Biotechnological production and insecticidal activity of the overexpressed vegetative insecticidal *vip3Ah1* gene in *Escherichia coli*

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Abstract. The most important research objectives concerning *Bacillus thuringiensis* (Bt) are to find more potent strains and to identify new insecticidal genes with broad host ranges. A local isolate of subspecies *aegypti*, BtC18, showed broad insecticidal activity against lepidopteran, dipteran, and coleopteran insects. Vegetative insecticidal proteins (Vips) are toxins isolated from Bt. Here, we report the cloning and overexpression of the *vip3Ah1* gene in *E. coli* and provide analysis of its insecticidal activity. An 89.5-kDa Vip3Ah1 protein is secreted by Bt during the vegetative growth phase. The full 2.3-kbp length of *vip3Ah1* coding region was isolated from genomic DNA, cloned into pCR2.1 vector, subcloned into pET-30a expression vector, and overexpressed in *E. coli* under the control of the inducible T7 promoter. The heterologously produced Vip3Ah1 protein ($\approx$ 30% of total protein) was found in both soluble and insoluble forms. The nucleotide sequence had 99% identity with that of previously-isolated *vip3Aa* genes. Expressed protein was purified, blotted, and assayed against the lepidopteran pests black cutworm (BCW) (*Agrotis ipsilon*; LC_{50} 46 ng cm^{-2}) and tobacco hornworm (THW) (*Manduca sexta*; LC_{50} 27 ng cm^{-2}). The overexpressed Vip3Ah1 protein showed significant insecticidal activity against black cutworm and tobacco hornworm.

INTRODUCTION

Bacillus thuringiensis (Bt) were recognized many years before the bacterium itself was identified, with some accounts suggesting that Bt spores may have been used in ancient Egypt (Sanahuja *et al.*, 2011). The gram positive bacterium produces Crystal and vegetative insecticidal proteins (Vips) (Maagd *et al.*, 2003; Bravo *et al.*, 2011). Crystals are made of Cry protein synthesized during sporulation. These inclusions are solubilized in insect midguts, releasing δ-endotoxins that, upon proteolytic activation, exhibit a highly specific insecticidal activity (Andrews *et al.*, 1987; Höfte & Whiteley 1989). In recent decades, many Bt strains with different insect host spectra have been identified, and their δ-endotoxins have been used in biopesticide formulations (Assaeedi *et al.*, 2011; Palma Jesús *et al.*, 2014). To date, more than 300 insecticidal crystal protein (ICP) genes have been cloned, sequenced, and classified into 73 Cry-gene and three Cyt-gene groups based on amino acid similarity (Schnepf *et al.*, 1998; Crickmore *et al.*, 2004). Although Bt Cry proteins showed toxicity against many insect pests, several insect pests are not sensitive to their action (Schnepf *et al.*, 1998; Crickmore *et al.*, 2004). *Agrotis ipsilon* (BCW) is one of these insect that not affected by Cry protein toxins. BCW

is a worldwide insect pest that causing serious damage for many crops such as, grains (Estruch *et al.*, 1996; EL-Ghareeb *et al.*, 2012). Vegetative insecticidal proteins (Vips), which are secreted during the vegetative growth of certain Bt strains, are insecticidal proteins with no homology to ICPs (Osman 2012). These proteins include the binary Vip1/Vip2, which is toxic to coleopterans such as the western corn rootworm and northern corn rootworm (Han *et al.*, 1999). Both Vip1 and Vip2 are required for maximal activity against the western corn rootworm (Shi *et al.*, 2004; Jucovic1 *et al.*, 2008; Prashant *et al.*, 2013; Bi *et al.*, 2015). Vip3 was shown to be toxic to lepidopterans (Estruch *et al.*, 1997), and Vip3A is toxic against a wide spectrum of lepidopteran insects, including *Agrotis ipsilon*, *Spodoptera exigua*, *Chilo partellus*, *Helicoverpa punctigera*, and *Diatraea saccharalis* (Dossa *et al.*, 2002; Wu *et al.*, 2004; Jun *et al.*, 2007; Hernández-Rodríguez *et al.*, 2009). More than 90 different *vip* genes that have been identified to date, including 11 *vip1*, 15 *vip2*, and 70 *vip3A*. The Vip3A class is a 89.5 kDa protein that is active against a wide spectrum of lepidopteran insects (Lee *et al.*, 2003; Cheryl *et al.*, 2008; Ramasamy *et al.*, 2008). Vip3A has a different mode of action from that of Cry proteins. The onset of symptoms provoked by ingestion of Vip3A was delayed 36–48 h compared with that of δ-endotoxins (Dossa *et al.*, 2002; Zeng *et al.*, 2004). Furthermore, Vip3A binds different receptors in brush border membrane vesicles than does Cry1A (Yu *et al.*, 1997; Abdelkefi-Mesrati *et al.*, 2009 & 2011), and Vip3A has at least 260-fold higher toxicity against *A. ipsilon* than some Cry1A proteins (Yu *et al.*, 1997). Vip3A insecticidal proteins are expressed in the vegetative stage of growth starting at mid-log phase as well as during sporulation. These proteins can be classified into three groups, eight subgroups, 25 classes, and 82 subclasses according to the amino acid sequence similarity (Yu *et al.*, 1997) (http://www.lifesci.sussex.ac.uk/home/ Neil_Crickmore/Bt/vip.html/).

Isolation of novel Bt toxins molecules or synthetic Bt genes should help circumvent the potentially serious problem of insect resistance to Bt. In this study, the *vip3Ah1* gene was isolated from a local Bt isolate, cloned, sequenced, heterologously expressed, and the purified protein was assayed against BCW and THW.

MATERIALS AND METHODS

Bacterial strain
Bacillus thuringiensis subsp. *aegypti* (BtaC18) was isolated on LB medium as described by (Abulreesh *et al.*, 2012), characterized, and patented in the USA (No. 5986177) by Prof. Y. A. Osman. *Escherichia coli* strains XL1-blue and JM 109 (Stratagene, Santa Clara, CA, USA) were used as hosts for DNA propagation and preparation while strain BL21 DE3 (Novagen, Tokyo, Japan) was used as expression host. The *E. coli* strains were grown in LB broth with 100 µg mL^{-1} ampicillin and kanamycin for bacterial selection.

Genomic DNA isolation
Genomic DNA was isolated as described by (Sambrook *et al.*, 2001) and analyzed by horizontal gel electrophoresis in 1% agarose.

PCR screening of the *vip3Ah1* gene
The vip3Ah1F and vip3Ah1R primers were used to check for the presence of *vip3Ah1* and to isolate the 2.3 kbp gene from the local Bt strain (Table 1). Primers were designed as (EL-Ghareeb *et al.*, 2012), and synthesized at Bioneer (Daejon, South Korea) vipint1F, vipint2F, and vipint1R plus M13F and M13R were used only in sequencing reactions to obtain the full nucleotide sequence of *vip3Ah1* (Table 1). Genomic DNA extracted from the isolates were used as templates. The PCR reaction mixture (50 uL total volume) contained 200 mM of each dNTP, 0.5 mM primers, 10 mM Tris HCl (pH 8.3), 1.5 mM MgCl$_2$, 50 mMKCl, 2.5 U Taq polymerase (ABgene, Surry, UK), and 100 ng of template DNA. The PCR temperature cycles were as follows: 3 min initial denaturation at 94ºC, followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2.5 min. Final extension was carried out for 7 min at 72°C. The full-

length PCR product of *vip3Ah1* was investigated using electrophoresis in a 1% agarose gel.

Cloning of the *vip3Ah1* gene

The amplified fragment was purified using the DNA Purification Kit (Fermentas, Vilnius, Lithuania) and ligated with T4 ligase enzyme into the pCR2.1 cloning vector (Invitrogen, Carlsbad, CA, USA). *Escherichia coli* XL1-blue and Jm 109 cells were used for transformation and selected on LB plates containing ampicillin (100 ug mL^{-1}), X-Gal (20 ug mL^{-1}), and isopropyl-b-d-thiogalactopyranoside (IPTG; 200 ug mL^{-1}). White recombinant colonies grown on fresh plates were screened and verified by PCR and restriction enzyme analysis. All DNA manipulations, including ligation, transformation, and restriction digestion, were carried out as described by (Sambrook *et al.*, 2001).

Sequencing of the *vip3Ah1* gene

Nucleotide sequencing of the cloned *vip3Ah1* gene (2.3 kb) was performed by Bioneer company according to (Sanger *et al.*, 1977) using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit in conjunction with an ABI PRISM 310 Genetic Analyzer and PCR 9700 instrument (Applied Biosystems, Foster City, CA, USA). The reactions were conducted in total volumes of 20 uL, containing 8 uL of terminator ready reaction mix, 250 ng of PCR product, and 2 pmol of primer (m13 forward, m13 reverse, vipint1F, vipint2F, or vipint1R; five reactions total). The sequences were then assembled into a single non-overlapping contiguous sequence using the Fragment Assembly Program of Genetics Computer. The *vip3Ah1* gene was analyzed and compared with updated GenBank data using BLAST (http://www.ncbi.nlm.nih.gov/blast). The isolated sequence of the *vip3Ah1* gene was submitted to GenBank and accessioned as KJ603457.

Cloning in expression vector and protein expression

The *vip3Ah1* 2.3-kbp fragment was sub-cloned into the pET30a expression vector (Invitrogen) and and overexpressed in *E. coli* (≈ 30% of total protein) host BL21DE3 (Novagen). A single colony was used to inoculate 5 mL LB containing 100 ug mL^{-1} ampicillin and then incubated at 37ºC with vigorous agitation in a shaking incubator. One milliliter of overnight culture was used to inoculate 100 mL of LB containing 100 ug mL^{-1} ampicillin in a 250 mL culture flask, and the culture was grown at 37ºC with vigorous agitation. When cells reached an optical density of 0.6 at 600 nm, IPTG (1 mM) was added. After 4 h of induction at 37°C, cells were harvested by centrifugation at $3000 \times g$ for 10 min at 4°C and frozen. The clone was streaked onto LB agar plate containing 100 µg/ml ampicillin and the plate was incubated at 37°C for 16 h. A single colony was used to inoculate 5ml LB broth containing 100 µg/ml ampicillin-kanamycin followed by its incubation at 37°C with vigorous agitation in a shaking incubator. One milliliter of overnight culture was used to inoculate 100 ml of LB broth containing 100 µg/ml ampicillin in a 250 ml culture flask and the culture was grown at 37°C with vigorous agitation. When cells reached an optical density (OD) 0.6 at 600 nm, Isopropyl-beta-D-thiogalactopyranoside (IPTG; 0.25mM) was added. After 2h of induction at 37°C, cells were harvested by centrifugation at 3000g for 10 min at 4°C and frozen. All steps were carried out at 4°C. After two freeze–thaw cycles, cells were resuspended in lysis buffer (50mM Tris/HCl, pH7.8, 1mM EDTA) and homogenized by sonic disruption for a total of 20 min, with pulse and interval time of 1min and 30 s, respectively for each duty cycle. The mixture was centrifuged at 13,500 g for 10 min at 4°C to remove the unbroken cells and the supernatant. The pellet containing the inclusion bodies was washed in three volumes of washing buffer (10mM Tris–HCl, pH 7.5, 300 mM NaCl, 1mM EDTA and 1% Triton X- 100), kept at room temperature for 5 min, and centrifuged as above. The supernatant was filtered in a clean tube and 1 mL of the 50% Ni-NTA slurry was added and mixed gently by shaking for 15–60 min at room temperature. Lysate resin mixture was loaded into an empty column. Endogenous proteins with histidine residues were washed out of the matrix twice using 4

mL of washing buffer C (100 mM NaH$_2$PO$_4$, 10 mM Tris HCl, 8 M urea, pH 6.3). The 6-His-tagged fusion protein was eluted four times with 0.5 mL buffer D (100 mM NaH$_2$PO$_4$, 10 mM Tris HCl, 8 M urea, pH 5.9) followed by four times with buffer E (100 mM NaH$_2$PO$_4$, 10 mM Tris HCl, 8 M urea, pH 4.5). Finally, the fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined by the BCA protein assay system (Sigma, St. Louis, MO, USA) using bovine serum albumin as a standard as described by (Bradford 1970).

Protein separation on SDS-PAGE and western blotting
Protein analysis was performed on 10% SDS-PAGE. Protein samples were reduced by boiling for 5 min in loading buffer containing 5% δ-mercaptoethanol then centrifuged at 10,000 × g for 3 min and directly loaded onto the gel. Protein electrophoresis was performed in vertical sub-cells (Bio-Rad, Hercules, CA, USA). Slab gels containing 10% (w/v) resolving gel and 5% stacking gel concentrations of acrylamide were run at a constant current of 80 V for 2 h. The proteins were fixed in 45% methanol and 10% acetic acid in distilled water and stained in 0.25% Coomassie brilliant blue R-250 previously dissolved in 10% acetic acid, 50% methanol, and water. The gels were destained for 3 h in 5% methanol and 7% acetic acid in distilled water (Laemmli 1970). For immunodetection, proteins were transferred in a semidry blotter (Bio-Rad) onto PVDF membrane (Millipore, Billerica, MA, USA) in transfer buffer (14.42 g L^{-1} glycine, 2.90 g L^{-1} Tris base) then electrophoresed for 1 h at 100 V. After transfer, the blots were blocked in milk-based blocking buffer (5% w/v non-fat milk, 0.5% Tween 20, 100 mL TBS, pH 7.4) for 1 h at room temperature and then probed with the 6-His-tagged antibody (1:1000 dilution in blocking buffer) as the primary antiserum for 4 h at room temperature. The membrane was washed three times with TBS (10 mM Tris base, 0.9% NaCl; 50 mL, 15 min per wash) to remove excess antibodies. The immune reactive proteins were then visualized using alkaline phosphatase conjugated goat IgG as secondary antibody (1:10,000 dilution in blocking buffer) for 2 h at room temperature. The membrane was washed three times with TBS (50 mL, 15 min per wash) to remove excess antibodies and then with TBS three times (50 mL, 15 min per wash). The alkaline phosphate (100 mM Tris base, 100 mM sodium chloride, 25 mM MgCl$_2$, pH 9.5) activities were determined using a Nitro Blue Tetrazolium (NBT) Tablet Western Blotting Detection Kit (Sigma; Cat. No. N5514) by mixing 330 mL of NBT stock solution with 10 mL of substrate buffer, then adding 33 mL of 5-bromo-4-chloro-3-indolyl phosphate stock solution. The membrane was incubated for 1–10 min. This substrate system produces an insoluble end product that was determined using an ECL kit from Amersham Pharmacia (Uppsala, Sweden) (Towbin *et al.*, 1979; Rajamohan *et al.*, 1999).

Insect toxicity assay
The toxicity of the purified Vip3Ah1 protein was determined by a bioassay against first instar larvae of BCW and THW and compared with that of the wild type strain. Several concentrations were applied to the surface of a semi-artificial insect diet. Thirty larvae of first instar BCW or THW were placed into a cup and incubated at 26°C for 3 days as described by (Levinson & Navon 1969). Each bioassay was repeated three times. Mortality was scored daily until death or pupation. The LC$_{50}$ was determined by probit analysis (Finny 1962; Cutting & Vander Horn 1990) using 'LdPLine†' software (http://embakr.tripod.com/ldpline/ldpline.htm). The control treatments consisted of the semi-artificial diet without addition of the toxin.

<center>RESULTS</center>

PCR screening of the *vip3Ah1* gene
The presence of the *vip3Ah1* gene in our local Bt isolate, BtC18, was confirmed by PCR using the Vip3F/Vip3R primers. The expected 2.3-kb fragment was amplified (Fig. 1). This entire amplicon then was used to express the protein.
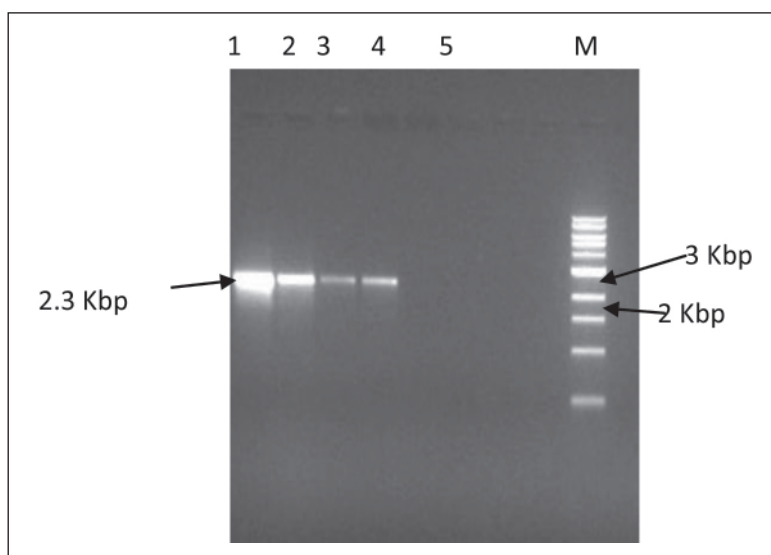
Fig. 1. Ethidium bromide-stained agarose gel resolving the PCR-amplified fragments of the 2.3-Kb *vip3Ah1* gene from local isolates of *Bacillus thuringiensis*. Lanes 1–4 are the PCR produce of *vip3Ah1*. Lane 5 is a negative control. M: DNA marker (1 Kbp).

Cloning of the *vip3Ah1* coding sequence

The amplified fragment was cloned into pCR2.1 vector. The cloning was confirmed by PCR and restriction digestion and sequencing. Nucleotide sequence BLAST analysis showed a 99% identity to the known *vip3Ah1* coding sequence. The *vip3Ah1* coding sequence was 2388 nucleotides in length and encoded a protein composed of 795 amino acids with an estimated molecular weight 89.5 kDa and a calculated isoelectric point (pI) of 5.1. Its amino acid composition revealed 196 aliphatic, 75 aromatic, 84 positive, 104 negative, and 136 tiny amino acids. The instability index (II) was computed to be 31.22, classifying the protein as stable. The nucleotide sequence comprised 906 A, 437 G, 751 T, and 294 C nucleotides (69% A+T and 31% G+C). Thus, *vip3Ah1* is a typical bacterial gene with low G+C content and high A+T content.

Cloning of *vip3Ah1* in expression vector and protein expression

Bt vegetative insecticidal protein encoding the 2.3-kbp *vip3Ah1* gene was subcloned into the pET-30a expression vector under control of the T7 promoter (Fig. 2), and its ATG initiation codon was fused in frame and in its correct orientation. The resulting construct, *vip3Ah1*–pET-30a, was transformed into *E. coli* strain BL21. The transformants were plated on LB ampicillin–kanamycin plates. Positive clones were selected based on PCR amplification and restriction digestion. Induction of *vip3Ah1* with 1 mM IPTG for 4 h produced a milligram amount of the 89.5 kDa Vip3Ah1 protein (Fig. 3). The insoluble nature of the recombinant protein synthesized in *E. coli* facilitated its purification. A single centrifugation of the bacterial lysate was used to isolate the inclusion bodies in which the recombinant protein was only slightly contaminated. In order to eliminate these contaminating bacterial proteins that copurify with the recombinant protein, inclusion bodies were washed with washing buffer containing 10mM Tris HCl pH 7.5, 300mM NaCl, 1mM EDTA and 1% Triton X-100, which was effective and yields high purity of recombinant protein. In order to solubilize the recombinant protein several buffers were used and prepared at various pH tested for their ability to solubilize the Vip3Ah and release it from the inclusion bodies. A Tris-HCL buffer at pH 10.0, with low
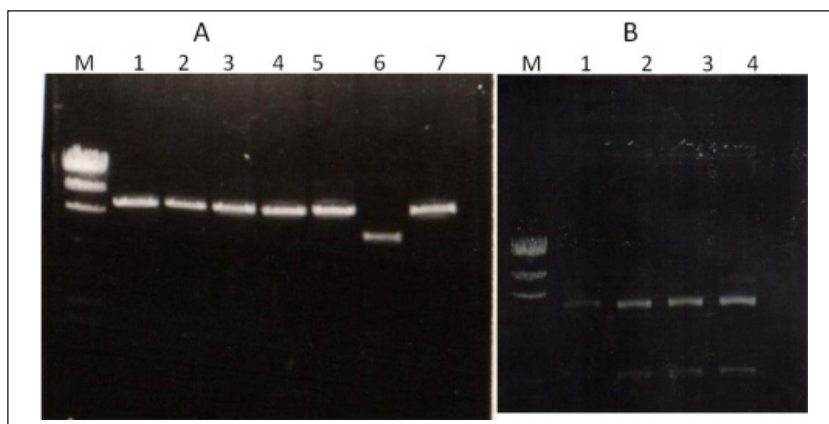
Fig. 2. Ethidium bromide-stained agarose gels resolving single digestion using *sal*1 enzyme panel (A): lanes 1–5 and 7 are positive clones; lane 6 is a negative clone. panel B: double digestion using using *Bam*H1/*note*1 enzymes: lanes 1–4 are positive clones. Lane M in both Panel A and B is a λ *Hind*III marker.
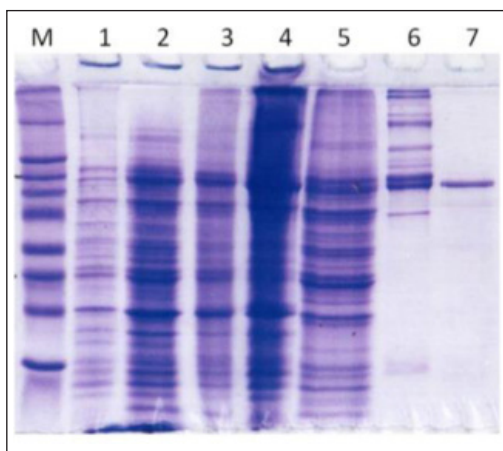

Fig. 3. Expression and purification of toxin protein Vip3Ah1. Lane M: wide range molecular weight protein marker; lanes 1–4: recombinant BL21 (DE3) cells at 0, 1, 2 and 4 h after induction with IPTG; lane 5: supernatant; lane 6: cell pellet; lane 7: affinity-purified Vip3h1 using Ni+2 column.

concentration of a reducing agent such as DTT was optimum for Vip3Ah solubilization. Inclusion bodies that were prepared from the clone in pET-30a consist mainly of Vip3Ah among other proteins. The Vip3Ah was released from the inclusion bodies at pH 10, and the remnant insoluble fraction was collected by centrifugation and an aliquot was separated in SDSPAGE (Fig. 3).

The first step in this study was to clone the *vip3Ah* coding sequence in a pET-30a for high level gene expression and efficient purification of Vip3Ah protein. Purification and western blotting of the 6-His-tagged fusion protein using Ni-NTA batch chromatography was accomplished. Western blot analysis of the protein transferred to the membraneshowed that antibody reacted with a protein of the expected size, and we concluded it was the expressed Vip3Ah protein (Fig. 4).

Insect Toxicity assay

The toxicity of the expression product against BCW and THW was assessed using several concentrations of the purified Vip3Ah1 toxin incorporated into artificial insect diets and poured into bioassay cups. First instar larvae of BCW and THW were placed into each cup and incubated at 26ºC for 3–7 days before assessing larval mortality. The LC_{50} values for BCW and THW were 46 and 27 ng cm^{-2}, respectively (Fig. 5).

DISCUSSION

Bta-C18 is an local Egyptian strain, showed insecticidal activity against lepidopteran, dipteran, and coleopteran insects. In recent years, *vip* genes have been cloned, characterized, and either expressed in *E. coli* cells to generate biopesticides or transformed into transgenic plants to enhance their ability
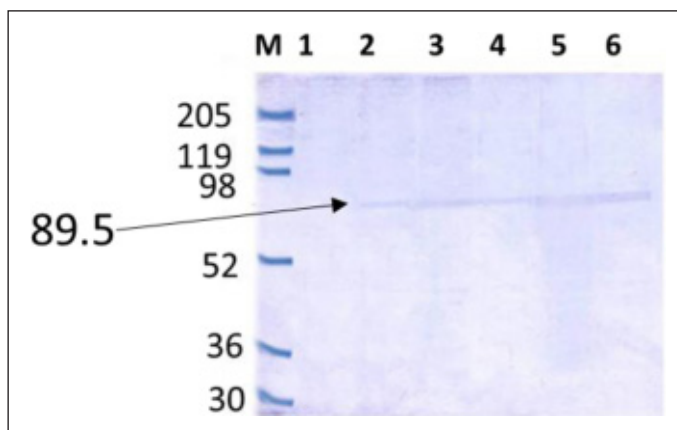
Fig. 4. Immunoblot of Vip3Ah1 protein expressed in *E. coli*. Lanes 1, 2 and 3 are BL21 (DE3) recombinant cells after 0, 1, and 2 of IPTG induction. Lane 4: supernatant; lane 5: cell pellet Lane; lane 6: affinity-purified Vip3h1 using Ni+2 column. Lane M: broad range protein Marker (Bio-Rad).
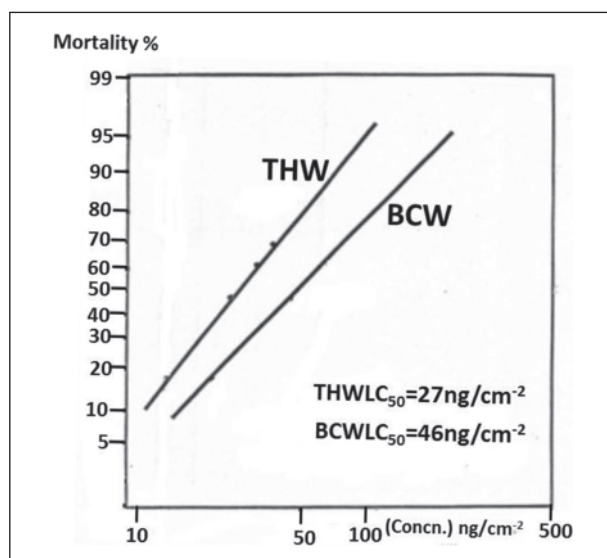

Fig. 5. Insecticidal activity of the recombinant *vip3Ah1* purified protein against first instar larvae of BCW and THW.

to control pests (Chen *et al.*, 2003 & 2005). Vip3Ah1 is a 89.5 kDa secreted protein that has no similarities with Cry proteins (Yu *et al.*, 1997). The first step in this study was to target the *vip3Ah1* gene. PCR screening detected *vip3Ah1* in genomic DNA, in agreement with some studies (EL-Ghareeb *et al.*, 2012) but not another (Zeng *et al.*, 2004). The *vip3Ah1* gene was cloned in pCR2.1 vector and then in expression vector pET-30a, with high-level expression under control of the T7 promoter. One major problem associated with over production of recombinant proteins in *E. coli* in general is the improper folding and formation of highly insoluble inclusion bodies (Elmenofy *et al.*, 2014). High molar concentration of denaturing agents such as Urea and Guanidine

HCL are routinely used to dissociate the inclusion bodies and release protein of interest into the solution. Treating the inclusion bodies with such denaturing agents truly affect the secondary and tertiary structural integrity of recombinant proteins. Subsequently, successive steps of dialysis in the presence of decreasing concentration of denaturing reagents are essential to renature the recombinant protein. Despite these laborious and time consuming successive dialysis steps, obtaining properly folded recombinant proteins are always not guaranteed. Since the insecticidal activity of Vip3Ah protein should be restored during purification, it was necessary to minimize the damage of recombinant protein during purification by using mild and less harsh condition. A Tris-HCL buffer at pH 10.0 with low concentration of a reducing agent such as DTT was optimum for Vip3Ah solubilization. The clones expressed an insoluble protein of 89.5kDa. Furthermore, the expression product was found in the supernatant of the recombinant culture. There are a lot of reports on the toxicity of Vip3A to BCW (Abdelkefi-Mesrati *et al.*, 2009 & 2011). Interestingly, the purified protein showed activity against both BCW and THW. The LC_{50} values for the wild strain and purified protein were 46 and 27 ng cm^{-2}, respectively. Interestingly, the wild strain was more toxic than the recombinant purified protein (Osman *et al.*, 2013). This difference in mortality may be attributed to the nature of the expression. Also, recombinant genes that are over-expressed in bacteria often form primarily insoluble proteins (Osman *et al.*, 2013). Treating these insoluble recombinant proteins with denaturing agents affects their secondary and tertiary structural integrity, affecting their subsequent toxicity. Overall, the toxicity of the recombinant protein was satisfactory and comparable to that of the native one (Osman *et al.*, 2013). Moreover, the toxicity is an indicator of the successful cloning of the functional gene. Results presented in this study can be used to develop a biopesticide or to design appropriate strategies for transgenic crop plants resistant to lepidopteran pests.

REFERENCES

Abdelkefi-Mesrati, L., Boukedi, H., Dammak-Karray, M., Sellami-Boudawara, T. & Jaoua, S. (2011). Study of the *Bacillus thuringiensis* Vip3Aa16 histopathological effects and determination of its putative binding proteins in the midgut of *Spodoptera littoralis*. *J Invertebr Pathol*. **106**: 250-4.

Abdelkefi-Mesrati, L., Rouis, S., Sellami, S. & Jaoua, S. (2009). *Prays oleae* midgut putative receptor of *Bacillus thuringiensis* vegetative insecticidal protein Vip3LB differs from that of Cry1Ac toxin. *Molec Biotechnol*. **43**: 15-9.

Abulreesh, H., Osman, G. & Assaeedi, S. (2012). Characterization of insecticidal genes of *Bacillus thuringiensis* strains isolated from arid environments. *Indian J of Microbiol*. **52**: 500-503.

Andrews, R., Fausr, R., Wabiko, M., Roymond, K. & Bulla, I. (1987). Biotechnology of Bt: A critical review. *Electron J Biotechnol*. **6**: 163-232.

Assaeedi, A., Osman, G. & Abulreesh, H. (2011). The occurrence and insecticidal activity of *Bacillus thuringiensis* in the arid environments. *AJCS*. **5**: 1185-1190.

Bi, Y., Zhang, Y., Shu, C., Crickmore, N., Wang, Q., Du, L., Song, F. & Zhang, J. (2015). Genomic sequencing identifies novel *Bacillus thuringiensis* Vip1/Vip2 binary and Cry8 toxins that have high toxicity to Scarabaeoidea larvae. *Appl Microbiol Biotechnol*. **99**: 753 -60.

Bradford, M. (1976). A rapid and sensitive method for the quantitative of microgram quantities in protei utilizing the principle of protein dye binding. *Anal Biochem*. **72**: 248-254.

Bravo, A., Likitvivatanavong, S., Gill, S. & Soberón, M. (2011). *Bacillus thuringiensis*: A story of a successful bioinsecticide. *Insect Biochem Molec Biol*. **41**: 423-431.

Chen, J., Sun, Fan., Tang, Lixia., Tang, Mujin., Yongxia, Shi., Jianxiu, Yu. & Pang, Yi. (2003). Expression of *Bacillus thuringiensis* full-length and N-terminally truncated vip184 gene in an

acrystalliferous strain of subspecies kurstaki. *World J Microbiol Biotechnol.* **19**: 883-889.

Chen, J., Sun, Y., Shi, W., Xu, W. & Pang, Y. (2005). Efficient expression of vip184DP gene under the control of promoters plus Shine-Dalgarno (SD) sequences of cry genesfrom *Bacillus thuringiensis*. *J Appl Microbiol*. **99**: 426-434.

Cheryl, E., Court, BeardLeon., Boets, Annemie., Mourant, Roslyn., Van Rie, Jeroen. & Raymond, J. (2008). Unusually High Frequency of Genes Encoding Vegetative Insecticidal Proteins in an Australian *Bacillus thuringiensis* Collection. *Curr Microbiol*. **57**: 195-199.

Crickmore, N., Zeigler, D., Feitelson , J., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J. & Dean, J. (2004). *Bacillus thuringiensis* toxin nomenclature.; [Online.] http://www.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index.htm.

Cutting, S. & Vander Horn, P.B. (1990). Genetic analysis. In: Cutting SM, Vander Horn PB. Molecular Biological Methods for *Bacillus*. London: John Wiley & Sons, pp. 65. 1

Dossa, K., Anup Kumar, R., Jayakumar, A. & Vaithilingam, Sekara. (2002). Cloning and expression of the vegetative insecticidal protein(vip3V) gene of *Bacillus thuringiensis* in *Escherichia coli*. *Protein expres purif*. **26**: 82-88.

EL-Ghareeb, D., Osman, G. & El baz, A. (2012). Isolation, cloning, and overexpression of vip3Aa gene isolated from a local *Bacillus thuringiensis*. *Biocont Sci Technol*. **22**: 11-21.

El-Menofy, W.H., Osman, G.H., Assaeedi, A. & Salama, M.S. (2014). Construction of A Novel Recombinant Baculovirus Containing Cry1Ab Insecticidal Protein from *Bacillus thuringiensis*. *Biol Online Proced. J*. **4**: 7-16

Estruch, J., Carozzi, N., Desai, N., Duck, N., Warren, G. & Koziel, M. (1997). Transgenic plants:an emerging approach to pest control. *Nat Biotechnol*. **15**: 137-141.

Estruch, J., Warren, W., Mullins, M., Nye, G., Craig, J. & Koziel, M. (1996). Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. *PNAS*. **93**: 5389-5394.

Finny (1962). Probit analysis. Cambridge: Cambridge University Press.

Han, S., Craig, J., Putnam, C., Carozzi, N. & Tainer, J. (1999). Evolution and mechanism from structures of an ADP-ribosylating toxin and NAD complex. *Nat Struct Biol*. **6**: 932-936.

Hernández-Rodríguez, S., Boets, A., Van Rie, J. & Ferré, J. (2009). Screening and identification of vip genes in *Bacillus thuringiensis* strains. *J Appl Microbiol*. **107**: 219-25.

Höfte, H. & Whiteley, H. (1989). Insecticidal crystal proteins of *Bacillus thuringiensis. Microbiol Rev*. **53**: 242-255.

Jucovic1, M., Walters, F., Gregory, W., Palekar, N. & Chen, J. (2008). From enzyme to zymogen: Engineering Vip2, an ADP-ribosyltransferase from *Bacillus cereus*, for conditional toxicity. *Protein Eng Des Sel*. **21**: 631-638.

Jun, F., Xiaoli, X., Ping, W., Jian-Zhou, Z., Anthony, M. & Jiaan, C. (2007). Characterization of Chimeric *Bacillus thuringiensis* Vip3 Toxins. *Appl Environ Microbiol*. **73**: 956-961.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. **227**: 680-685.

Lee, M., Walters, F., Hart, H., Palekar, N. & Chen, J. (2003). The mode of action of the *Bacillus thuringiensis* vegetative insecticidal protein Vip3A differs from that of Cry1Ab endotoxin. *Appl Environ Microbiol*. **69**: 4648-4657.

Levinson, Z. & Navon, A. (1969). Ascorbic acid and unsaturated fatty acids in the nutrition of the Egyptian cotton leaf worm. *J Insect Physiol*. **15**: 591-595.

Maagd, R., Bravo, A., Berry, C., Crickmore, N. & Schnepf, H. (2003). Structure diversity and evaluation of protein toxin from spore forming entomopathogenic bacteria. *Annu Rev Genet*. **37**: 409-33.

Osman, G. (2012). Detection,Cloning and Bioinformatics Analysis of vip1/vip2 Genes from Local *Bacillus thuringiensis*. *Afr J Biotechnol*. **11**: 11678-11685.

Osman, G., Assaeedi, A., Osman, Y., El-Ghareeb, D. & Alreedy, R. (2013). Purification and characterization of *Bacillus thuringiensis* vegetative insecticidal toxin protein(s). *Lett Appl Microbiol*. **57**: 310-316.

Palma, Jesús., Murillo, L., Caballero, P., Berry, C. & Muñoz, D. (2014). *Bacillus thuringiensis* Toxins: An Overview of Their Biocidal Activity. *Toxins*. **6**: 3296-3325.

Prashant, R., Mangesh, P., Dipti, R., Pravin, V., Niraj, S. & Mahendra, S. (2013). Screening of vip1/vip2 binary toxin gene and its isolation and cloning from local *Bacillus thuringiensis* isolates. *Sci Asia*. **39**: 620-624.

Rajamohan, F., Engstrom, C., Denton, T., Engen, L., Kourinov, I. & Uckun, F. (1999). High-level expression an purification of biological active recombinant pokeweed antiviral protein. *Protein Expres Purif*. **16**: 359-368.

Ramasamy, B., Nadarajah, V., Soong, Z., Lee, H. & Mohammad, S. (2008). A preliminary study of the bioactivity of vegetative proteins extracted from Malaysian *Bacillus thuringiensis* isolates. *Trop Biomed*. **25**: 64-74.

Sambrook, J.W. & Russell, D. (2001). Molecular Cloning A Laboratory Manual; 3rd edn. New York: Cold Spring Harbor Laboratory Press, pp. 1.32–1.34, 1.116–1.118, 1.123–1.125.

Sanahuja, G., Banakar, R., Twyman, R., Capell, M. & Christou, P. (2011). *Bacillus thuringiensis*: a century of research, development and commercial applications. *Plant Biotechnol J*. **9**: 283-300.

Sanger, F., Nicklen, S. & Coulson, R. (1977). Biochemistry DNA Sequencing with chain-terminating inhibitors (DNA Polymerase/nucleotide sequence/ bacteriophage 4X174). *PNAS*. **74**: 5463-5467.

Schnepf, H., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J. & Feitelson, J. (1998). *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. Mol Biol Rev*. **62**: 775-806.

Shi, Y., Xu, W., Yuan, M., Tang, M., Chen, J. & Pang, Y. (2004). Expression of vip1/vip2 genes in Escherichia coli and *Bacillus thuringiensis* and the analysis of their signal peptides. *J Appl Microbiol*. **97**: 757-65.

Towbin, H., Staehelin, T. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *PNAS*. **76**: 4350-4354.

Wu, Z., Wen, Yi., Guo, Jun., ZhiQiu, Tian., Pei, Huang., Xun, Bo Li. & Xiong, Guan. (2004). Cloning and localization of vip3A gene of *Bacillus thuringiensis. Biotechnol Lett*. **26**: 1425-1428.

Yu, G., Mullins, M., Warren, W., Koziel, M. & Estruch, J. (1997). The *Bacillus thuringiensis* vegetative insecticidal protein Vip3A lyses midgut epithelium cells of susceptible insects. *Appl Environ Microbiol*. **63**: 532-536.

Zeng, W., Wen, Y., Jun, Z., Tian, P., Xun, L. & Xiong, G. (2004). Cloning and localization of vip3A gene of *Bacillus thuringiensis. Biotechnol Lett*. **26**: 1425-1428.