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# Expression and Biochemical Characterization of the *Bacillus thuringiensis* Cry4B α1-α5 Pore-forming Fragment

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**Tryptic activation of the 130-kDa *Bacillus thuringiensis* Cry4B δ-endotoxin produced protease-resistant products of ca. 47 kDa and ca. 21 kDa. The 21-kDa fragment was identified as the N-terminal five-helix bundle (α1-α5), which is a potential candidate for membrane insertion and pore formation. In this study, we constructed the recombinant clone over-expressing this putative pore-forming (PPF) fragment as inclusion bodies in *Escherichia coli*. The partially purified inclusions were composed of a 23-kDa protein, which cross-reacted with Cry4B antibodies, and whose N-terminus was identical to that of the 130-kDa protein. Dissimilar to protoxin inclusions, the PPF inclusions were only soluble when the carbonate buffer, pH 9.0, was supplemented with 6 M urea. After renaturation *via* a stepwise dialysis, the refolded PPF protein appeared to exist as an oligomer and was structurally stable upon trypsin treatment. Unlike the 130-kDa protoxin, the refolded protein was able to release entrapped glucose from liposomes, and showed comparable activity to the full-length activated toxin, although it lacks larvicidal activity. These results, therefore, support the notion that the PPF fragment that consists of α1-α5 of the activated Cry4B toxin is involved in membrane pore-formation.**

**Keywords:** *Bacillus thuringiensis*, δ-endotoxins, Liposomes, Pore formation, Refolding

## Introduction

The different δ-endotoxins that are produced as cytoplasmic inclusions during sporulation by *Bacillus thuringiensis* (*Bt*) are variously active against the larvae of major insect agricultural pests and disease vectors (Aronson *et al.*, 1986; Schnepf *et al.*, 1998). These parasporal crystalline inclusions are composed of one or more polypeptides of varying molecular mass that have been classified as Cry and/or Cyt δ-endotoxins, according to the similarity of their deduced amino acid sequences (Hofte and Whiteley 1989; Crickmore *et al.*, 1998). For instance, the 130-kDa mosquito-larvicidal protein from *Bt* subsp. *israelensis*, is identified as the Cry4B toxin (Hofte and Whiteley, 1989; Schnepf *et al.*, 1998).

The general mechanism of gut epithelial cell disruption by the different *Bt* δ-endotoxins is thought to be the formation of lytic pores in the susceptible insect membrane (Knowles and Ellar, 1987). Upon ingestion, the inclusions are solubilized and the protoxins activated at the alkaline pH by proteases of the larval midgut. The activated toxins then bind to insect specific receptors and insert into the cell membrane to form leakage pores. Then, this results in cell death by colloid osmotic lysis (see Knowles, 1994 for reviews). However, an entire molecular characterization of the pore-forming process, mediated by these insecticidal proteins, has not yet been obtained.

To date, two tertiary structures of different Cry toxins, Cry1Aa and Cry3A, have been resolved by X-ray crystallography (Li *et al.*, 1991; Grochulski *et al.*, 1995). Both structures display a possible apparatus for pore formation in the form of a bundle of long amphipathic and hydrophobic helices in the N-terminal seven-helix domain (α1-α7), which could penetrate the membrane to form a transmembrane pore. The possibility that this helical structure is essential for pore formation is supported by the feature that it is conserved throughout the Cry toxin family (Hofte and Whiteley, 1989; Li *et al.*, 1991). This is also supported by analogies with the pore-forming domain structure of two other well-characterized bacterial toxins; colicin A that is comprised of a bundle of ten helices, and diphtheria toxin that consists of a nine-helix bundle (Parker and Pattus, 1993). Two studies with truncated proteins, corresponding to the seven-helix bundle of

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Cry1Ac and Cry3B2, demonstrated the pore-forming activity in planar lipid bilayers (Walters *et al.*, 1993; Von Tersch *et al.*, 1994). In addition, a number of studies *via* synthetic peptides or site-directed mutagenesis provided evidence to support the conclusion that α4 and α5 of several Cry toxins are involved in membrane penetration and pore formation (Cummings *et al.*, 1994; Schwartz *et al.*, 1997; Gazit *et al.*, 1998; Kumar and Aronson, 1999; Masson *et al.*, 1999; Gerber and Shai, 2000).

Recently, we demonstrated that α4 and α5 of the Cry4B toxin are important determinants of mosquito-larvicidal activity. They are likely involved in pore formation, rather than in receptor binding (Uawithya *et al.*, 1998; Sramala *et al.*, 2000). The elimination of one charged residue (Arg-158) in α4 of Cry4B was recently found to considerably affect toxicity (Sramala *et al.*, 2001). We also found that, in addition to removal of the C-terminal half of the 130-kDa Cry4B protoxin, the activated molecule had undergone proteolytic activation producing two main sets of cleavage products at ca. 47 kDa and ca. 21 kDa (Angsuthanasombat *et al.*, 1991). Aligning these positions with the Cry3A crystal structure (Li *et al.*, 1991) suggested that one cleavage occurred in a region before the start of the N-terminal helical bundle, and the other occurred in a predicted loop joining helices 5 and 6 in the bundle (Angsuthanasombat *et al.*, 1993). This putative N-terminal five-helix bundle (α1-α5) was isolated as a protease-resistant fragment of ca. 21 kDa under denaturing conditions (Angsuthanasombat *et al.*, 1993). Presently, the role of this α1-α5 fragment in the Cry4B mechanism of toxicity is still not clearly understood. In this report, a recombinant clone that highly expresses this putative pore-forming (PPF) fragment was successfully constructed. Subsequent investigation revealed that the refolded PPF protein was able to induce liposome permeability, suggesting that the α1-α5 fragment is an essential component for the pore-forming activity of the Cry4B toxin.

## Materials and Methods

**Construction of the plasmid expressing the PPF protein** A gene segment, encoding the PPF region (Met-1 to Leu-209; see Fig. 1) of the Cry4B toxin, was generated by a polymerase chain reaction (PCR) using the plasmid template pMU388 that contained the full-length 130-kDa Cry4B toxin gene (Angsuthanasombat *et al.*, 1987). Oligonucleotide primers (*universal primer*-f: 5'-TT GTGAGCGGATAACAATTTC-3'; *H5PPF*-r: 5'-GTGTACTGCA CCATGGTTTATTATAGTTGGTCACCAGA-3') were purchased from Genset Inc. (Singapore). The introduced *Nco*I site is underlined and two stop codons are shown as boldface. The PCR fragment with the end-repaired *BamH*I-5' and *Nco*I-3' termini was directionally cloned into the end-repaired *EcoR*I and *Nco*I sites of the expression vector pMEx8 that contained the *tac* promoter (Buttcher *et al.*, 1990), thus producing the recombinant plasmid pM4BH1-5. The PPF-coding sequence was verified by DNA sequencing using an ABI prism 377 sequencer (Perkin Elmer, Norwalk, USA).

**Expression and preparation of the PPF protein** The PPF protein was over-expressed in the *E. coli* strain JM109 by addition of isopropyl-β-D-thiogalactopyranoside (IPTG, 0.1 mM) to the mid-exponential phase cultures, and partially purified as described previously (Uawithya *et al.*, 1998). Protein concentrations were determined using a Bio-Rad protein quantitation kit. The protein inclusions (1 mg · $ml^{-1}$) were solubilized at 37°C for 1 hr in 50 mM $Na_2CO_3$ buffer, pH 9.0 supplemented with 6 M urea (Fluka Chemie AG, Switzerland), and any insoluble protein was removed by centrifugation in a bench minifuge at 16,000 × *g* for 15 min. The proteins were refolded by stepwise dialysis against 300 volumes of carbonate buffers with decreasing urea concentrations of 3 M, 1.5 M, 0.75 M, 0.5 M, 0.25 M, and 0.1 M at 25°C for 2-3 hr each. They were finally dialyzed twice against 300 volumes of a carbonate buffer.

Proteolytic stability of the refolded PPF protein was analyzed by digestion with 1 : 50 (w/w) trypsin (L-1-tosylamide-2-phenylethyl chloromethyl ketone treated, Sigma Chemical Co., St. Louis, USA): protein at 37°C for 1 hr. The samples were then analyzed by SDS 15% (w/v) PAGE. Immunoblotting was performed with polyclonal rabbit antibodies against the full length activated Cry4B toxin. Immunocomplexes were detected with an anti-rabbit antibody-alkaline phosphatase conjugate (Sigma). An ABI 492 automated sequencer (Perkin Elmer) was used to determine the N-terminal sequences of the electroblotted proteins on a polyvinylidene difluoride (PVDF) membrane (Problott, Applied Biosystems, USA).

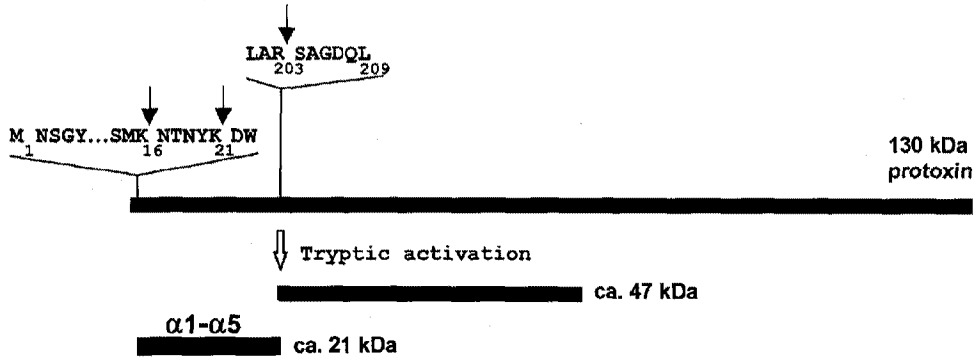


**Fig. 1.** Tryptic processing of the 130-kDa Cry4B protoxin. An overview of the results obtained from N-terminal sequencing of trypsin-treated products of Cry4B. The arrows indicate the known cleavage sites within the toxin. The region between Met-1 and Leu-209 represent the PPF fragment.

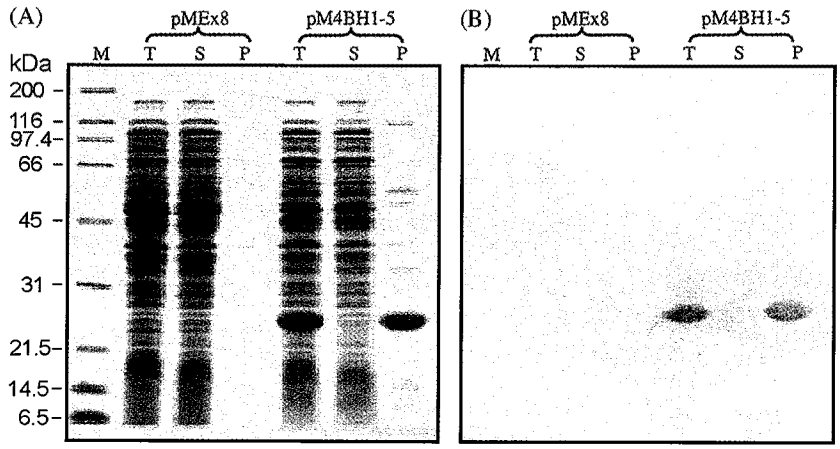


**Fig. 2.** Expression of the PPF protein in *E. coli.* (A) A Coomassie brilliant blue-strained SDS-15% polyacrylamide gel showing the total crude extracts (T) of *E. coli* cells harboring the pMEx8 vector or the pM4BH1-5 recombinant encoding the PPF protein. (B) Immunoblot analysis of (A) showing the 23-kDa PPF protein that cross-reacted with the Cry4B antibodies. (M) represents molecular mass standards. (S) and (P) are the supernatant and pellet fractions, respectively.

**Liposome entrapped glucose release assays** Liposomes with trapped glucose were prepared by a modified method of Kinsky (1974). A lipid mixture (Sigma) of 12.5 μmole phosphatidylcholine (PC), 3.6 μmole dicetyl phosphate, and 1.8 μmole cholesterol in 3 ml chloroform/methanol (2 : 1, v/v) was placed in a round bottomed flask. The solvent was then removed under vacuum at 37°C. The resulting lipid film was resuspended in 0.5 ml of 300 mM glucose/10 mM HEPES, pH 8.0. Small unilamellar vesicles (SUVs) were prepared by squeezing the suspension through the extruder membrane (0.1 μm in diameter, Avanti Polar Lipid, USA) for a minimum of 11 passes. Unentrapped glucose was removed from the SUV suspension by gel filtration on a PD-10 column (Sephadex G-25, Pharmacia, Uppsala, Sweden), equilibrated 150 mM KCl/10 mM HEPES, pH 8.0. An aliquot of washed liposomes (100 nmole PC) was placed in a 1 ml disposable polymethyl methacrylate cuvette (Brand, Germany) containing 1 unit of hexokinase (Sigma), 1 unit of glucose-6-phosphate dehydrogenase (Sigma), 1 mM ATP, 0.5 mM NADP, 2 mM Mg $(OAc)_2$ in 150 mM KCl/10 mM HEPES, pH 8.0. Glucose release, detected as an increase in absorbance of NADPH at 340 nm, was monitored at 25°C on a HP 8453 spectrophotometer (Hewlett Packard, USA). The relative glucose-release activities are indicated as a fraction of maximum release, which is defined as the amount released by 0.1% Triton X-100.

**Mosquito-larvicidal activity assays** Bioassays were performed as previously described using 2-day old *Aedes aegypti* larvae (Sramala *et al.*, 2000). In the assays, 1 ml of *E. coli* suspension (ca. $10^8$ cells) was added to a 48-well microtitre plate (11.3 mm well diameter) with 10 larvae per well, and a total of 100 larvae for each type of *E. coli* samples. *E. coli* cells containing the recombinant plasmid pMU388 were used as a positive control. Mortality was recorded after incubation for 24 hr at room temperature (25-30°C).

## Results and Discussion

In preliminary experiments, the results of the size-exclusion FPLC analysis under non-denaturing conditions (carbonate buffer, pH 9.0) indicated that the 21-kDa N-terminal helix bundle (α1-α5) of the Cry4B toxin remains non-covalently associated with the C-terminal portion (ca. 47 kDa) of the activated toxin after proteolytic activation with trypsin (Angsuthanasombat, unpublished data). Attempts made to isolate this α1-α5 fragment by FPLC under denaturing conditions (8 M urea, carbonate buffer, pH 9.0) were unsuccessful. In this study, cloning of the PPF fragment in *E. coli*, and over-expression of the protein, allowed the isolation and biochemical characterization of this PPF protein.

Upon induction with IPTG, the *E. coli* cells harboring the plasmid clone (pM4BH1-5) expressed the PPF protein at high levels in the form of sedimentable inclusion bodies with a yield of approximately 15 mg per liter of culture. An analysis of the inclusion preparation by SDS-PAGE revealed a major band at 23 kDa (see Fig. 2A), which specifically cross-reacted in Western blots that were probed with antibodies raised against the activated Cry4B toxin (Fig. 2B). The N-terminal sequence of the 23-kDa protein, obtained by automated Edman degradation, was found to be identical to the N-terminus of the 130-kDa protoxin (MNSGY). Since the predicted molecular mass of the polypeptide encoded in the cloned PPF fragment is 23.587 kDa, these results provide evidence that the 23-kDa product we obtained from over-expressing *E. coli* corresponds to the N-terminal α1-α5 segment of the Cry4B toxin.

Unlike the inclusions of the full length protoxin (1 mg · $ml^{-1}$), which are soluble (up to 80%) in a carbonate buffer, pH 9.0 (Uawithya *et al.*, 1998), the PPF inclusions (1 mg · $ml^{-1}$) were readily soluble only in the presence of 6 M urea, giving at least 60% solubility. The PPF protein was refolded by a stepwise dialysis against buffers with decreasing urea concentrations. Finally the protein was obtained in an urea-free carbonate buffer with pH 9.0 at a concentration of 0.5 mg · $ml^{-1}$. The refolded PPF preparation (Fig. 3, lane 3) was

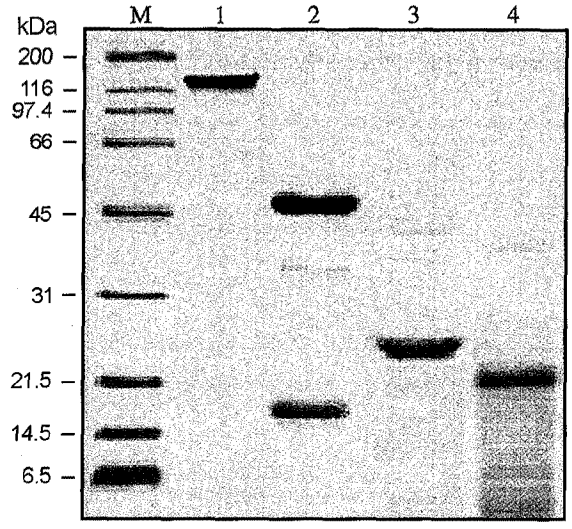


**Fig. 3.** SDS-PAGE (15% gel) analysis of tryptic processing of Cry4B and the PPF protein. M represents molecular mass standards. Lane 1 is the 130-kDa solubilized protoxin. Lane 2 is the activated toxin. Lanes 3 and 4 are refolded PPF and the refolded protein treated with trypsin (1 : 50 enzyme:protein, w/w), respectively.

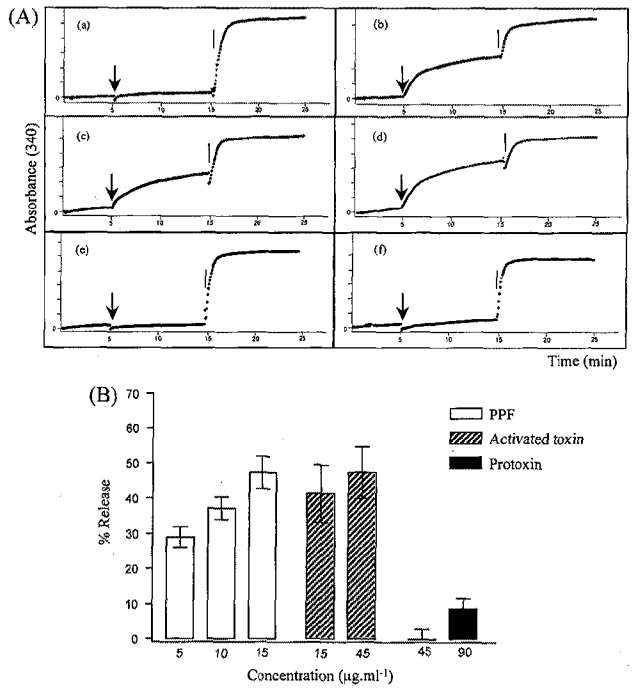


**Fig. 4.** Effect on glucose release from liposomes. (A) Release of entrapped glucose was monitored by using NADP-linked enzyme-linked assays at 25°C. The traces represent absorbance at 340 nm, which was continuously recorded for 5 min prior to adding each protein sample, as indicated by an arrow. The maximum release was obtained by adding 0.1% Triton X-100 (indicated by a vertical bar) after 10 min incubation with samples; (a) 100 µl carbonate buffer, (b) 15 µg · ml$^{-1}$ refolded PPF, (c and d) 15 and 45 µg · ml$^{-1}$ activated Cry4B and (e and f) 45 and 90 µg · ml$^{-1}$ 130-kDa protoxin. (B) The relative release activities of each protein sample with varying concentrations that are indicated as a fraction of the 100% release induced by Triton X-100. Error bars represent standard error of the mean from the five separate experiments. The release in the control sample incubated with carbonate buffer rarely exceeded 10% and these values have been subtracted in the figure.

subjected to size-exclusion FPLC using a Sephacryl S-100 column (Pharmacia) with a carbonate buffer, pH 9.0 as eluent at a flow rate of 0.5 ml · min$^{-1}$. Elution of the protein was monitored at 280 nm, and the 23-kDa PPF protein was eluted in the void volume of the column (data not shown). This suggested that a high molecular mass (>300 kDa) oligomer, or aggregate, was present rather than the monomeric form of the refolded PPF protein. Notwithstanding this observation, this aggregate could represent a stable and functional oligomerisation state of the PPF fragment with the ability to intercalate into lipid membranes to form a pore. Interestingly, treatment of the refolded 23-kDa PPF protein with trypsin resulted in a 21-kDa protein fragment that was detectable by SDS-PAGE (see Fig. 3, lane 4), which specifically cross-reacted with the Cry4B toxin antibodies (data not shown). We conclude that this 21-kDa PPF protein is structurally resistant to further proteolysis. Also, the refolded 23-kDa PPF protein likely exists in its native folded conformation. Proteins, which are extensively resistant to proteolysis, have been shown to maintain a significant portion of native-like conformation (Fontana *et al.*, 1997).

No significant activity was observed in bioassays against *Aedes aegypti* larvae using the *E. coli* cells that expressed the PPF protein. A lack of toxicity is most likely due to the absence of a three-β-sheet domain (Li *et al.*, 1991), which is required for binding of the activated toxin to the larval midgut epithelial cells *via* specific receptors. It is still an open question whether, after proteolytic activation *in vivo*, the bundle of five helices remain associated with the 47-kDa fragment that contains the receptor-binding domain in the pore-forming process. In other studies with the Cry4A cleavage products genetically fused with glutathione S-transferase (GST), significant larvicidal activity was observed only when both of the fusion proteins, GST-20-kDa (α1-α5) and GST-45-kDa containing the receptor-binding domain, were co-ingested by the larvae (Yamagiwa *et al.*, 1999).

The membrane lytic capability of the refolded PPF protein was assayed for its ability to release glucose from receptor-free phospholipid vesicles. Under the conditions used in this assay, the refolded PPF fragment was able to induce the release of entrapped glucose from liposomes, and the release activity depended on the concentration of the protein used (see Fig. 4). Approximately 47% of maximum release (when compared to Triton X-100 as a positive control) was observed within 10 min after the addition of the protein (15 µg · ml$^{-1}$ or ca. 0.6 mM). These results are consistent with former findings that the N-terminal α-helical bundles of the Cry1Ac and

Cry3B2 toxins were able to mediate the release of $^{86}Rb$ cation from phospholipid vesicles, and form cation selective channels in planar lipid bilayers (Walters *et al.*, 1993; Von Tersch *et al.*, 1994). At the equivalent molar concentration to that of the PPF protein, the full length activated Cry4B toxin (45 μg · $ml^{-1}$) exhibited similar activity in the glucose release assays; whereas, the 130-kDa protoxin (90 μg · $ml^{-1}$), and a control sample containing the carbonate buffer (50 mM $Na_2CO_3$, pH 9.0), showed little glucose release (<10%). Similarly, it was reported by other workers that the full length activated toxins of Cry1Aa (ca. 65 kDa), Cry1Ac (ca. 55 kDa), and Cry1C (ca. 67 kDa) were able to form channels in planar bilayers in the concentration range of 15-32 μg · $ml^{-1}$ (Slatin *et al.*, 1990; Schwartz *et al.*, 1993; Schwartz *et al.*, 1997). However, the stimulus for the channel formation is still unclear. High toxin concentrations might be sufficient to obviate the requirement for a receptor, which might favor spontaneous membrane insertion of the toxins.

In summary, our results demonstrate that the 23-kDa α1-α5 fragment is fully capable of permeabilising the membrane liposomes *in vitro*, apart from the rest of the activated Cry4B toxin. It, therefore, constitutes the region that is responsible for membrane insertion and pore formation within the Cry toxin molecules. The cloned PPF fragment will facilitate further investigations on the pore-forming mechanism of the Cry insecticidal toxins.

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