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Histopathological effects and determination of the putative receptor of *Bacillus thuringiensis* Cry1Da toxin in *Spodoptera littoralis* midgut

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ABSTRACT

Bacillus thuringiensis subsp. *aizawai* strain HD133, known by its effectiveness against *Spodoptera* species, produces many insecticidal proteins including Cry1Ab, Cry1Ca and Cry1Da. In the present study, the insecticidal activity of Cry1Da against *Spodoptera littoralis* was investigated. It showed toxicity with an LC_{50} of 224.4 ng/cm^2 with 95% confidence limits of (178.61–270.19) and an LC_{90} of 467.77 ng/cm^2 with 95% confidence limits of (392.89–542.65). The midgut histopathology of Cry1Da fed larvae showed vesicle formation in the apical region, vacuolization and destruction of epithelial cells. Biotinylated-activated Cry1Da toxin bound protein of about 65 kDa on blots of *S. littoralis* brush border membrane preparations. This putative receptor differs in molecular size from those recognized by Cry1C and Vip3A which are active against this polyphagous insect. This difference in midgut receptors strongly supports the use of Cry1Da as insecticidal agent, particularly in case of Cry and/or Vip-resistance management.

1. Introduction

Bacillus thuringiensis, a Gram-positive bacterium that produces regular parasporal crystals formed by one or several proteins (known as Cry proteins or δ-endotoxins) during the sporulation phase, is the most widely used microbiological insecticide in the world (Kaur, 2000; Qiu et al., 2003). The insecticidal activity of *B. thuringiensis* is due to the parasporal crystals which are specific and lethal to larvae in orders Lepidoptera, Diptera, Coleoptera, and Orthoptera. However, recent studies reported the emergence of resistance to some Cry toxins in pest populations (Wirth et al., 2012). Understanding the mechanisms of resistance to *B. thuringiensis* toxins could be helpful for management of rapid onset of insect resistance. Two steps in the mode of action of the Cry proteins are considered key steps in their toxicity against susceptible larvae, the alteration in the proteolytic activation of the protoxin and the loss of binding of these proteins to receptors located in the brush border membrane. In fact, both have been described as mechanisms of resistance to these toxins (Ferré and Van Rie, 2002; Ferré et al., 2008).

Although *B. thuringiensis* Cry toxins are effective insecticidal proteins, several agronomically important insects such as black cut worm, *Agrotis ipsilon* (Abdullah et al., 2009) and *Spodoptera* species are less sensitive to their action. For example, the Egyptian cotton leaf worm, *Spodoptera littoralis*, is not susceptible to most of the δ-endotoxins (Sadek, 2007).

Cry1Da toxins are known by their insecticidal activities against a large spectrum of lepidoptera especially that of *Spodoptera* species. In fact, they are classified in the category of the very few δ-endotoxins active against these polyphagous insects (Hernández-Martínez et al., 2008). In addition of its insecticidal activity, Cry1Da is endowed with an antimicrobial activity (Yudina et al., 2006). Although its efficiency against *Spodoptera* species (Porcar et al., 2000), none work was focused on the interaction of this toxin with its receptor. However, numerous reports identified the receptors of different toxins in this pest midgut. In fact, Sanchis and Ellar (1993) identified two receptors of 120 kDa and 40 kDa specific of Cry1Ac and Cry1Ca toxins in *S. littoralis*, respectively. In the present work, we studied the histopathological effects, and determined, for the first time, the molecular weight of the putative receptor of Cry1Da in *S. littoralis* midgut.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture media

HD133, a *B. thuringiensis* strain subsp. *aizawai*, (kindly provided by Pr. Raj Kamal Bhatnagar, ICGEB, India) was used in the present study to amplify the *cry1Da* gene. *Escherichia coli* strain Top10 was

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used as the cloning host and was grown in Luria Bertani (LB) medium at 37 °C. Plasmid pGEM-T Easy (Promega) was used as the cloning vector. *E. coli* strain Top10 containing the plasmid pGEM-T Easy, named Top(pGEM-T easy), was used as negative control in *cry1Da* expression and bioassay experiments. Plasmid pG-*cry1Da* and strain Top(pG*cry1Da*) were constructed in the present study.

2.2. Insects

S. littoralis larvae were reared with artificial diet in climatic room at 23 °C, 65% relative humidity and with 16:8 light/dark photoperiods. The diet 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, wessan salt, nipagine, sorbic acid, agar and water. Diet was poured into rearing cup, allowed to cool thoroughly and then stored in the refrigerator in plastic bags for up to 7 days. The adults were fed by 10% sucrose solution.

2.3. Cloning of insecticidal crystal protein gene

Plasmid DNA was extracted from *B. thuringiensis* subsp. *aizawai* HD133 using the alkali lysis method including the step involving lysozyme treatment (Sambrook et al., 1989). The Cry1Da-encoding gene was amplified by PCR using this DNA as template, Taq DNA polymerase (Amersham) and primers DF1D (5′-TATGGCTA GCATG GAAATAAATAATCAAAACC-3′) and DF1Dinv (5′-CTATACCCGGGT GGTCCTA T-3′). The amplified fragment was purified and ligated into pGEM-T plasmid vector (Promega) and the resulting plasmid named pG-*cry1Da* was transformed in *E. coli* strain Top10.

2.4. Expression of cry1Da in E. coli

Recombinant clone carrying the *cry1Da* gene, named Top(pG-*cry1Da*), was grown overnight at 37 °C in LB medium supplemented with 100 μg/ml of ampicillin. Then, a fresh culture with an initial absorbance at 600 nm of 0.05 was prepared. When the optical density of the culture reached 0.6 at 600 nm, *cry1Da* gene expression was induced by isopropyl thiogalactoside (IPTG) 0.4 mM, and cells were grown for further 4 h at 37 °C. After centrifugation, the weight of cell pellet was estimated then incubated for 30 min at −20 °C. Then, 3 ml of lyses buffer/g of *E. coli* cells were added and mixed vigorously. After adding 800 μg of lyzozymes/g of *E. coli* cells, the mixture was incubated for 20 min at room temperature then supplemented with 4 mg of Sodium desoxycholate/g of *E. coli* cells. After incubation for 10 min at 37 °C, 200 μl of DNase ((1 mg/ml)/g of *E. coli* cells) were added and the lysate was incubated for further 2 h at 37 °C. The suspension was then centrifuged for 20 min at 18,000 rpm, and then rinsed twice with wash buffer and twice with PBS 1X for 10 min. The resulting inclusions bodies contained the Cry1Da proteins.

2.5. Proteolysis assay and toxin purification

After *cry1Da* expression in *E. coli*, inclusion bodies containing Cry1Da δ-endotoxins were solubilized in 50 mM sodium carbonate buffer (pH 9.5) containing 10 mM dithiothreitol, at 37 °C for 3 h with agitation. After centrifugation for 15 min at 18,000 rpm, the solubilized protoxins were dialyzed against 20 mM Tris–HCl (pH 6.8) and digested with trypsin at a trypsin/protoxin ratio 1:10 (by mass) at 37 °C for 3 h with agitation. Finally, activated toxins were purified by FPLC (Fast Protein Liquid Chromatography Mono Q exchange column). Chromatographic separations were achieved with a linear gradient of *B* in *A* (*A* = 50 mM Tris–HCl, pH7.4; *B* = Buffer *A* containing 1 M NaCl). Proteins were eluted between 20–50 mM *B* gradient at a flow rate of 1 ml/min (data not shown). Eluted fractions containing the 65 kDa protein were pooled and separated by electrophoresis in 10% polyacrylamide gel and purified Cry1Da toxins were used for binding assays. Cry1Da concentration was measured with the Bradford assay, using bovine serum albumin as a standard (Bradford, 1967).

2.6. Bioassays

Bioassays were carried out using second instars larvae of *S. littoralis*. Overlay bioassays in the artificial diet was accomplished using seven concentrations of Cry1Da protoxin and 16 neonatal larvae for each concentration. The plates were incubated for 6 days in the insect culture room under the same controlled conditions of temperature, relative humidity and photoperiod as those used for larvae rearing. As negative control, 16 larvae were fed with diet supplemented with Top(pGEM-T easy) protein extracts. 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).

2.7. Cry1Da labeling

Trypsin-activated pure Cry1Da 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.

2.8. S. littoralis tissue preparation and BBMV preparation

Midguts (3–5 mg) were dissected from last-instar (L5) larvae, washed in ice-cold METbuffer (250 mM Mannitol, 17 mM Tris–HCl, 5 mM EGTA [pH 7.5]), frozen in liquid nitrogen, and kept at −80 °C until required. Using the differential magnesium precipitation method (Wolfersberger et al., 1987), *S. littoralis* brush border membrane vesicles (BBMV) were prepared and protein concentration was determined by the method of Bradford with BSA as a standard.

2.9. Ligand blotting

After separation by SDS–PAGE, *S. littoralis* brush border membrane proteins were 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 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).

2.10. Homologous competition experiments

Biotinylated-trysinized Cry1Da toxins were incubated with 40 μg of BBMV in phosphate-buffered saline, pH 7.6, for 1 h in the absence or presence of an excess of unlabeled toxins. Subsequently, the sample was centrifuged for 10 min at 14,000*g* to separate bound from free toxins, the pellets containing the bound toxins were washed twice with 500 μl of the same buffer then suspended in 20 μl of phosphate-buffered saline. Samples were boiled for 5 min, loaded onto an SDS–PAGE gel, and electrotransferred to a