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Cry4Ba toxin of *Bacillus thuringiensis* subsp. *israelensis* uses both domains II and III to bind to its receptor—*Aedes aegypti* alkaline phosphatase

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ABSTRACT

Receptor binding is one of the crucial steps to exhibit the insecticidal activity of Cry toxins. In addition, binding to receptors is a determining step for the specificity of toxins. In this work, receptor binding domain II was cloned from the full-length Cry4Ba toxin and heterologously expressed in *Escherichia coli*. The 21 kDa purified protein was characterized as Cry4Ba domain II using Western blotting and tandem mass spectrometry coupled to liquid chromatography. Circular dichroism revealed the correct folding of the isolated domain II fragment, similar to that found in the Cry4Ba protein. Binding analysis using an enzyme-linked immunosorbent assay revealed that the purified Cry4Ba-domain II had bound to the 54 kDa alkaline phosphatase cloned from *Aedes aegypti* (Aa-mALP) with a dissociation constant of approximately 116.27 ± 11.09 nM. The binding affinity of Cry4Ba-domain II to Aa-mALP was comparable to that of Cry4Ba domain III, suggesting that both domains II and III of the Cry4Ba contributed equally in binding to the Aa-mALP protein. Our findings should provide more valuable insight on the molecular mechanisms in the toxin-receptor interaction of the Cry4Ba toxin.

1. Introduction

Intensive use of chemical insecticides leads to leaching of toxic residues into the environment which can have adverse effects on other non-target organisms—not only to beneficial insects but also to humans. Each year, more than 26 million people suffer from direct or indirect contact with pesticides, resulting in nearly 220,000 deaths [1]. In addition, there are significant increased risks associated with several type of cancers that have been reported to be associated with exposure to pesticides (for example, see Ref. [2]). In addition, the continuous use of chemical insecticides may lead to the development of tolerance in insect pest populations. Increasing concerns regarding environmental damage, human health problems, and insect resistance have paved the way for biological insecticide alternatives that could offer sustainable insect control instead of using chemicals.

Safety and specificity are major advantages of microbial insecticides over chemical agents to control insect pests. *Bacillus thuringiensis* (*Bt*) is one of the most successful microorganisms that has been used as a bioinsecticide to control the larvae of agricultural insect pests and insect vectors of human disease. *Bt* is a Gram-positive bacteria that produces proteinaceous crystalline inclusions

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known as δ-endotoxins or insecticidal crystal proteins, which are composed of crystal (Cry) and cytolytic (Cyt) toxins during the sporulation phase [3]. After 100 years since its discovery and more than 80 years since its first commercial production, *Bt* has been increasingly used inform of its crystal toxins or the heterologous expression of its toxin-encoding genes in transgenic plants (see Refs. [4,5]). Cry proteins are the most explored and studied of *Bt* toxins. Up until now, more than 800 Cry proteins (grouped into 78 subfamilies) have been discovered from different subspecies of *Bt* (available at http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/) [6]. Although *Bt* has been successfully used to control the larvae of insect pests and vectors for a long time, the mechanisms of its toxins are still not fully understood.

Cry toxins share a similar fold structure that is composed of three functional domains [7]. The most approved and widely accepted mode of action of Cry toxins is the lysis of epithelial cells in the insect midgut (see Ref. [8]). Domain I is composed of seven alpha helices and has been shown to be involved in membrane insertion and pore formation. Domains II and III are a beta prism and beta sandwich, respectively that have been reported to function in interaction with midgut receptor proteins [7]. Domains II and III were shown to remain on the membrane surface after insertion of the domain I into the membrane [9]. Specificity determinants of Cry toxins toward target insects rely on several steps, such as exposure to the insecticidal toxins, crystal solubilization, toxin process and stability, toxin sequestration, crossing the peritrophic matrix, and binding to receptors (for a review, see Ref. [10]). Among the factors involved in target specificity, receptor binding is one of the critical steps to exert larvicidal activity [10]. Several midgut proteins, such as aminopeptidase N (APN), alkaline phosphatase (ALP), and cadherin-like protein, and glycolipids have been reported as functional receptors for Cry toxins [11].

For the Cry4Ba toxin, a mosquitocidal toxin produced by *B. thuringiensis* subsp. *israelensis,* membrane-bound ALP (Aa-mALP) and APN (Aa-mAPN) located in the *Aedes aegypti* midgut were reported as its functional receptors [12–15]. In our recent studies, we showed that mutation on surface residues on the Cry4Ba domains II and III affected binding to the Aa-mALP receptor and hence toxicity toward *A. aegypti* larvae [16,17]. In addition, we showed that the isolated domain III fragment was bound to the Aa-mALP receptor with a dissociation constant (K_d) of about 110 nM [17]. However, the binding affinity of the isolated domain II fragment to the Aa-mALP receptor remains to be explored. Therefore, in the current work, we subcloned and expressed the domain II fragment from the Cry4Ba toxin and studied its binding to the Aa-mALP receptor using enzyme-linked immunosorbent assay (ELISA) and pulldown assay.

2. Materials and methods

2.1. Materials

Oligonucleotide primers for amplification of the Cry4Ba-domain II were purchased from Macrogen (Seoul, South Korea). *Nde*I (#RE1266) and *Xho*I (#RE1332) restriction enzymes, isopropyl-β-D-thiogalactopyranoside (IPTG) (#PC0708), and *pfu* DNA polymerase (#PL5201) were purchased from Vivantis Technologies (Selangor, Malaysia). The 96-well immunoplates (#32296) were purchased from SPL Life Science (Gyeonggi, South Korea). ProFound™ Pull-Down Poly His protein: protein interaction kit (#21277), tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (#20233), Zeba spin desalting columns (7 kDa molecular weight cut-off, MWCO; #89889), silver staining kit (#K0681), and nickel-nitrilotriacetic acid (Ni-NTA) affinity columns (#88225) were purchased from Thermo Fisher Scientific (Rockford, USA). The rabbit polyclonal antibody against Cry4Ba was kindly provided by the Bacterial Toxin Research Innovation Cluster (BRIC), Institute of Molecular Biosciences, Mahidol University (Bangkok, Thailand). Goat anti-rabbit horseradish peroxidase (HRP)-linked IgG (#7074S) and 3,3,5,5-tetramethylbenzidine (TMB; #7004S) were purchased from Cell Signaling Technology (Beverly, USA). Goat anti-rabbit alkaline phosphatase (ALP) -linked IgG (#AP132A) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium liquid substrate reagent (BCIP/NBT) (#B1911) were purchased from Sigma-Aldrich (St. Louis, USA). Bradford's reagent (#ML106) was purchased from HiMedia Laboratories (Maharashtra, India). Vivaspin concentrator columns (10 kDa MWCO) (#28932296) were purchased from Cytiva (Uppsala, Sweden). All other reagents used in the study were of analytical grade.

2.2. Cloning of Cry4Ba-domain II

The DNA fragment of 555 bp encoding the Cry4Ba-domain II (from Glu_{286} to Asp_{470}) was amplified using high-fidelity *pfu* DNA polymerase with the DII-F forward primer, containing an additional *Nde*I recognition site, with the start codon (5′-CCCCC**CATATG***GAATTTACAAGAGAGATTTATACA*-3′, where bold letters indicate the *Nde*I recognition site, underlined letters indicate the additional start codon, and the 5′ specific Cry4Ba-domain II sequence is shown in italic type), and the DII-R reverse primer containing the additional stop codon and *Xho*I recognition (5′-AAAAA**CTCGAG**CTA*GTCAACAATC TTATGTGTCC*-3′, where bold letters indicate the *Xho*I recognition site, underlined letters indicate the additional stop codon, and the 3′ specific Cry4Ba-domain II sequence is shown in italic type). The p4Ba-R203Q plasmid containing the full-length Cry4Ba toxin gene was used as a template. Thirty cycles of amplification were performed with denaturation at 94 °C for 20 s, annealing at 52 °C for 30 s, and extension at 72 °C for 1 min. The PCR products were double digested with *Nde*I and *Xho*I and ligated into the pET-17b vector and then transformed into *E. coli* strain BL21 (DE3). Recombinant clones on Luria-Bertani (LB) agar containing 100 μg/mL ampicillin were selected for further characterization using restriction digestion. The correction of sequences of the Cry4Ba-domain II was confirmed using the nucleotide sequencing services of First BASE Laboratories (Selangor, Malaysia).

2.3. Expression and purification of Cry4Ba-domain II

Recombinant clone containing pET17b-Cry4Ba-DII was grown in 500 mL of LB broth, supplemented with 100 μg/mL ampicillin, at 37 °C with shaking at 100 rpm until the optical density at 600 nm (OD_{600}) reached 0.3–0.5 (approximately 2 h). Expression of the Cry4Ba-domain II fragment was induced with 0.1 mM IPTG for 4 h; then, it was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a vertical minigel Hoefer SE260 Mighty Small II system (Hoefer; Holliston, USA). Cells were harvested using centrifugation at 6000×*g* for 10 min at 4 °C and the pellets were resuspended in 30 mL of 0.1 M KH_2PO_4 pH 5.0 containing 0.5% Triton X-100. Resuspended cells were lysed on ice with five cycles of 20 s pulses and 30 s rest using a Sonics Vibra cell sonicator (Bioblock Scientific; Illkirch, France) set at 40% amplitude and centrifuged at 12,000×*g* for 10 min. Inclusion bodies were washed sequentially with washing buffer I (0.1 M KH_2PO_4, pH 5.0, 0.1% Triton X-100, 0.8 M NaCl), washing buffer II (0.1 M KH_2PO_4, pH 5.0), and diH_2O, respectively. The obtained inclusion bodies were further dissolved in phosphate buffered saline (PBS) containing 8 M urea (pH 7.5) for 1 h at 25 °C. The suspension was centrifuged at 12,000×*g* for 10 min to separate the solubilized protein. Refolding of Cry4Ba-DII was carried out using serial dialysis in refolding buffers (PBS buffer at pH 8.0 containing different concentrations of urea: 6 M, 4 M, 2 M, 1 M, 0.5 M, and 0 M) for 12 h each at 4 °C. Then, the refolded sample was centrifuged at 12,000×*g* for 30 min at 4 °C to separate the soluble and refolded proteins. Next, the refolded Cry4Ba-DII was purified using a fast protein liquid chromatography (FPLC) system on a Superdex-200 HR gel filtration column (Amersham-Pharmacia Biotech; Piscataway, USA). Then, the purified Cry4Ba-DII was concentrated and the buffer was replaced with 50 mM carbonate buffer (50 mM Na_2CO_3/$NaHCO_3$, pH 9.2) using Vivaspin (10 kDa MWCO) concentrators. The purified Cry4Ba-domain II was analyzed using SDS-PAGE and its concentration was determined based on Bradford assay.

2.4. Characterization of the Cry4Ba-domain II fragment

2.4.1. Western blot analysis

Western bot analysis was performed to analyze the expression of the Cry4Ba-domain II fragment. After analysis of the purified Cry4Ba-domain II fragment using SDS-PAGE, the purified Cry4Ba-domain II protein on polyacrylamide gel was transferred onto a nitrocellulose membrane using a Hoefer TE Series Transphor electrophoresis unit (Hoefer; Holliston, USA). The membrane was blocked with 5% skimmed milk in PBS (pH 7.4) for 1 h at room temperature (RT). The membrane was washed 3 times with PBS containing 0.1% Tween 20 (PBS-T) and incubated overnight at 4 °C with anti-Cry4Ba antibody at a 1:1000 dilution in the blocking solution. After washing 3 times with PBS-T, the membrane was incubated with ALP-conjugated anti-rabbit IgG at a 1:5000 dilution in the blocking solution for 30 min at RT. The membrane was washed 3 times with PBS-T and then the phosphatase activity was developed using the BCIP/NBT chromogenic substrate.

2.4.2. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

LC-MS/MS was performed to verify the heterologous expression Cry4Ba-domain II protein. The purified Cry4Ba-domain II protein was analyzed on SDS-PAGE and excised from the gel using a razor blade. In-gel digestion and LC-MS/MS analysis of the tryptic peptides were performed using standard methods serviced by the Research Instrument Center, Khon Kaen University, Thailand. Briefly, protein in the gel piece was reduced, alkylated, and then subjected to in-gel protease digestion with sequencing grade trypsin. The tryptic peptides were analyzed using an EASY-nLC II nano-liquid chromatography system (Bruker Daltonics; Bremen, Germany) coupled to a MicrOTOF-Q II hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer (Bruker Daltonics; Bremen, Germany) equipped with captive spray ionization (Bruker Daltonics; Bremen, Germany). One microliter (~1000 ng) of sample was resolved in an Easy-Column (10 cm × 75 μm, 3 μm particle size, C18; Thermo Fisher Scientific; Waltham, USA) at a flow rate of 300 nL/min. The gradient mobile phase consisted of solution A (0.1% formic acid) and solution B (acetonitrile containing 0.1% formic acid). The gradient mode was set as solution B concentration from 30% to 60% for 20 min and from 60% to 80% for 10 min. Mass spectrometry was performed in the positive-ion mode using a range of 50–3000 *m/z*. The obtained data were analyzed using the Mascot MS/MS ions search procedure (https://www.matrixscience.com/search_form_select.html) against the SWISS-PROT protein database.

2.4.3. Secondary structural analysis using far-ultraviolet (UV) circular dichroism (CD) spectroscopy

The CD spectrum was analyzed to evaluate the changes in the secondary structure of the Cry4Ba-domain II fragment. The purified Cry4Ba-domain II was diluted to 1 mg/mL in 50 mM carbonate buffer (pH 9.2). The spectrum of the far-UV region (190–250 nm) was recorded at 25 °C in a quartz cuvette (1.0 cm path length) at a scan speed of 20 nm/min using a J-815 spectropolarimeter (Jasco Analytical Instruments; Tokyo, Japan). A baseline measurement using 50 mM carbonate buffer (pH 9.2) was performed for subsequent subtraction from the spectrum. The measured ellipticity was converted to molar ellipticity [θ] expressed as deg cm^2 /dmol and plotted versus the wavelength.

2.5. Preparation of Cry4Ba active toxin

A sample of 65-kDa Cry4Ba active toxin was prepared according to Ref. [18], with some modifications. In brief, *E. coli* JM109 containing pCry4Ba-R203Q plasmid encoding the 130 kDa full length toxin, in which a trypsin-sensitive residue Arg_{203} was mutated to Gln for easy purification of a 65 kDa trypsin-activated, single polypeptide chain (see Ref. [18]), was cultivated in LB broth containing 100 μg/mL ampicillin until the OD_{600} nm reached 0.3–0.5; then, the Cry4Ba-R203Q protein expression was induced with 0.1 mM IPTG for 4 h. Inclusion bodies were prepared as mentioned above and then solubilized in 50 mM carbonate buffer (pH 9.2) at 37 °C for 1 h.

The solubilized protoxins were activated proteolytically by incubating with TPCK-treated trypsin (at a ratio 1:20 (w/w) enzyme/toxin in carbonate buffer, pH 9.2) at 37 °C for 16 h. The 65 kDa-activated toxin was purified using an FPLC system on a Superdex-200 HR gel filtration column (Amersham-Pharmacia Biotech; Piscataway, USA) as described by Ref. [19] and then concentrated using Vivaspin (10 kDa MWCO) concentrators. The purified Cry4Ba-R203Q protein was determined for its concentration using the Bradford assay and then analyzed using SDS-PAGE.

2.6. Preparation of Cry4Ba-domain III fragment

The Cry4Ba-domain III protein was prepared according to Ref. [20]. In brief, *E. coli* JM109 containing pMEx-4BD3 plasmid encoding the Cry4Ba domain III protein was cultivated at 37 °C in LB broth containing 100 μg/mL ampicillin with shaking at 100 rpm until the OD_{600} reached 0.3–0.5. Protein expression of Cry4Ba-domain III was induced with 0.1 mM IPTG for 4 h. After induction, the cells were harvested using centrifugation at 6000×*g* for 10 min at 4 °C and resuspended in 50 mM Tris-HCl, pH 7.4. The cells were broken using sonication on ice, as described above. The sonicated lysate was centrifuged at 10,000×*g* for 60 min at 4 °C. The supernatant was collected to purify the 21 kDa Cry4Ba-domain III fragment using anion-exchange with a Resource Q column (GE Healthcare Bio-Sciences; Piscataway, USA) and size-exclusion FPLC, as described by Ref. [20]. The purified Cry4Ba domain-III fragment was determined for its concentration using the Bradford assay and analyzed using SDS-PAGE.

2.7. Preparation of Aedes aegypti alkaline phosphatase−Cry4Ba toxin receptor

Membrane-bound alkaline phosphatase from the *A. aegypti* midgut (Aa-mALP), functioning as a Cry4Ba toxin receptor, was prepared according to Ref. [15], with some modifications. In brief, *E. coli* BL21(DE3) containing pET-Aa-mALP encoding C-terminal 6× Histidine-tagged Aa-mALP protein was grown at 30 °C in LB broth containing 100 μg/mL ampicillin with shaking at 100 rpm. When the OD_{600} nm of the culture reached 0.3–0.5, the expression of Aa-mALP protein was induced with 0.1 mM IPTG for 4 h. Cells were harvested, and inclusion proteins were prepared using the method described above. The His-tag fused Aa-mALP inclusion bodies were solubilized in PBS buffer (pH 7.5) containing 8 M urea for 1 h at 25 °C. The Aa-ALP protein was simultaneously purified and refolded in an Ni-NTA affinity column. Aa-ALP protein was eluted from the column using PBS buffer containing 500 mM imidazole. Desalting and changing to 50 mM carbonate buffer (pH 9.2) were performed in Zeba spin desalting columns and concentrated on 10 kDa MWCO concentrator columns, according to the manufacturer's protocols. The purified alkaline phosphatase was analyzed using SDS-PAGE and its concentration was determined based on Bradford assay.

2.8. Enzyme-linked immunosorbent assays for Cry4Ba domain II-Aa-mALP receptor interaction

ELISAs were performed to study the interaction between the Cry4Ba-domain II and the Aa-mALP receptor compared to those of the 65 kDa full-length Cry4Ba toxin and the Cry4Ba-domain III, based on methods in Ref. [17], with slight modifications. In brief, 96-well Maxi-binding microplates were coated with Aa-mALP receptor (2.5 μg in 50 mM carbonate buffer, pH 9.0) and left overnight at 4 °C. The plates were washed 3 times with PBS-T (PBS pH 7.4, 0.05% Tween-20) and blocked with 5% skimmed milk in PBS-T, pH 7.4 for 1 h at RT. After washing 3 times with PBS-T, the wells were incubated with either purified full-length Cry4Ba protein, domain II fragment, domain III fragment, or bovine serum albumin (BSA) at different concentrations (100, 200, 300, or 400 nM) at 37 °C for 2 h. After washing, the Cry4Ba protein or its domain II or III fragments that had bonded to the immobilized Aa-mALP receptor were detected by incubating for 1 h at 37 °C with rabbit anti Cry4Ba antibody (1:10,000 dilution), followed by incubating for 1 h at 37 °C with goat anti-rabbit HRP-linked IgG (1:5000). After washing, the wells were developed using incubation for 10 min with 0.1 mL of TMB substrate; this reaction was terminated by adding 0.1 mL of 1 N HCl and the resulting absorbance was determined at 450 nm using a 96-well microplate reader (Multiskan; Vienna, USA).

2.9. Pulldown assay for Cry4Ba-Domain II-Aa-mALP receptor interaction

Pulldown assays were performed to study the interactions between hexahistidine-tagged Aa-mALP as the bait protein and Cry4Ba or its domains II or III as the prey protein using a ProFound™ Pull-Down Poly His protein: protein interaction kit according to the manufacturer's protocol. In brief, 100 μg (1.8 nmol) of purified 6-His tagged Aa-mALP was incubated with 50 μL of HisPur cobalt resin in the Pierce spin columns for 1 h at 4 °C and washed 5 times with washing buffer. Immobilized Aa-mALP was incubated with 31.5 μg (1.5 nmol) of domain II, 31.5 μg (1.5 nmol) of domain III, or 100 μg (1.5 nmol) full-length Cry4Ba proteins for 2 h at 4 °C and then washed 5 times with washing buffer. Next, the bait and prey proteins were eluted from the columns with 250 μL of elution buffer containing 290 mM imidazole. After desalting and concentration, the eluted proteins were analyzed using SDS-PAGE, and LC-MS/MS.

2.10. Statistical analysis

The experiments were done in triplicate and repeated three times. Student's *t*-test was used to identify significant differences between the binding of full-length Cry4Ba or its domains DII or DIII to the Aa-mALP receptor based on a significance test level of $p < 0.05$.

3. Results

3.1. Expression and purification of Cry4Ba-domain II fragment

The Cry4Ba-domain II encoding region was successfully amplified, cloned into the *Nde*I-*Xho*I sites of the pET-17b vector, and transformed into *E. coli* BL21. After IPTG induction at 37 °C for 4 h, the Cry4Ba-domain II was heterologously expressed as approximately 21 kDa (calculated molecular mass = 21.02 kDa, as shown in Fig. 1A, lane 1). The Cry4Ba-domain II was expressed as insoluble protein and accumulated as inclusion bodies in *E. coli* (Fig. 1A, lanes 4). The inclusion bodies were dissolved in PBS buffer containing 8 M urea, refolded using serial dialysis, and purified using size exclusion chromatography. The FPLC elution profile revealed that 21-kDa Cry4Ba-DII was eluted as a single peak (Fig. 1B). The SDS-PAGE result of the purified domain II is shown in Fig. 1A, lane 5.

3.2. Characterization of Cry4Ba-domain II fragment

The heterologously expressed Cry4Ba-domain II was further characterized using Western blot, CD spectroscopy, and LC-MS/MS analyses. The 21 kDa purified Cry4Ba-DII was separated on SDS-PAGE (Fig. 2A) and analyzed using Western blot analysis with anti Cry4Ba polyclonal antibody. The Western blot revealed a specific band (about 21 kDa) of the purified Cry4Ba domain II which was similar to that expressed in *E. coli* (Fig. 2B). CD spectroscopy analysis was used to analyze the secondary structure of the refolded Cry4Ba-domain II protein. The CD spectrum of the refolded Cry4Ba-domain II is shown in Fig. 2C. The peptide mass fingerprint from a tryptic digest gel-purified 21 kDa band was analyzed using electrospray mass spectrometry. The mass spectrum of the tryptic digest of Cry4Ba domain II is shown in Fig. 3A. MS/MS ions were searched against the SWISS-PROT protein database using a Mascot MS/MS

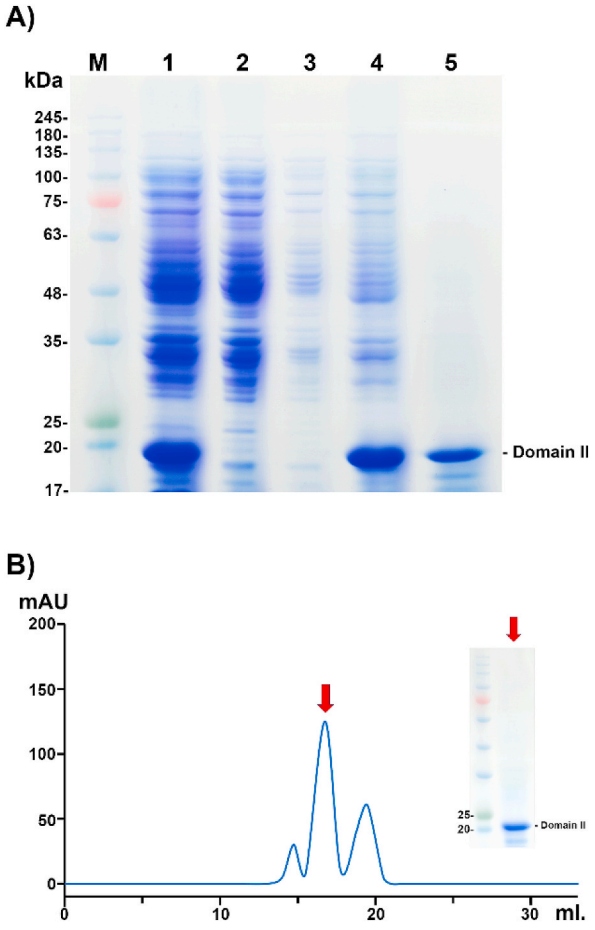


Fig. 1. Expression and purification of Cry4Ba domain II (A) SDS-PAGE (Coomassie brilliant blue-stained 10% gel) analysis of expression and purification of Cry4Ba-domain II, where lane M: molecular mass standard; lane 1: protein expression of *E. coli* harboring pET17b-Cry4Ba-DII; lane 2: protein expression of *E. coli* harboring pET17b (negative control); lane 3: soluble fraction of cell lysate of induced *E. coli* harboring pET17b-Cry4Ba-DII; lane 4: insoluble fraction of cell lysate of induced *E. coli* harboring pET17b-Cry4Ba-DII; lane 5: purified Cry4Ba domain II. Uncropped image is available as supplementary file S1. (B) Size exclusion elution profile of the Cry4Ba-domain II on a Superdex 200 column. Inset, SDS-PAGE (Coomassie brilliant blue-stained 10% gel) analysis of a selected peak (arrow) containing approximately 21 kDa Cry4Ba domain II.

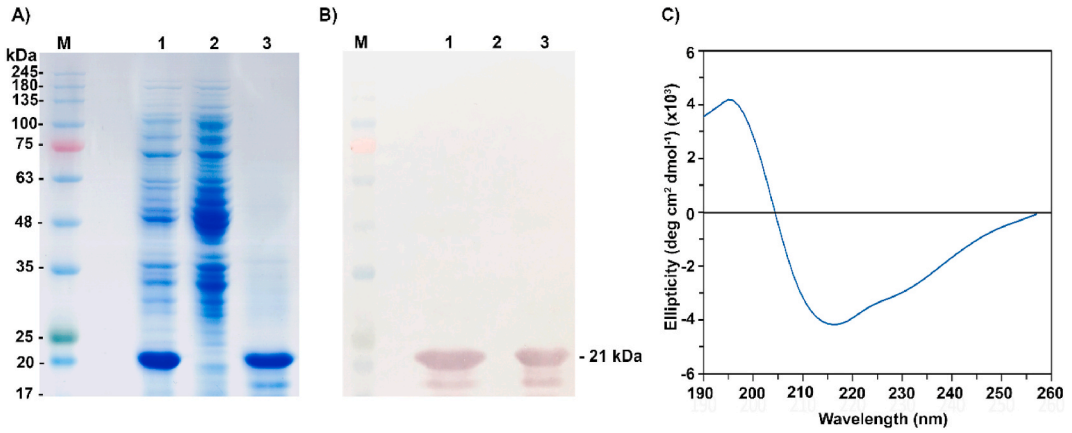
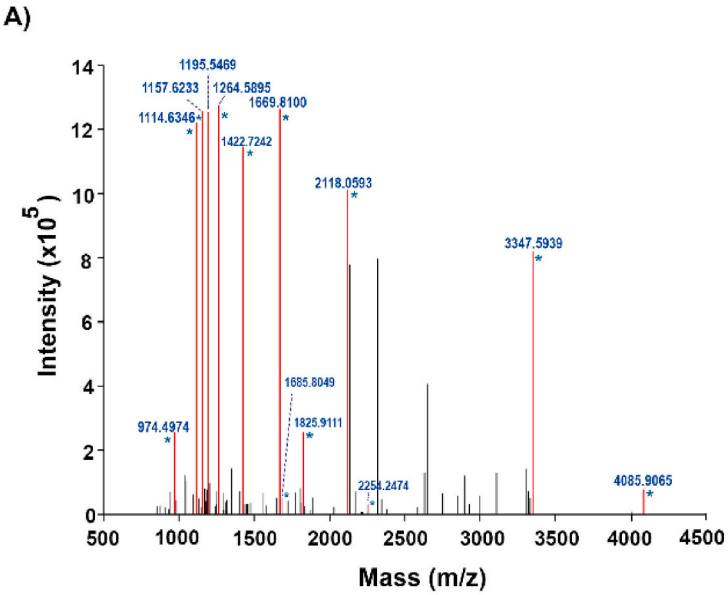


Fig. 2. Characterization of purified Cry4Ba-domain II (A) SDS-PAGE (Coomassie brilliant blue-stained 10% gel) analysis and (B) Western blot analysis using anti Cry4Ba antibody, where lane M: molecular mass standard; lane 1: induced *E. coli* harboring pET17b-Cry4Ba-DII (positive control); lane 2: induced *E. coli* harboring pET17b (negative control); lane 3: purified Cry4Ba-DII protein. Uncropped images are available as supplementary file S1. (C) CD spectrum of the purified Cry4Ba-domain II in 50 mM carbonate buffer (pH 9.2).



B)

1 EFTR**EIYTAL VESPSSKSIA ALEAALTRDV HLFTWLKRVD FWTNTIYQDL**

51 **R**FLSAN**KIGF SYTNSSAMQE SGIYGSSGFG SNLTHQIQLN SNVYKTSITD**

101 **TSSPSNR**VTK MDFYK**IDGTL ASYNSNITPT PEGLRTTFFG FSTNENTPNQ**

151 **PTVNDYTHIL SYIKTDVIDY NSNRVSFAWT HK**IVD

Fig. 3. (A) Mass spectrum of the tryptic digest of Cry4Ba domain II, where matched peaks in peptide mass fingerprinting are highlighted in red color and marked using asterisks (B) Peptide sequences matching with domain II of the Cry4Ba (SWISS-PROT accession number P05519: pesticidal crystal protein Cry4Ba from *Bacillus thuringiensis* subsp. *israelensis*). Red bold letters show the peptides matched. Sequence coverage is 89% (164/185 amino acids of Cry4Ba domain II).

ions search. The results revealed 71 matching peptides covering 89% (164/185 amino acids) of the domain II of the Cry4Ba toxin (SWISS-PROT accession number P05519: pesticidal crystal protein Cry4Ba from *Bacillus thuringiensis* subsp. *israelensis*), as shown in Fig. 3B, with a Mascot score of 2858. The MS/MS spectra of matched peptides are shown in supplementary file S2.

3.3. Binding analysis of Cry4Ba-domain II to Aa-mALP receptor

Interactions between the Cry4Ba-domain II and the Aa-mALP receptor were studied using ELISA and pulldown assays. Cry4Ba full-length (65 kDa) and its domain III truncated fragment (21 kDa) were successfully obtained (supplementary Fig. S1) and used for binding comparison. $His_{(6)}$-tagged Aa-mALP (54 kDa)—a functional receptor for Cry4Ba toxin—was efficiently prepared (supplementary Fig. S2) and used as a partner protein for binding analysis. The ELISA assays revealed saturation binding between the Cry4Ba-domain II and the Aa-mALP receptor similar to those of the full-length Cry4Ba-R203Q and domain III fragment (Fig. 4A). The dissociation constant (K_d) of binding between the Cry4Ba-domain II and the Aa-mALP receptor was approximately 116.27 ± 11.09 nM, which was not significantly different ($p = 0.8787$) to that of Cry4Ba-domain III ($K_d = 117.56 \pm 8.10$ nM); however, it was significantly

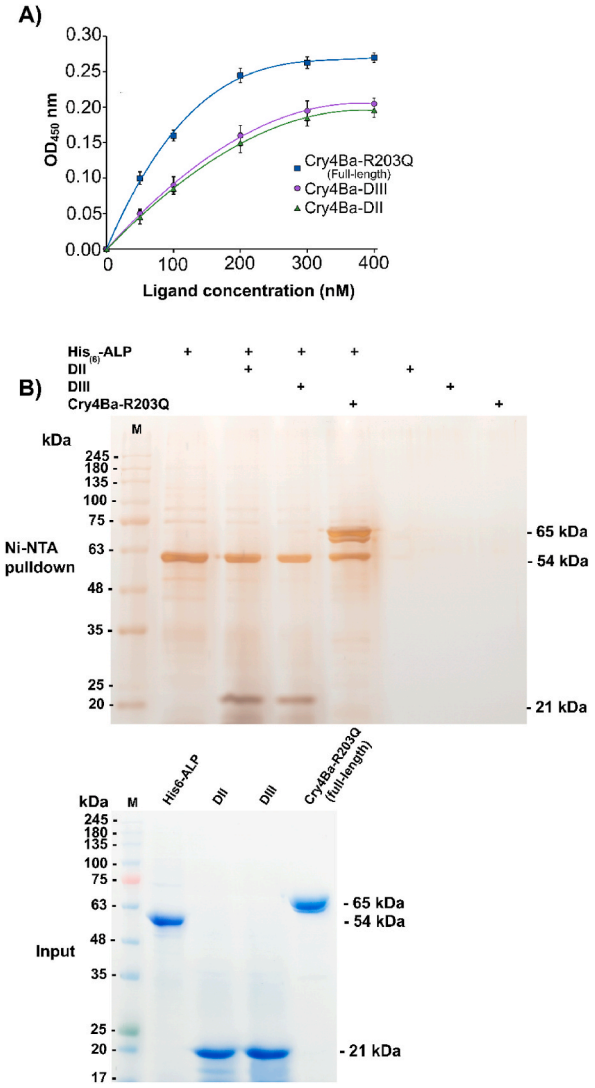


Fig. 4. A) ELISA binding assay of Cry4Ba-R203Q (full-length toxin) or its domain II or domain III fragments with *A. aegypti* alkaline phosphatase (Aa-mALP) receptor. Error bars represent the standard error of three independent experiments, with the obtained data already subtracted for nonspecific binding controls. B) Pulldown interaction analysis of Cry4Ba-R203Q or its domain II or domain III with Aa-mALP. Silver stained SDS-PAGE (10% gel) of a pulldown assays using $His_{(6)}$-Aa-mALP (bait) and Cry4Ba or its domain II or III (prey) (10% of total elutes were load in each lane), Coomassie brilliant blue stained SDS-PAGE (10% gel) of $His_{(6)}$-Aa-mALP (load 10% of input), Cry4Ba full-length (load 10% of input), and Cry4Ba domains II and III (load 32% of input) used for pulldown experiments, lane M represents the molecular mass standard proteins. Uncropped images are available as supplementary file S1.

different ($p = 0.004$) to that of the Cry4Ba full-length toxin (K_d 76.49 ± 4.26 nM). Binding of domain II to the Aa-mALP receptor can be saturated, implying that the binding is specific. Pulldown assay revealed that the Cry4Ba-domain II fragments were co-eluted with the $His_{(6)}$-tagged Aa-mALP receptor from the HisPur cobalt resin column (Fig. 4B) at a comparable level to that of Cry4Ba-DIII, which were lower than that observed in the full-length Cry4Ba protein (Fig. 4B). The mass spectrometry results revealed that the proteins collected from the pulldown assay were Aa-mALP and Cry4Ba-domain II, with sequence coverages of 22% and 31%, respectively (supplementary file S3).

4. Discussion

Binding to specific receptors on the midgut epithelial cells is one of the crucial steps to exert larvicidal activity of Cry toxins. Several pieces of evidence supported that domains II and III of several Cry toxins are the main determinants of Cry toxin binding specificity (see Ref. [10]). Even the ALP receptor was proposed as less important for the toxicity of lepidopteran-specific Cry1A toxin [21], where this receptor was observed to be crucial for dipteran-specific Cry4Ba toxin as heterologous expression of the Aa-mALP receptor on *Spodoptera frugiperda* (*Sf9*) insect cells revealed sufficient to exert toxicity of the Cry4Ba [22]. In addition, silencing of ALP1 led to tolerance to Cry4Ba and Cry11Aa of *A. aegypti* larvae [13]. Amino acid substitution of some residues on the apical loops of domains II and III of the Cry4Ba toxin showed effects on both binding to the ALP receptor and toxicity against *A. aegypti* larvae [13,16,17], which implied that the specific determinant binding to the Aa-mALP receptor relied on both domains II and III. Previously, domain II–III and III fragments, which were cloned from the Cry4Ba toxin, showed binding to midgut tissue sections and the brush border membrane proteins of *A. aegypti* [20,23]. In addition, our recent work revealed that the Cry4Ba-domain III fragment was bound to the Aa-mALP receptor with a K_d value of 110 nM [17]. In the current work, the 21 kDa domain II fragment was successfully cloned from the full-length Cry4Ba toxin. The FPLC elution profile revealed a single peak elution of Cry4Ba-domain II which corresponded to an elution volume of 21 kDa Cry4Ba-DIII and 23 kDa lysozyme [20], suggesting that its exists as a monomer. Western blot analysis and LC-MS/MS revealed that the 21 kDa heterologously expressed protein was the Cry4Ba-domain II. The CD spectrum revealed a positive peak at 195 nm and a negative peak at 216 nm which are typical for antiparallel β-pleated sheets [24], implying that the isolated Cry4Ba-domian II fragment was refolded into an antiparallel β-sheet structure, as observed in the native full-length Cry4Ba toxin [25]. Therefore, this domain II fragment would be suitable for further analysis of its interaction with the Aa-mALP receptor.

The saturation binding observed in the Cry4Ba-domain II-Aa-mALP receptor interaction revealed using ELISA implied that the isolated Cry4Ba-domain II fragment was bound specifically to the receptor. The binding affinity of the isolate domain II fragment to the Aa-mALP (116.27 ± 11.09 nM) was close to that of the domain III fragment (117.5 ± 8.10 nM), implying that both domains of Cry4Ba likely equally contributed to binding to the Aa-mALP receptor, even though binding to the apical microvilli of the *A. aegypti* midgut was suggested mostly by the function of Cry4Ba-domain II [23]. In addition, our results showed that the binding affinity of each domain II or domain III fragment to the Aa-mALP receptor was lower than that of the full-length toxin containing both domains ($K_d = 76.19 \pm 4.26$ nM), implying that the Cry4Ba used both domains II and III to bind to the Aa-mALP receptor. This finding, together with our other works (that reported mutations on some residues on the surface of domains II and III affected binding to the Aa-mALP receptor [16,17]), strengthened the proposition that both domains II and III of the Cry4Ba toxin contributed to binding to the Aa-mALP receptor. In addition, the pulldown assay results revealed binding between the Cry4Ba domains II or III to the Aa-mALP receptor, supporting the interaction between the Aa-mALP receptor and both domains of the Cry4Ba toxin. In contrast, for Cry1Ie toxin, the isolated domain III fragment was reported to contribute to binding to the peritrophic membrane of the Asian corn borer, a lepidopteran insect, more than that of the domain II fragment [26]. It was possible that the specific determinant in receptor binding is a complex process depending on the types of toxins and specific receptors located in the midgut of insect larvae.

Our findings provided more insight into the interaction between the Cry4Ba and the Aa-mALP receptor, broadening knowledge about the possible binding mechanisms of mosquito-specific toxins, as shown in Table 1. Each receptor binding domain of Cry4Ba was successfully isolated from the full-length toxin and showed interaction with the purified Aa-mALP receptor. Even binding interaction assays were performed *in vitro*, with the results supporting our previous studies in which amino acid residues on domains II and III were shown to involve receptor binding and hence toxicity toward *A. aegypti* larvae [16,17]. Both domains of the Cry4Ba toxin seemed to be related in receptor binding to exhibit the larvicidal activity of the toxin.

5. Conclusion

In this study, the domain II of the Cry4Ba was cloned and heterologously expressed in *E. coli*. The purified 21 kDa domain II was shown to interact with the purified Aa-mALP receptor with a K_d value of 118 nM that was comparable with the binding of the Cry4Ba-domain III and the Aa-mALP. Our results suggested that Cry4Ba toxin utilized both domains II and III equally to bind to the Aa-mALP receptor. The results from this work have provided more specific detail about the possible binding mechanisms of mosquito-specific Cry4Ba toxin.

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Table 1
Receptor binding analysis of some mosquito-specific Cry toxins.

Cry toxin	Part of toxin (full-length/ domain)	Receptor/Mosquito source	Dissociation constant (K_d, nM)	Technique for binding analysis	Reference
Cry4Ba	Full-length	ALP/*A. aegypti*	14	Quartz crystal microbalance	[15]
Cry4Ba	Full-length	ALP/*A. aegypti*	80	ELISA	[17]
Cry4Ba	Full-length	Cadherin fragment CR11-MPED/*Anopheles gambiae*	23	ELISA	[27]
Cry4Ba	Domain II	ALP/*A. aegypti*	116	ELISA	This study
Cry4Ba	Domain III	ALP/*A. aegypti*	110	ELISA	[17]
Cry11Ba	Full-length	Brush border membrane vesicles (BBMV)/ *A. aegypti*	8.2	ELISA	[28]
Cry11Ba	Full-length	APN/*An. gambiae*	6.4	ELISA	[29]
Cry48Aa	Full-length	Alpha-glucosidase/*Culex quinquefasciatus*	48.7	ELISA	[30]

Author contribution statement

Anon Thammasittirong, Sutticha Na-Ranong Thammasittirong: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix ASupplementary data

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