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Bacillus thuringiensis kurstaki strains produce vegetative insecticidal proteins (Vip 3) with high potential

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Abstract

Bacillus thuringiensis (*Bt*) produces vegetative insecticidal proteins (Vip) during its vegetative growth stage. Vip3 proteins have a significant role in insecticidal activity of this bacterium. Each new bacterial isolate may encode Vip with different significance. The Vip protein coding by vip genes of two *Bt* strains (BnBt and MnD) with high insecticidal activity was characterized in this study. Polymerase chain reaction (PCR)-based screening for Vip genes of these *Bt* isolates expressed the detected Vip gene and tested the protein for insecticidal activity against the cotton leafworm, *Spodoptera littoralis* larvae. As a result of the screening tests, Vip3 genes were determined in MnD and BnBt isolates. Vip3 genes of both isolates were expressed and confirmed by 90 kDa proteins. Partially purified and trypsin-activated protein samples of BnBt and MnD isolates were tested against the second instar larvae of *S. littoralis*. The results showed that the highest insecticidal activity of the Vip3 proteins of BnBt and MnD was 86.66% and 83.33% mortality in 10 days, respectively. The median lethal concentrations (LC_{50}) of BnBt and MnD were determined as 41.860 and 55.154 ng/μl, respectively. The results suggest that Vip3 protein is effective for preventing resistance in various insect-pest species. The expressed proteins may be utilized as a biopesticide against nocuous insects.

Keywords: *Bacillus thuringiensis kurstaki*, Insecticidal activity, Microbial control, Vip3 gene

Background

In most of the developed and developing countries, *Bacillus thuringiensis* (*Bt*) preparations are used commercially against pests belong mainly to orders Lepidoptera, Diptera, and Coleoptera. *Bt* toxins are highly specific for their pests and have great importance as an alternative to chemical insecticides all over the world. Despite the wide success of Cry proteins in agricultural insect control, increased insect resistance and narrow insecticidal spectrum threaten the long-term sustainability of *Bt* technology (Kurt et al. 2005; Sezen et al. 2007 and 2008). Therefore, it is necessary to identify new insecticidal proteins with different characteristics other than known Cry proteins, i.e., vegetative insecticidal proteins (Vip, etc.). Towards this aim, several studies have been conducted to identify and characterize new Vips from new *Bt* isolates which exist in various habitats (Chakroun 2015).

There are many lepidopteran species which are resistant to Cry proteins and *Spodoptera littoralis* (Boisd.) is one of the most important of these pests. In addition, it has been reported that Vip3 proteins against this pest have been highly effective (Chakroun 2015; Chakroun et al. 2016; Van Frankenhuyzen and Nystrom 2018).

Vip proteins are a family of insecticidal proteins secreted by different *Bacillus* species (Bhalla et al. 2005). They are classified into four families Vip1, Vip2, Vip3, and Vip4, according to their sequence similarity. Vip1 and Vip2 have activity against coleopteran and homopteran insects; however, Vip3 has insecticidal activity against lepidopteran pests (Warren 1997; Crickmore et al. 2017).

Because *Bt kurstaki* isolates have Vip3 gene, the present study aimed to investigate Vip3 gene in two different *Bt kurstaki* isolates (MnD, BnBt) expressing Vip3

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gene and test the protein for its insecticidal activity against *S. littoralis* larvae.

Materials and methods

Bacillus thuringiensis isolates and growth conditions

Local *B. thuringiensis kurstaki* strains (MnD and BnBt) obtained from the culture collection at Department of Biology, Faculty of Science, Karadeniz Technical University, Turkey, and *B. thuringiensis kurstaki* HD-1 strain (positive control) obtained from *Bacillus* Genetic Stock Center were used in this study (Kati et al. 2005, 2007; Sezen et al. 2010). *Bt* isolates were grown in a nutrient agar (NA; Merck) and Luria Bertani media at 30 °C (for 1 l LB; 10 g tryptone, 5 g NaCl, 5 g yeast extract).

Insect colonies

Spodoptera littoralis was grown in the Microbiology Laboratory, Karadeniz Technical University, Trabzon, Turkey, under controlled conditions of 25 ± 2 °C, 60 ± 5% RH, and L16:D8 h. *S. littoralis* originated from a standardized culture line. Larvae were reared on semi-synthetic diet (266.5 g dried bean, 4 g ascorbic acid, 2.5 g methyl 4 hydroxybenzoate, 1.25 g sorbic acid, 0.5 g B vitamin, 3 g wheat germ, 35 g yeast extract, 14 g agar).

Detection of Vip3 gene by polymerase chain reaction

Total DNA was extracted as explained earlier (Ferrandis et al. 1999). For the screening of the Vip3 gene, degenerate primers were used (Hernández-Rodríguez et al. 2009). Polymerase chain reaction (PCR) was prepared in a final volume of 50 μl. The PCR mixtures included 3 μl of the DNA template, 1.25 U of *Taq* DNA polymerase (New England BioLabs), 5 μl of 10× reaction buffer, 3 μl 1.5 mM $MgCl_2$, 1 μl 10 mM of each dNTP, and 1 μl 10 mM primers for Vip3 gene screening. The PCR conditions were as follows: 5 min denaturation at 95 °C, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 45 °C for 1 min, and extension at 72 °C for 2 min. Final extension was carried out at 72 °C for 10 min. The products were separated by electrophoresis in 1% agarose gel.

Cloning and sequencing of Vip3 gene

The amplified Vip3 DNA fragment was purified, using the NucleoSpin Extract DNA Purification Kit (from Macherey-Nagel), ligated with pGEM-T cloning vector (from Promega) using T4 DNA ligase enzyme, and transformed into *Escherichia coli* JM101 cells. The clones were selected on LB plates containing ampicillin (50 mg/ml), X-Gal (40 mg/ml), and IPTG (24 mg/ml). The Vip3 gene was analyzed and compared to the updated GenBank data by using BLAST program (http://www.ncbi.nlm.nih.gov/blast).

Purification of Vip3 proteins

Culture of B. thuringiensis kurstaki isolates (BnBt, MnD)

B. thuringiensis isolates were re-inoculated to a 0.1 OD_{600} nm from an overnight culture on a Teriffic (12% tryptone, 2.4% yeast extract, 0.04% glycerol, 0.17 M KH_2PO4, 0.72 M K_2HPO_4) liquid medium to determine the bacterial growth phase and vegetative phase and were maintained at 30 °C in a shaking incubator. Measurements were taken at OD_{600} nm with samples taken at specific time intervals for 36 h, and the vegetative phase was determined by plotting the data on the graph.

Isolation of Vip3 proteins from cultured supernatants

Bacterial cultures grown for 24 h were centrifuged at 12.000*g* for 10 min at 4 °C, and supernatants containing Vip3 proteins were collected (Sattar et al. 2008).

Ammonium sulfate precipitation

Ammonium sulfate [$(NH_4)_2SO_4$] precipitation method (REF) was followed to precipitate the protein from the culture supernatant. First of all, solid ammonium sulfate was added to the bacterial supernatant at 60% saturation (Sattar et al. 2008). Ammonium sulfate was added to the supernatant slowly at 4 °C by mixing on a magnetic stirrer. Precipitation was followed by centrifugation at 10.000 rpm for 10 min at 4 °C. After centrifugation, the pellets were resolved by using 1 ml of 100 mM pH 7.5 Tris-HCl buffer.

The ammonium sulfate precipitation was subjected to overnight dialysis against 20 mM pH 7.5 Tris-HCl buffer to remove especially the salts and small molecules. Following dialysis, the samples were centrifuged at the highest speed for 10 min to allow the degradation of the proteins.

Ion-exchange chromatography

A column of 50 cm in length and 1.5 cm in diameter was used for ion-exchange chromatography. For this purpose, Q-Sepharose (anion exchanger), an anionic ion exchanger, was used in this process. Tris-HCI buffer (20 mM pH 8) was used as the mobile phase. The gasses of the column material and all buffers used in the experiment were taken in a vacuum pump and then slowly filled into the collar using a pasteurized pipette. After filling, the column was equilibrated with 500 ml of Tris-HCI buffer. The extract was passed through the column to allow the contained proteins to be attached to the column packing material. Subsequently, 50 ml of buffer was passed through the column to remove proteins that were not attached to the column. The salt (NaCl) content of the column was then increased from 0 to 0.6 M. A 200 ml NaCl gradient bridge was used for this. The buffer flow rate was set to 1 ml/min, and the fractions from the column were collected in glass tubes to be 3.5 ml.

The fractions obtained were run on 12% SDS-PAGE. The determined tubes containing protein extract were combined and selected for purity on SDS-PAGE. After completion of the SDS-PAGE run, the gels were stained with fast silver staining technique. The gel image was transferred to the computer using a scanner.

Estimation of the protein

The amount of protein was determined according to the Bradford method (Bradford 1976). Bovine serum albumin (BSA) was used as standard in the calibration curve prepared. The prepared standards and samples were transferred onto 96-well microplates, and measurements were made at 595 nm, using a UV-visible spectroscopy system (Bio-rad).

Trypsin activation of Vip3 proteins

Partially purified Vip3 proteins were activated by commercial trypsin (10%) at 37 °C for 2 h.

Determination of insecticidal activities of Vip3 proteins

The second instar larvae of *S. littoralis* were used to test the activity of partially purified and trypsin-activated Vip3 proteins of *Bt kurstaki* isolates (BnBt, MnD). Protein samples were prepared at the concentrations of 10, 25, 50, 100, and 200 ng/μl from both Vip3 proteins. In the experiments, water and NaCl were used as negative controls. Samples containing Vip3 proteins of BnBt and MnD isolates were spread with 10 μl of sample on the 1 cm^2 of lettuce leaf for testing on each one larva of *S. littoralis*. The concentrations of toxin samples were applied per insect. Larvae were starved for 4 h before application. For each concentration of Vip3 protein, 30 larvae were placed in test vessels one by one, and tests were repeated three times. Biotests were carried out at 25 ± 2 °C and 60% RH. Larval mortality was followed up to 10 days and corrected for control mortality, using Abbott's formula (Abbott 1925).

The data were subjected to ANOVA and subsequently to LSD. The lethal concentrations (LC_{50} and LC_{90}) were estimated by probit regression analysis (Finney 1971). Statistical analyses were performed using SPSS 20.0 software.

Results and discussion

Molecular analysis of Vip3 gene from local *Bt* isolates

The Vip3 gene was amplified by polymerase chain reaction (PCR) and electrophoresed on a 1% agarose gel. As a result, 1.621 bp of Vip3 gene of both isolates was detected. The presence of the Vip3 gene in the HD-1 isolate used as a control was also observed. The presence of Vip3 gene was detected in *Bt kurstaki* BnBt and MnD strains (Fig. 1).

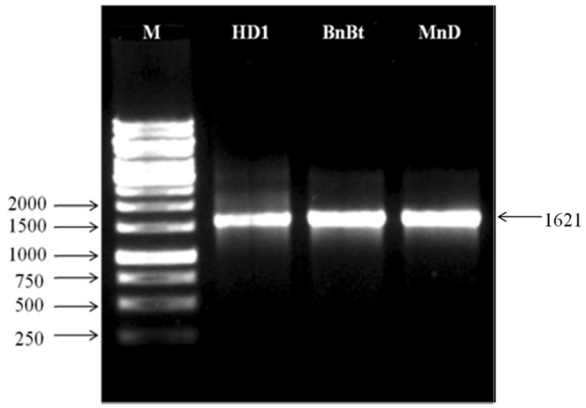

Fig. 1 The presence of Vip3 gene of *Bacillus thuringiensis kurstaki* BnBt and MnD strains and HD-1 strain as a positive control

Nucleotide sequence accession number

The sequence of the gene encoding the Vip3 protein was deposited in the GenBank database (http://www.ncbi.nlm.nih.gov) under the accession number MH318013 for BnBt and MH318014 for MnD.

Isolation of Vip3 proteins of *B. thuringiensis kurstaki* strains

After purification and SDS-PAGE analysis of Vip3 proteins, a band of ~ 90 kDa was detected for Vip3 proteins (Fig. 2).

Determination of the potential of the obtained Vip3 proteins on *S. littoralis* larvae

The highest larval mortalities were observed by the application of 200 ng/μl concentration. The mortalities were determined on the 10th day as 86.66 and 83.33% for BnBt and MnD isolates, respectively (Fig. 3). In the negative control, 13.3% of mortality rate was observed. The median lethal concentrations (LC_{50}) of Vip3 of BnBt and MnD were determined as 41.860 and 55.154 ng/μl, respectively. LC_{90} of BnBt and MnD were determined as 367.204 and 420.978 ng/μl, respectively (Table 1).

Overlapping of fiducial limits of LC_{50}s means insignificant differences among the tested strains at 5% level of probability.

During the vegetative growth phase of *Bt*, it has been found that some strains produce medium-secreted proteins called Vip that show insecticidal activity against pests (Donovan et al. 2001; Milne et al. 2008).

Analysis of the local *Bt* collections may help to illuminate the dispersion of Vip genes and the stock of novel Vip genes. To date, there are a lot of studies on Vip genes of *Bt* in the literature; however, there is no study regarding this issue in the Eurasia region (Milne et al. 2008). So, this study has a unique value as the first investigation for the presence of Vip3 gene in local *Bt*

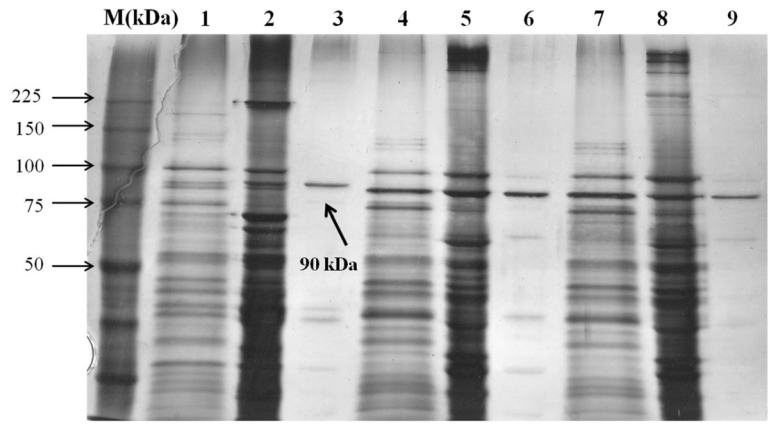

Fig. 2 SDS-PAGE analysis of partially purified Vip3 proteins from *Bacillus thuringiensis* isolates. 1, 4, and 7 indicate crude extract of HD-1 as positive control, BnBt, and MnD strains. 2, 5, and 8 indicate 60% ammonium sulfate precipitation of HD-1, BnBt, and MnD supernatants. 3, 6, and 9 indicate ion-exchange column chromatography

kurstaki (BnBt, MnD) strains and for the application of Vip3A proteins against larvae of *S. littoralis*.

PCR studies were initially performed to determine the presence of Vip genes in the local *Bt kurstaki* (BnBt, MnD) isolates and in standard/control strain HD-1. For this purpose, Vip1 (585 bp), Vip2 (845 bp), and Vip3 (1621 bp) gene regions were partially amplified and sequenced as indicated in earlier studies (Hernández-Rodríguez et al. 2009). According to the obtained results, the *Bt kurstaki* (BnBt, MnD) isolates contained only the Vip3 gene.

Hernández-Rodríguez et al. (2009) conducted a study to identify known Vip genes and potentially identify new Vip genes in the *Bt* collection of 507 strains. The screening of the Vip genes was performed by primers designed from the appropriate regions in the Vip gene families. By screening, 507 strains detected 54 (10.7%) strains of Vip1 gene, 46 (9.1%) strains of Vip2 gene, and 248 strains detected Vip3 (48.9%) gene. They then used the PCR-RFLP method to identify the different Vip genes of the same family.

As a result of this study, the presence of ~ 90 kDa protein was observed by SDS-PAGE analysis. These results were consistent with the results of earlier studies (Bhalla et al. 2005; Mesrati et al. 2005a, 2005b; Sattar et al. 2008).

As a result of bioassays, 86.6 and 83.3% of insecticidal activities of the Vip3 proteins were obtained from the BnBt and MnD isolates, respectively, at the concentration of 200 ng/μl. Lethal concentrations (LC_{50} and LC_{90}) of BnBt and MnD were determined as 41.860, 55.154 ng/μl and 367.204, 420.978 ng/μl, respectively.

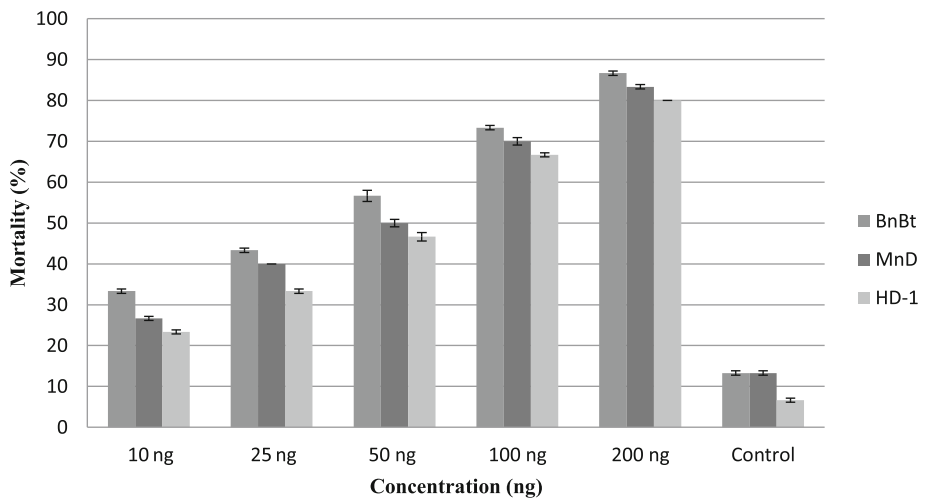

Fig. 3 Potential of Vip3 proteins of BnBt and MnD versus second instar larvae of *Spodoptera littoralis*

Table 1 LC_{50} and LC_{90} values of Vip3 proteins of BnBt and MnD against *Spodoptera littoralis* larvae

Strains	LC_{50} (FL, 95%)	Slope ± SE	LC_{90} (FL, 95%)	df	X^2
BnBt	41.860 ng/μl (22.355–78.383)	1.365 ± 0.139	367.204 ng/μl (196.102–687.592)	3	0.972
MnD	55.154 ng/μl (30.455–99.882)	1.454 ± 0.132	420.978 ng/μl (232.460–762.381)	3	0.989
HD-1	68.066 ng/μl (34.596–133.917)	1.258 ± 0.150	714.561 ng/μl (363.188–1405.878)	3	0.983

LSD multiple comparison test ($p < 0.05$)
FL fiducial limit, *SE* standard error, *df* degree of freedom, X^2 chi-square

Activity of Vip3A-activated toxins at 2.5 μg/cm^2 against *S. littoralis* larvae after 10 days was 100% mortality (Escudero et al. 2014). In another study, LC_{25}, LC_{50}, LC_{75}, and LC_{90} values for the purified proteins were 118.9, 142.4, 170.5, and 200.5 μg/ml against the first larval instar of *S. littoralis*, respectively (El-Ghareeb et al. 2012). Sattar et al. (2008) conducted a two-step ion-exchange chromatography in order to obtain Vip3 protein as a single band. In the same study, crude protein extracts obtained from the supernatants of different *Bt* isolates were bioassayed against *Agrotis ipsilon*, *Helicoverpa armigera*, and *S. littoralis* larvae with Vip3 proteins, partially purified by ion-exchange chromatography. Deaths were observed for 7 days and mortality rates ranged between 11 and 95% versus the first instar neonate larvae with 25 μg/ml crude protein extract. The *Bt* isolate with the highest toxicity was selected, the Vip3 protein was purified by a two-step DEAE-Sephacel anion exchange column, and LC_{50} values were determined by testing against lepidopteran pest larvae. Comparing the present results to the results of Vip3 activity of other *Bt* strains described by other authors showed very similar effects.

Conclusion

The results of the study confirm the significance of continuous exploration of new *Bt* strains from different ecological regions of the world. In addition, the Vip3A protein was responsible for the toxicity of the *Bt* isolates BnBt and MnD culture supernatants. Also, gene may be effective for preventing resistance in various insect-pest species. This protein may be used to develop a biopesticide.

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Authors' contributions
EG and AA performed all the experiments. ZD and KS conceived and designed all the experiments. All authors read and approved the final manuscript.

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Availability of data and materials
The dataset(s) supporting the conclusions of this article is (are) included within the article (and its additional file(s)).

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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