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Study of the *Bacillus thuringiensis* Vip3Aa16 histopathological effects and determination of its putative binding proteins in the midgut of *Spodoptera littoralis*

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ABSTRACT

The bacterium *Bacillus thuringiensis* produces, at the vegetative stage of its growth, Vip3A proteins with activity against a broad spectrum of lepidopteran insects. The Egyptian cotton leaf worm (*Spodoptera littoralis*) is an important agricultural pest that is susceptible to the Vip3Aa16 protein of *Bacillus thuringiensis kurstaki* strain BUPM95. The midgut histopathology of Vip3Aa fed larvae showed vacuolization of the cytoplasm, brush border membrane destruction, vesicle formation in the apical region and cellular disintegration. Biotinylated Vip3Aa toxin bound proteins of 55- and 100-kDa on blots of *S. littoralis* brush border membrane preparations. These binding proteins differ in molecular size from those recognized by Cry1C, one of the very few Cry proteins active against the polyphagous *S. littoralis*. This result supports the use of Vip3Aa16 proteins as insecticidal agent, especially in case of Cry-resistance management.
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1. Introduction

Bacillus thuringiensis is a Gram positive soil bacterium with insecticidal activity against many agriculturally important pests (Schnepf et al., 1998). This microorganism produces at the sporulation stage of its growth an intracellular crystal composed of one or more δ-endotoxins (Höfte and Whiteley, 1989). These Cry toxins are toxic to a variety of insect orders including Lepidoptera, Diptera and Coleoptera (Schnepf et al., 1998; De Maagd et al., 2001). However, recent reports suggest that many pests have developed resistance against some Cry toxins (Tabashnik et al., 2000).

B. thuringiensis protoxins activation by proteolysis in the larvae midgut (Lightwood et al., 2000; Lee et al., 2003; Rausell et al., 2004) and the binding of these toxins to the receptors located on the brush border membrane vesicles (BBMV) have been classified as key steps in their action against susceptible larvae (Schnepf et al., 1998). The alteration of one of these two steps may be a cause of resistance emergence (Schnepf et al., 1998; Ferré and Van Rie, 2002). In fact, field-evolved resistance of *Spodoptera frugiperda* to *B. thuringiensis* corn producing Cry1C and Cry1F occurred in the United States territory of Pierto Rico (Matten, 2007; Matten et al., 2008). Moreover, the insecticidal crystal proteins are not very effective in controlling some agronomically important pests such as the polyphagous *S. littoralis* which is relatively resistant to Cry1A (MacIntosh et al., 1990). In addition, *S. littoralis* developed resistance against Cry1C (Müller-Cohn et al., 1996), one of the very few δ-endotoxins active against this insect (Kalfon and de Barjac, 1985; Visser, 1990; Avisar et al., 2009).

To resolve these problems, screening programs are carried out by various groups to search *B. thuringiensis* toxins with new insecticidal spectra. Thus, a second family of *B. thuringiensis* toxins, represented by the Vegetative Insecticidal Proteins (Vip), has been identified (Estruch et al., 1996). It includes the binary toxin Vip1 and Vip2 with Coleopteran specificity and Vip3 with a wide activity spectrum against Lepidoptera (Estruch et al., 1996; Warren, 1997; Shi et al., 2004; Abdelkefi-Mesrati et al., 2005a). The Vip3 bear no similarity to Cry toxins. Moreover, Vip3 and Cry toxins recognize different receptors in the midgut of susceptible larvae (Lee et al., 2003; Abdelkefi-Mesrati et al., 2009). In *Prays oleae* midgut, Vip3A toxin recognizes a putative binding receptor of about 65 kDa which differs from that recognized by Cry1Ac (210 kDa) (Abdelkefi-Mesrati et al., 2009). These differences between these *B. thuringiensis* toxins are very important, and support the use of Vip3 toxins especially for pest Cry-resistance management.

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Numerous reports described the effect of different Cry toxins on the midgut of susceptible and nonsusceptible larvae (Bravo et al., 1992; Escriche et al., 1995; Rodrigo-Simon et al., 2006; Rouis et al., 2007, 2008). However, little is known about the histopathological effects of Vip3 toxins on larvae. In the present work, we investigate the activation process, the histopathological effects and the putative receptors of Vip3Aa16 in the midgut of the Egyptian cotton leaf worm, *S. littoralis*, which causes damage in many crops and is not very sensitive to *B. thuringiensis* Cry toxins.

2. Materials and methods

2.1. Over-expression of Vip3Aa16 and protein purification

BUPM95 is a *B. thuringiensis* subsp. *kurstaki* strain producing the Vip3Aa16 (Vip3LB) protoxin (Abdelkefi-Mesrati et al., 2005a). After *vip3Aa16* gene cloning, the corresponding protein was over-expressed in recombinant *E. coli* (Abdelkefi-Mesrati et al., 2009). Then the cell pellet was suspended in sonication buffer [PBS (1X, pH 7.5); 4 mM 4-(2-amino-ethyl)-benzenesulfonyl fluoride] and sonicated as described by Abdelkefi-Mesrati et al. (2009). The supernatant, collected after centrifugation and containing the Vip3Aa16 fused with six histidine, was loaded onto His-Trap column (Amersham) preequilibrated with a binding buffer (PBS 1X, imidazole 40 mM). 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 1X.

2.2. Bioassays

The diet, used in *S. littoralis* bioassays, is a derivative of the artificial semi-solid diet (Poitout and Bues, 1970) consisting of mixture of wheat germ, beer yeast, maize semolina, ascorbic acid, nipagine, agar and water. Overlay bioassays in this diet were carried out using seven concentrations of purified protoxin and 16 neonatal larvae for each concentration. The plates were incubated for 6 days in the insect culture room under controlled conditions of temperature 23 °C, relative humidity of 65% and a photoperiod of 18 h light and 6 h dark. A control set devoid of the protoxin but containing the buffer solution was maintained in the same conditions and used as negative control. The experiment was replicated three times. Larval mortality was scored after 6 days. Fifty and ninety percent lethal concentrations (LC_{50} and LC_{90}) were calculated by probit analysis using programs written in the R. language (Venables and Smith, 2004).

2.3. Gut juice preparation, proteolysis assays and SDS–PAGE

Gut juice was collected by inducing regurgitation. Fifth instar larvae of *S. littoralis* were given shocks with an electric current at 20–30 V and the vomited gut juices were collected and recovered as a supernatant by a centrifugation at 13,000*g* for 10 min at 4 °C. The concentration of the gut juice extract was determined by the method of Bradford (Bradford, 1976), using bovine serum albumin as a standard. The amount of midgut juice collected was approximately 80 μg protein/larvae.

Purified Vip3Aa16 protoxins (20 μg) were mixed with soluble proteins in 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 using PBS 1X buffer. The mixtures were incubated at 37 °C with constant agitation for 2 h. Then, the proteolysis was stopped by addition of 0.1 mM PMSF final concentration. Samples were separated by sodium dodecyl sulfate 10% polyacrylamide gel electrophoresis (SDS–PAGE) and stained with Coomassie blue dye.

2.4. Preparation and sectioning of insect tissues

After exposure of 3 days to the Vip3Aa16 protoxins (100 ng/cm^2 of diet), 3rd instars *S. littoralis* larvae were chilled on ice during 30 min. The guts were then excised and placed in 10% formol then dehydrated in increasing ethanol concentrations, rinsed in 100% toluene, and embedded in paraffin wax. Sections (5 μm) were placed in carriers loaded with a mix of 1.5% egg albumin and 3% glycerol in distilled water. For histopathological localization of the effects of toxins, the sections already de-paraffinated in 100% toluene were stained with hematoxylineosin as reported by Ruiz et al. (2004).

2.5. S. littoralis BBMV preparation and binding assays

Midguts (3–5 mg) were dissected from last-instar (L5) larvae, washed in ice-cold MET buffer (250 mM Mannitol, 17 mM Tris–HCl, 5 mM EGTA [pH 7.5]), frozen in liquid nitrogen, 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 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 diluted in bicarbonate buffer (40 mM) 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 operated by loading the mixture on G25 column and elution using PBS 1X, pH 7.5.

For competition experiments, biotinylated trypsinized Vip3Aa16 toxin (40 nM) was incubated with BBMV (40 μg) in PBS (1X, pH 7.6) buffer for 1 h at 28 °C in the absence or presence of unlabelled trypsinized toxins (50, 100, 400 and 1000 fold). Subsequently, unbound toxin was removed by centrifugation (10 min at 14,000*g*) and BBMV were washed with the same buffer. Then, BBMV were suspended in 20 μl of PBS (1X), loaded in a SDS–PAGE and electrotransferred to a nitrocellulose membrane. The biotinylated proteins that were bound to BBMV were visualized by incubating with streptavidin-peroxydase conjugate (1:1500 dilutions) supplied in ECL protein biotinylation module for 1 h, followed by three 5-min washing cycles. Binding was visualized using luminol according to manufacturer's protocol (ECL; Amersham Pharmacia Biotech, France).

2.6. Ligand-blotting

BBMV (40 μg) prepared from *S. littoralis* were separated via SDS–PAGE and blotted onto a nitrocellulose membrane by electrotransfer (Bio-Rad, France). The membranes were blocked with 5% milk for 1 h then reacted with biotinylated trypsinized toxins (40 nM) for 2 h at room temperature. After three 5-min washing cycles, membranes were incubated with streptavidin-peroxidase conjugate (1:1500 dilutions) supplied in ECL protein biotinylation module for 1 h, followed by washing as described above. Binding was visualized using luminol according to manufacturer's protocol (ECL; Amersham Pharmacia Biotech, France).

3. Results

3.1. Bioassays

When tested against first instars larvae of *S. littoralis*, Vip3Aa16 showed toxicity with an LC_{50} of 305 ng/cm^2 with 95% confidence

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