



Research article

Oligomerization of Cry9Aa in solution without receptor binding, is not related with insecticidal activity



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ABSTRACT

Background: *Bacillus thuringiensis* Cry toxins bind with different insect midgut proteins leading to toxin oligomerization, membrane insertion and pore formation. However, different Cry toxins had been shown to readily form high molecular weight oligomers or aggregates in solution in the absence of receptor interaction. The role of Cry oligomers formed in solution remains uncertain. The Cry9A proteins show high toxicity against different Lepidoptera, and no-cross resistance with Cry1A.

Results: Cry9Aa655 protein formed oligomers easily in solution mediated by disulfide bonds, according to SDS-PAGE analysis under non-reducing and reducing conditions. However, oligomerization is not observed if Cry9Aa655 is activated with trypsin, suggesting that cysteine residues, C14 and C16, located in the N-terminal end that is processed during activation participate in this oligomerization. To determine the role of these residues on oligomerization and in toxicity single and double alanine substitution were constructed. In contrast to single C14A and C16A mutants, the double C14A–C16A mutant did not form oligomers in solution. Toxicity assays against *Plutella xylostella* showed that the C14A–C16A mutant had a similar insecticidal activity as the Cry9Aa655 protein indicating the oligomers of Cry9Aa formed in solution in the absence of receptor binding are not related with toxicity.

Conclusions: The aggregation of Cry9Aa655 polypeptides was mediated by disulfide bonds. Cry9Aa655 C14 and C16C are involved in oligomerization in solution. These aggregate forms are not related to the mode of action of Cry9Aa leading to toxicity.

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1. Introduction

Bacillus thuringiensis (Bt), a gram-positive bacterium, produces insecticidal crystal proteins during its sporulation phase of growth [1]. These crystal proteins known as Cry toxins have been widely used to control insect pests, including lepidoptera, diptera, and coleoptera [2,3], mainly because they are non-toxic to non-target pests, and it is safe to the environment [4,5,6].

The cry9Aa genes encoding a 130 kDa protein have been cloned from several different *B. thuringiensis* strains [7,8]. Cry9-type proteins show high toxic effect against several lepidoptera including *Ostrinia furnacalis*, *Plutella xylostella*, *Spodoptera exigua*, *Exorista larvarum*, *Thaumetopoea pityocampa* [9,10,11] and no-cross resistance with Cry1A proteins [12]. An important additional information is that Cry9Aa synergizes with Cry1Ca against *Helicoverpa armigera*, and Cry1Ea

against *P. xylostella* [13]. All these data indicate that Cry9Aa proteins may be considered as good and wide prospects of commercial application to control the Cry1A-resistance insects, and to reduce the risk of insect resistance development by pyramiding them with other cry genes. However, more information at the basic level still requires to be done to evaluate the potential value of Cry9Aa proteins in the biological control of insect pests.

In a previous work 12 truncated recombinants containing all three domains of the Cry9Aa toxin were cloned in *Escherichia coli*, and we found that the truncated recombinant Cry9Aa 1-655 (Cry9Aa655), was the only one which was expressed as a primarily soluble form at 18°C, and showed insecticidal activity against *P. xylostella* similar to the full-length Cry9Aa protein [14]. In this work we report that this truncated protein is able to form oligomers in the solution.

Oligomerization of Cry1A toxins is triggered by receptor binding promoting removal of helix alpha-1 [15,16]. Cry1Ab oligomers formed after receptor binding have been shown to be involved in toxicity since some single helix alpha-3 mutations severely affected oligomerization and were not toxic to *Manduca sexta* [17]. However, different Cry

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toxins have been shown to form high molecular weight oligomers in solution in the absence of receptor interaction. Güereca and Bravo [18] reported that Cry1Aa, Cry1Ac, Cry1C, and Cry1D toxins are soluble and forms monomers and high molecular mass aggregates of more than ten monomers; Walters et al. [19] reported that alkaline-solubilized Cry3A toxin formed a stable dimer in solution. Besides, Cry1Ie toxin and Cry1Ah toxin can form oligomer in the solution that resulted in the decrease of insecticidal activity compared with the monomer [20,21].

Although the oligomerization in solution of Cry9Aa655 did not affect the toxicity against *P. xylostella* [14], the role of these oligomeric forms on toxicity remains uncertain. In this manuscript we report that Cry9Aa655 oligomerization depends on disulfide bonds, according to the SDS-PAGE analysis of Cry9Aa655 polypeptides under non-reducing and reducing conditions. Furthermore, mutational analysis of cysteine residues C14 and C16 showed that a double C14A–C16A mutant was affected in oligomer formation in solution. Cry9Aa655 C14A–C16A mutant showed similar toxicity as Cry9Aa655 indicating that oligomers formed in solution are not related with toxicity.

2. Materials and methods

2.1. Strains and Materials

The *E. coli* Rosetta (DE3) harboring the recombinant plasmid pEB-cry9Aa655 was stored by the Institute of Plant Protection at the Chinese Academy of Agricultural Sciences. The pEB vector (lac operator, T7 promoter, multiple cloning site, His-Tag, HSV-Tag, lacZ start codon, *E. coli* promoter, Amp^r) is an expression vector in *E. coli* constructed by our lab [22].

Ni-NTA agarose was purchased from General Electric Company. All other chemical reagents were local products of analytical grade.

2.2. N terminal amino acids sequencing of cleavage activated Cry9Aa655 polypeptides by trypsin

The Cry9Aa655 polypeptides were digested by trypsin (Sigma) with ratio of 1:10 (trypsin:protein,w/w) at 37°C for 3 h. The activated polypeptides were purified by Superdex 75 (GE Healthcare) and stored at 4°C. N-terminal amino acids sequencing was attempted initially with this sample after being dissolved in water with limited success. This sample was then run on SDS-PAGE gel and transferred into PVDF membrane and sent to Huada Protein Research Center for N-terminal sequencing.

2.3. Construction and expression of Cry9Aa655 mutants in *E. coli*

Single and double mutants of Cry9Aa655 (Table 1) were constructed by Fast Mutagenesis System (TransGen Biotech) using plasmid pEB-cry9Aa655 with His-tag after the cry9Aa655 gene as template. The preparation of Cry9Aa655 wild type and mutant proteins were as follows. The cells were grown in 300 mL of Luria–Bertani (LB) medium until the optical density of 0.5 at 600 nm, and the expression of the recombinants genes was induced using isopropyl- β -D-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM. The cells were centrifuged after 10 h of incubation with IPTG at 16°C. The cell pellet was resuspended in 20 mM Tris–HCl buffer (pH 8.0), sonicated (Ultrasonic processor,

Ningbo Scientz Biotechnology Co., LTD) for 5 min (75% power), and centrifuged at 23,000 \times g for 15 min at 4°C. The supernatants were collected, and the pellet was resuspended in 20 mM Tris–HCl buffer (pH 8.0). These samples were analyzed by SDS-PAGE (8%), and the protein concentrations were determined using ImageJ software (National Institutes of Health) with bovine serum albumin (BSA) as standard.

2.4. Purification of proteins

The supernatant of Cry9Aa655 wild type and mutations prepared as described above was loaded onto a Ni-chelating sepharose column previously equilibrated with binding buffer (20 mM Tris–HCl, 500 mM NaCl, 50 mM imidazole, pH 8.0). The fraction that passed through was collected and reloaded into the column to improve recovery of the protein. The protein that bound to the column was eluted with elution buffer 1 (20 mM Tris–HCl, 500 mM NaCl, 250 mM imidazole, pH 8.0), followed by elution buffer 2 (20 mM Tris–HCl, 500 mM NaCl, 500 mM imidazole, pH 8.0). The proteins collected were analyzed by SDS-PAGE.

2.5. The SDS-PAGE analysis of Cry9Aa655 polypeptides under non-reducing and reducing conditions

The purified Cry9Aa655 was dialyzed in different pH buffers (20 mM Na₂CO₃/NaHCO₃ pH 9.0–11.5). Dialysis buffer was change for every 3 h. After 12 h of dialysis, the samples were divided into two parts, one sample was prepared under reducing condition (mixed with loading buffer containing β -mercaptoethanol) while other samples were prepared under non-reducing condition (mixed with loading buffer without β -mercaptoethanol). Both samples were analyzed by SDS-PAGE and gels stained using Coomassie Brilliant Blue R-250 stain solution.

2.6. Bioassay

The insecticidal activities of the Cry9Aa655 mutations against *P. xylostella* larvae were measured by fresh leaf disks using the leaf-dip bioassay as described previously [23]. All mutant serial dilutions were accurately prepared to final concentrations of 0.03, 0.10, 0.30, 0.90, and 2.70 μ g/mL respectively with 20 mM Tris–HCl (pH 8.0) buffer, all of the treatments were divided into three replicates in Petri dishes (5 cm in diameter), and the products of pEB vector expressed in Rosetta (DE3) strain were used as negative control. Thirty 2nd-instar larvae were then placed onto each Petri dish with fresh leaf. The numbers of surviving and dead larvae were recorded after 48 h. The median lethal concentration (LC₅₀) values were calculated using SPSS Statistics 13.0 software.

3. Results

3.1. SDS-PAGE analysis of Cry9Aa655 dialyzed at different pHs

SDS-PAGE analysis of Cry9Aa655 under reducing (samples prepared with β -mercaptoethanol, Fig. 1a) or non-reducing conditions (samples prepared without β -mercaptoethanol, Fig. 1b) is shown in Fig. 1. Under reducing condition, Cry9Aa655 at different pH's showed a single band of approximately 66 kDa (Fig. 1a). However, under non-reducing condition, besides the 66 kDa band (band 1), another four bands of higher molecular weights were observed at the different pH's analyzed (bands 2 to 5 in Fig. 1b). The formation of aggregates with high molecular mass weight (>200 kDa, band 2 in Fig. 1b) disappears at high pH (pH 11.0 to pH 11.5). These data suggest that the formation of these Cry9Aa655-oligomers or -aggregates probably involved disulfide bonds since they were dissociable under reducing conditions.

Table 1

Characterization of mutations of Cry9Aa655.

Mutations	Characterization
Mutant C14A	Single mutant: amino acid C14 on Cry9Aa was changed to alanine
Mutant C16A	Single mutant: amino acid C16 on Cry9Aa was changed to alanine
Mutant C14A–C16A	Double mutants: both C14 and C16 were changed to alanine

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