

AN ABSTRACT OF THE THESIS OF

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Title: Development of an Enzyme-Linked Immunosorbent Assay to Detect Antibodies against *Bacillus thuringiensis* subspecies *israelensis* in Mallard Ducks (*Anas platyrhynchos*)

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To develop an assay to detect antibodies to *Bacillus thuringiensis* subsp. *israelensis* in mallard ducks, a growth curve was first established for the bacterium. The growth curve indicated that the crystal delta endotoxin would be best harvested from the rest of the cell material after 12 hours of growth. The delta endotoxin was solubilized in alkaline conditions followed by treatment with proteases or no treatment. The two differently treated delta endotoxins were purified by column chromatography. Fractions were assayed for duck erythrocyte lysis and cytotoxicity to a mosquito cell line. The proteolyzed sample gave four protein peaks with gel filtration, and the fourth peak containing biological activity was further separated into three protein fractions by anion exchange chromatography; two of the three showed biological activity. These two

fractions contained 22 and 23 kD proteins species. The nonproteolyzed sample was separated into two protein fractions by gel filtration; only the first peak contained the biological activity. This fraction was further separated into two fractions by anion exchange chromatography; only the second fraction, containing a 28 kD protein, exhibited the activity. This fraction contained a 28 kD protein. However, the fractions containing 22 or 23 kD proteins originating from the proteolyzed sample showed the highest biological activity.

Mallard ducks were repeatedly exposed to an aerosolized commercial preparation of the organism. Sera were collected periodically and tested for the antibody by an enzyme-linked immunosorbent assay (ELISA). Those toxic antigens containing 22 or 23 kD proteins were unsuitable for the assay. The exposed ducks were found to produce antibodies against the first fraction from anion exchange chromatography of the proteolyzed sample. The antibody titres increased as the number of exposures increased. The results suggest that ELISA is applicable for detecting antibodies against *B.t.i.* in wild ducks using the fraction containing a 50 kD protein.

Development of an Enzyme-Linked Immunosorbent Assay
to Detect Antibodies against *Bacillus*
thuringiensis subspecies *israelensis* in
Mallard Ducks (*Anas platyrhynchos*)

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This thesis is dedicated to Sue Nelson because she is pretty cool and I love her.

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Development of an Enzyme-linked Immunosorbent
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Chapter 1

Literature Review

Bacillus thuringiensis, an insecticidal bacterium

Bacillus thuringiensis (B.t.) is a gram positive soil bacterium that has the ability to kill larvae of lepidopteran insects. It was discovered in 1955 that the larvicidal activity of this novel bacterium was due to an intracellular crystal protein (Hannay and Fitz-James, 1955). Many different strains of B.t. have been discovered and the classification of 27 different serovars, using flagellar antigens, was recently described by de Barjac and Frachton (1990). Reviews of the structure and characteristics of B.t. organisms have been done by Aronson et. al. (1986) and Hofte and Whiteley (1989). The applications of B.t. as a pesticide includes control of agricultural pests such as the gypsy moth (Burges, 1982). The bacteria was found to provide long-term control of moths that infest storage grains. This effect was probably due to spores and crystals which are able to survive long periods of time in dry places. Other experimental applications of B.t. include the formation of transgenic plants that produce the crystal protein, which have been shown to protect the plants from potential pests (Vaeck et. al., 1987; Fischhoff et. al., 1987; Barton et. al., 1987)

Goldberg and Margalit (1977) isolated a mosquito larvicidal bacterium out of soil taken from known mosquito breeding grounds in Israel. Only one isolate out of 1,000 showed rapid larvicidal activity to mosquitos from four

different genera. This newly isolated bacterium, named *Bacillus thuringiensis* subspecies *israelensis* (*B.t.i.*) was also found to be lethal to black-fly larvae (Undeen and Nagel, 1978; Undeen and Berl, 1979). Since *B.t.i.* had the ability to kill dipteran larvae, it was recognized to be a legitimate tool to help control vector-borne diseases.

Structure of the crystal inclusion body of B.t.i.

The characterization of *B.t.i.* showed that it contained a spore and a crystal inclusion like other strains of *Bacillus thuringiensis* (Tyrell et. al., 1981). The crystal inclusion was produced at the same time as the spore (Bechtel and Bulla, 1976), and was shown to be composed of several proteins (Tyrell et. al., 1981). The *israelensis* subspecies contained proteins of 135 kD and 68 kD, but unlike the other *B.t.* species it contained a major protein of 28 kD. Lee et. al. (1985) isolated inclusion bodies from *B.t.i.* by gradient centrifugation. Morphologically, they increased in size from small dots, to large dots, to small refractile bodies, to large refractile bodies. These various inclusion bodies differed in their protein content, with the small dots and large dots consisting of mostly 38 kD and 65 kD protein, and the small and large refractile bodies containing 135, 65, 38, and 28 kD proteins. The 28 and 65 kD proteins became the major proteins the more time expired after sporulation began, with concomitant decrease of the 38 kD protein in the inclusions.

**Larvicidal action and toxicity
caused by the delta endotoxin**

The larvicidal action of *B.t.i.* was determined to be in the crystal protein, therefore it was called the delta endotoxin (Tyrell et. al., 1979). Further analysis of the individual proteins of the delta endotoxin implied that a 230 and 130 kD protein were important for its larvicidal activity (Visser et. al., 1986). Others determined the 65 kD protein to be the larvicidal component of the delta endotoxin (Lee et. al., 1985; Hurley et. al., 1985), while still others suggested that the 28 kD protein worked synergistically with the 65 kD protein to cause the larvicidal activity of *B.t.i.* (Wu and Chang, 1985; Chilcott and Ellar, 1988). It was later shown that the 28 kD protein was not the primary larvicidal component of the delta endotoxin (Held et. al., 1986).

The larvicidal action was described as occurring due to solubilization of the crystal protein by the high pH of the gut of target insects, allowing the proteins to disrupt the gut epithelial cells (Lilley et. al., 1980). Through a specific protein-receptor interaction (Van Rie et. al., 1990). It was further suggested that there may be a population of receptors to the larvicidal protein that exist, which would explain differences in insecticidal spectrum of *B.t.* crystal proteins. The specific larvicidal action was suggested to be the protein-receptor interaction

followed by integration of the protein into the lipid bilayer of the target cells forming a pore after aggregation in the membrane (Haider and Ellar, 1989). The pores allowed an influx of ions from the colloid-osmotic equilibrium, with water following the ions, which caused the cells to swell and lyse (Knowles and Ellar, 1987).

Exposure of *in vitro* cell lines to the delta endotoxin caused rapid cell changes and cell death, along with lysis of erythrocytes (Thomas and Ellar, 1983). Death was observed when mice were injected intravenously with 15-30 μ g of protein per gram of body weight of the entire solubilized delta endotoxin. The 28 kD protein was implicated as the protein containing the cytolytic and hemolytic activity (Armstrong et. al., 1985; Hurley et. al., 1985; Held et. al., 1986; Chilcott and Ellar, 1988). When Armstrong et. al. (1985) tried to produce monoclonal antibody to the 28 kD protein, mice injected intraperitoneally with 100 μ g of protein died after 6 hours.

The mechanism of the cell death by the 28 kD protein was also suggested to be integration of the protein into the cell lipid bilayer to form aggregates of the proteins, resulting in formation of membrane pores (Ellar et. al., 1985). The protein was able to target primarily unsaturated phospholipids of insect and mammalian cells, but not bacterial lipids. This is in contrast to the suggested target of the lepidopteran toxic proteins, which targeted a

glycoconjugate in the membrane and then caused cytotoxicity by pore formation. Haider and Ellar (1989) suggested the 28 kD protein also bound to a nonphospholipid receptor because of slower pore formation and disruption of liposomes when compared to disruption of insect cells, which have the cell receptors. Aggregation of toxin proteins to form pores would be promoted by binding more than one toxin molecule by the receptors.

Aggregates of a proteolyzed product of the 28 kD toxin were isolated from insect cells using detergent and a sucrose density gradient (Chow et. al., 1989). The concentration of aggregates isolated increased with the amount of toxin attached to the cells, and there was a threshold amount of toxin necessary to form the 400 kD aggregates. The hemolysis caused by the 25 kD protein could be inhibited by the cysteine modifier HgCl₂, indicating that the protein contains a cysteine residue in an essential binding portion of the molecule. Monoclonal antibody to this same portion of the molecule also blocked the hemolytic activity.

Molecular biology of the delta endotoxin proteins

The 28 kD protein of B.t.i. was found to be coded in plasmid DNA as shown by inserting the plasmid into *E. coli* and immunoprecipitating the protein using polyclonal serum to the protein (Ellar et. al., 1985). The nucleotide sequence of the DNA fragment coding the 28 kD protein was determined (Waalwijk et. al., 1985). This allowed the open reading frame encoding the protein to be determined, and within it there was a coding capacity for a 249 amino acid polypeptide. The amino acid sequence of the protein was deduced from the nucleotide sequence, and it had the almost identical sequence as that determined by tryptic peptide analysis (Armstrong et. al., 1985). The protein was shown to contain mostly hydrophobic amino acids, as determined by a hydropathicity plot (Ward and Ellar, 1986). It was also shown that the transcripts of the 28 kD protein were produced during the early stages of sporulation (Waalwijk et. al., 1985). After the stop codon in the sequence there exists two more stop codons and regions of symmetrical sequences that allow formation of two hairpins in the transcript.

With the nucleotide sequence in hand it was shown that site specific mutagenesis of the 28 kD protein changed the larvicidal, *in vitro*, and *in vivo* activity (Ward et. al., 1988). When the Glu45 residue was changed to Ala45 the hemolytic activity was reduced eightfold, but not the

larvicidal activity. This change in the charge at this position apparently changes the conformation of the protein not allowing the phospholipid-protein interaction. It was suggested that the larvicidal action is maintained because cleavage by gut proteases would result in the original toxic conformation of the protein. One mutant that contained the change of Lys124 to Ala124 showed a threefold increase in *in vitro* activity, but a threefold decrease in *in vivo* activity. The possible explanation of these results is that the crystal protein of this mutant does not assemble large crystal inclusion bodies, therefore the mosquitos probably were not ingesting the small proteins as well as they were ingesting large proteins. This amino acid change may also expose sites on the protein that would allow the proteases of the insect gut to cleave the protein to an inactive form. The results allowed Ward et. al. (1988) to propose a model of the 27 kD protein.

The nucleotide sequence of the 130 kD protein of *B.t.i.* has also been determined (Chungjatupornchai et. al., 1988). The amino acid sequence deduced from the DNA sequence showed greater than 50% homology with a 130 kD protein gene sequence of a lepidopteran specific subspecies (*B.t. berliner*), with most of the homology occurring in the C-terminal portion of the protein. The 130 kD protein of *B.t.i.* also reacted with anti-*B.t. berliner* sera. The similar sequence homology was not seen with the 28 kD and 65

kD proteins of *B.t.i.* The hydropathicity plot showed two strong hydrophobic regions in the N-terminal end of the protein, which were also seen with the *B.t. berliner* protein.

The gene encoding the 58 kD protein was cloned and expressed in mutants of *B.t.i.* that did not have the plasmid encoding the delta endotoxin (Garduno et. al., 1988). The mutant strain did produce the 58 kD protein, which was shown to be structurally related to the 135 kD. The sequence of the 58 kD protein gene did not have any similarities with the 28 kD protein gene (Thorne et. al., 1986).

An interesting 20 kD protein was shown to be important in the production of the 28 kD protein (Adams et. al., 1989). The gene of the 20 kD protein was sequenced and demonstrated to act in a post-transcriptional manner to enhance the production of the 28 kD protein. It was shown that this gene lies upstream to the gene encoding the 28 kD protein about four kilobases and is adjacent to the gene encoding the 68 kD protein. When a transposon was inserted into the 20 kD protein gene the expression of the 28 kD protein was reduced without a reduction in its mRNA indicating the post-transcriptional action. The exact mechanism of action of the 20 kD protein has not yet been elucidated. It is interesting to note that the appearance of this protein was not seen by electrophoresis unless high concentrations of proteins were separated. The small

amounts of the 20 kD protein were observed early in the sporulation process as the 28 kD protein was beginning to be produced.

Nontarget toxicity of B.t.i.

Since *B.t.i.* has the ability to cause toxicity to cells of organisms not targeted by applications of the pesticide, a few studies have been conducted on different organisms to determine the effects of exposure. It was first shown that exposure of mice to the crystal delta endotoxin killed mice when they were challenged intravenously (Thomas and Ellar, 1983). Mice, rats, and rabbits were challenged with the *B.t.i.* insecticide formulation through several routes and only intraperitoneal injection with 10^7 CFU/athymic mouse and intracerebral injection with 10^7 CFU/rat caused mortality (Siegel et. al., 1987). No mortality was seen in the rabbits. When mice and rats were challenged with an intraperitoneal (2.5 mg/kg) inoculation of the 28 kD protein there was no cytolysis of red and white blood cells, which would be expected from the *in vitro* assay results (Mayes et. al., 1989). Pathogenic effects were observed in the jejunum and liver of the rats and mice. The histopathological observations revealed epithelial necrosis and sloughing of cells of the villi of the intestine, and centrilobular congestion of the liver.

Clearance of *B.t.i.* from mice and rabbits exposed by intraperitoneal and topical ocular application was studied by Siegel and Shaddock (1990). They found that the organisms applied were cleared from the eyes of the rabbits after one week when exposed to 5.43×10^6 CFU. The bacteria

were consistently recovered from the spleens of mice exposed to *B.t.i.* for up to 80 days. This result suggested that the bacteria was multiplying in the mice, and that the ability to multiply was dependent on the spore to vegetative cell ratio that was in the exposure material. It was hypothesized that spores are more likely to be cleared because of three phases that must occur for multiplication: spore phase, germination phase, and developed vegetative phase. This would allow phagocytosis to occur more readily than with the single vegetative cycle. Even though it was suggested the organisms multiplied in the mice, there was no visible toxicity caused by the *B.t.i.*

Another study testing the exposure to a vertebrate was the challenge of minnows with *B.t.i.* (Snarski, 1990). No adverse effects were observed after placing low concentrations of commercial preparations of *B.t.i.* pesticide into the water containing fish, even though spores were detected in the animals. There was mortality observed when a high concentration was introduced into the water, but this was attributed to a severe dissolved oxygen depletion.

An invertebrate toxicity study was done on the eggs of *Trichostrongylus colubriformis*, a ruminant nematode (Wharton and Bone, 1989). They showed that exposure to *B.t.i.* crystal endotoxin disrupted the egg-shell at the lipid layer and caused autolysis of the embryo.

Aerosol exposure to birds

Stearns et. al. (1987) investigated the deposition of iron oxide dust particles in the lungs of mallard ducks. They recognized that deposition of particles would probably differ from mammalian lung deposition patterns because of a different anatomy. Inflation and deflation does not occur in the lungs of birds; air is pumped through parallel bronchi into atria, then enters smaller passages called infundibula which radiate into the gas exchange tissue. They found that when the birds were exposed to the dust with a mean diameter of $0.18\ \mu\text{m}$ there was deposition within the epithelial cells of the atria and the infundibula, and within a thick surfactant that coats the epithelial cells called the trilaminar substance. No free macrophages were observed within the atria to phagocytize the particles caught in the trilaminar substance. Instead the particles phagocytized by the epithelial cells were probably transported across to the interstitial macrophages and cleared.

Aerosol exposure of bobwhite quail chicks with polystyrene microspheres ranging from 0.2 to $20.0\ \mu\text{m}$ showed that particles greater than $2\ \mu\text{m}$ in diameter were rarely found in the air sacs or lungs of the birds (Driver et. al., 1990). Most of the deposition of the larger particles occurred in the nasal area and trachea of the birds. The location that particles are deposited will determine if

antigen presenting cells (macrophages) will phagocytize the foreign particles.

Since *B.t.i.* has the potential to cause toxicity in non-target species the amount of contamination that occurs in the environment should be monitored. Mallard ducks may be exposed to *B.t.i.* because they are a species that is often found in areas that mosquito larvae would mature. For this reason mallard ducks could be used as a sentinel species to provide indication of non-target species exposure by the bacterial pesticide *B.t.i.*

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Chapter 2

Growth Curve and Sporulation Determination of *B.t.i.*

Summary

A growth curve of *B.t.i.* was determined in the GGYS medium. The bacteria reached exponential growth four hours after the inoculation when incubated at 28°C. Sporulation was first observed eight hours after the inoculation when the bacteria were beginning lag phase of growth. Establishing when spores were present during the growth of *B.t.i.* indicated the optimal time to harvest the delta endotoxin from the bacteria.

Introduction

Bacillus thuringiensis produces a spore and a proteinaceous crystal delta endotoxin during its growth cycle (Bechtel and Bulla, 1976). Both the spore and the parasporal crystal were produced after seven hours of growth. The mature spore and proteinaceous crystal in an unlysed sporangium were observed 12 hours after the growth began.

The purpose of the present study was to determine the growth characteristics of *B.t.i.* so that the mature crystal inclusion could be harvested for maximum yield.

Materials and methods

A growth curve was established for *B.t.i.* by inoculating a 100 ml starter culture of GGYS with a loopful of a colony from a culture grown on a TGY agar plate (Haynes et. al., 1955). The GGYS medium was the same as the basal medium used by Nickerson and Bulla (1974) except that it contained yeast extract (0.2%) and glutamic acid (1%). The TGY medium was inoculated with *B.t.i.* (Vectobac Technical Material; Abbott Laboratories, lot no. 27-801-CD). The starter culture was inoculated into 500 ml of GGYS media at 6% (v/v), then incubated at 28°C and shaken at 100 rpm. At one or two hour intervals 10 ml samples of the culture were taken for pH, for absorbance readings at 280 nm, and for colony forming units (CFU) determinations. Counts for CFU were done by the standard dilution and plating method. The samples were also Gram-stained to determine the bacterial sporulation.

Results and discussion

The growth curve shown by the *B.t.i.* indicated that logarithmic growth of the bacteria started about four hours after the medium was inoculated (Fig. 1). This continued through eight hours after the inoculation where the number of bacteria became more constant. At this lag phase of growth spores began to appear (Table 1). The pH increased in the first two hours and then consistently decreased until it reached 4.6-4.7 during lag phase of growth.

The results are similar to the results shown by Tyrell et. al. (1981), where sporulation was complete after 10 hours, although the pH of the growth medium differed. The Tyrell group saw the pH of the culture medium decrease from 7.0 to 6.6, then increase to 8.0. The results of this study showed an increase from pH 6.3 to 6.6, then a consistent decrease to 4.7. This discrepancy in the pH changes could be attributed to a difference in the growth media. The medium used for this experiment contained glutamic acid and yeast extract.

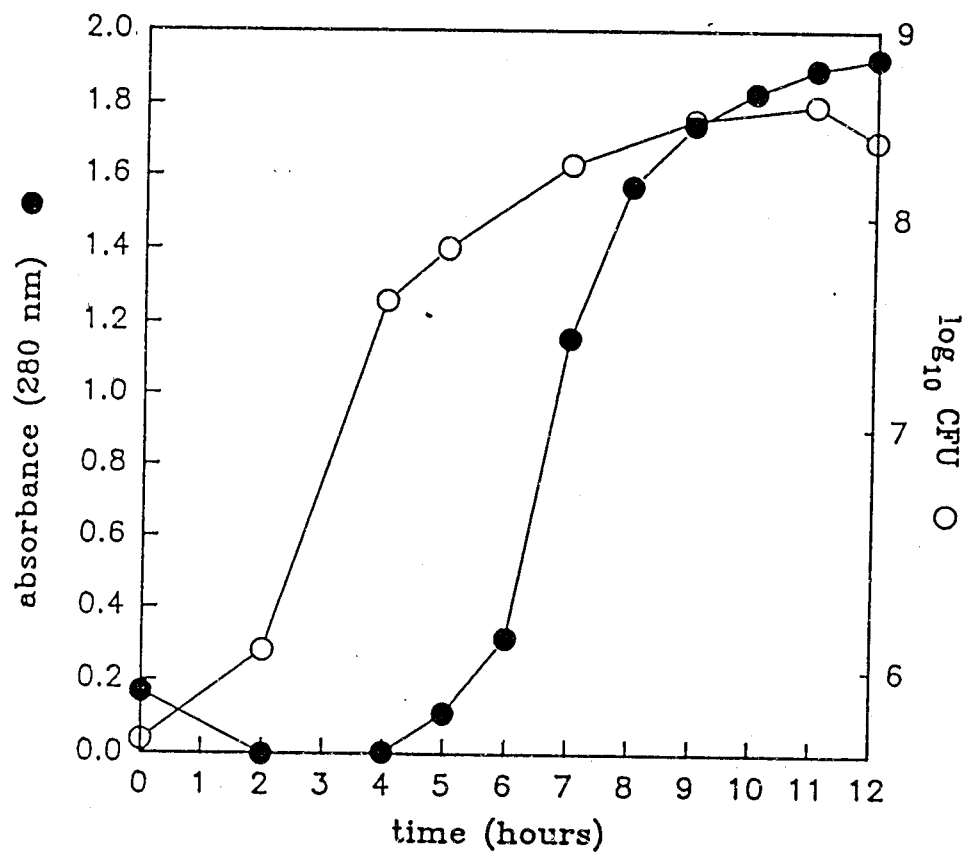


Fig. 1 Growth curve of *B.t.i.* in GGYS. The open circles are log₁₀ CFU of samples taken from the culture medium. The closed circles are absorbance readings at 280 nm of samples taken from the culture medium.

Table 1 Crystal inclusion formation and pH readings of samples from the *B.t.i.* growth curve culture medium.

time	pH	crystals
0	6.3	
1	--	
2	6.6	+
3	--	
4	6.4	-
5	5.9	-
6	5.5	-
7	5.1	-
8	4.7	+
9	4.6	
10	4.6	++
11	4.7	
12	4.7	++

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Chapter 3

Purification of the Delta Endotoxin Proteins of *B.t.i.*

Summary

The proteins of the delta endotoxin of *Bacillus thuringiensis* subsp. *israelensis* were purified by gel filtration and anion exchange chromatography. Two different preparations of alkali solubilized delta endotoxin were used; one of the preparations was treated with proteases, while the other was left untreated. The protease treated sample separated into four peaks (PS-1, PS-2, PS-3, and PS-4) when passed through a gel filtration column with Sephadex G-150. The fourth peak (PS-4) from this separation was further separated into three peaks (PD-1, PD-2, and PD-3) when passed through an anion exchange column. The nonprotease treated sample separated into two peaks (NS-1 and NS-2) when chromatographed by gel filtration. The first peak separated into two more peaks (ND-1 and ND-2) when run through the anion exchange column.

The separated peaks were analyzed by SDS-PAGE, Western blot, and immunoelectrophoresis. The PS-4 sample showed proteins of 23 and 22 kD on the Western blot using a monoclonal antibody to the 28 kD protein. The PD-2 and PD-3 peaks also reacted with the monoclonal antibody showing 23 and 22 kD bands, respectively. The protease separated peaks did not react with a monoclonal antibody specific for the 65 kD protein. The fractions from the untreated solubilized delta endotoxin showed multiple proteins on the SDS-PAGE analysis. The NS-1 and ND-2 fractions reacted with both the

28 kD and 65 kD protein monoclonal antibodies. The PD-2 and PD-3 fractions showed single bands that migrated toward the cathode in the immunoelectrophoresis results. The ND-2 fraction also showed a band that migrated toward the cathode but was longer in length. The separated proteins could be used as antigen for ELISAs to test animal sera for antibodies to the crude pesticide.

Introduction

Bacillus thuringiensis subsp. *israelensis* has toxicity against mosquito and blackfly larvae (Goldberg and Margalit, 1976; Undeen and Nagel, 1978). Previous studies demonstrated that proteins of the delta endotoxin are responsible for the toxicity (Hurley et. al., 1985; Held et. al., 1986). The delta endotoxin has been separated from the other cellular material using gradient centrifugation (Sharpe et. al., 1975; Ang and Nickerson, 1978). It was later shown that specific proteins of the delta endotoxin could be isolated using column chromatography (Armstrong et. al., 1985; Hurley et. al., 1985; Held et. al., 1986). In this study, proteins of the delta endotoxin were solubilized by alkali conditions and were either treated with proteases or were non-treated. The two differently treated solubilized delta endotoxins were then fractionated using gel filtration and anion exchange chromatography. The various fractions were characterized by SDS-PAGE, Western blot, and immunoelectrophoresis.

Materials and methods

Alkali Solubilization and Proteolysis of B.t.i. Delta Endotoxin. The alkali solubilization and proteolysis of the B.t.i. was similar to the method used by Armstrong, et. al. (1985), with some modifications. Sixty grams of B.t.i. was suspended in 1200 ml of distilled water and centrifuged for 20 minutes at 15,000 x g at 4°C. The pellets were resuspended in 500 ml of distilled water then sonicated for 5 minutes in 30 to 40 ml aliquots using a Biosonik sonicator (Bronwill). The sonicated material was pooled and the pH was raised to 12 using 1 N NaOH followed by incubation for 3 hours at 37°C. After its pH was lowered to 7.5 with 1 N HCl, the material was centrifuged at 15,000 x g for 15 minutes at 4°C and the supernatant saved. Trypsin (Millipore) was added to the alkali-treated supernatant at a concentration of 15 µg/ml followed by incubation for 2 hours at 37°C. Proteinase K (Sigma) was added at 15 µg/ml and the mixture was further incubated at 37°C for 2 hours. Phenyl methyl sulfonyl fluoride (PMSF; Sigma) was added to the solution to a final concentration of 0.1 mM. Saturated ammonium sulfate was added to 15% saturation with mixing at 4°C for 30 minutes followed by centrifugation at 12,000 x g for 15 minutes. The supernatant was brought up to 35% saturation of ammonium sulfate and incubated at 4°C for 30 minutes, followed by centrifugation for 15 minutes at 12,000 x g. The pellet was dissolved in 60 ml 5mM NaH₂PO₄ (pH 7.5)

and is referred to as soluble endotoxin or proteolyzed crude extract (PCE).

Alkali Solubilization Without Proteolysis. The B.t.i. Vectobac Technical Material (20 g) was suspended in 400 ml of sterile distilled water and incubated at 28°C for 4 days to cause autolysis of the bacteria (Mahillon and Delcour, 1984). The incubated cell material was centrifuged for 20 minutes at 16,000 x g. The pellet was resuspended in 160 ml of distilled water and sonicated, followed by raising the pH to 12 with 1 N NaOH. The material was incubated for 3 hours at 37°C. The pH was brought down to 7.5 with 1 N HCl and then centrifuged at 15,000 x g for 20 minutes. The pellet, supernatant, and a soft pellet were analyzed for protein concentration and molecular weight as described below.

Column Chromatography. Column chromatography using Sephadex G-150 and DEAE Bio-Gel A was performed to separate the alkali solubilized-proteolyzed and nonproteolyzed proteins. Sephadex G-150 (Pharmacia) was equilibrated in 50 mM NH_4HCO_3 and packed in a 78 x 2.75 cm column. The flow rate of the 50 mM ammonium carbonate buffer (pH 8.5) was set at 10 ml/hour. A 0.33 ml sample of the alkali-proteolyzed B.t.i. material was diluted 1:3 in the ammonium carbonate buffer and applied to the column. The fractions were collected in 2.3 ml volumes. The fractions that were eluted

from the column were monitored by UV absorption at a wavelength of 280 nm. Four different samples of the cell material were run through the column. Fractions of peaks were pooled and concentrated by lyophilization followed by analysis described below.

The alkali-solubilized, non-proteolyzed cell material (NCE) in 1.0 ml was chromatographed through the Sephadex G-150 column. Each fraction from four runs was measured for the absorption at 280 nm, followed by each peak being pooled and lyophilized.

A 28 x 1.0 cm column of DEAE Bio-Gel A (Bio-Rad) was equilibrated with 50mM ammonium carbonate buffer, and approximately 30 mg of pooled-lyophilized fractions off the Sephadex G-150 column were run through the column. The pooled peaks from the Sephadex G-150 chromatography were dialyzed in 50mM NH_4HCO_3 overnight. The column was washed with 50mM ammonium carbonate before a linear gradient (total volume=400 ml) from 50-200 mM NH_4HCO_3 (pH 8.5) applied. The ionic strength of the eluate was monitored using a conductivity bridge (Beckman, model RC 16B2). The fraction absorption at 280 nm was measured, and fractions of protein peaks were pooled, lyophilized, then analyzed as described below.

Protein Assay. The protein concentration for solutions was determined following the Lowry method using crystallized

bovine serum albumin (Sigma) for the standard (Lowry et. al., 1951).

Monoclonal Antibody Production. Hybridomas that produced monoclonal antibody to the 28 kD protein of B.t.i. (monoclonal antibody B.t.i. clone 102) were obtained from Dr. Armstrong, USEPA, Corvallis, OR, and recovered using Dulbecco's modification of Eagles medium with glutamine and 25 mM HEPES buffer containing 0.25 μ l/ml of gentamicine, 20% fetal bovine serum, and 30 μ g/ml endothelial cell growth supplement. Mice (Balb/c) were primed with 0.5 ml of pristane 10 days prior to inoculation with hybridomas. Hybridomas were injected (6.5×10^5 cells/mouse) and mouse ascitic fluid was harvested 10 days later.

Dr. Kawanishi, USEPA, Research Triangle Park, NC, graciously donated B.t.i. monoclonal antibody 135-5.

SDS-PAGE. Separating mini gels composed of 12% polyacrylamide (Bio-Rad) were made with 4% stacking gels on top of the separating gels. Samples were diluted in the sample buffer (40% glycerol, 0.25M Tris, 8% SDS, 10% β -mercaptoethanol, and 0.002% bromophenol blue) and boiled for 5 minutes. Samples, usually 15 μ l, were placed in the wells of the stacking gel and electrophoresed for 45 minutes at 185 volts.

Western Blot. SDS-PAGE gels were equilibrated in a transfer buffer (20% methanol, 1.44% glycine, and 0.3% tris base, pH 8.3) for 30 minutes and then transblotted onto nitrocellulose overnight at 50 volts and 4°C. The electrophoresed nitrocellulose was then equilibrated in tris-buffered saline (TBS) for ten minutes and then incubated in the blocking solution (3% BSA in TBS) for one hour at room temperature. Membranes were washed twice using Tween 20-TBS (TTBS) for two minutes for each wash followed by two-minute washes with TBS two times. The primary antibody (1:500 dilution of ascites in TTBS) was applied to the nitrocellulose and incubated for 3 hours at room temperature. The nitrocellulose was then washed with TTBS and TBS three times with each solution. The secondary antibody (1:1000 dilution of horse radish peroxidase labeled goat-anti-mouse antibody in TTBS; BioRad) was incubated on the membranes for one hour at room temperature followed by another wash. The blots were developed by placing 0.05% horseradish peroxidase color developing reagent (4-chloro-1-naphthol; BioRad), 17% methanol, and 0.05% H₂O₂ in TBS on the nitrocellulose until all the bands were developed.

Polyclonal Antibody Production. Rabbits were inoculated subcutaneously with 2.5 ml of a 2 mg/ml preparation of B.t.i. Vectobac Technical Material in 50 mM NaHCO₃-Na₂CO₃ buffer at pH 9.5 with Freund's complete

adjuvant. They were boosted subcutaneously after four weeks with the same concentration of B.t.i. using Freund's incomplete adjuvant, then were bled after 3 weeks.

Immunoelectrophoresis. Agarose (1% ME agarose; SeaKem) was made in barbital-lactate buffer (0.088% sodium barbital, 0.004% calcium lactate, 0.014% barbital, and 0.031% chloroform) and poured onto microscope slides. Wells were cut into the agarose and samples of column purified proteins were loaded into the wells. The slides were electrophoresed for two hours at 5.5 volts/cm. After the electrophoresis a long trough in the center of the slide was cut and loaded with polyclonal antibody against crude B.t.i. toxin, and allowed to diffuse for 24 hours.

Results

Column Chromatography of Alkali-Solubilized-Proteolyzed

B.t.i. Proteins. Column chromatography was used to purify proteins of the delta endotoxin of *B.t.i.* The soluble endotoxin was chromatographed with a Sephadex G-150 column. Four protein peaks were obtained (Fig. 2). There were two major peaks, PS-1 and PS-4, and two minor peaks, PS-2 and PS-3. Only the last peak (PS-4) was shown to contain a protein that reacted with the *B.t.i.* clone 102 monoclonal antibody. This fraction also showed toxic activity (see below). The pooled PS-4 fraction was subjected to a DEAE Bio-Gel A column. Three major peaks, PD-1, PD-2, and PD-3 were obtained (Fig. 3).

Column Chromatography of Alkali Solubilized-

Nonproteolyzed B.t.i. Proteins. Column chromatography of a nonproteolyzed sample of *B.t.i.* was also used to purify proteins of the soluble endotoxin. The technical material of *B.t.i.* was solubilized under alkaline conditions and analyzed by Western blot to verify the isolation of the 28 kD protein from the crystal endotoxin. A soft upper portion of the pellet, after alkali solubilization and centrifugation, contained the 28 kD protein. The soft pellet material (NCE) gave two peaks, NS-1 and NS-2, after passing through the Sephadex G-150 column (Fig. 4). Column chromatography with DEAE Bio-Gel A of NS-1, which contained

proteins that reacted with the B.t.i. clone 102 antibodies (see below), gave two fractions (Fig. 5). The first fraction (ND-1) did not absorb to the column, while the second fraction (ND-2) was eluted with the linear gradient.

SDS-PAGE Analysis of Column Separated Proteins. The proteolyzed crude extract material showed multiple bands between 50 and 70 kD, along with multiple bands between 28 and 40 kD (Fig. 6, lane b). There was also a diffuse band observed at 23 kD. The PS-1 fraction showed similar bands to the crude extract, while PS-2 showed the 50-70 kD bands along with densely stained bands at 23, 28, and 34 kD (lanes c and d). The fraction labelled PS-3 had multiple proteins from 30 to 60 kD (lane e). The three peaks from the DEAE Bio-Gel A separation of PS-4 fraction showed proteins at 66 down to 50 kD for PD-1, a light band at 23 kD for PD-2, and a light band at 22 kD for PD-3 (lanes f-h).

The nonproteolyzed material showed protein bands at 66, 50, 36, and a strong band at 28 kD for the soft pellet material (Fig. 7, lane 2). NS-1 showed protein bands similar to the soft pellet material (lane 3). Only light bands around 66 kD were seen for NS-2 (lane 4). The ND-1 peak showed proteins from 30 to 34 kD, along with bands at 22, 46, and 56-66 kD (lane 5). Fraction ND-2 showed single bands at 28 kD and 66 kD (lane 6).

Western Blot Analysis of Column Separated Proteins.

Monoclonal antibody clone 102 reacted with multiple proteins in the various fractions of proteolyzed soluble *B.t.i.* (Fig. 8). Fractions PS-1 through 3 did not react with the antibody (lanes 3 through 5). The fraction PS-4 showed several bands smaller than 23 kD (lane 2), as did the crude proteolyzed soluble delta endotoxin (lane 6). The peaks from the DEAE Bio-Gel A column also showed distinct bands on the Western blot; PD-2 had a band at 23 kD, and PD-3 showed one band at 22 kD (lanes 8 and 9).

None of the proteolyzed *B.t.i.* proteins, including the solubilized delta endotoxin, showed any band formation when monoclonal antibody against the 68 kD protein, *B.t.i.* 135-5, was used for the Western blot.

The NS-1 fraction showed strong reactivity at 48 and 28 kD using *B.t.i.* 102, while the NS-2 peak showed no bands (Fig. 9, lanes 3 and 4 respectively). The soft pellet material also showed distinct bands at 28 and 48 kD (lane 2). This band formation was also exhibited by ND-2 (lane 6) and the crude pesticide used to expose the ducks (lane 7). The pesticide also exhibited a band at 44 kD.

Western blot of the nonproteolyzed proteins using *B.t.i.* monoclonal antibody 135-5 showed bands for the soft pellet around 68 kD, two bands probably around 56 kD, and one band around 34 kD (Fig. 10, lane a). The NS-1 material only exhibited a single band at 68 kD, while the ND-2

material showed bands at both 68 kD and 34 kD (lanes b and e respectively). The NS-2 and ND-1 samples did not show any bands (lanes c and d). The crude pesticide (Vectobac 12-AS) showed multiple bands (lane g).

Immuno-electrophoresis of Alkali Solubilized-Proteolyzed B.t.i. Separated Proteins. Immuno-electrophoresis was used to help verify proteins that were observed by SDS-PAGE and Western blot. Immuno-electrophoresis of the column separated *B.t.i.* proteins showed several bands with the rabbit antiserum against crude *B.t.i.* (Fig. 11). Several of these proteins migrated toward the cathode, while three bands migrated toward the anode. The PS-1 material exhibited one long band that migrated toward the anode and one short band that migrated toward the cathode. The PS-2 material showed only a short band that precipitated around the sample well. PS-3 sample precipitated a long diffuse band toward the cathode, while PS-4 had a band that migrated very far toward the anode and had one faint band that migrated midway from the sample well. PD-1 had a strong band that precipitated similar to the latter band described for PS-4, while PD-2 and PD-3 had precipitated bands that migrated far toward the anode.

Immunoelectrophoresis of Alkali Solubilized-Nonproteolyzed B.t.i. Column Separated Proteins. The precipitation of proteins that were separated on the Sephadex G-150 column showed a long band that migrated toward the anode and a short band that migrated toward the cathode for NS-1 (Fig. 12). The NS-1 band formations were very similar to the bands that formed for the soft pellet material. NS-2 did not show a significant band that precipitated. The DEAE Bio-Gel A column separated proteins of the NS-1 material showed a similar banding pattern for ND-2. ND-1 did not show significant precipitated bands.

Discussion

The use of the cross-linked dextran (Sephadex G-150) provided four fractionated peaks from the alkali solubilized delta endotoxin of *B.t.i.* Armstrong et. al. (1985) separated three peaks using the polyacrylamide-based Bio-Gel P-60. In our experience, Sephadex G-150 gave more reproducible results with simpler handling than Bio-Gel P-60.

Anion chromatography separated the PS-4 fraction from the Sephadex G-150 column into three peaks (Fig. 3). The biological activity was detected in the PD-2 and PD-3 fraction, both of which reacted with monoclonal antibody against the 28 kD protein (Fig. 8). The band formations were around 22 kD and 23 kD and may be breakdown products of the 28 kD protein. Proteins of these sizes were also observed by Pfannenstiel et. al. (1986).

In an attempt to isolate the intact 28 kD protein, nonproteolyzed crude material was examined by chromatography. The thick cell wall layer of the bacteria was autolysed by incubation in sterile distilled water for four days at 37°C (Mahillon and Delcour, 1984). A soft pellet after alkali solubilization and centrifugation was found to contain the intact 28 kD protein. This material was run through the Sephadex G-150 column giving only two peaks; only the first one (NS-1) still maintained the 28 kD protein (Figs. 7 and 9). Nishiura (1988) isolated a 27 kD

protein from a non-proteolyzed sample and reported its spontaneous breakdown to a 25 kD protein by endogenous proteases.

The anion chromatography of NS-1 eluted two more peaks with the fraction ND-2 containing the 28 kD protein. The *B.t.i.* clone 102 showed the 28 kD protein on a Western blot, along with a 48 kD protein (Fig. 9). This latter band may have been a dimer of the 28 kD protein or the 28 kD protein attached to other portions of the crystal endotoxin.

The separation of the nonproteolyzed soluble material into only two peaks with gel filtration (Fig. 4) indicates that the 28 kD protein was probably remaining intact with the other proteins, such as the 130 and 65 kD proteins, that form the crystalline endotoxin. This was verified by the SDS-PAGE total protein stain which showed multiple bands greater than the 28 kD protein (Fig. 7, lane 3), and in the long bands observed in immunoelectrophoresis (Fig. 12, well 3). This is in contrast to the proteolyzed samples where the trypsin and proteinase K cleaved the 28 kD protein from the crystal endotoxin allowing elution of the components of this protein in the last peak off of the Sephadex G-150 column (PS-4, Fig. 2).

In conclusion, employment of Sephadex G-150 made the separation of the delta endotoxin proteins easier because of simplified handling and the rigidity the material maintained. The use of proteases to cleave the delta

endotoxin was necessary to isolate individual proteins of the delta endotoxin. But when the solubilized delta endotoxin was treated with proteases the intact 28 kD protein was not isolated.

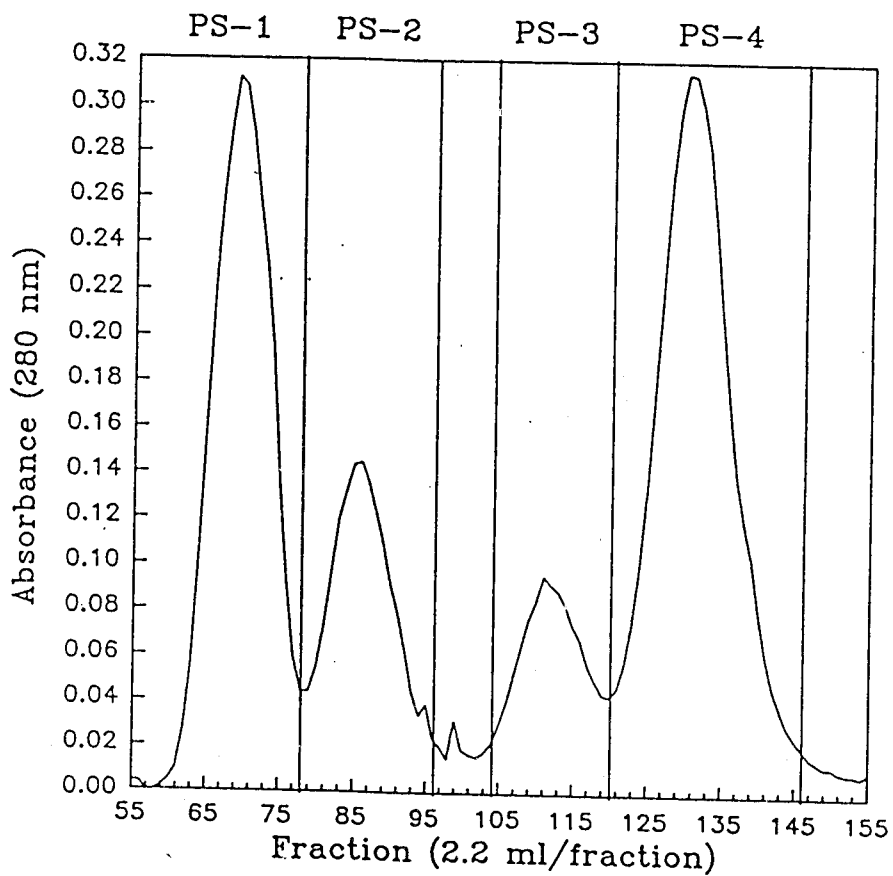


Fig. 2 Column chromatography of proteolyzed soluble delta endotoxin on Sephadex G-150. The sample was separated into four fractions labelled PS-1, PS-2, PS-3, and PS-4.

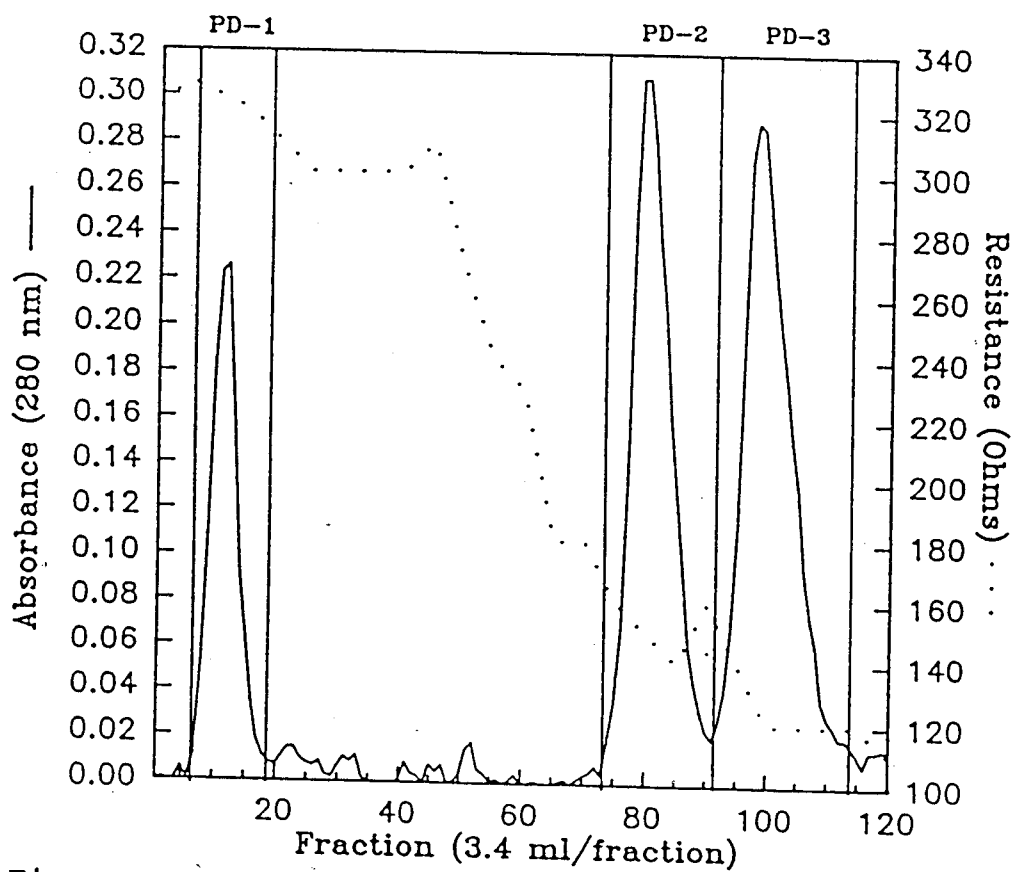


Fig. 3 Column chromatography of PS-4 on DEAE Bio-Gel A. The solid line is the separated fractions and the dotted line represents the salt gradient as measured by a conductivity bridge. The three fractions were labelled PD-1, PD-2, and PD-3.

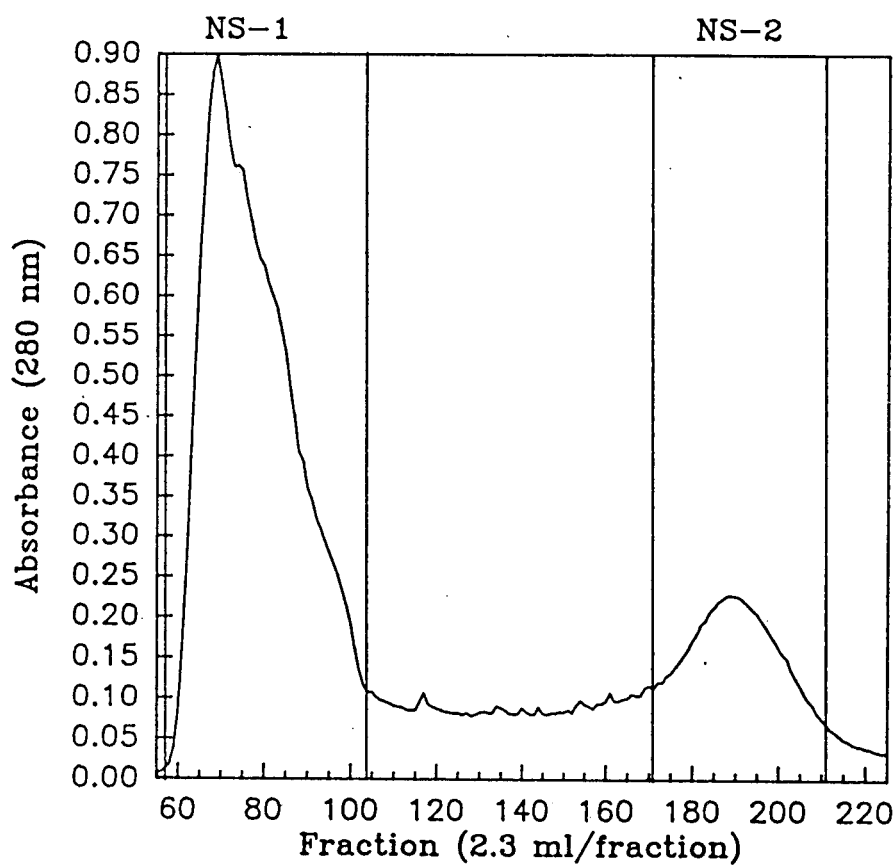


Fig. 4 Column chromatography of nonproteolyzed soluble delta endotoxin of *B.t.i.* on Sephadex G-150. The two fractions were labelled NS-1 and NS-2.

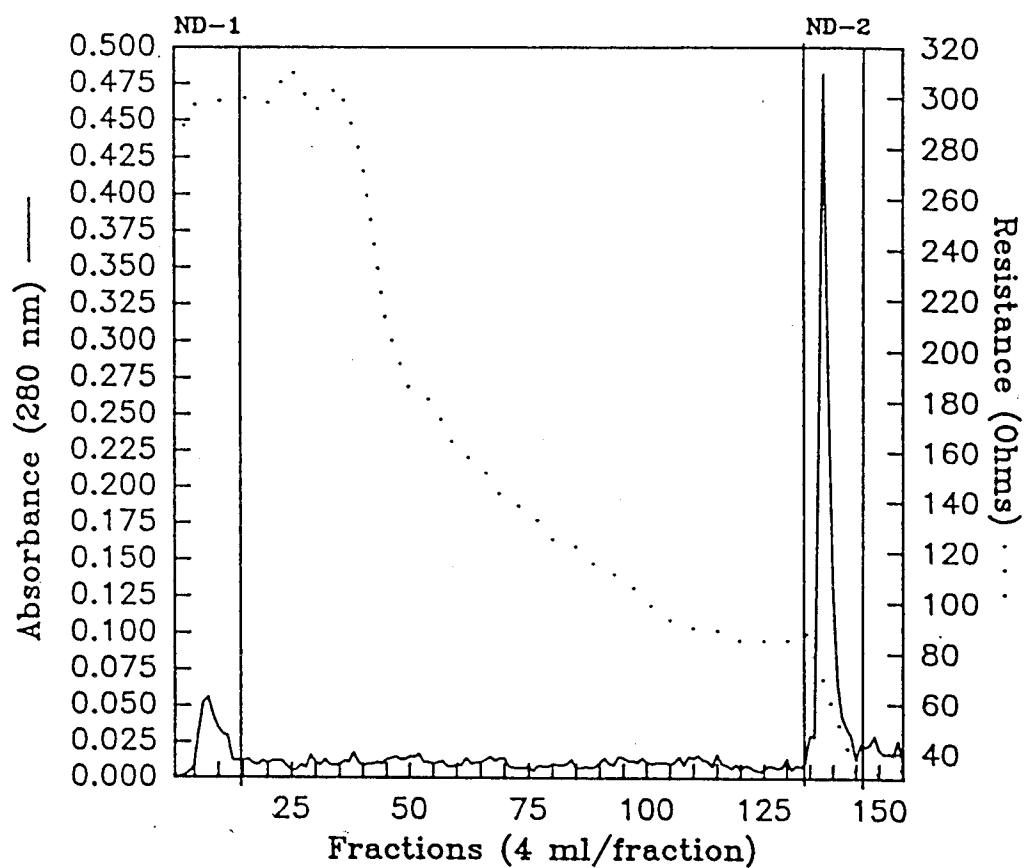


Fig. 5 Column chromatography of NS-1 on DEAE Bio-Gel A. The two fractions were labelled ND-1 and ND-2.

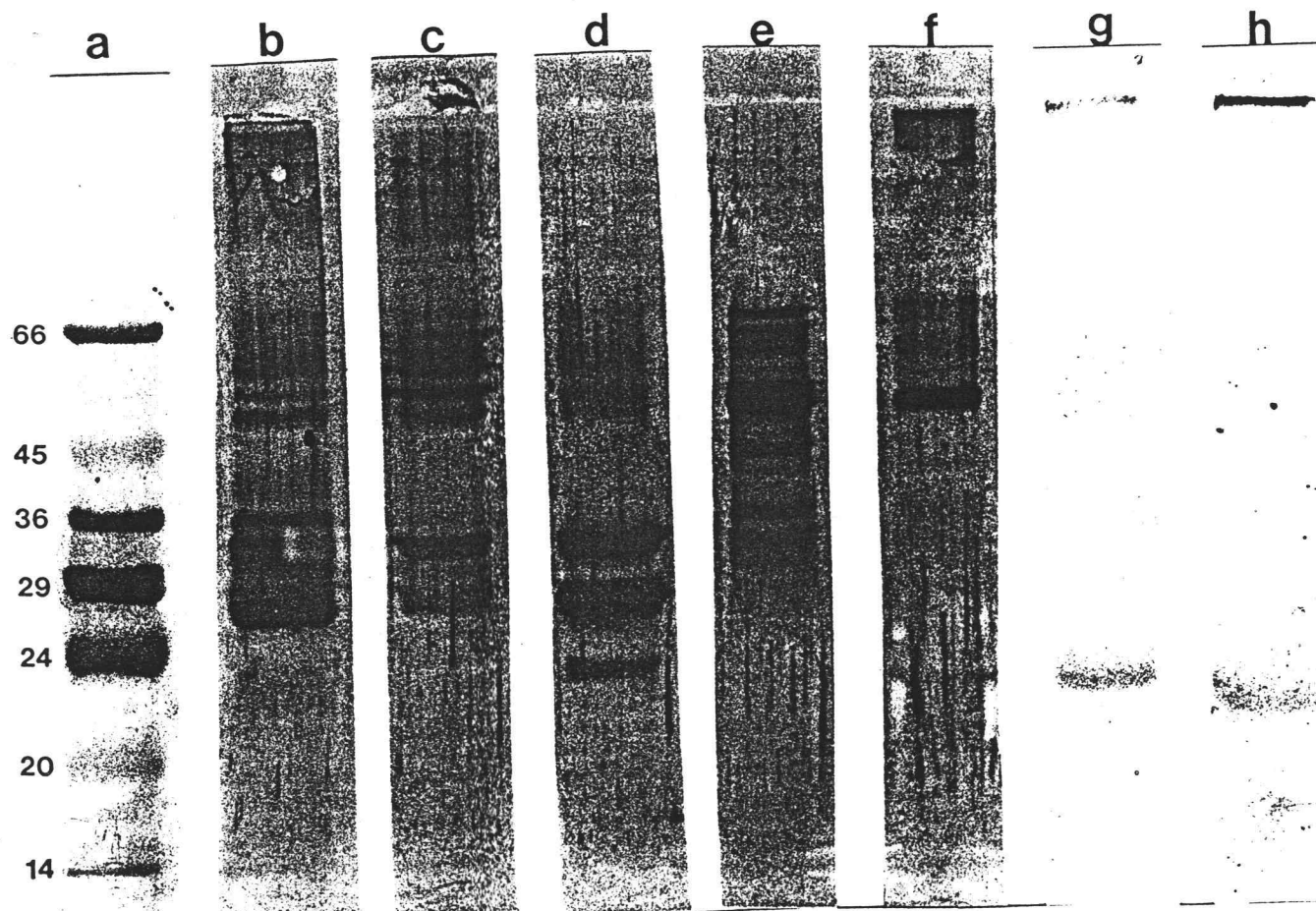


Fig. 6 Colloidal gold total protein stain of transblotted column purified proteolyzed proteins: lane a, 10 μ l of molecular weight standard; lane b, 33.8 μ g of PCE; lane c, 5 μ g of PS-1; lane d, 5 μ g of PS-2, lane e, 5 μ g of PS-3; lane f, 5 μ g of PD-1; lane g, 5 μ g of PD-2; and lane h, 5 μ g of PD-3.

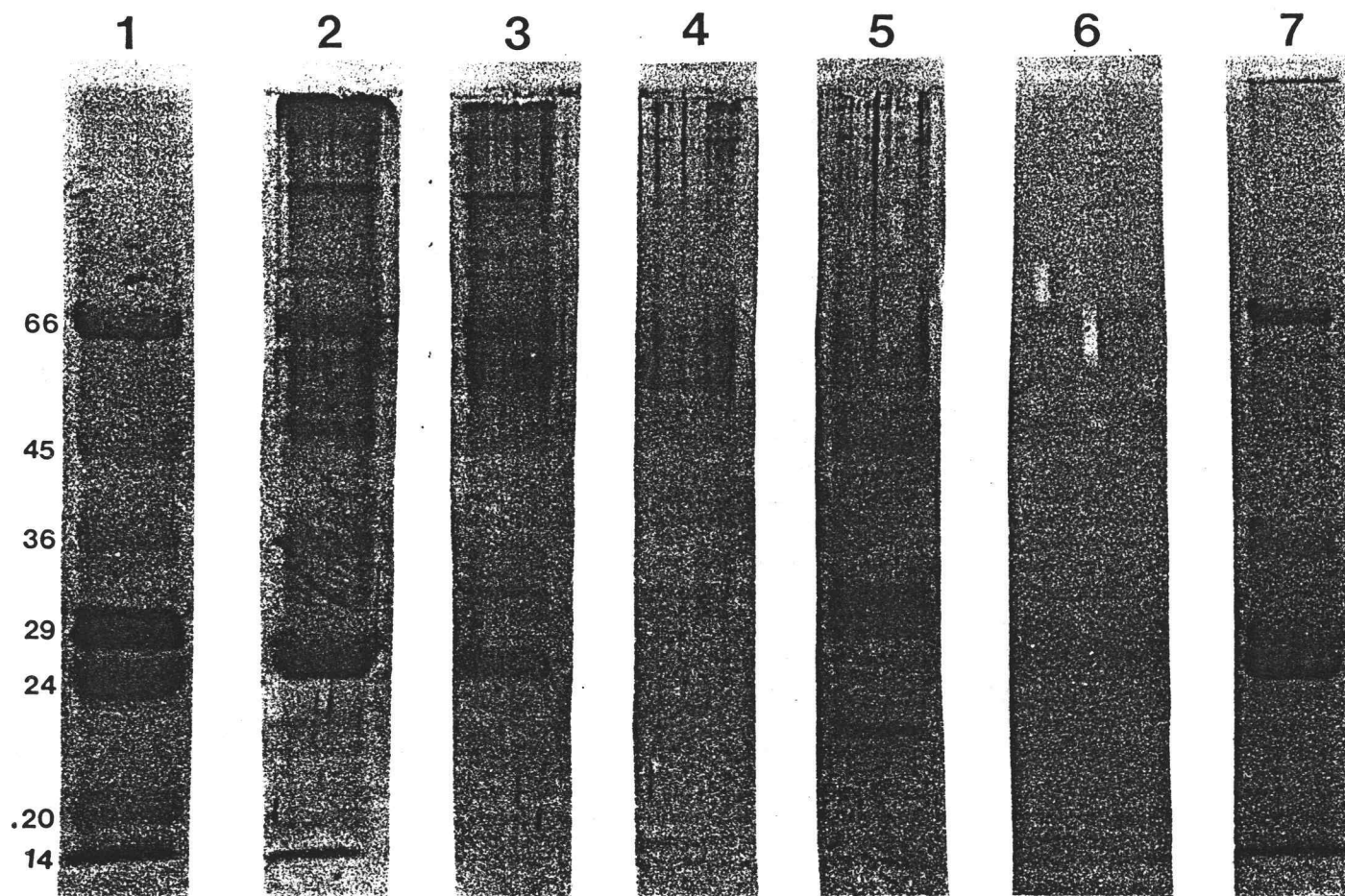


Fig. 7 Colloidal gold total protein stain of nonproteolyzed *B.t.i.* proteins: lane 1, 10 μ l of molecular weight standard; lane 2, 34 μ g of NCE; lane 3, 5 μ g of NS-1; lane 4, 5 μ g of NS-2; lane 5, 2.3 μ g of ND-1; lane 6, 1.4 μ g of ND-2; and lane 7, Vectobac 12-AS.

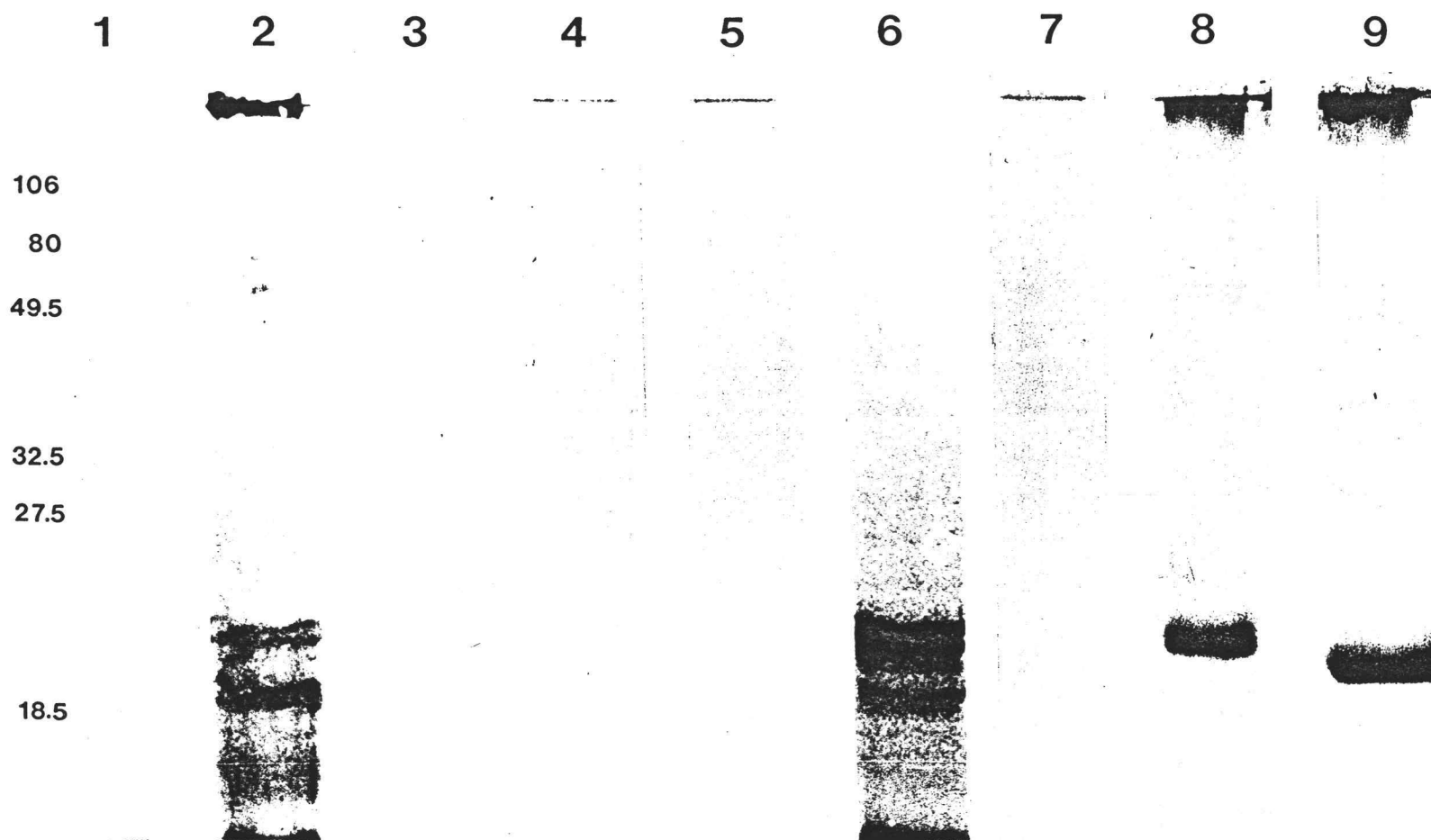


Fig. 8 Western blot of proteolyzed column purified *B.t.i.* proteins using monoclonal antibody *B.t.i.* clone 102. The proteins were as follows: lane 1, 8 μ l of molecular weight standard; lane 2, 22.5 μ g of PCE; lane 3, 5 μ g of PS-1; lane 4, 5 μ g of PS-2; lane 5, 5 μ g of PS-3; lane 6, 5 μ g of PS-4; lane 7, 5 μ g of PD-1; lane 8, 5 μ g of PD-2; and lane 9, 5 μ g of PD-3.

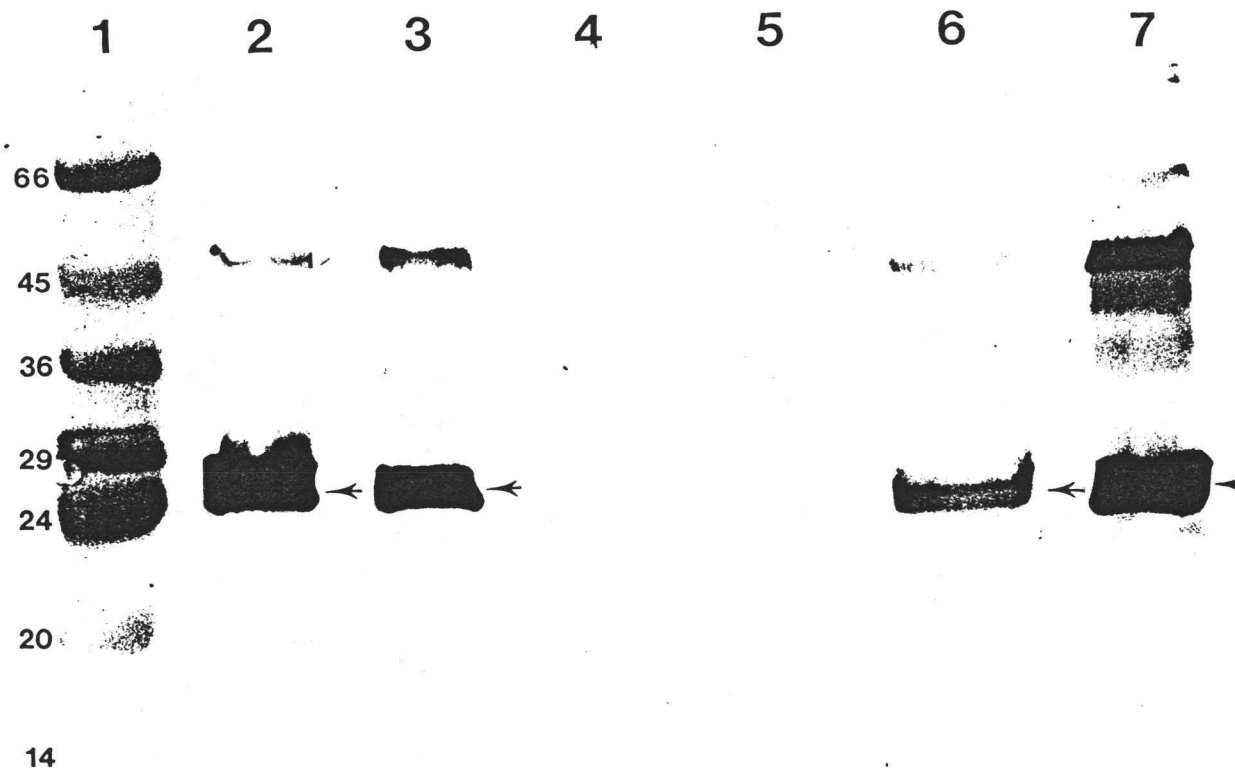


Fig. 9 Western blot of nonproteolyzed column purified proteins of *B.t.i.* using monoclonal antibody *B.t.i.* clone 102. The samples were as follows: lane 1, 10 μ l of molecular weight standard; lane 2, 34 μ g of NCE; lane 3, 5 μ g of NS-1; lane 4, 5 μ g of NS-2; lane 5, 2.3 μ g of ND-1; lane 6, 1.4 μ g of ND-2; and lane 7, Vectobac 12-AS. The arrows indicate the 28 kD protein.

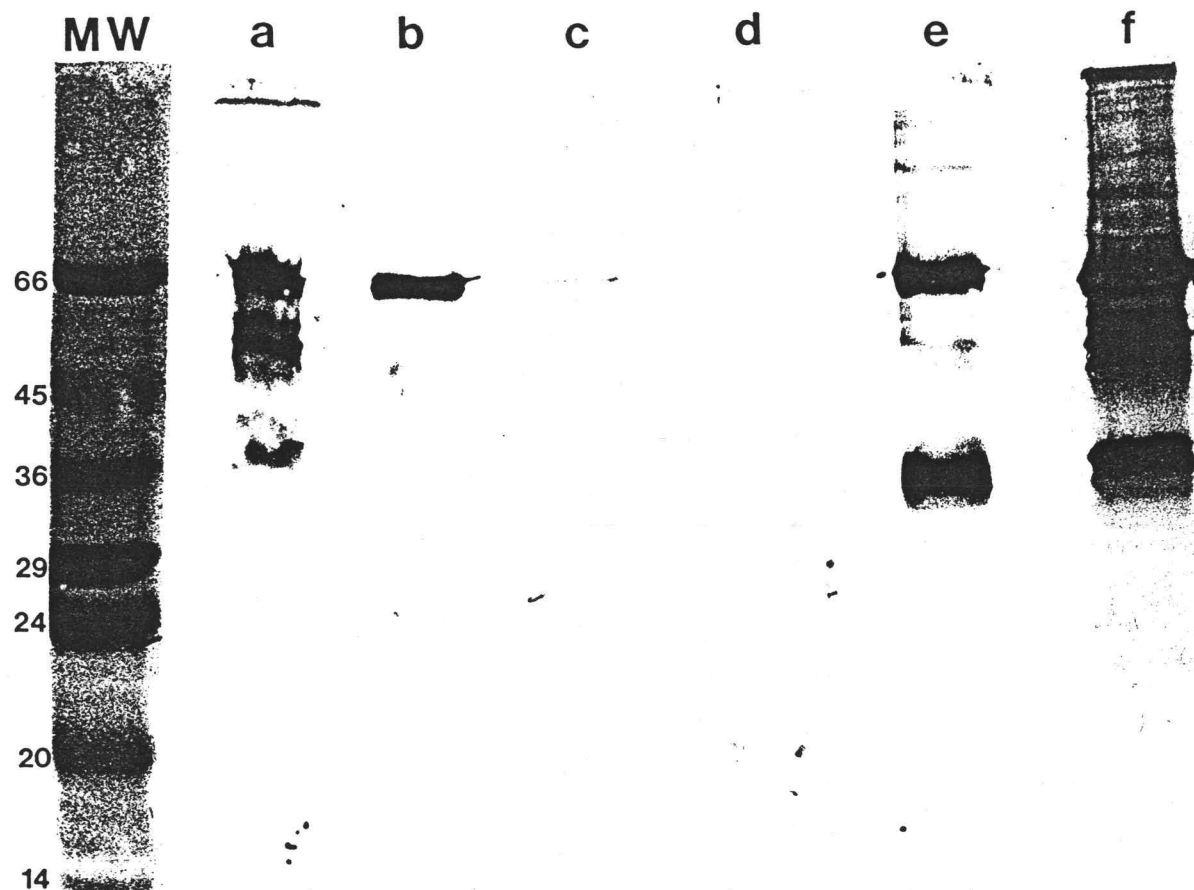


Fig. 10 Western blot of nonproteolyzed *B.t.i.* column purified proteins using monoclonal antibody *B.t.i.* 135-5. The proteins were as follows: lane a, 34 μ g of NCE; lane b, 5 μ g of NS-1; lane c, 5 μ g of NS-2; lane d, 2.3 μ g of ND-1; lane e, 1.4 μ g of ND-2; and lane f, Vectobac 12-AS. Protein standards are in lane MW.

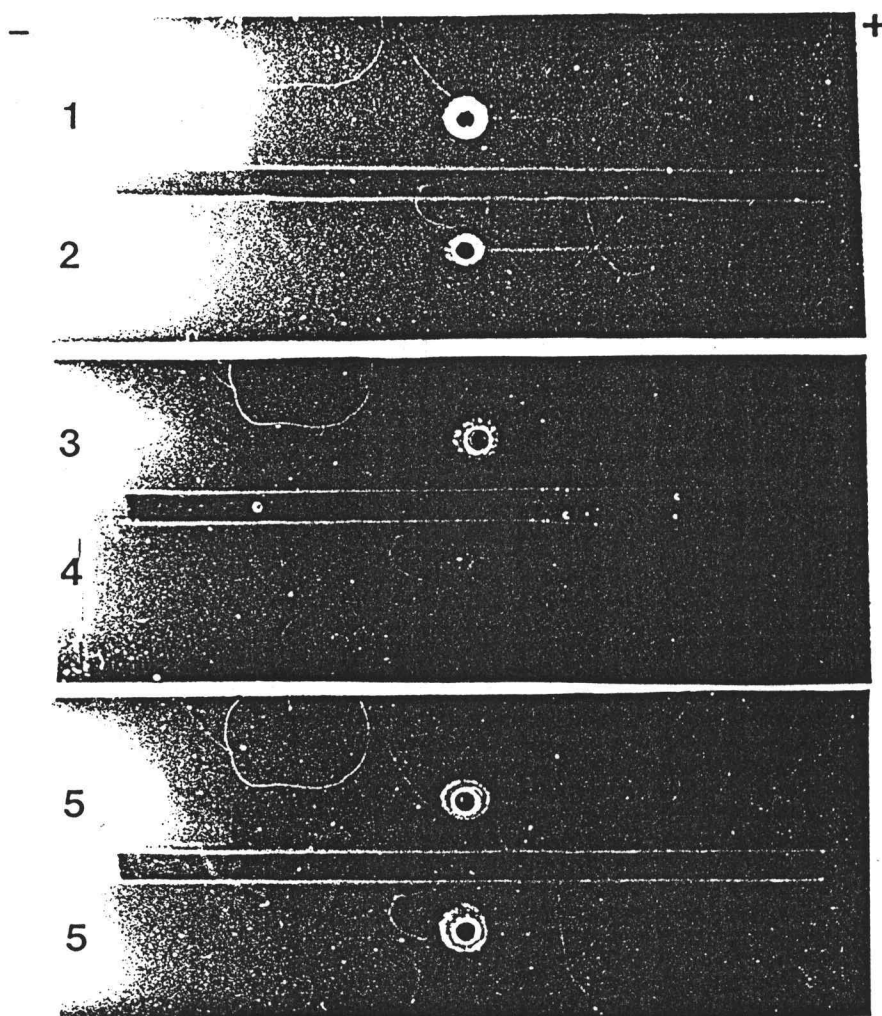


Fig. 11 Immunoelectrophoresis of proteolyzed column fractionated delta endotoxin proteins of *B.t.i.* The wells were loaded with the following samples: PCE in well 1; PS-1 in well 2; PS-2 in well 3; PS-3 in well 4; PS-4 in well 5; PD-1 in well 6; PD-2 in well 7; and PD-3 in well 8.

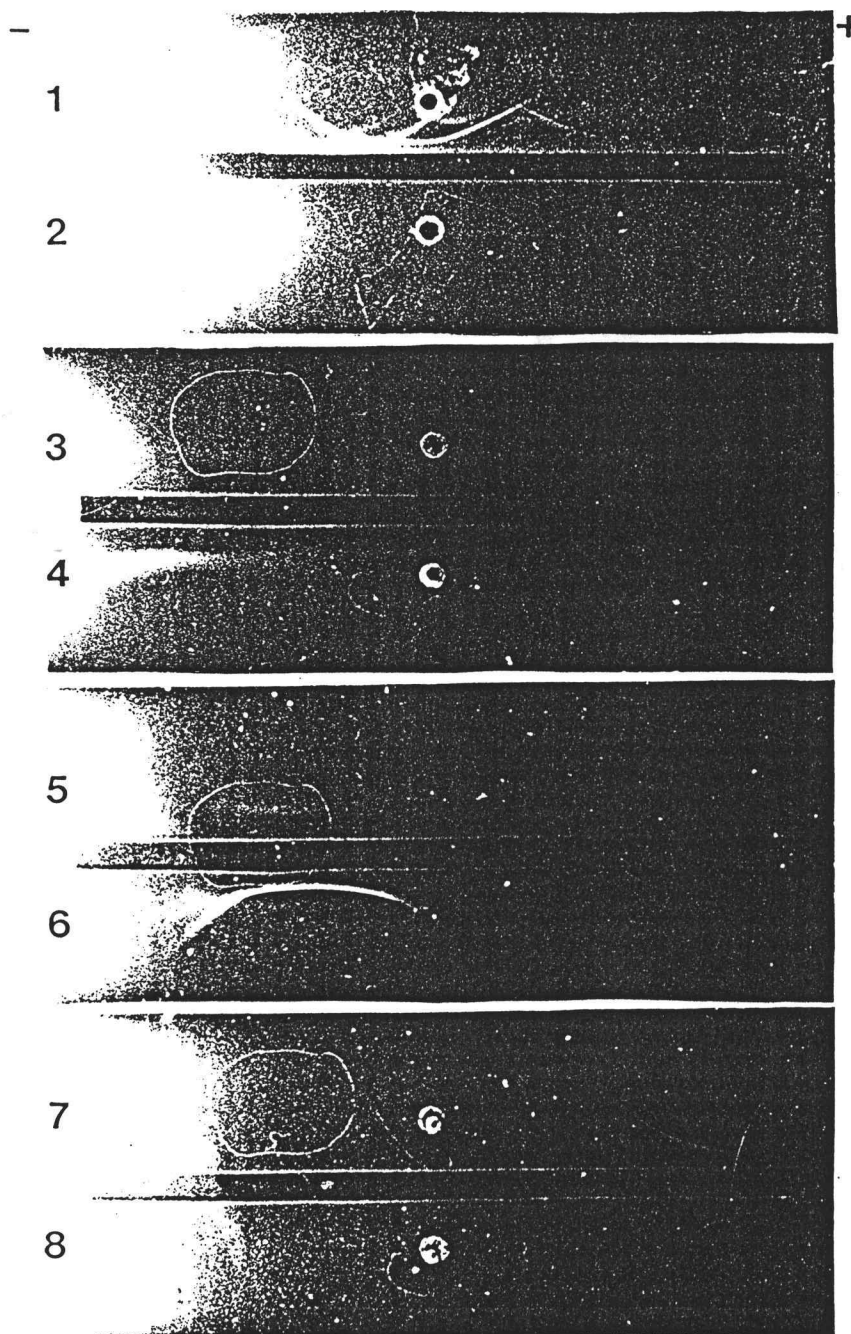


Fig. 12 Immunoelectrophoresis of nonproteolyzed column fractionated delta endotoxin proteins of *B.t.i.* The samples were loaded in the following manner: NCE in well 1; NS-1 in well 2; NS-2 in well 3; ND-1 in well 4; and ND-2 in well 5.

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Chapter 4

An Enzyme-linked Immunosorbent Assay to Detect the
Antibodies to *B.t.i.* in Pesticide-Exposed Mallard Ducks
and the *in vitro* Toxicity of the Delta Endotoxin Proteins

Summary

Two groups of mallard ducks were exposed to two different concentrations of a commercial preparation of *B.t.i.* every 14 days for 84 days while being bled every 28 days. The sera of the ducks were tested on an ELISA using the PD-1 antigen. Both groups showed an increase in antibody titres to the antigen with each successive exposure.

The column-chromatographed delta endotoxin proteins were also tested for their ability to cause hemolysis to duck erythrocytes and cytolysis to *Aedes aegypti* cells (mosquito cells). The PS-4, PD-2, and PD-3 protein fractions had the highest biological activity and caused hemolysis and cytotoxicity at concentrations of 7-8 μg of protein/ml. The NS-1 fraction needed a concentration of approximately 200 μg of protein/ml to cause hemolysis, while the ND-2 fraction caused cytotoxicity at 24 μg of protein/ml.

Introduction

Bacillus thuringiensis subsp. *israelensis* is a gram positive bacterium that produces a proteinaceous delta endotoxin when it sporulates (Tyrell et. al., 1981). The delta endotoxin kills mosquito and blackfly larva when it is ingested (Goldberg and Margalit, 1977; de Barjac and Coz, 1979; Undeen and Nagel, 1978). The delta endotoxin was also shown to be cytotoxic to mouse fibroblasts, primary pig lymphocytes, and mouse epithelial carcinoma cell types under alkaline conditions (Thomas and Ellar, 1983a). The delta endotoxin could be separated into protein species that exhibit toxic effects upon different targets. For instance, a 130 kD protein from the crystal endotoxin has been shown to kill mosquito larvae by binding receptors in the brush border of the intestinal gut (Van Rie et. al., 1989). A 65 kD protein kills mosquito and blackfly larvae (Hurley et. al., 1985; Lee et. al. 1985), while a 28 kD protein in alkaline conditions causes lysis of sheep and human erythrocytes (Held et. al., 1986; Armstrong et. al., 1985). A synergistic effect was indicated between the 28 and 65 kD proteins in targeting mosquito and blackfly larva (Wu and Chang, 1985). The 28 kD protein killed *in vitro* cell lines, while the 130 kD and 65 kD proteins showed more specific toxicity to insect cell lines (Chilcott and Ellar, 1988). These observations suggest that the delta endotoxin is potentially toxic to non-target species.

Many species of *B. thuringiensis* have been used to control insects in agricultural productions. In comparison with other chemical insecticides, the biological pesticide is considered safe and compatible with the environment (Miller et. al., 1983). The endotoxin, live *B.t.*, or combination of both have been routinely used for the past decade; in many instances, commercially produced toxins have been sprayed over large areas by the use of airplanes and other mechanical devices. Such routine use of massive application is undoubtedly causing the exposure of non-target animal species in the environment. Data is lacking to assess the amount of non-target species exposure in the wild.

In the present study an ELISA was developed to quantitatively detect antibodies in mallard ducks exposed to *B.t.i.* antigen in the commercially available pesticide Vectobac 12-AS. We hypothesized that the level of the antibody correlates with the amount of exposure to the pesticide. Column-chromatographed proteins of the delta endotoxin of *B.t.i.* were tested for use as the antigen in the ELISA. The potential toxicity to avian species from *B.t.i.* endotoxin proteins was tested by performing a hemolytic assay on erythrocytes of mallard ducks using the column purified proteins.

Materials and methods

Mallard Duck Exposure to B.t.i. A group of 25 mallard ducks (Whistling Wings, Hanover, Illinois) was randomly divided into three groups: one group of ten ducks was exposed to a 1:10 dilution of Vectobac 12-AS (Abbott Labs, Chicago, IL) in water, one group of ten ducks was exposed to a 1:100 dilution of Vectobac 12-AS (recommended application rate was 0.029-0.235 ml/m²), and one group of five ducks were kept as unexposed controls. The two groups of treated ducks were exposed by placing ten ducks in a 44 x 58 x 98 cm plastic crate, which was placed under a 66 x 92 x 117 cm wooden frame covered with sheet plastic. A 145 ml volume of the diluted Vectobac 12-AS was aerosolized using an ultrasonic nebulizer (DeVilbiss Ultrasonic Nebulizer 65) at a setting of 6. The mist of pesticide entered the plastic covered box through a hose with the end placed through a hole in the sheet plastic. The material was sonicated for one hour for the 1:100 dilution and 1.5 hours for the 1:10 dilution to allow equal volumes of pesticide to be sonicated.

The exposure scheme for the two groups of ducks was every 14 days followed by bleeding every 28 days before the exposure. The birds were bled from the jugular vein on days 0, 28, 56, 84, 113, and 207. They were exposed on days 0, 14, 28, 42, 56, 70, and 84. The sera were separated and stored at -20°C.

The amount of nebulized delta endotoxin was roughly estimated by plating the dilutions of Vectobac 12-AS on TGY agar and counting colony forming units (CFU). This gave CFU/ml which was multiplied times the amount of pesticide nebulized, giving the total number of CFU nebulized.

ELISA Plate Coating Procedure. Antigens (PD-1 fractions) were prepared as described in chapter 3, then diluted in 0.2 M carbonate-bicarbonate coating buffer (pH 9.8), and 200 μ l was dispensed in each well of a 96-well polystyrene microtitration plate (Flow/ICN). The plates were placed in a 37°C incubator and the coating buffer was allowed to evaporate to dryness.

ELISA Procedure. Coated ELISA plates were post coated with 200 μ l of 0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), pH 7.2, and incubated for one hour at 37°C. The plates were then washed four times with PBS with Tween 20 (0.005%; Sigma). The test sera (250 μ l) were then applied to the top wells of the plate after being diluted 1:10 in 0.1% BSA in the washing buffer. The serum samples were serially diluted five fold down the plate using an automatic diluter (Automatic Pipette, Model No. 25004; Micromedic Systems). The plates were incubated for one hour at 37°C, followed by the washing procedure. The alkaline phosphatase-conjugated goat secondary antibody against duck

IgG (Kirkegaard Perry Laboratories) was diluted in conjugate diluent (serum diluent buffer containing 2mM MgCl_2) and then added to the plate in 200 μ l per well. The plates were incubated at 37°C for one hour and then washed followed by application of the substrate (3.5mM p-nitrophenyl phosphate disodium; Sigma, in diethanolamine buffer; diethanolamine buffer is 9.7% diethanolamine and 2mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, pH9.8). The substrate was incubated for one hour at 37°C and the plates were read at 405 nm (Titertek Multiskan MC, Model No. 340; Flow Laboratories).

The positive control serum was taken from a bird injected subcutaneously with 10 μ g/ml of whole bacteria suspended in 50 mM NaHCO_3 - Na_2CO_3 buffer (pH 9.5) and mixed 1:2 in Complete Freund's Adjuvant (Difco). The bird was boosted with 10 g/ml of whole bacterial suspension mixed 1:2 in Incomplete Freund's Adjuvant (Difco). The negative control serum was taken from the blood of the same bird prior to inoculating with the bacterial suspension.

The antibody titre was determined from the 70% reduction of the logit transformation of the ELISA data, $\text{logit} = \log_{10}(X/100-X)$ (Hwang et. al., 1986). The 100% value used for the logit transformation was the highest absorbance reading that the ELISA reader could make, and the X value was the absorbance reading of the sample in the well.

Hemolytic Assay. The assay was similar to the assay

described by Thomas and Ellar, (1983a). The erythrocytes were collected in 0.05 units/ml heparin and washed three times with 0.12 M glucose in PBS. The washed cells were suspended in the washing buffer containing 0.05% gelatin. The column-purified proteins were diluted 1:2 and dispensed in 100 μ l/well in a round bottom microtiter plate. The erythrocyte solution was added to each well (100 μ l) and then incubated at room temperature for 3 hours. No button formation in the bottom of the wells indicated hemolysis.

Cytolytic Activity by Column Purified Proteins. Aedes aegypti (ATCC CCL125) cells were grown in Mitsuhashi and Maramorosch medium (Sigma), with 20% fetal bovine serum (FBS; Hyclone) at 28°C. The cells were maintained in 25 cm² culture flasks with 5 ml of media. The media was replaced with 2.5 ml of fresh media, and cells were detached mechanically from the flask. Two drops of the cell suspension (approximately 50 μ l) were pipetted into each well of a flat bottom 96-well plate. The plate was incubated at 28°C for one hour to allow cells to attach to the plate. The media was siphoned out of each well and 100 μ l/well of PBS was added. The PBS was immediately removed and 200 μ l of the test proteins in PBS were placed in the top well of each column on the plate. The test materials were serially diluted 1:2 down the plate and the plate was then incubated at 28°C for one hour. The test material was

removed and each well was rinsed with 100 μ l of PBS. The cells were stained with 0.25% Safranin in 10% ethanol for one minute. The cells were dried at room temperature and viewed microscopically for cytopathic effects caused by the protein fractions.

Results

Hemolytic assay. The column purified proteins were tested for their toxicity in a hemolysis assay with erythrocytes of mallard ducks (Table 2). The alkali-solubilized proteolyzed crude extract (PCE) showed hemolysis with 7.8 μg protein/ml. The PS-4 fraction, as well as PD-2 and PD-3 fractions, showed hemolysis down to 7.8 μg protein/ml. The nonproteolyzed crude extract (NCE) showed hemolysis at 250 μg while the NS-1 fraction showed hemolysis down to 200 μg protein/ml. Fraction NS-2 did not show hemolysis at the highest concentration (800 μg protein/ml) placed with the erythrocytes. The ND-1 fraction showed hemolysis at a concentration of 150 μg /ml, while the ND-2 fraction did not show hemolysis at its highest concentration of 24 μg /ml.

Cytolytic Assay. The PS-3 and PS-4 protein fractions of caused cell death to the *Aedes aegypti* cells (Table 2). The lowest concentration of protein fraction PS-3 that caused cytolytic activity was 146 μg protein/ml, while the PS-4 caused cytolysis with only 11 μg protein/ml. The DEAE Bio-Gel A purified peaks from PS-4 showed cytolysis for all three peaks. The cytolytic activity was observed down to 216 μg protein/ml for PD-1, at least 8 μg protein/ml for PD-2, and at least 7 μg protein/ml for PD-3.

The nonproteolyzed soft pellet material showed

cytolytic activity with 2282 μg protein/ml. The DEAE Bio-Gel A peaks from the NS-1 material showed cytolytic activity at 150 μg protein/ml for ND-1 and down to 24 μg protein/ml for ND-2.

ELISA. Preliminary results using the PD-3 protein fraction as antigen did not show antibody titre values in pesticide-exposed birds above the values for unexposed birds. This led to the use of the PD-1 protein fraction for the antigen on the ELISA. The plates were coated with 1 μg protein/ml (200 μl /well). The 70% logit reduction of the ELISA results showed an increase in the antibody titres in the ducks exposed to the bacterial pesticide (Fig. 13). The unexposed ducks showed a relatively constant antibody titre to PD-1 antigen (not shown). The antibody titres of the group of ducks exposed to the 1:10 pesticide dilution showed a larger increase and higher values than titres in sera of the 1:100 exposure group. The antibody titres of both exposed groups were much lower than that of the positive control. The unexposed antibody titres (day 0) were similar to the titre of the negative control.

Western Blot of Exposed Duck Sera. A Western blot was run to verify if exposed ducks produced antibodies to the whole 28 kD protein. The sera samples from a duck exposed to 1:10 dilutions of pesticide began to develop antibodies

to the NS-1 and ND-2 fractions after two exposures (Fig. 14). The antibodies became more reactive to the 28 kD protein after four exposures and increased in their reactivity the more the bird was exposed. The antibody reactivity decreased after the exposures were stopped. Sera from the positive (hyperimmunized) and negative control ducks showed high antibody reactivity and some background band formation, respectively.

Table 2 Biological activity of column purified alkali solubilized delta endotoxin proteins of *B.t.i.*

Fraction	Lowest conc. (μ g/ml protein)	
	hemolysis ^a	cytotoxicity ^b
PCE	<8	N.D.
PS-1	500	>400
PS-2	1000	>530
PS-3	580	146
PS-4	<8	<11
PD-1	>1000	216
PD-2	<8	8
PD-3	<8	7
NCE	250	2282
NS-1	200	N.D.
NS-2	>800	N.D.
ND-1	150	150
ND-2	>24	24

^aThe lowest concentration of *B.t.i.* proteins that caused hemolysis of mallard duck erythrocytes.

^bThe lowest concentration of *B.t.i.* proteins that caused cytotoxicity to *Aedes aegypti* cells.

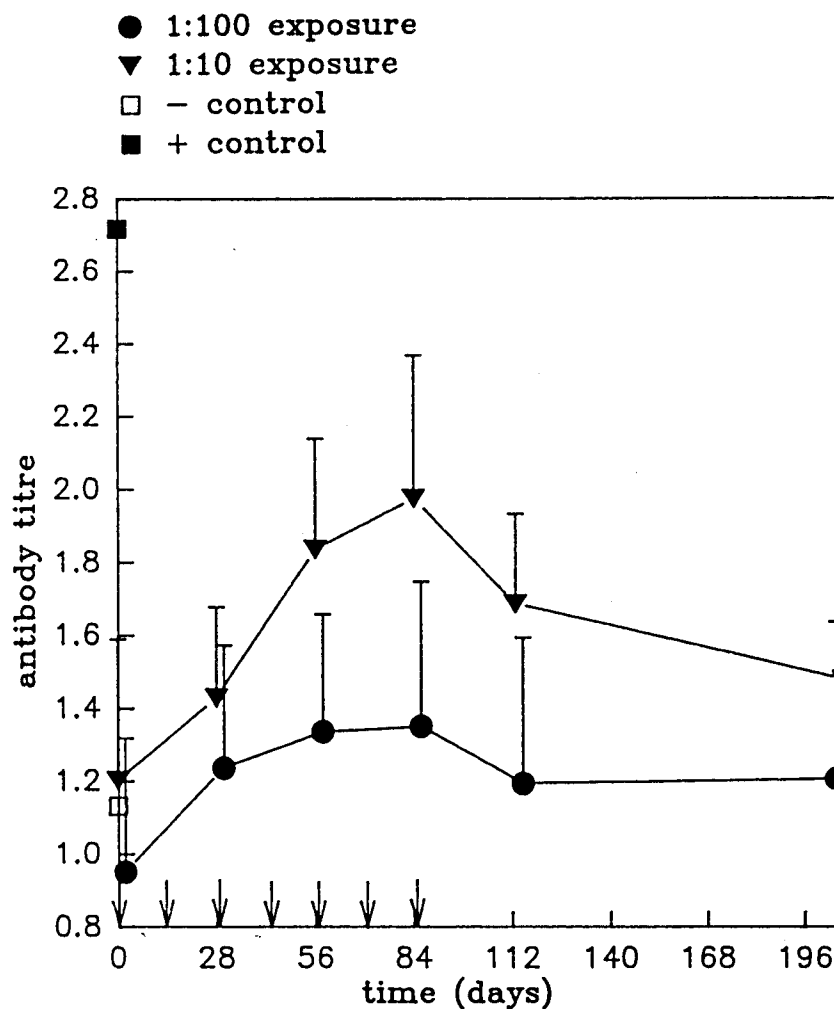


Fig. 13 The mean antibody titres of two groups of ten mallard ducks exposed to *B.t.i.* pesticide (Vectobac 12-AS). The arrows indicate the days the birds were exposed. Antibody titres were derived from the 70% logit conversion of the ELISA data. The error bars are the standard deviation of the mean values of the calculated antibody titres. The negative control is the sera of an unexposed mallard duck. The positive control is the sera from the same duck after hyperimmunization.

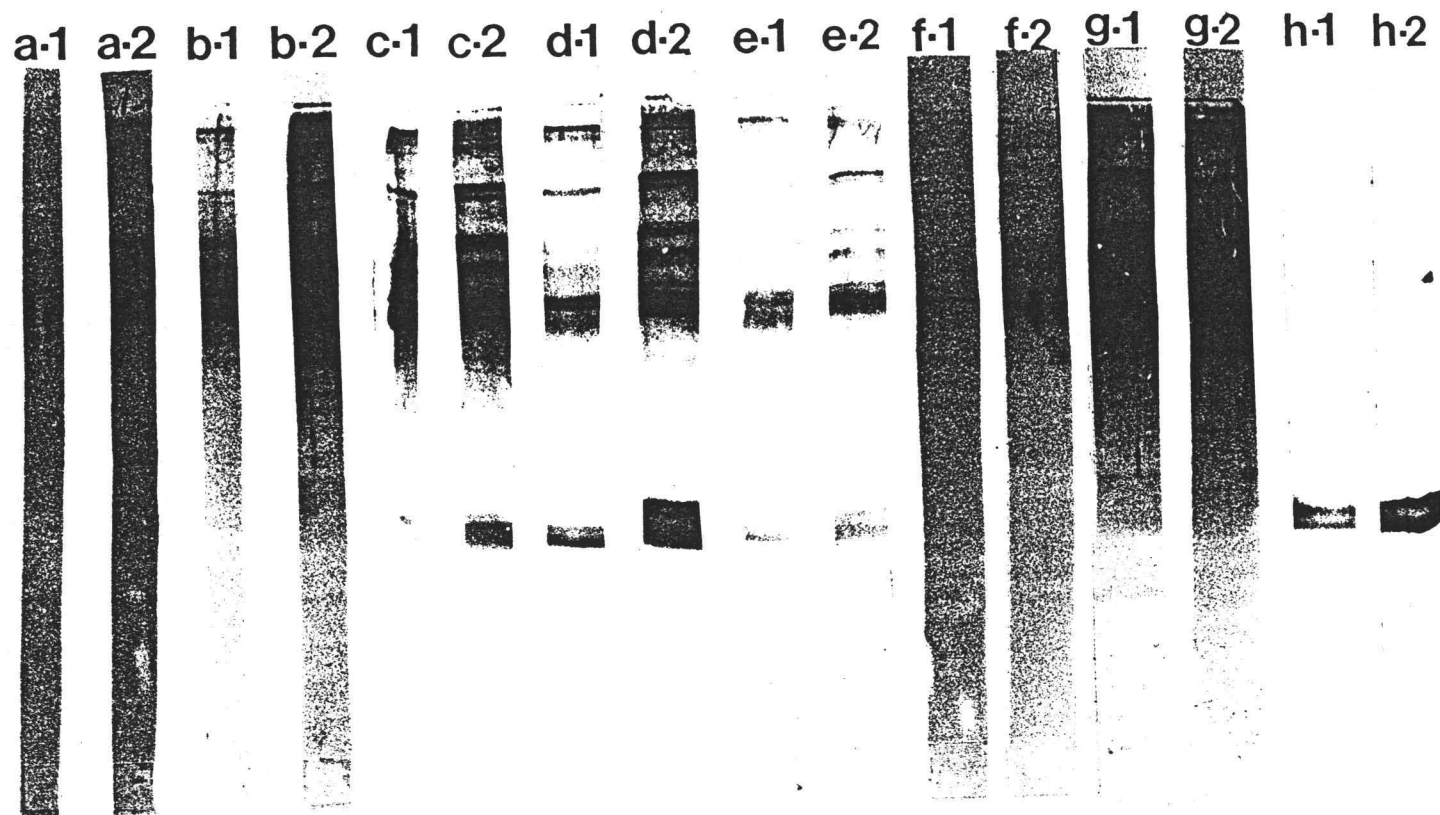


Fig. 14 Western blot of duck sera from a duck exposed to a 1:10 dilution of Vectobac 12-AS over time on 1.4 μ g of ND-2 fraction (all lanes labelled 1) and 5 μ g of NS-1 fraction (all lanes labelled 2). The test sera was as follows: lane a, day 0; lane b, day 28; lane c, day 56; lane d, day 84; lane e, day 113; lane f, unexposed duck sera (negative control); lane g, hyperimmunized duck sera (positive control); and lane h, B.t.i. clone 102 monoclonal antibody.

Discussion

The ducks produced antibodies against *B.t.i.* after the repeated aerosol exposure with the pesticide. Although Western blot analysis showed that the exposure resulted in antibody production against 28 kD protein (Fig. 14), preliminary investigation demonstrated that neither the PD-2 nor the PD-3 fraction detected temporal production of antibodies in the ducks (data not shown). On the other hand, PD-1 fraction, which does not contain the toxin component, efficiently detected antibodies in ELISA (Fig. 13). A dose and time dependent antibody response was observed after the exposure, and the low level of antibody titre persisted longer than 6 months after the initial exposure. The results indicate that this ELISA is a valid method of detecting the antibody against *B.t.i.* It was not determined whether it requires the toxin only, whole cells of *B.t.i.*, or both to induce antibody against the PD-1 antigen.

The method of exposure of the ducks to the *B.t.i.* pesticide was designed to provide a plausible approach to how the birds would be exposed in the wild, along with moderately realistic amounts of exposure to pesticide. The two groups of birds were exposed every 14 days because the pesticide would probably be applied in this manner. Field trials show that *B.t.i.* has a 10 day period of time where it is effective in killing mosquito larva (Shannon et. al.,

1989; Perich et. al., 1990).

The amount of pesticide to which the ducks were exposed was 1.35×10^{-3} ml/cm² for the 1:10 exposed group, and 1.35×10^{-4} ml/cm² for the 1:100 exposed group. They are approximately 100- and 10-fold greater concentrations than that suggested by the application directions. These values are for area of the base of the box in which the ducks were placed. The volume of the box was 7.10×10^5 ml. Within the volume of pesticide that was sonic nebulized there were 4.35×10^{11} CFU for the 1:10 group, and 4.35×10^{10} CFU for the 1:100 group. According to the literature, the resting respiratory rate of pekin ducks is 15.6 breaths/minute with an expiration volume of 910 ml/minute (Powell and Scheid, 1989). Therefore, in an hour, one duck should have inspired 54.6 liters of air and pesticide mixture. According to Yates et. al. (1981) droplets from the sonic nebulizer average 1.8 μ m in diameter when the nebulizer is on a setting of six. With this particle size sufficient deposition of the bacteria should have occurred in the birds (Driver et. al., 1990; Stearns et. al., 1987).

The ducks did not exhibit any clinical problems from exposure to the pesticide. One duck that was found dead did not contain lesions in its respiratory tract, even though a swab of the lung tissue did produce colonies of Gram positive bacteria that contained spores and inclusion bodies. The death of this bird was not attributed to

exposure by the pesticide. These results are reminiscent of a study conducted with flathead minnows where the fish did not exhibit adverse effects when exposed to commercial preparations of *B.t.i.* (Snarski, 1990).

The *in vitro* toxicity of the proteins purified from the delta endotoxin of *B.t.i.* varied in the concentration necessary to cause death to *Aedes aegypti* cells and lysis of duck erythrocytes. The toxicity observed in the present study with the 28 kD protein and its proteolytic products was comparable to the results seen by others (Armstrong et. al., 1985; Thomas and Ellar, 1983a). The 22 and 23 kD proteins from the proteolyzed cell material were more toxic to the mosquito cells and duck erythrocytes than was the entire 28 kD protein. The delta endotoxin exhibits its toxicity by incorporating into the lipid bilayer of cells forming a pore, and causing an influx of solutes into the cell causing lysis (Thomas and Ellar, 1983b; Ellar et. al., 1985). The 26 kD protein was demonstrated to be inserted into liposomes of certain lipid species and may be proteolytically cleaved into a 12 kD protein which may be optimal for aggregation of proteins into pores. The increased toxicity of the 22 and 23 kD proteins in comparison to the 28 kD protein shown in the present study could be due to increased ability of the toxins to form pores in the plasma membrane of cells. The smaller proteins (22 and 23 kD) may have lost a portion of the protein that

hinders their integration into the membrane of cells. The smaller proteins also may allow easier cleavage into the 12 kD protein to form the aggregates of the pore. The smaller proteins also may be more easily cleaved into the 12 kD protein that forms the aggregates of the pore. Another explanation is that the 28 kD protein may still be bound to proteins of the crystalline delta endotoxin, hindering the integration of the protein into the membrane of the cell. The 28 kD protein in the NS-2 fraction is not as pure as the 22 and 23 kD proteins, and the other proteins within the fraction may be part of the crystal delta endotoxin to which the 28 kD protein remains attached. This is indicated in the gel filtration (Fig. 3), total protein stains (Fig. 5) and immunoelectrophoresis (Fig. 10) of the untreated fractionated proteins.

The current unrestricted use of commercial preparations of *B.t.i.* as a pesticide will undoubtedly result in the exposure of non-target species in the environment. The development of the ELISA will provide a tool to examine if exposure to mallard ducks is occurring in the wild. However, further development of ELISAs to detect antibodies to other strains of *Bacillus thuringiensis* would help determine the extent of non-target species exposure to these biological pesticides. Antibodies in different species could also be detected by variation in the ELISA (i.e. use of a different secondary antibody). This would provide a

whole library of usable ELISAs that could be used to monitor non-target species exposure to *B.t.* and help in regulating the application of the pesticides.

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