Search for Vegetative Insecticidal Proteins (VIPs) from local isolates of *Bacillus thuringiensis* effective against lepidopteran and homopteran insect pests

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ABSTRACT

Certain strains of *Bacillus thuringiensis* (Bt) have shown to produce secretary proteins during their vegetative growth phase. In recent years, these proteins have shown remarkable insecticidal properties against several lepidopteran and coleopteran pests, and were named as vegetative insecticidal proteins (VIPs). Wide spread use of transgenic Bt crops which produces the well known insecticidal crystal protein (ICP) of Bt has increased the chances of resistance among pests. In this situation, the advent of the VIP is considered as a possible way to overcome the limitations of the first generation Bt toxins. The current study is aimed to search for novel VIPs or VIP-like proteins with effect towards several economically important pests that could not be controlled by the ICPs. A fairly large number of Bt isolates were screened in order to find a suitable VIP-like protein that might offer novelty in their insect mortality potential and newer sensitivity range amongst the target insects to be used in future transgenic approach. The toxic protein from the different strains were isolated and partially purified, and the susceptible insects from the lepidopteran and homopteran group were confirmed through bioassay. We were able to identify two VIP toxins; one was found to be effective against lepidopteran pests and the other to be effective against homopteran insect pests. This is the first study demonstrating activity of a Vip toxin against homopteran pests.

Key words: *Bacillus thuringiensis,* VIP, lepidopteran and homopteran insect pests

INTRODUCTION

Bacillus thuringiensis (Bt) derived biopesticides have been in extensive use worldwide during the past decade. The insecticidal crystal proteins (ICPs) derived from the sporulating stage of this gram positive soil bacterium has revolutionized the insect pest management strategies worldwide, thus using in a new era of chemical free insect control in agricultural systems. However the ICPs are not the sole answer to this crucial problem, as recent reports suggest that many pests have already developed resistance against the continuous use of crystal proteins (Tabashnik *et al.*, 2000). Moreover, these crystal proteins have not been able to provide protection against all the groups of the agronomically harmful pests (Estruch *et al*., 1997). In view of this situation the recently reported vegetative insecticidal proteins (VIPs) seem to provide the possible solution. VIPs with their significantly different mode of action than the ICPs (Lee *et al*., 2003) and their broad range of host specificity seem to be the potential candidate for gene pyramiding, and is being regarded as the second generation insecticidal toxins (Estruch *et al*., 1997).

VIPs as the name suggest is secreted into the culture medium during the vegetative growth phase of the bacterium. These proteins have shown toxicity against certain important lepidopteran pests like black cut worm (*Agrotis ipsilon*) and cotton leaf roller (*Spodoptera littolaris*), which could not be curbed earlier by the crystal toxins. Moreover, some of the lepidopteran insects that are susceptible to the Cry toxin at microgram level could be killed completely by the VIPs at nanogram level (Doss *et al.*, 2002). The known VIP toxins can be classified into two groups – the first group comprises a binary toxin, Vip1A(a) and Vip2A(a) which are known to be coleopteran specific and were the first reported VIPs secreted by *B. cereus* (Carozzi *et al.*, 1997). A homologue of the same toxin i.e., VIP1A(b) and Vip2A(b) were also reported from *B. thuringiensis*. Both of these were reported to be coleopteran specific and showed no effect against lepidopterans. The second group of VIPs are dominated by the Lepidoptera specific toxins- VIP3a was the first to be reported which showed activity against several lepidopteran insect larvae including black cutworm, fall

Table1. LC_{50} values (ng/cm^2 or *ng/ml) of different Vip toxins against some lepidopteran insect pests.

Toxin	Reference	LC_{50} Values			
		A. ipsilon	*H. armigera*	*S. littolaris*	*P. xylostella*
Vip3a	Estruch *et al.*,1996	31	-	-	-
VipS	Selvapandiyan *et al.*,2001	2165	-	5ng/cm^2	36
Vip14	Cai *et al.*, 2002	-	-	-	32
Vip83	Cai *et al.*, 2002	-	-	-	29
Vip3V	Doss *et al.*, 2002	81	325	45	221
Vip3A-S184	Chen *et al.*, 2003	-	160*	270*	-
Vip3Aa14	Bhalla *et al.*, 2005	-	-	12	120
Vip3LB	Mesrati *et al.*, 2005	-	-	-	-

- No data available,

army worm, tobacco budworm etc. (Estruch *et al.*, 1996). In the following years many Lepidoptera specific VIPs have been reported (see Table 1). However, these toxins have already been patented and cannot be easily commercialized in our country; thus it is desirable to search for such VIP like toxins among the local isolates so that they can be easily made available for the agriculturists. However, the agronomically important insect pests that wreck havoc in the farmer's field are not limited to the lepidopteran and coleopteran order only. Some of the devastating pests of economically important crop plants belong to the homopteran or sap sucking group. They not only directly affect the plants by sucking the phloem sap, but are also vectors of several notorious viral plant pathogens. Among the most important sap sucking pest that cause wide spread destruction to crop plants are the mustard aphids and the cotton aphids. The control of these sap sucking insects seem to be a major problem as they have the ability to rebound quickly even after repeated application of chemical pesticides, a phenomenon supported by their huge reproductive potential and an overall physiology suitable for developing resistance to chemical pesticides. Till date only the monocot mannose binding plant lectins have been able to offer some protection against the sap sucking group of pests (Rahbé and Febvay, 1993). But the very high lethal dose required by these lectins proves to be disadvantage in future transgenic applications. Therefore, we are on a lookout for an effective insecticidal agent against the sap sucking pest. Among the known Bt cry toxins none have shown any effect on this group of sap sucking pests. Moreover, none of the known VIPs have any effect on the homopteran group of insects. Therefore the search for novel toxins with antifeedant properties against lepidopteran and homopteran groups of insect pests is under investigation. The current report presents the results of screening of various vegetative culture supernatants from *Bacillus thuringiensis*, to search for a novel insecticidal toxic protein against homopteran group of insects as well as lepidopteran group of insects.

MATERIAL AND METHODS

Bacterial strains – growth media and growth curve

Twenty eight local isolates of *Bacillus thuringiensis* (Bt) designated as #BREF1 to #BREF28 (Biotechnological Research Extension Foundation - BREF) were used for screening for the presence of novel vegetative insecticidal toxins effective against lepidopteran and homopteran group of insect pests. The bacterial culture was grown in Teriffic broth medium (12% tryptone, 2.4% yeast extract, 0.4% glycerol, 0.17M KH_2PO_4, 0.72M K_2HPO_4) at 28ºC. To determine the vegetative growth phase a growth curve experiment was performed. The OD at $ë_{600nm}$ was monitored for 72 h, and the data was plotted on a graph with O.D vs. time, and the vegetative growth phase which is equivalent to the exponential phase was determined.

Isolation of secretary protein from the culture supernatant

The *B. thuringiensis* culture was grown in the above mentioned medium for 24 h in order to harvest the vegetative insecticidal proteins after centrifugation at 12,000 x g for 10 min at 4ºC, the cell pellet was discarded and the supernatant containing the proteins of interest was retained. Proteins present in the supernatant were precipitated with ammonium sulphate [$(NH_4)_2SO_4$] (80% saturation) and collected by centrifugation at 12,000 x g for 10 min at 4 ºC. The pellet was re-suspended in minimum

volume of 20 mM of Tris HCl buffer (pH 7.4) and dialyzed overnight at 4°C. The crude protein so obtained was subjected to ultra centrifugation at 56,000 x g for 30 min at 4ºC to remove traces of suspended matter.

Estimation of the protein

The amount of crude protein so obtained was estimated by Bradford's method, taking BSA as the standard (Bradford,1976).

Insect Bioassay with the crude protein extracts

Lepidopteran insect pests

Lepidopteran insects like black cut worm (*Agrotis ipsilon* Hfn.) cotton boll worm (*Helicoverpa armigera* Hubner), and cotton leaf roller (*Spodoptera littolaris* Fab.) were maintained in semi-synthetic diet 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. Each experimental set was replicated thrice.

Homopteran insect pests

To identify the most toxic strain, insect feeding assays were performed with the different crude extracts. Early instar nymphs of cotton aphids (*Aphis gossypii*) was fed with artificial diet (Dadd and Mittler., 1976) supplemented with crude protein extracts (50 µg ml^{-1}) from the culture supernatants of different Bt isolates. The assay sets for aphid bioassay were prepared with a little modification of the procedure described in Hossain *et al.* (2006). A control set up, devoid of the protein fractions, containing the buffer only was set as the standard. The mortality of the nymph was scored by counting the number of live insects present in the bioassay set up till 48 h of exposure.

Table 2. Percentage mortality irresponse to culture supernatant total protein of different Bt strains against lepidopterans and homopteran pests

Bt strains	Lepidopteran Pests	*A. gossyphi*
#BREF3	11.7	0
#BREF4	55	30.2
#BREF9	0	30.16
#BREF13	11.66	0
#BREF14	30.16	12.3
#BREF17	95	45.65
#BREF19	75	0
#BREF24	0	70.85

Table 3. Toxicity of partially purified toxin from BREF17 for lepidopteran pests and BREF24 (ng ml^{-1}) for *Aphis gossypii*

Insect pests	Lower fiducial limits	LC_{50}	Upper Fiducial Limits
Helicoverpa armigera	64.09	89.06	116.0 [b]
Agrotis ipsilon	45.41	58.99	80.11
Spodoptera littoralis	10.98	21.04	31.24
Aphis gossypii	81.41	154.98	247.25

Isolation and partial purification the toxic protein(s)

Isolation of the crude protein

The culture supernatants from the chosen strains were subjected to different $(NH_4)_2SO_4$ fractionation, namely 0-30%, 30-60% and 60-80%. The SDS PAGE (10%) analysis was carried out with all the $(NH_4)_2SO_4$ fractionates and simultaneously an insect bioassay was conducted with the same. On the basis of the insect feeding results, a further purification of the protein extract was carried out.

Partial purification of the protein entomotoxic to lepidopteran insect pests

The pellet containing the 30-60% $(NH_4)_2SO_4$ fractionated protein from Bt strain #BREF17 was resuspended in 100 mM phosphate buffer (pH-7.4) containing 0.01% Triton-X-100 and dialyzed extensively against the latter. It was then subjected to ultra centrifugation at 56,000 x g for 30 min at 4ºC to remove traces of suspended matter. The protein was thereafter fractionated through two-step DEAE-Sephacel anion exchange column, pre-equilibrated with 100 mM phosphate buffer (pH-7.4). Flow rate was maintained at 4 ml per hour. Flow through was collected and kept separately for insect bioassay and gel analysis purposes. The column-bound proteins were eluted in a stepwise manner with different concentrations namely 100-500 mM NaCl in 100 mM phosphate buffer (pH-7.4). Elution volume was 15 ml per salt wash and volume of each fraction collected was kept at 1 ml. Different fractions were collected, and dialyzed extensively against 100 mM phosphate buffer (pH-7.4) with repeated changes at 4°C. Insect feeding assay with different lepidopteran insects

was performed with the column fractions as described earlier, and subsequently the column fraction that showed most potent bioactivity was analyzed by spectrometry and gel electrophoresis and chosen for further studies. This fraction was further fractionated again on DEAE-Sephacel column and eluted using a NaCl gradient of 25-100mM in 100mM sodium phosphate buffer (pH 7.4). Fraction having 90 KDa toxin was used at 50 ng ml^{-1} for lepidopteran insect pests.

Partial purification of the protein entomotoxic to homopteran insect pest

The 60-80% $(NH_4)_2SO_4$ protein fraction of Bt strain # BREF24 was selected by bioassay as the most effective one and was subsequently applied to a cation exchanger, SP-Sepharose. The elution was carried out using a step gradient of 25mM, 50mM, 75mM and 100mM NaCl in 20 mM MOPSO (pH 6.8) at a flow rate of 4ml/hr. Insect feeding assay against the homopteran insects namely cotton aphids and mustard aphids were done with the column fractions (47 KDa at ng ml^{-1}) as described earlier, and subsequently the column fraction that showed most potent bioactivity was selected for further studies.

Bioassay with the partially purified toxins and determination of the lethal dose

Insect feeding assay was performed with the column fractions as described earlier, and subsequently the column fraction(s) that showed most potent bioactivity was analyzed by spectrometry and gel electrophoresis and chosen for further studies. A control set up, devoid of the protein fractions, containing only buffer was set as the standard. The mortality of the larva was scored by counting the number of live insects that remain in the bioassay after a given period of time. Probit regression analysis (Finey, 1971) was carried out with computer program Biostat® (www.Analystsoft.com) and the LC_{50} as well as lower and higher fiducial limits were determined.

RESULTS AND DISCUSSION

Growth curve of *Bacillus thuringiensis*

The bacterial growth curve with identifiable lag, exponential and stationary phases were obtained as OD was measured at regular intervals. The growth curve analysis was performed to determine the time required by the bacterium to reach its vegetative phase in a batch culture with shaking. The growth of the bacterium was monitored for 72 h, as *B. thuringiensis* (Bt) reaches its sporulating stage within that time. From the growth curve (Fig. 1) it is evident that the bacterium remains in its vegetative or exponential phase from 8- 24 h. The result of this experiment was very much in accordance to the growth pattern demonstrated by Estruch *et al.* (1996) for a different Bt strain. Thus the protein was harvested from the culture supernatant after 24 h of culture, expecting maximum yield of vegetative proteins secreted by the Bt strain.

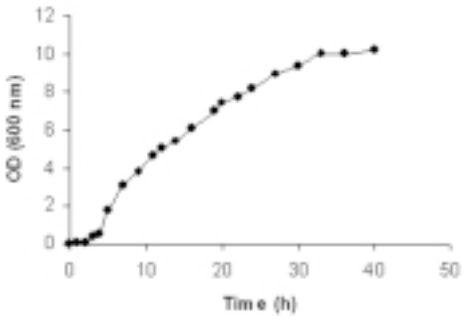

Fig 1. Growth curve of *Bacillus thuringiensis* 28ºC with constant shaking for different period of time (n = 3)

Screening of Bt strains entomotoxic protein (s)

Lepidopterans

Bioassay of lepidopteran insects with the proteins secreted into the culture supernatant during the vegetative growth showed promising results. Among twenty eight strains used for preliminary round of screening, only six isolates showed antifeeding property towards the lepidopteran larvae (Table 2). Moreover, similar results were obtained when tested with the other lepidopteran insects such as *A.ipsilon* and *S.littolaris*. This effect was observed when first instar neonate larvae were feed with crude protein isolate at a dose of 25 µg ml^{-1} of artificial diet and the mortality was recorded till 7 days of the exposure. Maximum toxicity was achieved by Bt isolate #BREF17, followed by #BREF19 (Table 2).

Homopteran

Among the twenty-eight strains used, only five strains *viz.*, #BREF4, #BREF9, #BREF14, #BREF17, and #BREF24 showed antifeedant effect on cotton aphid, *A. gossypii*. The rest of the strains had no such effect. It was observed that cotton aphids fed with a diet supplemented with 50µg ml^{-1} of crude protein of these strains showed differential toxicity within 48 h. About 84% mortality was achieved

with the strain #BREF24, followed by a toxicity of 46.66% with the strain #BREF17 (Table 2).

Toxicity of the ammonium sulphate fractionate(s)

The Bt isolate #BREF17 was chosen as most effective strain against lepidopteran insect pests. In order to isolate the toxic component secreted by this isolate, several ammonium sulphate fractionations *viz*. 0-30%, 30-60% and 60-80% were done. Among these, the 30-60% fractionate was found to be the most potent one as determined by the bioassay results against the lepidopteran insect pests (Fig 2A). Similar work was carried out with #BREF24 which was established as the most effective strain against homopteran sucking pest. For #BREF24 the toxic component was limited to the 60-80% ammonium sulphate fractionate (Fig 2B), as indicated by the bioassay data.

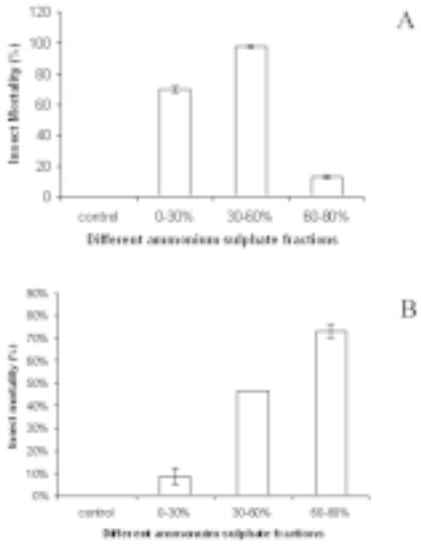

Fig 2. Insect mortality with different ammonium sulphate fractionates of the culture supernatant proteins obtained from various isolates of *Bacillus thuringiensis*. Determination of the most potent fractionate of #BREF17 against lepidopteran insect pests after 7 days of assay (A) and effect of different fractionates of #BREF24 against cotton aphids after 2 days of assay (B).

Partially purified toxic protein(s)
Lepidoptera specific toxin

The toxic protein from the 30-60% fractionate of Bt strain #BREF17 was purified by a two step process. Firstly it was applied to weak anion exchanger DEAE-Sephacel and eluted using a step gradient of 100-500 mM NaCL in 100mM sodium phosphate buffer (pH 7.4).The fractionates were analyzed spectrophotometrically at 280nm for protein content and a peak was found in the 100 mM NaCl fraction which corresponded to the most toxic fraction in the bioassay result. Thus it was confirmed that the toxic protein was of anionic nature. A 10% SDS PAGE analysis of the same showed a protein of ~90kDa to be the major constituent of this fraction, along with many contaminating bands (Fig 3). This ~90kDa was further purified by another step of ion–exchange chromatography. The second step involved the application of the 100mM NaCl fraction of the previous column eluate to another DEAE-Sephacel column, and elution by a step gradient of 25-100mM NaCl in 100mM sodium phosphate buffer (pH 7.4).A bioassay carried out with the eluates of the second column, confirmed the toxic potential to be present in the 25mM fraction. A 10% SDS PAGE of the same showed the presence of a single protein of ~90kDa (Fig 3). As reported earlier by various groups (Estruch *et al.*, 1996; Selvapandiyan *et al.*, 2001; Doss *et al*., 2002; Cai *et al.*, 2003; Bhalla *et al.*, 2005; Mesrati *et al.*, 2005) the entomocidal protein Vip3a and its various homologues are also of the similar size (around 90kDa). Therefore it can be speculated that this newly isolated 90kDa maybe a homologue to these proteins, a definite answer to which can be found only after amino acid sequencing of the protein.

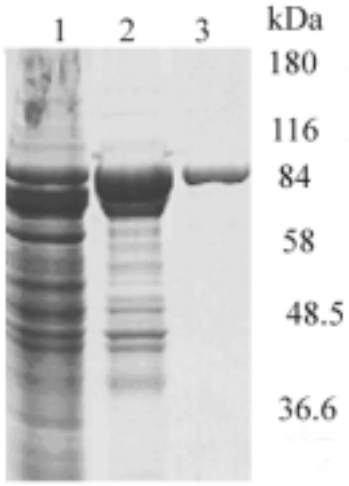

Fig 3. Coomassie blue stained SDS PAGE (10%) analysis of the partially purified toxin from #BREF17 effective against different lepidopteran insects. Lane 1 -crude toxin extract; Lane 2 -100mM NaCl eluate after 1st fractionation on DEAE Sephacel column; Lane 3 -purified 90 kDa protein present in the 25mM NaCl elution after re-fractionionation of the first eluant.

Homoptera specific toxin

The effective 60-80% $(NH_4)_2SO_4$ fraction of the culture supernatant of #BREF24 was applied to a cation-exchanger SP-Sepharose (pH 6.8) and eluted using a step gradient of 25, 50, 75 and 100mM NaCl in 20 mM MOPS buffer (pH 6.8) at a flow rate of 4ml/hr. The individual step gradient fractions were collected and analyzed for protein content at 280nm. A major protein peak obtained at 50 mM NaCl contributed to the most toxic effect in the feeding assay. The silver stained SDS-PAGE analysis revealed that the protein was not completely purified, along with a 47 kDa major band; the fraction also contained a minor protein of size ~ 94 kDa (Fig 4). Thus it is being speculated this insecticidal protein may belong to the class of binary toxins, Vip1 and Vip2.

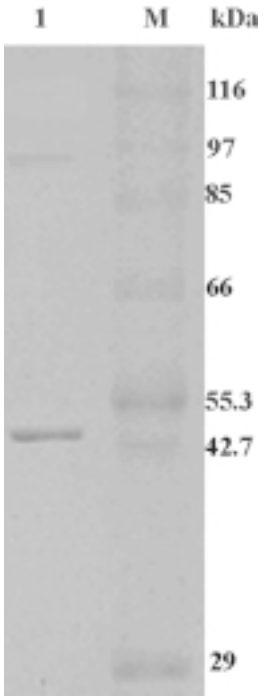

Fig 4. Silver stained SDS PAGE (10%) showing the 50mM NaCl elution (Lane 1) containing the ~47 kDa Vip2A(BR) as the major protein and ~94.5 kDa Vip1A(BR) as the minor protein, Lane M indicated molecular weight marker.

LC_{50} of the purified toxin(s) for pests

LC_{50} value is the amount of toxin required to cause 50% mortality in the given insect population. The newly purified 90kDa toxin could cause mortality in all the tested lepidopteran insect pests. The LC_{50} for *H. armigera* was 89.06 ng ml^{-1} of artificial diet, where as for *A. ipsilon* and *S. littolaris* the value was determined to be 21 ng ml^{-1} and 58.99 ng ml^{-1} respectively (Table 3). It may be noted that the LC_{50} for *A. ipsilon* is the lowest reported till date. The specificity of these toxins to certain lepidopteran insects and their intensively to others may be due to the change in some amino acids residues in the polypeptide chain. The major protein peak, obtained from the column purification of the culture supernatant proteins from #BREF24 at 50 mM NaCl contributed to the most toxic effect in the feeding assay against cotton aphids and showed a LC_{50} of 154.98 ng ml^{-1} of artificial diet (Table 3). As mentioned earlier this fraction contains a 47kDa protein as the major constituent and a 97 kDa protein as the minor band, thus giving arise to the speculation that these may be homologous to the binary toxin duo of Vip1-Vip2 as reported in other cases. However, no definite conclusion can be inferred till both the toxins are subjected to amino acid sequencing and further characterization. Nevertheless this a novel finding as none of the VIP toxins reported so far have shown any effect on the sap sucking insect pests. In conclusion, this study reported here reveals that an efficient strategy has been adapted to identify local Bt isolates that are effective against lepidopteran and homopteran insect pests that are devastating to several economically important crop plants. Further characterization of the two genes encoding the respective toxins will be useful in future development of transgenic insect resistant crop plants, which is universally recognised as an effective strategy in crop protection by means of biological control. In addition, recent studies of Wei *et al*. (2007) and Sobernon *et al*. (2007) reported that cry proteins were non-toxic to *Chrysoperla cornea* and *Manduca sexta* respectively.

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