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Toxicity and mode of action of insecticidal Cry1A proteins from *Bacillus thuringiensis* in an insect cell line, CF-1

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ABSTRACT

Bacillus thuringiensis Cry toxins are insecticidal proteins used to control insect pests. The interaction of Cry toxins with the midgut of susceptible insects is a dynamic process involving activation of the toxin, binding to midgut receptors in the apical epithelium and conformational changes in the toxin molecule, leading to pore formation and cell lysis. An understanding of the molecular events underlying toxin mode of action is essential for the continued use of Cry toxins. In this work, we examined the mechanism of action of Cry1A toxins in the lepidopteran cell line CF-1, using native Cry1Ab and mutant forms of this protein that interfer with different steps in the mechanism of action, specifically, receptor binding, oligomerization or pore formation. These mutants lost activity against both *Manduca sexta* larvae and CF-1 cells. We also analyzed a mutation created in domain I of Cry1Ab, in which helix α-1 and part of helix α-2 were deleted (Cry1AbMod). Cry1AbMod is able to oligomerize in the absence of toxin receptors, and although it shows reduced activity against some susceptible insects, it kills insect pests that have developed resistance to native Cry1Ab. Cry1AbMod showed enhanced toxicity to CF-1, suggesting that oligomerization of native Cry1Ab may be a limiting step in its activity against CF-1 cells. The toxicity of Cry1Ac and Cry1AcMod were also analyzed. Our results suggest that some of the steps in the mode of action of Cry1A toxins are conserved *in vivo* in insect midgut cells and *in vitro* in an established cell line, CF-1.

1. Introduction

Many subspecies of *Bacillus thuringiensis* (Bt) produce delta-endotoxin crystal (Cry) proteins during sporulation and are insecticidal when ingested by susceptible hosts. Lepidopteran larvae are particularly vulnerable to Cry1A toxins. The proteins are formed as 130 kDa protoxins and are proteolytically activated by the insect's midgut enzymes, resulting in the production of a protease-resistant, 60 kDa fragment that is capable of destroying insect midgut cells [28].

Bt toxins have been used in the control of insect pests for more than 50 years, either in the form of commercial sprays or genetically modified crops [5]. Cry toxins typically display a relatively narrow spectrum of activity against different insect hosts. Transgenic crops expressing various Cry toxins now cover more than 160 million hectares worldwide, resulting in effective protection against insect damage and reducing the reliance on chemical insecticides [15].

The mechanism of action of Cry1A toxins in insects is a complex process that involves contact with toxin receptors in the midgut epithelium, triggering conformational changes in the toxin molecules necessary for oligomerization and insertion into the cell membrane to form pores, eventually killing the larvae [28,39]. Trypsin-activated monomeric forms of Cry1A toxins bind to different receptors, such as aminopeptidase N (APN), alkaline phosphatase (ALP) and cadherin, located in the apical membrane of insect midgut cells [2,20,28,31,42]. It has been proposed that binding with cadherin facilitates the cleavage of helix α-1 in domain I, and the assembly of an oligomeric structure of the toxin [4,10]. Cry1AMod toxins, engineered forms of native Cry1A toxins, in which helix α-1 and part of helix α-2 have been removed, readily form oligomers without the cadherin interaction when the toxin is

Abbreviations: Cry, crystal proteins; Bt, *Bacillus thuringiensis*; GPI, glycosyl phosphatidyl-inositol; APN, aminopeptidase N; ALP, alkaline phosphatase; FBS, fetal bovine serum; LDH, lactate dehydrogenase; BBMV, brush border membrane vesicles; LC_{50}, median lethal concentration; ED_{50}, median effective dose; M_r, molecular mass.

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activated, in contrast to Cry1A toxins, which do not oligomerize in the absence of cadherin [26,36]. Cry1AMod toxins can kill different species of lepidopteran insects, including *Pectinophora gossypiella* and *Manduca sexta*, that are resistant or tolerant to the native toxin as a result of mutations in the cadherin receptor or cadherin silencing by RNAi [32,37], although they show reduced toxicity compared with their corresponding native toxins against susceptible insect populations [32,36,37]. Cry1A oligomerization appears to be a key limiting step in the mode of action of the native toxins [37].

Pore formation of Cry toxins in the insect midgut causes numerous intracellular changes, with cell lysis occurring from a combination of alkalization [14] and colloid osmotic shock [21], leading to insect death. It has been shown that helix α-3 of domain I is involved in toxin oligomerization [17,40] and helix α-4 in membrane insertion and pore formation [9,34,41]. Mutations in helices α-3 or α-4 thereby inhibit pore formation and such mutants completely lose toxicity against their target insects [9,17,34,40,41]. In addition, specific mutations in domains II and III that prevent toxin interaction with midgut receptors also prevent toxicity [2,27,35].

The insecticidal Cry1A toxins are non-toxic against mammalian epithelial cell lines, fibroblasts and lymphocytes [38]. However, some insect cell lines are susceptible to Cry toxins, but show distinct differences in sensitivity [11,22,23]. CF-1, an established cell line from larvae of the spruce budworm, *Choristoneura fumiferana*, is sensitive to certain Cry1A toxins and not to others [11]. Other insect cell lines from *C. fumiferana*, such as CF-200 from larval midgut and CF-70 from pupal ovary, show no sensitivity to Cry1A toxins [11]. Activity spectra of Bt toxins against insect cell lines have been the subject of numerous reports [7,11,18,19,22]. The intracellular changes that occur in cultured insect cells exposed to Bt toxins include enhanced ion channel activity, Ca^{2+} release, uncoupling of oxidative phosphorylation and swelling [12,25].

In this work we analyzed the susceptibility of CF-1 cells to Cry1A mutant toxins previously shown to affect specific steps of the intoxication process in the insect, namely, receptor binding, oligomerization and pore formation. Our data show that the effects of the mutant toxins are the same in CF-1 as in susceptible insects, indicating that some steps in the mode of action of Cry1A toxins are conserved in the CF-1 cell line. We also analyzed the toxicity of Cry1AMod toxins, which showed higher mortality than their corresponding native toxins, suggesting that oligomerization of native toxins may be a limiting step in their activity against CF-1 cells.

2. Materials and methods

2.1. Purification of Cry1Ab native and mutant toxins

Bt HD73 strain harboring Cry1Ac was grown in HCT sporulation medium [26]. The construction of the different *cry1Ab* mutants in pHT315, such as G439D [34,35], R99E [17], E129K [34], F371A [2,33], as well as Cry1AbMod and Cry1AcMod [36], were previously described. The Bt transformant strains were grown at 30 °C in the same medium supplemented with 10 μg/ml erythromycin until sporulation was completed. Crystal inclusions were observed under phase contrast microscopy. The harvested product was washed twice in 300 mM NaCl, 10 mM EDTA with centrifugation at 10,000 rpm, 4 °C for 10 min. The crystals were purified in discontinuous sucrose gradients [38], followed by solubilization in 100 mM Na_2CO_3, 0.2% β-mercaptoethanol, pH 10.5. The pH was reduced to 8.5 by adding an equal volume of 1 M Tris buffer, pH 8.5. Monomeric activated toxins were obtained by trypsin digestion in a mass ratio of 1:50 (trypsin/toxin) for 2 h at 37 °C. Phenylmethylsulfonyl fluoride (PMSF) at 1 mM final concentration was added to stop proteolysis. An Amicon Ultra centrifugal filter unit (Millipore) was used to transfer the activated toxins to PBS buffer, pH 8.5, adjusted to 340 mOsm with KCl to be isosmotic with the medium used for the insect cells. Protein concentration was determined by the Bradford assay, using bovine serum albumin as standard.

2.2. Toxicity assays against Manduca sexta larvae

Bioassays were performed with *M. sexta* neonate larvae by the surface contamination method. Toxin solution was poured on the diet surface and allowed to dry. Larvae were placed on the dried surface and mortality monitored after 7 days. The median lethal concentration (LC_{50}) of the Cry1A proteins and mutants against *M. sexta* was estimated by probit analysis in four independent assays (POLO-PC, LeOra Software).

2.3. Insect cell line

IPRI CF-1, an established cell line derived from minced neonates of the spruce budworm (*C. fumiferana* Clemens) [13] was obtained as a seed culture from the Great Lakes Forestry Centre, Sault Ste. Marie, Canada, and grown at UNAM in Grace's insect cell culture medium, pH 6.8, supplemented with 10% fetal bovine serum (FBS) and 0.25% tryptose. The cells were grown at 27 °C in 25 cm^2 tissue culture flasks and transferred to fresh medium every 3–4 days. Cells were counted with a haemocytometer counting chamber.

2.4. Toxicity assays against CF-1

Toxicity was determined with the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI), which measures the release of lactate dehydrogenase (LDH) from cells that have undergone lysis. Cells were suspended in Grace's medium supplemented with 2.5% FBS at a concentration of 6×10^5 cells/ml and 0.1 ml aliquots transferred to 96-well ELISA plates (Costar 3596, Corning, Corning, NY). The plates were incubated for 3 h at 27 °C. Twenty μl of the activated Cry proteins at different concentrations was added to the cells and the plates incubated for 1 h at 27 °C, with agitation at 300 rpm (Eppendorf Thermomixer). For a positive control, 10 μl of 0.8% Triton X-100 was added, and for a negative control, the same volume of PBS buffer was used. Fifty microliters of medium was transferred to a clean ELISA plate to which 50 μl of assay solution was added and incubated for 30 min at room temperature. Fifty microliters of stop solution was added per well and absorbance at 490 nm recorded with a microplate reader (Molecular Devices, Sunnyvale, CA). Four replicates per treatment were performed and the median effective dose (ED_{50}) estimated by Hill's three parameters equation.

The toxicity of Cry toxins to CF-1 was also analyzed in the presence of 10 mM EDTA or 10 mM EGTA at pH 7.0. Cells were treated with a single dose of toxin as follows: 305 nM Cry1Ab, 100 nM Cry1AbMod, 21 nM Cry1Ac or 63 nM Cry1AcMod and the response recorded by LDH release assay. For these assays, cells were suspended in Solution M-1 that has an equivalent salt composition of Grace's medium but containes reduced amounts of Mg^{2+}: 54.7 mM KCl, 70 mM NaCl, 7 mM $MgCl_2$, 7 mM $CaCl_2$, 11.3 mM CH_3NaO_3S, 25 mM sucrose, and 10 mM HEPES, pH 6.8. The pH of the medium was readjusted to pH 7.0 after addition of the chelator. Osmolality in these solutions was maintained at 340 mOsm. Viability of cells incubated in these solutions for 6 h at 27 °C was not affected (not shown). Four replicates were performed. The responses were normalized to that of the toxin that gave the strongest response, which was assigned a relative value of 1. Standard deviations of the data sets were calculated and error bars included in the figures. These data were analyzed by a two-way ANOVA, with significant differences $P < 0.001$, using SigmaPlot (version 10, Systat Software Inc).

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