Electronic Journal of Biotechnology 21 (2016) 54–57

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research article

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

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ARTICLE INFO

Article history:
Received 15 October 2015
Accepted 24 February 2016
Available online 6 April 2016

Keywords:
Cry9Aa655
Disulfide bonds
Oligomerization
Insecticidal activity

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.

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 promoving 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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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2016.02.005

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-*cry*9Aa655 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-*cry*9Aa655 with His-tag after the *cry*9Aa655 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 ×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.

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

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 Na_2CO_3/$NaHCO_3$ 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 μg/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 (LC_{50}) 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.

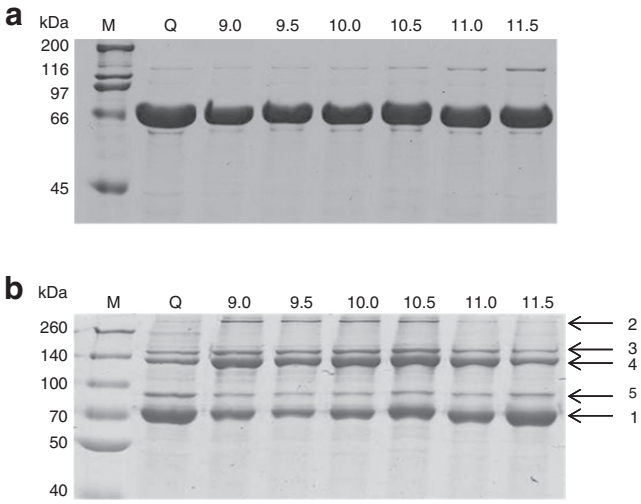

Fig. 1. SDS-PAGE analysis of Cry9Aa655 protein after dialysis in different pH buffer (20 mM Na_2CO_3/$NaHCO_3$ pH 9.0–11.5) for 12 h using reducing (with β-mercaptoethanol, (a)) and non-reducing conditions (without β-mercaptoethanol, (b)), Q indicates sample before dialysis. The numerals shown above each lane represent samples with different pH.

3.2. N terminal sequencing of activated Cry9Aa655 digested by trypsin

Cry9Aa655 contains six cysteines: C14, C16, C39, C104, C241 and C413. To determine which cysteine residues are involved in Cry9Aa655 oligomerization, Cry9Aa655 was activated for 3 h with trypsin and the oligomerization of the trypsin activated Cry9Aa655 was analyzed. Fig. 2 shows that the trypsin-activated protein was unable to form oligomers in non-reducing conditions (lane 3) in contrast with Cry9Aa655 that was not treated with trypsin (lane 4). Amino-terminal end sequence of the trypsin activated protein was determined. The results of N terminal sequencing of the activated Cry9Aa655 indicated two possible N-terminal sequences ^{36}NLNSCQNSSI^{45} (80% of sequence obtained) ^{27}ANNPYSSALNL^{37} (20% of sequence obtained) showing that C14 and C16 are not present in the trypsin activated fragment.

3.3. The SDS-PAGE analysis of oligomerization of mutations

To determine if C14 and C16 are involved in Cry9Aa655 oligomerization, single and double alanine substitutions in C14 and C16 were constructed. After expression and purification of the mutants, the oligomerization was analyzed. Fig. 3 shows that single mutants on either C14A or C16A still formed oligomers (Fig. 3, lane 2 and lane 3) as Cry9Aa655. However, the double C14A–C16A mutant didn't form oligomers, demonstrating that C14 and C16 are involved in oligomerization of Cry9Aa655.

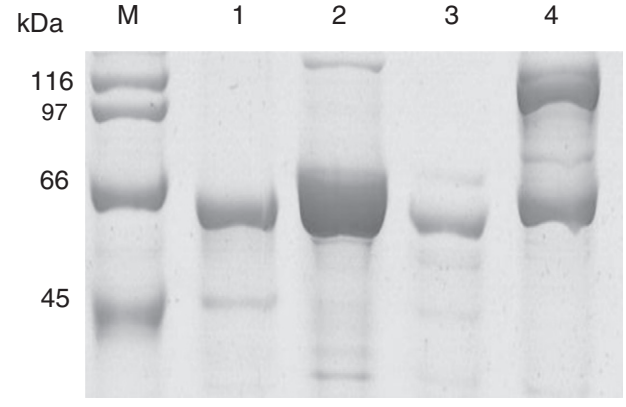

Fig. 2. SDS-PAGE analysis of oligomerization of truncated Cry9Aa655 protein and activated fragment. Trypsin-activated truncated Cry9Aa655 protein mixed with Laemmli sample buffer with β-mercaptoethanol (lane 1) or without β-mercaptoethanol (lane 3). Truncated Cry9Aa655 protein mixed with the Laemmli sample buffer with β-mercaptoethanol (lane 2) or without β-mercaptoethanol (lane 4).

3.4. Toxicity of different cysteine mutants against P. xylostella

The LC_{50} values of the different mutants against *P. xylostella* larvae are listed in Table 2. The Cry9Aa655 exhibited high toxicity against *P. xylostella* (LC_{50} = 1.73 μg/mL, 1.04–4.15). All Cry9Aa655 mutants, Cry9Aa655 C14A (LC_{50} = 1.33 μg/mL, 0.89–2.35), Cry9Aa655 C16A (LC_{50} = 1.41 μg/mL, 0.89–2.83) and Cry9Aa655 C14A–C16A (LC_{50} = 2.26 μg/mL, 1.45–4.87) exhibited similar levels of insecticidal activity as the Cry9Aa655. This bioassay result indicates that the Cry9Aa655 oligomerization in solution is not involved in toxicity.

4. Discussion

The mechanism of action of Cry toxins is a multistep process, which includes activating by trypsin-like gut proteases, binding to midgut receptor(s), oligomerization and insertion into the membrane to form lytic pores [24]. Oligomerization has been shown to be an important step involved in this mechanism [16,17].

Some studies have found that oligomerization or aggregation of different Cry toxins in solution decreases their insecticidal activity or have no effect on toxicity. However the relevance of these oligomeric forms formed in the absence of receptor binding remains uncertain. To better understand whether oligomerization or aggregation of Cry9Aa655 toxin in solution is important for toxicity, SDS-PAGE analysis of Cry9Aa655 protein under reducing and non-reducing conditions was conducted. Our data shows that the Cry9Aa655 toxin produced high molecular mass bands when samples were prepared without β-mercaptoethanol. However, oligomers were not observed in samples prepared with β-mercaptoethanol. These results suggest that the high molecular mass bands of Cry9Aa655 toxin observed in SDS-PAGE under non-reducing conditions could be mediated by disulfide bond formation. Disulfide bonds can be divided into two kinds: Intra-molecular and Inter-molecular disulfide bonds [25]. The disulfide bonds mentioned above may be the inter-molecular disulfide bonds, which will be easy to break by β-mercaptoethanol at low concentration compared to the intra-molecular disulfide bonds [26]. Most Cry toxins can be activated by trypsin proteases to produce a mature toxin, leading to insect death [1]. The Cry9Aa655 protein also can be digested by trypsin to form activated toxin with a size of 60–65 kDa. After SDS-PAGE analysis of activated toxin under non-reducing condition, we speculate that the fragment released by trypsin could be involved in the oligomerization of Cry9Aa655.

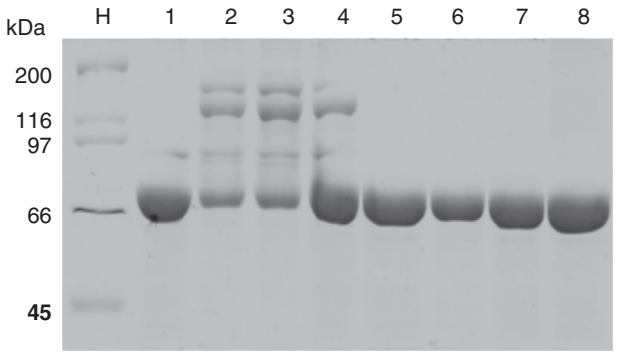

Fig. 3. SDS-PAGE analysis of oligomerization of truncated Cry9Aa655 protein and mutants. Samples mixed with Laemmli sample buffer without β-mercaptoethanol (lanes 1–4) or with β-mercaptoethanol (lanes 5–9). Mutant C14A–C16A (lanes 1, 5), Mutant C14A (lanes 2, 6), Mutant C16A polypeptides (lanes 3, 7), Cry9Aa 655 polypeptides (lanes 4, 8).

Table 2
Bioassy results of mutants against *P. xylostella* larvae.

Treatment for insects	LC_{50} (μg/mL)	95% confidence interval
Cry9Aa655	1.73	1.04–4.15
Mutant C14A	1.33	0.89–2.35
Mutant C16A	1.41	0.89–2.83
Mutant C14A-C16A	2.26	1.45–4.87
Trypsin-activated fragment	2.80	2.25–3.84

Considering the N-terminal end after trypsin treatment, two cysteines C14 and C16 were inferred to be involved in oligomerization of Cry9Aa655 protein. The analysis of oligomerization of C14A and C16A mutations demonstrated that both cysteine residues are involved in oligomerization of Cry9Aa655 since a double Cry9Aa655 C14A–C16A did not formed oligomers in solution.

Some studies have found that nonfunctional oligomerization mediated by inter-molecular disulfide bond will affect the function of the protein [27]. Fortunately, the oligomerization of Cry9Aa655 protein didn't affect the insecticidal activities according to the results of bioassay against *P. xylostella*, unlike the oligomerization of activated Cry1Ie and Cry1Ah [20,21]. The 3D structure of Cry9Aa predicted by SWISS-MODLE workspace showed that the initiating amino acid on N terminal end was residue 54, which indicated that although C14 and C16 were involved in oligomerization, they were cut away during activation with trypsin, and resulted in no affect to the toxicity against *P. xylostella*. Our results indicate that Cry9Aa655 oligomers formed in solution represent aggregates that are not related with toxicity.

5. 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.

Conflict of interest

There was no conflict of interest.

Financial support

This study was supported by the National Natural Science Foundation of China (31272115), the Key Project of Chinese National Programs for Fundamental Research and Development (973 Program, 2009CB118902).

Acknowledgments

We thank Mr. Xiaoqi Wang, Ms. Yingping Liang and Shuqin Liu for their assistance with insect rearing and the bioassays.

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