

Bacillus thuringiensis var. *israelensis* δ -Endotoxin: Evidence of Neurotoxic Action

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The neurotoxic, cytolytic, and lethal properties of a 25-kDa protein isolated from the δ -endotoxin of *Bacillus thuringiensis* subsp. *israelensis* (Bti) were compared with those of the alkaline-dissolved whole extract of the δ -endotoxin and the cytolytic enzyme phospholipase A₂. *In situ* electrophysiological measurements of the ventral nerve cord of larval *Trichoplusia ni* treated at 15 and 25°C with various dosages of the three protein preparations showed that only the whole extract of the δ -endotoxin induced hyperexcitation. When the appearance of lactate dehydrogenase activity in the haemolymph of toxin-injected larvae was used to quantitate cell lysis, the cytolytic 25-kDa protein was found to be a potent cytotoxic factor like phospholipase A₂, whereas cytotoxicity of the whole δ -endotoxin extract was relatively low. Bioassays at 28, 17, and 9°C demonstrated a positive temperature correlation with mortality for the whole toxin extract, but showed inverse correlations for the two cytolytic materials. This and previous studies indicate that insect mortality is not solely due to the cytolytic activity of the Bti δ -endotoxin upon injection into *T. ni*. An unidentified neurotoxic factor(s) exists within the Bti δ -endotoxin which induces hyperexcited activity in the insect nervous system. This and/or other factors impart the δ -endotoxin with biological and physiological activities different from the isolated cytolytic component. © 1987 Academic Press, Inc.

INTRODUCTION

The insecticidal properties of the *Bacillus thuringiensis* species are promising for the control of insect pests, as targets of genetic manipulation and as probes to characterize new targets for toxin action. First isolated by Goldberg and Margalit (1), the dipteran larvicidal activity of *B. thuringiensis* subsp. *israelensis* (Bti)¹ was later demonstrated to reside within the bacillus' inclusion body formed during sporulation (2-6). Histopathological studies showed that the δ -endotoxin of Bti is cytotoxic against the gut epithelium of the mosquito and black-fly larvae (7, 8). Furthermore, the toxin was observed to lyse a wide variety of tissue culture cell lines and mammalian erythrocytes (9-13). This cytolytic

activity of the Bti δ -endotoxin was presumed to be the cause of toxicity in susceptible host insects.

Recently, the δ -endotoxin of Bti was demonstrated to possess other toxicities in addition to its general cytolytic activity. Broad spectrum toxicity was reported against both insects and mammals when the toxin was injected (12, 14). Alkaline-dissolved δ -endotoxin was shown to possess neurotoxic effects and to cause dysfunction in insect nerve preparations (14, 15), as well as myotoxic effect (16). The cytolytic and mosquito toxicity of the δ -endotoxin was further demonstrated to reside in separate components of the bacterium's parasporal crystal (10, 17).

In this article, the neurophysiology of Bti poisoning is studied. The effects of the isolated cytolytic agent were compared to those of the whole crystal extract *in vivo* as well as with *in situ* electrophysiological

¹ Abbreviations used: Bti, *Bacillus thuringiensis* subsp. *israelensis*; VNC, ventral nerve cord; LDH, lactate dehydrogenase.

techniques. The roles of cytotoxicity and neurotoxicity in insect mortality are evaluated.

MATERIALS AND METHODS

Crystal purification and solubilization. *B. thuringiensis* subsp. *israelensis* (HD-567, Dulmage) was provided by Dr. Bruce Carlton, then of the University of Georgia. Spores and crystals were harvested from a 72-hr submerged culture in GYS medium (18) at 30°C with rotary agitation. Crystals were first separated from the spores using a biphasic extraction technique described by Goodman *et al.* (19). Sucrose density gradient centrifugation was then used to purify the crystals (20). Purified crystals were washed with distilled water, lyophilized, weighed, and stored at -60°C. Dissolution of the crystals was achieved by suspending the lyophilized materials in 0.05% sodium carbonate (pH 11.0) and 0.02% sodium azide for 3 hr at 4°C with sonication at 30-min intervals. The solubilized toxin was then dialyzed against 0.025 M phosphate buffer, pH 8.0. After the protein concentration was determined by the method of Lowry *et al.* (21), the solubilized toxin was divided into aliquots and stored at -20°C.

Cytolytic protein. From a previous study (10), a protein was separated from the alkaline-dissolved δ -endotoxin using DEAE-Sephacel (Pharmacia) anion-exchange chromatography with a very shallow salt gradient (0.0 to 0.2 M NaCl, 200 ml each). This protein (25,000 Da) exhibited lytic activity toward human erythrocytes and haemocytes of *Trichoplusia ni*. The proportion of this protein increased within the whole alkaline extract of the Bti δ -endotoxin as the time of dissolution increased. This cytolytic protein showed no toxicity toward mosquito larvae when ingested, and it had reduced toxicity by injection toward the larvae of *T. ni* when compared with the whole toxin extract. The neurophysiological and biological properties of this cyto-

lytic factor are further investigated in this study.

Test animals. Cabbage looper larvae, *T. ni*, were reared on a pinto bean artificial diet at 28°C with a 14-hr light and 10-hr dark photoperiod (22). Larvae at the wandering stage were selected for bioassay and lactate dehydrogenase assays. Two-day-old fifth stadium larvae weighing 160–190 mg were selected for electrophysiological experiments.

Electrophysiology. The head and thorax of the larvae were removed with one cut. The digestive system and its contents were pulled from the abdomen of the decapitated animal. After rinsing with insect Ringer's (23), the carcass was placed in an environmental chamber overnight at 10°C with 100% relative humidity. Overnight incubation minimized spontaneous background activity so that it would not interfere with electrophysiological recordings. Prior to the electrophysiological experiments, the insect preparation was pinned ventral side up in a paraffin-filled watchglass. Small incisions were made at the mid-ventral line just anterior to the abdominal prolegs and between the last pair of abdominal prolegs. Mineral oil- and petroleum jelly-insulated tungsten electrodes were inserted (using micromanipulators) at the incisions adjacent to the ventral nerve cord (VNC), and 30 min was allowed for acclimation. The anal proleg was then mechanically stimulated with a glass microelectrode to establish pre- and postinjection reference activity levels. Larval preparations that did not respond to this stimulation with gross muscular movement were selected for injection with toxin preparations.

Ventral nerve cord activity was monitored using the Frederick Haer and Company (Brunswick, Maine) high-impedance amplification system with a slope/height window discriminator to reduce baseline noise. Signals were monitored on a Tektronix storage oscilloscope (Beaverton, Ore.) and recorded on an Akai stereo tape deck (Compton, Calif.).

By use of a glass needle, 1 μ l of a test solution was injected into the last abdominal proleg adjacent to the recording electrode. Materials injected in saline included alkaline-dissolved Bti δ -endotoxin, the 25-kDa cytolytic protein from the δ -endotoxin, and bee venom phospholipase A₂ (Sigma Chemical Co., St. Louis, Mo.). Dose- and time-dependent spontaneous activities were measured for each toxin. At 1 hr postinjection, the anal proleg was mechanically stimulated and the response was recorded and compared to the pretreatment response. Observations through a dissecting microscope verified that no visible muscle movement occurred during each successful recording period. Tests were conducted at 25°C for all treatments. Additional measurements were taken at 15°C for the alkaline-dissolved toxin.

Bioassays. The alkaline-dissolved toxin, cytolytic protein, and phospholipase A₂ were injected directly into the haemocoel of the cabbage looper larvae at the base of the last abdominal proleg. Mortalities at 28, 17, and 9°C were scored at 24 hr postinjection. LDH levels in the haemolymph were also determined at 15 min and 12 hr postinjection.

Lactate dehydrogenase (LDH). The appearance of LDH (a cytosolic enzyme released during cell lysis) in the haemolymph of *T. ni* larvae after injection with Bti toxin preparations was used as a marker for the cytolytic activity of the toxin. Pooled cell-free haemolymph from treated larvae was assayed for LDH activity according to the method described by Bergmeyer and Bernt (24). Values of LDH activity were the average of at least two replications each with three determinations.

RESULTS

Electrophysiology. Examples of electrophysiological responses of control and treated VNCs are shown in Fig. 1. Neurophysiological responses to toxin treatments can be divided into two categories. One was spontaneous repetitive discharge or

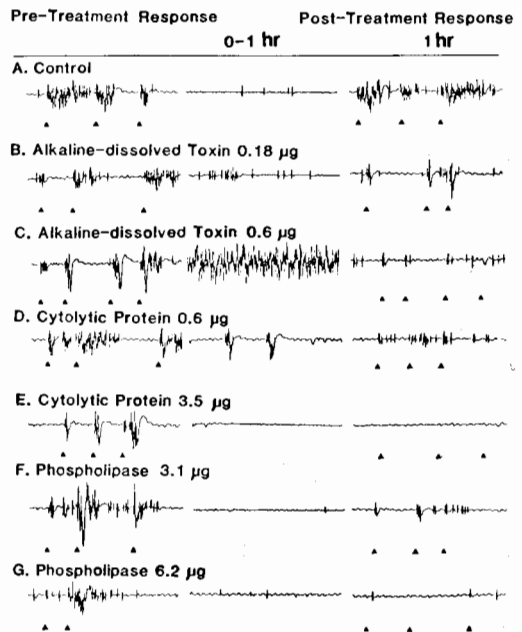


FIG. 1. Electrophysiological tracings of the nervous activity of *Trichoplusia ni* ventral nerve cord preparations injected with saline (A), alkaline-dissolved Bti δ -endotoxin (B,C), the 25-kDa cytolytic protein from the Bti toxin (D,E), and phospholipase A₂ (F,G). Mechanical sensory stimulation of the anal proleg is given at pointers (▲). The results for other doses are discussed under Results.

“hyperexcited activity.” Onset of hyperexcitability usually followed a short latency period after toxin application. The second effect was failure to respond to anal proleg stimulation.

At a dosage of 0.18 μ g, the alkaline-dissolved toxin did not elicit any hyperexcited activity in the VNC. At 0.6 μ g treatment, hyperexcitability occurred within 30 min (Fig. 1C), and the same was observed with a dosage of 1.8 μ g (not shown). The duration of these spontaneous repetitive discharges ranged from 5 to 17 min, and at 1 hr post-treatment responses to anal proleg stimulation were reduced but not lost in all specimens tested.

With the cytolytic protein of Bti δ -endotoxin at 0.6- (Fig. 1D) and 1.8- μ g (not shown) doses, no hyperexcited activities were observed. But spontaneous activity was apparent in short bursts of action po-

tentials occurring throughout the postinjection period. The anal proleg stimulatory response was also reduced after 1 hr. Increasing the treatments with the cytolytic protein to 2.6 μg (not shown) and 3.5 μg (Fig. 1E) did not induce hyperexcitability within an hour in any specimen. However, there was an immediate (i.e., <1 min) loss of spontaneous activity and stimulatory responses.

No dosage of phospholipase A_2 tested elicited VNC hyperexcitability (Figs. 1F and G). The dosage of 3.1 μg reduced the anal proleg stimulatory response during the post-treatment period, and increasing the dosage to 6.2 μg eliminated the stimulatory response within 30 min.

The VNC preparations treated with 0.6 and 1.8 μg of the alkaline-dissolved Bti δ -endotoxin elicited hyperexcited activity at 15°C as they did at 25°C. But the initiation of the response was delayed considerably at the lower temperature (Table 1). The onset of repetitive discharges occurred at between 78 and 122 min post-treatment, which is approximately four times longer than at 25°C. The 0.18- μg dose did not elicit hyperactivity with 3 hr at 15°C. There was no apparent correlation between the dosage and the onset of hyperexcited activity at either temperature.

LDH level in haemolymph. Because the haemolymph-collecting procedure inevitably resulted in lysing some haemocytes, control larvae were injected with saline and bled, and the LDH level in the haemolymph was determined. This background level of LDH (21.1 ± 7.6 Wroblewski unit/ml, $n = 6$) was subtracted from all the LDH values of the treated animals. The haemolymph LDH level increased when larvae of *T. ni* were injected with either the alkaline-dissolved δ -endotoxin or the cytolytic protein. A direct correlation was observed between LDH levels and dosages with both toxin preparations (Table 2). However, LDH levels were much lower in the larvae injected with the alkaline-dissolved toxin than in those injected with the

TABLE 1
Temperature Dependency of *B. thuringiensis subsp. israelensis* δ -Endotoxin Elicited Ventral Nerve Cord Hyperexcited Activity

Dosage (μg)	Temperature (°C)	Mean initiation time (min)
0.18	25	No hyperactivity ($n = 10$)
	15	No hyperactivity ($n = 5$)
0.6	25	21.4 ($n = 12$)
	15	96.6 ($n = 5$)
1.8	25	19.5 ($n = 10$)
	15	92.0 ($n = 5$)

cytolytic protein. The initial (15 min) LDH levels in the haemolymph of larvae treated with the cytolytic protein were one to two orders of magnitude higher than those of the alkaline-dissolved toxin-treated larvae. Incubation at 17 and 9°C after injection with the two toxin preparations did not significantly alter the LDH levels of the treated larvae from those incubated at 28°C (Table 3).

Bioassays. The results of bioassay of the alkaline-dissolved toxin and the 25 kDa cytolytic protein are shown in Table 2. In addition to a threefold decrease in the 50% lethal dose (LD50), the two toxin preparations of Bti also exhibited different symptoms. Injection with the alkaline-dissolved toxin resulted in many neuromuscular symptoms which included mouth palpation of injection site, heart arrest, listing when the insect crawled, paralysis, and flaccidity. Localized blackening of the insect occurred at advanced stages (20–24 hr postinjection), and this eventually spread to the entire insect. Although the cytolytic protein also caused mouth palpation of the injection site and heart arrest, animals were rarely paralyzed. Blackening of the treated larvae never occurred. Larvae maintained their green coloration even when they no longer responded to mechanical stimulation.

Effect of temperature on the toxicity of

TABLE 2
Comparison of Lepidopteran Insecticidal Activity and Cytolytic Activity of the δ -Endotoxin and a 25-kDa Haemolytic Factor from *B. thuringiensis* subsp. *israelensis*

Conc. ($\mu\text{g/g}$)	<i>T. ni</i> mortality at 24 hr PI ^a		LDH activity ^b in haemolymph at 15 min PI ^a	
	Alkaline extract (%)	25-kDa protein (%)	Alkaline extract	25-kDa protein
1	35	0	7 \pm 3	104 \pm 45
2	75	0	8 \pm 5	168 \pm 53
3	87	0	87 \pm 46	320 \pm 136
4	100	0	128 \pm 41	275 \pm 61
5	100	35	481 \pm 60	436 \pm 33
6	/	60	/	1008 \pm 48
7.5	/	70	/	1041 \pm 271
10	/	100	/	2660 \pm 151

^a Postinjection.

^b LDH activity is in Wroblewski units/ml haemolymph (Bergmeyer and Bernt, 1974).

the two toxin preparations is shown in Table 4. A positive temperature dependency was observed with the alkaline-dissolved toxin. Mortality decreased when the temperature was lowered from 28 to 17 and 9°C. On the other hand, mortality of the cytolytic protein showed an inverse correlation with the temperatures tested. A similar inverse relationship between temperature and dosage was found with phospholipase A₂.

DISCUSSION

Electrophysiological techniques are the most straightforward means of studying functional changes of the insect nervous system. Many classical insecticides disrupt the nervous system through inhibition (i.e.,

organophosphates and carbamates) or by interference (i.e., DDT) (25). Spontaneous repetitive discharges, or hyperexcitability, in the abdominal nerve cord is a characteristic symptom of nerve poisoning. It is commonly used to examine the direct action of insecticides, as it reflects the level of excitation in the insect nervous system (26–28).

Hyperexcitability was elicited by alkaline-dissolved Bti δ -endotoxin when injected at dosages of 0.6 and 1.8 μg , but not at 0.18 μg . Contrary to observations with other nerve poisons such as pyrethroids (29), the latency period between the time of injection and the onset of hyperexcited activity did not change for the 0.6- and 1.8- μg

TABLE 4
Temperature Dependence of Insecticidal Activities of *B. thuringiensis* subsp. *israelensis* δ -Endotoxin and Phospholipase A₂

TABLE 3
In Vivo Cytolytic Activities of *B. thuringiensis* subsp. *israelensis* δ -Endotoxin in Larvae of *T. ni*

Temperature (°C)	LDH level ^a in haemolymph at 12 hr postinjection	
	Alkaline extract	25-kDa protein
28	563 \pm 80	2454 \pm 311
17	438 \pm 78	2336 \pm 399
9	403 \pm 94	1979 \pm 692

^a LDH activity is in Wroblewski units/ml haemolymph (Bergmeyer and Bernt, 1974).

Temperature (°C)	<i>T. ni</i> mortality at 24 hr ^a (n = 20)		
	Alkaline extract (%)	25-kDa protein (%)	Phospholipase A ₂ (%)
28	87	35	30
17	0	60	55
9	0	85	85

^a Concentration of alkaline extract is at 3.5 $\mu\text{g/g}$, 25-kDa protein is at 5 $\mu\text{g/g}$, and phospholipase A₂ is at 30 $\mu\text{g/ml}$.

doses. This would suggest a very narrow dose-dependent toxicity for the toxin. Because normal haemolymph circulation was disrupted in our preparations, one would suspect that the delay was caused by delay in the distribution of the toxin. Injections were therefore made at the abdominal proleg adjacent to the VNC and the recording electrodes to minimize the diffusion distance. However, considering the almost immediate response to injections of the cytolytic protein and the increased delay in onset of hyperexcited activity at a lower temperature, it appears that hyperexcitability was elicited by a component(s) of the δ -endotoxin which did not penetrate the nervous system barriers rapidly. Since neither the cytolytic protein nor the phospholipase A₂ induced any hyperexcited activity, our observations would indicate a different mode of action for the alkaline-dissolved toxin.

To study the role of the cytolytic protein in Bti δ -endotoxin, effects of phospholipase A₂ (a potent cytolytic enzyme from bee venom) were compared to the effects of the two Bti preparations. The alkaline-dissolved toxin, the cytolytic protein, and phospholipase A₂ all caused a reduction or loss of response to the anal proleg stimulations. However, high dosages of both the 25-kDa protein and phospholipase A₂ elicited immediate reductions in the stimulatory responses without inducing hyperexcitation in the larval VNC. In larval preparations that still possessed gross muscular activity when mechanically stimulated, injection with the 25-kDa protein at high dose resulted in a total cessation of all stimulatory responses (e.g., nerve blockage). These electrophysiological data and the neuromuscular effects observed in bioassays suggested a myotoxic effect for the Bti δ -endotoxin that may be manifested by a combination of the cytolytic factor and an unidentified hyperactivity-inducing component(s). On the other hand, it is possible that hyperexcitability may result in permanent damage to the insect nervous

system regardless of the presence of the cytolytic factor. Nerve blockage was also observed with high dosages of alkaline extract in cockroach nerve preparations by Chilcott *et al.* (30) and Singh and Gill (16). With Ca²⁺ ionophore treatment, this blockage was alleviated. However, desheathed ganglia showed hyperactivity upon Bti treatment followed by complete blockage which was not abolished by Ca²⁺ ionophore treatments. This observation would suggest that gross membrane damage (by the 25-kDa cytolytic protein) was probably not the cause of nerve blockage in intact nerve preparations; otherwise, the ionophore treatment should have made no differences as with the desheathed ganglia.

Previous probit analysis (10) of the mortality data for the alkaline-dissolved toxin (LC₅₀ = 1.95 μ g/ml, slope = 4.00) and the cytolytic protein (LC₅₀ = 5.97 μ g/ml, slope = 8.87) indicated that the mode of action is very different for the two toxin preparations. In this study, the LDH level in haemolymph was used as a marker for cell lysis resulting from Bti intoxication. On the basis of general cytolysis alone, we cannot account for the high toxicity of Bti in larvae of *T. ni*. Densitometer scanning of the two toxin preparations separated on SDS—PAGE showed that the purified cytolytic factor is about 90% pure and that <20% of the alkaline-dissolved toxin consists of the cytolytic protein. Therefore, at any of the concentrations of the alkaline-dissolved toxin tested (1 to 5 ppm, Table 2), the cytolytic protein (at <20%, or 0.2 to 1 ppm) was not present at a high enough concentration to cause the observed mortality. In other words, it appears that mortality with the alkaline-dissolved toxin is not directly related to the degree of cell lysis as measured by LDH activity.

The effect of temperature on the toxicity of Bti δ -endotoxin has been studied by a number of investigators. Increased toxicity at higher temperatures has been reported for mosquito (31) and black-fly (32, 33)

larvae. Our observations regarding the temperature-dependent toxicity of Bti with *T. ni* showed the same positive correlation. The alkaline-dissolved toxin had a positive correlation with temperature whereas the cytolytic protein showed a negative temperature correlation.

This study demonstrates that the toxic effects resulting from cytolysis are apparently not sufficient to cause the observed toxicity with the alkaline-dissolved Bti toxin when injected into *T. ni*. The mode of action of the cytolytic factor of Bti δ -endotoxin is clearly different from that of the whole toxin extract. This conclusion is supported by the differences in bioassays, temperature-dependent toxicity, and neurophysiological studies. The presence of a hyperactivity-inducing component(s) within Bti δ -endotoxin is indicated by the neurophysiological data. However, the identity of this component is not apparent at this time. Furthermore, the relative contributions of the cytolytic factor and putative neurotoxin(s) to the toxicity of Bti δ -endotoxin remain to be determined. Whether these components act in concert or act independently of each other warrants further investigation.

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