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Original article

First report of detection of the putative receptor of *Bacillus thuringiensis* toxin Vip3Aa from black cutworm (*Agrotis ipsilon*)

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ABSTRACT

Black cutworm (BCW) *Agrotis ipsilon*, an economically important lepidopteran insect, has attracted a great attention. *Bacillus thuringiensis* (Bt) is spore forming soil bacteria and is an excellent environment-friendly approach for the control of phytophagous and disease-transmitting insects. In fact, bio-pesticide formulations and insect resistant transgenic plants based on the bacterium Bt delta-endotoxin have attracted worldwide attention as a safer alternative to harmful chemical pesticides. The major objective of the current study was to understand the mechanism of interaction of Bt toxin with its receptor molecule (s). The investigation involved the isolation, identification, and characterization of a putative receptor – vip3Aa. In addition, the kinetics of vip toxin binding to its receptor molecule was also studied. The present data suggest that Vip3Aa toxin bound specifically with high affinity to a 48-kDa protein present at the brush border membrane vesicles (BBMV) prepared from the midgut epithelial cells of BCW larvae.

1. Introduction

Bacillus thuringiensis (Bt) technology has been proved universally as a valuable alternative to the conventional synthetic insecticides. The use of Bt as a microbial insecticide offers several advantages over the harmful chemical insecticides. The species-specific action of the insecticidal crystal (Cry) proteins (ICPs) or vegetative insecticidal proteins (Vips) makes them safe to non-target or beneficial insects, vertebrates, environment, and the users (El-Menofy et al., 2014; Osman et al., 2015). The Bt toxins become active only after ingestion by insects. The alkaline pH and proteolytic enzymes present in the midgut solubilize the endotoxin protein and convert it to an active toxic form. These toxic compounds disrupt the midgut epithelial cells resulting in cessation of feeding and consequent insect mortality. The first step in the insecticidal action of Bt toxin is the recognition of target molecules inside the midgut of the insect host (Assaeedi et al., 2011; Abulreesh et al., 2012). Existing reports indicate that the target receptor molecules are located within or on the membrane surface of epithelial cells of the midgut of a susceptible insect. Although, the specific binding of these toxins to the brush border membrane vesicles (BBMV) of midgut has been reported for Cry toxin proteins (Hofmann et al., 1988; Osman, 2012), little is known about the molecular attributes of the toxin binding proteins of Vip 3Aa toxin proteins.

1.1. Insect receptors for cry toxins

The first step in the action mechanism of Bt toxins is the recognition and binding to the high-affinity site of specific receptors on the brush border surface of the midgut of susceptible insect host. Several immunological methods have been used to identify Bt

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toxin targets, which apparently reside in the luminal brush border of the larval midgut (Bravo et al., 2011; El-Ghareeb et al., 2012). The *B. thuringiensis* toxin receptor, BT-R_1, present in the midgut brush border membrane was purified and respective cDNA was cloned from the larvae of lepidopteran *Manduca sexta* (Vadlamudi et al., 1993, 1995). As determined by SDS-PAGE, the molecular mass of BT-R_1 is approximately 210 kDa with an isoelectric point of ~5.5. The BT-R_1 cDNA encodes a polypeptide with a predicted molecular mass of 192 kDa. The BT-R_1 is a cadherin-like glycoprotein. Recently, two other cadherin-like proteins of 175 and 180 kDa, showing amino acid sequence homology to BT-R_1 have also been identified, purified, and cloned from the lepidopteran *Bombyx mori* (silkworm) (Nagamatsu et al., 1998; Ihara et al., 1998). Moreover, a 210-kDa protein from the lepidopteran *Lymantria dispar* (Gypsy moth) was reported to bind Cry1Aa and Cry1Ab in ligand blot studies Valaitis et al., 1997. A 175-kDa protein from *B. mori* binds specifically to Cry1Aa with high affinity. Anti-BT-R antibodies were capable of reducing toxicity *in vivo* as well as blocking binding to the membrane vesicles *in vitro*. The study showed that the 175-kDa BT-R receptor was found only in the gut. Similarly, Midboe et al., (2001) also detected BT-R_1 exclusively in the midgut of *M. sexta*. Conserved features shared between protocadherins and the cadherin-like proteins along with the amino acid sequence homology between the toxin-binding proteins suggest that these integral midgut membrane proteins represent a novel class of invertebrate protocadherins and therefore were labeled as epithelial invertebrate protocadherins (E-IVPs). Recently, the midgut epithelial cells of *M. sexta* were shown to possess a putative Cry 1Ac toxin receptor, which is a 120-kDa glycoprotein (Yaoi et al., 1997).

1.2. Insect resistance mechanisms

In order to understand the mechanism of development of resistance, it is imperative to examine the fate of crystal proteins within the midgut of insects. In the first step, the crystal protein is solubilized and proteolytically degraded by proteinases in the gut of susceptible insect host. Consequently, a 60–70 kDa core proteinase-resistant toxin is produced. The toxin eventually interacts with a specific receptor(s) presumably creating pores in the midgut cell membranes and causing an ionic imbalance. This cascade of events may lead to septicemia in the insect. Given the multiple steps involved in the processing of the Cry protein to form an active toxin, it is not very surprising that the insect populations might develop various means of resistance against the toxin. Any alteration in insect gut physiology or biochemical system could disrupt the consecutive process and may result in toxin resistance and, therefore, several mechanisms of insect resistance to Bt toxins have been proposed (Osman et al., 2013). The current study was aimed at the identification and molecular characterization of Bt toxin Vip3Aa and its putative receptor in black cutworm, *Agrotis ipsilon,* a lepidopteran corn pest.

2. Materials and methods

2.1. Preparation of Brush Border Membrane Vesicles (BBMV)

The early 4th instar larvae of black cutworm were placed on ice for 1 h and were dissected to remove the midguts. Then, BBMV were prepared from the midgut tissues by following differential magnesium precipitation method (Wolfersberger et al., 1987) in the presence of protease inhibitors (5 mg/mL pepstatin, antipain, aprotonin, leupeptin, 1 mM PMSF, and 5 mM benzamidine). The midgut tissue was homogenized in nine volume of buffer "A" (300 mM Mannitol, 5 mM EGTA and 17 mM Tris–HCl, pH 7.5) using a glass Teflon homogenizer (9 strokes up and down at 3000 rpm). An equal volume of 24 mM $MgCl_2$ solution was added to the homogenate and further re-homogenized. The homogenate was incubated on ice for 15 min and centrifuged at 4500 rpm for 15 min at 4 °C. The supernatant was transferred to a fresh tube and again centrifuged at 31,000 *g* for 30 min at 4 °C. The supernatant was discarded and the pellet was re-suspended in half the volume of buffer "A" and this step was repeated. The final pellet was re-suspended in buffer "A" containing protease inhibitors, flash frozen in liquid nitrogen, and stored at −85 °C.

2.2. Protein analysis of BBMV by SDS-PAGE

The integument or the extracted fluid was dissolved in sample buffer (0.06 M Tris HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and bromophenol blue). The samples were heated for 5 min in boiling water bath and separated on 10% polyacrylamide gel as described by Laemmli (1970). The gels were stained with 0.1% Coomassie brilliant blue R–250 and de-stained in 7% (v/v) acetic acid containing 50% methanol.

2.3. Protein iodination

Purified Vip3Aa toxin used in this work was radioiodinated using the chloramine-T method (Hunter and Greenwood, 1962) with ^{125}I-Na (NEN Dupont, Billerica MA) Carrier free. About 10 μg of toxin was mixed with 5 μL of ^{125}I-Na (approximately 0.5 mCi) in 100 μL of $NaHPO_4$ buffer (0.5 M, pH 7.4) with 25 μL of Chloramine-T (4 mg/mL). The reaction mixture was vortexed for 20–25 s at 23 °C and the reaction was stopped by adding 50 μL of $Na_2S_2O_5$ (4.4 mg/mL). Free iodine was removed by gel filtration on a Sephadex G–50 column equilibrated with PBS containing 10 mg/mL BSA.

2.4. Toxin binding assays

Homologous competition inhibition binding assays were performed as described by Keeton and Bulla (1998). About 25 μg of BBMV were incubated with 1.2 nM^{125}I-Vip3Aa toxin in the presence of different concentrations (0–1000 nM) of appropriate unlabeled homologous toxin (Vip3Aa) in 100 μL of binding buffer (PBS/0.2% BSA) at 25 °C for 30 min. The radiolabeled and unlabeled toxins were mixed together before adding to the BBMV. The unbound toxins were separated from BBMV-bound toxin by centrifugation at 14,000*g* for 10 min. The pellet containing bound toxin was washed three times with ice-cold binding buffer by gentle vortexing and radioactivity in the resultant pellet was measured using a Beckman Gamma 5500 counter. The binding data were analyzed by using the PRISM program (GraphPad Software Inc., San Diego, CA, USA).

2.5. Radioligand blotting

About 200 μg of BBMV protein was solubilized, separated by 7.5% SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membrane as described by Francis and Bulla (1997). The blots were blocked with TBS (10 mM Tris–HCl and 0.9% NaCl) containing 5% non-fat dry milk powder, 5% glycerol, 0.5% Tween–20, and 0.025% sodium azide for 2 h at 25 °C. Blocking buffer was removed and the membranes were incubated for 2 h at 25 °C in an equal volume of fresh blocking buffer containing 2×10^5 cpm/mL (1–1.25 nM) of ^{125}I-Vip3Aa toxin either in the presence or absence of unlabeled toxins. Finally, the membranes were washed thrice with fresh blocking buffer for 10 min each, rinsed once with TBS, dried, and exposed to Kodak X-ray film at 80 °C.

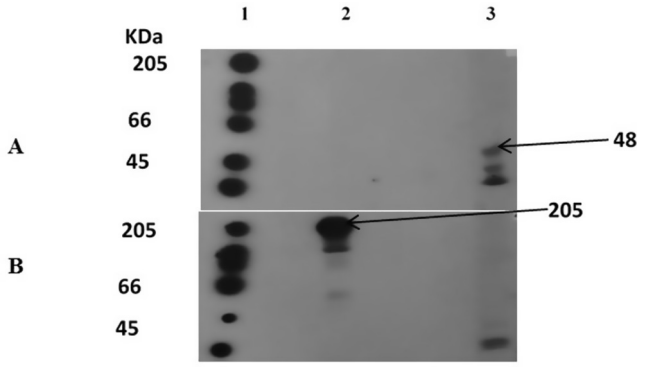

Fig. 1. Ligand blotting of BCW BBMV. Lane 1A and B. High molecular weight markers indicated in kDa; lane 2. BCWBBMV and THW proteins (200 μg) separated by 10% SDS-PAGE, blotted and probed with 1.0–1.25 nM of ^{125}I-Vip3Aa; lane 3A and B ^{125}I-Cry1Ab.

2.6. Immunoprecipitation of Vip3Aa binding protein

Immunoprecipitation was carried out according to Vadlamudi et al. (1993). About 25 μL of Vip3Aa antiserum was added to 1 mL of protein A-Sepharose CL–4B equilibrated in washing buffer (1% Nonidet P–40, 6 mM EDTA, 50 mM Tris–HCl, 250 mM NaCl) and mixed for one hour at 4 °C. After washing the blot three times with washing buffer, about 700 μg of Vip3Aa toxin was added and the mixture was incubated for another 1 h at 4 °C and washed again three times with washing buffer. The BCW-BBMV proteins (6 mg) were solubilized in 1 mL of washing buffer containing 1% NP–40 and protease inhibitors (10 μg/mL pepstatin, antipain, aprotonin, and leupeptin; 5 mM iodoacetamide; and 1 mM PMSF). The insoluble proteins were removed by centrifugation at 15,000 rpm for 30 min. The soluble proteins were filtered through a 0.45-μm filter and added to 1 mL of Sepharose-protein A beads linked to Vip3Aa toxins, and the sample was stirred gently for 1 h at 4 °C. The Sepharose beads were centrifuged and washed four times with washing buffer containing 0.25% NP–40 and 0.02% SDS. The toxin-binding protein complex was dissociated from the beads by heating in the sample buffer (Laemmli, 1970) and the binding proteins were stained with Coomassie brilliant blue and detected by ligand blotting with ^{125}I-Vip3Aa toxin and Western blot using Vip3Aa antiserum (Provided by Microbial Genetics lab, AGERI – Egypt). The proteins were resolved by SDS-PAGE, transferred to the PVDF membrane, which was raised in mice by immunize BALB-C mice upon the approval of AGERI internal biosafety and bioethics committee by the typical protocol used by Salem et al. (2017).

3. Results

3.1. Identification of ^{125}I-Vip3Aa binding proteins (putative receptors)

One aspect of characterizing toxins such as insecticidal proteins, which has recently become very important, is the identification of the appropriate receptor molecules in the target host. Besides this, the identification of insect midgut proteins that bind to a specific Bt toxin is also a part of the characterization strategy for newly discovered Bt toxins. The Vip3Aa receptor molecule in the BCW midgut epithelial cells was characterized using Western blot analysis and ligand blots with anti-Vip3Aa and ^{125}I–Vip3Aa, respectively. In both the approaches, the solubilized BBMVs from the BCW midgut were fractionated by SDS-PAGE and blotted onto PVDF membranes. In the Western blot analysis (Fig. 1) Vip3Aa bound firmly to a 48 kDa protein band. Moreover, the ^{125}I Vip3Aa protein bound to 40 and 31 kDa bands in ligand blot with BBMV of the BCW (Fig. 1). Both the experimental approaches produced similar results suggesting that Vip3Aa from *B. thuringiensis* binds to more than one protein present on the BCW midgut epithelial cells, however, it showed a higher affinity to the 48 kDa protein. The ligand blots of BBMV obtained from tobacco hornworm (*M. sexta*) and iodinated with Cry1Ab were used as positive controls (Fig. 1) for the reference and quality control of our procedures.

3.2. Competitive inhibition of binding assay

Competitive inhibition of binding assays is used in ligand blots not only to confirm the strength of the ligand binding to its respective receptor but also to check the specificity of the two molecules. Therefore, ^{125}I-labeled Vip3Aa proteins were used in binding assays with the BBMV from the BCW midgut epithelial cells. Competition binding of ^{125}I-Vip3Aa toxin to BCW was carried out in the presence of increasing concentrations of unlabeled Vip3Aa toxin that varied from 0.01 to 1 μM. A 50% inhibition of ^{125}I-Vip3Aa binding was observed at 10 nM concentration of unlabeled Vip3Aa (Table 1). The competitive inhibition assay data were analyzed using the non-linear regression model. The K_d value was calculated using Prism program (GraphPad Software Inc., San Diego, CA, USA) (Fig. 2). Data analysis revealed that the dissociation constant (K_d) of the toxin is 163 nM. It is a relatively rational value and an indicator of a high-affinity binding between Vip3Aa toxin protein and its receptor in BCW midgut tissues. The values were estimated as means ±SEM of three independent experiments.

3.3. Immunoprecipitation of the Vip3Aa binding protein

Immunoprecipitation experiments were conducted using Vip3Aa toxin, which has the highest binding affinity and the

Table 1
Homologous competition inhibition binding assays of ^{125}I-labeled Vip3Aa toxin to BCWBBMV proteins: Reading of the δ counter.

Reading of Gamma counter					
Replicates reading					
Concentration (nM)	Serial	Normalization factor	First reading	Second reading	Maximum % binding
0	32	1.00	382,022	382,822	100
0.01	33	1.00	294,217	294,749	76.99375
0.1	34	1.00	232,912	233,281	60.9372
1	35	1.00	213,631	213,996	55.89961
10	36	1.00	180,107	180,393	47.1219
100	37	1.00	113,556	113,859	29.74202
1000	38	1.00	104,854	105,112	27.45715

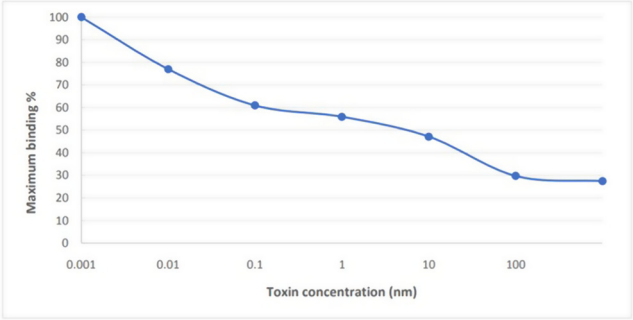

Fig. 2. Homologous competition inhibition binding assays of ^{125}I-labeled Vip3Aa toxin to BCWBBMV proteins. BBMV (25 μg) was incubated with the radiolabeled toxin in the presence of increasing concentrations of the corresponding unlabeled toxin (0.01–1 μM). Each point represents the mean of three independent experiments. A: The reading of δ counter, B: the decrease in the binding with increasing unlabeled toxin; Kd = 163 nm. $^{+}K_d$ = equilibrium dissociation constant. K_d was determined using nonlinear regression. Values are means ±SEM of three independent experiments.

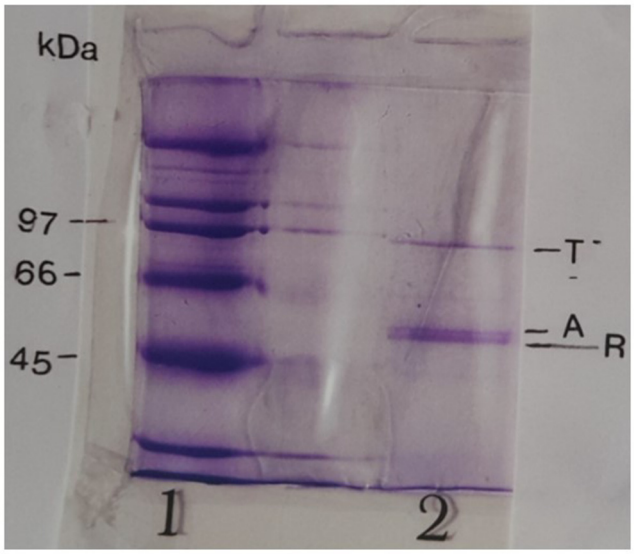

Fig. 3. Immuno-precipitation of the Vip3Aa toxin binding protein. The Vipp3Aa binding protein was precipitated by adding anti Vip3Aa serum to Sepharose protein-A. Vip3Aa was added followed by the incubation with the solubilized BCW BBMV proteins. The bound proteins were separated by 10% SDS-PAGE, as shown in lane 2, while lane 1 contains high molecular weight protein marker.

solubilized BBMV prepared from the midgut epithelial cells of the BCW larvae. The Vip3Aa toxin binding protein was precipitated by adding anti-Vip3Aa toxin serum to Sepharose protein A. The Vip3Aa toxin was added and incubated with the solubilized BBMV proteins from BCW. The bound proteins were precipitated by centrifugation and separated on 10% denatured SDS-PAGE (Fig. 3). It demonstrated the selective precipitation of ~48 kDa protein from the solubilized BBMV of the BCW with Vip3Aa toxin. The radioligand blotting with ^{125}I-Vip3Aa toxin showed that it binds to the same ~48 kDa protein (Fig. 3) from the BBMV of the BCW suggesting that the precipitated protein formed a complex with the Vip3Aa toxin and anti-Vip3Aa toxin is the only binding protein and it is the same binding protein on the epithelial cells of the BCW (Fig. 3).

4. Discussion

The main objectives of the present study were identification, isolation, and characterization of a putative receptor protein from *Agrotis ipsilon*, the black cutworm (BCW), corresponding to an insecticidal protein expressed during the vegetative growth of *B. thuringiensis*. The scientific research in this particular area opens up the avenues toward the biological control of insect pests and further alleviates the hazardous effects of the chemical pesticides on humans and the environment. The characterization of the tissue targeted by Bt toxins and the post-ingestion histopathological examination of susceptible insect host is extremely important (Hofmann et al., 1988; Estruch et al., 1996; Osman et al., 2016). The *in vitro* studies conducted previously on the mechanism of toxicity have revealed a variety of symptoms including paralysis of insect gut, disruption of midgut epithelial cell structure and function, and cessation of feeding followed by death (Vadlamudi et al., 1993, 1995). There has been observed an inhibition of K^+ dependent amino acid transport when the BBMV were inoculated with activated toxins. The formation of pores and colloids and osmotic lysis of midgut epithelial cells have also been proposed as a possible mechanism of toxin action (Hofmann et al., 1988; Zhou et al., 2016). If the toxin induces pores, it is still not known whether the toxin does so by interacting with the protein present in the BBMV or by the insertion into the BBMV (Vadlamudi et al., 1993). Several factors have been determined, which may influence the insect host range of the insecticidal proteins produced by Bt subspecies. These factors include (i) solubilization of the crystal protein, (ii) processing by midgut proteases, (iii) presence of specific binding sites on susceptible cells, and (iv) the insertion of the bound toxin into the membrane (Vadlamudi et al., 1993, 1995). In this study, several proteins of 48, 40 and 31 kDa from the BBMV of *A. ipsilon* (BCW) were identified and purified. These proteins specifically recognize and bind to the Vip3Aa toxins of *B. thuringiensis* subsp. *aegypti* strain C18 (BtaC18). However, the binding to the 48-kDa proteins was the strongest. The radiolabeled Vip3Aa protein bound firmly to the BBMV that was prepared from the BCW midgut. The equilibrium dissociation constant (K_d) was calculated as 163 nm using the ligand binding and ligand blotting assays. This result suggests the high affinity and firm binding of the Vip3Aa toxin to the receptor sites in *A. ipsilon* BBMV. It also explains the high specificity and toxicity of the Vip3Aa to BCW. It was also noticed that the 48 kDa protein recognized and bound to the Vip3Aa under both reducing as well as nonreducing conditions. Moreover, if the BBMV preparation was carried out at room temperature without protease inhibitors, the degradation and loss of binding to Vip3Aa were observed when BBMV proteins were ligand blotted with unlabeled Vip3Aa toxin, and the binding complex was visualized using anti-Vip3Aa antiserum. Immunoprecipitation with the same antiserum and subsequent ligand blotting with Vip3Aa toxin also showed binding of the toxin to the 48 kDa protein from the BBMV of BCW midgut. All the above-mentioned evidence unequivocally demonstrates that the 48 kDa protein is a specific binding protein only for the Vip3Aa toxin. These findings strongly suggest that the insect host range specificity of the Bt toxins is highly dependent on receptor-mediated differences in the midgut of the insect as well as on the nature and type of the toxins (Maaty, 1999; Hua et al., 2001). On the other hand, several researchers have purified toxin binding proteins from BBMVs of insect midgut cells. In *M. sexta*, a 210-kDa cadherin-like protein and 120-kDa aglycosylphosphatidyle inositol-anchored (GPI) aminopeptidase N (APN) were identified, which bound Cry1Ab and Cry1Ac, respectively with (Knight et al., 1995) and 106 kDa aminopeptidase was identified as a Cry1C receptor in *M. sexta* (Luo et al., 1996, 1997). The Cry1Ac-binding APN proteins have also been purified from the BBMVs of *Lymantria dispar*, *Heliothis virescens,* and *Plutella xylostella* (Gill et al., 1995; Palma et al., 2014). However, few reports suggest that not all APN surface enzymes act as toxin binding proteins (Maaty, 1999; Assaeedi and Osman, 2017). From the current and earlier findings, it may

be inferred that the interference with the midgut function seems to be the common mechanism by which most effective insecticidal proteins, including the Vip3Aa toxin, kill their target insect.

5. Conclusion

The important findings of the present study are the identification, isolation, and characterization of a receptor molecule in BBMV of the BCW that bound specifically to the Vip3Aa toxin. However, the actual mechanism by which Vip3Aa toxin affects its target insect (BCW) has yet to be elucidated. The current piece of work scrutinized and narrowed down the spectrum of the mechanism by which Vip3Aa toxin kills BCW as an interaction between the toxin and the receptor molecule mediated by binding to the specific receptor. Moreover, the strength of binding determines the specificity of the toxin. It was hypothesized that the receptor molecule may aggregate to form a channel that transport nutrients into the insect gut and the Bt toxins probably interfere with this function either during channel formation or nutrient transport. The Vip3Aa is also envisioned to exert its toxicity in a similar manner.

Conflicts of interest

No conflict of interest exists.

Acknowledgment

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