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Employing phage display to study the mode of action of *Bacillus thuringiensis* Cry toxins

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Abstract

Phage display is an *in vitro* method for selecting polypeptides with desired properties from a large collections of variants. The insecticidal Cry toxins produced by *Bacillus thuringiensis* are highly specific to different insects. Various proteins such as cadherin, aminopeptidase-N (APN) and alkaline phosphatase (ALP) have been characterized as potential Cry-receptors. We used phage display to characterize the Cry toxin-receptor interaction(s). By employing phage-libraries that display single-chain antibodies (scFv) from humans or from immunized rabbits with Cry1Ab toxin or random 12-residues peptides, we have identified the epitopes that mediate binding of lepidopteran Cry1Ab toxin with cadherin and APN receptors from *Manduca sexta* and the interaction of dipteran Cry11Aa toxin with the ALP receptor from *Aedes aegypti*. Finally we displayed in phages the Cry1Ac toxin and discuss the potential for selecting Cry variants with improved toxicity or different specificity.

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1. Introduction

Bacillus thuringiensis (Bt) is an aerobic, spore-forming bacterium from the *Bacillus cereus* group that is distinguished due to its capacity to produce δ -endotoxins that have insecticidal activity. δ -endotoxins are produced as crystalline inclusions during the sporulation phase [4]. The δ -endotoxins, or Cry proteins, are specifically toxic to the following insect Orders: Lepidoptera, Coleoptera, Hymenoptera and Diptera [4,31]. One feature that distinguishes Cry toxins is their remarkable specificity, and therefore they are harmless to non-target insects or vertebrates. Cry toxins are being used world-wide for the control of vectors of human diseases or insect agricultural pests as insecticidal sprays or in transgenic plants [4].

2. Bt Cry toxins

Cry toxins belong to the group of pore forming toxins (PFT) and it is widely accepted that their toxic effect is due to the formation of ionic pores in the membrane of insect epithelial midgut cells, which leads to cell swelling and death. To exert its toxic effect, crystals are ingested by susceptible larvae and solubilized by the alkaline pH and reducing conditions of the midgut. Midgut proteases act on the protoxin giving rise to a protease-resistant 55 to 60 kDa toxin fragment. The toxin fragment binds to specific midgut membrane associated proteins resulting in the oligomerization and membrane insertion of the toxin [4]. Toxin receptor interaction is a key step that determines insect specificity [4,31]. Even more, the principal mechanism of resistance to Cry toxins are mutations that affect toxin-receptor interaction [8]. Different proteins such as cadherins, aminopeptidase-N (APN), and alkaline phosphatase (ALP) have been characterized as Cry-receptors in different insect species [17,18,21,33]. Thus, understanding the molecular basis of the interaction of Cry toxins with their receptor molecules would be useful not only for engineering Cry proteins with different specificities or with enhanced insecticidal activity but also for coping with the problem of insect resistance in the field.

The three dimensional structures of six Cry toxins with different insect specificities have been solved [2,3,9,15,22,26]. These toxins are composed of three domains – domain I, a seven α -helix bundle involved in membrane insertion, oligomer formation and pore formation [4]; domain II, a three anti-parallel β -sheets packed around a hydrophobic core in a “beta-prism” involved in receptor interaction [4]; and domain III, a β -sandwich of two antiparallel β -sheets also involved in receptor interaction [4].

3. Phage display

For the past few years we characterized Cry toxin-receptor interaction in lepidopteran and dipteran insects to understand the molecular basis of insect specificity of these toxins. For this purpose we have employed a molecular technique known as phage display. Phage display, first developed in 1985 [32], displays recombinant peptides or proteins on the surface of phage particles, that can be screened by enabling the phage to interact with ligands that are immobilized in tubes (panning). This is a very powerful technique since the selected phages maintain a physical link between the displayed protein (phenotype) and the encoding gene (genotype). Filamentous phages, like M13, have been extensively used to develop different types of phage display libraries that display millions of variants of peptides or antibodies. Phage display involves the fusion of foreign DNA sequences to a coat protein gene enabling the fusion protein to be displayed on the surface of the phage. Most commonly, phage display libraries are constructed using vectors called phagemids, which are hybrids of phage and plasmid vectors. These phagemids contain the origins of replication from the M13 phage and *E. coli*; the gene coat protein III or gene coat IV for protein fusion, and an antibiotic resistance gene [1,25]. To display the fusion protein, *E. coli* cells harboring the phagemids are infected with a helper phage that provides all the necessary components for phage assembly.

In this review we will summarize the types of libraries and the panning procedures used to characterize the Cry toxin-receptor interaction. Our experimental approach has been to select, by panning, ligands of the toxin or the receptor that compete the toxin-receptor interaction. This approach has enabled us to map the binding epitopes in the Cry toxins and in the receptors, identify novel receptor molecules, study receptor localization in the insect gut and most importantly, to determine how receptors promote toxicity. Finally, we will discuss the potential of displaying Cry toxins on the surface of phages as a way to select toxins with different specificities or that overcome insect resistance to Cry toxins.

4. A synthetic human scFv library- selection of an scFv molecule that mimics a cadherin receptor

Our first attempt to characterize the amino acid epitopes involved in the interaction of Cry toxins with their receptor molecules was with the lepidopteran insect *Manduca sexta* and the Cry1Ab toxin [10]. At that time, two *M. sexta* proteins that bind Cry1A toxins had been cloned and characterized, a 210 kDa cadherin protein known as Bt-R₁ and a 120 kDa glycosylphosphatidy-linositol (GPI) anchored aminopeptidase N (APN) [21,33]. As a way to determine which was the functional receptor and the amino acid epitopes involved in the toxin-receptor interaction, we decided to select by phage display peptides that bind Cry1Ab toxin and identify those that competed toxin interaction with both receptor molecules, then we used these phages as tools to determine the role of both *M. sexta* proteins in toxicity. Also, we expected that some of the peptides that competed the interaction of Cry toxins with their receptor could share sequence similarity with discrete receptor amino acid sequences that were involved in toxin binding as has been shown for several examples of interacting proteins [20]. For the first panning procedure we used the Nissim synthetic phage-antibody library. This library, with a diversity of 1×10^8 , contains a diverse repertoire of *in vitro* rearranged human variable heavy (VH) genes containing a random VH-CDR3 of 4 to 12 amino acids residues in length [27]. Antigen-recognition by antibodies is determined mainly by CDR regions in the variable light (VL) and VH fragments. To facilitate the display of antibody molecules in phages, the VL and VH fragments are assembled as single chain molecules with a (Gly₄Ser)₃ linker peptide resulting in the construction of scFv genes fused to the pIII gene in phagemids [1,27]. scFv antibodies are likely to retain the binding properties of the whole antibody molecule [1,27]. For the panning procedure, we immobilized Cry1Ab toxin in immunotubes and after several panning rounds of selection we identified three scFv-phages that bound specifically to Cry1Ab toxin. Using ligand blots assays and by competing the binding of Cry1Ab toxin with *M. sexta* brush border membrane vesicles (BBMV) blotted proteins, we identified one scFv (scFv73) that competed the binding of Cry1Ab with the 210 kDa Bt-R₁ molecule but not with the 120 kDa APN [10]. Antibody scFv73 inhibited the toxicity of Cry1Ab toxin in bioassays. Sequence analysis of the CDR3 region of scFv73 led to the identification of a eight amino acid region (₈₆₉HITDTNNK₈₇₆) in cadherin repeat 7 of Bt-R₁ [10]. Synthetic peptides corresponding to the Bt-R₁ amino acid region or to scFv73 CDR3 competed the binding of Cry1Ab with Bt-R₁ [10].

Use of the scFv73 antibody was also very important to map the cognate-binding region in the Cry1Ab toxin [11]. Previous work of other groups demonstrated by site directed mutagenesis that domain II loops located in the apex of the β -prism were important for receptor recognition [4,31]. We determined if these loop regions were involved in the interaction of Cry1Ab toxin with scFv73. To do this, we performed binding competition of scFv73 binding with Cry1Ab toxin in Western blots using synthetic peptides corresponding to each of the Cry1Ab loop regions (loop 1, loop 2 and loop 3) [11]. Only loop 2 peptide competed the binding of scFv73 with Cry1Ab toxin [11]. Interestingly a mutant loop 2 synthetic peptide that contained two amino acid residue changes that in the context of the whole toxin affected binding and toxicity did not compete the binding of scFv73 with Cry1Ab [11]. These results indicate that the

Cry1Ab domain II loop 2 region interacts with the Bt-R₁ 869HITDTNNK₈₇₆ amino acid region [11] (Fig. 1).

Antibody scFv73 was also very useful to determine the role of the cadherin receptor in promoting toxicity. For several other pore forming toxins, binding to a receptor facilitates a proteolytic cleavage that finally induces the formation of oligomeric structures that are capable of inserting into membranes [29]. Using scFv73 as a surrogate of Bt-R₁, we demonstrated that Cry1Ab toxin forms a soluble 250 kDa oligomer when activated *in vitro* by *M. sexta* midgut-proteases in the presence of scFv73 [12]. Subsequently, we demonstrated that the 250 kDa oligomer is membrane-insertion-competent and forms stable pores with high conductances and open probability, in contrast to the 60 kDa monomeric structure that barely inserts into membranes and induces unstable low-conductance pores in black lipid bilayers [30]. Also, we demonstrated that the 250 kDa oligomer is formed in the presence of cadherin fragments containing the toxin binding regions [13]. These results led us to propose that binding to cadherin facilitates the formation of a pre-pore oligomer that is membrane insertion competent (Fig. 2).

5. An immune rabbit scFv library-mapping the binding epitopes of oligomeric toxin and APN receptor

In order to determine the role of other Cry1Ab toxin regions in binding to the receptor molecules, we attempted to isolate other scFv antibodies that recognize other domain II loop regions or domain III, previously shown to be involved in receptor interaction [4,31]. Nevertheless, different attempts of panning using the Nissim library were unsuccessful. Therefore we decided to construct a new scFv phage display library that would be enriched in scFv molecules that recognize Cry1Ab toxin. For this purpose, phage display libraries constructed from a host immunized with the target antigen favors selection of high-affinity, specific antibodies generated during immunization process [14]. A Cry1Ab-immune M13 phage-repertoire was constructed using antibody gene transcripts of bone marrow and spleen from a rabbit immunized with Cry1Ab toxin [14]. These were the first immune-libraries described against Cry toxins.

We also used novel panning protocols to identify antibodies that recognize exposed regions of domain II and III. scFv-antibodies to domain II loop 3 were selected by panning against a domain II loop 3 synthetic peptide. Anti-domain III-scFv molecules were identified by selecting scFv-phages that bound Cry1Ab toxin in the presence of soluble Cry1Ac toxin, which has similar domains I and II to Cry1Ab, but a non-related domain III. Then, toxin overlay binding competition assays in the presence of synthetic peptides were used to show that domain II loop 3 of Cry1Ab is an important epitope for interaction with the Bt-R₁ receptor. This interaction between the toxin and the anti-domain II loop 3 scFv antibody also promoted the formation of the pre-pore oligomer as a previously observed with an anti-domain II loop 2 scFv73 antibody [14]. The selected anti-loop 3 antibody (scFvL3-3) molecule lowered the toxicity of Cry1Ab to *M. sexta* larvae in bioassays.

The Cry1Ab epitopes involved in the interaction of the oligomeric structure with APN were also mapped by ELISA and toxin overlay binding competition assays. The β 16 and β 22 from domain III play an important role in this interaction since a scFv-antibody that recognizes these regions (scFvM22) inhibited the interaction of the pre-pore oligomeric structure with APN and lowered the toxicity of Cry1Ab to *M. sexta* larvae in bioassays [14]. scFvM22 did not promote the *in vitro* formation of a pre-pore oligomeric structure. Our results show that interaction with both receptors is important for *in vivo* toxicity.

Finally, we found that scFvL3-3 and scFv73 antibodies preferentially recognized the monomeric toxin rather than the pre-pore structure, suggesting a conformational change in the domain II loop regions is recognized by these antibodies [14]. Overall, these results indicated that the first interaction of Cry1Ab with Bt-R₁ through domain II loop regions, promotes the formation of the pre-pore oligomeric structure with a subtle change in the structure of these exposed domain II loops; then the pre-pore interacts with APN through a different domain III region (β 16) (Fig. 3).

6. Random peptide library- identification of an ALP receptor and its interaction with Cry toxins in mosquitoes

In the case of mosquitocidal toxins we decided to work with a Cry11Aa toxin that is particularly toxic to *Aedes aegypti* that is the vector of dengue in humans. *Bacillus thuringiensis* subs *israelensis* (Bti) produces a crystal inclusion composed of six toxins: Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, Cyt1Aa and Cyt2Ba that are highly active against dipteran insects, vectors of human diseases [23]. In contrast to *M. sexta* and Cry1Ab, no receptor molecules had been identified in *Ae. aegypti* nor the binding regions in the toxin. We decided to perform panning against Cry11Aa to select molecules that competed for the binding of the toxin with *Ae. aegypti* BBMV and also panning against the BBMV to select molecules that bind the receptor. This is an example that shows that molecules that bind to a receptor molecule can be selected without the need of having a pure preparation of this receptor [7]. We used a commercial phage-peptides library obtained from New England BioLabs Inc. (Ph.D.-12 Phage Display Peptide Library Kit). This library has a complexity of 2.7×10^9 transformants, and is based on a combinatorial library of random 12-mers fused to the coat protein pIII of the M13 phage. We demonstrated that the putative exposed loop α -8 of Cry11Aa toxin, located in domain II, is an important epitope involved in receptor interaction and toxicity. Four exposed loops and three putative exposed regions in domain II of Cry11Aa were predicted by modeling the three-dimensional structure of the mosquitocidal Cry11Aa toxin [6]. Synthetic peptides corresponding to these regions were used in heterologous binding competition assays of Cry11Aa to *Ae. aegypti* BBMV. We identified three regions of Cry11Aa toxin, corresponding to loop α -8, β 4 and loop 3 that are involved in binding of this toxin to the BBMV [6]. We isolated peptide-displaying phages that bind to Cry11Aa toxin (P5.tox) or to *Ae. aegypti* BBMV (P1.BBMV) [6,7]. These phages interfered with the interaction of the toxin with the BBMV and attenuated the toxicity of Cry11Aa in bioassays [6,7]. The binding of both phages was competed by loop α -8 synthetic peptide, suggesting that phage P5.tox binds the toxin by interacting with loop α -8 while P1.BBMV interacts with the receptor by using an epitope similar to this region [6,7].

A 65 kDa GPI-anchored ALP was identified as a binding Cry11Aa protein in *Ae. aegypti* BBMV. P1.BBMV bound specifically to ALP demonstrating that ALP is a functional receptor of Cry11Aa [7]. On the other hand, immunocytochemical localization of the binding of phage P1.BBMV and Cry11Aa toxin to midgut tissue sections from the mosquito revealed that Cry11Aa and P1.BBMV strongly recognized the same regions in the midgut tissue. These regions corresponded to the microvilli membrane from the caeca and from the posterior part of the midgut indicating that ALP colocalizes with the binding sites of Cry11Aa [7]. Our results identify domain II loop α -8 as a key determinant for the binding of Cry11Aa toxin to GPI-anchored ALP [6,7] (Fig. 4).

7. Displaying Cry toxins in phages and selection of novel variants

One of the most important features of phage display is that it allows a rapid selection of variants with improved binding characteristics [1,25]. Even more, further mutagenesis and selection by panning allows *in vitro* molecular evolution of proteins [5]. This seems particularly interesting

for Cry toxins since for many insect pests there are no Cry toxins available for their control. Also, a major threat for the use of Bt toxins in transgenic plants is the appearance of insect resistance. It has been demonstrated that a single nucleotide change in the *Heliothis virescens* cadherin receptor gene produces an amino acid change that abolishes Cry1A toxin binding [35]. Therefore the need of developing an efficient method that allows genetic evolution of Cry toxins to kill novel targets or to recover toxicity to resistant insects, in the case of the appearance of resistance in the field, will be greatly desirable.

Cry1A toxins have been displayed in three different phages. The first attempts to display Cry1A toxins were using M13 phages [19,24]. In the case of the filamentous M13 experiments, the Cry1Aa toxin was not properly displayed resulting in deletions of the fused protein [24]. A complete Cry1Ac was efficiently displayed in M13 showing toxicity to *M. sexta* larvae, however, the displayed Cry1Ac protein did not bind to functional receptors *in vitro* suggesting structural constraints of the displayed toxin [19]. M13 phage-display systems have an intrinsic problem in displaying big proteins since fusion proteins have to be transported into the *E. coli* periplasm where phage assembly occurs.

To circumvent this problem, λ and T7 phages have been used to display Cry1Ac toxin since the assembling of phage particles in both systems occur in the cytoplasm of bacterial cells, thus allowing the display of bigger proteins [28,34]. In the case of λ , the Cry1Ac protein was fused with the capsid protein D and displayed on the surface of phage particles. The displayed Cry1Ac toxin retained toxicity and the capacity to interact with the *M. sexta* APN receptor [34]. More recently we described the use of T7-phage display system to display Cry1Ac toxin [28]. The *cry1Ac* gene was fused to the 3' end of the T7 10B capsid protein gene and the chimeric protein was displayed on the surface of T7 phage. The T7-Cry1Ac bind receptors recognized by Cry1Ac toxin and retained toxicity against *M. sexta* larvae. Also, we showed that T7-Cry1Ac phage particles bind specifically to *M. sexta* BBMV that contain the native receptor molecules [28]. Nevertheless, a problem with both λ and T7 displaying systems is that for displaying the fusion protein both systems rely on *in vitro* packaging systems that under the best scenario allow for the production of up to 10^7 to 10^8 recombinant phage particles/ μ g of DNA, making the construction of libraries with large number of variants inefficient. Recently the T7 system was used successfully to select Cry1Aa toxin variants with higher toxicity by selecting phages by panning domain II loop 2 variants that bound the *B. mori* cadherin protein (Bt-R₁₇₅) with higher affinity [16]. This result shows that it is possible to select novel Cry toxins with desirable binding properties from a pool of variants.

8. Concluding remarks

To exert their toxic effect, Cry toxins interact with several receptor proteins and undergo drastic structural changes allowing a soluble protoxin molecule to insert as oligomers in the membranes of their target cells, thus forming ionic pores. Several receptor molecules in different insect species have been shown to be involved in this complex process. We have exploited the potential of phage display methodology to determine the identity and localization of one receptor molecule in mosquitoes and to determine the sequential participation of two receptor molecules leading to pore formation in lepidopteran insects. We believe that phage display will also be useful for the *in vitro* evolution of Cry toxins for selecting toxins with novel specificities and for selecting toxin mutants that kill resistant insects. Such a system will be of great value to assure long-term use of Bt toxins for insect management; this is highly desirable since the use of these toxins is recognized as an environmentally friendly technology.

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Cry1Ab monomer

Figure 1.
Cry1Ab toxin binds *Manduca sexta* cadherin repeat 7 by means of domain II loop 2.

Figure 2. Binding of Cry1Ab toxin to the *Manduca sexta* cadherin receptor promotes cleavage of helix α -1 in domain I and the subsequent formation of a pre-pore oligomeric structure that is membrane-insertion-competent.

Figure 3.
The Cry1Ab pre-pore oligomer binds aminopeptidase-N (APN) from *Manduca sexta* by means of β -16 in domain III.

Figure 4.
The Cry11Aa toxin binds *Aedes aegypti* alkaline phosphatase (ALP) by means of the α -8 loop in domain II.