Novel Preparation and Characterization of the $\alpha 4$-loop-$\alpha 5$ Membrane-perturbing Peptide from the *Bacillus thuringiensis* Cry4Ba $\delta$-endotoxin

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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) $\delta$-endotoxins, based on the similarity of their deduced amino acid sequences (Crickmore *et al.*, 1998). Up to now, the Cry $\delta$-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 $\delta$-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 Wingard, 2003). However, the underlying molecular basis of this toxicity process is not yet completely described.

Currently, five X-ray crystal structures of *Bt* Cry $\delta$-
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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 α3-α7. The tryptic cleavage sites at Arg170 and Arg201 on the exposed loops are indicated with arrows. The α4-loop-α5 hairpin is shaded. The 3D structure was generated by Molscript (http://www.ks.uiuc.edu/Research/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 (Galtisky 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 organization. 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 (Angsuthanasombut et al., 1993). This interhelical cleavage was demonstrated to be crucial for cytotoxicity of the activated Cry4Ba toxin against mosquito cell lines (Angsuthanasombut et al., 1993). In addition, the 20-kDa fragment which maps to the first five helices, α1-α5, 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 α4 and α5 into the lipid bilayers as a helical hairpin structure, with the remaining helices spread over the membrane surface (Gazi et al., 1998). Subsequent oligomerisation of the transmembrane hairpins occurs to form an oligomeric pore with a diameter of 10-20Å (Schwartz et al., 1997). Several reports supporting this model have suggested that α4 arranges in the aqueous interface of the pore and participates in ion conduction (Masson et al., 1999; Srama et al., 2001), whilst the more hydrophobic α5 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 α5 of the Cry4Ba toxin plays an essential role in toxicity and toxin oligomerisation (Likivivatanavong et al., 2006).

Furthermore, it has been demonstrated that the loop connecting α4 and α5 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, 2000c). In our earlier studies, we have strengthened this notion by demonstrating that one highly conserved tyrosine residue in this critical loop of both the two closely related dipteran-specific toxins (Cry4Aa: Tyr18; Cry4Ba: Tyr170) is an important determinant for larvicidal activity, conceivably being involved in an interaction with lipid head groups stabilising the oligomeric pore structure (Kaniruncul et al., 2005; Pottmwanun et al., 2004). In addition, we have further provided biologically relevant evidence for a structural requirement of both the disulphide bridge (C192-C200) and the proline-rich motif (P20-PNP25) (Tapaneeyakorn et al., 2005), which are exclusively found within the α4-α5 loop of the Cry4Aa toxin (Angsuthanasombut et al., 2004). In the present report, we describe for the first time a reliable semipreparative production of the full-size α4-α5 loop, using 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 α4-α5 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 harboring the recombinant plasmid (pS136NSSSNP) which encodes the Cry4Ba mutant toxin designated as T6, containing an additional tryptic cleavage site in the α3-α4 loop (Krittanai 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 10' 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 K₃HPO₄, 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 10.5, 0.8 M NaCl, 0.1% Triton X-100 at 4°C for 30 min, and washed 3 times with 100 mM K₃HPO₄, 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 toxin. 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 x g for 10 min, the supernatant and inclusion suspension were analysed by SDS-PAGE. Solubilised protoxins (4 mg/ml) were digested with trypsin (1:1000 weight-to-weight) and incubated at 20°C as previously described (Singh et al., 2004). After incubation for 15 min, the mixture was treated with 200 µg/ml aprotinin (Sigma), pH 7.5, 4°C, and subjected to 5 cycles of freezing and thawing, the lipid suspension was applied to a ion-exchange column (Avanti Polar Lipid) for 30 min. The peptide sample was eluted with carbonate buffer (50 mM, 25°C, 0.2-mm optical path-length). The peptide sample was prepared in methanol with a concentration of 1.95 x 10⁶ 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 ms. 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 Lα-phosphatidylcholine (PC)/Lα-phosphatidylethanolamine (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 calcium (pre-dissolved in 100 mM NaCl, pH 9.0)/10 mM Tris-Cl, 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-yring syringe extender (Avanti Polar Lipid). The unentrapped 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-Cl, pH 9.0. Lipoconcentrations were estimated by measuring the lipid phosphorus content (Mrosovsky 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 poly(methyl 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 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 as or 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
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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 α4-loop-α5 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; Samala et al., 2000; Angsuthanasombat et al., 2001; Kaninmakul et al., 2003; Pornwiroon et al., 2004; Tapaneejakorn et al., 2005; Tuntipipawan 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 (Krittananit 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 α4-loop-α5 hairpin peptide. The T6 protein (N256SSRN) possesses both the engineered tryptic cleavage site (Arg159) in the α3-α4 loop and the pre-existing trypsin site (Arg204) in the α5-α6 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 (Krittananit 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 (Boonsri 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 proteins for subsequent investigations.

We have shown earlier that the 130-kDa T6 mutant protein 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 toxin (Krittananit et al., 2001). In addition, it is noteworthy that the 7-kDa fragment was produced by cleavage at Arg159 and Arg204 located in the α3-α4 and α5-α6 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 semi-preparative 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 µg/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 α4-loop-α5 peptide (calculated molecular mass = 7403.05 Da).
7388.55 Da). It should be noted that the molecular mass of the α4-loop-α5 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 α4-loop-α5 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 Arg<sup>190</sup> during sample preparation as previously suggested (Krittanai et al., 2001).

Moreover, we examined the purified α4-loop-α5 peptide for secondary structural elements by far-UV CD spectroscopy. Repeated scans from 190 to 260 nm demonstrated that the α4-loop-α5 peptide exhibited a spectrum (Fig. 4) with two minima at 208 and 222 nm.

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<sub>18</sub> 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 purified fragment.

Fig. 4. The far-UV CD spectrum of the 7-kDa purified α4-α5 hairpin peptide (n = 3) in methanol, showing two minima at 208 and 222 nm.
cell membranes (Marheineke et al., 1998) were created with fluorometric 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 α4-loop-α5 peptide (4.5 μg/ml or ca. 0.6 μM) 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 α4-loop-α5 synthetic peptide of Cry1Ac is capable of permeabilising phospholipid vesicles (Gerber and Shai, 2000). Dissimilar to the isolated membrane-active α4-loop-α5 hairpin, the ability of the 65-kDa full-length activated T6 protein (at the equivalent molar concentration of 0.6 μM) to induce the release was much weaker, with maximally release of only 20% (Fig. 5B). This indicates that the purified α4-loop-α5 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 α4-loop-α5 hairpin from the activated T6 toxin to perturb the membranes. In addition, the 47-kDa purified toxin fragment, which corresponds to α6-loop-α7 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 Brnco 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 α1-α3 is capable of induce calcein permeability of lipid vesicles.

In conclusion, we have succeeded in production and purification of the full-size α4-loop-α5 membrane active peptide, alternatively to conventional chemical synthesis, by utilizing the Cry4Ba mutant toxin containing an engineered tryptic cleavage site. We have also presented evidence that the α4-loop-α5 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

![Fig. 5. Effects on calcein release from LUVs.](image)

(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 μM): (a), the purified α4-loop-α5 hairpin peptide; (b), the 65-kDa full-length T6 protein; (c), the 47-kDa purified fragment; (d and e), 5 μl of carbonate buffer and DMSO, respectively. (B) The relative release activities of each protein sample, the α4-loop-α5 peptide (●), the 65-kDa full-length T6 protein (○), the 47-kDa fragment (▼), 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 α4-loop-α5 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

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of inserting itself into the lipid monolayers (Kanintronkul 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 (Pantheeranurak et al., 2005). Further investigation of the insertion behavior and molecular organisation within lipid membranes of the Cry4Ba α4-loop-α5 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.

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