Ex vivo Cytotoxicity of the *Bacillus thuringiensis* Cry4B δ-Endotoxin to Isolated Midguts of *Aedes aegypti* Larvae

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The pathological effect of the *Bacillus thuringiensis* Cry δ-endotoxins on susceptible insect larvae had extensive damage on the midgut epithelial cells. In this study, an *ex vivo* assay was devised for assessing the insecticidal potency of the cloned Cry4B mosquito-larvicidal protein that is expressed in *Escherichia coli*. Determination of toxicity was carried out by using a cell viability assay on the midguts that were dissected from 5-day old *Aedes aegypti* mosquito larvae. After incubation with the toxin proteins, the number of viable epithelial cells was determined photometrically by monitoring the quantity of the bioreduced formazan product at 490 nm. The results showed that the 65-kDa trypsin-activated Cry4B toxin exhibited toxic potency ca. 3.5 times higher than the 130-kDa Cry4B protoxin. However, the trypsin-treated products of the non-bioactive Cry4B mutant (R158A) and the lepidopteran-specific Cry1Aa toxin displayed relatively no *ex vivo* activity on the mosquito-larval midguts. The *ex vivo* cytotoxicity studies presented here confirms data that was obtained in bioassays.

Keywords: *Bacillus thuringiensis*, Cell viability assay, δ-Endotoxin, *Ex vivo* toxicity, Mosquito-larval midgut, Toxic potency

Introduction

During sporulation, *Bacillus thuringiensis* (*Bt*) synthesizes cytoplasmic crystalline inclusions, which contain one or more proteins of varying molecular mass (Aronson *et al*., 1986).

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These crystal proteins, which have been classified as Cry and Cyt δ-endotoxins, are specifically toxic to the larvae of insect crop pests and disease vectors (Hofte and Whiteley, 1989; Crickmore *et al*., 1998; Schnepf *et al*., 1998). For instance, the 130-kDa Cry4B δ-endotoxin, one of the four major insecticidal proteins that are produced by *Bt* subsp. *israelensis* (*Bti*), is highly toxic to mosquito larvae, including the *Aedes* and *Anopheles* species (Hofte and Whiteley, 1989; Angsuthanasombat *et al.,* 1992; Schnepf *et al*., 1998).

In the *Bt* inclusion bodies, the δ-endotoxins exist as inactive protoxins that are dependent on the larval midgut environment for solubilization and proteolytic activation. When ingested by susceptible insect larvae, the protein inclusions are solubilized and the protoxins activated by the alkaline pH and proteases of the larval midgut. The activated toxins first recognize and bind to specific receptors that line the microvillus surface of midgut epithelial cells, and then insert into the plasma membrane of the epithelial cells, causing them to swell and lyse. The larvae then stop feeding and eventually die (Aronson *et al*, 1986; Knowles, 1994).

The cytolytic activity of a number of the Cry toxins has been demonstrated *in vitro* using a wide range of lepidopteran and dipteran cell lines (Knowles and Ellar, 1987; Thomas and Ellar, 1993). Our earlier work revealed that the *in vitro* cytotoxicity of the cloned Cry4B toxin to mosquito cell lines is not entirely consistent with its toxicity *in vivo* (Angsuthanasombat *et al*., 1993). Poor correlations, observed between *in vivo* and *in vitro* assays of the Cry2Aa toxin, have been reported (Nicholls *et al*., 1989). Even considering these observations, the insect cell line assay might not be a good model system for studying toxin mechanism *in vitro* since the cell lines that were used did not originate from the midgut epithelial cell targets of these *Bt* toxins (Hink, 1972). Presently, no insect midgut epithelial cell line has been successfully cultured *in vitro*. It would therefore be interesting to employ the isolated larval midguts for investigating this

underlying toxicity mechanism. In this report, we describe a microscale *ex vivo* toxicity assay for demonstration of the cytolytic activity of the cloned Cry4B toxin on isolated midguts from *Aedes aegypti* mosquito larvae.

Materials and Methods

Toxin expression, inclusion solubilization, and proteolytic activation The cloned Cry4B toxin (Angsuthanasombat *et al.*, 1987), its non-toxic R158A mutant (Sramala *et al.*, 2001), and the cloned Cry1Aa toxin (a generous gift of Dr. J-L Schwartz, National Research Council of Canada) were over-expressed in *E. coli* as inclusion bodies upon IPTG (isopropyl-β-D-thiogalactopyranoside) induction at 37°C. After disruption in a French Press Cell, protoxin inclusions in the crude lysates were obtained by centrifugation, as described earlier (Uawithya *et al.*, 1998). Protein concentrations were determined by using a dye-binding method (BioRad, Hercules, USA), with bovine serum albumin (Sigma, St. Louis, USA) as a standard. Protoxin inclusions (3-5 mg ml^{-1}) were solubilized in 50 mM Na$_2$CO$_3$, pH 10.0 at 37°C for 60 min. The solubilized protoxins were digested with trypsin (*N*-tosyl-*L*-phenylalanine chloromethyl ketone treated, Sigma) at a toxin:enzyme ratio of 20 : 1 (w/w) for 12 h, and were analyzed by 12.5% (w/v) SDS polyacrylamide gel electrophoresis as described previously (Uawithya *et al.*, 1998).

Larval midgut preparation *Aedes aegypti* and *Culex quinquefasciatus* mosquito eggs that were obtained from the mosquito-rearing facility (Institute of Molecular Biology and Genetics, Mahidol University) were hatched in a plastic container (22 × 30 × 10 cm deep) with 500 ml of fresh tap water that was supplemented with 2 pieces of fish diet (0.12 g per piece). After a 24 h incubation, approximately 200 larvae were placed into a new container with 1,000 ml of water and 3 pieces of diet pellets. Each day, the 1,000 ml of water and 3 pieces of diet were replaced, and rearing was carried out at 30°C. Before being used for the midgut preparation, 4-d old healthy larvae were selected for a fast of 24 h in 1,500 ml of fresh water.

The 5-d old larvae were then chilled in an ice-cold dissecting buffer (5 mM KCl, 0.5 mM MgSO$_4$, 100 mM NaCl, 20 mM NaHCO$_3$, 5 mM Na$_2$HPO$_4$, 10 mM glucose, pH 8.6) for 5 min. Midguts were dissected after the removal of peritrophic membranes and malpighian tubules. Freshly prepared midguts were kept in the dissecting buffer and used for the *ex vivo* assay within 2 h after dissection.

Ex vivo toxicity assay Cytotoxicity was assessed *via* a cell viability assay using the CellTiter96®AQ$_{ueous}$ kit (Promega Corporation, Madison, USA), which is based on the quantitative determination of the conversion of a tetrazolium compound to a formazan product that is directly proportional to the number of living cells. The assay was performed in a 96-well flat-bottomed microtitre plate containing 70 μl of the dissecting buffer with 5 isolated midguts. Three wells were used for each toxin in each experiment. After adding 10 μl of the toxin samples (3-6 μg) and 20 μl of the CellTiter96 solution, formation of the reduced formazan product in each well was simultaneously monitored at 490 nm by using a SpectraMax 250 microtitre plate reader (Molecular Devices Corporation, Sunnyvale, USA), and the background signal that measured at A$_{650}$ was subtracted. A$_{490}$ readings were recorded at intervals of 1 min for a period of 3 h at room temperature. Three independent experiments were carried out for each toxin sample.

Each reaction curve was analyzed by nonlinear regression using GraphPad PRISM version 2.0. Net toxic potency value (T_{net}) for each toxin was calculated by subtraction of the specific cell death factor with that of the buffer. A statistical analysis was carried out by using one-way analysis of variance (ANOVA) tests for comparing all the group means. The Tukey-type multiple comparison testing among pairs of group means (Zar, 1984) was used as post tests to determine which groups are statistically significantly different from other groups. Probabilities <0.05 were considered as being significant.

***In vivo* larvicidal activity assays** A mosquito-larvicidal activity test was performed, as described previously, using 2-d old *A. aegypti* larvae (Sramala *et al.*, 2000). The assay was carried out with 1 ml of *E. coli* suspension (10^8 cells suspended in distilled water) in a 48-well mirotitre plate (11.3 mm well diameter), with 10 larvae per well and a total of 100 larvae for each *E. coli* clone. Mortality was recorded after a 24-h incubation.

Results and Discussion

The *in vivo* bioassays that are presented here (Fig. 1) clearly confirm our previous report (Angsuthanasombat *et al.*, 1992) that *E. coli* cells expressing the 130-kDa Cry4B toxin were highly active (95.7 ± 1.8% mortality) against *A. aegypti* larvae, but not against *C. quinquefasciatus* larvae. The *E. coli* cells expressing the mutant-R158A, whose Arg-158 in helix 4 was mutated to alanine (Sramala *et al.*, 2001), or the lepidopteran-specific Cry1Aa toxin were relatively non-toxic to *Aedes* larvae (Fig. 1). Examination of insect larval gut
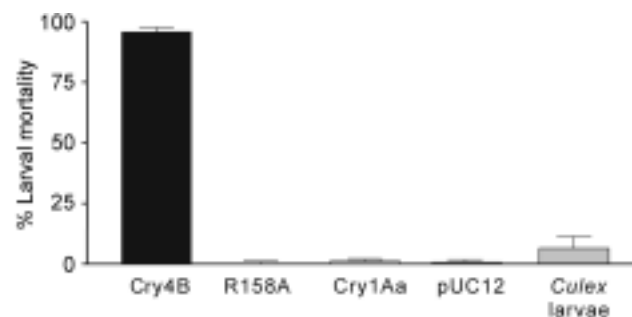

Fig. 1. Mosquito-larvicidal activity of *E. coli* cells expressing one of Cry4B, its mutant (R158A), and Cry1Aa against *A. aegypti* larvae. *E. coli* cells containing the pUC12 vector were used as negative control cells. *C. quinquefasciatus* larvae were used as negative control larvae. Error bars indicate standard error of the means from three independent experiments.
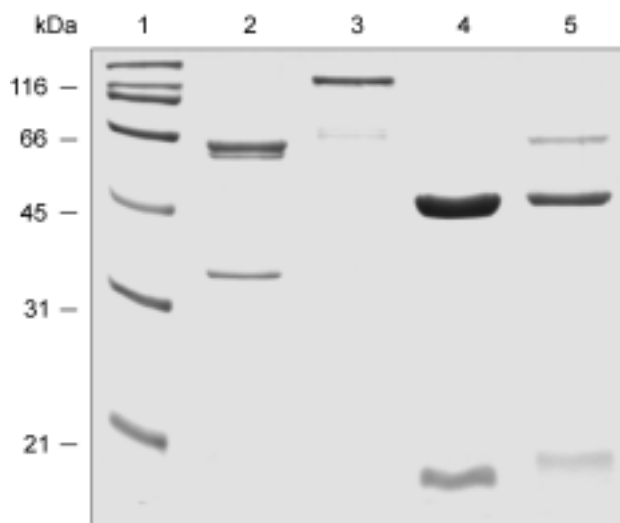
Fig. 2. Coomassie brilliant blue-stained 12.5% SDS of PAGE of solubilized protoxins and trypsin-treated toxins. Lane 1 represents standard molecular mass markers. Lane 2 is the trypsin-treated Cry1Aa toxin. Lanes 3, 4, and 5 are the 130-kDa solubilized Cry4B protoxin, trypsin-treated products of Cry4B, and its mutant (R158A), respectively.

tissue following ingestion of the *Bt* toxins revealed that midgut epithelial cells are the primary action sites (Aronson *et al.*, 1986). In this context, the isolated midgut tissue of susceptible mosquito-larvae would, therefore, represent a good model system for directly investigating the underlying toxicity mechanism of the cloned Cry4B toxin.

As described previously, toxin activation of the cloned Cry4B protein that is expressed in the *Bti* strain IPS-78/11 can be simulated *in vitro* by solubilizing the protoxin inclusions in alkaline buffers and subsequently digesting with trypsin (Angsuthanasombat *et al.*, 1991). In this study, the Cry4B toxin, its non-toxic R158A mutant, and the Cry1Aa toxin (over-expressed in *E. coli* as inclusion bodies) were solubilized in a carbonate buffer, pH 10.0, and treated with trypsin. An SDS-PAGE analysis revealed that the 130-kDa Cry4B protoxin and the R-158 mutant were proteolytically processed to stable bands of ca. 47 and ca. 20 kDa (Fig. 2, lanes 3-5). These two fragments are produced by cleavage in the exposed loop linking helices 5 and 6 within the pore-forming domain in addition to the removal of the C-terminal half of the Cry4B protein molecule; they remained associated under physiological conditions (Angsuthanasombat *et al.*, 1993). Unlike the Cry4B toxin, the 130-kDa Cry1Aa protoxin, upon trypsin digestion, showed a dominant protease-resistant polypeptide of ca. 65 kDa (Fig. 2, lane 2). Although there are no reports of interhelical proteolysis for the lepidopteran-specific Cry1 toxins, the 65-kDa activation products of Cry1Ac or Cry1C were able to lyse susceptible insect cell lines *in vitro* (Knowles and Ellar, 1987; Schwartz *et al.*, 1991).

Previous studies showed that the Cry4B toxin could bind

only to the apical brush-border membranes of midgut cells of *Anopheles gambiae* mosquito larvae, but exhibited weak binding to gastric caecae and anterior gut regions (Ravoahangimalala and Charles, 1995). In our study, *Aedes* larval epithelial cells were therefore prepared from isolated midguts for the assessment of Cry4B toxicity *via* a cell viability assay. After incubating the dissected midguts with the toxin samples, the number of viable epithelial cells was determined by a colorimetric method that monitors the quantity of water-soluble formazan products by photometric measurement at 490 nm (Cory *et al.*, 1991). Changes in A_{490} were assumed to be directly proportional to the number of viable cells (Fig. 3). It should be noted that a small decline of A_{490}, which is due to the degradation of formazan that is possibly caused by oxidative agents from the dissected tissues, was observed in the assays. Therefore, the *ex vivo* data were corrected for the formazan degradation by the decay factor D, as shown in the following equation:

$$\frac{dA490}{dt} = k\,Nt - D\,A_{490} \tag{1}$$

in which A_{490} is the absorbance at 490 nm, t is the reaction time, k is the formazan generation constant, and D is the correction factor for formazan degradation. N_t represents the number of cells at the time t. The decrease in the number of viable cells can be described by an exponential decay model as follows:

$$Nt = No\,e^{-Tt} \tag{2}$$

in which N_O is the number of viable cells at $t = 0$, and T represents the specific cell death factor or toxic potency. Therefore, experimentally observed changes at A_{490} can be expressed as a function of time. After substituting for Nt in equation 1, this gives, on integration:

$$A_{490} = \left(\frac{kN_o e^{-Tt}}{D-T}\right) + (C\,e^{-Dt}) \tag{3}$$

where C is a constant of integration, which is approximately equal to $A_{490\,max} - A_{490\,min}$. The decay factor D for formazan degradation was experimentally determined to be 0.15 h^{-1} by fitting with decreasing A_{490} values that were obtained after a 4-h incubation. Since the kN_0 parameter was used as a constant of 1 (an averaged experimental value of initial velocity of formazan development as $No = Nt$; $t \to 0$), equation 3 may be simply written as follows:

$$A_{490} = \left(\frac{e^{-Tt}}{D-T}\right) + (C\,e^{-Dt}) \tag{4}$$

The reaction curves (Fig. 3) revealed that the number of viable cells after a 1 h exposure to the 65-kDa trypsin-activated Cry4B toxin was significantly lower than that of the cells that were incubated with the protoxin or the trypsin-treated products of Cry4B-R158A and Cry1Aa toxins. This
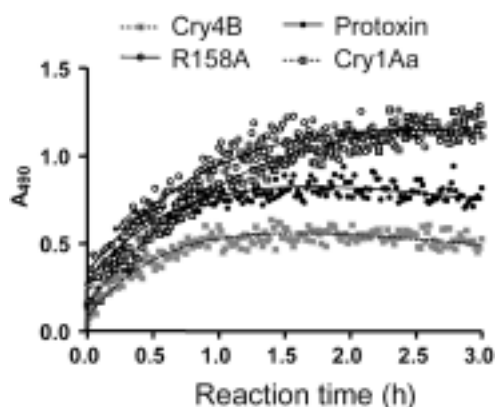
Fig. 3. Reaction progress curves of the *ex vivo* toxicity assay. Example traces represent absorbance at 490 nm, which is directly proportional to the number of viable cells of the isolated larval midguts after incubation with each toxin sample of the Cry4B toxin (30 µg ml^{-1}) and its mutant-R158A (30 µg ml^{-1}) that was treated with trypsin, the 130-kDa solubilized Cry4B protoxin (60 µg ml^{-1}) and the trypsin treated Cry1Aa toxin (30 µg ml^{-1}), as determined by monitoring the absorbance at 490 nm. Reaction curves were analyzed to obtain the potency parameter by using a nonlinear regression curve fitting method with GraphPad PRISM version 2.0.

result suggests that the toxic effect of the trypsin-treated Cry4B toxin on the *Aedes* midgut cells is comparatively higher than the protoxin and mutant, or trypsin-treated Cry1Aa toxin. These observations are almost consistent with the data that was obtained in bioassays as *E. coli* cells expressing the Cry4B-R158A mutant or the lepidopteran-specific Cry1Aa toxin was relatively non-toxic to *Aedes* larvae (Fig. 1).

Each reaction curve was analyzed for the potency parameter by nonlinear regression curve fitting (Fig. 4) and the net toxic potency (T_{net}) of the samples was compared. The analysis shows that the activated Cry4B toxin (30 µg ml^{-1}) exhibits toxic potency, which is approximately 3.5 times (P < 0.01, Tukeys test) higher than the value for the 130-kDa protoxin (60 µg ml^{-1}). The T_{net} value (0.12 ± 0.06 h^{-1}) that was observed for the Cry4B protoxin is conceivably due to the activity of the protoxin being activated by proteases that must occur in the isolated larval midguts. However, the trypsin-treated products of both the non-bioactive Cry4B-R158A mutant and lepidopteran-active Cry1Aa toxin (30 µg ml^{-1}) displayed relatively low or no *ex vivo* cytotoxicity against the isolated larval midguts, by giving T_{net} values of 0.06 ± 0.05 h^{-1} and 0.05 ± 0.07 h^{-1}, respectively (P value < 0.01). Consistent with the results of the *in vivo* activity that was mentioned earlier, an equivalent concentration (3 µg ml^{-1}) of the activated Cry4B toxin exhibited very low *ex vivo* activity against the isolated *Culex* midgut cells (T_{net} 0.04 ± 0.02 h^{-1}). This indicates a very small population or the absence of Cry4B toxin-specific receptors on the midgut epithelial cell membranes of this non-susceptible insect.
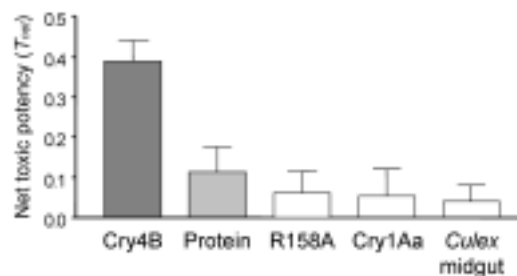

Fig. 4. Comparison of net toxic potency, T_{net} (h^{-1}), of the Cry4B toxin and its mutant (R158A) treated with trypsin, the 130-kDa solubilized Cry4B protoxin, and the trypsin-treated Cry1Aa toxin on isolated *A. aegypti* larval midguts *via* the *ex vivo* cytotoxicity assay. The isolated *C. quinquefasciatus* larval midguts were used as negative control midgut cells for the activated Cry4B toxin. Error bars indicate standard error of the means from three independent experiments.

In conclusion, we devised a microscale *ex vivo* cytotoxicity assay for assessing the insecticidal potency of the Cry4B mosquito-larvicidal proteins, and demonstrated that the trypsin-activated Cry4B toxin specifically exerted its cytolytic activity towards the isolated midguts from *Aedes aegypti* larvae. The availability of this *ex vivo* system will allow further investigation of the mechanism of action and the nature of the specific receptors on the *Aedes* midgut cell surface.

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