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Cloning and expression of the vegetative insecticidal protein (*vip3V*) gene of *Bacillus thuringiensis* in *Escherichia coli*

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Abstract

A genomic library of *Bacillus thuringiensis* var. *kurstaki* (*B.t.k.*) was constructed and a positive clone harboring the full-length gene encoding a novel vegetative insecticidal protein (Vip3V) was characterized. The *vip3V* gene was subcloned into pET-22b(+) vector and overexpressed in *Escherichia coli* to an extent of about 30% of the total protein. While transcription was influenced by the T7 promoter of the vector, synthesis of Vip3V in *E. coli* host occurred from the *B.t.k.* ribosomal binding site (rbs) found 917 bp downstream of the insert and not from the *E. coli* rbs of the vector. The expressed Vip3V protein was found in the soluble and periplasmic fractions as well as in the inclusion bodies. A simplified anion-exchange chromatographic method for the purification of Vip3V using step gradient or one-step elution was developed. The purified protein showed broad-spectrum activity against some of the lepidopteran larvae tested and did not show any activity against the larvae of silkworm (*Bombyx mori*) and mosquito (*Culex quinquefasciatus*). © 2002 Elsevier Science (USA). All rights reserved.

Keywords: *Bacillus thuringiensis*; Vegetative insecticidal protein; Biopesticide; Biocontrol agent; Overexpression; Secretory protein

Bacillus thuringiensis (*B.t.*), a well-known gram-positive bacterium, produces crystalline inclusions during sporulation. These inclusions contain highly insecticidal proteins (Cry proteins), which solublize in the insect midgut. The dissolved Cry protein is proteolytically processed to become 'activated toxin,' which binds to the high affinity sites present in the epithelial midgut cells of the susceptible insects. These bound toxins generate pores in the cell membrane, thus, disturbing the cellular osmotic balance [1]. This leads to the swelling and lysis of the cells, which eventually results in cessation of feeding and the death of the larvae [2]. In the past decades, many *B.t.* strains with different insect host spectra have been identified and their *cry* genes have been cloned in several microbes, including cry negative *B.t.* [3] and *E. coli*. [4,5]. Although industrial formulations of the Cry proteins have been used as biopesticides [6], most Cry proteins are not very effective in controlling some of the agronomically important insects [7]. The lepidopteran BCW[1], a worldwide pest that attacks more than 50 crops including cereal grains [8], is one such example.

A novel class of proteins called vegetative insecticidal proteins (Vips) produced by *B.t.* during its vegetative stages of growth have been identified [9,10]. Unlike the Cry proteins, the Vip3 is about 88.6 kDa in size [10]. The Vip3A shows acute bioactivity against BCW, 260 times higher than some of the Cry 1A proteins [8,10], and has broad-spectrum insecticidal activity against lepidopteran insects such as FAW, BAW, TBW, and CEW.

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[1] *Abbreviations used:* BAW, beet armyworm (*Spodoptera exigua*); BCIP, 5-bromo-4-chloro-3-indolyl phosphate, BCW, black cutworm (*Agrotis ipsilon*); CEW, corn earworm (*Helicoverpa zea*); CQ, *Culex quinquefasciatus* (mosquito); DBM, diamond black moth (*Plutella xyllostella*); DEAE, diethyl aminoethyl); DIG, Digoxigenin; EDTA, ethylenediaminetetraacetic acid; FAW, fall armyworm (*Spodoptera frugiperda*); GPB, gram pod borer (*Helicoverpa armigera*); IPTG, isopropyl-β-thiogalactopyranoside; NBT, *p*-nitroblue tetrazolium; O/N, overnight; SDS–PAGE sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SW, silkworm (*Bombyx mori*); TBW, tobacco budworm (*Heliothis virescens*); TCP, tobacco caterpillar (*Spodoptera litura*); X-Gal, 5-bromo-4-chloro-3-indolyl-β-galactoside.

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Though symptoms produced by Vips are similar to those caused by the Cry proteins, the symptoms develop over a period of 48–72 h after ingestion whereas it takes only 16–24 h for the symptoms to appear in the case of Cry proteins [11].

We have cloned and overexpressed a variant of *vip3* gene, *vip3V*, in the pET-22(b)+ vector. A simplified anion-exchange method for the purification [9] of the Vip3V protein and its effect on a few lepidopteran and dipteran insects hitherto unreported are also presented.

Materials and methods

PCR screening of vip3A gene

Primers (GCCCATGCAAAGAATAAT, GAACTA GTTTCTGTAGC) that correspond to 584 bp, coding region of the *vip3A*(*a*) gene [9] (GenBank Accession No. L48811) were used to check for the presence of *vip3A* gene in *B.t.* subsp *kurstaki* (*B.t.k.*), and *B.t.* subsp *israelensis* (*B.t.i.*) strains. Cells grown in 2 ml Luria–Bertani (LB) medium at 30 °C for 4–6 h were pelleted (5000*g* for 5 min), resuspended in 100 μl sterile distilled water, and boiled vigorously for 5 min. Ten μl of it was used as template DNA for the PCR using the conditions: 2 min initial denaturation at 95 °C, followed by 35 cycles of denaturation at 92 °C for 1 min, annealing at 55 °C for 1 min, and synthesis at 72 °C for $1\frac{1}{2}$ min. Final extension was carried out for 10 min at 74 °C [12]. The amplified fragment was cloned into pGEM-T vector and sequenced.

Dot-blot hybridization

Total DNA of PCR positive and negative *B.t.* strains [13] was blotted onto Hybond N^+ Nylon membrane, baked [12], and probed with the DIG-labelled amplification product [14].

Construction of genomic library

Total DNA of *B.t.* strain harboring the *vip3V* gene was digested with *Xba*I to completion and ligated with similarly digested pUC18 vector. *E. coli* DH5α cells were transformed with the ligation mix and selected on LB plates containing ampicillin (100 μg/ml), X-Gal (40 μg/ml), and IPTG (1 mM). White recombinant colonies grown on fresh plates were lifted onto nitrocellulose disks [12] and probed with DIG-labelled probe.

Restriction mapping

One of the clones (pVS647) showing positive signals in the colony blots was analyzed by restriction mapping, using the enzymes *Xba*I, *Acc*I, *Pst*I, and *Hin*dIII [14]. The digested fragments were checked on 1% agarose gel using λ DNA digested with *Hin*dIII and 1 kb Plus ladder as markers.

Sequencing of pVS647

The nucleotide sequencing of both strands of the insert DNA (4.4 kb) of pVS647 was performed using M13 forward, M13 reverse, and 25 internal primers in 13 sequencing reactions. The contigs were then assembled into a single non-overlapping contiguous sequence using the Fragment Assembly Program of Genetics Computer Group (GCG) of University of Wisconsin, Madison.

Subcloning and protein expression

The *vip3* insert was subcloned into pET-22b(+) vector as *Eco*RI–*Sal*I fragment (clone pVSBLD5) and transformed into *E. coli* host BL21(DE3). For expression, cells grown O/N in LB medium were used as seed culture to inoculate fresh cultures and when the optical density reached 0.6 at 600 nm, the cells were induced with IPTG (1 mM final concentration) and grown for 4 h post-induction at 37 °C and 250 rotations per minute (rpm).

SDS–PAGE and protein localization

Induced and uninduced cells were pelleted at 5000*g* for 5 min, resuspended in 50 mM Tris–HCl and 1 mM EDTA buffer (pH 7.4). These were sonicated six times at maximum power for 60 s each on ice. After a brief spin, the protein content was measured [15] and 50 μg of each of the protein samples was used for SDS–PAGE. Aliquots of induced and uninduced cultures were used to check for the presence of the expressed protein in the cytosol, periplasmic fractions and in the inclusion bodies [12].

Anion-exchange chromatography

Induced *E. coli* culture (1.5 litre) was centrifuged and the pellet (about 0.9 g wet weight) was suspended in Tris–Hcl (50 mM), EDTA (1 mM) buffer (pH 7.4). Cells were lysed by sonication and the pH of the supernatant was brought down to 4.5 using 20 mM sodium citrate buffer (pH 2.5). The resultant protein (isoelectric) precipitate was dissolved in 7 ml of 20 mM phosphate buffer (PB), pH 9.4, and loaded onto DEAE-Sepharose 6B anion-exchange column (8 ml matrix volume) pre-equilibrated with PB [16]. After washing the column once with 5 bed volumes of PB, bound proteins were eluted using a step gradient of 50, 100, 150, 200, 250, and 300 mM Nacl in PB (3 bed volumes each).

For batch elution, the protein in PB was mixed with equal volume of pre-equilibrated anion-exchange resin for 15 min. The slurry was washed once with 20 mM PB

and shaken with equal matrix volume of PB (250 mM NaCl concentration) for 15 min. The Vip3V-containing eluent was dialyzed O/N against 20 mM PB, pH 7.5.

Antisera production and immunoblotting

SDS–PAGE bands containing the induced proteins were injected intra-dermally to immunize two New Zealand white rabbits of about 1.5 kg [17], followed by two booster injections given subcutaneously within a gap of 15 days. Western blots were developed using the primary antisera treated with *E. coli* cell lysate for background reduction [12], secondary monoclonal anti-rabbit IgG–alkaline phosphatase conjugate, and the BCIP/NBT substrates.

ELISA and densitometric scanning

The total protein from induced and uninduced *E. coli* cells and the purified protein (50 μg each) were used to coat the wells of the ELISA plate [16]. The amount of expressed recombinant protein in the total *E. coli* protein was determined using the purified protein as the standard. One-dimensional lane (multi) densitometry and spot-densitometry software tools of the Alpha imager gel documentation system (Alpha Innotech, USA) were used to quantify and estimate the percentage of the expressed protein in the Coomassie brilliant blue stained gel.

Bioassay

Known amount of Vip3V was either coated onto fresh leaves and artificial diet cubes after measuring the surface area (cm^2) of each side and painting the appropriate volume of protein solution (concentration in nanograms) using a paint brush for bioassay on insects or by simply mixing it with water in the case of bioassays against mosquito larvae [18]. Late neonatal or first instar larvae were allowed to feed on such Vip3V-coated leaves or diet cubes (ng/cm^2) and maintained at 28 °C. Culex mosquitoes were maintained in water into which known amounts of Vip3V, *pET*/*E. coli* extracts (concentrations in micrograms) and buffer were added. Mortality was recorded up to 6 days.

Results and discussion

PCR screening of B.t. strains

Out of 11 *B.t.k.* serovars tested by PCR, only two strains produced the expected (584 bp-long) amplification product and the DNA template from *B.t.i.* did not produce any amplification product (Table 1). Amplified fragment was cloned into pGEM-T vector, sequenced, and found to be 99% identical to the fragment found in *vip3A*(*a*). The presence of *vip3A*(*a*) like gene in the PCR positive *B.t.k.* strains and absence of the same in the *B.t.i.* strain were confirmed by dot-blot hybridization (Fig. 1).

Table 1
PCR Analysis for the presence of *vip3A*(*a*) gene in *B.t.* strains

Strain	Amplification product
B.t.k. HD1(4D1)	–
B.t.k. HD2(4D2)	–
B.t.k. HD3(4D3)	–
HD73(4D4)	–
HD164(4D5)	+
HD1(4D6)	+
HD1Cry-1(4D7)	–
HD1Cry-6(4D8)	–
HD1Cry-7(4D9)	–
HD1Cry-8(4D10)	–
Cry(–)B(4D11)	–
B.t.i. HD567(4Q1)	–

PCR was carried out as described in the Materials and methods section. The strain designations of Bacillus Genetic Stock Center, Ohio State University, USA are given in parentheses.

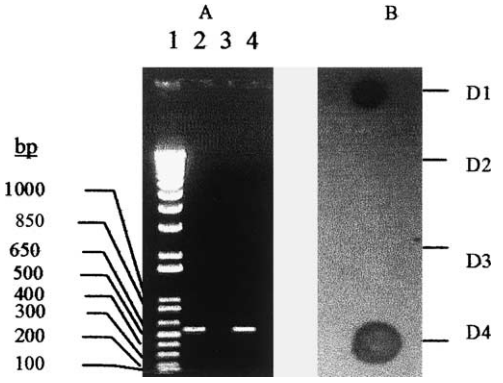

Fig. 1. *vip3A* gene analysis. (A) PCR analysis of *B.t.* strains. Lanes: 1, DNA marker (1 kb plus ladder); 2, *B.t.k.* HD1(4D5); 3, *B.t.i.* HD567; 4, *B.t.k.* HD1(4D6). (B) Dot-blot analysis of the *B.t.* strains used in PCR. D1, D2, D3, and D4 correspond to *B.t.k.* HD164, *DH5α*, *B.t.i.* HD567, and *B.t.k.* HD1(4D6) strains, respectively.

Genomic cloning of vip gene

When 900 clones of pUC18-based *B.t.k.* genomic library were probed with DIG-labelled probe, four cells gave positive signals. Restriction mapping of one of the clones (pVS647) using the enzymes *Xba*I, *Acc*I, *Pst*I, and *Hin*dIII showed that the cloned gene fragment (4.4 kb) was in the same orientation as that of the *lacZ* gene of the pUC18 vector and the restriction pattern obtained was similar to that of the *vip3A*(*a*) gene (2.4 kb) flanked by 0.7 and 1.3 kb up- and downstream sequences, respectively (Fig. 2).

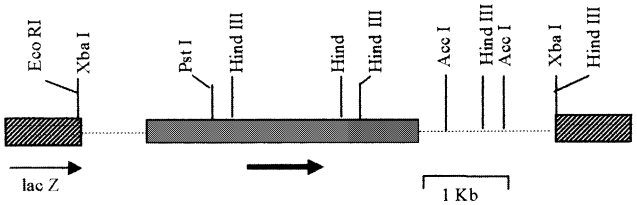

Fig. 2. Partial restriction map of pVS647. Hatched bars indicate pUC18 vector ends. Shaded bar indicates the putative coding region of the gene. Bold arrow indicates the orientation of the cloned *vip3V* gene. Dotted lines indicate the up- and downstream flanking regions.

```
502  TAGGGATAAATGAATTTTTGTGAATAGGAAGACCATTTGATAGGTAAATATTATAATTCA   561

562  AAAAGTAGAATAAGCAAAATTGAGTAACACATTAATGATATAAACTAATTTTATACAATT   621

622  GAAATTGATAAAAAGTTATGAGTGTTTAATAATCAGTAATTACCAATAAAGAATTAAGAA   681

682  TACAAGTTTACAAGAAATAAGTGTTACAAAAAATAGCTGAAAAGGAAGATGACATGAAC   741
  1                                              sd   M  N        2

742  AAGAATAATACTAAATTAAGCACAAGAGCCTTACCAAGTTTTATTGATTATTTTAATGGC   801
  3    K  N  N  T  K  L  S  T  R  A  L  P  S  F  I  D  Y  F  N  G   22
```

Fig. 3. Partial nucleotide and amino acid sequences of the *vip3V* gene encoding the 88.6 kDa insecticidal protein. The deduced amino acid sequence for the ORF is presented underneath the nucleotide sequence. The putative −35 and −10 regions are shown by single and double underlines. The Shine–Dalgarno (SD) sequence is shown inside the box.

Sequence analysis of the insecticidal gene

Sequence analysis of the 4.4 kb of the clone pVS647 revealed an open reading frame (ORF) of 2367 bases from nucleotides (nt) 736 to 3103 that encode a protein of 789 amino acids with about 88.6 kDa deduced molecular mass. A putative *B.t.* consensus −35 region [19] at nt 538–543, a −10 sequence at 553–558 and a terminator sequence at nt 3447–3474 were found (Fig. 3). A Shine–Dalgarno (SD) sequence found at nt 723–729 matches with that of a strong SD sequence of *B.t.* [9,20]. The translated sequence of the gene *vip3V* (GenBank Accession No. AF 373030) differs from those of the three previously reported *vip3* genes (L48811, L448812, and Y17158) at five amino acid residues: with Vip3A(a) at Q284 → k, with Vip3A(b) at R206 → L, P291 → T, G406 → E, and with Vip-S at G742 → E (Table 2).

Table 2
Amino acids showing regions of variations among the Vip proteins

	Positions of amino acids				
	206	284	291	406	742
Vip3A(a)	L	**Q**	T	E	E
Vip3A(b)	**R**	K	**P**	**G**	E
Vip-S	L	K	T	E	**G**
Vip3V	L	K	T	E	E

Shaded boxes indicate the differences found in the amino acids of previously reported Vip3 proteins with respect to Vip3V protein.

Transcription and overexpression in E. coli

B.t. insecticidal crystal protein encoding genes with or without their native promoters have been cloned and expressed in *E. coli* cells [5,6]. There are reports that deletion of sequences 15–145 bases upstream of −35 region of certain promoters [21] and deletion of 12–52 bases upstream of the crystal protein promoters [5] decrease transcription levels. In previous studies, the coding sequence of *vip3A*(*a*) gene was cloned into pET3d vector as two fragments by a three-way ligation with an introduction of *Nco*I site at the start codon, which changed the amino acids Asn(AAC) to Asp(GAC) [11]. Hence, we cloned the entire 4.4 kb *Xba*I fragment comprising 2.4 kb coding sequence and the 0.7 and 1.3 kb up- and downstream sequences into the pET-22b(+) vector.

Thus, our chimaeric vector pVSBLD5 (9.9 kb) contained *T7* promoter, lac operator, *E. coli* rbs, start codon, followed by the pelB leader sequence and the multiple cloning site of the vector wherein the *vip3V* gene was inserted as an *Eco*RI–*Sal*I fragment (Fig. 4). Only on induction with IPTG, this construct in BL21(DE3) expressed Vip3V protein of about 88.6 kDa protein to an extent of about 30% of the total protein (Fig. 5A). The expressed protein was found in the soluble, periplasmic fractions and in the inclusion bodies (Fig. 6) and was similar to Vip3A(a) protein in size, in its biochemical and biological properties (Table 4). There are no significant transcriptional termination sequences found in the 5′-flanking sequences and only on IPTG induction, overexpression of Vip3V protein corresponding to the longest ORF starting at 917 bp downstream of the transcript occurs. This is suggestive of the initiation of translation occurring at this region from the native *B.t.* rbs present and not from the *E. coli* rbs of the pET vector (Fig. 4). Whether the ORF1 beginning from the *E. coli* rbs, encoding 42 amino acids, is also

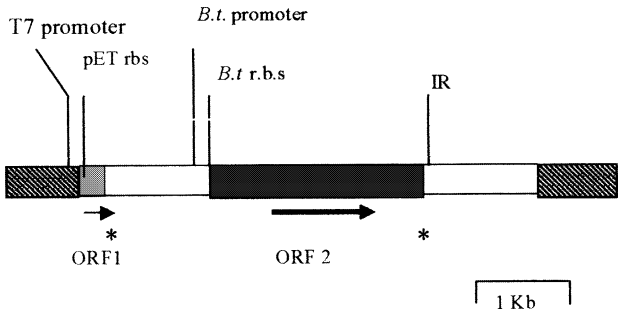

Fig. 4. Schematic map of pVSBLD5. Full-length *vip3V* gene in the expression cassette of pET 22-b(+) vector. Hatched bars indicate vector ends. Unshaded region shows the 5′ and 3′ flanking regions of the cloned gene. Shaded bar indicates the putative ORF of the pET vector. Solid bar shows the putative ORF of *vip3V* gene. Asterisk indicates the stop codon on both ORFs. Promoter sequences, rbs, and the inverted repeats (IR) are marked with vertical lines.

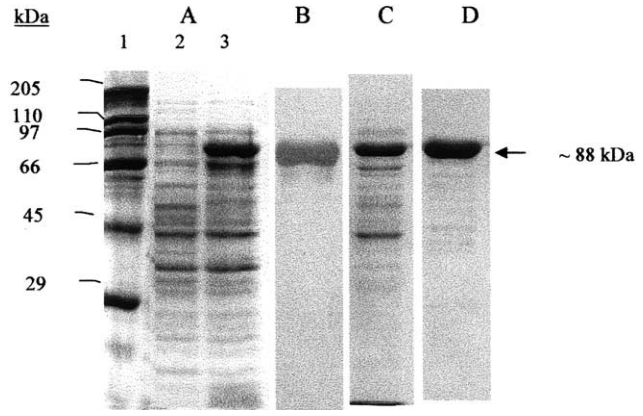

Fig. 5. Electrophoretic analysis and immunoblot of Vip3V protein expressed in *E. coli*. (A) SDS–PAGE (10% gel) stained with Coomassie stain. Lanes 1–3 correspond to protein molecular weight standards, 50 μg sonicated crude extract of uninduced and induced cells containing pVSBLD5, respectively. (B) Western blot analysis of the proteins transferred to the membrane. Rabbit anti-Vip3Vserum treated for background reduction was used in the blot. (C) and (D) are Coomassie stained SDS–PAGE (10%) gels of isoelectric precipitate and purified Vip3V insecticidal protein (88.6 kDa) fractions.

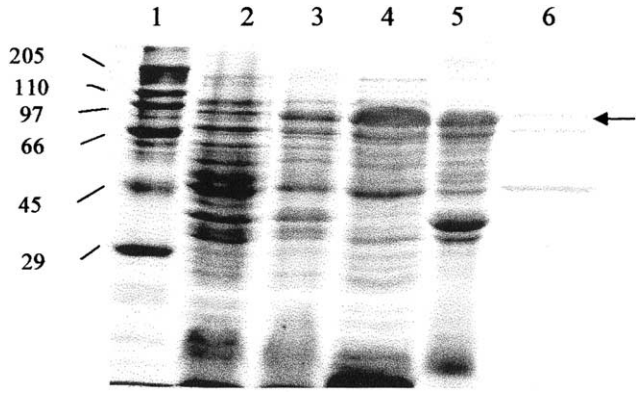

Fig. 6. Electrophoretic analysis and subcellular localization of Vip3V protein expressed in *E. coli* cells. Lanes: 1, protein molecular weight standards; 2, crude extract of uninduced cells with pVSBLD5; 3, induced cell extract; 4, *E. coli* cytosol fraction; 5, inclusion bodies; 6, periplasmic fraction.

translated [22,23] from the same transcript (as bicistronic) has not been verified. The transcriptional regulatory role, if any, of the upstream elements of the *vip* gene remains to be assigned.

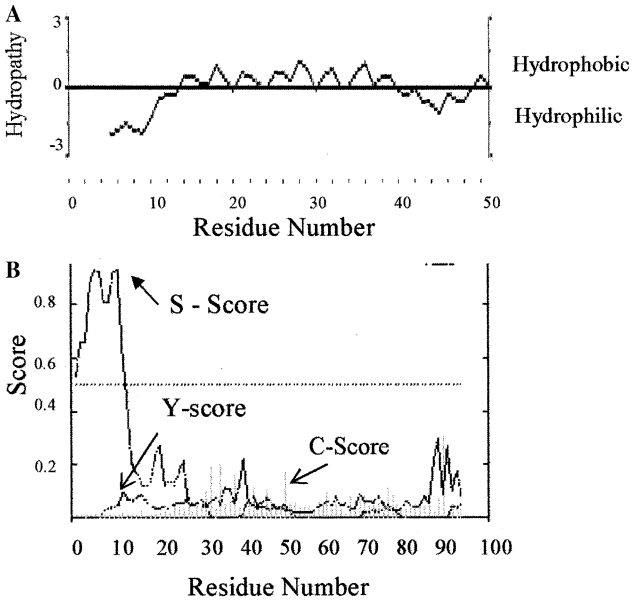

Fig. 7. (A) Hydropathic profiles of first 50 aminoacids of deduced Vip3V amino acid sequences, as calculated by the method of Kyte and Doolittle. In Vip3V, the hydrophilic nature of the first eight amino acids followed by hydrophobic core of about 31 amino acids is apparent. (B) "SignalP" prediction plot of the first 100 amino acids of Vip3V Protein. The S (signal peptide) score marked with arrow is apparently high between 10 and 11 amino acids and low thereafter, which is characteristic of signal sequences. The C (raw cleavage site) score and Y score (geometric average between the C score and S score) are marked by arrows.

Isoelectric precipitation and ion-exchange purification

When the pH of the *E. coli* lysate was brought down to 4.5 (its predicted isoelectric pH), Vip3V protein precipitated (Table 3) as a major constituent of the flocculent precipitate (Fig. 5C). This upon purification in the DEAE anion-exchange column eluted at near homogeneity (Fig. 5D) at 250 mM NaCl concentration both in 50 mM step gradient starting from 50 to 300 mM or in batch elution using a single ionic buffer.

Secretion of Vip protein

Though protein secretion from the *Bacillus* cell is mediated by the signal peptide, which is cleaved during the export [24], the Vip3A protein is secreted out by *B.t.* into the culture medium without N-terminal processing [9,25]. As shown in the hydropathy plot (Fig. 7A) of

Table 3
Purification of Vip3V protein by anion-exchange chromatography

	Total protein (mg)	Vip3V concentration (mg)	Recovery (%)	Purity (%)
Sonicated *E. coli* extract	158	47	100	30
Isoelectric precipitate	70	35	74	50
Anion-exchange column eluent	47	27	57	57
250 mM fraction	27	25.7	54	95

Table 4
LD_{50} values of Vip3V protein on Lepidopteran insects

	LD_{50} (95% fiducial limits) (ng)
BCW	80.7 (18–361)
GPB	325.20 (119–881)
DBM	220.73 (99–494)
TCP	45.41 (37.4–55.2)

Mortality was recorded up to 6 days. The results are the average of at least three trials with a minimum of 15 larvae per trial. Probit Analysis Software program was used to derive at the LD_{50} values (dosages of the protein required to bring out 50% mortality rates of the insects tested). The basal levels of mortality observed in the buffer and the *pET*/*E. coli* extract controls were also included for deriving the lethal doses.

Kyte and Doolittle [26], Vip3V contains hydrophilic residues of amino acids (a.a.) 2–8 followed by a hydrophobic core region from a.a. 9 to 34, which is similar to other signal peptides described for *Bacillus* [27]. As predicted by the signal peptide prediction program "SignalP" [28], Vip3V contains a putative signal peptide cleavage site between a.a. 10 and 11 (Fig. 7B) with an S score (signal peptide score) being high and low before and after this site. While secretion of the Vip3V into the culture supernatants of the *vip*-containing *B.t.* strains has been verified by immunoblot analysis (data not shown), N-terminal processing of Vip3V, if any, needs to be analyzed.

Insect bioassays

Bioefficacy of the Vip3V on few of the known, Vip3A(a)-sensitive lepidopteran insects and geographically distinct insects of the same family were tested (Table 4). Likewise, the effect of Vip3V on lepidoteran insects such as SW, DBM, and CQ larvae has been checked. Unlike Cry toxins, Vip3V is not toxic to SW and CQ, even at concentrations of $10\,\mu g/cm^2$ and $10\,\mu g/ml$, respectively (data not shown).

Vip3V protein reported here causes 50% mortality (LD_{50}) in BCW at a concentration of $80\,ng/cm^2$, whereas $2165\,ng/cm^2$ of the VipS and $62\,ng/cm^2$ of Vip3A(a) cause 50% and 100% mortality, respectively [9,29]. Similarly, LD_{50} doses of 5 and $36\,ng/cm^2$ of VipS are reported for TCP and DBM [29], while they are 45 and $220\,ng/cm^2$, respectively, for Vip3V. Whether the differences in the activity among these proteins are due to the changes in the amino acids (Table 2) or due to other factors such as altered sensitivity of the same but geographically distinct insects or the differences in larval stages used by each group remains to be verified.

The efficacy of Vip3V protein in controlling some of the agronomically important lepidopteran pests in the laboratory has been established and efforts are underway to develop this overexpressed protein as a biocontrol agent for field applications.

Acknowledgments

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