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δ-Endotoxin of *Bacillus thuringiensis* var. *israelensis*

Broad-Spectrum Toxicity and Neural Response Elicited in Mice and Insects

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The alkaline-dissolved Bacillus thuringiensis israelensis (BTI) δ-endotoxin when introduced by injection was biologically active against a wide spectrum of host animals including insects from four orders and mice. The LD_{50} for dissolved BTI δ-endotoxin in mice was 1.31 PPM and in Trichoplusia ni (Lepidoptera: Noctuidae) 3.71 PPM. Neuromuscular effects like heart cessation, lost coordination, tremor, and paralysis were observed in test animals. Using the appearance of lactate dehydrogenase in insect hemolymph post-injection as a cytosolic marker, we found that dissolved BTI δ-endotoxin was cytotoxic. In vivo recordings of activity in the ventral nerve cord post-injection indicated that dissolved BTI δ-endotoxin at the T. ni LD_{50} elicited hyperexcitability and then nerve death as was also the case for the organophosphate, methamidophos. The cytotoxin phospholipase-A_2 when injected at its LD_{50} elicited no neural response. BTI poisoning was also temperature dependent while BTI cytotoxicity was not. Proteins at 24, 27, 35, 49 and 68K daltons were resolved from the dissolved BTI δ-endotoxin. These were introduced in various combinations by injection and ingestion into mice and insects, and compared to the alkaline-dissolved Bacillus thuringiensis kurstaki δ-endotoxin.

Within the sporangium of the bacterium Bacillus thuringiensis (BT) is synthesized a parasporal, proteinaceous crystal (1-2) that has found widespread use as a biological control agent (3). This crystal is commonly referred to as the "δ-endotoxin" as suggested by Heimpel (4). The taxonomy of BT is based on the serology of the flagellar H antigen (5), and 29 subspecies and 26 serotypes have been identified (6). The δ-endotoxins from the

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majority of the serotypes are toxic when ingested by more than 182 species of insects, particularly in the economically important order, Lepidoptera (3). The majority of the research has centered on Bacillus thuringiensis subspecies kurstaki (BTK) because of its larvicidal activity against major agricultural pests in the order Lepidoptera. The serotype H14, Bacillus thuringiensis subspecies israelensis (BTI), differs from the other serotypes, however, by being highly toxic to members of the insect order Diptera (7-8) and yet has little known toxicity to Lepidoptera. The prospect of employing BTI to control mosquitoes, blackflies, or other medically important insect pests has stimulated great interest in elucidating the molecular basis for its mode of action (3). All of the BT δ-endotoxins are also of special interest because they appear to be highly selective against insects and seem to pose no health risks to humans or livestock.

The δ-endotoxin of BTK upon ingestion by larval Lepidoptera is quickly activated by high gut pH and gut proteolytic activity (9-11); gut epithelial cells swell, vacuoles form, and then the cells separate from the basement membrane and each other ultimately disrupting the gut-hemocoel barrier (12-15). Similar observations in mosquito larvae fed BTI (16) led to the general acceptance that the BT insecticidal activity was directed against, if not restricted to the gut epithelium of the host (12-13). An initial symptom of BTK poisoning is gut paralysis (17) and it was hypothesized that an increase in the hemolymph pH from the leakage of alkaline gut contents (17) or the influx of K^+ into the hemocoel caused this paralysis (18-19). These studies led to the discovery that BTK digests applied to the ventral nerve cord of the cockroach, Periplaneta americana (Orthoptera: Blattidae) caused excitation and then nerve blockage (20-21) which appeared to be presynaptic in origin (20). Other studies have shown that the alkaline-dissolved BTI δ-endotoxin is cytotoxic to a number of different cell lines from insects and mammals (3,22-24) and has a high affinity for specific phospholipids in the plasma membrane (25). Thus, the objective of this study is to assess the toxicity of alkaline-dissolved BTI introduced into insects and mice by feeding and injection and to assess the role of cytotoxicity and neurotoxicity in mortality when dissolved δ-endotoxin is injected into insects.

SDS-PAGE Analysis of BTK and BTI δ-Endotoxin

BTK and BTI strain IFC-1 were provided by Biochem Products - US Division (Salsbury Labs., Inc.). BTI was also isolated from a commercial preparation provided by Sandoz Inc., cultured on GYS medium (26). BTK and BTI toxin was prepared in an analogous manner. Spores and crystals were separated from cell debris by repeated washing with water and centrifugation. BTI crystals were subsequently separated from spores by Renografin density gradient centrifugation (27) and BTK crystals by discontinuous sucrose gradient centrifugation (3). Crystals were then dissolved by incubation for 3 h in 0.5% Na_2CO_3 (pH 11.0) and dialyzed into 0.025 M sodium phosphate (pH 8.0) for storage at

-60°C. BTI (Sandoz) was further purified by DEAE-Cellulose (DE-52, Whatman, 30 ml) in 0.025 M Tris-HCL (pH 8.00), eluted with a 10 h, 100 ml, 0.0-0.5 M NaCl linear gradient; by acid precipitation where the DEAE elutant was dialyzed into 0.05 M sodium acetate (pH 4.5) and the percipitate removed by centrifugation; and by Sephadex G-75 super fine (Pharmacia) gel permeation chromatography (95 x 1.3 cm i.d. column) in 0.025 M sodium phosphate (pH 8.0). Alkaline-dissolved and partially purified δ-endotoxin was analyzed by 12.5% SDS-PAGE (28), stained with Coomassie brilliant blue (Figure 1).

Incubation of BTK and BTI crystals in 0.5% Na_2CO_3 (pH 11.0) solubilized a number of protein components (Figure 1). For BTK, there were a number of proteins at 64K daltons and higher (Track 1). The standard procedure of washing BTK crystals with 1 M NaCl before solubilization removes endogenous proteinases and results in an enriched 130K dalton protein as the predominant component. This procedure had little effect on the immunoreactivity of the solubilized crystal or its toxicity in our studies. For BTI (Sandoz, Track 2) there were proteins at 24, 27, 35, 49 and 68K daltons. Of the lower molecular weight Sandoz BTI components, the 27K proteins were the predominent component in the Salsbury BTI (Track 8). Differences in the protein profile of the δ-endotoxin from different BT varieties have been reported previously (11,30-31). All alkaline-solubilized δ-endotoxin of BTI (Sandoz) adsorbed to DE-52 and eluted in one peak (Track 3) with an apparent concentration of the 68K component. The acid precipitate (Track 4) was enriched with the 35K component which was re-solubilized only at high pH. Because of its limited solubility, the acid precipitate could not be bioassayed in later studies. The soluble fraction was enriched with the 24K and 27K components (Track 5). Gel permeation chromatography enriched the 27 and 24K proteins (Tracks 6 and 7, respectively).

δ-Endotoxin Toxicity in Mice and Insects

BTI and BTK alkaline-dissolved and partially purified δ-endotoxins were injected and/or fed to insects of 6 orders and to mice (Tables I and II). The δ-endotoxin was injected in 0.15 M NaCl, 0.05 M Na_2HPO_4, and 0.02 M KH_2PO_4 at pH 7.2 into the insect hemocoel or intraperitoneally into mice. In feeding experiments, the toxin was dissolved in 5% sucrose and force-fed in 2 µl volumes, to Trichoplusia ni and Heliothis zea (Lepidoptera: Noctuidae). Aedes aegypti (Diptera: Culicidae) larvae fed for a standard incubation period in water containing BT toxin preparations; adults were given rectal injections (32). Mice were also given BT by gavage. Data collected were subjected to Probit analysis (33).

The alkaline-dissolved δ-endotoxin of BTI (Sandoz, Track 2) was toxic by injection to all animals tested except Tenebrio molitor (Coleoptera: Tenebrionidae) (Table I). Mice, Trichoplusia ni, and Periplaneta americana were the most sensitive, the LD_{50} being 1.3, 3.7 and 4.4 PPM, respectively. The susceptibility to BTI poisoning also varied significantly within a single insect family (the Noctuidae) with the LD_{50}

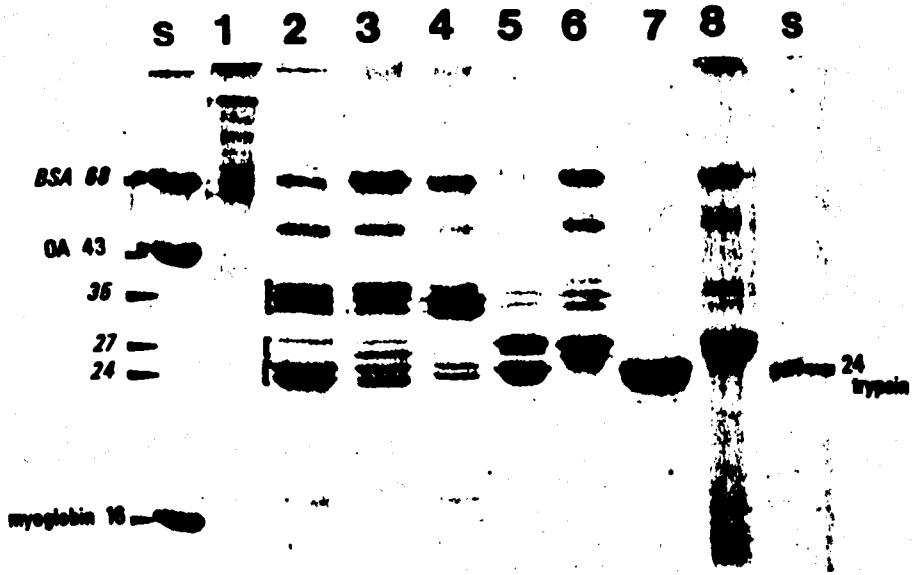

Figure 1. SDS-PAGE analysis of alkaline-dissolved Bacillus thuringiensis subspecies kurstaki (BTK) and israelensis (BTI) δ-endotoxin at 25 μg per track: (1) BTK δ-endotoxin from Biochem Products - US Division (Salsbury Labs., Inc.), (2) BTI δ-endotoxin from Sandoz Inc., (3) BTI (Sandoz) δ-endotoxin purified by DEAE-anion exchange chromatography, (4) percipitate formed after dialysis of BTI (Sandoz) δ-endotoxin into pH 4.5 sodium acetate buffer, (5) soluble fraction after dialysis of BTI (Sandoz) δ-endotoxin into pH 4.5 sodium acetate buffer, (6) BTI (Sandoz) δ-endotoxin purified by Sephadex G-75 gel filtration chromatography at R_f 1.35, (7) at R_f 1.58, and (8) BTI strain IFC-1 δ-endotoxin from Biochem Products - US Division (Salsbury Labs., Inc.). S, molecular weights as indicated X1000 for bovine serum albumin (BSA), ovalbumin (OA), trypsin, and myoglobin. Reproduced with permission from Ref. 29. Copyright 1984, Academic Press, Inc.

Table I. Injected toxicity of alkaline-solubilized BTI (Sandoz, Track 2) and BTK (Salsbury, Track 1) δ-endotoxin.

Animal - A or L*	24h BTI LD_{50}+	24h BTK LD_{50}+
Aedes aegypti - A (Diptera: Culicidae)	11.6 ± 2.2 (3.16)	>900
Musca domesticus - A (Diptera: Muscidae)	10.9 ± 2.2 (2.17)	>150
Trichoplusia ni - L (Lepidoptera: Noctuidae)	3.71 ± 0.32 (3.22)	>130
Heliothis sea - L (Lepidoptera: Noctuidae)	73.6 ± 3.0 (19.23)	>100
Tenebrio molitor - L (Coleoptera: Tenebrionidae)	>100	>100
Oncopeltus fasciatus - A (Hemiptera: Lygaeidae)	27.7 ± 7.0 (1.96)	>300
Periplaneta americana - A (Orthoptera: Blattidae)	4.42 ± 0.36 (7.04)	>20
Swiss-Webster Mice	1.31 ± 0.23 (4.47)	>30

*Adult or Larva.

+PPM or mg/kg body weight ± 1 S.D. with slope of probit analysis in parenthesis.

ranging from 3.7 PPM for T. ni to 73.6 PPM for Heliothis sea. The basis for this difference is unknown but these species differences could be useful in the elucidation of the mechanism for toxicity. BTI toxicity by injection also was not peculiar to the Sandoz strain but was also noted for the IFC-1 strain from Salsbury (Table III). By contrast the alkaline-dissolved δ-endotoxin of BTK (Track 1) showed no toxicity when injectad into the same species (Tabla I). Obvious fundamental differences exist between the dissolved δ-endotoxin of BTI and BTK.

When the purified, parasporal crystal of BTI (Sandoz) was fed to A. aegypti larvae, the LC_{50} was 2.95 ± 0.59 ng/ml. Alkaline-solubilization decreased the toxicity significantly to an LC_{50} of 2.29 ± 0.06 μg/ml and rectal injections in adults produced a LD_{50} of 54.5 ± 3.1 PPM (Table II). These findings were consistent with previous work (34). Dissolved BTI δ-endotoxin when fed to Lepidoptera and mice as expected (3) was not toxic (Table II). The BTI IFC-1 strain was also similar to the Sandoz strain in that both were toxic when fed to A. aegypti (Table III). BTK dissolved δ-endotoxin when given orally was toxic only to the lepidopteran, T. ni (Table II). The results from all of these feeding experiments were consistent with previous reports that BTK when fed to lepidopterans is active while BTI is toxic to only certain dipterans (3) and is supportive evidence that the BTI and BTK preparations used in our studies were similar to preparations previously used by other investigators. Furthermore, cross-contamination between BTI and

BTK as determined by ELISA was less than 0.01%, the detectable limit of the assay (35).

The combined 24, 27, and 35K components of BTI (Sandoz) δ-endotoxin (Figure 1, Tracks 2 and 3) had an equivalent toxicity

Table II. Oral toxicity of alkaline-solubilized BTI (Sandoz, Track 2) and BTK (Salsbury, Track 1) δ-endotoxin.

Animal	24h BTI LD_{50}+	24h BTK LD_{50}+
A. aegypti		
Larva	2.29 ± 0.06* (12.41)	>40*
Adult (ENEMA)	54.5 ± 3.1 (6.37)	>900
T. ni	>50	2.30 ± 0.27 (2.85)
Larva		
H. zea	>35	>35
Larva		
Swiss-Webster Mice	>30	>30

+PPM or mg/kg body weight ± 1 S.D. with slope of probit analysis in parenthesis.

*μg/ml of water in which larvae were incubated.

Table III. Toxicity of partially purified alkaline-solubilized BTI δ-endotoxin fed and injected into A. aegypti and T. ni, respectively.

Toxin	A. aegypti 24h LC_{50}*	T. ni 24h LD_{50}+
Alkaline dissolved, Sandoz (Track 2)	2.29 ± 0.06 (12.4)	3.71 ± 0.32 (3.22)
DEAE (Track 3)	1.91 ± 0.30 (2.92)	1.96 ± 0.64 (2.16)
pH 4.5 soluble (Track 5, 27 & 24K)	2.02 ± 0.54 (2.06)	1.95 ± 0.14 (4.32)
G-75, R_f 1.35 (Track 6, 27K)	2.71 ± 0.12 (5.72)	3.54 ± 0.50 (3.28)
G-75, R_f 1.53 (Track 7, 24K)	>20	2.97 ± 0.18 (7.00)
Alkaline dissolved, Salsbury (Track 8, 27K)	5.78 ± 0.42 (4.76)	6.09 ± 0.46 (4.67)

*μg/ml ± 1 S.D. with slope of probit analysis in parenthesis. Larvae were incubated in water with BTI δ-endotoxin added.

+PPM or mg/kg body weight ± 1 S.D. with slope of probit

before and after DEAE (Table III for both A. aegypti and T. ni), even though there appeared to be a concentration of the 68K component in this purification step. The 24 and 27K component individually (Figure 1, Tracks 7 and 6, respectively) also had an equivalent toxicity when injected into T. ni (Table III) but the 24K component (Figure 1, Track 7) was not toxic when fed to A. aegypti whereas the 27K component was toxic (Table III). This inactivity cannot be explained by the absence of the 35 and 68K components in Track 7 (Figure 1) because these same components are also absent in Track 5 and yet Track 5 retained oral toxicity to A. aegypti (Table III). In fact the absence of the 68 and 35K components (Figure 1, Tracks 5 and 7) likewise did not effect the T. ni activity (Table III). Thus it appears that at least the 27K proteins are necessary for A. aegypti oral toxicity while both the 24 and 27K components can impart toxicity to T. ni when injected. The 27K component was also toxic in both A. aegypti and T. ni regardless of the source (Track 6 and Track 8, Figure 1 and Table III).

Neural Toxicity of BTI δ-Endotoxin

The injection of alkaline-dissolved BTI δ-endotoxin led to a number of immediate neuromuscular effects (Table IV) including,

Table IV. Symptoms elicited after the injection of alkaline-dissolved BTI δ-endotoxin (Track 2) into mice and insects

Time Post-Injection	Trichoplusia ni (5 PPM)	Periplaneta americana (6 PPM)	Swiss-Webster Mice (1.5 PPM)
0-1h	Mouth palpation of injection site Increased wandering Heart arrest Abdominal paralysis List side to side when crawling Total paralysis & flaccidity	Loss of motor activity	Ruffled fur Lost alertness Not inquisitive Reduced responsiveness Slow in righting themselves Breathing shallow Lost activity in hind legs
20-24h	Localized blackening of the body Total blackening of the body No response to head stimulation	In Survivors Failure to right themselves Tremor	In Survivors, Constipation Dead animals with a pinched waist

in the insects tested, listing from side to side when crawling, heart arrest, paralysis, and tremors. These symptoms were observed for both the Sandoz and Salsbury BTI δ-endotoxin and were also observed for partially purified BTI δ-endotoxin (Table III). The symptoms observed in mice were somewhat similar to those observed in botulism poisoning (a neurotoxin), which included a loss of alertness, shallow breathing, and in some cases lost activity in the hind legs. Dead mice had a pinched waist, a sign of diaphram arrest. The symptoms observed in insects following the injection of BTI δ-endotoxin were clearly different from those following the ingestion of BTK δ-endotoxin. When T. ni were fed BTK δ-endotoxin, there was regurgitation within 15 min, a total cessation of feeding until death, and no overt neuromuscular anomalies. The injection of alkaline-dissolved BTK δ-endotoxin produced no obvious adverse effects in insects or mice.

A number of other lines of evidence also suggested that there may be another mode-of-action for BTI poisoning by injection other than its known, general cytolytic activity (3,22-24). Using the appearance of cytosolic lactate dehydrogenase (LDH) in insect hemolymph post-injection as a marker for cytotoxicity (36-37), we found that dissolved BTI δ-endotoxin was a potent cytotoxin. When T. ni, however, were injected with dissolved BTI δ-endotoxin and then incubated at 28, 15, and 9°, there was an increase in the LD_{50} with a decrease in temperature (Figure 2) but the LDH levels at 3.5 PPM BTI were unaffected by temperature.

A pharmocological study of ventral nerve cord function in T. ni also suggested that alkaline-dissolved BTI δ-endotoxin was affecting the insect nervous system as a nerve poison (Figures 3 and 4). After 7-60 min post-injection of alkaline-dissolved BTI δ-endotoxin at the LD_{50} concentration of 3.7 PPM, the vantral nerve cord of T. ni exhibited spontaneous-high frequency discharges. This was followed by a reduced baseline activity and sensitivity to sensory stimulation (S, Figure 4) at 24 h post-treatment. By 2-90 min post-injection of methamidophos, there also was spontaneous-high frequency discharge which lasted 20 min - 6 h and was followed by a reduced baseline activity and sensitivity to sensory stimulation (S, Figure 4) by 24 h. The response to methamidophos was slightly more rapid and sustained than with BTI toxin. Studies also suggested that the primary site of action for BTI might be the peripheral nervous system and that the mode-of-action of BTI and methamidophos on the insect nervous system were probably different. When the peripheral nervous system is severed from the T. ni ventral nerve cord, the methamidophos application still elicits spontaneous discharge, but there is no response to BTI toxin. When the cytotoxin, phospholipase-A_2, is injected into T. ni at its LD_{50} of 35 PPM, there is no spontaneous-high frequency discharge in the ventral nerve cord and no reduction in the stimulus-response (S, Figure 4) as was the case for BTI.

A 25K dalton component was isolated at relatively high purity as determined by SDS-PAGE (Figure 5, Track 3) from alkaline-dissolved BTI δ-endotoxin (Figure 5, Track 1). The

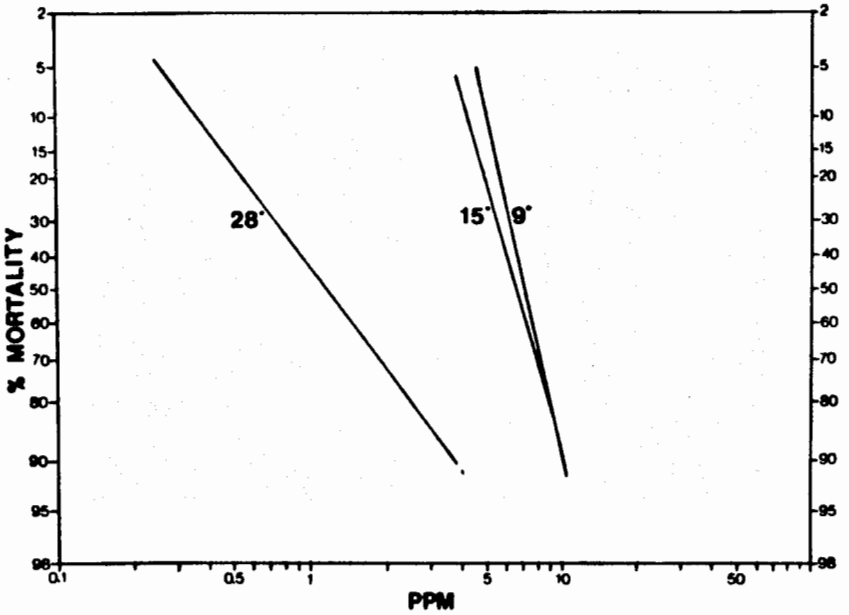

Figure 2. The temperature dependency of alkaline-dissolved BTI δ-endotoxin injected into Trichoplusia ni.

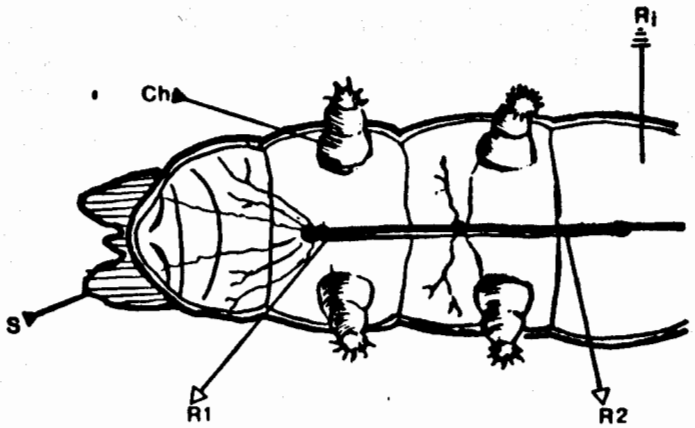

Figure 3. Neurophysiological preparation of Trichoplusia ni. Head, thorax and gut are removed. Tungstan electrodes were placed into the hemocoel along side abdominal ganglion VIII (at R1), the vantral nerva cord (at R2) and the abdominal wall (ground, R_i). Injections of alkaline-dissolved BTI δ-endotoxin, methamidophos and phospholipase-A_2 were into the second pair of abdominal prolegs (Ch). Mechanical sensory stimulation with a glass probe was at the anal proleg (S). Activity in the ventral nerve cord was monitored through 24 h post-treatment (38-40) (see Figure 4).

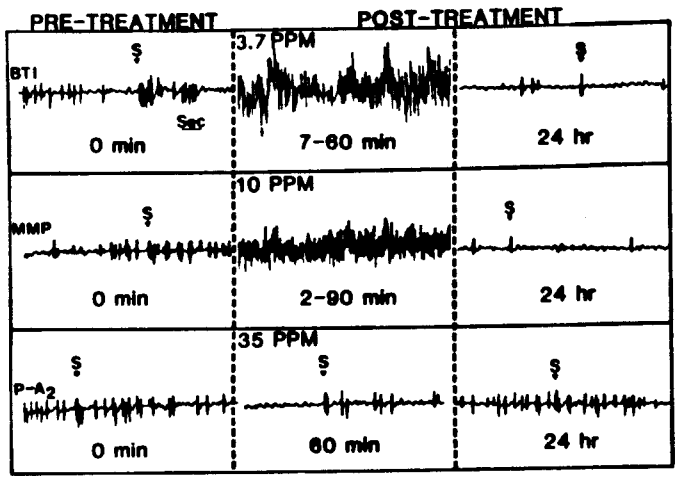

Figure 4. Time dependency of nervous activity in the ventral nerve cord of Trichoplusia ni injected with 3.7 PPM alkaline-dissolved BTI δ-endotoxin (Sandoz), with 10 PPM methamidophos (MMP) and with 35 PPM phospholipase-A_2 (P-A_2). Mechanical sensory stimulation is given at arrow S. The control response was the same as the recording for P-A_2. BTI and P-A_2 were injected into T. ni at their respective LD_{50}.

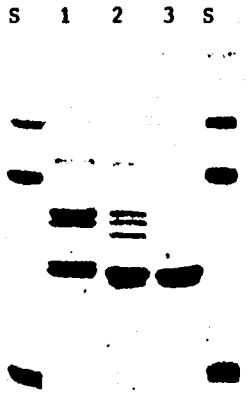

Figure 5. SDS-Page analysis of alkaline-dissolved Bacillus thuringiensis israelensis (BTI) δ-endotoxin from Sandoz Inc. at 25 μg per track: (1) BTI δ-endotoxin as prepared in Figure 1 (Track 2), (2) soluble fraction after dialysis of BTI δ-endotoxin into pH 4.5 sodium acetate buffer, and (3) 25K component from BTI δ-endotoxin after pH 4.5 percipitation and DEAE-anion exchange chromatography. S, molecular weight markers from top to bottom bovine serum albumin (68K daltons), ovalbumin (43K), and myoglobin (16K).

supernatant (Figure 5, Track 2) after pH 4.5 percipitation of the crude endotoxin was further purified by DEAE-Cellulose (DE-52, Whatman, 30 ml) in 0.025 M Tris-HCl (pH 8.00), eluted with a 48 h, 400 ml, 0.0-0.2 M NaCl linear gradient (Figure 5, Track 3). The resulting 25K dalton component (Figure 5, Track 3) at 0.85 µg/ml produced 50% cell lysis in a 1% human red blood cell solution (25°C, 15 min) and was also hemolytic against sheep and rabbit red blood cells. Hemolytic activity increased for the 25K component (Figure 5, Track 3) after a 15 sec incubation at 70°C and was inactivated at 90°C as was also the case for the crude toxin (Figure 5, Track 1). At 25 µg/ml, however, the 25K protein was not toxic orally to A. aegypti even after 15 sec heat treatments at 45, 70 or 90°C nor was it lethal when injected into T. ni larvae at 4.6 PPM (the 48 h LD_{50} > 4.6 PPM). Injections did disrupt larval-pupal metamorphosis later in development. The crude toxin (Figure 5, Track 1), however, was toxic to A. aegypti and T. ni at 45 and 70° but not after the 90° treatment. So the 25K component is cytotoxic but does not have either oral toxicity to A. aegypti or injected toxicity to T. ni.

Conclusions

It appears from our studies and reports in the literature that the parasporal crystal of BTI has gut-toxicity when fed to the mosquito (16), both in vitro and in vivo cytotoxicity (3,22-24) and in vivo neurotoxicity. Injected toxicity occurred in a number of insect species. The crude alkaline-dissolved δ-endotoxin of BTI when injected into T. ni was strongly cytotoxic and at its LD_{50} also neurotoxic. This was unlike the cytotoxin, phospholipase-A_2 which demonstrated no neurotoxicity at its LD_{50}. The toxicity of BTI δ-endotoxin injected was also temperature-dependent while its cytolytic activity was unchanged in the same temperature range. A 25K component isolated from BTI (Figure 5, Track 3) was also found to be cytolytic but when injected had no toxicity.

Until each of the components from the alkaline-dissolved δ-endotoxin of BTI (Figure 1, Track 2) can be purified and separately tested for cytotoxicity and neurotoxicity, the interrelationships of these modes-of-action to the many polypeptides found in the δ-endotoxin of BTI will be in question. The variations obtained with different BTI δ-endotoxin preparations and BTI strains will magnify the complexity of the problem. Nevertheless, a number of lines of evidence now exists to suggest that injected toxicity and neurotoxicity are not necessary a function of general cytolytic activity. The evidence is clear that there are also cytolytic components that demonstrate no gut toxicity. The finding of species difference within a single family of Lepidoptera to the susceptibility of BTI δ-endotoxin poisoning by injection will be an important tool for studying mode-of-action in the future. Reports on the nerve blocking action from digests of the δ-endotoxin of BTK (20-21), suggest that a neurotoxic element may be common to the action of other members of the BT complex.

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