Cys183, and Cys258 in Cry49Aa Toxin From *Lysinibacillus Sphaericus* Are Essential for Toxicity to *Culex Quinquefasciatus* Larvae

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Research Article

Keywords: *Lysinibacillus sphaericus*, Disulfide bond, Cry48Aa/Cry49Aa toxin, Larvicidal activity, Mutagenesis

Posted Date: May 27th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-478739/v1>

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Abstract

The two-component Cry48Aa/Cry49Aa toxin produced by *Lysinibacillus sphaericus* shows specifically toxic to *Culex quinquefasciatus* mosquito larvae. Cry49Aa C-terminal domain is responsible for specific binding to the larval gut cell membrane, while its N-terminal domain is required for interaction with Cry48Aa. To investigate functional role of cysteine in Cry49Aa, four cysteine residues at positions 70, 91, 183, and 258 were substituted by alanine. All mutants showed similar crystalline morphology and comparable yield to that of the wild type except that the yield of the C91A mutant was low. These cysteine residues did not involve in disulfide bond formation within or between Cry49Aa molecules. Cys91, Cys183 and Cys258 are essential for toxicity against *C. quinquefasciatus* larvae, while Cys70 is not. Substitution at C91, C183 and C258 caused weak Cry48Aa- Cry49Aa interaction, while mutations at C183 and C258 reduced the binding capacities to the larval gut cell membrane. Thus, Cysteine residues at position 91, 183 and 258 in Cry49Aa are required for full toxicity of Cry48Aa/Cry49Aa toxin.

Introduction

The Cry48Aa (135 kDa) and Cry49Aa (53 kDa) two-component toxin produced by some strains of *Lysinibacillus sphaericus* during sporulation phase as crystalline inclusion is shows specifically toxic to *Culex quinquefasciatus* mosquito larvae ([Jones et al., 2007](#)). This toxin represents a new insecticidal combination that acts in synergy and presents optimal toxicity at equimolar concentration different from Bin toxin (BinA/ BinB), since neither Cry48Aa nor Cry49Aa component is toxic to the larvae alone ([Jones et al., 2007, 2008](#)). After Cry48Aa/Cry49Aa crystals ingested by mosquito larvae and solubilized in the larval gut alkaline pH condition, following activation by gut proteases, then the activated toxin binding to a specific receptor located on the surface of midgut epithelium cell of susceptible larvae, destroying the cells and killing the larvae ([de Melo et al., 2009](#); [Guo et al., 2016](#); [Rezende et al., 2017, 2019](#)). However, the mode of action of Cry48Aa/Cry49Aa has not been elucidated.

Three dimensional models demonstrate that the Cry48Aa shows a typical three-domain Cry toxin structure ([Jones et al., 2008](#)). However, the three-dimensional (3D) structure of the Cry49Aa had not been elucidated, although it is a member of a family of Bin toxin-like proteins (Toxin-10 family) that includes the Bin toxin from *L. sphaericus* ([Srisucharitpanit et al., 2014](#)), as well as the Cry36 and Cry35 proteins from *Bacillus thuringiensis* ([Kelker et al., 2014](#)). Previous studies indicate that the 3D structures of Cry49Aa is similar to the BinB and Cry35Ab1, containing two distinct structural domains in its N- and C-terminus ([Kelker et al., 2014](#)). The N-terminal trefoil domain is composed of several α -helices and three β -sheets. The C-terminal domain consists of six α -helices and three antiparallel β -sheets. The theoretical model of the Cry49Aa1 protein missing the N-terminal 40 amino acids exhibits significant similarity to Cry35Aa1, particularly in the C-terminal β -sheet region ([Kelker et al., 2014](#)).

Generally, the insecticidal toxins solubilized in the insect gut and undergo proteolytic processing into active toxins before receptor binding and membrane pore formation occurs ([Charles et al., 1996](#); [Bravo et al. 2007](#); [Pardo-López et al., 2013](#)). The activated Cry49Aa fragment of approximately 47-kDa is produced

through chymotrypsin-like cleavage by *C. quinquefasciatus* gut extract between F48 and N49 (Jones et al., 2008). Previous studies have indicated the Cry48Aa/Cry49Aa complex can be formed in synergy through the N-terminal portion of both Cry48Aa and Cry49Aa subunit (Guo et al., 2016, 2020). Individual Cry48Aa and Cry49Aa subunit was able to interact with receptors located on the midgut of *Culex* larvae (Guo et al., 2016), and then these specific receptors are being studied and putative candidates have been recently preliminarily identified (Rezende et al., 2017). Further, Guo et al demonstrated that the C-terminal domain of Cry49Aa between S349 and N464 may be responsible for receptor binding, and its N-terminal domain including about 100 amino acids residues was sufficient for interaction with Cry48Aa (Guo et al., 2016). However, both Cry49Aa N- and C-terminal deletions combined with Cry48Aa lost their larvicidal activity against *C. quinquefasciatus* (Guo et al., 2016). Hence, amino acids played an important role for these interactions. However, there is no clear evidence to explicate what residues are really required for the above functions.

Cysteine residues have critical roles in protein function, localization and/or stability (Girard et al., 2008). Mutations of cysteine residue at positions 31, 47, and 195 in BinA are required for full toxicity and play a critical role in the formation of active BinA- BinB complex (Promdonkoy et al., 2008). While, Cys67, Cys161 in BinB are required for toxicity (Boonyos et al., 2010). Preliminary studies show that Cry49Aa forms part of a Toxin-10 family and has similar 3D structures as BinA and BinB. Amino acid sequence analysis shows that activated Cry49Aa toxin contains 4 cysteine residues at positions 70, 91, 183 and 258. In this work, these cysteine residues were changed with alanine using overlapping PCR technique. Then we investigated their effects on protein production, crystal formation, interaction and biological activity of Cry49Aa toxin.

Materials And Methods

Bacterial strains, plasmids, and oligonucleotide primers

B. thuringiensis strains carries the plasmids of pBU- *cry48Aa* and pBU- *cry49Aa* stored in our laboratory (Guo et al., 2016). *B. thuringiensis* strain BMB171, a crystal-minus strain used for the transformation and expression of Cry49Aa proteins, was obtained from the laboratory of Professor M. Sun (Huazhong Agricultural University, Wuhan, China). The plasmids pBU4 (Delécluse et al., 1991) were used for cloning and expression of Cry49Aa PCR products and as an *E. coli*– *B. thuringiensis* shuttle vector, respectively. Oligonucleotide primers used for the construction of Cry49Aa and its site-directed mutants are given in Table 1. Sequences of the reversed primers of C70A, C91A, C183A, and C258A are complementary to the forward primers.

Table 1 Primers used for construction of Cry49Aa site-directed mutants

Primers	Sequences (5' to 3')	Enzyme sites
cry49Aa -Fwd	<u>CGGATCC</u> GTCGAGTGTAAC TTCGTTGTTT	<i>Bam</i> HI
cry49Aa -Rev	CCAAGCTTTATATTTGATTAAAAATGATTAATACACG	<i>Hind</i> III
C70A- Fwd	TCCAATACATTCAAACAAT <u>GCT</u> AGTTTTGAAATTCTCAATA	/
C91A-Fwd	GGATTCTGTAGATGGAGAAG <u>GCT</u> AGATTGCAGTAGCCACAG	/
C183A- Fwd	CTAATACTTTCTGCTACTGT <u>CGC</u> ITGGGCACAGTTCAACAGTTG	/
C258A -Fwd	TAGGAAGAACA CT TATTCCAG <u>C</u> TTTATTTGTAAATGATCCTG	/

Primers were designed based on DNA sequence of Cry49Aa from *L. sphaericus* strain IAB59 (Genbank accession no. AJ841948).

Construction of Cry49Aa mutants

Plasmid pBU- *cry49Aa* was used as a template for site-directed mutagenesis using overlapping PCR technique. The first PCR products were obtained using cry49Aa -Rev primers and the forward primers (C70A, C91A, C183A, and C258A), respectively. The second PCR products were obtained using cry49Aa- Fwd primers and the reversed primers (C70A, C91A, C183A, and C258A), respectively. Then, using the mixture of two above PCR products as template, the *cry49Aa* mutants were obtained through PCR amplification with the primers cry49Aa- Fwd and cry49Aa- Rev. The amplified mutant fragments were cloned into the pBU4 shuttle vector, resulting in the recombinant plasmids, and verified by DNA sequencing. The plasmids were transferred into *B. thuringiensis* BMB171 by electroporation ([Okamoto et al., 1997](#)) and the recombinant colonies were selected on Luria-Bertani (LB) agar containing 10 mg/ml of tetracycline.

Protein preparation

The expression and purification of the crystal proteins from *B. thuringiensis* BMB171 harbouring the corresponding plasmids were carried out as previously described ([Jones et al., 2007](#)). Purified toxin inclusions (1–2 mg/ml) were solubilized by incubation at 30 °C for 2–3 h in 50 mM NaOH and then dialysed in 50 mM Na₂CO₃ (pH 10.0) at 4 °C overnight. Protein concentrations were determined by using a bicinchoninic acid (BCA) kit (Thermo Fisher Scientific, New York, USA) and bovine serum albumin (BSA) as standard. All purified proteins were analyzed by 12% SDS-PAGE according to the standard procedures. Then, the purified Cry49Aa protein was biotinylated according to the method as previously described ([Guo et al., 2013](#)).

Mosquito colonies and preparation of BBMFs

A susceptible *C. quinquefasciatus* colony was reared without exposure to *L. sphaericus* for more than 10 years in our laboratory ([Pei et al., 2002](#)). BBMFs were prepared and evaluated as described previously

(Silva-Filha et al., 1997; Guo et al., 2013). Freshly prepared BBMF were kept on ice and used the same day.

Mosquito-larvicidal activity assays

The mosquito-larvicidal activity was tested by using 4nd-instar *C. quinquefasciatus* larvae according to the standard method recommended by the World Health Organization (WHO, 1985). Each experiment was tested by mixing Cry48Aa and Cry49Aa crystals at 1:1 molar ratio in 100 ml distilled water containing 20 early 4nd-instar larvae in 125-ml plastic cups and diluted as a 2-fold serial dilution. Six to eight concentrations were used for each toxin. Mortality was recorded after incubation

at room temperature for 48 h. The bioassays were performed three times, and the tests were replicated on three different days. The percentage of mortality was analyzed using SPSS software version 13.0 (SPSS Inc., Chicago, USA) with a program indicating standard error of the mean.

Cry48Aa- Cry49Aa interaction assay

Far-Western dot blot analysis was performed to detect Cry48Aa-Cry49Aa interaction. The purified Cry49Aa and its mutants were spotted onto nitrocellulose membrane at concentrations of 5, 1, 0.2, and 0.04 μ M. Far-Western dot blot analysis was carried out according to the method as described by Guo et al. (2016). Signals on the membrane were detected by using enhanced chemiluminescence substrate.

Binding of Cry49Aa toxin and its mutants to BBMFs

The binding affinities of Cry49Aa and its mutants to BBMFs were performed as described previously (Guo et al., 2013, 2016). The homologous competitive binding was performed using a modified enzyme linked immunosorbent assay (ELISA) (Bravo et al., 2004) Biotin-labelled Cry49Aa toxin (10 nM) was equilibrated with increasing amounts of unlabelled Cry49Aa or its mutants (0.01 to 1000 nM) in PBST (PBS add 0.1% Tween-20) (100 μ l) for 1 h at room temperature. The mixtures were then transferred to plates coated with BBMFs for 2 h. The plates were washed three times with PBST (100 μ l). Bound biotin-labelled Cry49Aa protein was detected as described above. The IC₅₀ was obtained from two independent experiments. The analysis of all binding assay data was carried out using GRAPHPAD PRISM 5.0 software (Graphpad, La Jolla, CA).

Results And Discussion

Mutations at Cys70, Cys91, Cys183, and Cys258 did not affect crystal formation except for protein production of Cry49Aa

The cysteine residues played an important role in participating in toxin formation, toxicity and interaction (Girard et al., 2008; Promdonkoy et al., 2008; Boonyos et al., 2010). Here, C70, C91, C183 and C258 in Cry49Aa were changed to alanine. Cry49Aa wild type and mutants were constructed and expressed in *B. thuringiensis* BMB171. All mutant proteins were produced small spore-associated crystals that showed a

bipyramidal morphology as the wild type. While, the mutant proteins showed comparable yield to that of the wild type except that the C91A mutant was low (Fig. 1A). These results clearly showed that replacements at Cys70, Cys91, Cys183, and Cys258 by alanine did not affect crystal formation and morphology of Cry49Aa.

Disulfide bonds are key characterizing structural and functional properties (Girard et al., 2008; Promdonkoy et al., 2008; Boonyos et al., 2010). The SDS-PAGE with and without a reducing agent (10 mm β-Mercaptoethanol) was used to analyze whether disulfide bond could be formed between cysteine residues at position 70, 91, 183, and 258 in Cry49Aa. The results showed that there was no difference in the mobility of the major bands formed as Cry49Aa monomers from the wild type and mutants with and without reducing agent, while, a weak dimer band was observed in the samples without reducing agent (Fig. 1A, B). These results demonstrated that Cys70, Cys91, Cys183, and Cys258 may not participate in the intra- or intermolecular disulfide bonds formation in Cry49Aa molecules. Amino acid sequence analysis showed there are 2 cysteine residues (Cys479, Cys562) in N-terminal domain of active Cry48Aa toxin is responsible for Cry48Aa–Cry49Aa interaction (Guo et al., 2016, 2020). Thus, it is supposed that one or more cysteine residues in Cry48Aa could result in disulfide bond formation with Cys70, Cys91, Cys183, and Cys258 in Cry49Aa.

Cys91, Cys183, and Cys258 in Cry49Aa are crucial for the mosquito-larvicidal activity

The mosquito-larvicidal activity of the Cry49Aa mutants against *C. quinquefasciatus* larvae was tested by mixing with equimolar amounts of Cry48Aa toxin from partially purified crystals. The C183A mutant and C258A mutant completely lost their total biological activity, while the C91A mutant significantly reduced the toxicity. However, the alanine substitution at Cys70 showed a comparable toxicity to that of the wild type (Table 2). These results revealed that the cysteine at the position 91, 183, and 258 in Cry49Aa is absolutely crucial for toxicity of the Cry49Aa/ Cry48Aa toxin, whereas Cys70 is less important. The cysteine replacement with alanine of Cry49Aa toxin did not affect the crystal formation and structure described as above, it is possible to affect hydrophobicity or polarity of these regions.

Table 2 Mosquito larvicidal activity of Cry49Aa wild type and mutants against *Culex quinquefasciatus* larvae

Cry48Aa +	LC ₅₀ (ng/mL)
Cry49Aa (wild type)	7.3(5.1-9.8)
Cry49Aa-C70A	6.8(5.1-8.9)
Cry49Aa-C91A	45.6(34.1-68.5)
Cry49Aa-C183A	inactive
Cry49Aa-C258A	inactive

Effect of cysteine substitutions on Cry48Aa- Cry49Aa interaction

Cry49Aa subunit plays a crucial role in the action mechanism of Cry48Aa/ Cry49Aa two-component toxins, while Cry48Aa-Cry49Aa interaction is one of the key steps in achieving their toxic activity against larvae (Jones et al., 2007; Guo et al., 2016, 2020). In our study, the far-Western dot blot analysis was used to explore the possible role of Cry49Aa cysteine in the intermolecular Cry49Aa- Cry48Aa interaction. The results showed the C70A mutant, interacted with Cry48Aa, displayed the strongest signal with comparable sensitivity to that of the wild type. The C91A and C258A mutants had moderately lower signals than that of the wild type, while the C183A mutant showed the considerably weakest signal compared to the wild type (Fig. 2). Thus, the Cys91 Cys183, and Cys258 located in the Cry49Aa N-terminus may be required for intermolecular Cry48Aa-Cry49Aa interaction. These results suggested that the weaker Cry49Aa- Cry48Aa interaction caused by the Cry49Aa N-terminal mutations at C91, C183 and C258 may contribute to the decrease or loss of toxicity of the toxin.

Effect of cysteine mutations on binding to *C. quinquefasciatus* BBMFs

The toxin interacted with receptor proteins present in insect larvae midgut cells, leading to membrane insertion and pore formation, then destroying the cells and killing the larvae (Bravo et al., 2007; Jones et al., 2008; de Melo et al., 2009). Previous studies had demonstrated that Cry49Aa had high binding capacity to *C. quinquefasciatus* BBMFs (Guo et al., 2016; Rezende et al., 2017). Here, the affinity of Cry49Aa mutants bound to BBMFs was determined by using 10 nM labelled Cry49Aa wild type toxin incubated with increasing concentrations of unlabelled mutant toxins. The results showed that the wild type Cry49Aa component had high binding capacity to *C. quinquefasciatus* midgut BBMFs with a half-maximal inhibitory concentration (IC₅₀) of 24.3± 5.7 nM. While, the C70A and C91A mutants had comparatively higher competitive binding capacities to BBMFs than that of the wild type with IC₅₀ of 9.5± 2.1 and 13.9± 3.2 nM, respectively. Whereas, the C183A and C258A mutants had poorer binding affinities for BBMFs than that of the wild type with IC₅₀ of 154.6± 20.3 and 186.2± 25.3 nM, respectively (Fig. 3). These results imply that mutations at C183 and C258 result in weaker interaction between toxin and its receptors which may contribute to loss of toxicity. Previous investigation suggesting that Cry49Aa C-terminal fragment located between S349 and N464 is essential and sufficient for receptor binding, and the N-terminal region is important for interacting to Cry48Aa (Guo et al., 2016). Although C183 and C258 are in the N-terminal region, but closer to the C-terminus, both residues may locate in a close proximity to the C-terminal part after protein folded into the functional 3D structure, leading to weaken the receptor binding ability and lose its toxicity. In addition, the C91A mutant has higher receptor binding capacity but weaker ability to form Cry48Aa-Cry49Aa complex, which may result in decreasing the virulence. Thus, toxin oligomerization was also a critical step in the mosquito- larvicidal activity, and other factors may also involve in the action mechanism of Cry49Aa/ Cry48Aa toxin.

In general, the replacements at Cys91, Cys183, and Cys258 by alanine did not affect crystal formation but significantly decreased its toxicity and reduced receptor binding and oligomer formation. These cysteine residues may not participate in disulfide bond formation in Cry49Aa, but may affect crystal 3D structures,

and then play an important role on Cry48Aa-Cry49Aa complex formation and toxin-receptor interaction. The Cry49Aa, as accessory protein, only combined with Cry48Aa could achieved the insecticidal activity (Jones et al., 2007, 2008; Guo et al., 2016, 2020). However, it is uncertain that mutations at these positions lead to weaken interaction between Cry49Aa mutants and Cry48Aa or BBMFs could account for the loss of virulence. Cys183 and Cys258 in Cry49Aa may play a critical role on conformational change, receptor binding, and/or membrane insertion, because mutations at both positions had more adverse effect than at Cys91. These results provided for useful information to explore the mechanism of action of *L. sphaericus* Cry48Aa/Cry49Aa toxins.

Declarations

Acknowledgements

This work was supported by a National Natural Science Foundation of China (NSFC) Grant (31702069).

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Figures

Figure 1

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of Cry49Aa and its mutants A. *B. thuringiensis* BMB171 harbouring the corresponding plasmids were grown in ICPM medium until the crystals were released from cells, and then 1 ml cultures were collected and analyzed by SDS-PAGE with reducing agent (present β -Mercaptoethanol) B. The crystals were solubilized and analyzed by SDS-PAGE without reducing agent (absent β -Mercaptoethanol).

Figure 2

The Cry48Aa- Cry49Aa interaction in vitro by Far-Western dot blot analysis. Various amounts of purified Cry49Aa and its mutants were immobilized on a nitrocellulose membrane. The membrane was blocked with skimmed milk and then overlaid with purified Cry48Aa. The presence of Cry48Aa was detected by rabbit anti-Cry48Aa and goat anti-rabbit conjugated with Horseradish Peroxidase (HRP).

Figure 3

Competition binding of biotinylated Cry49Aa to BBMFs was determined in the presence of increasing concentrations of unlabelled Cry49Aa and its mutants. Binding of wild-type Cry49Aa (●) and the four Cys mutants C70A (■), C91A(▲), C183A (▼), and C258A (⬢) is shown. The half-maximal inhibitory concentration (IC₅₀) of Cry49Aa for binding to *C. quinquefasciatus* BBMFs is 24.3 nM, and those for the four mutants were 9.5, 13.9, 154.6, and 186.2 nM, respectively.