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Separation of Three Biologically Distinct Activities from the Parasporal Crystal of *Bacillus thuringiensis* var. *israelensis*

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Abstract. Three fractions showing biologically distinct activities were isolated from the *Bacillus thuringiensis* var. *israelensis* crystal δ-endotoxin. Using a shallow sodium chloride gradient on a DEAE anion-exchange column, three pooled fractions were obtained consisting of the 25Kd peptide; 25,26Kd peptides; and 31,34,35Kd peptides, respectively. The 25Kd peptide was hemolytic against human erythrocytes but not mosquitocidal. The 25,26Kd peptide mixture was hemolytic, not mosquitocidal, but toxic to larvae of *Trichoplusia ni* when injected into the hemocoel of these animals. The 31,34,35Kd peptide mixture was toxic to mosquito larvae, but neither hemolytic nor toxic to larvae of *T. ni* upon injection. In addition, teratogenic effects were observed when wandering larvae of *T. ni* were injected with each of these three fractions. All of the biological activities described were destroyed when peptides were treated for 15 s at 95°C or greater. The hemolytic activity was elevated by heating the peptide(s) at 70°C for 15 s. On the other hand, this elevation was not observed with the two insecticidal activities.

After almost ten years of intense study on the mosquito larvicidal toxin of *Bacillus thuringiensis* var. *israelensis* (Bti), a rather complex picture has emerged. The organism was first isolated by Goldberg and Margalit [4] in 1977. There now is tremendous enthusiasm for using this organism for control of mosquito and black-fly larvae due to the toxin's (δ-endotoxin) specificity and potency.

At least three distinct types of biological activity have been reported with the Bti δ-endotoxin. First, and the most studied, is the mosquitocidal (and toxicity toward black-fly larvae) activity of the Bti δ-endotoxin [6, 21, 22]. It was concluded that lysis of epithelial cells and subsequent leakage of gut contents into the hemolymph was the mode of action for the observed toxicity [7, 9]. The second biological activity of the Bti toxin was its ability to lyse cultured cell lines and mammalian erythrocytes [3, 14, 17]. A biochemical basis for this phenomenon was put forward by Thomas and Ellar [18], who showed specific affinity of the soluble δ-endotoxin for certain phospholipids. Lipid bilayers of liposomes were further reported to be disrupted by the δ-endotoxin [19]. This ability to disrupt membrane bilayers was suggested as the insecticidal mechanism for the Bti δ-endotoxin. A third and less studied bioactivity of the δ-endotoxin is the neurotoxic effect of the toxin. This observation was described by Cheung et al. [2] to include both insects and mammals. This neurotoxicity is observed when injected directly into the body cavity of animals but not when administered orally. Although similar observations were reported by Thomas and Ellar in mice [17], these authors failed to note the similarity of symptoms between Bti and other bacterial neurotoxin intoxications (e.g., botulinum toxin). In addition, soluble Bti was shown to cause nerve damage [15] when applied to in situ preparations of the ventral nerve cord of *Trichoplusia ni*.

Unlike *Bacillus thuringiensis* var. *kurstaki*, whose crystal gives one major protein at 130 kilodaltons (Kd) when solubilized, the Bti crystal shows a host of peptides ranging from 10 to 120Kd upon alkaline dissolution [1, 13, 20]. It is only recently that investigators were able to separate these components of the crystal from one another. Mosquito toxicity and cytolytic activity were assigned to the 28Kd peptide [17, 23]. Our work has shown that a combination of the 27,28Kd peptides from the Bti crystal toxin exhibited both mosquito toxicity and neurotoxic activity in larvae of *T. ni*. On the other hand, a combination of the 25,26Kd peptides

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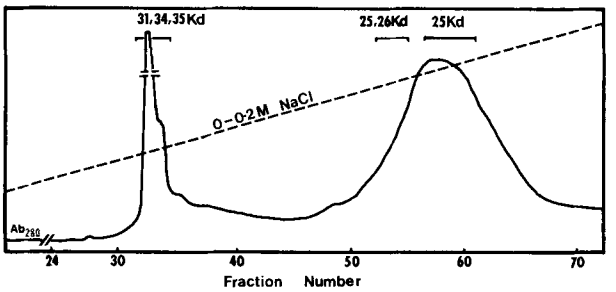

Fig. 1. Elution profile for alkaline-solubilized δ-endotoxin of Bti on a DEAE–Sephacel column. Fractions collected and pooled include fractions 33 and 34 (31–35Kd peptides), fractions 53–55 (25 and 26Kd peptides), and fractions 56–61 (25Kd peptides); pools are indicated by bars.

only showed neurotoxicity toward larvae of *T. ni* but had greatly reduced toxicity toward mosquito larvae [2, 15].

There is no solid evidence in the literature showing that the mosquitocidal, hemolytic (cytolytic), and the neurotoxic activities all reside in the same component. This report supports the belief that these activities, the hemolytic activity in particular, reside in individual distinct peptides of the Bti δ-endotoxin, and that they ultimately can be separated from one another. A hemolytic protein was isolated and the physical as well as biological properties of this protein are reported. In addition, two fractions exhibiting only mosquitocidal or *T. ni* lethal activity, respectively, are described.

Materials and Methods

Microorganism and growth conditions. *Bacillus thuringiensis* var. *israelensis* (HD-567, Dulmage) was obtained from Dr. Bruce Carlton at the University of Georgia, and designated HD-567-1 in their study [5]. The organism was maintained on a nutrient agar slant at 4°C and subcultured every three months. Cells for experimental use were cultured in a modified glucose–yeast-extract–salt medium [12] and collected as described previously [2].

Purification and solubilization of crystal δ-endotoxin. The Bti crystal toxin was separated from the spores by a discontinuous sucrose density gradient of 50%, 63%, and 73% (wt/vol) sucrose in 0.03% Triton X-100. After the spore–crystal suspension was layered on top of the gradient (30 ml), it was centrifuged at 3000 rpm/30 min, and then at 10,000 rpm/2 h in a Sorvall swinging-bucket rotor (HB-4). A band of bright reflective crystal was formed at the interface of the 63% and 73% sucrose. The crystals were removed, and washed three times with distilled water to remove the sucrose and Triton X-100. The pellet was then divided into aliquots, lyophilized to dryness, weighed, and stored at −60°C.

To solubilize the toxin, the dry crystal was resuspended in 0.5% sodium carbonate (pH 11.0) and 0.02% sodium azide for 3 h at 28°C with sonication at 30-min intervals. Nonsoluble matter was removed by centrifugation at 10,000 rpm/30 min (SS-34 rotor, Sorvall).

Protein determination. Protein concentrations of samples were determined by the method of Lowry [11] using bovine serum albumin (Gold label, Calbiochem) as the standard. Absorbance was measured at 620 nm on a Gilford EIA Manual Reader interfaced with an Atari 400 microcomputer.

Polyacrylamide gel electrophoresis. Discontinuous SDS–polyacrylamide gel electrophoresis, 12.5% (SDS–PAGE), was carout as described by Laemmli [8] with a Hoefer Vertical Slab Gel system (SE 600, Hoefer Scientific Instruments). Molecular markers included bovine serum albumin (68,000), egg albumin (43,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), chymotrypsinogen (26,000), soybean trypsin inhibitor (20,100), and α-lactoalbumin (14,000).

DEAE anion-exchange chromatography. The alkaline-dissolved Bti toxin (at pH 8.0) was applied onto a DEAE–Sephacel (Pharmacia) column (30-ml bed volume), preequilibrated at pH 8.0 with a 0.025-*M* Tris–HCl buffer. After washing with three bed volumes of the starting buffer, the column was eluted with a sodium chloride gradient of 0–0.2 *M* (200 ml of each) over a 48-h period.

Bioassays. Larvae of the yellow fever mosquito, *Aedes aegypti*, were reared at 28°C with a 16L:8D photoperiod, on a diet consisting of guinea pig chow (Geisler)–liver powder (Sigma)–Tetra Min (50 : 50 : 1, wt/wt). Third-stadium larvae were selected for the experiments. Ten larvae of *A. aegypti* were placed in each of several test tubes (16 × 125 mm) with 5 ml of water (distilled–tap, 1 : 1), to which were added the Bti toxin preparations. Larvae were then incubated at 28°C and mortality was scored at 24 h after inoculation (PI).

Larvae of the cabbage looper, *T. ni*, were reared on a diet modified from Shorey and Hale [16] at 27°C with a 14L:10D photoperiod. Three-day-old fifth-stadium wanderers, having an average weight of 0.25 ± 0.02 g, were selected for experiments. Toxin preparations were diluted with sterile physiological buffered saline (PBS–gelatin–glucose; 0.15 *M* NaCl, 0.05 *M* Na_2HPO_4, 0.02 *M* KH_2PO_4, 0.05% gelatin; 2.2% glucose; pH 7.2) and injected into the larval hemocoel directly in 2- to 3-μl aliquots at the base of the second abdominal proleg. Survivors at 24 h PI were further examined for teratogenic development at 48 h PI.

Four or more concentrations between 10% and 90% mortality, with at least ten animals per concentration, were performed for each analysis. The mortality data collected on larvae of *A. aegypti* and *T. ni* were subjected to analysis to determine the median lethal dose (LD_{50}) for all the Bti preparations using Lieberman's [10] computer adaption of Finney's probit analysis on an Apple IIe microcomputer.

Hemolytic assay. Human erythrocytes were collected from whole blood and washed twice with PBS–gelatin–glucose. The packed cells were then diluted with PBS–gelatin–glucose to make a 2% (volume of packed cells/final volume) red blood cell (rbc) suspension. Toxin preparations from Bti were made in PBS–gelatin–glucose, in a 250-μl volume. An equal volume of the 2% rbc suspension was added, and the suspension was incubated at room temperature for 14 min. After the incubation time, cells were removed by low-speed centrifugation for half a minute

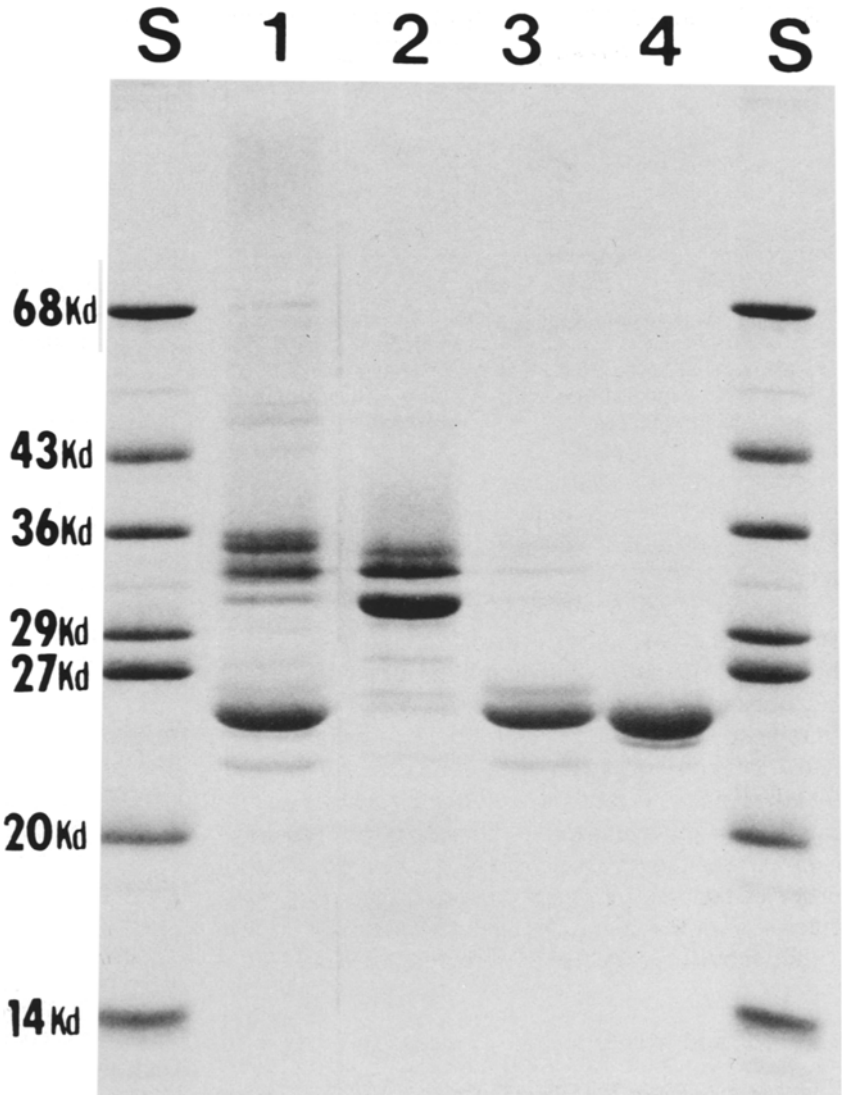

Fig. 2. SDS–PAGE analysis of pooled fractions from a DEAE anion-exchange column. Track 1, whole extract from alkaline dissolution; track 2, DEAE effluent (this fraction contains a mixture of the 31, 34, and 35Kd peptides); track 3, DEAE effluent (this fraction contains a mixture of the 25Kd and 26Kd peptides with a small amount of the 20Kd peptide); track 4, DEAE effluent (this fraction contains the 25Kd peptide as its major [>95%] component); and track S, molecular markers (see *Materials and Methods* for identities of proteins). Tracks 1–4 each have 25 μg of protein.

at room temperature. Absorbance of supernatant at 15 min after inoculation was measured at 500 nm. Percent rbc lysed was expressed as Ab_{500} of sample divided by Ab_{500} of total lysis. Concentrations at which 50% rbc lysis occurred were reported. Sodium dodecyl sulphate (SDS), one of the many potent non-ionic rbc lytic agents, was assayed and used as a positive control for hemolysis.

Heat treatment of Bti toxin. Toxin preparations in PBS–gelatin–glucose were heated at 45°C, 70°C, and 95°C for 15 s and chilled in an ice bath immediately. They were then assayed for activities against mosquito larvae, erthyrocytes, and larvae of *T. ni* to examine the effects of heat treatments on the biological effects of the peptides separated from the parasporal crystal.

Results and Discussion

DEAE anion-exchange chromatography. A total of three pooled fractions were collected from the effluent of the DEAE column following an extremely shallow NaCl gradient (Fig. 1). Of the three fractions, only one contained reasonably pure material, the 25Kd peptide. The other two fractions were composed of a mixture of peptides, 25,26Kd peptides and 31,34,35Kd peptides, respectively (Fig. 2). Biological activities of these three fractions are shown in Tables 1 and 2. Results of our bioassays and hemolytic assays indicate that each of these three fractions possesses a distinctly different biological activity.

Hemolytic assays. Hemolytic activity of the Bti δ-endotoxin resides in the 25Kd peptide (Table 1), and this activity is enhanced by heat treatment prior to the assay. The three samples showing hemolytic

Table 1. Hemolytic activities and temperature sensitivities of different preparations of alkaline-solubilized δ-endotoxin of Bti

Toxin preparations	Control not heat treated	15-s heat treatment at 45°C	70°C	95°C
Whole extracts	2.54 (2.62–2.45, 7.31)[a]	2.38 (2.49–2.29, 6.06)	1.46 (1.52–1.39, 7.11)	>48[b]
31,34,35Kd fraction	>40	>40	>59	ppt
25,26Kd fraction	1.59 (1.68–1.54, 8.01)	1.07 (1.10–1.03, 7.67)	0.69 (0.72–0.67, 7.22)	>20
25Kd protein	0.85 (0.88–0.82, 7.96)	0.79 (0.83–0.74, 6.22)	0.55 (0.58–0.53, 8.00)	>17

[a] Number represents concentration in μg/ml at which 50% of a 1% rbc suspension was lysed within 15 min at room temperature; 95% confidence intervals and slope of % lysis vs log concentrations of Bti are in parentheses [10].
[b] Indicates no rbc lysis up to that concentration.

Table 2. Biological activities of the different fractions of alkaline-solubilized δ-endotoxin of Bti

Toxin preparations	Mosquitocidal LC_{50} (μg/ml)	*T. ni* toxicity LD_{50} (ppm)[a]	*T. ni* teratogenic Effect ED_{50} (ppm)[a]
Whole extracts	3.63 (4.20–3.00, 3.70)[b]	1.95 (2.44–1.49, 4.00)	?[d]
31,34,35Kd fraction	6.68 (7.37–5.94, 4.95)	>20	3.32 (4.23–2.29, 450)
25,26Kd fraction	>25	3.51 (4.32–2.61, 3.74)	2.98 (3.64–2.39, 6.29)
25Kd protein	>25	5.97 (6.80–5.29, 8.87)[c]	2.94 (3.67–2.25, 7.33)

[a] ppm = mg toxin per kg body wet weight.
[b] Numbers represent median lethal dose with 95% confidence intervals and slope of probit analysis.
[c] Symptoms and slope of probit analysis are different from the 25,26Kd fraction and the whole extract (see text for detailed description).
[d] Not able to determine; doses tested either produced no teratogenic effect or >90% mortality.

activity—the whole extract, the 25,26Kd fraction, and the 25Kd peptide—all exhibited enhanced biological activities (by 1.5- to 2.3-fold) after they were heat treated at 70°C for 15 s. Temperatures higher than 90°C quickly inactivated the hemolytic activity in all the samples. Slopes of the hemolytic assays were very similar in all the samples tested, with or without heat treatment. This indicates that the same component probably is responsible for hemolysis in all three samples. Absence of hemolytic activity in the 31,34,35Kd fraction at concentrations 20 times higher than the whole extract and 50 times higher than the 25Kd fraction further supports the assignment of the hemolytic activity largely to the 25Kd peptide.

Bioassays. Toxicity toward mosquito larvae was found in the whole extract and the 31,34,35Kd fraction. On the other hand, no mosquito toxicity was found associated with the 25Kd peptide or the 25,26Kd fraction at concentrations five times the LC_{50} of the whole extract (Table 2, column 1). The 70°C/15-s treatment did not elevate the larvicidal activity and heat treatment at 95°C for 15 s destroyed any observed toxicity. It is generally accepted that a 28Kd peptide from the δ-endotoxin is responsible for mosquito toxicity [18, 23]. Our procedure (3-h extraction period) resulted in minimal amounts of the 28Kd peptide, but its concentration could be increased greatly at the expense of the 25,26Kd peptides by shorter periods of base treatment (i.e., 10 min).

When Bti toxin preparations were injected into larvae of *T. ni*, two types of lethal effects were observed. With the whole extract and the 25,26Kd fractions, immediate symptoms (~15 min PI) included mouth palpation of injection site, heart arrest, abdominal paralysis, listing when the insects

crawl, and total paralysis and flacidity at the LD_{90} or higher. Localized blackening of the body was observed in advanced stages with subsequent appearance throughout the entire body. This blackening of the body occurred much faster (within 10–12 h PI) when dosages were above the LD_{90}. With the 25Kd cytolytic factor, immediate palpation of injection site and heart arrest were observed, but animals were rarely paralyzed. The bodies of treated larvae were mushy but never blackened at 24 h PI. The carcasses maintained their green coloration up to 48 h PI even when the larvae no longer responded to mechanical stimulation. Possibly, the 26Kd peptide was responsible for the paralytic effect on the larvae of *T. ni*. Further separation and purification of the 25 and 26Kd peptides will be needed to elucidate the activities of these individual peptides.

A third biological phenomenon was observed when the three fractions were injected into larvae of *T. ni* that had entered the postfeeding wandering stage. Incomplete pupation was observed at 48 h PI with insects that had survived the injection at 24 h PI. As a result, live teratoids at various stages of pupation were observed. Teratogenic development occurred at concentrations higher than the LD_{50} of the whole extract, and all three fractions had approximately the same effective dosages (ED_{50}s between 2.9 and 3.3 ppm). It is unlikely that the teratogenic effects were the result of injuries due to injection, because heating at >95°C/15 s destroyed the activity in all three fractions. Furthermore, heating at 70°C/15 s prior to assay reduced the teratogenic effects.

The practical use of Bti as a mosquito control agent has recently come into question because of its demonstrated cytotoxicity toward mammalian cell lines and erythrocytes. Although the crystalline toxin has no demonstrable mammalian toxicity, the alkaline-solubilized material was shown to possess a wide toxicity spectrum. It is of environmental and human health concern that Bti materials can be accidentally solubilized and released into the environment. In this study, it was shown that the different biological activities associated with the δ-endotoxin of Bti can be separated from one another. A hemolytic factor (25Kd peptide) was isolated. This peptide had greatly reduced insecticidal activity against mosquito larvae. Its toxicity and mode of action appeared to be very different from those of the whole extract and a 25,26Kd fraction from the whole extract. Furthermore, toxicities toward larvae of *A. aegypti* and *T. ni* were separated from each other in fractions containing different peptides.

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