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Bacillus thuringiensis toxin, Cry1C interacts with 128HLHFHLP134 region of aminopeptidase N of agricultural pest, *Spodoptera litura*

Ravinder Kaur[a,1], Anil Sharma[a,1], Dinesh Gupta[b], Mridul Kalita[b], Raj K. Bhatnagar[a,*]

[a] *Insect Resistance Group, International Center for Genetic Engineering and Biotechnology, New Delhi 110067, India*
[b] *Bioinformatics Group, International Center for Genetic Engineering and Biotechnology, New Delhi 110067, India*

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ABSTRACT

We modeled Cry1C toxin and its Aminopeptidase-N receptor and *in silico* docking analysis was performed. Further, we utilized biopanning against Cry1C followed by blocking assays and mutagenesis analysis to identify the binding epitope of SlAPN. We have identified a putative SlAPN binding region, APN-CRY (128HLHFHLP134). A derivative of SlAPN carrying the 128HLHFHLP134 region termed as binding region of APN (BR-APN) was cloned and its involvement in Cry1C binding and toxicity was checked. Cry1C-BR-APN binding was competed by synthetic peptides homologous to loop2 and loop3 of domain II but not by that of loopα. Additionally, alanines substitution of residues H128, H130, H132 and P134 affect the binding efficiency of receptor to Cry1C toxin (upto 4-fold lower affinity).These residues are also implicated in Cry1C toxicity as shown by the reduced ability to affect the mortality of Cry1C on *S. litura* larvae when toxin was preincubated with a fragment of the receptor.

1. Introduction

The larvicidal inclusions (δ-endotoxins) of *Bacillus thuringiensis* (Bt) consist of one or more insecticidal crystal proteins (Cry) proteins which are classified into 72 classes according to their sequence similarity [1–4]. Upon ingestion the toxins are active against certain Lepidopteran, Dipteran, Coleopteran and Hymenopteran insect larvae [5–8].

Most of the Bt Cry toxins are synthesized as inactive protoxins which upon ingestion by a susceptible larvae, dissolve in the alkaline and reducing environment of the larval midgut, thereby releasing soluble proteins. The soluble protoxin is processed proteolytically by midgut enzymes, yielding 60–70 kDa protease-resistant toxic fragments [3]. The activated toxin then binds specific receptor molecules located in the midgut epithelial cell brush border membranes of the host insect [9]. The toxin molecules oligomerize to form of a pre-pore oligomeric structure. The specific binding involves two-steps, a reversible followed by an irreversible one leading to insertion of the toxin oligomer into the cell membrane, resulting in the formation of pores in the membrane and eventually cell lysis [10–12].

The receptors of the Cry toxins have been classified into five major groups: a cadherin like protein (CADR), a glycosyl phosphatidyl-inositol (GPI)-anchored aminopeptidase-N (APN), a GPI-anchored alkaline phosphatase (ALP), a 270 kDa glycoconjugate and glycolipids [13–17]. Since 1994, more than 60 different APNs from different Lepidopterans have been sequenced and registered in databases showing the high diversity in isoforms [18]. This exopeptidase is believed to be anchored in lipid rafts and serves as a binding molecule for Cry1A, Cry1C, Cry1F and Cry1J toxins in different Lepidopteran species [19–21]. The fact that it is implicated in toxin insertion [22] enhances Cry1Aa pore formation when incorporated into lipid bilayers [23]. Further, its inactivation by natural mutations [18] or by RNAi [24] makes the insect populations resistant to Bt toxins confirms its critical role as functional receptor in Bt toxicity. However, the details of APN-toxin interaction still need investigation. Although some APN binding epitopes in the toxin have been characterized, little is known about the APN domains involved in Cry toxin binding [25].

Previous work in our lab demonstrated that a 110 kDa recombinant APN is a receptor for Cry1C toxin in *Spodoptera litura* midgut and is responsible for Cry1C toxin sensitivity in the larvae [21]. The mapping of binding epitopes of APN is a challenging problem

Abbreviations: Bt, *Bacillus thuringiensis*; SlAPN, recombinantly expressed APN of *Spodoptera litura*; BBMV, brush border membrane vesicle.

* Corresponding author. Insect Resistance Group, International Center for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi, India.
Tel.: +91 11 26741153; fax: +91 11 26742316.
E-mail address: raj@icgeb.res.in (R.K. Bhatnagar).

[1] These authors contributed equally to the work.

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since mutagenesis studies of the receptor domains have never been performed as in case of toxins. We have earlier shown that the toxin binding and substrate binding regions of the SlAPN are different [26] and in this paper we took a more detailed look at the toxin binding domain of APN. In order to map the precise regions involved in SlAPN-Cry1C interactions, we decided to use the phage-display technique. This technology is highly successful in receptor identification [27] and epitope mapping [28,29]. The findings were further confirmed by alanine substitutions in the receptor protein aminopeptidase N.

2. Materials and methods

2.1. Modeling of Cry1C and SlAPN and Docking of the derived structures

A standalone version of BLAST 2.2.4 [30] was used to blast the target sequences against PDB database to retrieve the top hits to select respective templates for 3D-homology modeling. Using Clustal X 1.83 [31], an alignment was obtained between the best hit and the target sequence. We did extensive manual inspection and curation of alignments and used *alignment.check* script of MODELLER [32] to find any trivial alignment mistakes. These alignment files (after successful run of *alignment. check*) were given as input to the MODELLER 8v2, one at a time for model building. Using WHATIF programs [33], missing side chains were added to the respective models. Energy minimization (EM) was done *in vacuo* to relieve steric interactions within the model structure. The quality of energy minimized models was checked by various WHATIF programs. Stereochemical parameters of the model structures were analyzed with the PROCHECK program [34]. Local environment of atoms, packing quality and folding states of models were further studied using more advanced methods like PROSA II [35], Verify3D [36] and ANOLEA [37]. Finally, to access the deviation of modeled structure with their respective templates, we used STAMP based 3d-SS [38] and CE/CL based Compare3D. Using GRAMM v1.03 [39], we carried out the docking process in two modes, mode1: provided the HLHFHLP sequence moiety of APN receptor protein as interface residue constraints for consideration during docking and mode2: without any residue constraint mode. Top 10 complexes for each mode were studied for final analysis.

2.2. Purification of active recombinant Bt Cry1C toxin and production of polyclonal antibodies against it

The recombinant protoxin Cry1C expressing DH5α strain was obtained from the *Bacillus* Genetic Center, Ohio State University. Cry1C inclusion bodies were purified, solubilized and trypsin-activated by a modification of the procedure described by Lee et al. [40]. Briefly, inclusion bodies were purified and solubilized in 50 mM sodium carbonate buffer, pH 10.5, containing 10 mM dithiothreitol (solubilization buffer) at 37 °C. The solubilized crystal protein was activated by trypsin (United State Biochemical) at a trypsin: protoxin ratio of 1:10 (by mass) at 37 °C for 1 h. The activated toxin was purified by anion exchange liquid chromatography using Q-sepharose anion exchanger. The purity of the toxin was checked by SDS-PAGE and fractions containing the purified toxin were pooled and dialyzed against Tris buffered saline (TBS) for raising antibodies and binding studies.

To raise anti-Cry1C antiserum, a New Zealand White rabbit was immunized with 100 μg of purified active Cry1C administered in Freud's complete adjuvant (Sigma). After boosting the rabbit once with the toxin (administered in Freud's incomplete adjuvant), the rabbit serum was collected and assessed for its reactivity.

2.3. Biopanning and characterization of binding phage display clones

A synthetic combinatorial library of random disulphide linked hepta-peptides fused to the minor coat protein (pIII) of M13 phage was used in this work (New England Biolabs Inc Ph.D.-C7C phage display peptide library kit). This library size was 1.2×10^9 clones and was amplified once to yield approximately 200 copies of each sequence in 10 μl of the supplied phage. Each time 10 μl (2×10^{11} phages) of supplied library was used to select peptides binding to the coated active Cry1C as ligand. For each experiment, ligand-binding phages were isolated by panning using immunosorbent polystyrene 96-well plates (Costar NY, USA) which were coated with 5 μg of the ligand by overnight incubation at 4 °C. Three rounds of panning, with increasing concentrations of Tween20 in wash steps, were carried out according to the manufacturer's instructions. After each round of selection, phage titer was determined by plaque-assay method and enrichment of the eluted phage was analyzed by ELISA. After the final selection round DNA from individual phages was purified and sequenced using 28 gIII sequencing primer provided in the kit.

2.4. Cloning, expression and purification of SlAPN and BR-APN

SlAPN expressed in Sf21 cells was solubilized and purified as described previously [26]. Both biopanning and *in silico* docking analysis identified Cry1C interacting ^{128}HLHFHLP^{134} region of APN, named as APN-CRY. Thus, a 12 kDa protein fragment [Binding region of APN (BR-APN)] ranging from amino acid 51–162 with APN-CRY region in it (Table 1), was cloned in pQE expression vector and expressed in *Escherichia coli* M15 strain.

Production of soluble BR-APN fragment was obtained by incubating the log-phase M15 culture with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 16 °C for 16 h. Cells were collected by centrifugation and resuspended in 20 mM Tris buffer (pH 8) containing 150 mM NaCl (TBS). Total proteins were obtained by sonicating the cell suspension on ice (four 30-second pulses) and observed under microscope for complete lysis. The supernatant obtained after centrifuging the homogenate at low speed (5000 rpm at 4 °C for 10 min) was dialyzed against 50 mM sodium phosphate buffer (pH 8) (binding buffer) and loaded on Talon resin (Amersham) to purify the His-tagged BR-APN fragment. The recombinant BR-APN fragment protein bound to the resin were eluted according to the manufacturer's instructions.

2.5. Toxin-overlay assays

A total of 1 μg purified SlAPN or BR-APN was resolved on SDS-PAGE and electro blotted onto a nitrocellulose (NC) membrane. The blot was incubated with 2.5 μg/ml toxin and processed as described by Agrawal et al. [21]. Following Cry1C overlaying the membrane was washed with TBST (20 mM Tris buffer (pH 8) containing 150 mM NaCl and 0.5% Tween20) and incubated for 1 h in

Table 1
Summary of primers used in the study.

Name	Abbreviation	Primers	Base pairs	Amino acids	Mass (kDa)
Binding region of SlAPN	BR-APN	(primer 1) TGT GTA CCC TAC TGA TGT (primer 2) CGA CGC GGC CGC ACC TCT GTA GAA GCC	336	51–162	12