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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

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

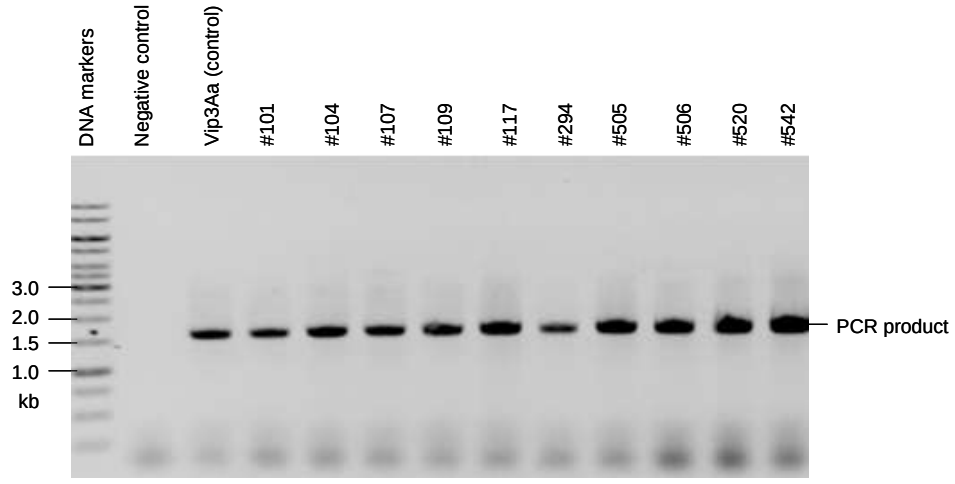
Primer name	DNA sequences (5′ to 3′)
Vip3 screening Fw	TGCCACTGGTATCAARGA
Vip3 screening Rv	TCCTCCTGTATGATCTACATATGCATTYTTRTTRTT
Vip3A full-length Fw	CCGCGGCCGCGGAGGATTAACATATGAACAAGAATAATACTAAATTAA
Vip3Aa full-length Rv	CTCGAGTTACTTAATAGAGACATCGGA
Vip3Ad full-length Rv	CTCGAGTTATTTAATAGAGAAATCATAAAAATGTA
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 2nd-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.



Veridian E-Journal, Science and Technology Silpakorn University
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Figure 2. Alignment of Vip3Aa, Vip3Ad and Vip3Af

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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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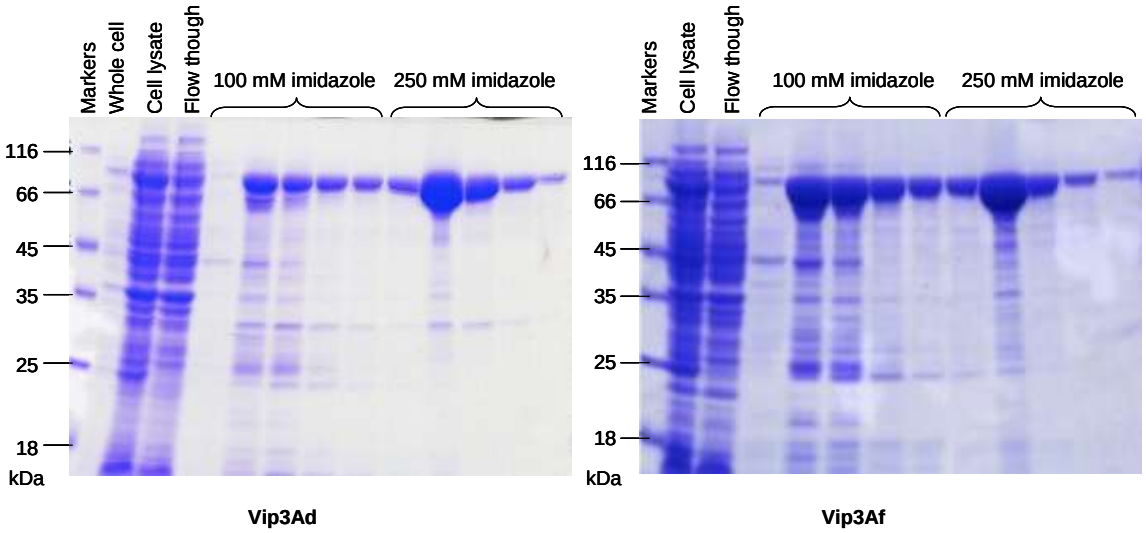
Figure 3. Coomassie blue stain of SDS-Polyacrylamide gel of the purified proteins from HisTrap FF column. Proteins were eluted in fraction containing 100-250 mM imidazole.



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