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Cloning, Expression and Bioassay of Vip3A Protein from an Indigenous *Bacillus thuringiensis* Isolate

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The present study was undertaken to test vip3A protein from *Bacillus thuringiensis* for use against lepidopteran pests especially *Spodoptera litura* and whether cloning and expression of the vip3A gene will help in formulating an improved bioinsecticide. Eight isolates that showed amplification of the vip3A gene (675bp) were selected from 150 *B. thuringiensis* isolates. The isolate BT-EG1 was selected for further studies as the vip3A protein extracts from this isolate was found to cause mortality of *Spodoptera litura* (LC_{50} of 9.09-9.92 μg/ml). The 2.367bp complete CDS was amplified using VCL1 and VCL2 specific primers and cloned into $_p$UC19 at NdeI and XhoI restriction sites. The positive clones were sequence confirmed. The vip3A was the successfully cloned into pET21a (NdeI/XhoI) expression vector and confirmed by restriction digestion and SDS-PAGE. The crude protein extracts obtained after 16h of IPTG induction showed a LC_{50} of 5.83 μg/ml against *S. litura* at 72h. The extracts were however highly toxic to *Plutella xylostella* (LC_{50} of 0.43 μg/ml after 16h of IPTG induction at 48h). The extracts however did not cause mortality of *Helicoverpa armigera* suggested the requirement of a combination of vip proteins for broad spectrum activity.

Keywords: Indigenous, *Bacillus thuringiensis*, cloning, expression,bioassay, vip3A gene, vip3A protein.

The capacity of *Bacillus thuringiensis* isolates to form crystal proteins that are made up of δ-endotoxins which are toxic to insects, during sporulation has been widely researched[1]. Vegetative insecticidal proteins (vips) are a different family of toxin proteins produced by *Bacillus* during vegetative phase or log phase of growth[2] and are totally different from δ-endotoxins. vip3A proteins are holotype toxins. The vip3A gene encodes an 88-kDa protein and is released into the supernatant fluid by *B. thuringiensis* cultures[3]. Research work has shown that the vip3A protein has broad spectrum insecticidal activity against lepidopteran pests and have been shown to be highly active against black cutworm (BCW; *Agrotis ipsilon*), fall armyworm (FAW; *Spodoptera frugiperda*), and beet armyworm (*Spodoptera exigua*)[4, 5]. Experiments have recorded that against BCW, vip3A exhibited 260-fold increased insecticidal activity when compared to some Cry1A proteins that were also active against the BCW[4]. Four main protein products with molecular weights of 22, 33, 45, and 66-kDa were obtained from trypsin treated vip3Aa1 protein[6]. Studies suggest that the 66-kDa fragment is the activated "trypsin resistant core" that is toxic and may vary in size from 62 to 66-kDa[6,7] and is conserved. However this 66-kDa

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fragment needs the 22-kDa N-terminal portion for folding since experiments have shown that the *E. coli* cloned with 66-kDa fragment was not toxic by itself[3,8,9]. In India research work on vip3A proteins is limited. We attempted PCR amplification, cloning and sequencing of vip3A gene (from a native *Bacillus thuringiensis* isolate (NBAIR-BTEG1)) in cloning vector, sub-cloning into pET expression vector, expression checking and bioassay against lepidopteran pests.

MATERIALS AND METHODS

At ICAR-National Bureau of Agricultural Insect Resources, native *Bacillus thuringiensis* isolates are being routinely isolated from soil / infected insect samples collected from different parts of India. The native *B. thuringiensis* isolates were isolated as per improved procedure of[10] and subjected to cry gene profiling[11,12]. PCR amplification of the vip3A gene was carried out as per the procedure of Rang *et al*. (2005) and primers were designed using DNASTAR (Version 2.5) based on available sequences at NCBI and synthesized from Chromous Biotech Pvt. Ltd.), for partial sequence (650-700bp). Chromosomal DNA was purified using extraction kit (HiPuria, Himedia). The PCR mixtures were prepared using 0.2-0.4 ¼M each of the forward and reverse primers (Forward primer: 5' -CTCAATGGGACGCATTTCTT- 3 Revered primers: 5' -CGGTTGTAAGG GCACTGTTC- 3').), 0.5U of Taq DNA polymerase (GeNei), 200 ¼M dNTP, 10 mM Tris, 50 mM KCl and 1.5mM MgCl2, 50ng of template DNA. PCR was run using the following conditions, 30 cycles of 5 min initial denaturation at 94°C, denaturation at 94°C for 1 min, annealing at 50°C for 1 min and elongation at 68°C for 1 min. Final extension was for 7.30 min at 68°C. PCR amplification was carried out in a thermal cycler (Bio-Rad T100). PCR products were run using 1.2% agarose having 0.1% EtBr, DNA bands were visualized in a gel documentation system (DNR, Israel). Ten isolates that showed amplification of vip3a gene were identified, sequences analysed and submitted to GenBank.

Selection of virulent isolate

In order to select a virulent isolate, crude preparations of vip3A protein was obtained from 10 isolates and analysed for toxicity against second instar larvae of *Spodoptera litura* as our target was this difficult pest. Mortality was recorded at 48h and LC_{50} values recorded (Table 1). The potential toxic isolate BTEG1 was selected as most toxic based on LC_{50} values.

Protein isolation from *Bacillus thuringiensis* isolates

The Bt isolates were grown in Terrific Broth Medium (HiMedia) for 24h[13] and the vegetative insecticidal proteins were harvested by centrifugation at 12,000 x g (Sigma 3-30K) for 10 min at 4ºC, pellet was removed and the vegetative proteins present in the supernatant were precipitated with ammonium sulphate [(NH4)2SO4] (80% saturation). The precipitate was pelleted at 12,000 x g for 10 min at 4 ºC (Sigma 3-30K). The pellet was re-suspended in minimum volume of 20 mM of Tris HCl (pH 7.4) and dialyzed overnight at 4°C. The amount of crude protein so obtained was estimated by Bradford's method, taking BSA as the standard [14]. Bioassay of the crude protein was carried against the larvae of *Spodoptera litura* and *Helicoverpa armigera*.

Insect Bioassay with the crude protein extracts
Lepidopteran insect pests

Insects belonging to Lepidoptera *viz*., *Plutella xylostella*, *Helicoverpa armigera* and *Spodoptera litura* were obtained from the mass production unit of the institute, maintained under laboratory conditions (25ºC and 70% RH and 16 L : 8 D). The first instar neonate larvae were subjected to the toxicity assays separately in each type of experiment. Crude protein extracts isolated from the culture supernatants of different Bt strains (25 µg ml^{-1}), were mixed with the artificial diet and allowed to solidify. The first instar larvae were placed with a soft brush on each such experimental set, containing diet supplemented with crude proteins, and kept in the laboratory conditions for seven days. In each experimental set, ten such larvae were used and a control set devoid of the toxin, but containing the buffer solution was also maintained. For the larvae of *P. xylostella* unsprayed cabbage leaves were used. Leaf discs (2.5 cm. Diameter) cut from 5 weeks old cabbage plants were dipped in the diluted protein solutions and placed in the container and allowed to dry. Second instar larvae of *P. xylostella* were introduced in the container. The mortality was recorded up to 72h. Each experimental set was

replicated thrice. The data was subjected to probit analysis using the SPSS 10 software.

Amplification of full length vip3A gene

The isolate *B. thuringiensis* (NBAIR-BTEG1) was selected for further studies based on bioassay results. Genomic DNA was isolated using HiPura (HiMedia) DNA isolation kit. The 2.3 kb full-length vip3A gene was ampliûed with primers VCL1 (ATAAGAATGCGGCCGCATGAACAA GAAT AATACTAA) and VCL2 (ACCGCT CGAGTTA TCTAATAGAGACATCGT) [19] using a thermal cycler (Bio-Rad T100). The PCR mix consisted of template (gDNA) 1.0 µl, forward Primer (100ng/ µl) 2.0 ¼l, reverse primer (100ng/ µl) 2.0 µl, dNTPs (10mM) 2.0 µl, 10X Taq Assay Buffer 5.0 µl, Taq polymerase (3U/µl) 0.5 µl (from Chromous Biotech), water 37.5 µl, Total reaction volume 50.0 µl. PCR conditions included 5min initial denaturation at 94°C, followed by 35 cycles of denaturation at 94°Cfor 30sec, annealing at 50 °C for 30 sec and elongation at 72 for 2.30 min. Final extension was for 15 min at 72°C. The full-length vip3A gene was ampliûed and analysed by electrophoresis in a 1% agarose gel.

Vip3a gene cloning and expression studies

PCR amplicon (~2.3Kb) was gel purified and cloned into a cloning vector (pUC29) at NdeI and XhoI restriction sites. Probable clones were screened by colony PCR and further confirmed by restriction digestion. Positive clone was sequenced and confirmed. The full length gene was submitted to GenBank (KT985383) and processed further for Sub-Cloning in expression vector (pET21a). For sub cloning primers designed were NBFP 5' – GACTATCATATGAACAAGAATAATACTAAATTAAGCAC – 3' (NdeI) and NBRP1 5' – GTACATCTCGAGCT TAATAGAGACATCGTAAAAATGTAC – 3' (XhoI). The vip3a-pUC29 clone was restriction digested (NdeI/XhoI); released vip3a gene was then cloned into pET21a (NdeI/XhoI) (supplied by Chromous Biotech with transformation protocol). Probable clones were screened and confirmed by restriction digestion. Positive pET21a clone was then transformed in BL21.de3 expression

Table 1. Effect of vip3A protein extracts on mortality of *Spodoptera litura*

Sl No.	Isolate No.	LC_{50} value (µg/ml)	95% confidence limits Lower	Upper	Std. Error
1	TRBT-19	15.85	1.0	27.12	0.71
2	ASBT-15	29.00	8.35	42.88	0.63
3	BT-D2	17.70	5.31	26.14	0.83
4	NE-60	15.86	1.10	27.08	0.64
5	ASBT-25	16.49	0.219	29.58	0.66
6	BT-EG1	9.92	1.0	22.34	0.74
7	TTRBT-10	107.54	67.54	2341.97	0.49
8	BT-G4	154.16	90.29	4747.04	0.53

Table 2. Effect of vip3A extracts on mortality of *Helicoverpa armigera*

Sl No.	Isolate No.	LC_{50} value (µg/ml)	95% confidence limits Lower	Upper	Std. Error
1	TRBT-19	14.74	3.14	23.79	0. 55
2	ASBT-15	26.90	0.385	44.59	0.46
3	BT-D2	13.79	1.16	23.08	0.94
4	NE-60	42.02	18.36	64.27	0.62
5	AS-BT25	16.72	4.49	25.90	0. 53
6	BT-EG1	9.09	1.0	19.21	0.56
7	C3	15.91	1.16	23.78	0.80
8	TRBT-10	16.72	1.82	27.59	0.71

host as per supplier protocol (Chromous Biotech) and screened for protein expression. Single colonies were screened for checking vip3A gene expression. The culture was grown to an OD of 0.6 at 600nm and induced with 1mM IPTG. Before induction, 4 hour after induction and 16 hours (overnight) induction samples were processed for protein extraction and loaded on 12% SDS-PAGE.

Protein extract from BL21.de3 (*Escherichia coli*) for bioassay

Vip3A proteins was expressed in recombinant *E. coli* BL21.de3 cells after induction with 1 mM isopropyl-b-D-thiogalactopyranoside (IPTG). Transformed *E. coli* clones were grown at 37°C in LOC medium containing 100 µg/ml ampicillin till OD_{600} of 6.0 followed by IPTG (1mM) induction for 4 and 7 hrs (maintained separately). 10mL of the culture was subjected to medium intensity sonication of 3 pulses for 20sec. 1mL of the sonicated culture was serially diluted and added to diet or spread on diet leaf[3]. Bioassay against *S. litura, H. armigera* and *P. xylostella* were carried out as described previously. The amount of protein used in bioassays was determined according to Bradford[14].

RESULTS AND DISCUSSION

The present study was undertaken mainly to assess the possibility of use of vip3A proteins for wider use against lepidopteran pests especially *Spodoptera litura* and whether cloning and expression of the vip3A gene will help in formulating an improved biopesticide. In the first experiment, the isolates that showed amplification of the vip3A gene (675bp) were selected from 150 *B. thuringiensis* isolates (Fig. 1). Sequence analysis of the genes confirmed their vip3A identity

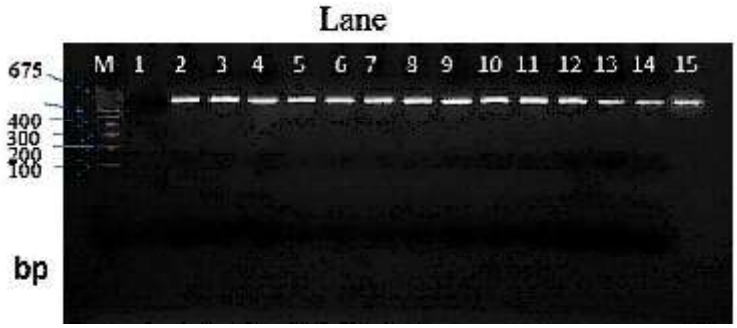

Fig. 1. Amplification of vip3A (675 bp) gene. M-1kb marker, Lane 1-Blank, Lane 2- HD1 (standard), Lane 3-BTEG1, Lane 4- BTD2, Lane 5-AsBt15, Lane 6-TRBT10, Lane 7- ASBT25, Lane 8-BTG4, Lane 9-BTNE60, Lane 10-C3, Lane 11-C3, Lane 12-TRBT19, Lane 13-N3

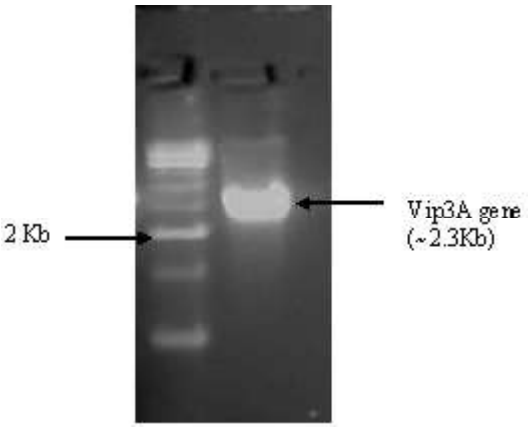

Fig. 2. PCR Amplified Vip3A gene from NBAIR-BTEG1 loaded on 1% Agarose Gel

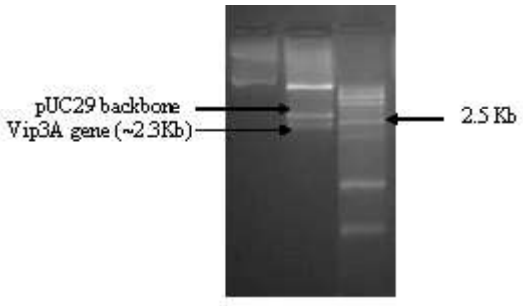

Fig. 3. Restriction digestion (NdeI/XhoI) of pUC29-Vip3a Clone

and 8 sequences were submitted to GenBank (KC596007, KC596008, KC596009, KC596010, KC596011, KC596012, KC596013 and KC596014). Identification of novel vip3 genes by PCR amplification using primers specific to conserved regions has been shown [15,16]. In our studies we confirmed the gene identity by blast analysis with NCBI. Bioassay of crude vip3A extracts from these eight isolates showed variation in their toxicity against *S. litura* and *Helicoverpa armigera* (Table 1 and Table 2). However for further studies the isolate BT-EG1 was selected as the vip3A protein extracts from this isolate was the most toxic exhibiting an LC_{50} value of 9.09-9.92 µg/ml. Crude extracts of vip3A protein was toxic to lepidopteran and homopteran insects when tested at 25 - 50 µg/ml[13]. [17] showed that protoxin crude extracts of vip3Aa and was toxic to *S. exigua* (LC_{50} 650 ng/cm^3) and *Agrotis epsilon* (LC_{50} 14 ng/cm^3). In our studies the crude extract of BT-EG1 was consistent in causing mortality to both *S. litura* and *H. armigera*. BT-EG1 was originally isolated from infected *Euthalia aconthea* (Hewitson, 1874) a lepidopteran pest of mango.

The 2.367bp complete CDS was amplified using VCL1 and VCL2 specific primers (Fig. 2). The PCR amplicon fragment was cloned into $_P$UC19 at NdeI and XhoI restriction sites. Probable clones were selected based on colony PCR and restriction digestion (Fig. 3). The positive clones were sequence confirmed. The sequence generated was confirmed as vip3A protein and submitted to GenBank (KT985383). The amino acid sequence analysis of the protein showed it composed mainly of leucine, asparagine, lysine, threonine, serine, isoleucine, glycine, glutamic acid, aspartic acid, valine and alanine (80%) with total mass of 92,602.76 Dalton. This protein undergoes processing to a 63kDa active form in the guts of susceptible and tolerant larvae[18]. The vip3A was

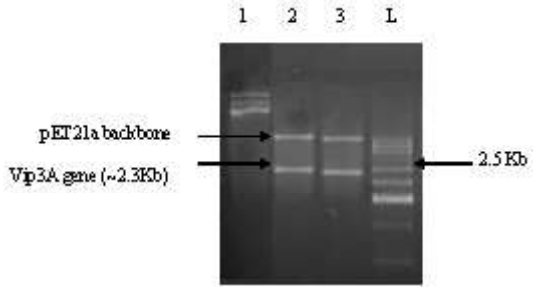

Fig. 4. Restriction digestion (NdeI/XhoI) of pET21a-Vip3A Clone {1 – Un-cut plasmid (pET21a-vip3a Clone, 2 – Digested clone (pET21a-vip3a), 3 – Digested clone (pET21a-vip3a), L – 500bp DNA Ladder}

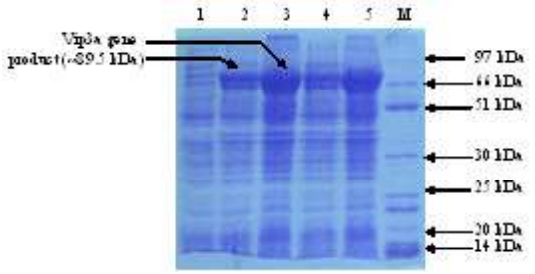

Fig. 5. Protein Expression Checking on 12% SDS-PAGE (Lanes 1 – Before induction sample; 2 – Colony 1: 4hours after induction sample; 3 – Colony 1: 16hours after induction sample; 4 – Colony 2: 4hours after induction sample; 5 – Colony 2: 16hours after induction sample; M – Protein Marker)

Table 3. Bioassay of recombinant vip3A protein extract against *Spodoptera litura* and *Plutella xylostella*.

IPTG induction time	LC_{50} (µg/ml)	95% confidence limits Lower	Upper	Std. Error
Spodoptera litura (at 72h)				
4 h	8.79	4.69	17.68	0.161
16h	5.83	2.94	11.20	0.160
Plutella xylostella (at 48h)				
4h	1.91	0.97	3.20	0.190
16h	0.43	0.10	0.86	0.252

successfully cloned into pET21a (NdeI/XhoI) expression vector and confirmed by restriction digestion (Fig. 4). Protein expression in BL21.de3 expression host was further confirmed by SDS-PAGE (Fig. 5). First successful cloning the vip3A gene in *E. coli* was reported[3]. For cloning of the 2460bp vip3A gene from plasmid of *B. thuringiensis* strain WB50 into the pMD18-T vector and *E. coli* JM109 cells were used for transformation and expression [19]. They reported that occurrence of vip3A in bacterial genome was not known. In this study we have shown that vip3A also occurs in bacterial genome and can be cloned.

Our next step was to assay the vip3A protein expressed in transformed BL21.de3 for insecticidal activity against insect pests. Reports suggest that purified vip3A proteins partially lose their activity after chromatography purification whereas crude extracts retain their activity[17]. Since our main aim was to come out with a cost effective product we evaluated the crude extracts for toxicity against the larvae of *S. litura, H. armigera* and *P. xyostella*. Extracts obtained after 16h of IPTG induction showed a LC_{50} of 5.83 µg/ml against *S. litura,* and extracts obtained after 4h of IPTG induction exhibited LC_{50} of 8.79 µg/ml (Table 3). Surprisingly no mortality was observed for *H. armigera*. However the extracts showed high toxicity to *P. xylostella* (Table 3) within 48h (LC_{50} of 0.43 µg/ml after16h IPTG induction). Vip3A extracts expressed by recombinant *E. coli* showed 100% mortality of *Agrotis epsilon* at 6 days (primary protein concentration of 1mg/ml) [3]. Experiments have shown that maximum mortality with vip3A crude extracts when fed to first instar neonate larvae of *A. ipsilon* and *S. littorallis* at a dose of 25 ¼g ml^{-1} of artificial diet [13]. In our study *H. armigera* was not found susceptible to the recombinant protein extract. Reports suggest that various types of vip proteins occur and vip3Ad, vip3Ae and vip3Af are new members of the vip3A family [17,20,21] sharing around 81-88%identity with vip3Aa. Vip3Ae protoxin was more toxic than vip3Aa and vip3Af protoxins [17]. Hence to achieve higher efficiency of vegetative insecticidal proteins a combination of the toxins needs to be present. Our studies have shown that the recombinant vip3A protein could be highly useful in combating *Plutella xylostella* as well as *Spodoptera litura.* We have currently formulated the protein for use in greenhouses.

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