Comparative Toxicity of *Bacillus thuringiensis* var. *israelensis* Crystal Proteins *in vivo* and *in vitro*

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Bacillus thuringiensis var. *israelensis* crystal proteins were purified by FPLC on a Mono Q column to yield 130, 65, 28, 53, 30–35 and 25 kDa proteins. All the purified proteins killed *Aedes aegypti* larvae after citrate precipitation, but the 65 kDa protein was the most toxic. A precipitated mixture of 27 and 130 kDa proteins was almost as toxic as solubilized crystals. In assays against a range of insect cell lines, the activated form (25 kDa) of the 27 kDa protein was generally cytotoxic with the lowest LC₅₀ values *in vitro*. By contrast, the activated forms of the 130 kDa and 65 kDa protoxins (53 kDa and 30–35 kDa proteins, respectively) were much more specific than the 25 kDa protein in their action on dipteran cells, and each showed a unique toxicity profile which, in the case of the 130 kDa preparation, was restricted to *Anopheles* and *Culex* cell lines.

INTRODUCTION

Bacillus thuringiensis var. *israelensis* (*Bti*), during sporulation, produces a protein crystal that is toxic to mosquito and blackfly larvae (Goldberg & Margalit, 1977). The *Bti* crystal contains three major proteins of 27 kDa, 65 kDa and a doublet of 130 kDa, but the role of each of these in determining the insecticidal specificity and potency of the crystal is not known. The toxicity of native crystals against mosquito larvae ranges from 0.2–5 ng ml⁻¹ (Thomas & Ellar, 1983; Yamamoto *et al.*, 1983). Because mosquito larvae are filter feeders, the soluble proteins have been attached to latex beads (Schnell *et al.*, 1984), encapsulated (Cheung & Hammock, 1985) or precipitated (Insell & Fitz-James, 1985) to increase the amount of the protein ingested by the larvae. Reported LC₅₀ values for the purified components are 40 ng ml⁻¹ for the 130 kDa doublet (Visser *et al.*, 1986), 43–600 ng ml⁻¹ for the 65 kDa protein (Insell & Fitz-James, 1985; Ibarra & Federici, 1986) and 125–1000 ng ml⁻¹ (Visser *et al.*, 1986; Ward *et al.*, 1986) for the 27 kDa protein. Wu & Chang (1985) reported that when 27 plus 65 kDa or 27 plus 130 kDa proteins were fed to mosquito larvae, the toxicity of each mixture was greater than if each protein had been fed alone, suggesting that they acted synergistically. The 25 kDa protein derived from proteolysis of the 27 kDa protein is cytolytic (Thomas & Ellar, 1983; Armstrong *et al.*, 1985), haemolytic, neurotoxic (Chilcott *et al.*, 1984; Singh & Gill, 1985) and toxic to suckling mice (Thomas & Ellar, 1983).

Here, we report the isolation of the crystal proteins (130, 65 and 28 kDa proteins) and their proteolytic products (53, 30–35 and 25 kDa proteins), comparing their toxicity, alone and in combination, to mosquito larvae and to insect cell lines.

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Abbreviations: LC₅₀, concentration of toxin required to kill 50% of the mosquito larvae or tissue culture cells; FPLC, fast protein liquid chromatography.

METHODS

Purification of *Bti* crystals. *Bacillus thuringiensis* var. *israelensis* (IPS78) was obtained from H. de Barjac (Institut Pasteur, Paris, France). Bacteria were grown in 3 l of casein yeast medium (Stewart *et al.*, 1981) at 30 °C for 24–36 h on an orbital shaker (200 r.p.m.). The sporulated culture was harvested by centrifugation (12000 g, 15 min) and the pellet was washed six times with distilled water. The crystals were purified by ultracentrifugation on discontinuous sucrose gradients as described by Thomas & Ellar (1983).

Protein estimation. The protein concentration was determined by the method of Petersen (1977) using bovine serum albumin (Sigma) as a standard.

SDS-PAGE. Separation of the proteins on 10 or 13% SDS-polyacrylamide gels was done as described by Thomas & Ellar (1983).

Insect cell lines. *Aedes albopictus* cells (Diptera, trypsinized larval tissue, obtained from Mrs T. Lescott, NERC, Institute of Virology, Oxford, UK), *Aedes aegypti* cells (Diptera, trypsinized larval tissue) and *Culex quinquefasciatus* cells (Diptera, larval tissue, obtained from Dr D. W. Roberts, Boyce Thompson Institute, Ithaca, USA), were grown in Mitsuhashi and Maramorosch medium (Hink, 1972). *Drosophila melanogaster* cells (Diptera, ovarian tissue, obtained from Mrs T. Lescott), were grown in Schneider's *Drosophila* medium (Hink, 1972). *Anopheles gambiae* and *Anopheles stephensi* (Diptera, trypsinized larval tissue, obtained from Dr Varma, School of Hygiene and Tropical Medicine, London, UK) were grown in MK/VP12 medium (Hink, 1972).

Choristoneura fumiferana CF1 and 63CF1 cells (Lepidoptera, spruce budworm, trypsinized larval tissue, obtained from Dr S. S. Sohi, Canadian Forest Pest Management Institute, Ontario, Canada) and *C. fumiferana* H11 cells (obtained from Dr D. Edwards, Mycogen Corporation, San Diego, USA) were grown in Grace's medium (Hink, 1972). *Spodoptera frugiperda* (Lepidoptera, fall armyworm, pupal ovary), *Heliothis zea* (Lepidoptera, cotton bollworm, adult ovary), *Trichoplusia ni* (Lepidoptera, cabbage looper, adult ovary) and *Mamestra brassicae* cells (Lepidoptera, cabbage moth) were obtained from Mrs T. Lescott and grown in TC100 medium (Gardiner & Stockdale, 1975). *Lymantria dispar* cells (Lepidoptera, adult ovary) were obtained from Biological Sciences Research Centre, Shell Development Company, and grown in TC100 medium. All media contained 10% (v/v) foetal calf serum (Gibco) and gentamycin (50 µg ml⁻¹). Cells were grown at 28 °C.

Purification of the crystal proteins. To isolate the crystal proteins several methods were used.

(a) **27 and 130 kDa proteins.** Purified crystals were incubated in 50 mM-Na₂CO₃/HCl, pH 10.3, at 37 °C for 60 min, followed by centrifugation at 10000 g for 10 min in an Eppendorf Minifuge. The pellet was incubated at 50 mM-Na₂CO₃/HCl, pH 9.5, containing 5 mM-phenylmethylsulphonyl fluoride and 10 mM-EDTA at 37 °C for 45 min. Dithiothreitol was added to a final concentration of 10 mM, and the sample incubated at 37 °C for a further 30 min. Purification of the 27 and 130 kDa proteins from this solution was done using fast protein liquid chromatography (FPLC) on a Mono Q column (Pharmacia). The column was equilibrated with 10 mM-NH₄HCO₃, pH 8.5 (pH adjusted with ammonia solution), and the protein was eluted using a 10–800 mM-NH₄HCO₃ (pH 8.5) gradient, over 40 min, at a flow rate of 1 ml min⁻¹.

(b) **25 kDa protein.** Fractions containing the 27 kDa protein peak from the FPLC were pooled and incubated with trypsin (20 µg ml⁻¹) at 37 °C for 30 min. The sample (3 ml) was applied to the Mono Q column and eluted using a 10–600 mM-NH₄HCO₃ (pH 8.5) gradient.

(c) **53 kDa protein.** The fractions containing the 130 kDa protein peak were incubated with trypsin (100 µg ml⁻¹) at 37 °C for 2 h. The sample was applied to the Mono Q column equilibrated with 10 mM-NH₄HCO₃, pH 10, and eluted (with a stepwise gradient) at 472 mM-NH₄HCO₃, pH 10.

(d) **65 kDa protein.** *Bti* crystals were incubated in 50 mM-Na₂CO₃/HCl, pH 9.5, containing 10 mM-dithiothreitol, at 37 °C for 60 min. The sample was centrifuged and the pellet incubated in 50 mM-Na₂CO₃, pH 11.3, at 37 °C for 60 min. The 65 kDa protein was purified on a Sephadex G-75 column equilibrated with 50 mM-NH₄HCO₃, pH 8.5, and eluted at a flow rate of 12 ml h⁻¹.

(e) **30–35 kDa proteins.** Fractions under the 65 kDa protein peak were pooled and incubated with trypsin (20 µg ml⁻¹) at 37 °C for 30 min. The 30–35 kDa proteins were eluted (with a stepwise gradient) at 407 mM-NH₄HCO₃, pH 10.

Mosquito bioassay. Mosquito bioassays were done using 5-d-old *Aedes aegypti* larvae grown from eggs, generously supplied by Mr D. Funnel (Shell Research). The purified proteins were precipitated by adjusting the pH of the sample to pH 4.5 with 12% (w/v) citric acid, and kept at –20 °C for 3 h. The precipitate was centrifuged at 15000 g for 15 min and washed with 2 mM-sodium citrate buffer, pH 4.5. Toxicity of solubilized *Bti* crystals was determined after the following treatment. The crystals were incubated in 50 mM-Na₂CO₃/HCl, pH 9.5, containing 10 mM-dithiothreitol at 37 °C for 60 min and centrifuged. The supernatant was collected and the pellet incubated in 50 mM-Na₂CO₃, pH 11.3, at 37 °C for 60 min. Both supernatants were pooled and precipitated as above. All bioassays were done in 2 mM-sodium citrate buffer, pH 4.5, to prevent solubilization of the precipitated protein, with appropriate controls. Mortality was recorded at 24 h and the LC₅₀ values calculated using interpolation.

Tissue culture assay. Insect cells were harvested and resuspended at 5 × 10⁵ cells ml⁻¹. Samples (100 µl) of the cell suspension were added to each well of a 96 well microtitre plate. The plates were pretreated with collagen (rat

tail, Sigma) at 1 mg ml⁻¹ or with 1% (w/v) gelatin for the lepidopteran cells. All cells were incubated at 28 °C for 2 h. The tissue culture medium was removed and 100 µl of the toxin, diluted in tissue culture medium, was added. Cells were incubated at 28 °C for 30 min (27, 25, 65 and 30–35 kDa proteins) or 90 min (130 and 53 kDa proteins). Cells were then washed twice with Dulbecco's phosphate-buffered saline (Oxoid) (PBS). Neutral red (100 µl, 0.5 mg ml⁻¹) solution was added to each well and the trays were incubated at 28 °C for 90 min. The cells were washed twice with PBS and 200 µl of acid-ethanol solution [equal volume of Sørensen citrate buffer (Perin & Dempsey, 1974), pH 4.2, and ethanol] was added to each well. Absorbance in each well was determined with an ELISA reader (Dynatech) at 540 nm.

RESULTS

The crystal proteins were purified by selective solubilization and ion-exchange chromatography (Fig. 1). FPLC of soluble *Bti* proteins on a Mono Q column at pH 8.5 resulted in the purification of the 27 and 130 kDa proteins (Fig. 2), and the 25 kDa protein following tryptic digestion of the 27 kDa protein (Fig. 3). The 65 kDa protein was purified by gel filtration on Sephadex G-75 column (Fig. 4). Following tryptic digestion of the 130 and 65 kDa proteins, the 53 kDa (Fig. 5) and 30–35 kDa proteins (Fig. 6) were purified on a Mono Q column equilibrated with NH₄HCO₃, pH 10.

The toxicity of the purified proteins against *Aedes aegypti* larvae is shown in Table 1. Mixtures of the 130, 65 and 27 kDa proteins were tested against the mosquito larvae to investigate a possible synergistic relationship between the proteins (Table 2). The ratios of the proteins tested were similar to those observed in the native crystals. A mixture of 130 and 27 kDa proteins (0.25:1, w/w) had an LC₅₀ value of 7.5 ng ml⁻¹, which was more toxic than if either alone had been fed to the larvae (LC₅₀ of 27 kDa is 115 ng ml⁻¹; LC₅₀ of 130 kDa is 32 ng ml⁻¹).

The toxicities of the purified proteins to five dipteran and seven lepidopteran cell lines were compared using the neutral red tissue culture assay (Table 3). Complete loss of viability occurred in 20–30 min when *Aedes aegypti* cells were incubated with 25 kDa protein (8 µg ml⁻¹) (Fig. 7) or when *Anopheles gambiae* cells were incubated with 30–35 kDa proteins (200 µg ml⁻¹) (Fig. 8). However, complete loss of viability of *Culex quinquefasciatus* cells incubated with 53 kDa protein (150 µg ml⁻¹) took 90 min to occur (Fig. 9).

Fig. 1. SDS-PAGE of *Bti* crystal proteins. Lane 1, molecular mass standards; lane 2, 100 µg native *Bti* crystals; lane 3, 50 µg 130 kDa doublet; lane 4, 50 µg 53 kDa protein; lane 5, 50 µg 65 kDa protein; lane 6, 40 µg 30–35 kDa proteins; lane 7, 30 µg 27 kDa protein; lane 8, 25 kDa protein.

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 2. Purification of the 27 and 130 kDa proteins by FPLC using a Mono Q column, equilibrated with 10 mM- NH_4HCO_3 , pH 8.5 (elution gradient 10–800 mM- NH_4HCO_3 ; flow rate 1 ml min^{-1} , 40 min). —, A_{280} ; ---, NH_4HCO_3 concn.

Fig. 3. Purification of the 25 kDa protein by FPLC using a Mono Q column (elution gradient of 10–600 mM- NH_4HCO_3 , pH 8.5). —, ---, As Fig. 2.

Fig. 4. Purification of the 53 kDa protein by FPLC using a Mono Q column (eluted at 472 mM- NH_4HCO_3 , pH 10). —, ---, As Fig. 2.

Fig. 5. Gel filtration chromatography of 65 kDa protein on a Sephadex G-75 column. Flow rate 12 ml h^{-1} ; column size 1.6 $\times$ 60 cm; eluant 50 mM- NH_4HCO_3 , pH 8.5.

Fig. 6. Purification of the 30–35 kDa proteins by FPLC using a Mono Q column (eluted at 407 mM- NH_4HCO_3 , pH 10). —, ---, As Fig. 2.

Fig. 7

Fig. 8

Fig. 9

Fig. 7. Time course of toxicity of *Aedes aegypti* cells incubated with the 25 kDa protein ($8 \mu\text{g ml}^{-1}$).

Fig. 8. Time course of toxicity of *Anopheles gambiae* cells incubated with the 30–35 kDa protein ($200 \mu\text{g ml}^{-1}$).

Fig. 9. Time course of toxicity of *Culex quinquefasciatus* cells incubated with the 53 kDa protein ($150 \mu\text{g ml}^{-1}$).

Table 1. Toxicity of *Bti* crystal proteins against *Ae. aegypti* larvae

Results are the means of four separate assays $\pm$ SD.

Preparation	LC ₅₀ (ng ml ⁻¹)
Native crystals	0.3 $\pm$ 0.08
Solubilized crystals*	1.0 $\pm$ 0.5
130 kDa protein	32.0 $\pm$ 4.0
65 kDa protein	4.0 $\pm$ 1.0
27 kDa protein	115.0 $\pm$ 12.5
53 kDa protein	40.0 $\pm$ 7.2
30–35 kDa protein	12.0 $\pm$ 2.8
25 kDa protein	74.0 $\pm$ 8.4

* As described in Methods.

Table 2. Synergism in mosquitocidal activity of purified 27, 65 and 130 kDa crystal proteins from *Bti*

Results are the means of four separate assays $\pm$ SD.

Protein combination	LC ₅₀ (ng ml ⁻¹)
130/65 (0.25:0.75, w/w)	15.0 $\pm$ 2.1
130/27 (0.25:1.0, w/w)	7.5 $\pm$ 1.8
65/27 (0.75:1.0, w/w)	2.0 $\pm$ 0.6
130/65/27 (0.25:0.75:1.0, by wt)	5.5 $\pm$ 1.1

DISCUSSION

It has been reported that after solubilization the toxicity of crystal protein is reduced markedly by fifty- to one hundred-fold (Insell & Fitz-James, 1985; Visser *et al.*, 1986). This reduction is not due to a real loss in toxicity but rather to a reduced level of toxin ingested by the larvae because of their filter feeding behaviour. To increase the uptake of the soluble proteins we precipitated

Table 3. *Toxicity of purified Bti crystals against insect cell lines*

Results are the means of four separate assays, each using a different batch of cells. In each case the SD values were not greater than 10% of the mean. —, No toxicity at 150 µg ml⁻¹ (130, 65, 27 and 53 kDa proteins) or no toxicity at 100 µg ml⁻¹ (30–35 and 25 kDa proteins).

Cell line	Crystal protein . . .	LC ₅₀ (µg ml ⁻¹)					
		130 kDa	65 kDa	27 kDa	53 kDa	30–35 kDa	25 kDa
<i>Aedes aegypti</i>	—	—	—	25	—	125	2.8
<i>Aedes albopictus</i>	—	—	—	20	—	—	3
<i>Anopheles stephensi</i>	—	—	—	9.1	35	79	3.3
<i>Anopheles gambiae</i>	—	—	—	9.5	23	78	2.8
<i>Culex quinquefasciatus</i>	—	—	—	20	34	90	3
<i>Drosophila melanogaster</i>	—	—	—	7.5	—	78	2.6
<i>Choristoneura fumiferana</i> CF1	—	—	—	98	—	—	34
<i>Choristoneura fumiferana</i> 63CF1	—	—	—	98	—	—	31
<i>Choristoneura fumiferana</i> HD11	—	—	—	88	—	—	36
<i>Heliothis zea</i>	—	—	—	—	—	—	68
<i>Spodoptera frugiperda</i>	—	—	—	98	115	—	11
<i>Mamestra brassicae</i>	—	86	—	45	44	94	2
<i>Lymantria dispar</i>	—	—	—	92	—	—	—

them at pH 4.5, resulting in a threefold drop in toxicity. The most toxic protein against mosquito larvae was the 65 kDa protein, with a LC₅₀ value of 4 ng ml⁻¹. Ibarra & Federici (1986) reported the LC₅₀ value of the 65 kDa protein as 43 ng ml⁻¹; they also showed that proteolysis of the 65 kDa protein with trypsin produced a 38 kDa protein. We found that tryptic digestion of the 65 kDa protein formed two proteins of 30 and 35 kDa. These may either come from the same 65 kDa protein or there may be two 65 kDa proteins in the crystal (i.e. one 65 kDa protein forming the 30 kDa protein and the other 65 kDa protein forming the 35 kDa protein). The LC₅₀ value of 35 ng ml⁻¹ for the 130 kDa protein is similar to the value of 40 ng ml⁻¹ reported by Visser *et al.* (1986) when the 130 kDa protein was attached to latex beads. However, the LC₅₀ value for the 28 and 25 kDa proteins (attached to latex beads) reported by Visser *et al.* (1986) and Wu & Chang (1985) is less than our value of 115 ng ml⁻¹. Ward *et al.* (1986) reported that inclusions of the 27 kDa protein produced by *Bacillus subtilis* cells, containing the gene coding for the 27 kDa protein, had an LC₅₀ value of 125 ng ml⁻¹.

Wu & Chang (1985) reported that mixtures of the 27 plus 65 kDa proteins and also of the 27 plus 130 kDa proteins were more toxic than if the two proteins were fed individually to the larvae, suggesting a synergistic relationship between these proteins. We observed a synergistic relationship between the 27 and 130 kDa proteins (Table 2) but no synergism between the 27 and 65 kDa proteins.

The use of a neutral red tissue culture assay allowed rapid comparison of the *Bti* crystal proteins against a range of insect cell lines. This assay is quantitative, rapid and less laborious than the trypan blue assay (Thomas & Ellar, 1983). The 130 kDa and 65 kDa proteins were not toxic to any of the cell lines except for the case of the 130 kDa protein against *M. brassicae* cells, suggesting that they are protoxins. However, the 27 kDa protein was toxic to the insect cell lines which could suggest either the presence of contaminating 25 kDa protein or that the 27 kDa protein was proteolysed by enzymes in the tissue culture assay. However, silver staining of SDS-polyacrylamide gels did not indicate the presence of any 25 kDa protein in the 27 kDa preparations, and 27 kDa protein incubated with culture medium in which cells had been incubated did not proteolyse the 27 kDa protein. The 25 kDa protein caused complete lysis of the dipteran cells in 30 min. Thomas & Ellar (1983) reported that *Ae. albopictus* cells incubated with alkali-solubilized *Bti* crystals (5 µg ml⁻¹) lysed in 30–40 min. Johnson & Davidson (1984) reported that alkali-solubilized *Bti* crystals had LC₅₀ values against *Ae. aegypti* and *Ae. gambiae* cells of 5.4 µg ml⁻¹ and against *C. fumiferana* cells of 47.2 µg ml⁻¹, but no toxicity against *Plodia interpunctella* cells. We also observed that the lepidopteran cells were tenfold less sensitive than the dipteran cells when incubated with 25 kDa protein, and the 25 kDa protein was not toxic to

Lymantria dispar cells. Both the 30–35 kDa proteins and 53 kDa proteins were toxic mainly to the dipteran cell lines showing specificity similar to that of native crystals (i.e. toxic to mosquito larvae). When the purified proteins were assayed individually against *Ae. aegypti* larvae, the 65 kDa protein was the most toxic protein in *Bti* crystals, while mixtures of the 27 and 130 kDa proteins were nearly as toxic as solubilized *Bti* crystals.

All the purified proteins were cytotoxic after trypsin activation when assayed *in vitro*, but distinct differences in specificity and potency were observed. Thus the 27 kDa protein and its 25 kDa product killed all cells with the exception of *L. dispar* and had the lowest LC₅₀ values. This broad cytotoxicity is consistent with the ubiquitous phospholipid receptor for this toxin (Thomas & Ellar, 1983). The 30–35 kDa products from the 65 kDa protoxin killed all dipteran cells tested except *Ae. albopictus* and only one lepidopteran cell type. The greatest dipteran specificity *in vitro* was shown by the 53 kDa products of the 130 kDa protoxin doublet. This preparation killed all Anopheline and Culex cells but had no effect on two Aedes and one Drosophila cell line. Interestingly, it was also distinguishable from the 30–35 kDa preparation by its activity against *S. frugiperda* cells. This restricted specificity of the activated forms of the 130 and 65 kDa protoxins compared to the 27 kDa toxin suggests that the receptors for these two toxins are unlikely to be invariant membrane components such as phospholipids. Although we are conscious of the need to exercise caution in relating the results of such assays *in vitro* to crystal activity *in vivo*, these findings coupled with the indications of possible synergism suggest that the host range and potency *in vivo* of this endotoxin may be determined by individual toxins with different insect specificity acting independently and/or synergistically.

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