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ENCODING OF BT (*Bacillus thuringiensis*) PROTEIN BY FUSION GENE

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ABSTRACT

A chimeric fusion gene was constructed with the coding regions of insecticidal crystal protein (Cry1Ac) and vegetative insecticidal protein (*vip3Aa14*) of *Bacillus thuringiensis*. Hyperexpression of the fusion gene in *E. coli* resulted in the synthesis of a protein of the size of ~140kDa as revealed by SDS-PAGE analysis. Stability of the fusion protein was studied by trypsin digestion. Western blotting against Cry1Ac and Vip3A polyclonal antisera showed the presence of a band corresponding to ~140 kDa. Insect bioassays of the fusion protein on three insect species viz., *Helicoverpa armigera*, *Spodoptera litura* and *Plutella xylostella* showed that the fusion protein retained the toxicity of Cry1Ac, but partially lost that of Vip3Aa14.

Key words : *Insecticidal protein and fusion gene, Bt*

Bacillus thuringiensis (Bt) is a gram-positive soil bacterium, naturally endowed with the capacity to produce insecticidal proteins, which are specifically toxic to insect pests (1). Bt produces a variety of toxins during vegetative as well as sporulation stages. Bt strains have been isolated and identified from variety of environmental locations (2). Under in vitro condition, using trypsin conversion of protoxin into toxin fragment is described for Cry and Vip toxins (3).

In recent years transgenic plants with various genes created a wave in agricultural Biotechnology across the world. In the past decade many Bt - endotoxins are cloned and economically important crop plants were transformed with them to produce insect resistant crop plants. Although Bt -endotoxins are effective insecticidal proteins, recent reports are showing resistant development in insects. Development of resistant is a major threat to the Bt usage in future. The reason identified for resistant development is loss of receptor binding and cross resistant to the -endotoxins (4). Recently Many strategies have been formulated to delay the resistant development in insects. Bt produces more than one kind of toxins, among them -endotoxins or cry toxins genes have bee widely utilized, recently explorations has been done on other toxins like Vip toxins.

Apart from -endotoxins, Bt produces many other toxins. Among them VIP toxins are valuable supplementary source. Vips are novel group of Bt toxins and reported to be present in 15% of the Bt strains. They are synthesized during vegetative phase of the Bt cells and secreted into the media. It has been reported that they have wider toxicity spectrum and more specifically toxic to Lepidoperan group. It has been demonstrated that like - endotoxins, they are non-toxic to beneficial insects like silkworm and Monarch butterfly (5). One among them being use of Bt toxins which are having novel structure and mode of action (6). Vips are known to bind to the receptors, which are not recognized by - endotoxins and their ion channel selectivity is different from that of cry toxins. Amino acid sequence predicted from the *vip* genes showed no homology with known Bt proteins. Studies shows that Vip3A binding is restricted to gut cells of the receptor binding is different from that of - endotoxins. Therefore, combination of Cry and Vip toxins might delay the resistant and cross-resistant development in the insects.

Cry1Ac belongs to Cry1 - endotoxin family and is active against *Helicoverpa armigera* and *Plutella xylostella* but it shows no mortality on *Spodoptera litura*. On the other hand recently discoveredVip3A is highly toxic to *Spodoptera litura*, but not to *Helicoverpa armigera.* Since *Helicoverpa armigera*, *Plutella xylostella S.litura* both are being notorious pest on some economically important crops like cotton, tomato, cabbage, cauliflower and insecticidal genes having toxicity on these pests is desirable. In the present study we describe cloning of two different Bt gene, *cry1Ac* and *Vip3A* fusion plasmid, its successful expression in *E.coli* cells and toxicity analysis was *P.xylostella*

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions : Plasmid pET29 (Stratagene,USA) was used as cloning and expression vector. *E. coli* strain DH5 (F^- gyrA96 (Nal^r) $recA_1$, endA, hsclR17,($r^-_k m^+_k$) gln44,LacZYA) was used for cloning experiments and BL21 (DE3) (F^- dcm OMPT $hsd(r^-_B m^-_B)$ was used as expression host as it is compatible and protease deficient host. *E. coli* cultures were grown in Luria–Bertani (LB) medium at 37^0C with vigorous shaking. Kanamycin (50 g/ml) was added wherever needed.

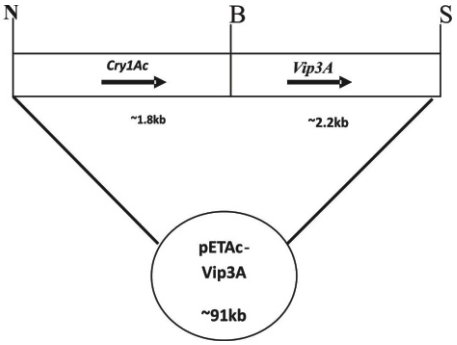

Figure-1 : Strategy used for cloning of cry1Ac and vip3A genes. The position of the cry1Ac, vip3A and direction of transcription are indicated by the arrows. Restriction sites; N-NcoI, B-BamHI and S-SalI

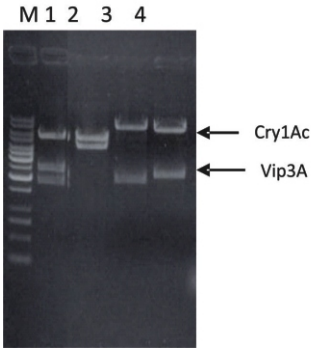

Figure-2 : Restriction analysis of fusion plasmid. Lane1: DNA Maker, Lane2: Fusion plasmid restricted with *NcoI+BamHI+SalI*, Lane3: Fusion plasmid restricted with *NcoI +SalI,* Lane4: fusion plasmid restricted with *NcoI+BamHI*.

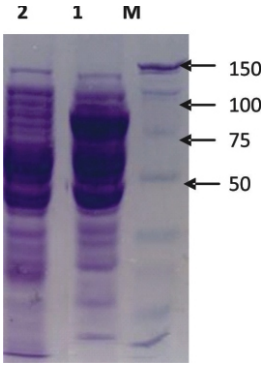

Figure-3 : Fusion protein expressed in *E.coli* containing plasmid encoding *cry1Ac-vip3A* fusion gene. 2-5 ug of protein was loaded per lane and stained with Coomassie brilliant blue. The position of the molecular marker (in kilo Daltons) is indicated on the right. Lane1:IPTG induced, lane2: Uninduced (control).

DNA manipulations : Ligation and restriction analysis was performed according to the manufacture's instruction. Plasmid DNA was prepared from *E. Coli* cells by the method of alkali lysis (6). Restriction and Ligation enzymes used were of from Promega, USA. Other recombinant DNA techniques were followed as described by (7). DNA fragments were separated by electrophoresis in 0.8% agarose gel electrophoresis and visualized under UV light. DNA fragments used for ligation was purified from agarose gel by using Gel elute mini kit from Qiagen.

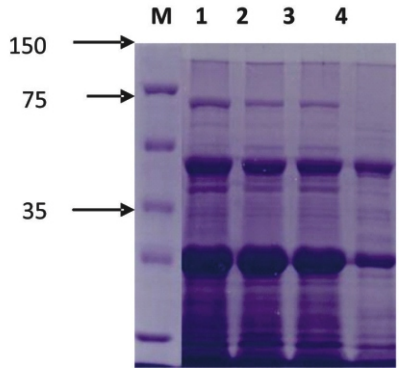

Figure-4 : SDS -PAGE analysis of trypsin activated fusion protein. Approximately 2 ug of total protein was loaded per lane. The position of the molecular weight marker (in kiloDaltons) is indicated on the left. Lanes1-4: 15min.30min.45min.and 90min incubation of the protein for trypsin digestion.

Cloning of *cry1Ac-vip3A* fusion gene : PCR amplification was done with high fidelity *Pfu* DNA polymerase (MBI Fermentas, Singapore) using forward primer (5'TG*CCATGG*ATGGATAACAATCCGAACATC 3') and reverse primer (CCTAATATTCAGCCTC GAGTG3') having *NcoI* and *BamHI* restriction sites respectively(restriction enzyme site sequence in the primer in italised). Primers were custom synthesized from Genset, Singapore. A 1.8 kb PCR product was amplified and purified using PCR purification kit (Qiagen). PCR product was restricted with *NcoI* and *BamHI* restriction enzymes and cloned in pET vector having vip3A gene in *BamHI* and *Sal I* sites. Fusion plasmid was transformed into DH5 *E.coli* strain and transformants were selected on LB solid medium containing kanamycin (50 g/ml). Plasmid DNA from selected colonies were isolated in miniprep and characterized by restriction pattern. One of the correct clones was transformed into *E. coli* strain BL21 (DE3) for protein expression studies.

Preparation and Purification of toxins from *E.coli* cell : Freshly grown single colony of BL21 (DE3) containing cry1Ac-vip3A plasmid was inoculated into 100ml LB broth and incubated at 37^0C in a water bath shaker with shaking speed of 200 rpm. When the optical density the culture reached one at 660nm, gene expression was induced isopropylthio-D-galactoside (IPTG) to a final concentration of 1 mM. The culture was grown for another 14-16 hours in the same condition. Cells were harvested by centrifugation at 8,000 rpm for 10 min and the supernatant was decanted. The pellet was resuspended in solution I (50 mM Tris-Cl pH8.0, 50mM EDTA, 100mM NaCl and 2% TritonX100). The suspension was subjected to sonication for 2 min with pulse rate of 20 (Brason, USA) by keeping the tube in ice. The cellular debris such as polysaccharides, membrane proteins, and lipids were removed by centrifugation. The pellet was washed with solution II (50mM Tris-Cl, (pH8.0), 50mm EDTA and 100mM NaCl) and finally with autoclaved

water. The pellet was resuspended in small volume of solubilization buffer (50 mM Sodium Carbonate and 0.1 mM dithiothreitol, (pH10.5) 0.1mM PhenyMethyl Sulphonyl Fluride (PMSF) to prevent proteolytic degradation of the proteins and incubated at 37^0C for 2 hours with gentle shaking. The solution was centrifuged at 10,000 rpm for 5 min; the supernatant was collected and stored at -20^0C for further analysis. The solubilized crystal protein was digested with trypsin (Sigma) at a trypsin/protein ratio of 1:50 (by mass) at 37^0C for different time periods (15min, 30min, 45min, 90min). Solubilized activated fusion protein was stored at -20^0c for further analysis.

SDS-PAGE analysis of Fusion protein : Total protein isolated from *E. coli* was subjected to SDS-Poly Acrylamide Gel Electrophoresis. A 25 l of the *E.coli* total protein was mixed with 5 l of SDS-PAGE loading buffer to a final volume of 30 l (2% SDS, 10 % glycerol, 5% 2-mercatoethonol and 62.5 mM Tris hydrochloride (pH 6.8) and boiled for 5 min, cooled, centrifuged at 10,000 rpm for 2 min. The clear supernatant obtained was analyzed on 7.5 % acrylamide gel according to the method described by Laemlli and Favr, 1973 using Bio Rad mini protein II electrophoresis apparatus, at 40 V initially for 30 min and then at 80 V for two hours. The proteins were stained with Coomassie Brilliant Blue. The molecular mass of proteins were estimated by comparison with precision stained protein marker (Bio Rad)

Immunoblotting : Proteins were separated by SDS-PAGE using according to the procedure Towbin et at 1979.Transfer of protein from the gel to the PVDF membrane was done with a electro blotting apparatus (Bio Rad) using a blot transfer buffer (39 mM Glycine, 48mM Tris (pH9.2), 0.00375 % (W/V) SDS-PAGE). After being blocked with 3% BSA in Tris-buffered saline (50 mM Tris-cl, pH 7.5), 150 mM NaCl) containing 0.1% Tween 20 (TBST), the membrane was incubated with polyclonal antisera of Cry1Ac /Vip3A in 1: 10,000 dilution in TBST containing 0.2 % BSA at 4^0C for overnight. The unbound antibodies were removed by three washes with TBST. The membrane was incubated with secondary antibody at room temperature for two hours. After three washes with TBST, the blot was incubated with alkaline phosphatase-conjugated anti-rabbit IgG for 1 hour and developed using p-nitroblue tetrazolium chloride (NBT) and 5-bromo-4chloro 3-indolylphosphate toluidine (BCIP) substrate as recommended by the manufacture (Boehringer Mannheim).

Larval Bioassay : Toxicity assay were performed with neonate larvae (except for *P. xylostella* for which third instar larvae were used). *H.armigera and S.litura* were reared on artificial nutrient medium with agar. The diet was dispensed in multiwell sterile plates. One ml of the diet was poured into the wells and kept for drying. The dilutions were made in Sodium carbonate buffer. A 50 ìl of volume of each of five dilutions of appropriate toxin was applied to the surface of the diet. The plates were dried in a flow hood. A total of 24 larvae (one per well) were released and covered with saran wrap and holes were made for aeration. Plates were kept under 16 h light and 8 h dark cycle at 25 ^{0}C –2 and relative humidity 0f 60 to 70 %. For *P. xylostella* assay, cabbage leaf dices were used made from young cabbage leaves. 20 ìl of each of dilutions were spread on both the sides of the leaf and kept in sterile plate wells of 3.5 cm dia. A group of five larvae were released per well and kept at the same conditions mentioned above. Observations were made after five days. LC_{50} value was calculated using probit analysis according to finney, 1970. For *S.litura* weight gain was recorded after one week and data were processed by one-way analysis of variance.

RESULTS AND DISCUSSION

Construction of *cry1Ac-vip3Aa14* fusion gene plasmid : We constructed translational fusion of two different Bt toxins, which are having different toxicity spectrum and evaluated its toxicity on target pests. Fig. 1 illustrates the overall strategy followed for the construction of fusion gene, pETcry1Ac-vip3A. Construction of the fusion gene cry1Ac-vip3A was started with cloning of cry1Ac gene fragment coding for toxic part of the protein in pET29 vector having vip3A gene in this vector described elsewhere (ref). DNA fragment comprising toxic part of *cry1Ac* gene was amplified from wild type full-length gene in plasmid pkk22-3 vector. The fusion gene was ligated together without altering their reading frame. A 1.8 kb fragment from bp 389 to 2256 (amino acids 29 to 463) coding for toxic part of the cry1Ac toxin was amplified and cloned into the pET vector. Cloning of the two genes in the pET29 vector was confirmed by agarose gel electrophoresis of restricted products (Fig.-2).

SDS-PAGE analysis : The *E. coli* cells harboring fusion gene plasmid grown for 48 hours with 0.1M IPTG to induce the gene expression. Total cell proteins isolated from these cells were solubilized in alkaline buffer (pH 10.5) and the toxin purity was checked by electrophoresis on SDS-PAGE gel (7.5% acrylamide) and stained with Coomassie brilliant blue R250 dye. SDS-PAGE separation of IPTG induced and uninduced (control) proteins showed that the over expression of ~ 140 kDa band which is predominant in induced culture and is absent in uninduced control, assuring fusion protein expression in *E.coli*. (Fig.3). Densitometric analysis showed that the fusion protein amounted approximately to 20-30% of the total protein (data not shown). This shows that the expressed protein accumulates without much degradation in the *E.coli* cells.

Western blotting : The total protein was isolated from

E.coli cells after IPTG induction. Proteins were separated on SDS-PAGE and transferred to PVDF membrane. Hybridized bands were visualized with Cry1Ac and Vip3A antibodies. These result confirmed the expression of fusion gene as ~140 kDa band which corresponds to the band, observed on SDS-PAGE gel (Fig.-4).

Trypsin digestion analysis : SDS-PAGE analysis of the trypsin digested samples at different period of time showed that the fusion protein is quite stable up to 60 min with accumulation of` ~ 70kDa and ~35 kDa protein bands after 15 min of incubation. The fusion protein was completely degraded into smaller protein bands at 90 min incubation time. This shows the overall stability of the fusion protein to the proteolytic digestion and correct processing of the fusion protein.

Effect of fusion protein on target pests : In order to determine the entomocidal activity of the fusion protein, which is expressed in *E. coli* cells, we performed insect feeding bioassays on the larvae of three-lepidopteron insect pests viz., *H. armigera, S.litura* and *P. xylostella*. Five dilutions of the toxin were made in Sodium Carbonate buffer and evenly poured on the artificial diet to feed *H. armigera* and *S.litura* larvae. A one-neonate larva per well was released and the mortality data was recorded after 72 hours. A group of five -third instar *P. xylostella* larvae was released on the disc coated with toxin. The mortality was recorded after five days. Table-1 shows the toxicity data recorded for cry1ac, Vip3A and Cry1Ac-Vip3A fusion proteins. It was expected that the fusion protein would be effective against three pests tested. But it retained the toxicity of Cry1Ac part and partially the Vip3A part since weight reduction was observed on *S. litura*.

There are compelling evidences showing the resistance development in insects against Bt toxins. In the recent study it has been shown that Vip3A toxin binds to 22 kDa receptor molecules which is different from that of Cry1Ab binds. Therefore, Vip toxin will be another weapon from the Bt to combat insect attack along with currently used Cry toxins. Already available reports show that it is possible to express the cry genes with other gene without altering their structure and function. This study shows that it possible to express two different Bt toxins, Cry and Vip under single promoter in a translation fusion manner. Here Vip3A was fused at C-terminal of Cry1Ac toxic part. In earlier attempts also Cry protein was successfully expressed in combination with other toxins (8).

Both SDS-PAGE and western blotting detected the expression of the full-length fusion protein (~140 kDa). This shows that the two genes fused are successfully expressed in the *E.coli* host. *E.coli* produces different intra cellular proteases and are capable of degrading the foreign protein and protein which are incorrectly folded (9). Further it gives the evidence that the globular structure of the protein is not drastically changed; otherwise *E.coli* machinery might have degraded the foreign protein during synthesis itself. Therefore, it is confirmed that the proteolytic cleavage sites are recognized appropriately by the larval mid gut proteases of these insects.

In the sequential step of the mode of action of Bt toxins, after ingestion of Bt toxin by susceptible larvae, it is subjected to proteolytic processing at N and C terminals. Therefore, it is important that the protolytic sites are exposed outside for proper cleavage. The stability of the fusion protein expressed in *E.coli* host was subjected to *in vitro* proteolytic digestion with trypsin. Trypsin digestion analysis gives the overall structure and stability of the Bt protein.

Since the fusion protein was showing stability and correct processing by trypsin, it was expected that the fusion protein would be toxic to three insects, viz., *H.armigera, P.xylostella, and S.litura*. But the larval bioassay data showed the fusion protein is toxic only to *H.armigera* and *P.xylostella*, which is similar to Cry1Ac toxicity. The toxicity of the fusion protein on *S.litura* larvae was not up to the level of Vip3A; instead it showed growth reduction on *S.litura* larvae. In the previous reports show that the activity of the C-terminally fused NPTII and Cry1B were showing their function without any reduction. Here, the partial activity of ip3A may due the proteolytic processing that results in degradation of the Vip3A in the *S.litura* mid gut.

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