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Identification of *Bacillus thuringiensis* Cry3Aa toxin domain II loop 1 as the binding site of *Tenebrio molitor* cadherin repeat CR12

Fernando Zúñiga-Navarrete [a], Isabel Gómez [a], Guadalupe Peña [b], Itzel Amaro [a, 1], Ernesto Ortíz [a], Baltazar Becerril [a], Jorge E. Ibarra [c], Alejandra Bravo [a], Mario Soberón [a, *]

[a] Instituto de Biotecnología, Universidad Nacional Autónoma de México, Apdo. postal 510-3, Cuernavaca 62250, Morelos, Mexico
[b] Centro de Investigaciones Biológicas, Universidad Autónoma del Estado de Morelos, Avenida Universidad 1001, Colonia Chamilpa, Cuernavaca 62209, Morelos, Mexico
[c] CINVESTAV IPN Unidad Irapuato, Apdo. Postal 629, 36500 Irapuato, Gto, Mexico

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ABSTRACT

Bacillus thuringiensis Cry toxins exert their toxic effect by specific recognition of larval midgut proteins leading to oligomerization of the toxin, membrane insertion and pore formation. The exposed domain II loop regions of Cry toxins have been shown to be involved in receptor binding. Insect cadherins have shown to be functionally involved in toxin binding facilitating toxin oligomerization. Here, we isolated a VHH (VHHA5) antibody by phage display that binds Cry3Aa loop 1 and competed with the binding of Cry3Aa to *Tenebrio molitor* brush border membranes. VHHA5 also competed with the binding of Cry3Aa to a cadherin fragment (CR12) that was previously shown to be involved in binding and toxicity of Cry3Aa, indicating that Cry3Aa binds CR12 through domain II loop 1. Moreover, we show that a loop 1 mutant, previously characterized to have increased toxicity to *T. molitor*, displayed a correlative enhanced binding affinity to *T. molitor* CR12 and to VHHA5. These results show that Cry3Aa domain II loop 1 is a binding site of CR12 *T. molitor* cadherin.

1. Introduction

Bacillus thuringiensis (Bt) is a gram-positive bacterium that produces insecticidal crystal inclusions upon sporulation. The insecticidal Cry proteins produced by Bt are grouped in four different families that are not related in primary sequence, structure and probably neither in their mode of action. The three-domain Cry (3d-Cry) group is the largest family of Cry proteins, with members that show toxicity against different insect orders, such as Lepidoptera, Diptera and Coleoptera (Bravo et al., 2011).

The 3d-Cry toxins are pore-forming toxins composed of three different domains. Cry3Aa was the first 3d-Cry toxin whose structure was solved (Li et al., 1991). Domain I is an alpha-helix bundle that is recognized as the pore-forming domain, since it has been shown to be involved in toxin insertion into the membrane and toxin oligomerization; domain II is a beta-prism with exposed loop regions that has shown to be involved in recognition of larval midgut proteins, while domain III is a beta-sandwich also involved in recognition of midgut proteins (Li et al., 1991; Bravo et al., 2011). Thus domains II and III determine the specificity of Cry toxins.

The conserved 3D structure of Cry toxins active against different insect orders suggests that the mode of action of 3d-Cry proteins is conserved. 3d-Cry toxins are produced as protoxins that can be classified as “truncated” (65–70 kDa) or “complete” (130 kDa) protoxins that, upon proteolytic activation by midgut proteases, release the active 3d-60-kDa toxin (Bravo et al., 2011; de Maagd et al., 2001). It has been proposed that 3d-Cry proteins undergo a sequential binding mechanism to different larval midgut proteins as aminopetidase-N (APN), alkaline phosphatase (ALP) and cadherin leading to toxin oligomerization, membrane insertion and pore formation bursting insect midgut cells by osmotic shock (Bravo et al., 2011).

Cry3 toxins are “truncated” coleopteran-specific 3d-Cry protoxins. However, their toxicity is low compared with lepidopteran-specific Cry1A toxins (Li et al., 1991; Wu et al., 2000). Different putative Cry3 binding proteins have been identified in different coleopteran species such as cadherins from *Tenebrio molitor* that bind Cry3Aa (Fabrick et al., 2009), or from *Alphitobius diaperinus*

* Corresponding author.
E-mail address: mario@ibt.unam.mx (M. Soberón).
[1] Present address: National Center for Disciplinary Rsearch in Veterinary Parasitology, INIFAP, Mexico.

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that bind Cry3Bb (Hua et al., 2014); an ADAM metalloprotease from *Lepinotarsa decemlineata* that binds Cry3Aa (Ochoa-Campuzano et al., 2007); a sodium solute symporter from *Tribolium castaneum* that binds Cry3Bb (Contreras et al., 2013) and an ALP from *T. molitor* that binds Cry3Aa (Zuñiga et al., 2013). In the case of *Diabrotica virgifera virgifera* a it was shown that a peptide of cadherin that bind Cry3Bb also enhanced toxicity of this toxin (Park et al., 2009).

In *T. molitor*, the cadherin protein is composed of 12 extracellular repeats (CR1-12), a membrane proximal region (MPED), a transmembrane region, and a cytoplasmic domain, which was shown to be a functional Cry3Aa receptor by RNAi silencing experiments (Fabrick et al., 2009). Moreover, the binding site for the Cry3Aa toxin was narrowed to CR12-MPED, a region shown to be involved in Cry3Aa toxin oligomerization (Fabrick et al., 2009).

Receptor binding has been reported to be a required step in the action mechanism of several Cry toxins, including Cry3Aa (Wu et al., 2000; Bravo et al., 2013). In the case of Cry3Aa, extensive mutagenesis of domain II loop regions identified loop 1 and loop 3 as important binding regions involved in toxicity (Wu and Dean, 1996; Wu et al., 2000). A Cry3Aa domain II loop 1 mutant was reported to have eleven-fold enhanced toxicity against *T. molitor* (Wu et al., 2000). However, the identification of the receptor involved in this enhanced toxicity remained unsolved. We had previously characterized scFv antibodies isolated by phage display that bound Cry1Ab domain II loop regions, which were shown to be useful tools for the identification of Cry1Ab receptors and mapping the binding regions (Gómez et al., 2001, 2006). In this article we report the isolation of a VHH (heavy chain variable region) antibody (VHHA5) from a naïve camelid library by phage display, which binds Cry3Aa loop 1. We used a library of camelid antibodies because, besides the conventional hetero-tetrameric IgG antibodies, camelids produce a special type of antibodies that do not contain the variable light chain region called HCab (Hamers-Casterman et al., 1993). Camelid variable heavy chain regions (VHHs) have been shown to retain high affinity interactions and are also robust molecules that are easily produced in *Escherichia coli* (Arbabi-Ghahroundii et al., 1997). The use of anti-Cry3Aa loop 1 VHHA5 led us to identify *T. molitor* cadherin CR12 as the loop 1 binding site. Moreover, we demonstrate that the loop 1 mutant of Cry3Aa, previously reported to have increased toxicity to *T. molitor*, showed a correlative enhanced binding affinity to *T. molitor* CR12 and to VHH5, supporting that Cry3Aa domain II loop 1 is a binding site of CR12 *T. molitor* cadherin.

2. Materials and methods

2.1. Construction of the phage-displayed llama VHH library

Two hundred fifty of peripheral whole blood were collected from a single llama (*Lama glama*) specimen. The collection was performed by veterinarians from the Camelid Division of the Mexico City Zoo, following the strict ethical and animal welfare procedures. Blood was diluted with PBS 1X, pH 7.4 in a one to one volume ratio. Mononuclear cells were isolated by gradient centrifugation using 6 ml of Ficoll-Paque (Amersham Biosciences) per 40 ml of diluted blood. After centrifugation for 10 min at 198 *g*, cells were collected from the cloudy layer over the dense Ficoll–Paque fraction. Cells were washed twice with 1 ml PBS 1X, pH 7.4 and collected by centrifugation at 469 *g* for 5 min. Cells were immediately used for total RNA extraction using the kit RNAgents Total RNA Isolation System (Promega) following the manufacturer's guidelines. One μg of total RNA was reverse-transcribed with the 1st Strand cDNA Synthesis Kit (Roche) as indicated by the manual. For this step, the mix of random hexameric oligonucleotides provided by the kit was employed. From the first strand cDNA, the repertoire of llama heavy-chain antibody variable regions (VHH) was amplified by PCR using the degenerate oligonucleotide VH1BACK (5′-AGG TSM ARC TGC AGS AGT CWG G-3′ in which S=C/G, M=A/C, R=A/G, and W=A/T) (Orlandi et al., 1989) in combination with one of the oligonucleotides Lam07 (5′-AAC AGT TAA GCT TCC GCT TGC GGC CGC GGA GCT GGG GTC TTC GCT GTG GTG CG-3′, where the *Not*I restriction site is underlined) or Lam08 (5′-AAC AGT TAA GCT TCC GCT TGC GGC CGC TGG TTG TGG TTT TGG TGT CTT GGG TT-3′, where the *Not*I restriction site is underlined) (van der Linden et al., 2000) in two independent reactions. In both cases the conditions were as follows: 5 min, 94 °C, with Hot Start; 30 cycles at 94 °C, 50 °C and 72 °C for 30, 40 and 40 s, respectively; a final extension of 5 min at 72 °C. Vent Polymerase (New England Biolabs) was used. The purified products were subjected to a new PCR with the oligonucleotide Lam01 (5′-GAG AGA GAG AGA GAG GCC CAG CCG GCC ATG GCC GAT GTS CAG CTG CAG SMR TCD GG-3′, where the *Sfi*I restriction site is underlined) (van der Linden et al., 2000) and Lam07 or Lam08 respectively. The PCR conditions were as above, except for the annealing temperature which was 55 °C. The ca. 450 bp DNA products were purified from agarose gels with the QIAquick Gel Extraction Kit (QIAGEN), sequentially digested with *Not*I and *Sfi*I (New England Biolabs) and purified from agarose gels as above. Equimolar amounts of the two PCR products were pooled and ligated to the *Not*I/*Sfi*I-restricted pSyn2 phagemid. The ligation reaction was performed with T4 DNA Ligase (New England Biolabs), using a molar ratio of vector:insert of 1:3. The ligated product was electroporated into electrocompetent *E. coli* XL1-Blue cells. 10 μl were plated into solid 2xYT medium, supplemented with ampicillin (200 μg/ml), tetracycline (15 μg/ml), and 1% glucose, to determine the library titer and to characterize its sequence variability. The remaining volume of the transformations was pooled and grown overnight in 100 ml 2xYT medium, ampicillin (200 μg/ml), tetracycline (15 μg/ml), and 1% glucose. Twenty ml of the culture were use to purify the phagemid library by the alkaline lysis method, and the remaining 80 ml were used to produce the phage-displayed VHHs library by infection with the M13KO7 helper phage (New England Biolabs). The resulting phages were purified by centrifugation with polyethylene glycol/NaCl and tittered. We were able to obtain a phage-displayed VHH library, representative of the naïve repertoire of the single-domain heavy-chain antibodies produced by a single llama specimen. The total library titer (maximum diversity) was determined to be 4×10^7 pfu. Twenty randomly selected clones were sequenced; all of them containing llama-related VHHs, resulting in 100% sequence diversity. After recovery with the helper phage, the final titer of the library was 7×10^{12} pfu/ml.

2.2. Cry3Aa toxins purification

Bt strains expressing Cry3Aa and A1 mutant were grown in SP medium (8 g/l Nutrient Broth, 1 mM $MgSO_4/H_2O$, 13.4 mM KCl, 0.01 mM $MnCl_2$, 0.2 mM $FeSO_4/7H_2O$, 0.5 mM $CaCl_2$, pH 7) at 30 °C until complete sporulation and autolysis (around 72 h) and then the cells and crystals were harvested by centrifugation and washed with TET buffer (10 mM Tris–HCl, 1 mM EDTA, 0.05% Triton X-100, pH 7.5). Spores and crystals were suspended in TTN buffer (20 mM Tris–HCl, 300 mM NaCl, 0.1% Triton X-100, pH 7.2), disrupted by sonication and separated in discontinuous sucrose gradients. Crystals were solubilized in carbonate buffer (50 mM Na_2CO_3, pH 10.2) plus 0.1% β-mercaptoethanol at 37 °C for 12 h. Soluble toxins were activated by addition of chymotrypsin in a 2:1 protease: toxin proportion and incubated at 37 °C for 12 h. The reaction was stopped by addition of 0.1 mM PMSF. Activated toxin was dialyzed in buffer 1 (50 mM Tris–HCl, 50 mM NaCl, pH 8.0) and purified by