,500 ng/cm$^2$. This result is in agreement to those observed previously which indicated that the deletion of 81 amino acids from the N-terminus of Vip3A resulted in the total loss of toxicity against *S. litura* larvae (Chen et al., 2003b). Naturally, Vip3A is produced by *B. thuringiensis* and secreted into the culture media without N-terminal processing (Estruch et al., 1996; Li et al., 2007). The N-terminus part might be required for the correct folding or maintaining the protein structure. In this work, we found that the production level of Vip3Aa-D199 is lower than that of its full-length protein (**Figure 2**). These results suggests that some of the 198 amino acids at the N-terminal part play a role during protein folding or might have a crucial effect in protecting Vip3A from degradation by the host cell endogenous proteases.

There was a report demonstrating that deletion at the N-terminal part of Vip3Aa14 up to 33 amino acids did not alter protein production level and its toxicity against *S. litura* larvae (Chen et al., 2003b). Interestingly, deletion of 39 amino acids at the N-terminus significantly decreased Vip3A's toxicity (Selvapandiyan et al., 2001). The deletion of 81 amino acids from the N-terminus of Vip3A resulted in the total loss of toxicity against *S. litura* larvae (Chen et al., 2003b). Thus, the finding of a total loss in toxicity of the Vip3Aa-D199 in our work in which 198 amino acids were deleted from the N-terminus was not unexpected.

The molecular mechanism in which Vip3A toxin employed to kill the insect cells is not clearly understood and difficult to predict since the lack of the toxin structure information. Furthermore, the functional receptor for Vip3A on the target insect cell membrane has not been identified. Amino acid sequence analysis revealed that all Vip3 proteins share homology to a carbohydrate binding module, CBM_4-9 superfamily that recognizes sugar molecules similar to that found in mannanase A (Bae et al., 2008). This CBM-like region is located around amino acid positions 530-650 which are at the C-terminal part of all Vip3 proteins. It might be possible that amino acids in this region interact to a specific receptor on the insect gut cell membrane.

Several bacterial toxins including *B. thuringiensis* crystal proteins bind to a sugar moiety of some GPI-anchor glycoproteins to initiate their activity against susceptible insects (Schnepf et al., 1998; Fernandez et al., 2006; Knight et al., 2004; Valaitis et al., 2001). The predicted CBM region is present in the full-length Vip3Aa as well as in the truncated fragment (Vip3Aa-D199). Therefore both proteins could bind to the same receptor on the larval gut cell membrane as a competitive fashion. In absent of the truncated fragment, the full-length toxin could bind to the receptor more efficiently and exhibits its full toxicity. Addition of the Vip3Aa-D199 might compete for the receptor binding of the Vip3A thus reducing its toxicity. Alternatively, the toxicity inhibition effect might occur from inter-molecular interaction or binding between the truncated fragment and the full-length Vip3Aa. This interaction may lead to protein accumulation or forming a protein complex thus reducing the amount of the Vip3Aa to bind to its receptor on the cell membrane. The toxicity inhibition effect was more obvious up on increasing the ratio of Vip3Aa-D199. The larvicidal activity of Vip3Aa might be completely abolished if the mixture composed of Vip3Aa:Vip3Aa-D199 at 1:100 (w/w) or higher. However, we were unable to prepare the truncated toxin at that high concentration (e.g. Vip3Aa:Vip3Aa-D199 at 200:20,000 ng/cm$^2$).

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**Figure legends**

**Figure 1.** Recombinant plasmids of pET-28b containing *vip3Aa* gene

The 2.4 kb fragment of *vip3Aa35* gene (**A**) and 1.7 kb fragment of *vip3Aa-D199* gene (**B**) obtained from *Bacillus thuringiensis* strain M190 was inserted into the *Nde*I-*BamH*I sites of pET-28b expression vector under control of T7 promoter. This map was generated using a plasmid editor (ApE) programme.

**Figure 2.** Protein profile of the full-length Vip3Aa and its C-terminal fragment (Vip3Aa-D199)

Full-length Vip3Aa and its truncated fragment were purified by Immobilized Metal ion Affinity Chromatography (IMAC). The fraction of full-length Vip3Aa (88 kDa) and its truncated fragment (62 kDa) was eluted from the column by using 250 mM and 100 mM imidazole as the eluent, respectively. Imidazole remained in concentrated protein was removed by gel filtration chromatography. The desalted full-length Vip3Aa and its N-terminally truncated fragment protein were analysed on 12% SDS-polyacrylamide gel. M: molecular weight markers; lane 1: the full-length Vip3Aa; lane 2: the truncated fragment of Vip3Aa (Vip3Aa-D199).

**Table 1.** Time required to kill 50% ($LT_{50}$) and 95% ($LT_{95}$) of the larvae fed with the mixtures between the full-length Vip3Aa and its C-terminal fragment (Vip3Aa-D199)

Mixture of Vip3Aa and Vip3Aa-D199 at 1:1, 1:5 and 1:25 (w/w) was overlaid on the surface of an artificial diet. The second instar *S. litura* larvae was placed in each well (1 larvae/ well) and kept at room temperature. The accumulated mortality was recorded daily for 7 days after feeding the mixture toxins. $LT_{50}$ and $LT_{95}$ were calculated from three independent experiments using Probit analysis (Finney, 1971). Fiducial limits at 95% confident are shown in parentheses. (NT: not toxic)

| **Vip3Aa:Vip3Aa-D199** | | **Toxicity** | |
|---|---|---|---|
| **(ng/cm$^2$)** | **Ratio (w/w)** | **$LT_{50}$ (Days)** | **$LT_{95}$ (Days)** |
| 200 : 0 | - | 2.3 (1.2-3.2) | 4.7 (3.4-16.1) |
| 200 : 200 | 1 : 1 | 3.2 (2.0-4.4) | 8.7 (5.8-34.7) |
| 200 : 1,000 | 1 : 5 | 3.1 (2.2-4.0) | 6.9 (5.2-15.3) |
| 200 : 5,000 | 1 : 25 | 3.9 (3.3-4.5) | 11.3 (8.6-18.2) |
| 0 : 5,000 | - | NT | NT |

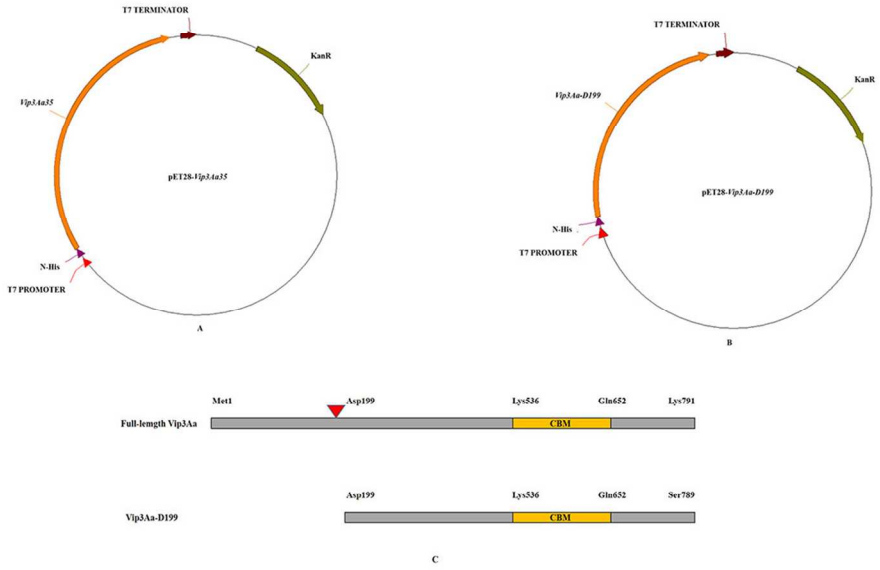



Figure 1

423x272mm (72 x 72 DPI)

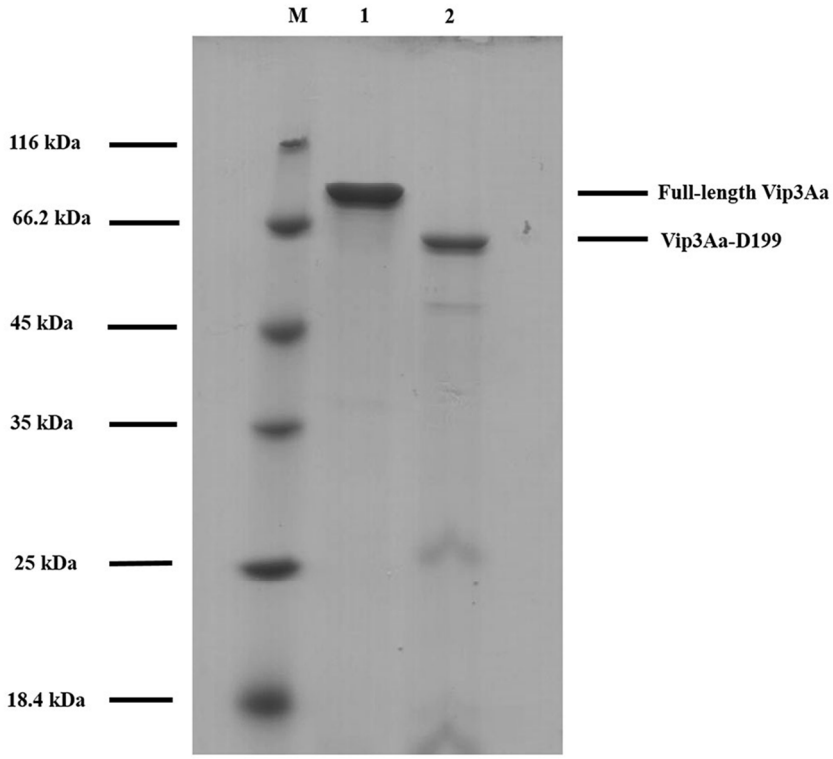



Figure 2

423x336mm (72 x 72 DPI)

**Supplementary 1.** Amino acid sequence of Vip3Aa (GenBank accession # GU733921)

The full-length Vip3Aa consists of 789 amino acids was produced in *E. coli* as a polyhistidine-tag fusion protein. Therefore 20 extra amino acids were added at its N-terminal end (underlined). The truncated fragment (Vip3Aa-D199) containing amino acid positions D199-K789 (**bold letters**) was also produced in *E. coli* with a polyhistidine-tag attached to its N-terminal end. The predicted carbohydrate binding motif (CBM) are highlighted. The protease cleavage site is indicated by an arrow.



MGSSHHHHHHSSGLVPRGSH

MNKNNTKLSTRALPSFIDYFNGIYGFATGIKDIMNMIFKTDTGGDLTLDEILKNQQLLND 60

ISGKLDGVNGSLNDLIAQGNLNTELSKEILKIANEQNQVLNDVNNKLDAINTMLRVYLPK 120

ITSMLSDVMKQNYALSLQIEYLSKQLQEISDKLDIINVNVLINSTLTEITPAYQRIKYVN 180

EKFEELTFATETSSKVKK↓**DGSPADILDELTELTELAKSVTKNDVDGFEFYLNTFHDVMVG** 240

**NNLFGRSALKTASELITKENVKTSGSEVGNVYNFLIVLTALQAKAFLTLTTCRKLLGLAD** 300

**IDYTSIMNEHLNKEKEEFRVNILPTLSNTFSNPNYAKVKGSDEDAKMIVEAKPGHALVGF** 360

**EISNDSITVLKVYEAKLKQNYQVDKDSLSEVIYGDMDKLLCPDQSEQIYYTNNIVFPNEY** 420

**VITKIDFTKKMKTLRYEVTANFYDSSTGEIDLNKKKVESSEAEYRTLSANDDGVYMPLGV** 480

**ISETFLTPINGFGLQADENSRLITLTCKSYLRELLLATDLSNKETKLIVPPSGFIKNIVE** 540

**NGSIEEDNLEPWKANNKNAYVDHTGGVNGTKALYVHKDGGISQFIGDKLKPKTEYVIQYT** 600

**VKGKPSIHLKDENTGYIHYEDTNNNLEDYQTITKRFTTGTDLKGVYLILKSQNGDEAWGD** 660

**NFIILEISPSEKLLSPELINTNNWTSTGSTNISGNTLTLYQGGRGILKQNLQLDSFSTYR** 720

**VYFSVSGDANVRIRNSREVLFEKRYMSGAKDVSEIFTTKLGKDNFYIELSQGNNLNGGPI** 780

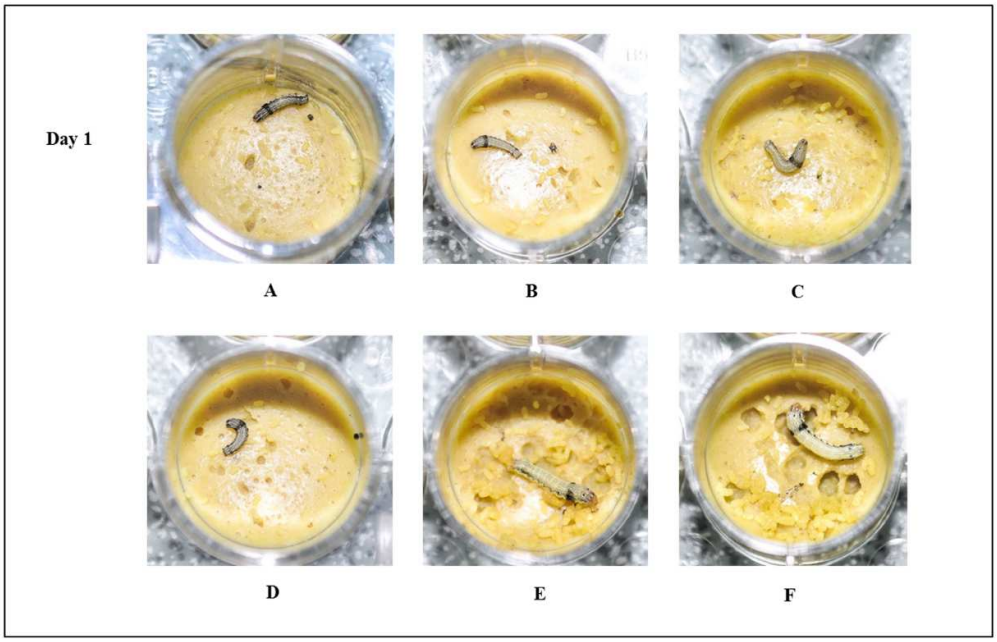
**VKFSDVSIK** 789

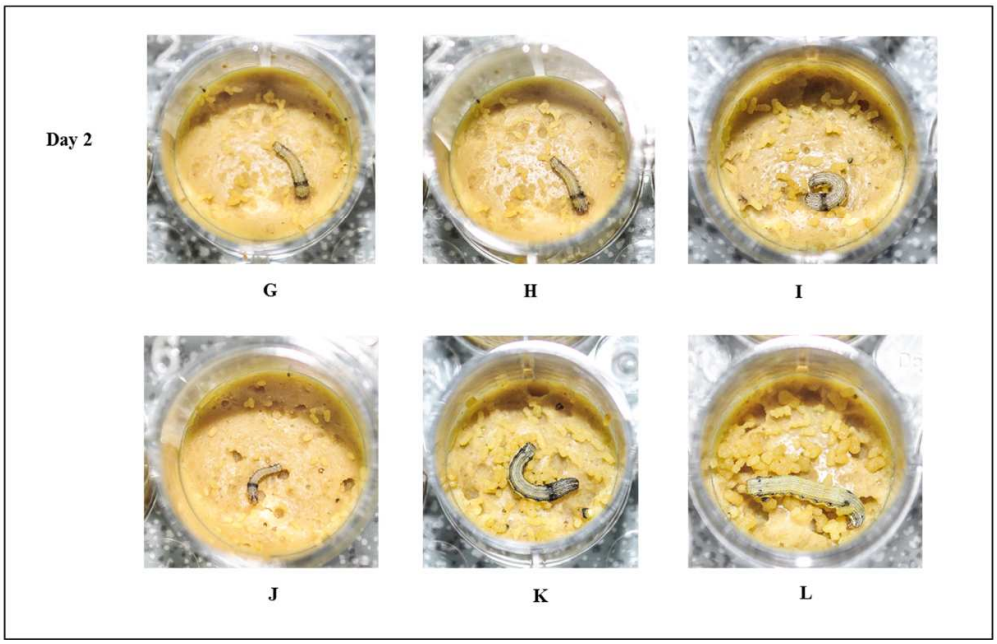
**Supplementary 2.** Mortality of larvae fed with different combinations of the full-length Vip3Aa and the truncated fragment (Vip3Aa-D199)

| Vip3Aa : Vip3Aa-D199 (ng/cm$^2$) | Protein ratio (w/w) | % mortality | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| 200 : 0 | - | 4.2 | 16.7 | 95.8 | 95.8 | 95.8 | 95.8 | 95.8 |
| 200 : 200 | 1 : 1 | 0 | 8.3 | 66.7 | 79.2 | 79.2 | 79.2 | 79.2 |
| 200 : 1,000 | 1 : 5 | 0 | 4.2 | 62.5 | 83.3 | 83.3 | 83.3 | 83.3 |
| 200 : 5,000 | 1 : 25 | 0 | 4.2 | 50.0 | 66.7 | 66.7 | 66.7 | 66.7 |
| 0 : 5,000 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**Supplementary 3.** Photographs showing different growth rate of *S. litura* larvae after feeding artificial diet overlaid with the toxin mixture between the full-length Vip3Aa and its C-terminal fragment (Vip3Aa-D199) at 200:200 ng/cm$^2$ (1:1 w/w), 200:1000 ng/cm$^2$ (1:5 w/w) and 200:5,000 ng/cm$^2$ (1:25 w/w).

The full-length Vip3Aa was mixed with the truncated protein (Vip3Aa-D199) at 1:1, 1:5, 1:25 (w/w) and overlaid on the surface of the artificial diet and fed to second instar *S. litura* larvae. The growth rate of larvae in each group was monitored and recorded by taking photographs after the larvae were challenged with the toxin for 1 and 2 days. Larvae fed with the mixture toxin at 1:1 (w/w) are shown in figure A (at day 1) and G (at day 2) whereas the larvae fed with the mixture toxin at 1:5 (w/w) are shown in figure B (at day 1) and H (at day 2). Figure C and I show larvae that was fed with the mixture toxin at 1:25 (w/w) at day 1 and day 2, respectively. Larvae fed with the full-length Vip3Aa alone (200 ng/cm$^2$) are shown in figures D (at day 1) and J (at day 2). Figures E and K represent larvae fed the truncated fragment alone (5,000 ng/cm$^2$) at day 1 and day 2, respectively. Larvae from the negative control group (without toxin) are shown in figures F and L for day 1 and day 2, respectively.


Day 1
A
B
C
D
E
F


Day 2
G
H
I
J
K
L

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# Cloning and characterization of vegetative insecticidal protein (Vip3A) from Thai isolated *Bacillus thuringiensis*

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**Abstract**

Vip3A is a group of vegetative insecticidal proteins produced during vegetative growth phase and secreted into the culture media by some strains of *Bacillus thuringiensis* (Bt). Vip3A proteins produced by different Bt strains show variable toxicity and specificity to different insects. Twenty Bt isolates collected from various locations in Thailand were screened for a new Vip3A by PCR using primers specific to *vip3* gene. Most of Bt isolates were found to carry *vip3Aa* gene. Only Bt isolates number 22 and 107 were found to carry *vip3A* genes similar to *vip3Af* and *vip3Ad*, respectively. Both genes were cloned into pET28b to obtained 6xHis-tag fusion proteins. The fusion proteins were highly produced in *Escherichia coli* and were purified using HisTrap FF column. Insect bioassays revealed that Vip3Ad from Bt#107 was not toxic to *Spodoptera exigua* and *Spodoptera litura* larvae since the mortality was only 8% and 2%, respectively when both species were fed with Vip3Ad up to 40 μg/cm$^2$. Vip3Af from Bt#22 caused mortality to 20% in *S. exigua* and 60% in *S. litura* when feeding both insects with the toxin at 2 μg/cm$^2$. Although the newly discovered Vip3Ad and Vip3Af were not as good as the benchmark protein (Vip3Aa) when tested against *S. exigua* and *S. litura*, both toxins might have higher activity against other insects.

**Keywords:** *Bacillus thuringiensis*, gene cloning, His-tag protein, insecticidal toxin, protein production, Vip3A

**Note:** GenBank accession number for *vip3Ad* from Bt#107 and *vip3Af* from Bt#22 are KX595193 and KX595192, respectively.

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## Introduction

*Bacillus thuringiensis* (Bt) is a soil bacterium that produces a wide variety of insecticidal proteins [1]. These proteins could be divided into two major groups, crystal proteins (Cry & Cyt) and vegetative insecticidal proteins (Vip). Crystal proteins are produced during sporulation phase as crystalline inclusions inside the mother cell. The crystal proteins will be solubilized in the insect gut lumen upon ingestion by susceptible larvae [1]. The toxins are active against insect larvae in several orders such as Lepidoptera (butterflies & moths), Coleoptera (beetle), Diptera (mosquitoes & flies) and Hemiptera (aphids). Some Cry toxins are also toxic to round worms [2], hook worms [3] and some cancer cells [4, 5]. These toxins are regarded as the first generation of Bt toxins which are extensively studied and applied in the field for decades. Selected *cry* genes have been cloned and expressed in economic crops such as potato, cotton, corn and soybean to protect those plants from insect pests [6]. Although they are effective, some insects develop resistant to those toxins [7]. Therefore it is necessary to find new insecticidal toxins with a different toxic spectrum.

Vegetative insecticidal proteins (Vip) are the second generation of Bt toxins. These proteins are produced during late exponential phase of growth and secreted into the culture medium as soluble proteins [8]. Vip proteins could be divided into 4 groups based on their amino acid sequence homology; Vip1, Vip2, Vip3 and Vip4. While Vip1 and Vip2 work together as a binary toxin and active against coleopterans and hemipterans [9], Vip3 works independently and shows high activity to lepidopteran larvae [10]. The target insect for the newly identified Vip4 is remained to be evaluated.

Vip3 proteins are the most commonly found in several *B. thuringiensis* strains. More than 100 different *vip3* genes have been identified ranging from *vip3Aa*, *vip3Ab*, *vip3Ac*,...to *vip3Ca*(http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/vip.html).Different*B.thuringiensis* strains produce different Vip3 toxins and show specific activity against different insects [8]. All Vip3 proteins share high homology to each other but show no homology to any known protein. Therefore it is difficult to predict the toxin 3D structure and its molecular mechanism employed to kill the insect. Previous reports found that Vip3 can kill insects that are resistant to Cry toxins [11]. This suggests that Vip3 recognizes different receptor to that of the Cry toxin and uses different mechanism to kill the insect cells. This finding leads to application of both Cry & Vip3 in the field to prevent insect resistant development [12, 13].

Thailand is rich in biodiversity and should have a large pool of *B. thuringiensis* strains producing novel Vip3 proteins that have high activity against major insect pests. This work aimed to screen for new *vip3* genes from *B. thuringiensis* local isolates. The identified genes were cloned and expressed in *Escherichia coli*. The proteins were purified and their toxicity against insect larvae was demonstrated.

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## Materials & Methods

Bacterial strains, plasmids and insect larvae

Twenty *Bacillus thuringiensis* isolates collected from various parts of Thailand were used in this work. *E. coli* JM109 was used as a cloning host and *E. coli* BL21(DE3)pLysS was used for production of Vip3A proteins. The plasmid pJET1_2 Blunt (Thermo Scientific®) and pET28b (Novagen®) were used for PCR cloning and expression of *vip3* genes, respectively. The laboratory reared *Spodopter exigua* and *Spodoptera litura* larvae were obtained from the Biotec NPV Pilot Plant.

Screening of *vip3* genes

Total genomic DNA from twenty *B. thuringiensis* isolates were extracted and purified using QIAGEN genomic extraction kit. The genomic DNA was used as a template for PCR screening using a pair of Vip3 screening primers as shown in table 1. Samples that showed positive results were selected for another PCR using specific primers for Vip3Aa, Vip3Ad and Vip3Af (table 1). The PCR products were cloned into pJET1_2 Blunt and transformed in to *E. coli* JM109. The recombinant plasmid was extracted and the DNA sequence of the entire insert gene was determined by automated DNA sequencer (Macrogen, Korea).

Cloning and expression in *E. coli*

In order to produce the new Vip3 protein in *E. coli*, the inserted gene in pJET1_2 Blunt was cut by *Nde*I and *Xho*I and ligated to pET28b that was digested with the same enzymes. This construct allows the inserted gene to be expressed as a 6xHis-tag at its N-terminus. The recombinant plasmid was transformed into *E. coli* BL21(DE3)pLysS for efficient expression of the target gene under T7 promoter. *E. coli* BL21(DE3)pLysS cells harboring pET28-Vip3Ad and pET28-Vip3Af were grown in LB medium supplemented with 34 μg chloramphenicol/ml plus 50 μg kanamycin/ml with shaking at 200 rpm, 37°C until the culture reach mid log phase ($OD_{600}$= 0.5-0.7). To induce the expression, 0.4 mM IPTG was added and the culture was further grown for 5 hours at 25°C, 200 rpm shaking. The induced culture was collected and stored at 4°C for further experiments.

Protein preparation

*E. coli* cells producing Vip3A proteins were collected by centrifugation at 10,000xg, 4°C for 5 minutes. The cell pellet was resuspended in PBS pH 7.4. Vip3A protein was released from the cell by ultrasonication. After centrifugation at 10,000xg, 4°C for 10 minutes, soluble Vip3A protein in the supernatant was purified by affinity chromatography using HisTrap® FF column following the manufacturer instruction. The 6xHis-Vip3A proteins were eluted using PBS pH 7.4 containing 100-250 mM immidazole and analyzed by SDS-PAGE.

Insect bioassay

Protein concentrations were quantified from the Coomassie blue stained SDS-polyacrylamide gel using densitometer with BSA as a standard. Proteins were diluted to the required concentration in PBS and applied to the surface of artificial insect diet in a 24-well tissue culture plate. After the protein solution was completely absorbed, two second-instar

larvae of *Spodopter exigua* or *Spodoptera litura* were added in each well. A total of 16 larvae were used for each toxin concentration and 3 independent experiments were performed. The larvae were kept at room temperature and mortality was recorded for 7 days after feeding the toxin. The 6xHis-Vip3Aa cloned from *B. thuringiensis* M190 (GenBank #GU733921) was used as a positive control and PBS was used as a negative control.

**Results & Discussion**

Cloning of new *vip3A* genes

Preliminary data from Biocontrol Research Laboratory, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency indicates that about ¼ of *B. thuringiensis* local isolates collected from various locations in Thailand are able to produce Vip3 protein. In this work, 20 isolates were selected from a total of 1,000 isolates to screen for new *vip3* genes. PCR screening from the genomic DNA of these isolates using a pair of Vip3 screening primers (table 1) revealed that all isolate carry *vip3* gene (figure 1). Since the primers were designed from the highly conserved region for all *vip3* genes that give the same PCR product size at 1.6 kb [14]. It is not possible to identify the subtype of those *vip3* genes.

It has been shown that all *vip3* genes are highly conserved at the 5′-end and highly variable at the 3′-end. Therefore it is possible to distinguish these genes by PCR using different primers at the 3′-end. Second PCR reactions were performed using genomic DNA from those 20 isolates as templates with the universal forward primer designed for all *vip3* genes and reversed primers specific for different subtype of *vip3* genes (table 1). This reaction could amplify the full-length *vip3* gene about 2.4 kb. Most of the samples tested gave a positive result when using primer for *vip3Aa*. This suggests that most *B. thuringiensis* isolates contain *vip3Aa* gene in agreement with previous reports. Only isolates no.22 and no.107 gave PCR product when using primers specific to *vip3Af* and *vip3Ad*, respectively. PCR products from both reactions were cloned into a PCR cloning vector, pJET1_2 Blunt and transformed into *E. coli* JM109. DNA sequencing analysis revealed that isolate no.22 contains a 2,367-bp fragment that showed 99.7% identity to *vip3Af1* gene (GenBank #AJ872070). This fragment could be translated to a 788-amino acid protein that has 99.7% identity to the full-length Vip3Af protein. Isolate no.107 carried a 2,361-bp fragment that is very similar to *vip3Ad2* gene (99.9% identity, GenBank #AJ872071). Amino acid sequence of this fragment shows 99.6% identity to the full-length Vip3Ad2 protein. DNA and amino acid sequences of the new *vip3Af* (isolate no.22) and *vip3Ad* (isolates no.107) were deposited in GenBank under accession number **KX595192** and **KX595193**, respectively. These new Vip3Ad and Vip3Af show some variation from the prototype Vip3Aa mostly at the C-terminal part as shown in figure 2.

Protein production level

The new *vip3Af* from isolate no.22 and *vip3Ad* from isolates no.107 were subcloned into pET28b in order to be expressed as 6xHis-tag fusion proteins. Both constructs were highly expressed in *E. coli* BL21(DE3)pLysS as soluble proteins. Both proteins could be purified using Ni-NTA affinity column (HisTrap FF column). The final yield of both proteins was comparable to that of the prototype toxin, Vip3Aa. It should be noted that both proteins could be eluted from the column when using buffer containing 100-250 mM imidazole although at 250 mM immidazole gave higher purity (figure 3).

Larvicidal activity of the new Vip3Ad and Vip3Af

To access larvicidal activity, both proteins were fed to *S. exigua* and *S. litura* larvae. These two species are major insect pests that infest many important crops in Thailand e.g. grape, shallot, cabbage, kale, asparagus and other vegetables and fruits. Vip3Ad showed very low activity to both larvae even when used at very high concentration up to 40 $\mu g/cm^2$ (table 2). The prototype toxin, Vip3Aa, was highly active against both larvae and could give 100% mortality when using the toxin more than 2 $\mu g/cm^2$. Vip3Af was active against *S. litura* more than *S. exigua*. Larvicidal activity of Vip3Af against *S. litura* was highly fluctuate and could give mortality up to 93.75% in one experiment and decreased to 43.75% in another experiment. Different lots of insect larvae may somehow contribute to this variation. Although our Vip3Ad and Vip3Af exhibited lower toxicity to *S. exigua* and *S. litura* comparing with that of the prototype Vip3Aa, both toxins might have high activity against other insects. There was a report demonstrating that Vip3Af exhibited higher toxicity than Vip3Aa when tested against *Spodoptera frugiperda* [15]. Vip3Af also showed comparable toxicity to Vip3Aa when tested with some insects such as *Helicoverpa armigera*, *Mamestra brassicae*, *Spodoptera littoralis* and *Lobesia botrana* larvae [16]. Vip3Ad was also showed some activity against *H. armigera* larvae [16]. It is remain to be investigated if our new Vip3Ad and Vip3Af are toxic to those insects.

**Acknowledgements**

This work was supported by the National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency (NSTDA), the Thailand Research Fund (TRF) and Silpakorn University. Support from the Young Scientist and Technologist Program (YSTP) to Siriruk Chimpalee is gratefully acknowledged.

**Table 1.** Primer sequences used for screening and cloning of *vip3* gene.

| Primer name | DNA sequences (5′ to 3′) |
|---|---|
| Vip3 screening Fw | TGCCACTGGTATCAARGA |
| Vip3 screening Rv | TCCTCCTGTATGATCTACATATGCATTYTTRTTRTT |
| Vip3A full-length Fw | CCGCGGCCGCGGAGGATTAACATATGAACAAGAATAATACTAAATTAA |
| Vip3Aa full-length Rv | CTCGAGTTACTTAATAGAGACATCGGA |
| Vip3Ad full-length Rv | CTCGAGTTATTTAATAGAGAAATCATAAAAATGTA |
| Vip3Af full-length Rv | CTCGAGTTATTTAATAGAAACGTTTTCAAAT |

**Table 2.** Larvicidal activity of purified proteins against *S. exigua* and *S. litura* larvae.

The purified protein was applied on the surface of artificial diet in each well. Two 2$^{nd}$-instar larvae were placed in each well and a total of 16 larvae were used for each sample. Three independent experiments were performed and mortality was recorded after 7 days.

| Sample | % mortality (average ± SD) | |
|---|---|---|
| | *S. exigua* | *S. litura* |
| 6xHis-Vip3Aa (2 μg/cm$^2$) | 83.33 ± 18.04 | 91.67 ± 9.55 |
| 6xHis-Vip3Ad (40 μg/cm$^2$) | 8.33 ± 7.22 | 2.08 ± 3.61 |
| 6xHis-Vip3Af (2 μg/cm$^2$) | 20.83 ± 3.61 | 60.42 ± 28.87 |
| PBS pH 7.4 (negative control) | 0 | 0 |

**Figure 1.** PCR products from reaction using genomic DNA from different *B. thuringiensis* isolates as templates with Vip3 screening primers. Lanes labeled with #101–#542 represent *B. thuringiensis* isolate number 101–542.

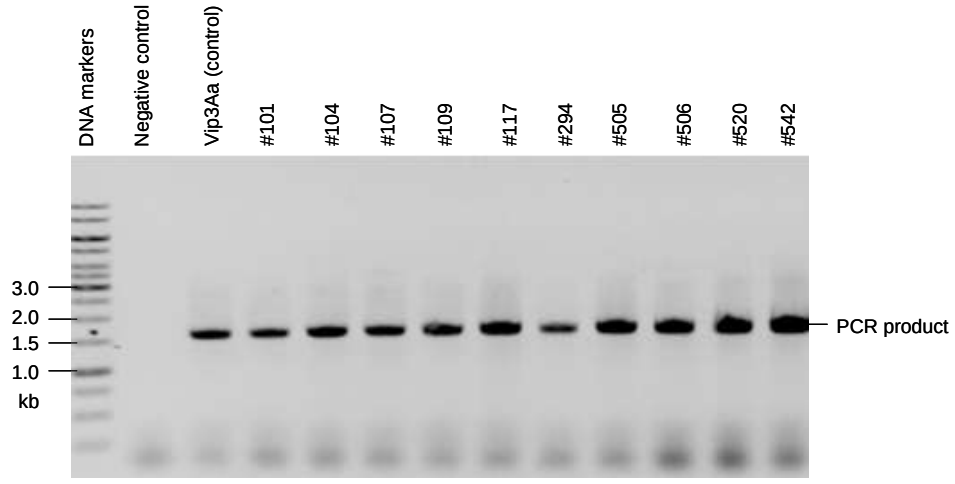

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**Figure 2.** Alignment of Vip3Aa, Vip3Ad and Vip3Af

```
Vip3Ad      MNKNNTKLNARALPSFIDYFNGIYGFATGIKDIMNMIFKTDTGSNLTLDEILKNQQLLNE   60
Vip3Aa      MNKNNTKLSTRALPSFIDYFNGIYGFATGIKDIMNMIFKTDTGGDLTLDEILKNQQLLND   60
Vip3Af      MNKNNTKLNARALPSFIDYFNGIYGFATGIKDIMNMIFKTDTGGNLTLDEILKNQQLLNE   60
            ********.:**********************************.:**************:

Vip3Ad      ISGKLDGVNGSLNDLIAQGNLNTELAKQILKVANEQNQVLNDVNNKLDAINSMLKIYLPK   120
Vip3Aa      ISGKLDGVNGSLNDLIAQGNLNTELSKEILKIANEQNQVLNDVNNKLDAINTMLRVYLPK   120
Vip3Af      ISGKLDGVNGSLNDLIAQGNLNTELSKEILKIANEQNQVLNDVNNKLDAINTMLHIYLPK   120
            *************************:*:***:********************:**::****

Vip3Ad      ITSMLSDVMKQNYVLSLQIEYLSKQLQEISDKLDIINVNVLINSTLTEITPAYQRMKYVN   180
Vip3Aa      ITSMLSDVMKQNYALSLQIEYLSKQLQEISDKLDIINVNVLINSTLTEITPAYQRIKYVN   180
Vip3Af      ITSMLSDVMKQNYALSLQIEYLSKQLQEISDKLDIINVNVLINSTLTEITPAYQRIKYVN   180
            *************.*********************************************:****

Vip3Ad      EKFEELTFATETTLKVKKDSPPADILDELTELTELAKSVTKNDVDGFEFYLNTFHDVMVG   240
Vip3Aa      EKFEELTFATETSSKVKKDGSPADILDELTELTELAKSVTKNDVDGFEFYLNTFHDVMVG   240
Vip3Af      EKFEELTFATETTLKVKKDSSPADILDELTELTELAKSVTKNDVDGFEFYLNTFHDVMVG   240
            ************: *****. ****************************************

Vip3Ad      NNLFGRSALKTASELIAKENVKTSGSEVGNVYNFLIVLTALQAKAFLTLTTCRKLLGLAD   300
Vip3Aa      NNLFGRSALKTASELITKENVKTSGSEVGNVYNFLIVLTALQAKAFLTLTTCRKLLGLAD   300
Vip3Af      NNLFGRSALKTASELIAKENVKTSGSEVGNVYNFLIVLTALQAKAFLTLTTCRKLLGLAD   300
            ****************:**********************************************

Vip3Ad      IDYTSIMNEHLNKEKEEFRVNILPTLSNTFSNPNYAKAKGSNEDTKMIVEAKPGYVLVGF   360
Vip3Aa      IDYTSIMNEHLNKEKEEFRVNILPTLSNTFSNPNYAKVKGSDEDAKMIVEAKPGHALVGF   360
Vip3Af      IDYTSIMNEHLNKEKEEFRVNILPTLSNTFSNPNYAKVKGSDEDAKMIVEAKPGHALVGF   360
            ************************************.***:**:*********:.****

Vip3Ad      EMSNDSITVLKAYQAKLKKDYQIDKDSLSEIIYSDTDKLLCPDQSEQIYYTKNIAFPNEY   420
Vip3Aa      EISNDSITVLKVYEAKLKQNYQVDKDSLSEVIYGDMDKLLCPDQSEQIYYTNNIVFPNEY   420
Vip3Af      EMSNDSITVLKVYEAKLKQNYQVDKDSLSEVIYGDTDKLLCPDQSEQIYYTNNIVFPNEY   420
            *:**********.*:****::**:*******:**.* ****************:**.*****

Vip3Ad      VITKIAFTKKMNSLRYEATANFYDSSTGDIDLNKTKVESSEAEYSMLKASDDEVYMPLGL   480
Vip3Aa      VITKIDFTKKMKTLRYEVTANFYDSSTGEIDLNKKKVESSEAEYRTLSANDDGVYMPLGV   480
Vip3Af      VITKIDFTKKMKTLRYEVTANFYDSSTGEIDLNKKKVESSEAEYRTLSANDDGVYMPLGV   480
            ***** *****::****.**********:*****.*********  *.*.** ******:

Vip3Ad      ISETFLNPINGFRLAVDENSRLVTLTCRSYLRETLLATDLNNKETKLIVPPNVFISNIVE   540
Vip3Aa      ISETFLTPINGPGLQADENSRLITLTCKSYLRELLLATDLSNKETKLIVPPSGFIKNIVE   540
Vip3Af      ISETFLTPINGFGLQADENSRLITLTCKSYLRELLLATDLSNKETKLIVPPSGFISNIVE   540
            ******.***** * .******:****:***** ******.**********. **.****

Vip3Ad      NGNIEMDTLEPWKANNENANVDYSGGVNGTRALYVHKDGEFSHFIGDKLKSKTEYLIRYI   600
Vip3Aa      NGSIEEDNLEPWKANNKNAYVDHTGGVNGTKALYVHKDGGISQFIGDKLKPKTEYVIQYT   600
Vip3Af      NGSIEEDNLEPWKANNKNAYVDHTGGVNGTKALYVHKDGGFSQFIGDKLKPKTEYVIQYT   600
            **.** *.********:** **::******:******** :*:******* ****:*:*

Vip3Ad      VKGKASIFLKDEKNENYIYEDTNNNLEDYQTITKRFTTGTDSTGVYLIFNSQNGDEAWGD   660
Vip3Aa      VKGKPSIHLKDENTGYIHYEDTNNNLEDYQTITKRFTTGTDLKGVYLILKSQNGDEAWGD   660
Vip3Af      VKGKPSIHLKDENTGYIHYEDTNNNLKDYQTITKRFTTGTDLKGVYLILKSQNGDEAWGD   660
            **** **.****:.    ********:*************** .*****::**********

Vip3Ad      NFIILEISPCEKLLSPELIKTDKWNSTGSTYISDDRLTLYRGGRGILKQNLQLDGFSTYR   720
Vip3Aa      NFIILEISPSEKLLSPELINTNNWTSTGSTNISGNTLTLYQGGRGILKQNLQLDSFSTYR   720
Vip3Af      KFTILEIKPAEDLLSPELINPNSWITTPGASISGNKLFINLGTNGTFRQSLSLNSYSTYS   720
            :* ****.*.*.*******: :.* :* .: ** : * :  * .* ::*.*.*:.:***

Vip3Ad      VNFSVDGDANVRIRNSREVLLEKRYLN--RKGVSEMPTTKFDKDNFYVELSQGDNLGTS-   777
Vip3Aa      VYFSVSGDANVRIRNSREVLPEKRY-MSGAKDVSEIPTTKLGKDNFYIELSQGNNLNGGP   779
Vip3Af      ISFTASGPFNVTVRNSREVLFERSNLMSSTSHISGTFKTESNNTGLYVELSRRS--GGGG   778
            : *:..*  ** :*******:*:       . :*  *.*:  :  :*:***: .    .

Vip3Ad      -VHFYDFSIK     786
Vip3Aa      IVKFSDVSIK     789
Vip3Af      HISFENVSIK     788
             : * :.***
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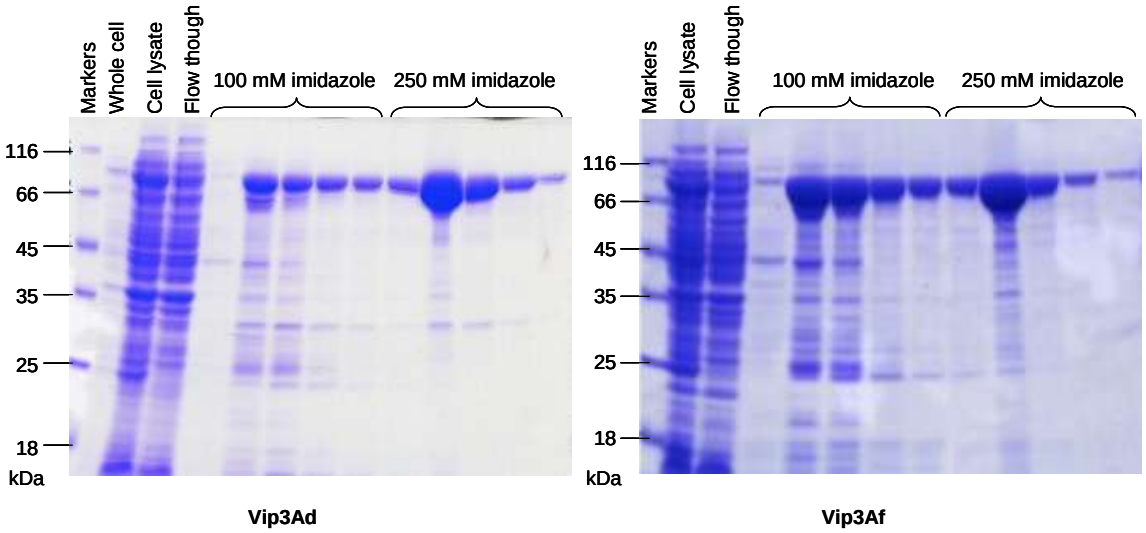
**Figure 3.** Coomassie blue stain of SDS-Polyacrylamide gel of the purified proteins from HisTrap FF column. Proteins were eluted in fraction containing 100-250 mM imidazole.

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# Amino acid substitutions in a putative carbohydrate binding module of Vip3Ag2 affect its larvicidal activity

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## ABSTRACT

Vip3Ag2 is an insecticidal protein produced by *Bacillus thuringiensis* M191 as a soluble protein during vegetative phase of growth and secreted into the culture medium as a 88 kDa protoxin. The protein is highly toxic to Lepidopteran insect larvae such as *Spodoptera exigua* and *Spodoptera litura*. Upon ingestion by susceptible larvae, the protein will be cleaved by gut proteases to yield an active toxin about 60 kDa. The activated toxin will then bind to a specific receptor on the gut cell membrane and subsequently induces cell death by an unknown mechanism. To identify amino acids playing important role during intoxication, single amino acid substitutions were employed at ten positions using site-directed mutagenesis technique (P551A, W552L, E594A, Q598A, D616A, N623A, T631A, F636I, W684L and W691L). These residues are located in a putative carbohydrate binding module that could play essential role during receptor binding. All mutant proteins were effectively produced in *Escherichia coli* and specifically reacted to anti-Vip3Ag2 antibody. Bioassays against *S. exigua* larvae revealed that most of the mutants retain their larvicidal activity comparable to that of the wild type except W552L, D616A and W684L which were unable to kill the larvae. These three residues might be important for interaction to a sugar moiety of a glycoprotein on the gut cell membrane that acts as a specific receptor for Vip3Ag2. Comparative binding assays between these mutants and the wild type to brush broader membrane vesicles (BBMV) from *S. exigua* larval guts are in progress.

**Keywords:** *Bacillus thuringiensis*, larvicidal protein, receptor binding, site-directed mutagenesis, Vip3Ag2

## INTRODUCTION

Vegetative insecticidal proteins (Vip) are a group of proteins present in some strains of *Bacillus thuringiensis* [1]. These proteins are produced during vegetative phase of growth and secreted to the culture medium. Different *B. thuringiensis* strains produce different Vip

proteins and toxic to different target insects [2]. Vip proteins could be divided into 3 major groups, Vip1, Vip2 and Vip3. While Vip1 and Vip2 function as a binary toxin and toxic to coleopteran larvae, Vip3 works independently and toxic to a wide range of Lepidopteran larvae [1,3]. The mechanism of action of Vip3 is not fully understood but the susceptible larvae stop feeding within a short time after treated with the toxin and eventually die a few days later [4]. Vip3 proteins are highly toxic to insect larvae and have $LD_{50}$ comparable to that of crystal proteins (Cry toxins) from *B. thuringiensis* [5,6]. In addition, Vip proteins are active against the Cry toxin-resistant insects [1]. Binding assays revealed that Vip3 proteins recognize different receptors to Cry toxins [4,7]. It is expected that proteins from both groups employ different mechanisms to kill the insects.

Recently, we have successfully identified Vip3Ag2 from *B. thuringiensis* local isolate no. M191 (GenBank # FJ556803). The toxin is highly active against many lepidoteran larvae such as beet armyworm (*Spodoptera exigua*) and common cutworm (*Spodoptera litula*) which are important pests for major economic crops in Thailand. The toxin is produced and secreted at high level up to 800 mg/liter in rich media. There is a potential to develop this protein as an effective bio-insecticide and our group is currently pursuing this goal. Although Vip3A proteins are highly toxic to insect larvae including those resistant to Cry toxins, their specific receptors on the target insects are remained unidentified. Amino acid sequences of all Vip3 proteins are highly conserved and share no homology to any protein with known 3D structure in the database. However, a putative carbohydrate binding module (CBM) has been identified in all Vip3 proteins. This part could bind to a sugar moiety of a glycoprotein that acts as a receptor on the insect gut cell membrane similar to other insecticidal proteins such as *B. thuringiensis* Cry4B [8].

Vip3Ag2 consists of 787 amino acids with molecular mass about 88 kDa (GenBank # ACL97352). A putative CBM is identified between amino acid positions 526-694. Homology modeling of this region using a CBM from *Thermoanaerobacterium polysaccharolyticum* ManA (PDB # 2ZEW) [9], as a template suggests specific amino acids that could make interaction to a sugar molecule in glycoproteins. Amino acid replacements at ten selected positions in a putative CBM of Vip3Ag2 were employed to verify if these residues are required for its activity and play a key role during receptor binding.

## MATERIALS AND METHODS

### Recombinant plasmid construction

The genomic DNA from *B. thuringiensis* M191 was extracted and purified using the genomic purification kit (QIAGEN). The *Vip3Ag2* gene (GenBank # FJ556803) was amplified by PCR using the genomic DNA as a template couple with a pair of primer; forward primer (*Nde*I): 5′-GCCATATGAACAAGAATAATACTAAATTAAGCGCA-3′, reverse primer (*Bam*HI): 5′-GCGGATCCTTACTTAATTGAAAAATCTCGGAAATTTAT-3′. The recombinant plasmid pET-Vip3Ag2 was generated by inserting the 2.4 kb PCR product into pET28b between *Nde*I and *Bam*HI sites. Vip3Ag2 produced from this plasmid will have a polyhistidine tag at its N-terminus to facilitate purification.

**Site-directed mutagenesis**

Ten Vip3Ag2 mutants (P551A, W552L, E594A, Q598A, D616A, F636I, T631A, N623A, W684L, and W691L) were generated based on Stratagene's QuikChange™ Site-Directed Mutagenesis (Strategenes). The recombinant plasmid pET28-Vip3Ag2 (wild type) was used as a template for single substitution together with appropriate primers shown in **Table 1**. The recombinant plasmids obtained for each mutant were distinguished from the template by restriction endonuclease digestion with the appropriate enzyme (**Table 1**). DNA sequences of the full-length *vip3Ag2* gene from all mutants were verified by automated DNA sequencer.

**Table 1** The mutagenic primers for generation of Vip3Ag2 mutant toxins

Recognition sites introduced in the primers for restriction endonuclease analysis are underlined. Mutated nucleotides are shown in bold; fw and rv represent forward and reverse primers, respectively.

| Primer | Sequence | Enzyme |
|---|---|---|
| P551Afw | 5'-GAAAACTTAGAG**G**C**C**TGGAAAGCAAATAAC-3' | *Stu*I |
| P551Arv | 5'-ATTTGCTTTCCA**GG**C**C**TCTAAGTTTTCTCC-3' | |
| E594Afw | 5'-TCGAAAACAG**C**ATATGTAATTCAATATATT-3' | *Nde*I |
| E594Arv | 5'-TTGAATTACATAT**G**CTGTTTTCGATTTCAA-3' | |
| Q598Afw | 5'-GAATATGTAATT**GC**ATATATTGTAAAGGGA-3' | - |
| Q598Arv | 5'-TTACAATATAT**GC**AATTACATATTCTGTTT-3' | |
| D616Afw | 5'-GAAAAAAATGGTG**CA**TGCATTTATGAAGAT-3' | *Sph*I |
| D616Arv | 5'-TTCATAAATGCA**TG**CACCATTTTTTTCATC-3' | |
| N623Afw | 5'-TATGAAGATAC**GGCC**AATGGTTTAGAAGAT-3' | *Eae*I |
| N623Arv | 5'-TCTAAACCATT**GGCC**GTATCTTCATAAATG-3' | |
| T631Afw | 5'-GAAGATTTTCA**GG**CCGTTACTAAAAGTTTT-3' | *Hae*III |
| T631Arv | 5'-TTTAGTAACGG**CC**TGAAAATCTTCTAATAA-3' | |
| F636Ifw | 5'-CCATTACTAAA**TCGA**TTATTACAGGAACGG-3' | *Cla*I |
| F636Irv | 5'-TCCTGTAATAA**TCGA**TTAGTAACGGTTTG-3' | |
| W684Lfw | 5'-TAAATTCAGATGC**ACTA**GTTGGATCTCAGG-3' | *Spe*I |
| W684Lrv | 5'-TGAGATCCAAC**TAGT**GCATCTGAATTTATC-3' | |
| W691Lfw | 5'-TCTCAGGGAAC**ACTA**GTTTCAGGAAATTCA-3' | *Spe*I |
| W691Lrv | 5'-AATTTCCTGAAAC**TAGT**GTTCCCTGAGATC-3' | |

**Protein production in *E. coli***

*E. coli* BL21(DE3)pLysS harboring pET28-*Vip3Ag2* was grown in 200 ml LB medium supplemented with 100 μg ampicillin/ml and 50 μg kanamycin/ml at 37°C, 200 rpm shaking until reaching mid-log phase. To induce Vip3Ag2 production, 0.2 mM IPTG was added and the culture was grown for additional 16-18 hours at 25°C, 200 rpm shaking. Cells were collected by centrifugation and resuspended in 50 ml PBS pH 7.4. Vip3Ag2 protein was

released from the cell by ultrasonication. Cell pellet and insoluble proteins were removed by centrifugation at 8000g, 4°C for 15 min. Vip3Ag2 in the supernatant was subjected to SDS-PAGE, western blot and bioassay.

**Larvicidal assay**

The insecticidal activity of Vip3Ag2 protein was tested against *Spodoptera exigua* larvae. Fifty μl of supernatants containing wild type toxin or its mutants from the previous step were applied onto the surface of a semisolid synthetic insect medium in a 24-well culture plate. After complete absorption of the supernatant into the medium, two second-instar larvae were placed in each well. The plate was kept at room temperature and insect mortality was recorded daily for 7 days. Bioassays were performed 3 times using 8 larvae for each sample.

**RESULTS AND DISCUSSION**

*Bacillus thuringiensis* M191 is a local isolate collected from soil sample in Thailand. This bacterium shows high toxicity to beet armyworm larvae. Further analysis found that it contains *Vip3Ag2* gene. In this study the full length *Vip3Ag2* gene was successfully amplified from the genomic DNA using primers designed based on 5′ and 3′ ends of the gene sequence (GenBank # FJ556803). The PCR product was cloned in to pET28b in such a way that the expressed product carries a polyhistidine tag at its N-terminus (6xHis-Vip3Ag2). DNA sequencing of the Vip3Ag2 in the recombinant plasmid pET-Vip3Ag2 was performed and found that it contains identical sequence to the original gene in the database.

To produce Vip3Ag2 protein, the plasmid pET-Vip3Ag2 was transformed into *E. coli* BL21(DE3)pLysS. After induction with IPTG, the protein was detected in the cell supernatant suggesting that the protein was produced as a soluble protein. All ten mutant proteins were also produced as soluble proteins similar to the wild type (**Fig.1**). Western blot and immuno-detection using polyclonal antibody against Vip3Ag2 demonstrated that all mutants could react to the antibody **(Fig.1**). It should be noted that some mutants e.g. W552L, N623A and W684L were produced at lower level than the wild type. Other mutants were produced at similar level to the wild type. It remains to be confirmed if these mutants are sensitive to proteolytic digestion by the host cell proteases.

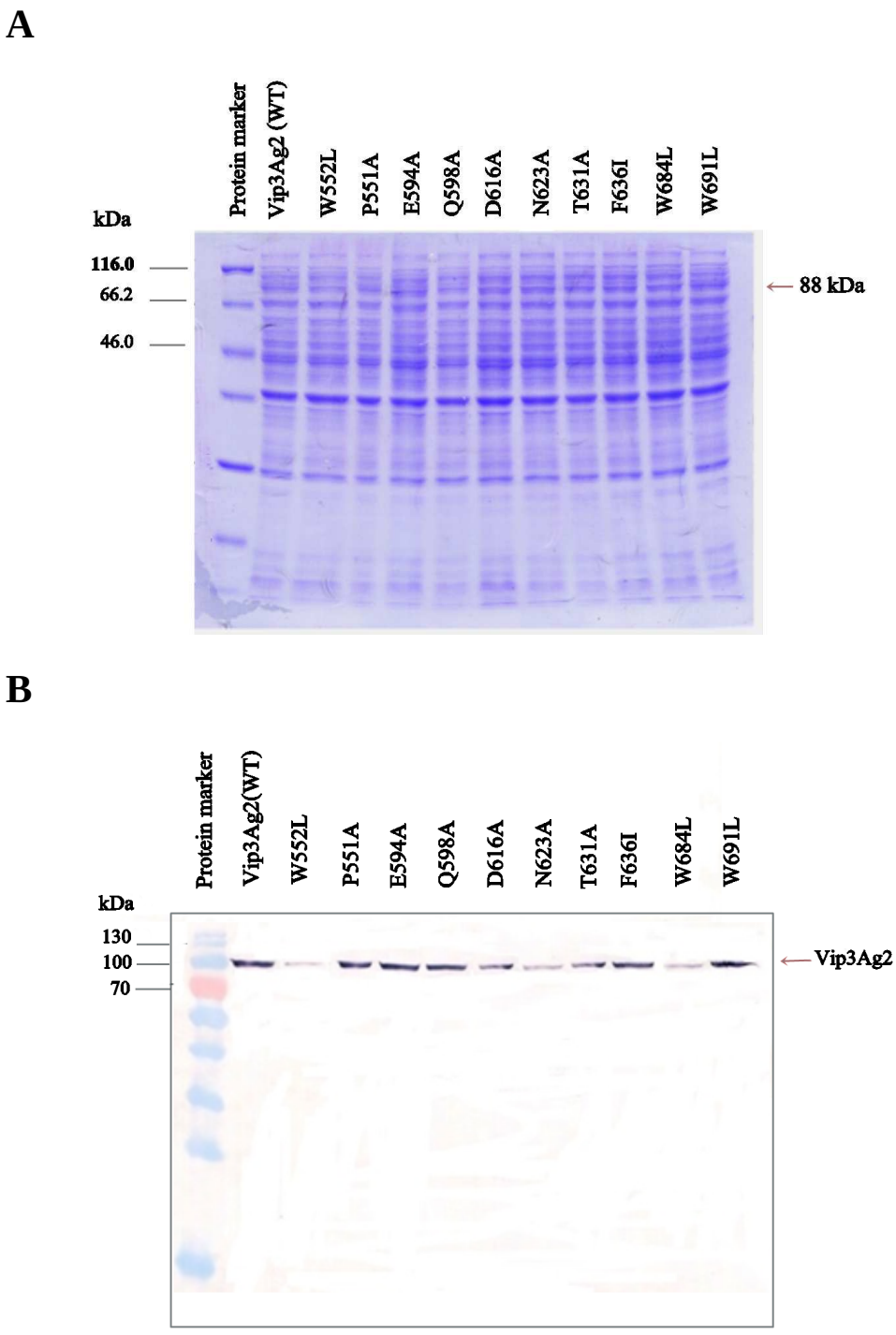


**Fig. 1** Production of Vip3Ag2. *E. coli* cells expressing the wild type Vip3Ag2 and its mutants were collected and lysed by ultrasonication. Soluble proteins from the cell lysate were collected after centrifugation and subjected to SDS-PAGE. Panel **A** represents a coomassie blue stained polyacrylamide gel and panel **B** is a western blot membrane detected by anti-Vip3Ag2 polyclonal antibody.

Bioassay results against *S. exigua* larvae were shown in **Table 2**. Seven mutants (P551A, E594A, Q598A, N623A, T631A, F636I and W691L) exhibited comparable larvicidal activity to the wild type. Three mutants (W552L, D616A and W684L) were unable

to kill the larvae. All mutants are predicted to be located in the CBM region and should play a part during receptor binding. Our results suggested that single amino acid substitution at P551, E594, Q598A, N623, T631, F636 and W691 did not affect receptor binding. However, different positions might contribute differently and could be compensated by amino acids at other positions. Receptor binding is generally involved interactions of many residues. Therefore double and triple mutations are required to elucidate the receptor binding region of Vip3Ag2. Larvicidal activity was completely abolished when amino acid replacements were made at positions W552, D616 and W684. This result indicates that amino acids in these positions are essential for the toxin action. They could play a key role during toxin binding to the sugar moiety of the glycoprotein in the larval gut cell membrane. By analogy to *T. polysaccharolyticum* ManA [9], W552, D616 and W684 in Vip3Ag2 might function similar to W20, Q81 and W125 in ManA in which they make direct interaction to a sugar residue. Nevertheless, structural alteration caused by the replacement in these positions should not be rule out for the loss of larvicidal activity. We are in the process to investigate the overall conformation of the wild type toxin and its mutants. Comparative binding between the wild type toxin and its mutants to BBMV from *S. exigua* larvae are also in progress.

**Table 2** Larvicidal activity of the wild-type toxin and its mutants against *S. exigua* larvae. Fifty μl of supernatant from cell lysate of *E. coli* expressing Vip3Ag2 was fed to 2$^{nd}$-instar larvae and mortality was recorded after incubation at room temperature for seven days.

| Sample | % Mortality |
|---|---|
| Wild type | 100 |
| P551A | 100 |
| W552L | 0 |
| P551A | 100 |
| E594A | 100 |
| Q598A | 100 |
| D616A | 0 |
| N623A | 100 |
| T631A | 100 |
| F636I | 100 |
| W684L | 0 |
| W691L | 100 |

## CONCLUSION

The plasmid pET-Vip3Ag2 was constructed by inserting *Vip3Ag2* gene from *B. thuringiensis* M191 into pET28b between *Nde*I and *Bam*HI sites. The plasmid was transform into *E. coli* BL21(DE3)pLysS to be expressed as a 6xHis-Vip3Ag2 fusion protein. The protein was highly toxic to *S. exigua* larvae. Single amino acid substitution was introduced into ten positions in the predicted CBM region of Vip3Ag2. All mutant proteins were successfully produced as soluble proteins similar to the wild type toxin. Seven mutants (P551A, E594A, Q598A, N623A, T631A, F636I and W691L) exhibited larvicidal acticity

comparable to the wild type and three mutants (W552L, D616A and W684L) were inactive. Therefore, W552, D616 and W684 are critical for the toxin activity possibly via the receptor binding.

## ACKNOWLEDGEMENTS

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# *Bacillus thuringiensis* strain M190 Vip190 gene (Vip3Aa35), complete cds

## GenBank: GU733921.1

FASTA Graphics

Go to:

LOCUS GU733921 2370 bp DNA linear BCT 29-MAR-2010

DEFINITION Bacillus thuringiensis strain M190 Vip190 gene, complete cds.

ACCESSION GU733921

VERSION GU733921.1

KEYWORDS .

SOURCE Bacillus thuringiensis

ORGANISM Bacillus thuringiensis

Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; Bacillus cereus group.

REFERENCE 1 (bases 1 to 2370)

AUTHORS Audtho,M., Ratlerdkarn,M. and Promdonkoy,B.

TITLE Vegetative protein from Bacillus thuringiensis M190 (local isolate)

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 2370)

AUTHORS Audtho,M., Ratlerdkarn,M. and Promdonkoy,B.

TITLE Direct Submission

JOURNAL Submitted (05-FEB-2010) National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, 113 Thailand Science Park, Phaholyothin Rd., Klong 1, Klong Luang, Pathumthani 12120, Thailand

FEATURES Location/Qualifiers

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# *Bacillus thuringiensis* strain 294 vegetative insecticidal protein (vip3Aa64) gene, complete cds

## GenBank: KY883694.1

FASTA Graphics

Go to:

LOCUS KY883694 2370 bp DNA linear BCT 17-JUL-2017

DEFINITION Bacillus thuringiensis strain 294 vegetative insecticidal protein (vip3Aa) gene, complete cds.

ACCESSION KY883694

VERSION KY883694.1

KEYWORDS .

SOURCE Bacillus thuringiensis

ORGANISM Bacillus thuringiensis

Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; Bacillus cereus group.

REFERENCE 1 (bases 1 to 2370)

AUTHORS Soonsanga,S., Rungrod,A., Autho,M. and Promdonkoy,B.

TITLE Tyrosine-776 influences Bacillus thuringiensis Vip3Aa thermostability

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 2370)

AUTHORS Soonsanga,S., Rungrod,A., Autho,M. and Promdonkoy,B.

TITLE Direct Submission

JOURNAL Submitted (04-APR-2017) Biocontrol Research Laboratory, National Center for Genetic Engineering and Biotechnology, 113 Thailand Science Park, Phahonyothin Road, Khlong Luang, Pathum Thani 12120, Thailand

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# *Bacillus thuringiensis* strain #107 Vip3Ad gene, complete cds

## GenBank: KX595193.1

FASTA Graphics

Go to:

LOCUS KX595193 2361 bp DNA linear BCT 10-MAY-2017

DEFINITION Bacillus thuringiensis strain #107 Vip3Ad gene, complete cds.

ACCESSION KX595193

VERSION KX595193.1

KEYWORDS .

SOURCE Bacillus thuringiensis

ORGANISM Bacillus thuringiensis

Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; Bacillus cereus group.

REFERENCE 1 (bases 1 to 2361)

AUTHORS Nimsanor,S., Chimpalee,S., Rungrod,A., Trakulnaleamsai,C., Jongsareejit,B. and Promdonkoy,B.

TITLE Cloning and characterization of vegetative insecticidal protein (Vip3A) from Thai isolated Bacillus thuringiensis

JOURNAL Veridian EJ Sci Technol Silpakorn Univ 3 (5), 228-236 (2016)

REFERENCE 2 (bases 1 to 2361)

AUTHORS Nimsanor,S., Rungrod,A. and Promdonkoy,B.

TITLE Direct Submission

JOURNAL Submitted (22-JUL-2016) National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, 113 Phahonyothin Road, Khlong Nueng, Khlong Luang, Pathumthani 12120, Thailand

FEATURES Location/Qualifiers

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# *Bacillus thuringiensis* strain M191 vegetative insecticidal protein (vip3Ag2) gene, complete cds

## GenBank: FJ556803.2

FASTA Graphics

Go to:

LOCUS FJ556803 2364 bp DNA linear BCT 20-MAR-2015

DEFINITION Bacillus thuringiensis strain M191 vegetative insecticidal protein (vip) gene, complete cds.

ACCESSION FJ556803

VERSION FJ556803.2

KEYWORDS .

SOURCE Bacillus thuringiensis

ORGANISM Bacillus thuringiensis

Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; Bacillus cereus group.

REFERENCE 1 (bases 1 to 2364)

AUTHORS Audtho,M., Ratlerdkarn,M. and Promdonkoy,B.

TITLE Direct Submission

JOURNAL Submitted (16-DEC-2008) National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, 113 Thailand Science Park, Phaholyothin Rd., Klong 1, Klong Luang, Pathumthani 12120, Thailand

REFERENCE 2 (bases 1 to 2364)

AUTHORS Audtho,M., Ratlerdkarn,M. and Promdonkoy,B.

TITLE Direct Submission

JOURNAL Submitted (20-MAR-2015) National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, 113 Thailand Science Park, Phaholyothin Rd., Klong 1, Klong Luang, Pathumthani 12120, Thailand

REMARK Nucleotide and amino acid sequences updated by submitter

COMMENT On Mar 20, 2015 this sequence version replaced gi:220966628.

FEATURES Location/Qualifiers

source 1..2364

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# *Bacillus thuringiensis* strain #22 Vip3Af gene, complete cds

## GenBank: KX595192.1

FASTA Graphics

Go to:

LOCUS KX595192 2367 bp DNA linear BCT 10-MAY-2017

DEFINITION Bacillus thuringiensis strain #22 Vip3Af gene, complete cds.

ACCESSION KX595192

VERSION KX595192.1

KEYWORDS .

SOURCE Bacillus thuringiensis

ORGANISM Bacillus thuringiensis

Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; Bacillus cereus group.

REFERENCE 1 (bases 1 to 2367)

AUTHORS Nimsanor,S., Chimpalee,S., Rungrod,A., Trakulnaleamsai,C., Jongsareejit,B. and Promdonkoy,B.

TITLE Cloning and characterization of vegetative insecticidal protein (Vip3A) from Thai isolated Bacillus thuringiensis

JOURNAL Veridian EJ Sci Technol Silpakorn Univ 3 (5), 228-236 (2016)

REFERENCE 2 (bases 1 to 2367)

AUTHORS Chimpalee,S., Rungrod,A. and Promdonkoy,B.

TITLE Direct Submission

JOURNAL Submitted (22-JUL-2016) National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, 113 Phahonyothin Road, Khlong Nueng, Khlong Luang, Pathumthani 12120, Thailand

FEATURES Location/Qualifiers

source 1..2367

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CDS 1..2367

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