Key Residues of *Bacillus Thuringiensis* Cry2Ab for Oligomerization and Pore-Formation Activity

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Abstract

As a pore-forming toxin, oligomerization and pore-formation were both required for the mode of action of Cry toxins. Previous studies revealed that Domain I helices α4-α5 were involved in oligomerization of Cry2Ab, while the active residues in charge of Cry2Ab aggregation remained ambiguous. In present studies, we built 20 Cry2Ab alanine mutants site directed in helices α4-α5 and demonstrated that mutants N151A, T152A, F157A, L183A, L185A and I188A blocked the assembly of 250 kDa oligomers, suggesting that those residues were key residues for Cry2Ab oligomerization. As expected, those variants severely reduced the insecticidal activity against *P. xylostella* which was similar to our previous reports. Furthermore, we found that the pore-forming activities of non-oligomerization mutants sharply decreased compared to wild-type Cry2Ab. Taken together, our data comprehensively identified key residues for Cry2Ab for the first time and emphasized that oligomerization was closely related to insecticidal activity and pore-forming activity in Cry2Ab.

Introduction

Cry toxin, an insecticidal crystal toxin derived from *Bacillus thuringiensis* (Bt), is widely applied as a bio-insecticides to control agricultural pests all over the world (Brave et al. 2011; Schnepf et al. 1998). Cry toxins are produced in an inactive form called protoxin and proteolytic activated by insect midgut protease. Activated Cry toxins interacted with midgut receptors in target insects, self-aggregated to form pre-pore oligomeric structures and inserted into the cell membrane to form pores, resulting in the death of insects (Pardo-Lopez et al. 2013; Bravo et al. 2007; Vachon et al. 2012).

At present, most researches have been devoted into “interaction of Cry toxins with midgut receptors” since which is an essential step for the insecticidal mechanism of Cry toxins (Pigott et al. 2007; Bravo et al. 2013). A number of insect midgut proteins such as cadherin, aminopeptidase-N (APN), alkaline phosphatase (ALP) and ATP-binding cassette transporter subfamily C member 2 (ABCC2) had been proposed as functional receptors for Cry toxins (Pigott et al. 2007; Zhou et al. 2017; Park et al. 2009; Guo et al. 2015; Chen et al. 2017). It was suggested that the mutation or down-regulation of Cry receptor genes were tightly linked with high level of Cry resistance in diverse insects (Ferré et al. 2002; Baxter et al. 2011). In most cases, Cry toxins were produced in an inactive protoxin and the proteolytic activation was required for insecticidal activity of Cry toxins (Xu et al. 2016). It was also reported that improper processing of Cry toxins was associated with Cry resistance in lepidopteran insects (Liu et al. 2014; Xia et al. 2016). Those studies made a closer look on the mechanism of Cry toxins and provided new strategies for agricultural pests control.

As a pore-forming toxin, Cry toxins kill insects by forming pore on their midgut cells. Assembly of pre-pore oligomers was required for pore-forming processing of Cry toxins (Jiménez-Juárez et al. 2013; Vachon et al. 2002). Domain I, a seven-helix bundle, was wildly reported involved in the assembly of pre-pore structure and membrane channel formation (Pardo-Lopez et al. 2013; Vachon et al. 2012). It was proposed that a binding step of cadherin receptors and proteolytic removal of helix α-1 were required for

oligomerization of Cry1A toxin (Soberón et al. 2007). Helix α-3 was found to participate in toxin oligomerization as the mutation on this region affected oligomerization, as well as toxicity of Cry toxin (Vachon et al. 2002; Muñoz-Garay et al. 2009). Recently, Pacheco et al. reported that an intramolecular salt bridge in helix α-3 was essential for the stability and oligomerization of Cry4Ba toxins (Pacheco et al. 2017). The helices α-4 and α-5 had been wildly reported as a pore-forming region, as they were the only helices capable of adopting a transmembrane orientation (Girard et al. 2008; Torres et al. 2008). An umbrella model was further proposed in which the helices α-4 and α-5 inserted into the membrane to form pores while other α-helices covered the surface of membrane (Pardo-Lopez et al. 2013).

Our previous study suggested that the exposure of helices α4-α5 was required for oligomerization and insecticidal activity of Cry2Ab (Xu et al. 2018). However, the key residues involved in the oligomerization activity of Cry2Ab were still unknown. In the present study, we sought to determine the key residues of Cry2Ab for the oligomerization activity by constructing 20 alanine mutants site directed on helices α4-α5. It revealed that residues N151, T152, F157, L183, L185 and I188 are involved in Cry2Ab oligomerization. The non-aggregation Cry2Ab mutants not only affected the insecticidal activities against *Plutella xylostella*, but also weakened the pore-forming activities on liposome. Our data firstly identified key residues of Cry2Ab for oligomerization activity and highlighted that oligomerization was tightly linked with the pore-forming activity of Cry2Ab.

Materials And Methods

Insects

A laboratory population of *P. xylostella* larvae was purchased from Henan Jiyuan Baiyun Industry Co., Ltd, China. *P. xylostella* larvae were fed with an artificial diet and maintained under environmental conditions of 27 ± 2 °C, 70% of humidity, and photoperiod of 14:10 h (light/dark).

Construction of Cry2Ab variants

Twenty Cry2Ab mutants site-directed on helices α4-α5 in Domain I were built by replacing V150, N151, T152, M153, Q154, Q155, L156, F157, L158, N159, R160, N182, L183, H184, L185, S186, F187, I188, R189, D190 with alanine. The Cry2Ab mutants were obtained by overlapping-extension PCR using wild-type Cry2Ab DNA fragment as template (Xu et al. 2018). Primers used for generation of site-directed mutagenesis in PCR fragments were listed in Supplementary Table S1. The mutated *cry2Ab* fragments were further digested by the restriction enzymes *Bgl* II and *Eco*R I, which were then ligated into the plasmid pET30a. Recombined plasmids were transformed into *Escherichia coli* BL21 (DE3) cells and positive clones were verified by PCR, restriction enzyme digestion and DNA sequencing (Xu et al. 2018).

Construction of Cry2Ab variants

The production and purification of wild-type and mutant Cry2Ab toxins were performed as previously described (Pan et al. 2014). *E. coli* BL21 (DE3) cells harbouring pET30a-*cry2Ab* were grown in LB medium

containing 35 μg/mL kanamycin. The expression of wild-type and mutants Cry2Ab protoxins were induced overnight at 25 ℃ with 0.5 mM isopropyl-B-D-thiogalactopyranoside (IPTG) after OD_{600nm} reached 0.4-0.6. Cry2Ab toxins were purified using a Ni-IDA Prepacked Column (Sangon, CHINA). Purified Cry2Ab were detected by 10% SDS-PAGE and western blotting using an anti-Cry2Ab antibody. To generate Cry2Ab activated-toxin, Cry2Ab protoxin was incubated with *P. xylostella* midgut juice (PxMJ) at 30℃ for 60 minutes at a mass ratio of 20:1 (m: m). Protein concentration of Cry2Ab toxins were determined using a BCA Protein Assay Kit (Beyotime, China).

Oligomerization assay

For oligomeric formation assays, wild-type and mutants Cry2Ab toxins were activated by PxMJ in the present of PxBBMV and exchanged into sodium carbonate buffer (50 mM, pH 9.5) using a PD-10 desalting column. The aggregation of Cry2Ab was processed at 30℃ overnight. The Cry2Ab proteins were mixed with 5×SDS-PAGE loading buffer and incubated at 60℃ for 10 min. The oligomeric formation of Cry2Ab was detected by 8% SDS-PAGE with Coomassie staining (Xu et al. 2018).

Bioassay

Bioassays of wild-type and mutants Cry2Ab toxins against second instar larvae of *P. xylostella* were estimated according to Pan et al. (Pan et al. 2014). Five or six concentrations of Cry2Ab toxins were set up and sodium carbonate buffer (50 mM, pH 9.5) was served as a negative control. Thirty second instar larvae of *P. xylostella* were used for each concentration assay and three independent replicates were performed. The median lethal concentration (LC_{50}) value were analyzed by SPSS 17.0 (Statistical Product and Service Solutions) using PROBIT analysis (Finney, 1971) (Finney et al. 1971). The relative potency (%) had been normalized to the LC_{50} value of wild-type Cry2Ab.

Liposome leakage assay

Liposome preparation was performed as described by Ding et al. (Ding et al. 2016). Phosphatidylcholine, phosphatidylethanolamine and cholesterol were dissolved in chloroform and mixed in a 4:4:2 proportion (molar mass). The mixed lipids were put in a glass vial and evaporated under a stream of nitrogen to form lipid film. The lipid film was then shaking with SUV-1 buffer (20 mM HEPES, 50 mM NaCl, 3 mM calcein, pH 7.5) at room temperature for 2 hours. Liposomes were generated by extrusion of the hydrated lipids through a 100-nm polycarbonate filter (Whatman) 35 times using a Mini-Extruder device (Avanti Polar Lipids Inc). Calcein, outside the liposome, were removed by exchanging the liposome into SUV-2 buffer (20 mM HEPES, 50 mM NaCl, pH 7.5) using a Sephadex G-50 column. Liposomes were stored at 4 °C and used within 48 h.

For liposome leakage assay, the liposome encapsulated calcein was diluted to 200 μM in SUV-2 buffer supplemented with 3 μM $MnCl_2$. The released calcein could be quenched by $MnCl_2$ in the solution. The excitation and emission wavelengths were set as 490 nm and 520 nm, respectively, to examine the fluorescence of calcein. 480 μL of liposome was added to the cuvette and the emission fluorescence was

readed as F_{t0}. 20 μL of Cry2Ab was then added to and the emission fluorescence was continuously recorded as Ft at 10s intervals. After 10 min, 20 μL of 10% Triton X-100 was added to achieve complete release of calcein and the fluorescence records were defined as F_{t100}. The percentage of liposome leakage at each time point is defined as: leakage (t) (%) = $(F_t - F_{t0}) \times 100 / (F_{t100} - F_{t0})$.

Results

Construction of Cry2Ab mutants sited-directed on helices α4-α5

Our previous studies demonstrated that helices α4-α5 in Domain I was involved in oligomerization of Cry2Ab since some Cry2Ab mutants (TM152153AA, LF156157AA, NR159160AA, LH183184AA, FI187188AA) failed to assemble 250 kDa oligomers (Fig 1A) (Xu et al., 2018). Those results suggested that the active regions for Cry2Ab oligomerization were limited to V^{150}-R^{160} in helix α-4 and N^{182}-D^{190} in helix α-5 (Fig. 1B). To further authenticate single residues for Cry2Ab oligomerization, 11 Cry2Ab mutants site-directed on helix α-4 (V150A, N151A, T152A, M153A, Q154A, Q155A, L156A, F157A, L158A, N159A, R160A) and 9 Cry2Ab mutants site-directed on helix α-5（N182A, L183A, H184A, L185A, S186A, F187A, I188A, R189A, D190A）were constructed using *Escherichia coli* expression system.

The upstream and downstream DNA fragments of Cry2Ab mutants contained mutation sites were amplified by PCR (Fig. S1A and S1B) and the full-length of DNA fragments of Cry2Ab mutants were obtained by overlap extension PCR (Fig. S1C). The Cry2Ab DNA was ligated to plasmid pET30a and transformation into BL21 competent cells. The recombinant plasmids were verified by PCR using T7 primers and a DNA product about 2300 bp could be amplified (Fig. S2). The recombinant plasmids were further digested with *Bgl* II and *Eco*R I and a pET30a fragment (about 5500 bp) and a Cry2Ab fragment (about 1900 bp) could be detected (Fig. S3). Those results suggested that the Cry2Ab DNA was ligated into pET30a. DNA sequencing revealed that the specific sites of 20 Cry2Ab mutants had been replaced by alanine.

Production and identification of Cry2Ab mutants

The expression of Cry2Ab mutants were performed under the induction of 0.5mM isopropyl-β-d-thiogalactoside. SDS-PAGE revealed that all Cry2Ab mutants could be induced-produced with a molecular weight of 65 kDa (Fig. 2A). Those Cry2Ab mutants further purified by a Ni-IDA prepacked column (Fig. 2B) and could be detected by an anti-Cry2Ab antibody (Fig. 2C). Furthermore, proteolysis assay indicated that all Cry2Ab variants could be processed into 50 kDa activated-toxins by PxMJ, which were similar to wild-type Cry2Ab (Fig. 2D). These results revealed that mutations in the helices α4-α5 did not cause a major structural disturbance in Cry2Ab.

Authentication of key residues for Cry2Ab oligomerization

The assembly of 250 kDa oligomer of variants Cry2Ab were evaluated by 8% SDS-PAGE (Fig. 3A). Wild-type Cry2Ab could form 250 kDa oligomers which was similar to our previous reports (Xu et al. 2018). Six

Cry2Ab mutants (N151A, T152A, F157A in helix α-4 and L183A, L185A, I188A in helix α-5) failed to assemble 250 kDa oligomers (defined as non-oligomerization group). Eight Cry2Ab mutants (M153A, Q154A, L156A, N159A, R160A in helix α-4 and N182A, H184A, R189A in helix α-5) greatly reduced the assembly of 250 kDa oligomers (defined as reduced oligomerization group). The remaining Cry2Ab variants (V150A, Q155A, L158A in helix α-4 and S186A, F187A, D190A in helix α-5) could aggregate and form 250 kDa pre-pore structure (defined as normal oligomerization group) which were similar to wild-type Cry2Ab.

To further assess the oligomers formation of Cry2Ab, we employed Image J software to evaluate the percentage of oligomer (oligomer / monomer × 100%). As shown in Fig 3B, the proportion of oligomer and monomer in normal oligomerization group was about 80%-110%, which was similar to wild-type Cry2Ab (about 95%). However, this value in reduced oligomerization group was about 40-60% and in non-aggregation group were less than 20%. Those results suggested that residues N151, T152, F157, L183, L185, I188 might serve as key residues for Cry2Ab oligomerization.

Oligomerization is associated with insecticidal activity of Cry2Ab

We further assessed the insecticidal activities of non-oligomerization group (N151A, T152A, F157A, L183A, L185A and I188A) Cry2Ab mutants against second instar larvae of *P. xylostella*, with wild-type Cry2Ab, V150A and S186A (two Cry2Ab mutants in normal oligomerization group) as positive control (Figure 4). The LC_{50} values of V150A and S186A was 1.978 and 1.432 μg/cm^2, which were close to that of wild-type Cry2Ab (1.458 μg/cm^2). However, the LC_{50} values of N151A, T152A, F157A, L183A, L185A and I188A were 5.097, 4.232, 3.234, 3.083, 3.579 and 3.545 μg/cm^2, respectively, all of which were higher than that of wild-type Cry2Ab (1.458 μg/cm^2).

Oligomerization is associated with pore-forming activity of Cry2Ab

The pore-forming activities of non-oligomerization group (N151A, T152A, F157A, L183A, L185A and I188A) Cry2Ab mutants were further evaluated by time course of liposome leakage assay. As shown in Figure 5, wild-type Cry2Ab could make pore on liposome and led to high leakage of calcein into solution, which was the same with V150A and S186A. The maximum calcein release percentage caused by wild-type Cry2Ab, V150A and S186A were 60.95%, 55.87%, and 58.73%. However, the leakage of calcein caused by N151A, T152A, F157A, L183A, L185A and I188A were much lower than wild-type Cry2Ab, their maximum calcein release percentage were 24.33%, 20.72%, 35.98%, 32.36%, 37.53% and 32.53%. Those data indicated that non-oligomerization mutants may weaken the pore-forming activities on liposome. Taken together, our data strongly suggested that oligomerization was tightly linked with the pore-forming activity of Cry2Ab.

Discussion

The helices α-4 and α-5 of Cry1A had been wildly reported as a pore-forming region (Girard et al. 2008; Torres et al. 2008). In other Cry toxin, helices α-4 and α-5 also reported in the oligomerization activity. Kanintronkul et al. reported that N166 and Y170 in helices α4-α5 was involved in the aggregation of Cry4B (Kanintronkul et al. 2003). Pornwiroon et al. reported that Y202 in helices α4 was responded to the oligomerization activity of Cry4A (Pornwiroon et al. 2004). In our previous study also suggested that helices α4-α5 participate in Cry2Ab self-aggregation (Xu et al., 2018). In this study, we further constructed 20 alanine mutants site directed on helices α4-α5 and showed that residues N151, T152, F157, L183, L185 and I188 may be key residues for Cry2Ab oligomerization and insecticidal activity.

The pore-formation of Cry toxins on the membrane was one of the less characterized steps and was indispensable to fully understand the mechanism action of Cry toxins (Muñoz-Garay et al. 2006). Zavala et al. demonstrated that only Domain I inserted into the liposome while Domain II and III remained in the surface of membrane (Zavala et al. 2011). This result was consistence with the umbrella model of toxin insertion. It also revealed that residues V171 and T122 involved in the insertion of Cry1A toxin into members by fluorescent studies. Angsuthanasombat et al. revealed that residue Arg-136 participated in the membrane channel formation of Cry4Ba (Angsuthanasombat et al. 2001). It was also reported that residue N183 located in the middle of helix α-5 was crucial for the insecticidal activity and pore-forming activity of Cry4Ba (Likitvivatanavong et al. 2006). Similarly, our data showed that mutations on helice α-4 and α-5 (N151A, T152A, F157A, L183A, L185A and I188A) also blocked the pore-formation of Cry2Ab on liposome.

It was worth mentioning that Cry2Ab mutants which failed to assemble 250 kDa oligomers, not only disrupted the insecticidal activity against *P. xylostella*, but also lost pore-forming activity of liposome. The results suggested that oligomerization and pore-formation was closely related in Cry2Ab. This mode of action was quite different from that of Cry1A toxin. For example, helices α-3 and α-6 of Cry1A was reported involved in oligomerization while helices α-4 and α-5 participated in pore-formation (Jiménez-Juárez et al. 2013; Lin et al. 2014). Furthermore, in Cry1A toxin, it was reported that toxins oligomerization required a binding step to cadherin, which promoted to cleavage of helix α-1 (Soberón et al. 2007). However, our previous data revealed that Cry2Ab could oligomerize in vitro after proteolysis by PxMJ in the absence of the cadherin (Xu et al. 2018). This diversity suggested that the mode of action of Cry2Ab might different from Cry1A toxin in some details and more researches were needed to clarify the elaborate difference. In conclusion, our study comprehensively identified key residues for Cry2Ab for the first time and demonstrated that non-oligomerization mutants affected the insecticidal activity as well as the pore-forming activity of Cry2Ab. It highlighted that oligomerization was closely related to insecticidal activity and pore-forming activity of Cry2Ab, which could make a closer look on the mode of action of Cry2Ab toxins.

Declarations

Availability of data and material

The supplementary materials are available online.

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Authors' contributions

YZ provided conceptual framework and technical oversight on all experiments; ZP and LX conducted the experiments and analyzed the experimental data. YZ, ZP and LX wrote the manuscript; QC and BL revised the manuscript. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

All authors have provided consent for this publication.

Competing interests

All the authors declare that they do not have any conflict of interest.

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Figures

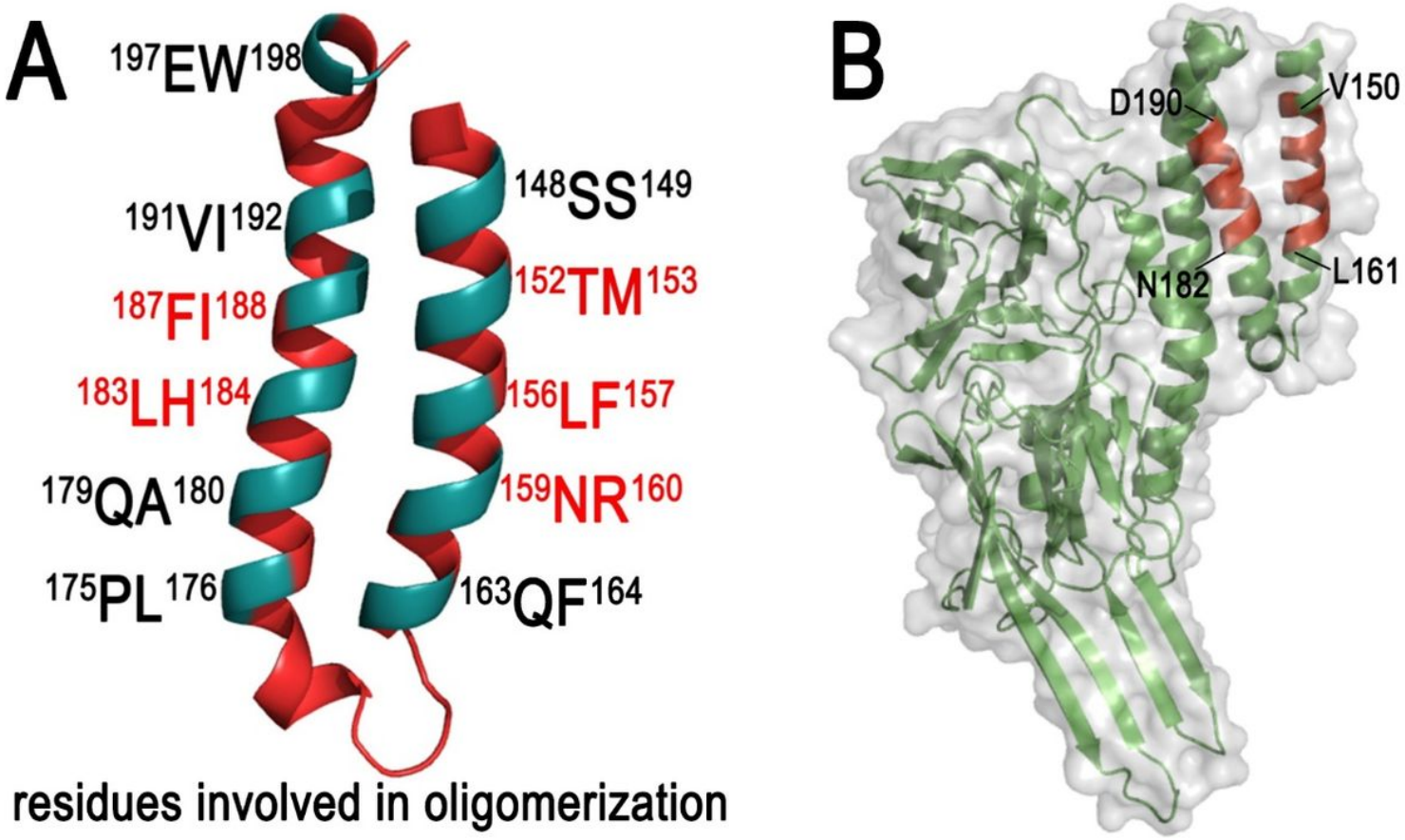

Figure 1

(A) Helices α4-α5 were involved in Cry2Ab oligomerization. (B)The design and construction of alanine mutants of Cry2Ab site-directed within the helices α4-α5.

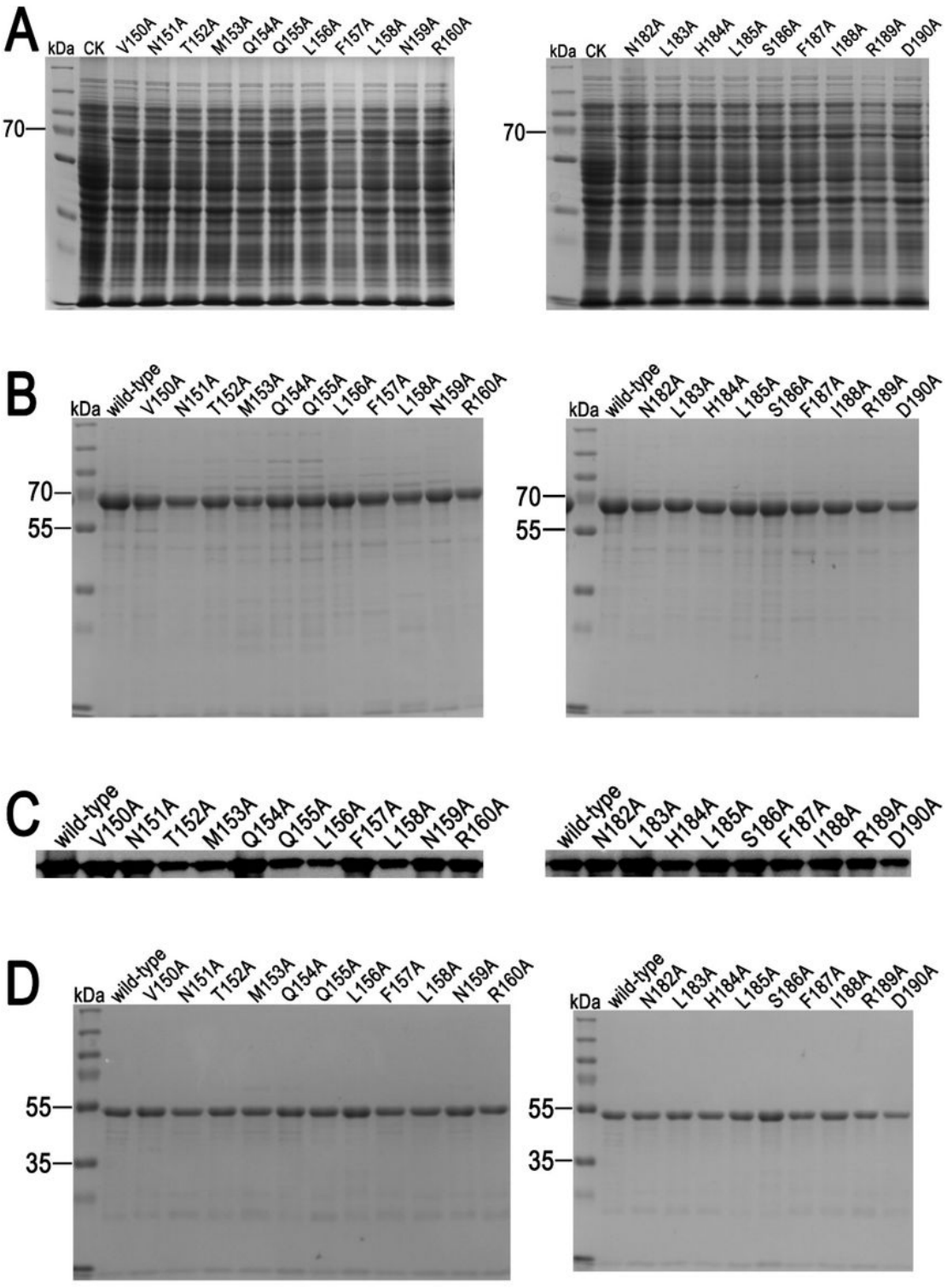

Figure 2

Expression, purification and identification of Cry2Ab mutants. (A)The induced expression of Cry2Ab variants site-directed on helices α4-α5. (B) The purity of Cry2Ab mutants detected by SDS-PAGE followed by Coomassie blue staining. (C) Identification of Cry2Ab mutants using an anti-Cry2Ab antibody. (D) The proteolytic activation of Cry2Ab mutants by PxMJ.

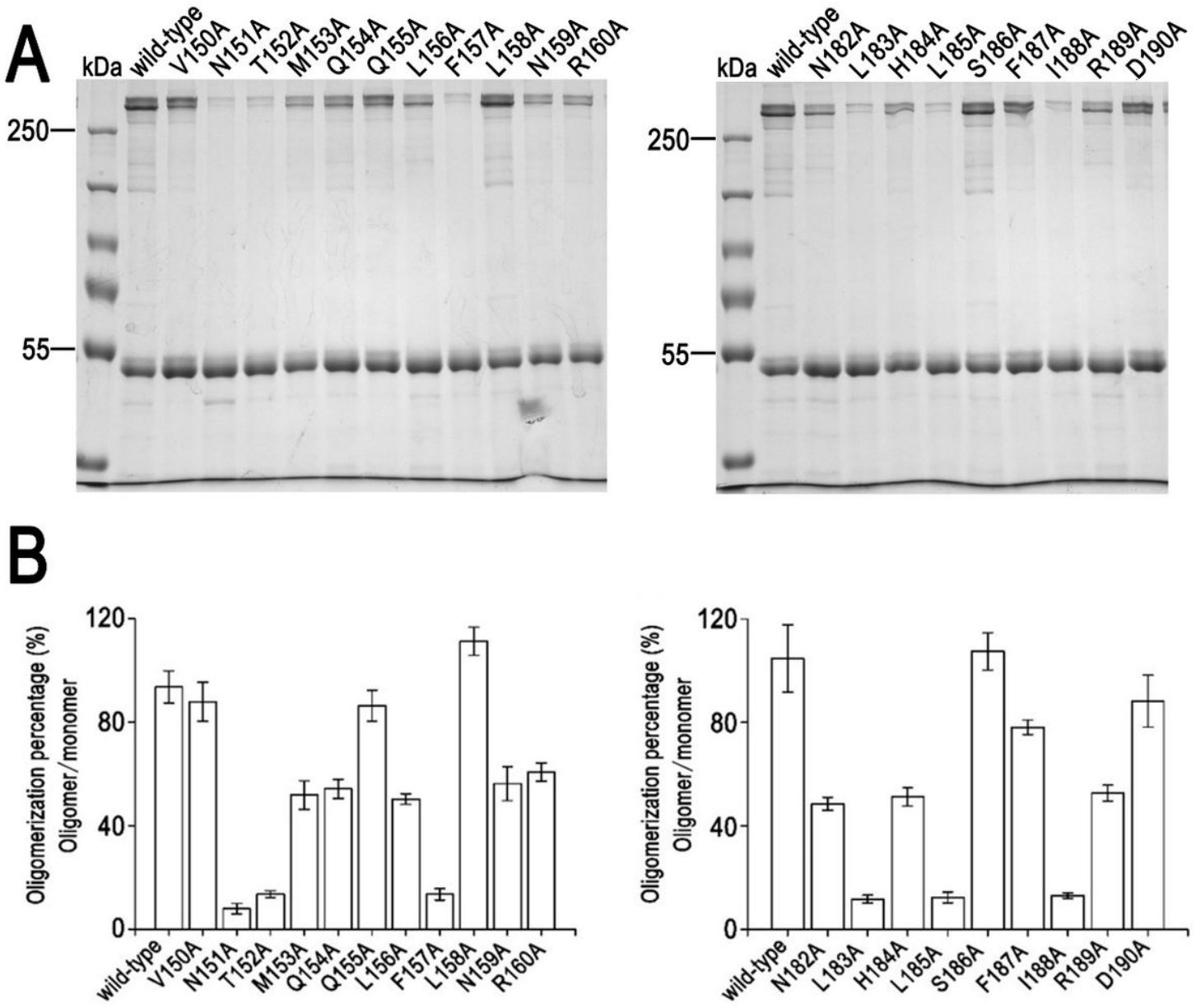

Figure 3

Oligomerization assay of Cry2Ab mutants. (A) Assemble of Cry2Ab oligomers detected by SDS-PAGE followed by Coomassie blue staining. (B) Calculation of the percentage of oligomer and monomer in Cry2Ab by image J.

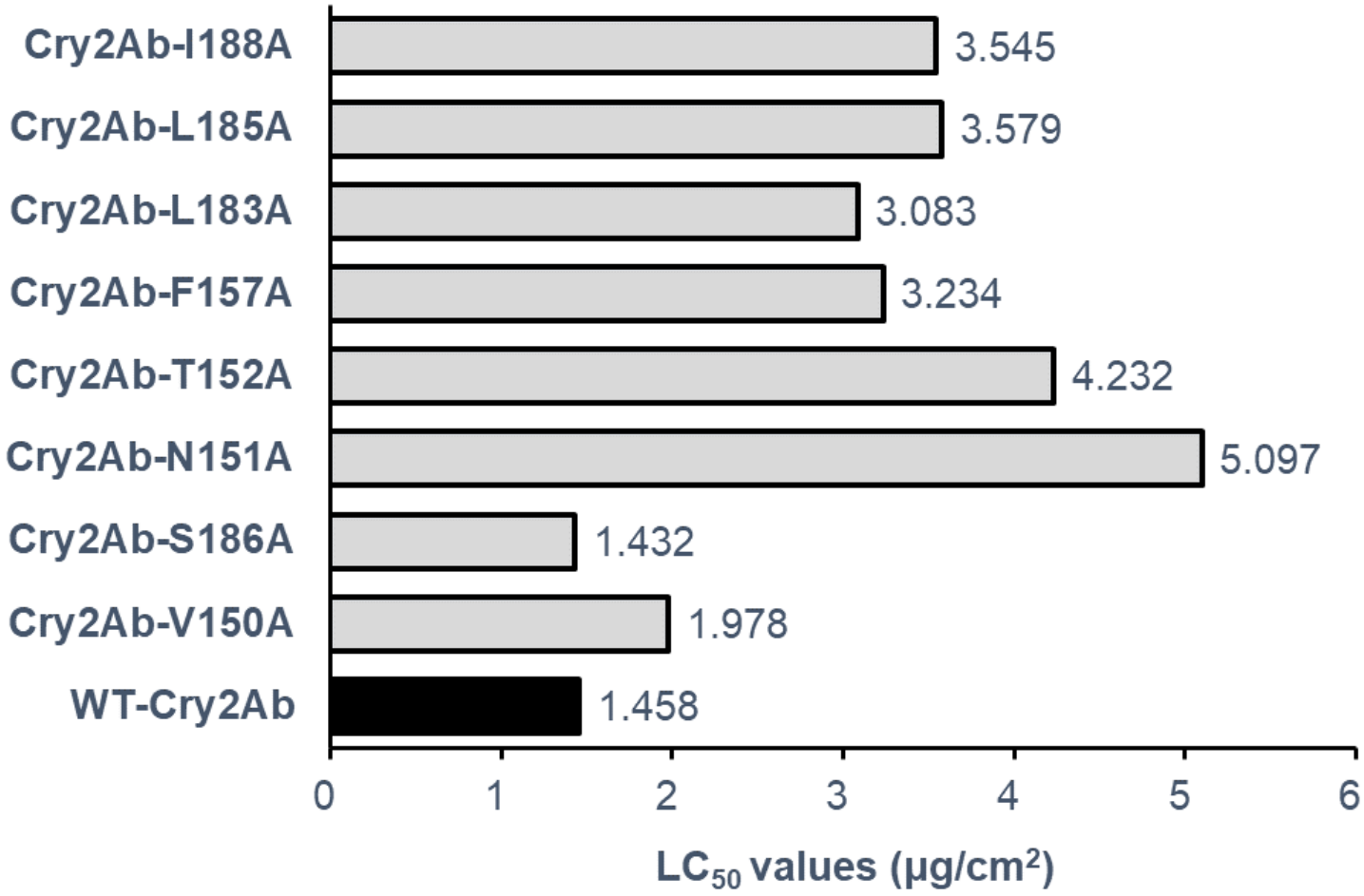

Figure 4

Comparison LC50 values of mutants Cry2Ab (site-directed on the helices α4-α5) against 2nd instar larvae of Plutella xylostella with wild-type Cry2Ab. WT-Cry2Ab means the wild-type Cry2Ab.

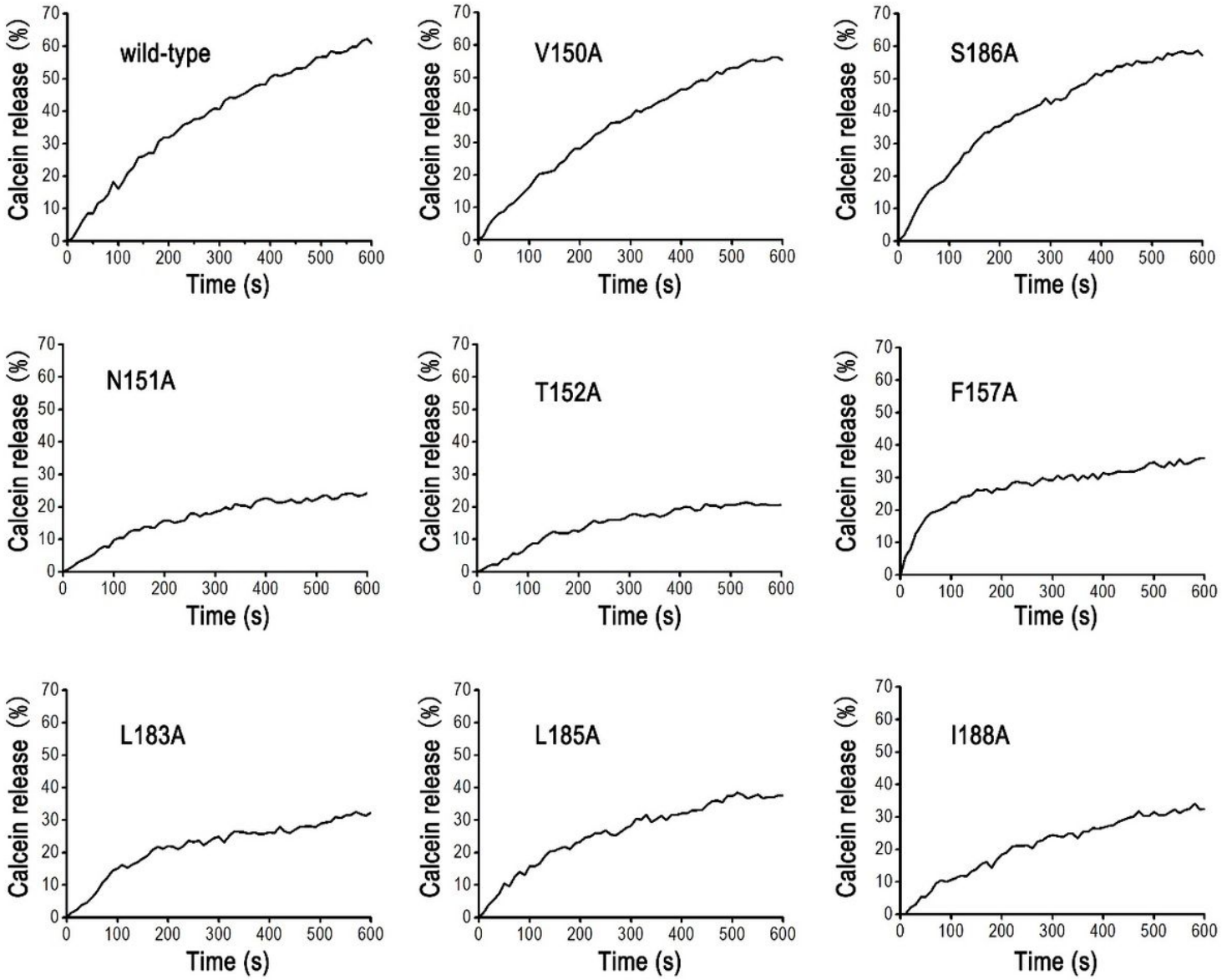

Figure 5

Liposome leakage assay detected pore-forming activity of Cry2Ab mutants.

Supplementary Files

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- SupplementaryMaterial.docx