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Agrotis segetum midgut putative receptor of *Bacillus thuringiensis* vegetative insecticidal protein Vip3Aa16 differs from that of Cry1Ac toxin

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ABSTRACT

Considering the fact that *Agrotis segetum* is one of the most pathogenic insects to vegetables and cereals in the world, particularly in Africa, the mode of action of Vip3Aa16 of *Bacillus thuringiensis* BUPM95 and Cry1Ac of the recombinant strain BNS3Cry-(pHT*cry1Ac*) has been examined in this crop pest. *A. segetum* proteases activated the Vip3Aa16 protoxin (90 kDa) yielding three bands of about 62, 45, 22 kDa and the activated form of the toxin was active against this pest with an LC_{50} of about 86 ng/cm^2. To be active against *A. segetum*, Cry1Ac protoxin was activated to three close bands of about 60–65 kDa. Homologous and heterologous competition binding experiments demonstrated that Vip3Aa16 bound specifically to brush border membrane vesicles (BBMV) prepared from *A. segetum* midgut and that it does not inhibit the binding of Cry1Ac. Moreover, BBMV protein blotting experiments showed that the receptor of Vip3Aa16 toxin in *A. segetum* midgut differs from that of Cry1Ac. In fact, the latter binds to a 120 kDa protein whereas the Vip3Aa16 binds to a 65 kDa putative receptor. The midgut histopathology of Vip3Aa16 fed larvae showed vacuolization of the cytoplasm, brush border membrane lysis, vesicle formation in the goblet cells and disintegration of the apical membrane. The distinct binding properties and the unique protein sequence of Vip3Aa16 support its use as a novel insecticidal agent to control the crop pest *A. segetum*.

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1. Introduction

Bacillus thuringiensis is a Gram positive bacterium with insecticidal activity due to its ability to synthesize large amounts of crystal proteins called delta-endotoxins or Cry proteins and it is the most widely used biocontrol agent (Schnepf et al., 1998). After ingestion by the insect, Cry proteins are solubilized and processed by the midgut juice to an active toxin core (Rouis et al., 2007). Active toxins bind to receptors on the brush border membrane and oligomerize to form pores that result in cell lysis, gut paralysis and larvae death (Van Rie et al., 1990). Although any variation in a step of the Cry toxin mode of action can potentially result in decreased susceptibility, alteration of toxin–receptor interactions is the most reported resistance mechanism (Ferré and Van Rie, 2002). Current "toxin-binding models" to explain these interactions are based on binding competition studies using radiolabeled toxins and insect midgut brush border membrane vesicles. On the basis of their toxin binding specificity, three populations of Cry1 toxin-binding sites (A, B, and C) were described in BBMV from *B. thuringiensis* susceptible insects (Pigott and Ellar, 2007). In addition to Cry, several strains of *B. thuringiensis* are known to produce vegetative insecticidal proteins (Vip). This class of insecticidal proteins includes the binary toxin Vip1-Vip2 active on Coleoptera and Vip3 toxin active on Lepidoptera (Warren, 1997). The secreted Vip protein is structurally, functionally and biochemically different from *B. thuringiensis* delta-endotoxins. One of the interesting features of the Vip proteins is that they do not share sequence homology with the known delta-endotoxins (Estruch et al., 1996). Shotkoshi and Chen (2003) showed that Vip3A protein is activated by proteolysis in the lepidopteran gut and that this activation, alone, was not considered sufficient for insect specificity. In fact, ion channel analysis demonstrated that the activated Vip3A toxin must bind to specific receptors in the BBMV in order to form stable ion channels and accomplish its insecticidal activity (Lee et al., 2006). In ligand blotting experiments with BBMV from the Lepidoptera *Prays oleae*, activated Cry1Ac and Vip3Aa16 bound to different molecules receptors (Abdelkefi-Mesrati et al., 2009). Histopathological observations indicate that Vip3A ingestion by susceptible insects such as the fall armyworm (*Spodoptera frugiperda*) causes gut paralysis at concentrations as low as 4 ng/cm^2 of diet and complete lysis of gut epithelial cells resulting in larvae death at concentrations above 40 ng/cm^2 (Yu et al., 1997). Compared to *B. thuringiensis* Cry toxins, Vip3A toxins have a mode of action that

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appears to be different especially in the binding step, and this supports its use as a novel insecticidal agent. *Agrotis segetum* is a pest that causes important damage on more than 50 plant crops including cereal grains (Esbjerg, 1989). BUPM95 is a *B. thuringiensis* subsp. *kurstaki* strain producing the Vip3Aa16 protoxin which is toxic against *P. oleae*, *Spodoptera exigua*, *S. frugiperda*, *Spodoptera littoralis* and *Ephestia kuehniella* (Abdelkefi-Mesrati et al., 2009, 2011a,b; Chakroun et al., 2012). In the present work, we studied the mode of action of Vip3Aa16 in the crop pest *A. segetum* in comparison with the Cry1Ac protoxin produced by the *B. thuringiensis* recombinant strain BNS3Cry-(pHT*cry*1Ac) (Tounsi et al., 2005).

2. Materials and methods

2.1. Bioassays

Bioassays were carried out using first instar larvae of *A. segetum*. Ten larvae were transferred to sterile petri dishes containing a 1 cm^2 of artificial diet. Protoxins concentrations were estimated using Bradford method (Bradford, 1976) with bovine serum albumin (BSA, Amersham) as a protein standard.

To test the efficiency of Cry1Ac against *A. segetum*, the diet was poured with crystal/spores mixture of the recombinant *B. thuringiensis* strain BNS3Cry-(pHT*cry1Ac*), expressing the Cry1Ac δ-endotoxin, previously constructed and investigated in our laboratory (Tounsi et al., 2005). Eight different concentrations (μg/cm^2) of Cry1Ac proteins were tested, ten larvae were used per concentration, and each test was done in triplicate. The acrystalliferous strain BNS3Cry- was used as a negative control and mortality was recorded after 3 days at 28 °C.

In the same conditions, five different concentrations (ng/cm^2) of purified Vip3Aa16, as reported in Section 2.4, were poured on the surface of the diet and incubated in the presence of 10 larvae (per toxin concentration). As negative control, 10 larvae were fed with artificial diet treated with buffer solution. The mortality was recorded after 6 days at 28 °C and results are the average of three repetitions.

Fifty and ninety percent lethal concentrations (LC_{50} and LC_{90}) were calculated from pooled raw data by probit analysis using programs written in the R language (Venables and Smith, 2004).

2.2. Preparation of whole larvae extract and proteolysis

Third instar *A. segetum* larvae were chilled on ice during 30 min. Then, in each 1.5 ml eppendorf tube, 10 whole larvae were collected in 100 μl MET buffer 1 (300 mM Mannitol, 5 mM EDTA, 20 mM Tris pH 7.2) as described by Dammak et al. (2010). After centrifugation for 10 min at 13,000*g*, supernatants were recovered and the protein concentration was determined by the method of Bradford (1976), using BSA as standard.

Purified Vip3Aa16 protoxins (20 μg) were mixed with soluble proteins from the larvae gut juice (3 μg) or with bovine pancreas trypsin (0.5 μg) (Amersham Pharmacia Biotech, France) in a final volume of 50 μl in PBS buffer (Phosphate buffer saline 1X). The mixtures were incubated at 37 °C with constant agitation for 1 h, 3 h, 5 h and overnight. Immunoblot analysis, with anti-Vip3Aa16 protein, was used for the proteolysis visualization.

Solubilized Cry1Ac proteins (20 μg) were mixed with soluble proteins from *A. segetum* larvae extracts (3 μg) or with trypsin in a final volume of 50 μl. The mixtures were incubated at 37 °C for 5–180 min and overnight. Samples were separated by sodium dodecyl sulfate 10% polyacrylamide gel electrophoresis (SDS–PAGE) and stained with Coomassie blue dye.

2.3. Preparation of Cry1Ac toxin

B. thuringiensis subsp. *kurstaki* BNS3Cry-(pHT*cry1Ac*) was grown in T3 medium (5 g peptone/1.5 g yeast extract/2 mg $MnSO_4$ $7H_2O$/20 mg $MgSO_4$ $7H_2O$/1.4 g Na_2HPO_4/1.2 g NaH_2PO_4 per liter) at 30 °C until cell lysis. The spore-crystal mixture was harvested and washed twice with 1 M NaCl, 0.01% Triton then twice with sterile water. Crystals were solubilized overnight at 37 °C in 50 mM Na_2CO_3, 10 mM DTT. Solubilized crystal protein was treated with trypsin (60:6-w/w) at 37 °C for 3 h. The 60-kDa Cry1Ac toxin was purified by Fast protein Liquid Chromatography (FPLC; Pharmacia) using a Mono Q anion exchange column equilibrated with 20 mM Tris–HCl, pH 8. Toxin was eluted with a linear gradient of 1 M NaCl in 20 mM Tris–HCl, pH 8. Activated pure toxin was diluted in bicarbonate buffer (40 mM, pH 8.6) in order to obtain a final concentration of about 1 mg/ml. Then, 40 μl of biotinylation substrate (ECL™ protein biotinylation module: Amersham Pharmacia Biotech, France) were added and the mixture was incubated at room temperature with constant agitation for 1 h. Purification of the biotinylated toxin was performed by loading the mixture on G25 column and elution using PBS (pH 7.5).

2.4. Preparation of Vip3Aa16 toxin

BUPM95 is a *B. thuringiensis* subsp. *kurstaki* strain producing the Vip3Aa16 protoxin (Abdelkefi-Mesrati et al., 2005). After *vip3Aa16* gene cloning, the corresponding protein was overexpressed in recombinant *E. coli* cells (Abdelkefi-Mesrati et al., 2009). Then, the cell pellet was suspended in PBS (pH 7.5) buffer and sonicated as described by Abdelkefi-Mesrati et al. (2009). The supernatant, collected after centrifugation and containing the Vip3Aa16 fused with six histidines, was loaded onto a His-Trap column (Amersham) preequilibrated with a binding buffer (PBS, 40 mM imidazole). After washing the column with 10 ml of the same binding buffer, the bound proteins were eluted using elution buffers containing increasing concentrations of imidazole in PBS.

For binding assays, purified Vip3Aa16 protoxins were activated by proteolysis using bovine pancreas trypsin (Amersham Pharmacia Biotech, France) with a 1:40 ratio of trypsin: protoxin and incubation at 37 °C for 2 h. Activated pure toxins were treated with biotin as described above.

2.5. *A. segetum* BBMV preparation and competition binding assays

Midguts were dissected from fifth instar (L_5) larvae, washed in ice-cold MET buffer 2 (250 mM Mannitol, 17 mM Tris–HCl, 5 mM EDTA [pH 7.5]) and kept at −80 until required. BBMV were prepared by the differential magnesium precipitation method (Wolfersberger et al., 1987) and the protein concentration was determined by the method of Bradford with BSA as a standard.

For homologous competition binding assay, biotinylated trypsinized Vip3Aa16 or Cry1Ac toxins (80 nM) were incubated with BBMV (80 μg) in PBS (pH 7.6) buffer for 1 h at room temperature in the absence or presence of unlabelled trypsinized toxins (50, 200 and 400 fold). For heterologous competition binding assay, biotinylated trypsinised Cry1Ac or Vip3Aa16 toxins (80 nM) were incubated with BBMV (80 μg) in PBS (pH 7.6) buffer for 1 h at room temperature in the absence or presence of unlabeled trypsinized Vip3Aa16 or Cry1Ac toxin (200 fold), respectively.

In both assays, the pellet obtained after centrifugation was suspended in 30 μl of PBS, loaded on SDS–PAGE and electrotransferred to a nitrocellulose membrane. The biotinylated proteins that were bound to BBMV were visualized by incubating with streptavidin- peroxidase conjugate (1:1500 dilutions) supplied in the

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