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Involvement of the processing step in the susceptibility/tolerance of two lepidopteran larvae to *Bacillus thuringiensis* Cry1Aa toxin

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ABSTRACT

Bacillus thuringiensis (Bt) Cry1A toxins are known for their effectiveness against lepidopteran insects. In this study, the entomopathogenic activity of Cry1Aa was investigated against two lepidopteran larvae causing serious threat to various crops, *Spodoptera littoralis* and *Tuta absoluta*. Contrarily to *S. littoralis*, which showed low susceptibility to Cry1Aa (40% mortality with 1 μg/cm^2), *T. absoluta* was very sensitive to this delta-endotoxin (LC_{50} of 95.8 ng/cm^2). The different steps in the mode of action of this toxin on the two larvae were studied with the aim to understand the origin of their difference of susceptibility. Activation of the 130 kDa Cry1Aa protein by *T. absoluta* larvae juice generated a 65 kDa active toxin, whereas *S. littoralis* gut juice led to a complete degradation of the protoxin. The study of the interaction of the brush border membrane vesicles (BBMV) with purified biotinylated Cry1Aa toxin revealed six and seven toxin binding proteins in *T. absoluta* and *S. littoralis* BBMV, respectively. Midgut histopathology of Cry1Aa fed larvae demonstrated approximately similar damage caused by the toxin in the two larvae midguts. These results suggest that the activation step was strongly involved in the difference of susceptibility of the two larvae to Cry1Aa.

1. Introduction

Bacillus thuringiensis produces during its sporulation phase of growth insecticidal proteins which are accumulated into regular crystal inclusions. The insecticidal Cry proteins are critical components of Bt biopesticides which are widely used as bioinsecticides since they are specific to target insects and are generally considered safe for mammals and birds. In spite of their different insect specificity (Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Orthoptera and Mallophaga orders) [1], Cry proteins share similar structure and mode of action. Three-dimensional resolved structures of some Cry toxins showed that the majority of *B. thuringiensis* toxins are composed of three domains [2] despite their different structures. The primary action of *B. thuringiensis* toxins is to lyse midgut epithelial cells by inserting into the target membrane and forming pores [3,4]. But to become active, crystal inclusions are dissolved in the alkaline environment of the gut, and the solubilized protoxins are cleaved by midgut proteases yielding 60–70 kDa protease resistant proteins [5]. This processing involves the removal of 600 amino acids corresponding to the C-terminal end for large Cry proteins and a peptide from the N-terminal end (20–60 amino acids). Solubilization and activation of Cry proteins are followed by another important step determining the specificity to insects. In fact the activated toxin interacts with specific membrane receptors on the apical membrane of midgut cells [6]. This is the most significant event since subsequent to binding to receptors, pre-pore oligomeric structure formation is initialized followed by insertion into the apical membrane and leading to epithelial cells lysis [7].

Toxicity of Cry proteins to lepidoptera was largely investigated and several species showed different susceptibilities to the same toxin [8]. Some factors such as variability of midgut proteases [9,10] and variability in cell membrane receptors have been proposed to account for this toxin sensitivity.

In the present paper, we studied the susceptibility of two lepidopteran species, the tomato leaf miner *Tuta absoluta* (Gelechiidae family) and the Egyptian cotton leaf worm *Spodoptera littoralis* (Noctuidae family), towards Cry1Aa11 that differs from the prototype Cry1Aa1 in two amino acids [11]. We investigated also the involvement of the different steps of the mode of action of this delta-endotoxin in the difference of susceptibility of the two studied insects.

2. Materials and methods:

2.1. Preparation of Cry1Aa protoxins

Recombinant cells DH5α(pBScry1Aa) of *E. coli* transformed with plasmid pBS*cry1Aa11* [11] were grown in LB medium supplemented with ampicillin (60 μg/ml) at 37 °C in a shaking incubator (200 rpm) for 16 h. Pre-culture was used to inoculate 200 ml of LB media containing ampicillin (60 μg/ml). Cultures were agitated until the OD_{600}

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reached 0.6. Cry protein expression was then induced by the addition of IPTG at a final concentration of 0.4 mM. The culture was maintained at 37 °C for four additional hours with agitation (200 rpm). Protein expression was verified by observing inclusion bodies by light microscopy. After harvesting by centrifugation, cell pellets were washed with cool water, pelleted by centrifugation, and frozen at −20 °C. Then the cell pellet was suspended in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4(7H_2O)$, 1.8 mM KH_2PO_4) and sonicated as described by Dammak et al. [12]. Inclusion bodies were harvested by centrifugation for 10 min at 6000 rpm, and washed twice with 1 M NaCl cold solution, then four times with cold distilled water. Inclusion bodies containing Cry1Aa δ-endotoxins were solubilized in 50 mM sodium carbonate buffer (pH 9.5).

2.2. Insects

The *T. absoluta* larvae used in the bioassays were reared, in the laboratory of Entomology (CRRHAB), on tomato plants inside insect proof cages and maintained under controlled environment conditions (28 °C, 75 ± 5% relative humidity and 12 h photoperiod).

S. littoralis larvae were reared as described by BenFarhat-Touzri et al. [13] on an artificial semi-solid diet [14], in climatic room at 23 °C, 65% relative humidity and with 16:8 light/dark photoperiods.

2.3. Bioassays

Bioassays were carried out using washed Cry1Aa inclusion bodies. Proteins concentrations were estimated using Bradford method [15] with bovine serum albumin (BSA, Amersham) as a protein standard.

Bioassays were carried out using first instars larvae of *S. littoralis*. Ten larvae were transferred to sterile petri dishes containing a 1 cm^3 of artificial diet impregnated with inclusions of Cry proteins at desired concentrations. 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.

For *T. absoluta* bioassay, first instar larvae were used. Tomato leaves were immersed in different Cry1Aa inclusions concentrations. The leaves were subsequently placed in Petri dishes. Ten larvae were placed in each petri dish, which was placed at 25 ± 0.5 °C, 75 ± 5% relative humidity and 12 h photoperiod. Mortality was recorded after 5 days.

A control set devoid of the inclusions but containing the buffer solution was maintained in the same conditions of each test and used as negative control. Experiments were replicated three times. Larval mortality was scored during 6 days. Fifty percent lethal concentrations (LC50) were calculated by probit analysis using programs written in the R. language [16].

2.4. Gut juice preparation and proteolysis assays

Tuta absoluta 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 (300 mM Mannitol, 5 mM EDTA, 20 mM Tris pH 7.2) as described by Dammak et al. [12].

The gut juice of *S. littoralis* is collected by regurgitation induced by applying an electric current at 20–30 V to the larvae. After centrifugation at 13,000 g for 10 min at 4 °C, the supernatant was recovered.

Protein concentration in the gut extracts was determined by the method of Bradford [15]. Solubilized inclusion bodies (50 μg) were mixed with soluble proteins in *T. absoluta* larvae extracts, *S. littoralis gut extract*, or with bovine pancreas trypsin (Amersham Pharmacia Biotech, France) in a final volume of 50 μl. The mixtures were incubated at 30 °C for 5–180 min. Samples were separated by sodium dodecyl sulfate 10% polyacrylamide gel electrophoresis (SDS–PAGE) and stained with Coomassie blue dye.

2.5. Toxin purification

Solubilized protoxins were digested with trypsin at a trypsin/protoxin ratio 1:20 (*w*/*w*) at 37 °C for 2 h with agitation then dialyzed against Na_2CO_3 20 mM, pH 9.6 and concentrated with PEG (Poly Ethylene Glycol). Activated toxins were purified by FPLC (Fast Protein Liquid Chromatography) using a Mono Q anion exchange column equilibrated with 20 mM Na_2CO_3, pH 9.6. Toxin was eluted with a linear gradient of 1 M NaCl in 20 mM Na_2CO_3, pH 9.6. Eluted fractions containing the active toxin were pooled and separated by electrophoresis in 10% polyacrylamide gel and purified Cry1Aa toxin was used for binding assays.

2.6. Cry1Aa labeling

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 1× (pH 7.5). Protein concentration was estimated by measuring the optical density of the eluted fractions at 280 nm.

2.7. BBMV collection and preparation

BBMVs were extracted according to the method described by Wolfersberger et al. [17]. Intestines were dissected from last-instar *S. littoralis* and *T. absoluta* larvae, washed in cold MET buffer (250 mM Mannitol, 17 mM Tris–HCl and 5 mM EGTA pH 7.5), frozen in liquid nitrogen and stored at −80 °C. One g of larval midgut was homogenized in MET buffer by a potter then the homogenate was diluted with an equal volume of ice-cold 24 mM $MgCl_2$. A low speed centrifugation (4500 rpm for 15 min at 4 °C) was applied and the supernatant from the initial centrifugation was further centrifuged at 13,000 rpm for 45 min at 4 °C then the resulting pellet was suspended in the MET buffer, diluted with an equal volume of $MgCl_2$ and centrifuged again at 4500 rpm for 15 min at 4 °C. The supernatant was recovered and also centrifuged at 13 000 rpm for 45 min at 4 °C. The resulting pellet (corresponding to the BBMVs preparation) was suspended in MET buffer (0.5×), flash-frozen in liquid nitrogen and stored at −80 °C.

2.8. BBMV ligand-blotting assay

Ligand-blotting was performed in accordance with the procedures reported by Abdelkefi-Mesrati et al. [18]. BBMV (40 μg) prepared from *S. littoralis* and *T. absoluta* were separated in 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 trypsinized and biotinylated Cry1Aa (40 nM) for 2 h at room temperature. Membranes were then, incubated for 1 h with peroxidase (HRP)-conjugated streptavidin (1:1500 dilution) supplied in ECL protein biotinylation module. Binding was visualized using luminol according to manufacturer's protocol (ECL; Amersham Pharmacia Biotech, France).

2.9. Preparation and sectioning of insect tissues

After exposure to the *B. thuringiensis* Cry1Aa toxin, killed larvae were placed in 10% formol then dehydrated in increasing ethanol concentrations, rinsed in 100% toluene, and embedded in paraffin wax. Sectioning of larvae tissues and preparation of slides were accomplished as described by Abdelkafi-Mesrati et al. [18].