Novel Preparation and Characterization of the α 4-loop- α 5 Membrane-perturbing Peptide from the *Bacillus thuringiensis* Cry4Ba δ -endotoxin

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Received 12 December 2005, Accepted 30 January 2006

Helices 4 and 5 of the *Bacillus thuringiensis* Cry4Ba δ -endotoxin have been shown to be important determinants for mosquito-larvicidal activity, likely being involved in membrane-pore formation. In this study, the Cry4Ba mutant protein containing an additional engineered tryptic cleavage site was used to produce the α 4- α 5 hairpin peptide by an efficient alternative strategy. Upon solubilization of toxin inclusions expressed in *Escherichia coli* and subsequent digestion with trypsin, the 130-kDa mutant protoxin was processed to protease-resistant fragments of ca. 47, 10 and 7 kDa. The 7-kDa fragment was identified as the α 4-loop- α 5 hairpin via N-terminal sequencing and mass spectrometry, and was successfully purified by size-exclusion FPLC and reversed-phase HPLC. Using circular dichroism spectroscopy, the 7-kDa peptide was found to exist predominantly as an α -helical structure. Membrane perturbation studies by using fluorimetric calcein-release assays revealed that the 7-kDa helical hairpin is highly active against unilamellar liposomes compared with the 65-kDa activated full-length toxin. These results directly support the role of the α 4-loop- α 5 hairpin in membrane perturbation and pore formation of the full-length Cry4Ba toxin.

Keywords: *Bacillus thuringiensis*, Calcein release, α -helical hairpin, Membrane-pore formation, Mosquito-larvicidal protein, Tryptic cleavage

Abbreviations: *Bt*, *Bacillus thuringiensis*; CD, circular dichroism; Ch, cholesterol; Cry, crystal; FPLC, fast-performance liquid chromatography; IPTG, isopropyl- β -D-thiogalactopyranoside; LUV, large unilamellar vesicle; PC, *L*- α -phosphatidylcholine; PE, *L*- α -phosphatidylethanol amine; PMSF, phenylmethane sulfonyl fluoride; RP-HPLC, reverse-phase high-performance liquid chromatography; TFA, trifluoroacetic acid

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Introduction

A number of bacteria are pathogenic to insect larvae and are in use as potential biocontrol agents (Aronson *et al.*, 1986). Among these, the most promising candidate is *Bacillus thuringiensis* (*Bt*), an aerobic, spore-forming Gram-positive soil bacterium, which can produce different forms of highly specific larvicidal proteins in large quantities as parasporal crystalline inclusions during sporulation (Aronson *et al.*, 1986; Schnepf *et al.*, 1998). These insecticidal proteins have been classified as Cry (crystal) and Cyt (cytolytic) δ -endotoxins, based on the similarity of their deduced amino acid sequences (Crickmore *et al.*, 1998). Up to now, the Cry δ -endotoxins have been shown to be toxic with different efficacies towards insect larvae in the orders Diptera (mosquitoes and flies), Lepidoptera (moths and butterflies), Coleoptera (beetles and weevils) and Hymenoptera (wasps and bees) (Schnepf *et al.*, 1998; De Maagd *et al.*, 2001).

The *Bt* Cry δ -endotoxins are synthesised as insoluble inactive protoxins (Aronson *et al.*, 1986). The active toxins are generated in the susceptible insect larvae after ingestion when protoxin inclusions are solubilised in the alkaline contents of the larval midgut (particularly in dipteran and lepidopteran) and are processed by gut proteases. Processing generally results in the formation of protease-resistant toxic core fragments. The activated toxins subsequently bind to specific receptors lining the apical brush-border membranes of the midgut epithelium, leading to a structural rearrangement of the toxin molecule. These conformational changes would trigger the insertion of their pore-forming portion into the target cell membrane to form ion-leakage pores. These leakage pores cause a net influx of ions and water which leads to osmotic cell lysis, resulting in serious destruction of the midgut and eventually death of the larvae (for reviews, see Knowles, 1994; Whalon and Wingerd, 2003). However, the underlying molecular basis of this toxicity process is not yet completely described.

Currently, five X-ray crystal structures of *Bt* Cry δ -

Fig. 1. (A) The 3D structure of the 65-kDa activated Cry4Ba toxin (Boonserm *et al.*, 2005), showing the three-domain organization (I-III). Domain I, a helical bundle, is illustrated in a schematic ribbon, while domains II and III are shown in a space-filling model. (B) Helical patterns of Cry4Ba α 3- α 7. The tryptic cleavage sites at Arg¹³⁹ and Arg²⁰⁴ on the exposed loops are indicated with arrows. The α 4-loop- α 5 hairpin is shaded. The 3D structure was generated by Molscript (<http://www.avatar.se/molscript/>) and VMD (<http://www.ks.uiuc.edu/Research/vmd/>).

endotoxins, Cry1Aa (Grochulski *et al.*, 1995), Cry2Aa (Morse *et al.*, 2001), Cry3Aa (Li *et al.*, 1991), Cry3Bb (Galitsky *et al.*, 2001) and Cry4Ba (Boonserm *et al.*, 2005), are available that all reveal a high degree of overall structural similarity with a three-distinct domain organisation. Of particular interest, the N-terminal helical domain comprising both amphipathic and relatively hydrophobic α -helices has been proved to be responsible for membrane insertion, leading to formation of the ion-leakage pores (Walters *et al.*, 1993; Von Tersch *et al.*, 1994; Puntheeranurak *et al.*, 2001; Puntheeranurak *et al.*, 2004). Despite the fact that the Cry4Ba structure bears a resemblance to the other known Cry structures, the finer features are rather different. For instance, there is additional *in vitro* proteolysis by trypsin occurring in the loop connecting

helices 5 and 6 of the Cry4Ba toxin (see Fig. 1), thus generating two non-covalently associated fragments of ca. 47 and 20 kDa (Angsuthanasombat *et al.*, 1993). This interhelical cleavage was demonstrated to be crucial for cytotoxicity of the activated Cry4Ba toxin against mosquito cell lines (Angsuthanasombat *et al.*, 1993). In addition, the 20-kDa fragment which maps to the first five helices, α 1- α 5, was shown to be capable of permeabilising liposomes (Puntheeranurak *et al.*, 2001) and inducing ion channels in planar lipid bilayers (Puntheeranurak *et al.*, 2004).

The “umbrella-like” model seems now to be widely accepted of the best description for the membrane-bound state of the Cry toxins, which involves an insertion of α 4 and α 5 into the lipid bilayers as a helical hairpin structure, with the remaining helices spread over the membrane surface (Gazit *et al.*, 1998). Subsequent oligomerisation of the transmembrane hairpins occurs to form an oligomeric pore with a diameter of 10-20 Å (Schwartz *et al.*, 1997). Several reports supporting this model have suggested that α 4 arranges in the aqueous interface of the pore and participates in ion conduction (Masson *et al.*, 1999; Sramala *et al.*, 2001), whilst the more hydrophobic α 5 interacts with the lipid bilayers and is involved in toxin oligomerisation (Nunez-Valdez *et al.*, 2001). Very recently, we have demonstrated that the highly conserved residue-Asn¹⁸³ located in the middle of α 5 of the Cry4Ba toxin plays an essential role in toxicity and toxin oligomerisation (Likitvivanavong *et al.*, 2006).

Furthermore, it has been demonstrated that the loop connecting α 4 and α 5 of the lepidopteran-active Cry1Ac toxin is needed for efficient penetration of these two transmembrane helices into the lipid bilayers to form lytic pores and subsequently cause toxicity (Gerber and Shai, 2000)c. In our earlier studies, we have strengthen this notion by demonstrating that one highly conserved tyrosine residue in this critical loop of both the two closely related dipteran-specific toxins (Cry4Aa: Tyr²⁰²; Cry4Ba: Tyr¹⁷⁰) is an important determinant for larvicidal activity, conceivably being involved in an interaction with lipid head groups stabilising the oligomeric pore structure (Kanintronkul *et al.*, 2003; Pornwiroon *et al.*, 2004). In addition, we have further provided biologically relevant evidence for a structural requirement of both the disulphide bridge (C₁₉₂-C₁₉₉) and the proline-rich motif (P₁₉₃PNP₁₉₆) (Tapaneyakorn *et al.*, 2005), which are exclusively found within the α 4- α 5 loop of the Cry4Aa toxin (Angsuthanasombat *et al.*, 2004). In the present report, we describe for the first time a reliable semipreparative production of the full-size α 4-loop- α 5 membrane-perturbing peptide. As an alternative to conventional solid-phase peptide synthesis, this method utilised the Cry4Ba mutant toxin containing an engineered tryptic cleavage site. Quantitative recovery of the desired peptide by semipreparative chromatographic purification allowed us to provide direct evidence for membrane-perturbing activity of the α 4-loop- α 5 hairpin that is conceivably the central transmembrane pore-forming element of the Cry4Ba toxin.

Materials and methods

Expression and purification of the mutant toxin inclusions. *E. coli* cells strain JM109 harbouring the recombinant plasmid (pS136NSSRNP) which encodes the Cry4Ba mutant toxin designated as T6, containing an additional tryptic cleavage site in the $\alpha 3$ - $\alpha 4$ loop (Krittana *et al.*, 2001) were grown at 30°C in Luria-Bertani medium supplemented with 100 µg/ml ampicillin. When the culture reached OD₆₀₀ 0.4–0.5, isopropyl- β -D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM, and incubation was continued for another 10 h. Samples containing 10⁷ cells/ml were analysed for toxin expression by using sodium dodecyl sulfate (15% w/v) polyacrylamide gel electrophoresis (SDS-PAGE).

Cells expressing the mutant toxins as inclusion bodies were harvested by centrifugation and resuspended in 100 mM K₂HPO₄, pH 6.5. The cell suspension was disrupted by using a French Press Cell at 20,000 psi. The crude lysate was centrifuged at 6,000 g, 4°C for 10 min. Following the method described previously (Moonsom, 2004), the pellets were incubated in 80 mM K₂HPO₄, pH 6.5, 0.8 M NaCl, 0.1% Triton X-100 at 4°C for 30 min, and washed 3 times with 100 mM K₂HPO₄, pH 6.5, followed by washing with cold distilled water. Protein concentrations of the partially purified toxin inclusions were determined by using the Bradford-based protein microassay (Bio-Rad), with bovine serum albumin fraction V (Sigma) as a standard.

Inclusion solubilization and proteolytic processing of the mutant protoxin. The T6 protoxin inclusions (5 mg/ml) were solubilised in 50 mM Na₂CO₃, pH 9.0 and incubated at 37°C for 1 h as previously described (Sramala *et al.*, 2000). After centrifugation at 8,000 × g for 10 min, the supernatant and inclusion suspension were analysed by SDS-PAGE. Solubilised protoxins (4 mg/ml) were digested with trypsin (*L*-1-tosylamide-2-phenylethyl chloromethyl ketone-treated, Sigma) at enzyme/toxin ratio of 1/20 (w/w) in 50 mM Na₂CO₃, pH 9.0, at 37°C for 16 h, and enzyme activity was inhibited by adding phenylmethane sulfonyl fluoride (PMSF) to give a final concentration of 1 mM.

Purification of the $\alpha 4$ -loop- $\alpha 5$ hairpin peptide. Initial purification of the 65-kDa trypsin-treated T6 mutant toxin was accomplished using a size-exclusion FPLC system (Superose 12/HR10 column, Amersham Pharmacia Biotech) eluted with carbonate buffer (50 mM Na₂CO₃, pH 9.0) at a flow rate of 0.4 ml/min and monitored by UV absorption at 280 nm. Elution fractions across the 65-kDa protein peak were pooled and subjected to further purification by a reverse-phase high-performance liquid chromatography (RP-HPLC) system using a Jupiter C₁₈ column (5 µm, 250 × 4.6 mm, Phenomenex, USA). Chromatographic separations were achieved with a linear gradient of B in A (A = 0.1% TFA in H₂O; B = 0.1% TFA/5% H₂O/5% isopropanol/90% acetonitrile). Proteins were eluted over 30 min between 30–80% B gradient at a flow rate of 1 ml/min.

Characterization of the purified $\alpha 4$ -loop- $\alpha 5$ hairpin peptide. The purified $\alpha 4$ - $\alpha 5$ hairpin peptide was further analysed by automated N-terminal sequencing. The peptide transferred onto a polyvinylidene difluoride membrane (Prosorpt[®], Applied Biosystems, USA) was subjected to automated Edman degradation using an

ABI 491cLC pulse-liquid phase sequencer (Applied Biosystems). Circular dichroism (CD) measurements of the purified peptide were performed on a Jasco J-715 CD spectropolarimeter (Jasco Inc.) in the far UV region (190–260 nm) at 25°C using a rectangular quartz cuvette (0.2-mm optical path-length). The peptide sample was prepared in methanol with a concentration of 1.95×10^{-4} M, as determined by far UV absorbance. The CD spectra were recorded at a scanning rate of 20 nm/min with a spectral bandwidth of 2 nm and response times of 2 msec. Three accumulations were taken and the results were averaged. After background subtraction, all CD data were converted from CD signal (mdeg) into mean residue ellipticity (deg cm²/dmole). Reference spectra were obtained and scale calibration was performed with camphorsulfonic acid. The secondary structure content was estimated from the CD spectra using the method of Greenfield and Fasman (Greenfield and Fasman, 1969). Matrix-assisted laser desorption time of flight mass spectrometry (Bruker Reflex IV MALDI-TOF) was employed to verify that the purified helical peptide corresponded to the predicted molecular mass.

Membrane perturbation of liposome vesicles. Large unilamellar vesicles (LUVs) were prepared from 2 mg/ml of a lipid mixture (Avanti Polar Lipid) of *L*- α -phosphatidylcholine (PC)/*L*- α -phosphatidyl ethanolamine (PE)/cholesterol (Ch) (10 : 10 : 1 w/w) dissolved in chloroform. The solvent was evaporated under a nitrogen stream and the resulting lipid film was resuspended in 200 µl of 60 mM calcein (pre-dissolved in 100 mM Na₂CO₃, pH 9.0)/10 mM Tris-HCl, pH 9.0. After subjected to 5 cycles of freezing and thawing, the lipid suspension was repeatedly squeezed through a polycarbonate membrane (0.1-µm pore size, Avanti Polar Lipid) for a minimum of 20 passes, using a two-syringe extruder (Avanti Polar Lipid). The untrapped calcein was removed from the LUV suspension by gel filtration on a HiTrap[™] desalting column (Amersham Pharmacia Biotech), equilibrated with 150 mM NaCl/10 mM Tris-HCl, pH 9.0. Liposome concentrations were estimated by measuring the lipid phosphorus content (Mrsny *et al.*, 1986), and a final concentration of 1.25 µM LUV was used for the calcein release assay (Allen and Cleland, 1980). After adding 5 µl of a tested sample (5–10 µg) into 400 µl of LUV solution placed in a 1-cm light-path polymethyl methacrylate cuvette (Brand), the degree of LUV perturbation was determined as an increase in the fluorescence intensity of the released calcein. Fluorescence was monitored at 25°C on a Perkin-Elmer LS50 spectrofluorimeter with excitation and emission wavelengths set at of 485 and 520 nm, respectively, and a slit width of 5 nm. The time-course of the percentage of calcein release was indicated as a fraction of maximum fluorescence release determined after addition of 0.1% (v/v) Triton X-100 to the liposome suspension.

Results and Discussion

Use of molecular biological tools to express only putative membrane-inserted segments of a protein, either directly or as chimeric fusion products, has a number of latent limitations. The peptide products may be lethal to the host cells, may form insoluble aggregates, or may be rapidly degraded thereby preventing accumulation of the polypeptides. Thus, chemical

Fig. 2. SDS-PAGE (Coomassie brilliant blue-stained 12% gel) analysis of lysates extracted from *E. coli* (10^7 cells) expressing 130-kDa protoxins of Cry4Ba-T6 mutant at 30°C (lane 1). Lanes 2 and 3 are the partially purified T6-protoxin inclusions and its solubilised fraction in carbonate buffer, pH 9.0, respectively. M represents molecular mass standards.

peptide synthesis becomes known as a desirable approach. By using conventional peptide synthesis, however, a long peptide (>50 residues in length) is still notoriously difficult to be prepared in quantities sufficient for biophysical characterization (Fisher and Engelman, 2001). Attempts made to produce the α 4-loop- α 5 hairpin (65 amino acid residues in length) of the Cry4Ba toxin *via* chemical synthesis were unsuccessful. In our earlier work, PCR-based site-directed mutagenesis has been employed to construct a number of mutant toxins for investigating its structure-function relationships of Cry4Ba and its related mosquito-larvicidal proteins (Uawithya *et al.*, 1998; Sramala *et al.*, 2000; Angsuthanasombat *et al.*, 2001; Kanintronkul *et al.*, 2003; Pornwiroon *et al.*, 2004; Tapaneeyakorn *et al.*, 2005; Tuntitippawan *et al.*, 2005). In particular, a series of Cry4Ba mutant toxins were generated to resemble the Cry3Aa toxin in which the loop connecting helices 3 and 4 is cleavable by trypsin *in vitro* (Krittana *et al.*, 2001). In the present study, we have utilised the Cry4Ba-T6 mutant toxin as a starting-material for the efficient production of the α 4-loop- α 5 hairpin peptide. The T6 protein (N¹³⁶SSRNP) possesses both the engineered tryptic cleavage site (Arg¹³⁹) in the α 3- α 4 loop and the pre-existing trypsin site (Arg²⁰⁴) in the α 5- α 6 loop within the N-terminal pore-forming domain (see Fig.1). This biosynthetic strategy would be therefore an alternative to the conventional solid-phase peptide synthesis.

Under experimental procedures described previously (Krittana *et al.*, 2001), the Cry4Ba-T6 mutant toxin was expressed as a cytoplasmic inclusion in *E. coli* upon cultivation at 37°C and IPTG induction for 4 h, albeit at a lower-level expression compared with the wild-type toxin. In addition, the solubility of the T6 toxin inclusion in carbonate buffer, pH 9.0, was found to be much lower than that of the

wild-type inclusion. We thereafter found that both the expression level and solubility of the T6 mutant inclusion were markedly enhanced with cultivation at 30°C together with prolonged-IPTG induction for 10 h (Fig. 2), as earlier demonstrated for its closely related dipteran-specific toxin, Cry4Aa (Boonserm *et al.*, 2004). We therefore conducted growth and induction for the T6 mutant production at 30°C rather than 37°C, yielding toxin inclusions of 0.1-0.2 mg/ 10^8 *E. coli* cells. The availability of the highly dissolvable toxin inclusion (see Fig. 2, lane 3) allowed us to obtain sufficient amounts of starting-soluble protoxins for subsequent investigations.

We have shown earlier that the 130-kDa T6 mutant protoxin was cleaved by trypsin into three protease-resistant polypeptides of ca. 47, 10 and 7 kDa, in addition to the removal of the C-terminal half of the protoxin (Krittana *et al.*, 2001). In addition, it is noteworthy that the 7-kDa fragment was produced by cleavage at Arg¹³⁹ and Arg²⁰⁴ located in the α 3- α 4 and α 5- α 6 loops, respectively (Fig. 1B). For purification, the trypsin-activated T6 fragments were initially subjected to FPLC-gel filtration and the eluted materials were analysed on SDS-PAGE (see Fig. 3C, lane 1). Similar to the two protease-resistant fragments (47 and 20 kDa) of the activated wild-type toxin (Angsuthanasombat *et al.*, 1993), the three T6 fragments of 47, 10 and 7 kDa were also found associated to each other under the non-denaturing condition used (50 mM Na₂CO₃ buffer, pH 9.0), which were eluted from the column in a distinct single peak corresponding to the eluted fractions of the 66-kDa BSA protein marker (see Fig. 3A). These three non-covalently linked polypeptides were subsequently subjected to semipreparative RP-HPLC for further separation (Fig. 3B). As can be seen that products were eluted with acetonitrile in less than 40 min, and the peaks were well separated. SDS-PAGE analysis of each eluted fraction revealed that the primary peak at retention time of 14 min corresponded to the 47-kDa proteolytic fragment (Fig. 3C, lane 2). Furthermore, the 7-kDa and 10-kDa fragments showed longer retention times of 24 and 30 min, respectively, indicating a higher hydrophobicity. It should be noted that elution of these hydrophobic peptide products required solvents such as isopropanol in addition to acetonitrile in order to increase the hydrophobicity of the organic phase (see Materials and methods). However, unlike the 7-kDa fragment, the 10-kDa fragment was eluted in broad overlapping peaks (see Fig. 3B, inset). This broad elution profile may reflect aggregation, conformational heterogeneity or impurities that each could give rise to adsorbed peptides on the column, requiring different elution conditions. Additional experiments are therefore needed to purify the 10-kDa proteolytic fragment for further characterization.

N-terminal sequencing of the 7-kDa purified fragment (approximate yields of 0.3-0.5 μ g/ 10^8 cells) confirmed that the peptide was pure and processed at the expected site as annotated in Fig. 3C. Mass spectral data (observed molecular mass = 7403.05 Da) were also found to correspond to the full-length α 4-loop- α 5 peptide (calculated molecular mass =

Fig. 3. Protein purification and SDS-PAGE analysis of the 65-kDa trypsin-activated Cry4Ba-T6 mutant protein (A) Chromatographic elution profile from the Superose 12/HR10 size-exclusion column, showing absorbance at 280 nm (AU) and elution time (min). (B) Elution profile from C_{18} Jupiter RP-HPLC column. Inset, magnified scale at retention time of 30 min. (C) SDS-PAGE (Coomassie brilliant blue-stained 14% gel) analysis of selected peak fractions (arrowed) obtained from A and B. M represents molecular mass standards. Lanes 1-4 are peak fractions 1-4 as indicated in A and B, respectively. NPSYR...SLAR is an annotated sequence of the 7-kDa purify fragment.

7388.55 Da). It should be noted that the molecular mass of the $\alpha 4$ -loop- $\alpha 5$ hairpin peptide obtained in the present study was rather different from the mass determined previously (5,914.72 Da) by Krittanai *et al.* (2001). The mass discrepancy of the hairpin peptide might be that in the digestion condition in our present work, PMSF was also added to a final concentration of 1 mM in order to inhibit remaining trypsin activity prior to purification of the helical hairpin peptide. In addition, the 7-kDa $\alpha 4$ -loop- $\alpha 5$ hairpin peptide that was purified from HPLC purification was used directly for mass determination. Therefore, in our previous report (Krittanai *et al.*, 2001), the mass (5,914.72 Da) of the ca. 6-kDa trypsin-digestion fragment which was purified from the SDS-gel would possibly correspond to the size of the truncated hairpin peptide which is likely due to further cleavage at Arg¹⁹⁰ during sample preparation as previously suggested (Krittanai *et al.*, 2001).

Moreover, we examined the purified $\alpha 4$ -loop- $\alpha 5$ peptide for secondary structural elements by far-UV CD spectroscopy. Repeated scans from 190 to 260 nm demonstrated that the $\alpha 4$ -

Fig. 4. The far-UV CD spectrum of the 7-kDa purified $\alpha 4$ - $\alpha 5$ hairpin peptide ($n = 3$) in methanol, showing two minima at 208 and 222 nm.

loop- $\alpha 5$ peptide exhibited a spectrum (Fig. 4) with two minima at 208 and 222 nm ($-21,687$ and $-20,647$ deg cm²/dmole, respectively). These spectral characteristics are typical

Fig. 5. Effects on calcein release from LUVs. (A) The traces represent fluorescence intensity of the entrapped calcein upon release from LUVs, which were continuously monitored as a function of time after incubation with samples (0.6 μ M); (a), the purified α 4-loop- α 5 hairpin peptide; (b), the 65-kDa full-length T6 protein; (c), the 47-kDa purified fragment; (d and e), 5 μ l of carbonate buffer and DMSO, respectively. (B) The relative release activities of each protein sample, the α 4-loop- α 5 peptide ($\bullet$), the 65-kDa full-length T6 protein ($\circ$), the 47-kDa fragment ($\blacktriangledown$), with varying concentrations that are indicated as fraction of 100% release induced by Triton X-100. Error bars represent standard error of the mean from three independent experiments. The release in the control sample incubated with carbonate buffer or DMSO rarely exceeded 5% and these values have been subtracted in the figure.

for an α -helical structure. Additionally, analysis by the method of Greenfield and Fasman (Greenfield and Fasman, 1969) designed to deconvolute spectra calculated an approximate amount of α -helical elements at 61%. As expected, no β -structural content was detected.

To investigate the potential effect of the purified α 4-loop- α 5 peptide on the integrity of lipid membrane vesicles, calcein-release experiments were performed in comparison to the 65-kDa full-length T6 mutant protein. Here, LUVs of PC/PE/Ch compositions, which are more relevant to the insect

cell membranes (Marheineke *et al.*, 1998), were created with fluorimetric calcein entrapped in their interior cavity. The release of the entrapped calcein from LUVs was measured as the 'dequenching' of the calcein fluorescence, and was thereby monitored continuously as an increase in the fluorescence intensity (see Fig. 5A). Under the conditions used in the assays, the α 4-loop- α 5 peptide (4.5 μ g/ml or ca. 0.6 μ M) was able to induce a fast and efficient release of the encapsulated calcein, with maximally 70% release (when compared to Triton X-100 as a positive control, see Fig. 5B) which was observed within 15 min (Fig. 5A, trace a). This result is consistent with data published by other workers that the α 4-loop- α 5 synthetic peptide of Cry1Ac is capable of permeabilising phospholipids vesicles (Gerber and Shai, 2000). Dissimilar to the isolated membrane-active α 4-loop- α 5 hairpin, the ability of the 65-kDa full-length activated T6 protein (at the equivalent molar concentration of 0.6 μ M) to induce the release was much weaker, with maximally release of only 20% (Fig. 5B). This indicates that the purified α 4-loop- α 5 hairpin could exert its permeabilising activity more readily in the receptor-free lipid membranes when it is present in its isolated form rather than association with the two other fragments in the full-length activated toxin. Also, this might be explained by the fact that these artificial membranes lack the toxin receptors required for toxin-membrane interactions. Although the high toxin concentrations used might have overcome the requirement for receptors, toxin-receptor binding could be one of the factors triggering conformational changes which might facilitate the release of the α 4-loop- α 5 hairpin from the activated T6 toxin to perturb the membranes. In addition, the 47-kDa purified toxin fragment, which corresponds to α 6-loop- α 7 linked with domains II and III, only marginally increased the calcein fluorescence when compared to the buffer control (see Fig. 5A, trace c). As was previously demonstrated by Bravo and co-workers (Flores *et al.*, 1997), the isolated domains II-III fragment of Cry1Ab was able to interact with its receptor in a way somewhat similar to the 65-kDa full-length toxin, confirming our studies that the 47-kDa Cry4Ba fragment is primarily involved in receptor-binding interactions rather than membrane insertion and pore formation. However, it remains to be tested whether the 10-kDa fragment which corresponds to α 1- α 3 is capable of induce calcein permeability of lipid vesicles.

In conclusion, we have succeeded in production and purification of the full-size α 4-loop- α 5 membrane active peptide, alternatively to conventional chemical synthesis, by utilising the Cry4Ba mutant toxin containing an engineered tryptic cleavage site. We have also presented evidence that the α 4-loop- α 5 hairpin is capable of perturbing the integrity of lipid membrane vesicles, supporting its role as a fundamental membrane-inserted pore-forming determinant of the full-length Cry4Ba toxin, and that it could be separated as an isolated helical hairpin that retains at least its functionality. Very recently, we have demonstrated *via* Langmuir-Blodgett technique that the 65-kDa activated Cry4Ba toxin is capable

of inserting itself into the lipid monolayers (Kanintrunkul *et al.*, 2005). By using atomic force microscopy, the activated Cry4Ba toxin was also found to form a symmetric pore-like structure in receptor-free lipid bilayers (Puntheeranurak *et al.*, 2005). Further investigation of the insertion behavior and molecular organisation within lipid membranes of the Cry4Ba α 4-loop- α 5 hairpin are of great interest, since this would shed light on the general principles that underlie protein-protein interactions of this membrane-perturbing peptide within the lipid bilayers.

Acknowledgments We are very grateful to Drs. Sakol Panyim, Prapon Wilairat, Jaime Torres and Panadda Boonserm for critical comments. We also thank Seangdeun Moonsom for technical assistance. This work was supported in part by the grant number BRG4780004 from the Thailand Research Fund (TRF). The Royal Golden Jubilee Ph.D. scholarship from TRF to S.L. is gratefully acknowledged.

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