UNIVERSITY OF OTTAWA

ACTIVATION OF *BACILLUS THURINGIENSIS* δ-ENDOTOXIN

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ABSTRACT

Bacillus thuringiensis (Bt) produces a proteinaceous parasporal crystalline inclusion which is pathogenic to insect larvae. The insecticidal activities of crystals from different subspecies of Bt are highly specific for larvae within a particular insect order and in some cases for specific larvae within a family. Lepidopteran-active Bt crystals are composed of 130 kDa δ-endotoxin (protoxin molecules, covalently linked by disulfide bridges). On ingestion by susceptible larvae, the δ-endotoxin is converted to a 58-68 kDa toxin by the action of the high pH gut juice (pH~10-11) containing proteolytic enzymes and other proteins which associate with the toxin. One of the objectives of the research was to determine the role of enzymes/proteins in the spruce budworm (*Choristoneura fumiferana*) gut in determining the activity/specificity of a toxin.

The gut juice of the spruce budworm larvae was found to contain a single trypsin-like serine protease (CFT-1) which was responsible for the activation of the δ-endotoxin. CFT-1 was purified from gut juice by a combination of size exclusion and ion exchange chromatography. Some characteristics, such as molecular mass, N-terminal and active site sequences, charge and substrate specificity were similar to mammalian pancreatic trypsins. However, differences were observed in the burst kinetics and pH dependence of the catalysis where the apparent ionization constant of the active site histidine was over 1 pH unit higher than in the mammalian serine proteases. Another significant difference was that CFT-1 was stable for long periods of time at pH values as high as 11 whereas mammalian trypsins rapidly autolyse at pH values greater than 8. Inhibition of CFT-1 in gut juice prevented activation of the δ-endotoxin. However, gut juice activation produced a slightly smaller toxin than the CFT-1 alone.

It was observed during the digestion of δ-endotoxins with gut juices that toxin was precipitated. This precipitate was shown to be caused by a protein in the gut juice. This protein has a molecular mass of 75 kDa and anionic character based on ion-exchange chromatography. A weak elastase-like activity was also shown to be associated with the precipitating protein. When the aggregate of toxin and gut juice protein was dissociated, it was observed that smaller toxin fragments were generated indicating that the toxin had been further proteolysed. The precipitation and degradation of toxin by gut juice may explain the observation that, when gut juice is used to activate the δ-endotoxin, the *in vitro* recovery of toxin is much lower than expected. Therefore precipitation may play a role in determining the toxicity towards various insect larvae.

Evidence had been obtained that DNA was associated with the δ-endotoxin and toxin *in vitro* after the usual purification procedures and this DNA appeared to play an important role in the activation of the δ-endotoxin. A study was undertaken to determine whether DNA found in purified Bt crystal preparations was also associated with crystals *in vivo*. The results obtained from photomicrography of Bt during its stages of development showed that DNA condenses in the region where the parasporal crystal forms. These results provided further evidence that the DNA found to be associated with purified crystals *in vitro* was not artifactual.

DEDICATION

To Dad

It was my father who taught me that "getting there is not half the fun.....it's all the fun!" Most of our journeys were camping or fishing trips and his philosophy indeed described those trips. Many years have now passed since I started this journey back to academia. Thanks to my Dad , I have not lost site of the purpose, and that was, to enjoy the trip.

Dad never missed an opportunity during those trips to ask about my studies, but I think his questions and my answers told us more about ourselves than it did about the work. Nevertheless, I was amazed at how much detail and perspective he recalled to those discussions. Our fishing trips are over now, but to this day I still feel I have to respond to him..... It's part of the journey..... the story..... the fun!

To Mom

I'm sure my mother is responsible for my life long interest in things biological. She tolerated the bugs, snakes, lizards, turtles and all kinds of furry and feathered creatures. Her advice on how to care for the young, the sick or the injured pets would suggest acceptance rather than tolerance. Come to think of it, she really enjoyed (and loved) some of them! At least that's the impression she gave me.....back then. Mom always had a way of encouraging those interests without totally letting her guard down.

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If one can reserve a special place for their supervisor this is it. Dr. Harvey Kaplan has been a mentor, a colleague and a friend. His mixture of science and humor is infectious and one cannot help but learn and enjoy the experience at the same time.

Many thanks to Dr. K. van Frankenhuyzen, Dr. L. Gringorten and Dr. A. Pang for their interest and suggestions in the development of this study.

I cannot adequately express the appreciation and thanks to my wife Shelagh and my sons Ryan and Teigan. They have encouraged me, supported me and suffered my absence. Most importantly they have provided the anchor of a family.

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LIST OF ABBREVIATIONS

AEBSF ..... 4-(2-aminoethyl)benzenesulfonyl fluoride

ATAPNA ..... N-acetyl-ala-ala-ala p-nitroaniline

BAEE ..... N-benzoyl-L-arginine ethyl ester

BAPNA ..... Nα-benzoyl-DL- arginine p-nitroanilide

Bt ..... *Bacillus thuringiensis*

CAPS ..... 3-(cyclohexylamino)-propanesulfonic acid

CF-1 ..... *Choristoneura fumiferana* cultured cells

CFT-1 ..... *Choristoneura fumiferana* trypsin

cry ..... crystal gene

Cry ..... crystal protein

Da ..... Daltons

DEAE ..... diethylaminoethyl

DFP ..... diisopropyl fluorophosphate

DIP ..... diisopropyl phosphate

DMSO ..... dimethylsulphoxide

DNA ..... deoxyribonucleic acid

DNase ..... deoxyribonuclease

Dns ..... dansyl

DTE ..... dithioerythritol

DTT ..... dithiothreitol

EDTA ..... ethylenediamine tetraacetate

GCG ..... Genetic Computer Group

HPLC ..... high pressure liquid chromatography

HVPE ..... high voltage paper electrophoresis

IEF ..... isoelectric focusing

K_m ..... Michaelis-Menten constant

LD_{50} ..... lethal dose for 50% mortality

LPNA ..... leucine p-nitroanilide

MWCO ..... molecular weight cutoff

NPGB ..... p-nitrophenyl p'-guanidino-benzoate

NRC ..... National Research Council

p-NPP ..... p-nitrophenyl phosphate

PAGE ..... polyacrylamide gel electrophoresis

PBS ..... phosphate buffered saline

pK_a ..... ionization constant

PTH ..... phenylthiohydantion

PVDF ..... polyvinylidene difluoride

SDS ..... sodium dodecyl sulfate

SP ..... sulfopropyl

T1 ..... toxin eluting at low salt concentration

T2 ..... toxin eluting at high salt concentration

TEA ..... Tris-EDTA-acetate

TLCK ..... Nα-p-tosyl-L-lysyl chloromethyl ketone

TPCK ..... Nα-p-tosyl-L-phenylalanyl chloromethyl ketone

TPP ..... toxin-precipitating protein

Tris ..... tris[hydroxymethyl]aminomethane

V_{max} ..... maximum (catalytic) velocity

CHAPTER 1

REVIEW OF *BACILLUS THURINGIENSIS* AND INSECT PATHOLOGY

On Silkworm Sericulture Maladies,

Come learn what healing helps should be prepar'd
When dire diseases threat the sick'ning herd:
Like us attack'd, their tender bodies know
Mortal mischance, and feel their lot of woe;
Pale sickness shakes alike their tiny frames;
Whether the tainted air's corrupting steams
Or noxious food the latent poison hold,
Whate'er the cause, infection thins the fold;
Fate triumphs, bodies stain'd with putrid gore
Deform the shelves and fun'rals strow the floor;
No flatt'ring prospect heaps the golden thread,
But ev'ry hope lies mingled with the dead.

Marcus Hieronymus Vida (1527)
in the poem "De Bombicum"

INTRODUCTION

A growing public perception, that the use of chemicals to control insects can be harmful to the environment, has accelerated the research for biological control alternatives. These alternatives range from the use of, pheromones for trapping or disruption of mating behavior, and insect growth regulators that interfere with larval development, to parasitoids, fungi, viruses and bacteria which debilitate or cause death in the infected insect. One of the more successful biological control organisms is a naturally occurring bacterial pathogen, *Bacillus thuringiensis* . The entomopathogenic toxins produced by *B. thuringiensis* (Bt), have gained worldwide acceptance as biological controls for certain insects. These toxins show a high level of specificity where only certain orders of insects are susceptible to specific toxins (Höfte and Whiteley, 1989). Although several toxins may be associated with a particular isolate of Bt some isolates only produce one toxin. These single toxin isolates have been shown to have a narrow range of specificity for certain species of lepidopteran larvae (van Frankenhuyzen et al. 1991).

Bt has been used in agriculture and forestry for decades and has more recently been used for vector control against mosquitoes and black flies (Becker and Margalit, 1993) and for household use. The expansion of world markets has seen a parallel growth in the number of companies both producing and researching this bacterium (van Frankenhuyzen, 1993). New products have sought to increase the efficacy, and depending on the strategy for use, increase or decrease the range of target species. The increases in efficacy include improvements in formulation for better spray atomization or increased persistence by, the addition of compounds, or encapsulation (van Frankenhuyzen, 1993). Changing the range of species targeted by the product, by changing the type of toxin/toxins in the formulation, has seen considerable interest and has posed many new questions regarding the mechanism and development of resistance (Marrone and MacIntosh, 1993).

The recent advances in genetic engineering have played a large role in the development of new products and delivery systems for Bt. Initially the molecular geneticists provided recombinant *Escherichia coli* which expressed a spore-free single toxin. Now the focus of much research is centered on transgenic plants which express these toxins in the plant tissues eaten by insects (Meeusen and Warren, 1989). The engineering of Bt toxin into plants has further complicated the question of resistance.

Although much progress has been made in the almost 100 years since Bt was first identified, we find that many aspects concerning this pathogen have not been elucidated. We know that the toxin must be solubilized from an inactive crystalline inclusion then enzymatically converted from a protoxin to the toxin (Tojo and Aizawa 1983). This solubilization and activation can be carried out *in vitro* using high alkaline buffers and commercially available mammalian proteases. It would seem that the normally high gut pH of many lepidopteran larva and the ever present proteases in their digestive systems would readily activate the toxin. Nevertheless some investigators reported that preactivating the toxin before administering to larva caused some larva to become susceptible to the toxin where feeding the crystalline inclusion gave little or no response (Jaquet et al., 1987). It was further shown that larval gut enzymes from one order of insect activated the toxin towards that order, whereas a second order's gut enzymes produced a toxin with different specificities (Haider et al., 1986). These observations have suggested that the larval gut enzymes could confer different specificity when the toxin was activated *in vivo*.

HISTORICAL REVIEW

Research on Bt has spanned almost a century and was carried out on all but the polar regions. To date the research and use of Bt continues to see a worldwide distribution. No doubt the student of Bt research from Asia or Australia would recount the history differently depending on

the available literature. This is an important caveat since much of the early literature may have remained obscure. In searching for some of this early literature one encounters difficulties in translations and interpretations and it would be inappropriate to make the same assumptions or draw the same conclusions today as one may have 90 years ago. Nevertheless, attention will be given to some research that has proved prophetic or showed unusual insight.

Early History of Insect Pathology

Much of the early history of insect pathology, recounted here, is based on a fascinating review by Steinhaus (1956). Readers are directed to this review for individual references.

Mention of insect maladies can be found in literature from as early as the 4th century BC when Aristotle (384-322 BC.) wrote of the destruction of honeycombs brought on by the wax moth. The naturalist Pliny (AD. 23-79) also refers to "the pestilence" which afflicted bees. The infections and maladies of the silkworm were also of great concern, first to the Chinese and Japanese and later to the Europeans, after monks pilfered silkworm eggs (in their hollowed-out pilgrim staffs!) from the Orient and introduced them to the West about AD. 555. Much of our understanding of the pathology of insects is a direct result of these early observations on the honey bee and the silkworm which were the basis for two major economies. The link between sericulture economies and the study of insect pathology was strengthened by Agostino Bassi (1773-1856) when he showed that a fungus *Beauveria bassiana* (Balsamo) was responsible for the "muscardine" contagium of silkworm. By many, Bassi is considered the founder of the doctrine of pathogenic microbes.

Although much of Louis Pasteur's (1822-1895) early work focused on problems of the wine industry, he reluctantly investigated the plagues that threatened the silk industry near Alès, France (silk production had fallen from 26 million kilograms in 1853 to 4 million kilograms in 1865).

Pasteur correctly diagnosed that the larvae were suffering from two different diseases. One disease caused the larvae to develop black spots or corpuscles and the second affliction left the larvae flaccid , black and decayed. However, it was Naegeli in 1857, who identified and named one of the offending organisms as *Nosema bombycis* (a microsporidian). Pasteur observed the transovarial nature of these infections and further showed that by selecting only disease free eggs the "pébrine" condition could be halted. The first of the two diseases had been successfully thwarted and the second which caused a "flacherie" was quickly identified as a bacterial infection. Actually two bacteria were initially isolated, *Streptococcus bombycis* and *Bacillus bombycis* aucct. The streptococcus was later shown to cause a different disease called "gattine", and the bacillus is now associated with the "true flacherie".

Early History of Bacillus thuringiensis

Although in no way complete, events in the preceding historical account helped set the stage for the scientific approach to the study of insect pathology. Research continued to focus on the economically important insects. The "flacherie" disease of the silkworm had not been completely described and many investigators thought a virus may also be involved. It was the continued interest in diseases of the silkworm that likely resulted in the first discovery of the bacillus organism that later became to be known as *Bacillus thuringiensis*. In 1901 and 1902 Ishiwata (see Steinhaus, 1961) reported the isolation and description of a spore forming bacillus that caused the "sotto" (sudden collapse) of infected silkworm larvae. He named the organism "sotto bacillus". A decade later Berliner isolated a bacterium which caused similar symptoms in *Anagasta kühniella* (flour moth) and in 1915 named the organism *Bacillus thuringiensis* after the province Thuringia in Germany where it was found.

Much controversy has centered around the proper naming of the bacterium. Heimpel and

Angus (1958), in proposing new nomenclature for several Bt species, argued that Aoki and Chigasaki had used the correct combination in naming "*B. sotto*" but that their article was not published until July of 1915 whereas the Berliner paper was published in April, 3 months earlier. Although translations of the Japanese phonetic syllabary showed that as early as 1906 Nomura, to whom Ishiwata refers, had used the phrase "....bacillus sotto discovered by Ishiwata." he had not used the proper Latin scientific name *Bacillus sotto*. According to Steinhaus's (1961) account of this controversy, in 1908 (preceding the Berliner report by 7 years) Iwabuchi may have unwittingly used the correct Latin binomial when referring to a Japanese translation of the Nomura paper which was, incidentally, originally published in Italian! Steinhaus finally suggested the matter be referred to the Judicial Commission of the International Committee on Bacterial Nomenclature. To date the name of the type species, *Bacillus thuringiensis* var. *thuringiensis* has prevailed. The simple naming of this organism holds little significance now, but this author feels that the controversy may have helped bring attention to much of the early Japanese and Chinese literature.

Ishiwata's 1902 report suggested a toxin may be associated with the bacterium. The idea was further investigated by Aoki and Chigasaki (1915a, b) and their conclusions were the same. In the meantime Berliner had identified the bacterium which infected *A. (Ephestia) kühniella* (Heimpel and Angus 1960) and his description mentioned a peculiar inclusion which he called a "Restkörper" (Andrews et al., 1987). Interestingly Berliner's original isolate was lost and Mattes (1927) reisolated the organism from *A. kühniella* (Heimpel and Angus 1960). Mattes (1927) also reported an inclusion which he observed after staining with Giemsa stain. His detailed drawings clearly showed this inclusion and based on the staining he suggested this may represent a secondary spore formation. Little significance was attributed to this inclusion for the next 25 years.

The "inclusion", "restkörper", or "parasporal body", as it was variously called, was found only in sporulating cultures of Bt.. Hannay (1953) pointed out that the inclusion was diamond-shaped and coined the name "crystalline inclusion". Hannay also reported the

solubilization of this structure with dilute alkali. To Hannay, the crystalline inclusion was only associated with bacteria pathogenic to insects and speculated that it may be a virus or phage or "in some way connected with the formation of a toxic substance". A year later Angus (1954) showed that using alkali (0.2M carbonate, pH 10.3) one could solubilize a toxic substance from old cultures of Bt. The toxic component was heat labile and the soluble sterile filtrate remained toxic to silkworm larvae. He also noted that the alkali treatment dissolved the crystals and proposed that the crystalline inclusions were responsible for the paralysis of the larvae. Angus was likely not aware that a similar study was reported by Mitani and Watari (1916) almost 40 years earlier! These authors had solubilized and filtered the toxic component from the same strain of bacillus (*B. sotto*), using dilute (1%) "natrium carbonicum". Angus' rediscovery of the soluble nature of the toxin marked the renewed biochemical interest in these entomopathogenic bacteria. Hannay and Fitz-James (1955) followed up on the initial report of the crystalline inclusion (Hannay, 1953) by providing remarkable electron micrographs of the crystalline structure. In that report they also provided the first evidence that the crystals were composed almost entirely of protein.

During the early 1900's several new entomogenous bacilli were identified including *B. entomocidus, B. cereus alesti, B. finitimus* and *B. entomocidus* var. *subtoxicus* (Heimpel and Angus, 1958). The classification of these new bacilli was often based on their pathogenicity more than anything else and this has proven to cause considerable discord in the scientific community as will be discussed later.

The Practice of Biological Control

Until the early 1920's the focus of insect diseases had been almost entirely on the effect of pathogens with regards to beneficial insects such as the honey bee and silkworm. Many of the early investigators however, recognized the potential of pathogenic microorganisms to combat

pests or harmful insects. Much of our knowledge of these early studies comes from field trials in France, Hungary, and Russia. The target insect was the corn borer and an international program to control the ravages of this pest helped promote the biological alternatives and the use of Bt. In a series of reports Husz (1928-1931) detailed field experiments in the south of Hungary. Similarly Metalnikov and Chorine, in collaborative work between The Pasteur Institute and the Botanical Gardens of Zagreb, Jugoslavia , reported on the field use of Bt on both the corn borer (Metalnikov and Chorine, 1929a) and the gypsy moth (Metalnikov and Chorine, 1929b). Metalnikov et al. (1930) outlined a series of experiments on corn borer which attempted to answer several problems "(1): A comparison of the effectiveness of various bacteria; (2) The duration of the effectiveness of the bacterial treatment of corn plants; (3) The relative merits of spraying and dusting with bacteria; (4) Methods of increasing the adherence of the spray; and (5) The relative merits of applying but a single virulent bacteria or of using a mixture of several species." These same problems are surprisingly familiar to investigators today.

The earliest report in the literature of a commercial Bt preparation was 1938 and the product was called "Sporeine" (see Jacobs, 1950). This product was still being used 13 years later in experiments to control the flour moth (*Ephestia Kuehniella Z.*) (Jacobs, 1950). Steinhaus (1951) is credited with raising the profile of Bt as a biological control alternative in the USA. His efforts resulted in the commercialization in 1957, of the product "Thuricide" (Bioferm Corp.), based on *B. thuringiensis var. thuringiensis.*

With commercialization came the necessity of a standard. In Europe (France) the standard was E-61 (produced from *B. thuringiensis var. thuringiensis*), and was assigned a potency of 1000 IU/mg in 1966 based on assays against *Pieris brassicae.* In the USA a separate standard was established in 1971 based on *B. thuringiensis* var. *kurstaki* and labelled HD-1-S-1971 after H. Dulmage (cited in Dulmage and Cooperators, 1981). This standard, when compared to E-61, was given a relative potency of 18,000 IU/mg based on assays against *Trichoplusia ni.* The confusion

in determining potencies, that has arisen from the use of different Bt strains vs different test insects, continues in the literature (Dulmage and Cooperators, 1981, Tompkins et al., 1990).

Briggs (1986) and Weiser (1986) have reviewed the early commercialization efforts and van Frankenhuyzen (1993) has reviewed the more recent companies and products available. Of significance is the recent resurgence of industrial interest in Bt.. Some of this interest is due to new Bt strains that are pathogenic to Dipterans and Coleopterans, but most of the impetus comes from a growing public concern over the use of traditional chemical insecticides.

PHYSICAL PROPERTIES OF CRYSTALLINE δ-ENDOTOXIN

The δ-endotoxin crystals are produced in the sporangium in a 1:1 ratio with the spore. Some investigators have reported more than one crystal per spore, however this seems to be the exception. Both spore and crystal are approximately 1μ in length and white in colour, but they exhibit different densities. This difference in density has allowed purification of the crystal from the spore and other cell debris by density gradient centrifugation. Numerous techniques have been reported for the separation of purified crystals. Monro (1961) estimated the crystal density at 1.41 g/cm^3 from sucrose gradient centrifugation. Fast (1972) utilized CsCl gradients to purify crystals and reported densities of 1.30 and 1.35 for crystals and spores respectively. Renografin -76 (66% N-methylglucamine salt and 9.4% sodium salt of 3, 5-diacetamido-2,4,6 -triiodobenzoic acid, supplied by E. R. Squibb and Sons) has been used in linear (Sharpe et al., 1975: Milne et al., 1977) and discontinuous gradients (Milne et al., 1977) and the buoyant densities for crystals and spores were reported as 1.27 and 1.32 respectively. Large scale purification of crystals based on zonal gradient centrifugation using NaBr (Ang and Nickerson, 1978) resulted in density estimates of 1.32 and 1.38 for crystals and spores respectively. More recently Ludox HS-40 (supplied by DuPont Co.) has been reported as a suitable medium for crystal purification (Zhu et al., 1989) but

buoyant density data for the crystal was not reported. The purification of crystals by other methods based on biphasic separations in organic solvents has been reviewed by Cooksey (1971).

A modification of the Renografin-76 discontinuous gradient method for crystal purification (Milne et al., 1977) was employed to obtain highly purified crystals for Raman spectroscopy (Carey et al., 1986). The Raman features indicated that the crystal protein from *B. thuringiensis* var. *kurstaki* HD-1 was composed of 25% α-helix, 21% β-sheet and 54% unordered structure. Interestingly the side chain Raman data suggested the crystal was quite open and that water had access to a large percentage of the molecule

The crystal protein has been characterized as having a bipyramidal shape as shown by electron micrographs (Hannay and Fitz-James, 1955). Labaw (1964) also presented a study of the surface structure of the crystal. The repeating subunits on the surface were interpreted as being spherical with a diameter D = 87 Å in a cubic-close-packed arrangement. Labaw noted that in spite of the high quality of the carbon-replica micrographs there appeared to be "debris-filled troughs" in the structure. Holmes and Munro (1965) performed powder x-ray diffraction on the crystals and indicated their results suggested a repeating unit in which the short dimension was, a = 90 Å and the long dimension was, c = 269 Å. They also compared their results to those of Labaw and reconciled their differences by suggesting Labaw's packing arrangement but with a dumbbell- or ellipsoid-shaped molecule instead of the spherical shape that Labaw suggested. Holmes and Munro (1965) also calculated that the minimal molecular weight of the asymmetric unit was 230,000, although as mentioned above their density measurement (1.41) may have been overestimated. Norris (1971) later reviewed their work and provided a diagram of the proposed surface structure (reproduced Fig 1).It was over 25 years later before the x-ray crystal structure was solved for the activated portion of the protein that comprised the parasporal inclusion. Several investigators had initially attempted to crystallize the δ-endotoxin from the lepidopteran specific

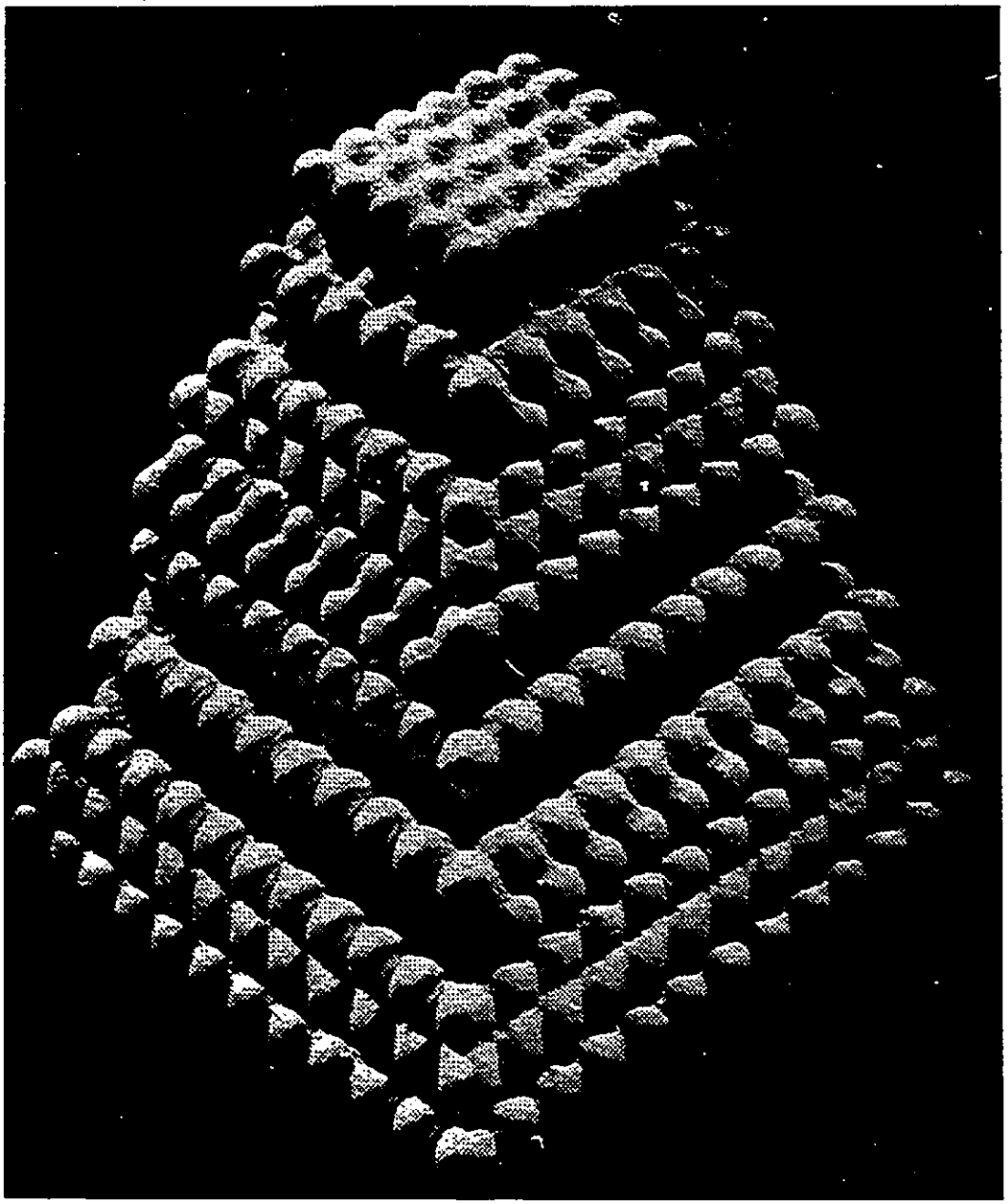

Figure 1. Model of *Bacillus thuringiensis* protein crystal constructed on the basis of X-ray diffraction data.
(taken from Norris, 1971)

toxin without success. Li et al. (1991) however, crystallized the coleopteran specific toxin from *tenebrionis* and provided the first 3-D images of the truncated protein (reproduced Fig. 2). The N-terminal half of the protein was characterized as having 6 helical structures arranged around a 7^{th} helix. The remaining C-terminal half was characterized as having two distinct regions both composed mostly of β-structures. A second x-ray structure has now been solved for one of the lepidopteran-specific toxins, $Cry1A_{(a)}$, (Borisova et al., 1994). Surprisingly the two crystallographic structures were superimposable with only a few deviations in the general backbone structure. This agreement of the two x-ray structures was originally suggested by Li et al. (1991), given the similarity in toxic action and gene sequence homologies between most of the δ-endotoxins.

CLASSIFICATION OF BACILLUS THURINGIENSIS

Bacillus thuringiensis is a gram +ve, aerobic, spore-forming, soil bacteria (Dulmage and Aizawai, 1982). This bacterium is unique in its ability to produce crystalline inclusions during sporulation. Bechtel and Bulla (1976) have provided an excellent overview of the events during the biogenesis of the crystal (Fig. 3). It is the these crystalline inclusions which have caused the dilemma in the classification of the different bacteria. As noted in the Early History section the naming of the type strain caused considerable controversy. That controversy was mild compared to the arguments presented for the proper classification of the different strains that have since been isolated. Heimpel and Angus (1958) concluded that previous attempts to establish a taxonomic status for the crystal-forming bacteria proved confusing. They proposed a classification based on similarities with the *Bacillus cereus* group but providing for a new species designation for the Bt's. Their criteria were based on traditional biochemical and morphological characteristics. A year later Toumanoff and Le Corroller (1959) proposed a scheme in which the "crystalliferous"

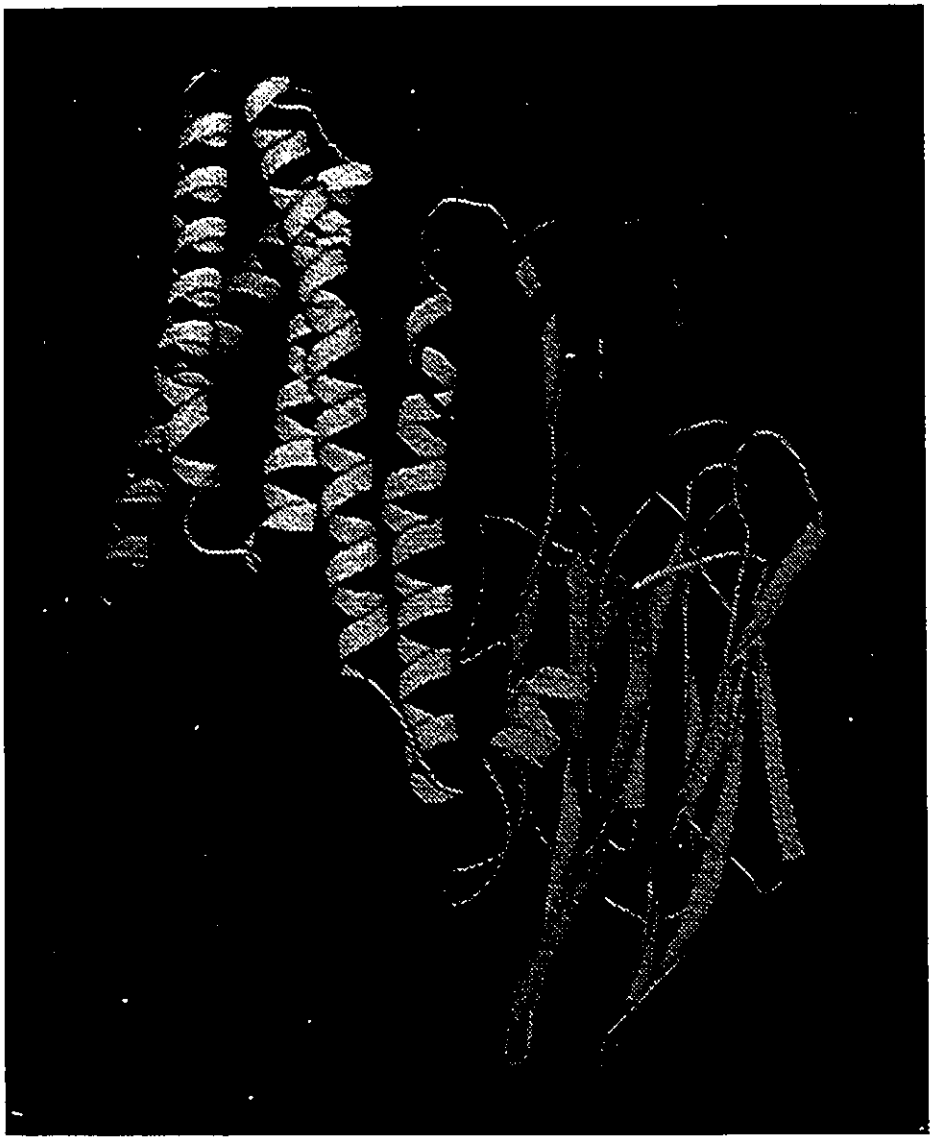

Figure 2. Schematic ribbon representation of the CryIIIA beetle toxin (taken from Li et al., 1991)

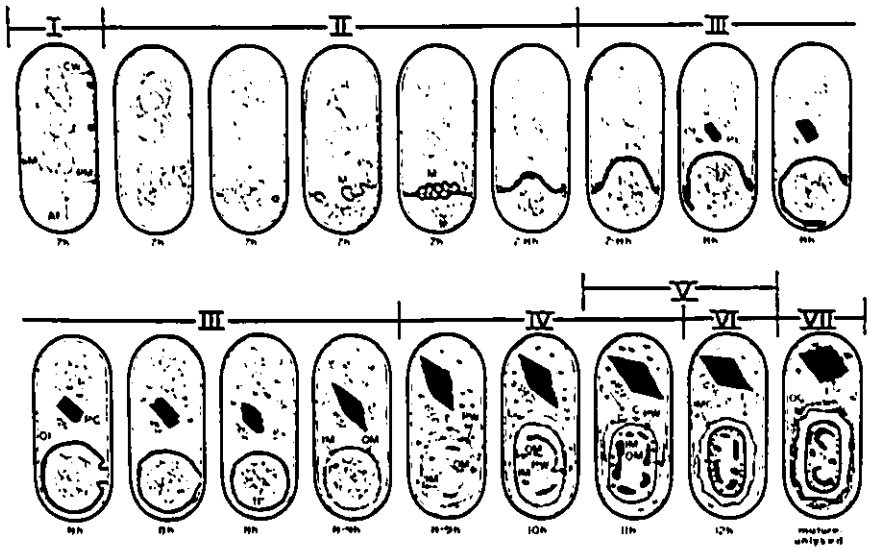

Figure 3. Diagram of sporulation in Bacillus thuringiensis.
M, mesosome; CW, cell wall; PM, plasma membrane; AF, axial filament; FS, forespore septum; IF, incipient forespore; OI, ovoid inclusion; PC, parasporal crystal; F, forespore; IM, inner membrane; OM, outer membrane; PW, primordial cell wall; E, exosporium; LC, lamella spore coat; OC, outer spore coat; C, cortex; IMC, incorporated mother cell cytoplasm; S, mature spore in an unlysed sporangium (taken from Bechtel and Bulla (1976))

group was differentiated, in some instances, based on the host from which the organism was isolated . They recognized that different characteristics appeared depending on the host and suggested that *B. thuringiensis* remain within the *B. cereus* group. Although this reasoning is flawed, it points out the early difficulties encountered in describing these bacteria. Heimpel (1967) presented yet another taxonomic key that proposed variety distinction based on toxicity. Rogoff questioned the usefulness of this new scheme in a letter to the journal (Rogoff 1968) and Heimpel's immediate reply (Heimpel, 1968) attests to the strong differences in opinion between the bacteriologists and the insect pathologists. In a series of papers, de Barjac and Bonnefoi (1962, 1973) and de Barjac and Frachon (1990, 1994) have classified these bacteria by H-antigen serotyping. Similarly Krewienczyk and Angus (1967) used the immunological approach to classify the various isolates by their crystal antigen profiles. The different approaches that have been proffered in an attempt to better describe these bacteria have resulted in references to serovars, biovars and crystovars.

Since the insecticidal activities of these bacteria were the main concern of the pathologist, the classifications based on crystovars and biovars proved most useful at the bench. Much of this early confusion has now been explained by the discovery that the genes that code for the crystal protein are often found on plasmids (Stahly et al., 1978). Gonzales et al. (1981) have shown that these conjugative plasmids can be lost or expressed depending on culture conditions. In at least one case the toxin gene has also been found in the chromosomal DNA (Aronson et al., 1986). Many of these genes have been sequenced and now form the basis of a classification system by crystal gene type (Höfte and Whiteley, 1989). Whereas 45 different serovars had been identified (de Barjac and Frachon, 1994), there are now 5 major gene classes that are subdivided to represent 18 different holotypes. Many of the previously identified serovars have now been shown to contain multiple toxin gene-types. These organisms, containing the multiple genes, may or may not express all gene types at all times. The criteria for different gene types is based in part, on the

specificity towards certain orders (Lepidoptera, Coleoptera or Diptera), and on the comparison of sequence homologies.

CHEMISTRY OF THE CRYSTAL

The amino acid analysis of crystals has been performed on several different strains of Bt by numerous authors (Angus, 1956; Lecadet, 1965; Holmes and Monroe, 1965; Spencer, 1968; Cooksey, 1968; Somerville et al., 1968; Bateson and Stainsby, 1970; Herbert et al., 1971; Lecadet et al., 1972; Bulla et al., 1977; Chestukina et al., 1977; Lilley et al., 1980). Fast (1981) reviewed these analyses and calculated a minimal molecular weight of 13,000 for a sub-unit of the toxin, assuming on average, one methionine residue per subunit. We are now aware that a protoxin may contain 10 methionine residues and a recalculation of Fast's estimate based on 10 MET residues is now shown in Table 1, column A. These averaged amino acid values are compared to the deduced amino acid sequence for the *kurstaki* HD-73 gene (Adang et al., 1985). Column C of the table shows the calculated difference between the two estimates of amino acid content and in brackets, the absolute difference as a percentage of the deduced amino acids (B). Except for the estimate of tryptophan residues, the average amino acid content for whole crystals compares favorably with the single-gene data. Of note is that tryptophan is normally difficult to quantify due to its propensity to degrade during acid hydrolysis prior to analysis.

The reaction of crystal protein with CNBr has confirmed that multiple methionine sites exist in both the whole crystal protein (Milne, 1989; Masson et al., 1990) and in the toxic N-terminal half of the protein (Chestukhina et al., 1982; Milne, 1989; Pang and Mathieson, 1991).Several reports have suggested that the δ-endotoxin may be glycosylated (Holmes and Munro, 1965; Bateson and Stainsby, 1970; Bulla et al., 1977; Tyrell et al., 1981; Insell and Fitz-James, 1985; Aronson and Arvidson, 1987; Phannenstiel et al., 1987). These authors have used different methods and report

Table 1. A comparison of the average numbers of amino acid residues in crystals with the number deduced from the gene sequence of kurstaki HD-73.

	A	B	C
Amino Acid	Avg. # of residues (from estimate of Fast, (1981))	# of residues deduced from gene sequence	A-B, (100[A-B]/B)
ASP	141	146	-5, (3.4)
THR	78	67	+11,(16.4)
SER	92	91	+1 (1.1)
GLU	134	136	-2 (1.5)
PRO	60	53	+8 (15.0)
GLY	111	82	+29 (35.4)
ALA	88	66	+22 (33.3)
VAL	79	84	-5 (5.9)
CYS	17	16	+1 (6.3)
MET	10	10	**
ILE	75	74	+1 (1.4)
LEU	113	94	+19 (20.2)
TYR	45	57	-13 (22.8)
PHE	48	54	-6 (11.1)
LYS	41	34	+7 (20.6)
HIS	23	22	+1 (4.5)
ARG	64	73	-9 (12.3)
TRY	10	19	-9 (47.4)
total	1229	1178	
MW	138.5 kDa	133 kDa	

from 0.5 % (Holmes and Munro, 1965) to 12 % (Bateson and Stainsby, 1970) carbohydrate associated with the crystal. The controversy over whether carbohydrate is a contaminant or covalently bound to the toxin and therefore functionally significant, appeared to be resolved when δ -endotoxin was expressed in *E. coli*, since proteins are not enzymatically glycosylated in these gram-negative hosts (Ylverton et al., 1984). Nevertheless Bhattacharya et al. (1993) has recently proposed that non-enzymatic glycosylation may account for the variability in the reported levels of sugar adducts. Using highly purified and washed crystals these authors found that for some of the sugars there was less than one residue per protoxin molecule. They have proposed that the

non-enzymatic glycosylation reaction may result in sugars covalently attached to lysine side chains at the surface of the crystal. Furthermore the glycosylation of the crystal protein and subsequent cross-linking (Nickerson, 1980; Thorpe and Baynes, 1982) also provides an explanation for the often reported high molecular weight aggregates of crystal protein (Fast, 1981). The mechanism whereby the crystal protein tends to aggregate has not been resolved. Whether due to cross linking of carbohydrate moieties or insolubility due to surface charge interactions, the resultant high molecular weight aggregates have proven problematical in chemical and biochemical studies. A method to re-dissolve these aggregates at pH 7, requires 1M KSCN (Fast and Milne, 1979), suggesting that the protein-protein interactions are associated with charges that can be effectively neutralized with chaotropic salts.

ACTIVATION OF THE TOXIN

The crystal is characterized by its relative insolubility but Tojo and Aizawai (1983) have shown that the crystal must first be solubilized before activation. The solubilization is normally carried out *in vivo* by exposure to the highly alkaline environment in the mid-gut of susceptible larvae. *In vitro* the solubilization can be effected by using high pH buffers (pH 10-11). The addition of reducing agents (DTT, DTE, thioglycolate or β-mercaptoethanol) has been shown to reduce the time required to solubilize the intact crystal (Fast, 1981). The protein released from the crystal is approximately 130 kDa (depending on the strain used) and represents a protoxin (Calabrese et al., 1980). Conversion of the protoxin to toxin is carried out *in vivo* by proteases in the mid-gut (Tojo and Aizawai, 1983). Relatively few studies have addressed the type, quantity and number of different enzymes which may act on the protoxin. Activation can be carried out *in vitro* using mammalian proteases or dilute gut-juice collected from the larvae. The action of proteases appears to result in the cleavage of discrete blocks of protein in the C-terminal portion of the

protoxin until approximately half the molecule has been removed (Chestukhina et al, 1982; Choma et al, 1990). This cleavage is reported to be sequential, beginning at the C-terminus and ending at or near amino acid position 623 for the HD-73 toxin (Bietlot et al., 1989). An additional cleavage occurs at the N-terminal of the protoxin removing the first 29 amino acids (Nagamatsu et al., 1984). In all cases the main enzyme activity appears to be attributed to a serine protease. N- and C-terminal sequencing indicates that arginine at position 29 and lysine at position 629 are the sites of attack for the mammalian trypsin. Similar studies delineating the toxin have not been reported for the action of mid-gut proteases. The proteases found in the mid-gut of lepidopteran larvae have been tentatively identified by several different investigators (see review by Applebaum, 1985). Both exo- and endoproteases are well represented. Although the initial activation appears to require only a trypsin-like or chymotrypsin-like enzyme there is some evidence that exopeptidases may also modify a previously activated toxin. Whether or not this type of enzymatic modification occurs *in vivo* remains to be determined.

Once activated the toxin is relatively soluble and stable to further enzymatic digestion. This stability has allowed the facile preparation of toxin from crude fermentation products by simply digesting with excess bovine trypsin (Bietlot et al., 1989). Most of the contaminating proteins are digested to low molecular weight peptides which can be removed by dialysis or column chromatography. The resultant partially purified toxin is 60-70 kDa in size and carries a net $+^{ve}$ charge, (pI = 4.5),. Most biochemical studies have use activated toxins prepared in this, or a similar, manner.

MODE OF ACTION OF THE TOXIN

Initially the action of toxin was studied using Bt preparations which contained both spores and crystals or purified crystals. Heimpel and Angus (1959) proposed that susceptible larvae

responded in different ways to these preparations: Type-I, insects which exhibit general paralysis and which show blood pH change to crystal protein alone (e.g. *B. mori, M. sexta*); Type-II, insects which are susceptible to crystal alone, suffer gut paralysis but no gut leakage, no change in blood pH or general paralysis (e.g. most *Lepidoptera*); and Type-III, insects which require both crystals and spores for pathogenesis (e.g. *A. kuehniella, L. dispar*). Fast (1977) reported that spores alone played little or no role in the mortality of *C. fumiferana* (a Type-II). However the addition of spores to *E. coli* produced toxins significantly enhanced the mortality of *C. fumiferana* larvae and changed the relative specificity of the different Cry1A toxins (Milne et al, 1990). It therefore appears that the role of the spore may not be just as an inactive spectator, but as an opportunistic pathogen.

The symptoms observed upon ingestion of δ-endotoxin by a susceptible larvae have been reviewed by Fast (1981) and Himeno (1987). Depending on the type of insect larva used, the following sequence of events may be observed: cessation of feeding and peristalsis in the mid-gut, vomiting, sluggish movement, diarrhea and death. Nishitsutsuji-Uwo and Endo (1980) proposed four successive stages of symptoms for *B. mori* based on locomotion, heartbeat and peristalsis in the gut. The four stages were characterized by the following observations: Stage-0; normal locomotion, feeding ceases. Stage-1; locomotion sluggish, heartbeat slows, no peristalsis in the mid-gut. Stage-2; very sluggish, mouth parts and legs respond only when stimulated, heartbeat irregular. Stage-3; complete paralysis (no reflex movement), heartbeat stopped, only for-gut and hind-gut show contractile response. These stages of symptoms were compared to the action of two organo-phosphate stomach poisons and two contact poisons. These chemicals caused total paralysis of the for-, mid- and hind-gut when observed just after the heartbeat stopped. They observed no paralysis of the for- or hind-gut in Bt treated larvae, suggesting that this unique mid-gut paralysis is indicative of Bt toxicity.

Angus (1968) proposed that the δ-endotoxin mimicked the action of the ionophore valinomycin causing cells to disrupt. A cytopathological study of the midgut from *B. mori* (Percy

and Fast, 1983)) clearly showed the progressive cell damage caused by the purified toxin and Fast et al., (1978) showed that toxin bound to Sephadex beads and unable to penetrate the cell membrane could still elicit the cell swelling response (Murphy et al., 1976) in CF-1 cells (cultured *C. fumiferana* cells from neonate larvae). Several investigators have proposed that δ-endotoxin causes an ionic imbalance due to interference with various active and passive pumps in the cell membrane (Knowles and Dow, 1993).

More recently the mode of action of toxin has focused at the molecular level. Knowles and Ellar, (1987) have proposed that cell disruption is the result of colloid osmotic lysis. Furthermore the toxin is recognized by a receptor/s isolated from susceptible cells (Knowles et al., 1991; Knight et al., 1994; Sangadala et al., 1994). The receptor is thought to provide the necessary specificity for different toxins towards different insects. Although considerable evidence would suggest a correlation between the existence of the receptor and resultant toxicity, there is also evidence that in the case of gypsy moth the receptor does not show a preferential binding affinity for the most specific toxin (Wolfersberger, 1990). It is proposed that both number of binding sites on susceptible cells and binding efficiency are important in the overall toxin vs cell interaction. The receptor from *M. sexta* brush border membrane has been identified as a leucine aminopeptidase (Sangadala et al., 1994; Knight et al., 1994) and is associated with the cell membrane through a glycosyl-phosphatidylinositol (GPI) anchor (Garczynski et al., 1995). Sangadala et al. (1994) were unable to separate all the alkaline phosphatase activity from their aminopeptidase fraction and Knight et al. (1994) appeared to have sequenced unrelated fragments in their preparation of aminopeptidase. It therefore remains possible that the receptor is an aggregate of different proteins and not just a single aminopeptidase protein.

English et al. (1991) have shown that the incorporation of brush border membrane proteins into artificial membrane reduces the concentration of toxin required for pore formation. This would suggest that receptor proteins help concentrate toxin at the cell surface. Walters et al. (1994) has

further proposed that some toxins may form oligomeric structures in order to sustain large pores in susceptible membranes. These pores or channels have been observed in patch clamp or planar lipid membrane experiments and depending on the conditions in the assay can be either cation or anion selective (Walters et al., 1993; Schwartz et al., 1993). Knowles and Dow (1993) have reviewed the recent mode of action work and proposed a model, which explains the stages of cell lysis: Stage 1; normal cells, electrogenic K^+ pump supplied by both goblet cells and columnar cells (via gap junctions). Stage 2; < 1 min, toxin pore allows rapid ion flux resulting in depolarization of apical membrane. Stage 3; 1 min., gap junction closes, cavity pump depletes goblet cell K^+. Stage 4; 1-5 min, transepithelial potential collapses. Stage 5; 5-30 min, V-ATPase shuts down, columnar cells swell, goblet cells shrink due to Donnan effects. Stage 6; > 1 H, columnar cells lyse, integrity of midgut is lost.

The current mode of action studies have concentrated on the toxin/receptor and pore forming/lysis aspects. However it remains unclear whether receptor type, receptor number or binding efficiency is the most critical factor in the overall pore forming action of the toxin.

RESISTANCE

Until McGaughey (1985) showed otherwise, many investigators mistakenly believed that insects would not develop resistance to Bt (Burges, 1971; Boman, 1981; Briese, 1981). Control of larval populations by individual applications of Bt allowed a considerable survival population and only a short (1 generation) exposure. This condition was altered drastically when stored grains were treated with Bt powders. Several generations of meal moth larvae were exposed to high levels of Bt and eventually resistance was developed (McGaughey (1985). Since this first report several other reports of resistant larvae have followed: *Plodia interpunctella* and *Cadra cautella* (McGaughey and Beeman, 1988); *Heliothis virescens*, (Stone et al., 1989); *Plutella xylostella*

(Tabashnik et al. 1990) *C. fumiferana* (van Frankenhuyzen and Milne, 1992). In each case the pattern of multiple generation exposure and isolated populations recurs. The development of resistance to Bt has also been shown for other orders of insects: *Leptinotarsa decemlineata* (Miller et al., 1990); *Culex quinquefasciatus* (Georghiou, 1984); *Aedes aegypti* (Goldman et al. 1986)

The mechanism by which insects develop resistance to Bt is thought to be linked to the number and type of receptors in the mid-gut epithelial cells (McGaughey, 1994). Several studies have shown a correlation between binding affinity and toxicity (Hofmann et al., 1988; van Rie et al., 1990b; Ferré et al., 1991). Although the correlation between binding affinity and toxicity is compelling, the experimental design was biased towards looking only at receptors. Others have shown that the binding affinity may be reversed for some insects (van Rie et al, 1990a; Wolfersberger, 1990. van Rie et al. 1990b) showed that in the case of the Indian meal moth the number of binding sites correlated with resistance. Johnson et al., (1990) reported that resistant *P. interpunctella* did not show an altered proteolytic processing of Bt. However in a subsequent study Oppert et al., (1994) concluded that some types of resistant *P. interpunctella* did show a slower proteolytic activation, depending on the mixture of toxins used to challenge the insect. Tabashnik et al., (1993) have shown a three-fold cross-resistance in diamond back moth challenged first with *kurstaki* (containing CrylA(a),(b),(c) and CryIIA toxins) then exposed to *aizawai* (containing CrylC and CrylA(a) toxins). They concluded that cross-resistance was likely due to the CrylA(a) toxin which both strains had in common. Rossiter et al., (1990) showed that with gypsy moth larva the development of resistance may be linked to both genotype and the maternally determined nutritional state of the eggs. Clearly the mechanism of resistance remains complex (Marrone and Macintosh, 1993) and it is likely that the resistant insects have revealed only a portion of their Bt defense arsenal.

MOLECULAR GENETICS

Several excellent reviews have followed the elucidation of the various toxin genes (Dean, 1984; Carlton and Gonzalez, 1984; Aronson et al. 1986; Whiteley and Schnepf, 1986; Andrews et al. 1987; Brousseau and Masson, 1988; Höfte and Whiteley, 1989; Lereclus et al. 1993). The following chronological account highlights the development of this aspect of Bt studies.

Stahly et al. (1978) used heat shock (80^{0}C /40 min) to select acrystalliferous mutants of Bt, suggesting that the gene coding for the toxin was plasmid-born. Gonzales et al. (1981) showed a correlation between toxin production and the presence of specific plasmids. Schnepf and Whiteley (1981) were the first to clone the plasmid-born crystal gene of Bt *kurstaki*-HD-1 using the vector pBR322 and the host *E. coli* strain HB101. In further studies, plasmids from different strains of Bt were digested with the restriction enzyme HIND III. The plasmid fragments were then hybridized with single stranded DNA from the recombinant crystal gene cloned in E. coli. The results of these hybridization studies indicated homologous sequences on plasmid fragments of different sizes. The homologous sequences were grouped according to the plasmid restriction fragment size and were referred to as the 4.5, 5.3 and 6.6 Kbp gene types (Schnepf and Whiteley, 1985).

The facile cloning of the protoxin gene resulted in several reports of the expression and sequencing of different gene types (Klier et al., 1982; Adang et al., 1985; Shibano et al., 1985; Thorne et al., 1986; Hefford et al., 1987). Comparison of the deduced amino acid sequences of three of the gene types (Andrews et al., 1987) showed considerable homology (85-99%). With the ever increasing number of toxin gene types being isolated the classification of genes required updating. Höfte and Whiteley (1989) proposed a classification of 42 reported genes based mainly on homology and susceptible species: cryI genes, lepidopteran active bipyramidal crystals; cryII genes, lepidopteran and dipteran active cuboidal crystals; cryIII genes, coleopteran active rhomboidal crystals; and cryIV genes, dipteran active ovoid crystals. These four major classes

were characterized as having similar structural features. A fifth minor gene type, cytA (dipteran active), was described as having little homology to the other four gene types. Currently there are now five major gene classes (Lereclus et al., 1993) with 18 subclasses (see Table 2). The fifth major class of genes, designated cryV (Tailor et al., 1992) is both lepidopteran and coleopteran active.

Table 2. Bacillus thuringiensis crystal protein genes and product specificity (taken in part from Lereclus et al. 1993)

Gene type	predicted molecular weight (kDa)	Host range§
cryIA(a)	132.2	L
cryIA(b)	131	L
cryIA(c)	130	L/D
	133.3	
cryIB	138	L
cryIC	134.8	L
cryID	132.5	L
cryIE	130	L
cryIF	133.6	L
cryIIA	70.9	L/D
cryIIB	70.8	L
cryIIC	69.5	L
cryIIIA	73.1	C
cryIIIB	74.2	C
cryIVA	134.4	D
cryIVB	127.8	D
cryIVC	78.8	D
vryIVD	72.4	D
cryVA	82.2	L/C
cytA	27.4	non-specific

§ L: lepidopteran, D: dipteran, C: coleopteran

Genetic manipulation of the δ-endotoxin has provided unique insights in structure function studies. Through deletion mutations it has been shown that the N-terminal portion of activated toxin is not required for the expression of activity. Using homolog scanning mutants, Ge et al.,

(1989, 1991) showed that the specificity region of the toxin for *B. mori* and *H. virescens* could be localized in the C-terminal portion of the activated toxin. Subsequently the same region has also been shown to affect receptor binding Lee et al. (1992). Numerous studies have reported the exchange of internal regions of the toxin (for review see Visser et al., 1993). However the interpretation of these experiments remains confusing. For example Ge et al., (1991) reported a 30-fold increase in activity of a chimeric toxin when tested against *H. virescens*. It was later found that this activity was specific to one colony of these larvae. When the same toxin was tested against a different colony the difference in activity was less dramatic.

The focus of recent genetic manipulation is in the area of delivery. That is, in which organism the Bt gene is expressed. There are reports of expressing the gene in root colonizing bacteria (Dimock et al., 1988; Stock et al., 1990; Waalwijk et al., 1991), cyanobacteria (Angsuthanasombat and Panyim, 1989), and in plant hosts such as tomato (Fischoff et al., 1987), tobacco (Adang et al., 1987), potato (Leemans et al., 1990), cotton (Perlak et al., 1990) and poplar (McCown et al., 1991). Ely, (1993) has listed the stable transformation of over 50 plant species which are hosts to Bt susceptible pests. The recent testing of transgenic plants (expressing one or more toxins) have raised the awareness and concerns of environmentalists and those studying the development of resistance (Brunke and Meeusen, 1991; Gould and Anderson, 1991).

The combination of numerous gene sequences and the recent X-ray crystallography of one of the coleopteran active toxins has provided the basis of structure-function models (Li et al., 1991). The N-terminal region of these toxins appears to be almost entirely α-helical structure with transmembrane insertion potential. The C-terminal region is composed of two β-sheet regions, the first of which has been implicated in a receptor binding function. The remaining region has as yet to be elucidated. This region may be required for structural and proteolytic stability.

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CHAPTER 2

ENZYMATIC PROCESSING OF δ-ENDOTOXIN

The result of a mathematical development should be continuously checked against one's own intuition about what constitutes reasonable biological behavior. When such a check reveals disagreement then the following possibilities must be considered:

a. *A mistake has been made in the formal mathematical development;*
b. *The starting assumptions are incorrect and/or constitute a too drastic oversimplification;*
c. *One's intuition about the biological field is inadequately developed;*
d. *A penetrating new principle has been discovered.*

Harvey J. Gold,
Mathematical Modeling of Biological Systems

INTRODUCTION

A variety of bioassays are required to determine the effects of Bt δ-endotoxin on forest defoliating Lepidoptera. These assays include diet incorporation and force feeding techniques (van Frankenhuyzen and Gringorten, 1991; van Frankenhuyzen et al., 1991, 1993) as well as *in vitro* methods using cultured insect cells (Murphy et al., 1976; Fast et al., 1978 ; Gringorten et al., 1990) . Each assay demands a different approach as to just how the δ-endotoxin will be presented to the target. In efforts to standardize and compare certain native and recombinant gene δ-endotoxin products one is forced to consider the repercussions of pre-activating toxin with enzymes. In the course of study it was noted that not all toxins behaved the same nor did all enzyme digesting systems yield the same product.

In order to determine whether the gut enzymes of susceptible larvae conferred some specificity in the action of the toxin, a study was undertaken to characterize the δ-endotoxin activation process by comparing the action of *C. fumiferana* and *B. mori* gut juice to a mammalian trypsin system.

METHODS

Aliquots of purified *sotto* crystals (Milne et al., 1977) at 2 mg/ml (protein concentration was determined by alkali solubilization and the dye binding method (Bradford, 1976)) in 0.1M CAPS, pH 10.5 buffer were incubated with dilutions of either bovine trypsin, silkworm gut-juice or spruce budworm gut-juice (lab-reared). After 2 h incubation at 30°C, the samples were centrifuged at 12400 x g (Beckman Microfuge-11 with a 13.2 fixed angle rotor at 13250 RPM). The soluble protein concentrations in the supernatant were measured using the dye-binding method (Bradford 1976). Enzyme blanks were similarly incubated and centrifuged to correct for proteins contributed by the different enzyme systems.

Activation of δ-endotoxins were repeated for both *sotto* ($Cry1A_{(a)}$) and HD-73 ($Cry1A_{(c)}$)separately (at 1.6 mg /ml) and in one to one combination (at 0.8 mg/ml each) using *C. fumiferana* gut juice (at 1/10 dilution). After activation, the products (supernatant and pellet) were analyzed by SDS-PAGE and bioassayed using the CF-1 cell lawn assay (Gringorten et al., 1990).

RESULTS AND DISCUSSION

Recovery of soluble protein during δ-endotoxin activation

Table 3 shows the comparison of yields of soluble protein after digestion with either bovine trypsin or larval gut-juices. In each case, the recovery of soluble protein was higher when the apparent enzyme levels were lower. Although little difference was seen on dilution of bovine trypsin, there was a 7.5 fold increase in protein recovered when the gut-juice from *C. fumiferana* was diluted 1000 times. For *C. fumiferana* this represents a 655% increase in protein recovery. *B. mori* gut-juice gave an intermediate recovery of protein throughout the dilution range. The low recovery of soluble protein correlated with high levels of precipitated protein observed as a pellet after centrifugation (not shown).

SDS PAGE analysis

The *C. fumiferana* gut juice activated δ-endotoxins from *sotto* and HD-73 were centrifuged after digestion. The whole sample (after digestion) and both the soluble and precipitated δ-endotoxin products were analyzed by SDS PAGE using 10% mini gels. Fig. 4, shows that recovery of toxin from HD-73 was mainly in the soluble fraction (lane, 11) whereas the recovery of *sotto* toxin was found in both the supernatant and the pellet (lanes 3 and 4). When the *sotto* and HD-73 δ-endotoxins were digested together, again a portion of toxin appeared in the pellet (lane 8).

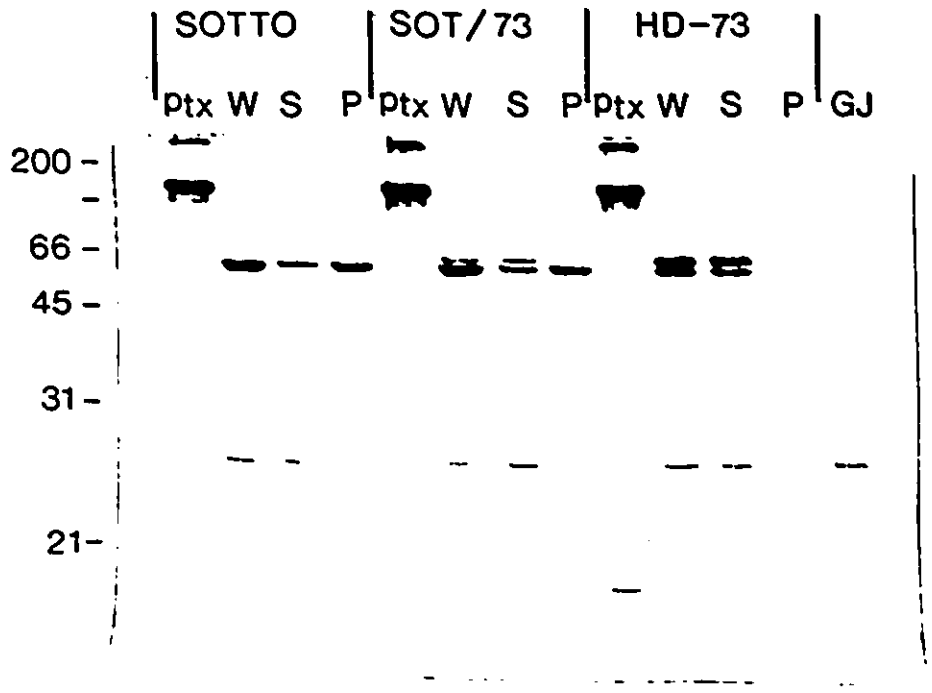

Figure 4. 10% SDS PAGE showing the activation of *sotto* and HD-73 protoxin with diluted *C. fumiferana* gut juice.
Lane 1, *sotto* protoxin (Ptx) at 1.6 mg/ml. Lanes 2-4 after digestion of *sotto* with 1/10 gut juice. Lane 5, *sotto* and HD-73 (sot/73) protoxins each at 0.8 mg/ml. Lanes 6-8, after digestion of sot/73 with 1/10 gut juice. Lane 9, HD-73 protoxin at 1.6 mg/ml. Lanes 10-12, after digestion of HD-73 with 1/10 gut juice. Lane 13, *C. fumiferana* gut juice (GJ)diluted 1:10 with CAPS buffer pH 10.5. W = whole sample before centrifugation, S = supernatant after centrifugation, P = pellet after centrifugation

From this analysis it was expected that the material in the pellet (lane 8) would be of *sotto* origin.

Table 3. Soluble protein *recovered from 2 mg/ml *sotto* crystals after 2 h digestion at 30°C with insect gut-juice or bovine trypsin.

Bovine trypsin		*B. mori* gut-juice		*C. fumiferana* gut-juice	
Conc. of enzyme (mg/ml)	Soluble protein (mg/ml)	Dilution of neat gut-juice	Soluble protein (mg/ml)	Dilution of neat gut-juice	Soluble protein (mg/ml)
1	0.92	1:1	0.76	1:1	0.18
0.02	1.15	1:40	1.47	1:100	0.44
0.002	1.59	1:400	2.13	1:1000	1.36

*Values corrected for trypsin or gut-juice proteins added

Bioassay

The toxin preparations analyzed by SDS-PAGE were also tested for activity using the CF-1 cell lawn assay (Table 4). The dose for threshold response indicates the lowest dose to yield a visible response on the cell lawn assay, where a higher dilution factor represents a higher level of toxin present in the stock. Soluble and precipitated fractions were compared to the whole sample (taken before centrifugation). The bioassay of the *sotto* digests shows that toxin is present in both the supernatant and pellet. The bioassay of HD-73 shows that the toxin is primarily found in the supernatant. These results confirm the SDS-PAGE analysis. In contrast, the products from the combined digestion of *sotto* and HD-73 did not confirm the SDS-PAGE analysis. Specifically, the pellet after digestion exhibited much more activity than could be contributed by *sotto* alone. Therefore it appeared that some of the precipitated toxin was of HD-73 origin.

Table 4. Recovery of toxin from *sotto* (Cry1A$_{(a)}$) and HD-73 (Cry1A$_{(c)}$) after 2 H digestion at 30°C with 10% *C. fumiferana* gut juice.

DILUTION FACTOR FOR THRESHOLD RESPONSE			
SAMPLE	SOTTO (1.6 MG/ML)	HD-73 (1.6 MG/ML)	SOTTO/HD-73 (0.8 MG/ML EACH)
WHOLE DIGEST	16§	800§	800
SUPERNATANT	8	800	400
PELLET	4	4	64

§ these dilution factors correspond to threshold doses for sotto and HD-73 of 100 ng and 2 ng respectively.

CONCLUSIONS

According to Andrews et al., (1985) the recovery of toxin after digestion should approach values determined by the stoichiometric conversion of protoxin to toxin. In contrast, we observed a loss of soluble toxin with the concomitant appearance of insoluble materials. In the case of *sotto* activation, the insoluble portion retains some activity. More significantly when digesting multiple toxin types the insoluble *sotto* (Cry1A$_{(a)}$) products appear capable of trapping or interacting with the other toxin types (HD-73 (Cry1A$_{(c)}$). The resultant precipitate contains (Cry1A$_{(c)}$toxins which one would otherwise expect to be soluble and in the supernatant.

During the preparation of toxin for bioassay (pre-activation) one should not expect the complete recovery of toxin. Similarly, when δ-endotoxins are administered to larvae and the activation of toxin occurs *in vivo* there may be much less available soluble toxin than previously thought and for the purpose of determining LD$_{50's}$ the calculation of toxin dose would be

overestimated.

Clearly the activation of toxin in the gut juice of spruce budworm is not straightforward and requires more investigation into the enzymes and mechanisms responsible for the apparent losses of toxin during activation. Chapter 3 deals with the enzymes responsible for activation. A review of the literature (Applebaum, S. W., 1985) suggested that serine proteases would likely be the major proteolytic enzymes present in the gut-juice. With that one assumption, a major trypsin-like enzyme was identified, purified and characterized. Although the trypsin-like enzyme appeared to be solely responsible for the activation of protoxin to toxin the mechanism whereby toxin was lost during activation was not due to this activating enzyme. Chapter 4 deals with the purification and characterization of a toxin-precipitating protein (TPP) which does appear to be responsible for the loss of soluble toxin during activation. This protein existed in extremely low levels in the gut-juice but had a profound and somewhat specific effect on the Cry1A$_{(a)}$ type toxin. Chapter 5 introduces yet another aspect of the activation of toxin. Whereas most of the work for this thesis focused on the proteolytic enzymes in the midgut, a study was undertaken to determine the *in vivo* significance of DNA associated with the toxin. The finding that toxin forms a strong association with DNA, forces one to rethink the activation processes.

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CHAPTER 3

PURIFICATION AND CHARACTERIZATION OF A TRYPSIN-LIKE DIGESTIVE ENZYME FROM SPRUCE BUDWORM (*CHORISTONEURA FUMIFERANA*) RESPONSIBLE FOR THE ACTIVATION OF δ-ENDOTOXIN FROM *BACILLUS THURINGIENSIS*

"All insects, without exception, die if they be smeared over with oil; and they die all the more rapidly if you smear their head with oil and lay them out in the sun"

D. W. Thompson's translation of Aristotle's *Historia Animalium*

INTRODUCTION

The proteolytic activities of gut enzymes from several different insect orders have been identified using synthetic and protein substrates (Applebaum, 1985). In the order Lepidoptera, most interest has focused on the serine proteases represented by chymotrypsin-like and trypsin-like enzymes (Applebaum, 1985). However, relatively few of these proteases have been highly purified and characterized. Eguchi and Kuriyama (1983) have isolated three trypsin-like proteases from *Bombyx mori* which show maximum casein hydrolysis at pH 11.2. Similarly, Ahmed *et al.* (1980) have identified three trypsin-like proteases from *Spodoptera litura* with optimum caseinolytic activities at pH 9.0, 10.5 and 11.0. The presence of trypsin-like and chymotrypsin-like enzymes have been reported for *Trichoplusia ni*, based on activity towards artificial ester substrates, (Pritchett *et al.*, 1981), and Lecadet and Dedonder (1966) reported chymotryptic and tryptic activity for gut enzymes from *Pieris brassicae*.

Several partially purified lepidopteran gut proteases have been characterized with regard to their pH dependence for maximal hydrolysis of synthetic amino acid ester and amide substrates or denatured proteins: *Heliothis zea* - pH 11 (Klocke and Chan, 1982); *Galleria mellonella* - pH 10.5 and 11 (Hamed and Attias, 1987); *Pieris brassicae* - pH 10.5 (Lecadet and Dedonder, 1966); *Ostrinia nubilalis* - pH 10 (Houseman *et al.*, 1989); *Trichoplusia ni* - pH 9.8 (Pritchett *et al.* 1981); *Erinnyis ello* - pH 9.5 and 10.0 (Santos and Terra, 1986). The pH of the midgut lumen has been reported to be in the range of 9.0 to 11.5 (Waterhouse, 1949; Berenbaum, 1980) and, therefore, the high pH optima observed for these enzymes is consistent with the natural environment of the lepidopteran midgut.

Larval gut enzymes have been studied primarily for their role in digestion and their

compartmentalization in the various regions of the gut (Terra, 1990; Applebaum, 1985). Our interest in the lepidopteran larval gut proteases was prompted by the essential role they play in the proteolytic activation of the δ-endotoxin from *Bacillus thuringiensis* (Bt), (Huber and Lüthy, 1981). Bai *et al.* (1990) have suggested that protease levels in the guts of *P. brassicae*, *Mamestra brassicae*, and *Spodoptera littoralis* may be responsible for the difference in susceptibility of these larva to δ-endotoxin. Jaquet *et al.* (1987) have shown that the crystalline δ-endotoxin and δ-endotoxins, pre-activated with bovine trypsin, have different toxicities and specificities when bioassayed against three susceptible larval species.

This observation also suggests that the toxicity of the δ-endotoxin towards various insect larvae may be partly dependent on a specific proteolytic action of the larval gut proteases (Milne *et al.*, 1990).

The spruce budworm larva has been shown to be susceptible to the δ-endotoxins associated with Bt (van Frankenhuyzen and Nystrom, 1987). However, little is known concerning the enzymes responsible for the proteolytic processing of the protoxin to the active toxin. The aim of the present study was to identify and characterize the enzymes responsible for protoxin activation as a first step towards elucidating their role in the action of this biological pesticide against forest insect pests.

MATERIALS AND METHODS

Chemicals

The following enzymes, substrates and inhibitors used were from Sigma unless otherwise noted: Trypsin TPCK treated #T8642, Elastase #E0127, Insulin B-chain oxidized, N-benzoyl-L-arginine ethyl ester, (BAEE), N-benzoyl-L-arginine p-nitroanilide (L-BAPNA), N-benzoyl-DL-arginine p-nitroanilide (DL-BAPNA), p-nitrophenyl p'-guanidino-benzoate (NPGB), Nα-p-tosyl-L-

lysine chloromethyl ketone (TLCK), Nα-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK), diisopropyl fluorophosphate (DFP), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), N-acety-L-trialanine p-nitroanilide (ATAPNA); Chymotrypsin (Worthington Biochemicals); δ-endotoxin crystals from *Bacillus thuringiensis* var. *kurstaki* HD-73 (van Frankenhuyzen *et al.* 1991), which produces the CrylA(c) toxin (Adang *et al.* 1985)

Insects

Gut juice was collected from early 6th instar spruce budworm larvae reared on artificial diet (Grisdale, 1984). The larvae fed *ad libitum* prior to the collection procedure. Larvae could be forced to expectorate gut juice by gentle massaging of the mouth parts with a capillary tube; 1-2 µl of collected gut juice was transferred to a vial on ice. The gut juice was then clarified by centrifugation and 250 µl aliquots were stored in a chest freezer at -20°C.

Protease Activities in Neat Gut Juice

The relative amounts of the trypsin-like, chymotrypsin-like and elastase-like activities in neat gut juice were determined using the chromogenic substrates L-BAPNA, DL-BAPNA, L-BTPNA and ATAPNA. Procedures outlined by Christeller *et al.* (1989) were changed as follows. Reactions were carried out at 25°C in 0.1M CAPS, pH 10, with substrate concentrations of 0.5-1.5 mM and 1.5% DMSO. Progress curves were followed using a Beckman DU-7 spectrophotometer with thermoregulated cell.

Identification of Crystal Activating Enzyme

Aliquots (4 µl) of gut juice were subjected to isoelectric focusing using pre-cast pH 3-10 Phast gels (Pharmacia) with the pH range extended by the addition of paper strips containing pH 9-11 ampholines (Kleine, 1988). After focusing, the gel was blotted onto a second gel made from 1.5% agarose (SeaKem ME) containing purified δ-endotoxin crystals (Milne et al., 1977) at 1 mg/ml in 0.1M CAPS, pH 10, (crystals were added at 40°C, just prior to gel setting, providing an opaque substrate). The focused enzyme acrylamide gel was incubated 30 min. with the undissolved crystal imbedded in the agarose gel. Enzyme activity was then recorded as a clearing of the agarose gel.

Inhibition of Crystal Activating Enzymes

The DFP stock was diluted tenfold with anhydrous isopropanol and stored at 4°C (DFP/10). Gut juice was thawed and diluted tenfold in 1% NH_4HCO_3, pH 8, then treated with either anhydrous isopropanol (as control) or DFP/10. Gut juice typically contained approximately 2 mg/ml total protein. We estimated that 1% of the protein was serine proteases from the SDS-PAGE gels (see Results Fig. 6). Gut juice was incubated 3 h at room temperature with 5µl/ml DFP/10 (approximately 10x molar excess of DFP based on enzyme concentration). Inactivation of treated and control enzyme solutions was allowed to continue for 24 h at 4°C. Control and DFP-treated gut juice were then incubated with an equal volume of purified δ-endotoxin crystals from *B. thuringiensis* (HD-73), made to 1 mg/ml in 0.1 M CAPS-KOH buffer pH 10.5. Aliquots were taken at various times during the incubation and immediately treated with a 10x molar excess of DFP. Treated aliquots were prepared for SDS-PAGE analysis by boiling samples in sample buffer for 2 min.

Active Site Labelling

Frozen gut juice was thawed and clarified by centrifugation. A 0.9-ml sample was buffered by the addition of 0.1 ml of 0.2M CAPS, pH 10, and incubated at 20°C for 30 min. with 25 µl ^{14}C-DFP (Specific Activity 125 mCi/mmol NEC-378, New England Nuclear). An additional 25 µl of unlabeled DFP/10 was added after 30 min. and incubation continued for 1 h. An aliquot of the diisopropyl phosphate (DIP) labeled proteins was taken for SDS-PAGE analysis and autoradiogaphy . The remaining sample was dialyzed using 3500 MWCO tubing (Spectra/por), in acidified water (pH 5) for 24 h. The retentate was shell-frozen and lyophilized.

High Voltage Paper Electrophoresis (HVPE)

Analysis of active site peptides was carried out according to procedures outlined by Bietlot *et al.*(1990). Lyophilized ^{14}C-DIP labeled gut juice proteins and similarly labeled mammalian serine proteases (chymotrypsin, trypsin and elastase), were denatured and carboxymethylated (Means and Feeney, 1971), then subjected to partial acid hydrolysis (5.7N HCl at 37°C for 3 days) as described by Naughten *et al.* (1960). The peptides were separated using HVPE at pH 2.1 and subjected to autoradiography in a cassette for 3 days .

Enzyme Purification

DEAE A-25 anion exchanger (Pharmacia) was prepared by equilibration in 0.1M CAPS-NaOH, pH 10, and stored at 4°C. Frozen gut juice was thawed and centrifuged to clarify. Aliquots of 250 µl were treated with 0.5 ml packed volume of exchanger for 30 min. on ice with occasional

mixing. The unretained fraction was aspirated from the slurry and combined with a 250 µl buffer wash of the slurry. The sample was then sterilized using a 0.22µ filter and injected on a SP-5-PW column (Bio Rad, 75mm x 7.5mm) that had been equilibrated with the same buffer but at 0.02M. Gradient elution with increasing NaCl was carried out using a Waters 625 HPLC with PDA detector. Slow gradient development was used in order to facilitate collection of fractions. Aliquots of fractions were immediately tested for DL-BAPNA activity by mixing 25µl fraction with 75 µl of 0.2 mg/ml DL-BAPNA in 0.02M CAPS-NaOH, pH 10, (DL-BAPNA stock was made 20 mg/ml in DMSO). The major active fraction was dialyzed using 3500 MWCO tubing in H_2O at 4°C for 24 h. The retentate was shell-frozen and lyophilized.

Isoelectric Point Determination

The isoelectric point of the enzyme was determined by injecting 250 µl of DEAE-treated gut juice on the SP-5-PW cation exchange column and eluting with a pH gradient. The starting buffer was 0.02M CAPS-NaOH, pH 9.5, and the eluting buffer was 0.02M CAPS-NaOH, pH 11.5. The fractions were tested for DL-BAPNA activity and the pH recorded for each fraction..

Amino Acid Analysis and N-Terminal Sequencing

Aliquots of gut juice were treated with DFP then column purified according to the procedure outline above. The purified fraction was dialyzed against water using 3500 MWCO tubing and lyophilized. After acid hydrolysis and phenylthiohydantion (PTH) derivatization, amino acid analysis was carried out using a Technicon TSM analyzer (an aliquot was also oxidized with performic acid to determine cysteine as cysteic acid). A second aliquot was subjected to SDS-PAGE analysis and the 25 kDa band was blotted then sequenced using a gas-phase sequencer.

Cyanogen Bromide Cleavage

A 20-fold molar excess of CNBr in 70% formic acid and formic acid alone, as control (Gross, 1967), were used to cleave purified CFT-1. The reaction was carried out at room temperature for 3 h, then an aliquot was removed and neutralized with 0.1N NaOH. Cleavage products were separated in an 18% SDS-PAGE gel and blotted for gas phase sequencing.

pH Stat Kinetics

Enzyme kinetics using the artificial substrate BAEE were carried out with some changes to the procedures outlined by Walsh and Wilcox (1970). Briefly, the purified lyophilized CFT-1 enzyme was resuspended in 0.1M CAPS-KOH, pH 10, at 50µg/ml, as determined by protein dye binding. No $CaCl_2$ was included in the reaction mixture. The enzyme solution was allowed to stand for 30 min. on ice before use. Typically 50 µl of enzyme was added to 5 ml of 2.5×10^{-3} M BAEE in 0.1M KCl. Reactions were carried out at 25°C and followed using a Radiometer Titrator-II and Ole Dich recorder. Data were analyzed using the Enzfitter program (Leatherbarrow, 1987)

Purified Enzyme Burst Kinetics

The titration of active sites was carried out according to the procedure outlined by Chase and Shaw (1970). Titrations of bovine trypsin and purified CFT-1 were carried out at pH 8.3 in Veronal buffer using the active site titrant NPGB ($\varepsilon_{410} = 16595$ at pH 8.3). Post burst production of p-nitrophenol was followed to ascertain whether deacylation was significant. Enzyme levels were determined empirically to yield comparable traces.

Insulin B-Chain Digestion

Proteolytic specificity of the CFT-1 enzyme and neat gut juice were determined by comparison of peptide profiles with those generated by bovine trypsin. Conditions for digestion were as follows: Insulin B-chain was made to 1 mg/ml in 0.5% NH_4HCO_3, pH 8.0, and freshly prepared enzyme was added (substrate to enzyme ratios were 50:1 and neat gut juice was added at a 5 X 10^{-3} final dilution). A time course for digestion was used from 2 to 60 minutes. At specified times a 20µl aliquot was taken and diluted with 80 µl of A-buffer (pH 2.5) to stop the reaction. The aliquot (100µl) was injected on a C-18 column (Delta-pac 3.9 X 150 mm, 5µ, Waters) equilibrated at 1 ml/min in A-buffer containing 0.1% TFA and 2.5% acetonitrile. Elution was carried out with a 25 min linear gradient to 60% of B-buffer containing 0.1% TFA and 80% acetonitrile beginning at 5 min.

Protoxin Preparation

Delta-endotoxin crystals were stored in 0.1M EDTA and 5µl/ml DFP/10 at 4°C. Protoxin was prepared from δ-endotoxin crystals by solubilizing 2-4 mg of spore-crystal mixture (in 1 ml 0.1M CAPS-KOH, pH 10.5 containing 0.4% DTT) (Gringorten *et al.* 1992). The mixture was incubated for 30 min at room temperature then centrifuged and the supernatant filtered at 0.2µ. Protoxin was diluted to 1 mg /ml in 0.1M CAPS-KOH, pH 10, and proteolysis was carried out in an incubator shaker at 30°C. See Figure 9 for digestion schedule.

SDS-Polyacrylamide Gel Electrophoresis and Autoradiography

SDS-PAGE analysis was carried out using a MINI-GEL or PROTEAN II system (Bio Rad). Bio Rad reagents, molecular weight standards and protocols were also followed. Destained gels were prepared for autoradiography using a slab-gel drier, then placed in a cassette with X-omat AR film (Kodak). Exposure times were determined empirically (usually 1-3 days).

Protein Determinations

Protein levels were determined by the dye binding method of Bradford (1976) using Bio Rad reagents. Samples at pH 10 were neutralized with 0.1M Tris, pH 7 prior to analysis.

RESULTS

Treatment of Gut Juice with DFP

Table 5, gives the activity of neat spruce budworm gut juice toward chromogenic substrates of trypsin (L-BAPNA and DL-BAPNA), chymotrypsin (L-BTPNA) and elastase (ATAPNA). Only a minor response was recorded for chymotryptic activity; no response was recorded for elastase activity. Trypsin-like activity predominated and treatment of the gut juice with DFP completely eliminated this activity. More significantly, DFP treatment destroyed the ability of gut juice proteases to convert the 130-kDa Cry1A type δ-endotoxin from *B. thuringiensis* to the 60-kDa toxin (Fig. 5). To determine which of the proteins in gut juice reacted with DFP, neat gut juice was treated with [^{14}C] DFP and a comparison was made between the silver-stained SDS gel and the corresponding autoradiogram (Fig. 6, lanes 1 and 3).

Table 5. . Serine protease activities in spruce budworm gut juice *

ACTIVITY	SUBSTRATE	RATE * (nmol/min/µl)
TRYPSIN	L-BAPNA	62.4
	DL-BAPNA	46.4
CHYMOTRYPSIN	L-BTPNA	1.0
ELASTASE	ATAPNA	0

* Gut juice from 200 larvae was pooled, clarified (see Methods) and used at 1 x 10^4 final dilution
* Rates given are for 1 µl neat gut juice

Only a single major radiolabeled band was found, which coincided with a protein band with an apparent molecular mass of 25 kDa. The remaining stained bands were variable in different gut juice preparations, and most likely were dietary proteins.

Proteolytic hydrolysis of the [^{14}C]DIP-labeled gut juice enzymes and representative [^{14}C]DIP-labeled mammalian serine-proteases did not yield a common pattern of radioactive peptides on high voltage paper electrophoresis. In contrast, a comparison of the radiolabeled peptides obtained after partial acid hydrolysis of the gut juice proteins with those from bovine trypsin, chymotrypsin and porcine elastase showed that identical peptides were produced (Fig. 7). The peptide profile shown (Fig. 7) is similar in the number, intensity and relative migration of bands to those reported by Hartley *et al.* (1959) for the comparison of elastase to trypsin and chymotrypsin.

Enzyme Purification

Attempts to estimate the isoelectric point of the crystal (δ-endotoxin) activating enzyme from gut juice on IEF gels (pH 3-11) indicated that all the activity migrated to the extreme cathode region

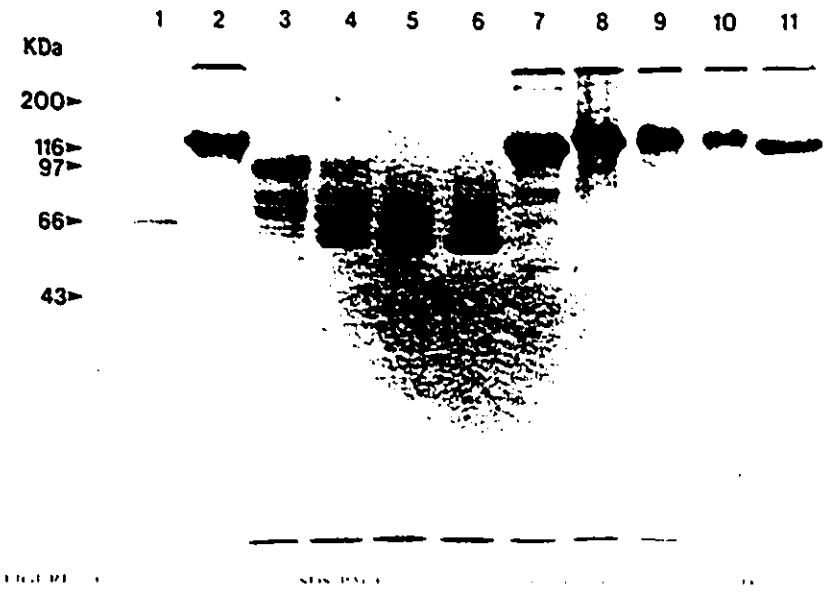

Figure 5. Coomassie blue stained 10% SDS PAGE gel showing the proteolytic activation of the 130 kDa δ-endotoxin and inhibition with DFP.
Protoxin at 1 mg/ml was digested with 2µl/ml neat gut juice. Lane 1, molecular mass markers; Lanes 2-6, are 0, 5, 10, 30 and 60 min. Lanes 7-11 same as lanes 2-6 except gut juice was pretreated with DFP. Precipitation at the top of the gel is due to the DFP treatment prior to SDS sample preparation.

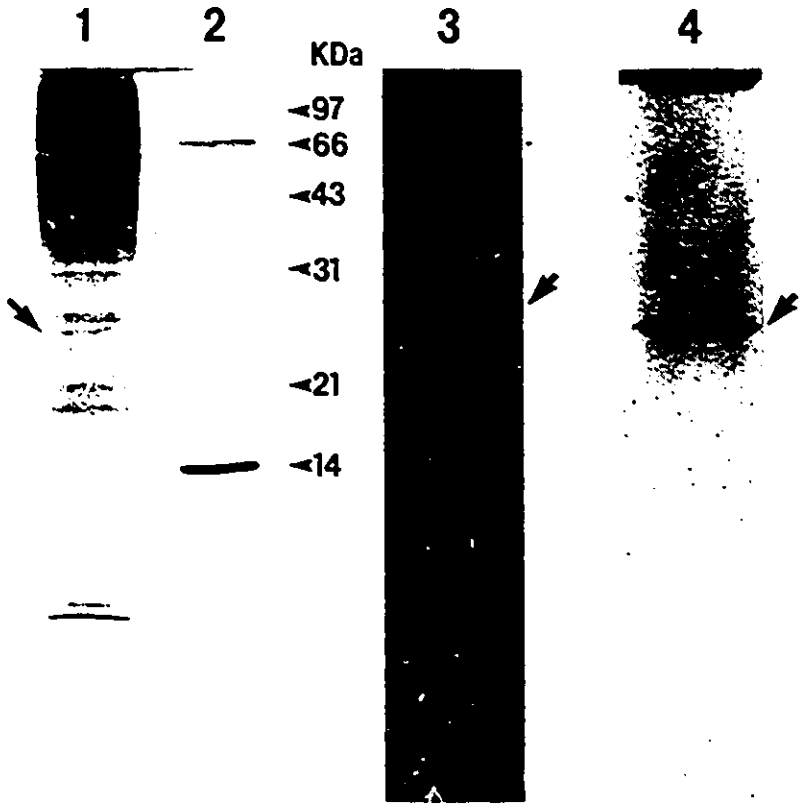

Figure 6. 15% SDS PAGE gel showing a comparison of silver stained ^{14}C-DIP labeled neat gut juice proteins, lane 1, with the corresponding autoradiogram, lane 3 ; lane 2, molecular weight markers; lane 4, non-radioactive DFP-treated purified gut enzyme (CFT-1). Arrows indicate alignment of bands.

of the gel but did not resolve, (the reaction was recorded but allowed to overdevelop to observe lower levels of activity, not shown). It appeared that the enzyme(s) of interest had an isoelectric point greater than 10. Therefore, the following purification strategy was adopted: 1) Batch anion exchange treatment at pH 10 to remove contaminating proteins with lower isolelectric points; 2) adsorption of the unretained fraction at pH 10 to a SP-5-PW cation exchanger and elution with a salt gradient (Fig. 8). A void volume peak of neutral material was obtained and a single major peak eluted at 17.5 min (approximately 0.06M salt). Although the minor peaks showed activity towards BAPNA, only the 17.5 min. peak had the properties of a homogeneous protein, as judged by spectral and SDS PAGE analysis (not shown),and was used for further characterization.

Characterization

The peak obtained from ion exchange chromatography gave a single protein band on SDS-PAGE using Coomassie blue and the more sensitive silver stain (see Fig. 6, lane 4, for comparison with neat gut juice and the radiolabeled fraction). A molecular mass of 25 kDa was determined for the purified enzyme using 15% acrylamide gel. Adsorption of the purified enzyme to the cation exchange column and elution with a pH gradient gave an apparent isoelectric point of 10.3 (Fig. 9). Amino terminal sequencing was carried out and the results are given in Table 6 along with the amino acid sequences from other trypsins for comparison.

Table 7 gives the amino acid composition of the purified spruce budworm protease. Miller *et al.* (1974) pointed to the large difference in possible dicarboxylic acid residues in the *Manduca sexta* midgut trypsin compared to other trypsins as an explanation for its lower pI (pH 8:4). This difference was not apparent for the CFT-1 enzyme and instead we observed a 2-3 fold higher level of the basic amino acids contributed mostly by lysine. Besides lysine only threonine and alanine showed increased residue values compared with the other trypsins. Only 0.3 residues of

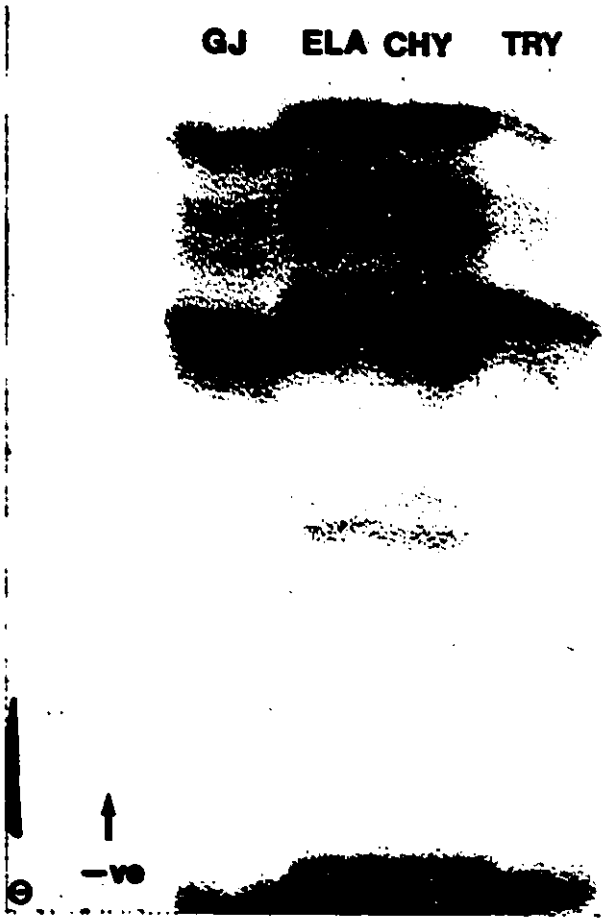

Figure 7. Autoradiogram of the partial acid hydrolysis generated peptides separated using HVPE at pH 2.1 .
Lane 1, neat gut juice; lane 2, elastase; lane 3, chymotrypsin; lane 4, trypsin. O - sample origin, arrow indicates direction of mobility. Note: Mobility in the gut juice lane was retarded due to the high level of contaminants.

methionine per molecule were obtained on analysis. This was confirmed by a single cleavage with cyanogen bromide which yielded two peptides with molecular masses of 18 kDa and 7 kDa and indicating the possible presence of one methionine.

Table 6. Comparison of the N-terminal sequence of CFT-1 with other trypsins[1]

```
CFT-1 IVGGSVTTIE Q*PSGSALLY S*NLVTYSQA AGGAILNTRS ILSAA-----
      ..... ...    .    .   .              .   .
Dtryp IVGGSATTIS SFPWQISLQR SGSHSCGGSI TSANIIVTAA HCLQSVSASV
      ....    .    .
Cocoo IVGGFTIGID TVPY-------------------------------------
      ....          .    .          .
Btryp IVGGYTCGAN TVPYQVSLNS GYHFCGGSLI NSQWVVTAAA HCYKSGIQVR
      ....          .    .
Hypod IVGGVEMKIE *FPWEI*LQ* P-----------------------------
```

A period (·) over an amino acid residue indicates homology with the CFT-1 sequence

* indicates residue not identified

1. Hypodermin, (Hypod), (*H. lineatum*), sequence from Tong *et al* (1981); Cocoonase, (Cocoo), (*A. polyphemus*), sequence from Law and Dunn (1977); Bovine, (Btryp) and Drosophila, (Dtryp), trypsin sequences from NBRF data bank accessed through the G.C.G. program (Devereux *et al.*, (1984)).

N-terminal sequence analysis of the 7 kDa peptide gave the following sequence THR-VAL-GLY-GLY-SER-PRO indicating that this peptide was the C-terminal fragment. The presence of a CNBr sensitive site that would yield peptides of 18 and 7 kDa is consistent with the known conserved methionine at position 166.

The purified enzyme had a high activity towards the trypsin substrates BAPNA and BAEE

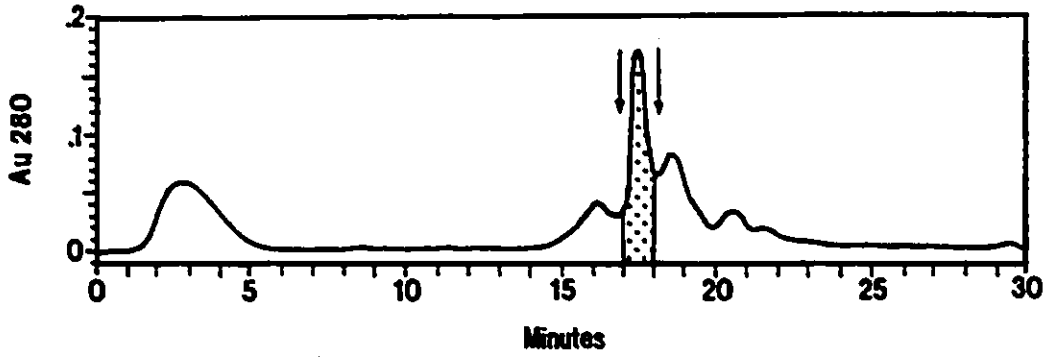

Figure 8. Chromatogram showing the HPLC cation exchange fractionation of gut juice. Initial conditions of 100% A-buffer were held for 5 min followed by a 25 min linear gradient to 20% of B buffer. A-buffer, 0.02M CAPS-NaOH, pH 10; B-buffer, 0.02M CAPS-NaOH, pH 10, containing 0.5 M NaCl; Flow rate 1 ml/min. Arrows indicate the purified fraction recovered.

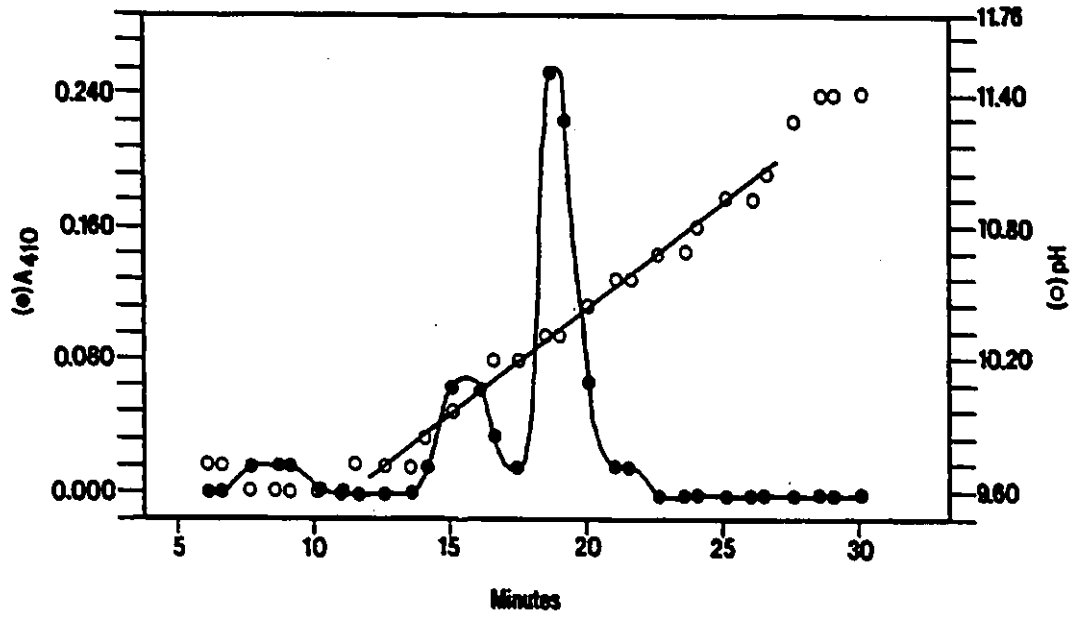

Figure 9. Chromatogram showing the HPLC cation exchange pH gradient elution of CFT-1.

Hydrolysis of DL-BAPNA was recorded for each fraction. Gradient conditions were as follows; 100% A-Buffer for 10 min then a linear gradient to 100% B-buffer at 30 min. with A-buffer at 0.02M CAPS-NaOH, pH 9.5 and B-buffer at 0.02M CAPS-NaOH, pH 11.5 and flow at 1 ml/min

(see below), but negligible activity towards BTPNA and ATAPNA. TLCK (a specific trypsin inhibitor), at a molar excess of 1.5:1, completely inhibited the enzymatic hydrolysis of BAEE, whereas TPCK (a specific chymotrypsin inhibitor) at the same excess gave no inhibition, indicating a narrow specificity for catalysis (data not shown). The K_m value for BAEE was less than 10^{-5} M and could not be accurately quantified due to the low concentration of substrate required. With DL-BAPNA as substrate a K_m value of 2.5×10^{-5} M was obtained. The pH-activity profile for V_{max} (Fig. 10) is sigmoidal with the rate dependent on an apparent ionization with a pK_a of 7.96 ± 0.19

Table 7. Comparison of amino acid analysis for purified CFT-1, Bovine trypsin and other insect trypsins

AMINO ACID	CFT-1	MANDUCA SEXTA[a]	HYPODERMIN A[b]	BOVINE TRYPSIN[a]
1/2 CYS	8	6	3-4	12
ASP	21	27	22	22
GLU	14	39	19	14
SER	18	20	22	33
GLY	30	28	20	25
HIS	5	3	5	3
ARG	13	14	11	2
THR	19	11	6	10
ALA	30	14	13	14
PRO	7	10	10	9
TYR	6	3	10	9
VAL	15	15	20	12
MET	1*	2	2	2
ILE	15	12	19	17
LEU	13	18	15	10
PHE	3	3	14	14
TRY	-	2	4	4
LYS	26	9	11	14

a Miller *et al.* (1974)
b Tong *et al.* (1981)
* less than one residue calculated per molecule
- not determined

(standard deviation of three determinations). Optimal activity is achieved at pH 9.5 and

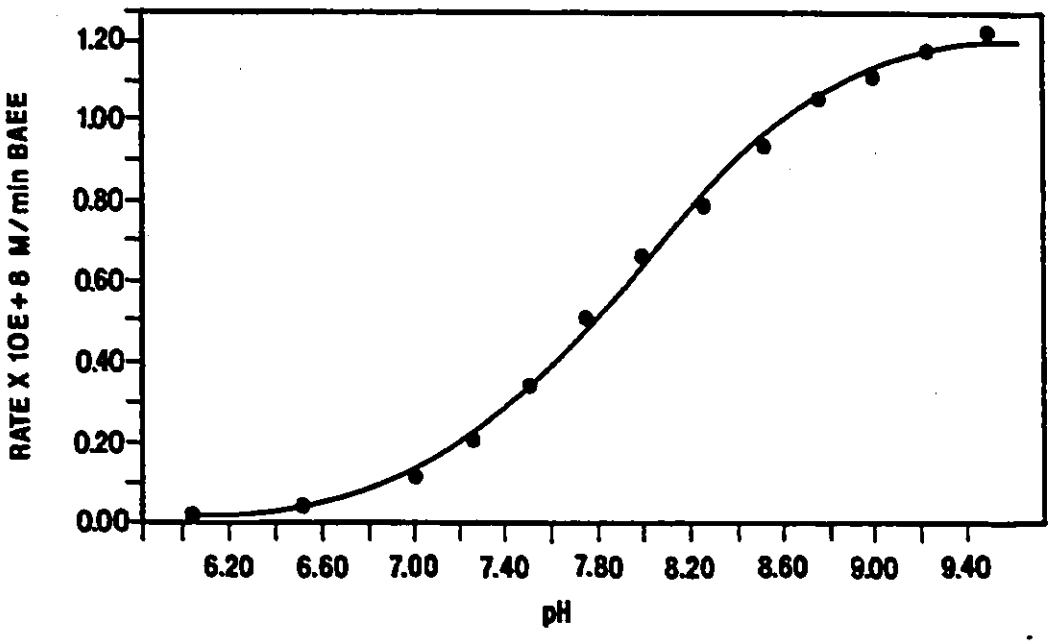

Figure 10. pH activity profile for the hydrolysis of BAEE with purified CFT-1 enzyme. The calculated pKa for the figure was 7.94. The reactions were carried out in 0.1N KCl at 25°C and the pH adjusted with NaOH

above. Based on these data the enzyme is trypsin-like in its activity and has been designated *Choristoneura fumiferana* Trypsin-1 (CFT-1). Burst kinetics were compared for bovine trypsin and CFT-1 using the trypsin specific titrant NPGB in an effort to establish a measure of active enzyme sites (Figure 11). Compared to bovine trypsin, significant deacylation occurs for the purified CFT-1 enzyme and the value of the "burst" was corrected according to the formula proposed by Bender *et al.*(1966). The calculation of active sites from burst kinetics and total protein determination by dye binding indicated that 70% of the purified protein fraction was active enzyme. This compares favorably with values reported for other purified serine proteases (Bender *et al.* 1966; Hruska *et al.* 1969)

A comparison of the peptide profiles from insulin B-chain digestion is given in Figure 12. The early digestion patterns showed that the major peptides produced were identical for trypsin, CFT-1 and gut juice. Interestingly the cleavage by bovine trypsin, which produced peak I I Ib was extremely slow for both insect enzyme preparations. A minor cleavage was also noted for the insect enzyme preparations, which produced peaks I Ib and I Ic. The time course indicated that these peptides coincided with a loss of peptide I Ia. Digestions were also carried out at pH 10 (0.1M CAPS-KOH) (not shown) to determine whether specificity was affected by the low pH of the conditions reported. Profiles were judged to be the same as for the pH 8 condition except for peptides initially produced by alkaline hydrolysis of the insulin B-chain.

Figure 13 shows a comparison of the proteolytic activation of the Bt protoxin with gut juice and the purified protease. Both enzyme samples were adjusted to the same level of BAPNA activity before comparison. Digestion of the Bt protoxin shows that the purified enzyme (lane 3) was not capable of producing the same end product of digestion (a single major protein band of approximately 60 kDa, lane 4) as gut juice, within the time constraints of the incubation. The rate of toxin production by the purified enzyme always lagged behind that of the whole gut juice (lane 5 compared to lane 6) in spite of the attempt to normalize the activity levels before hand. Limited

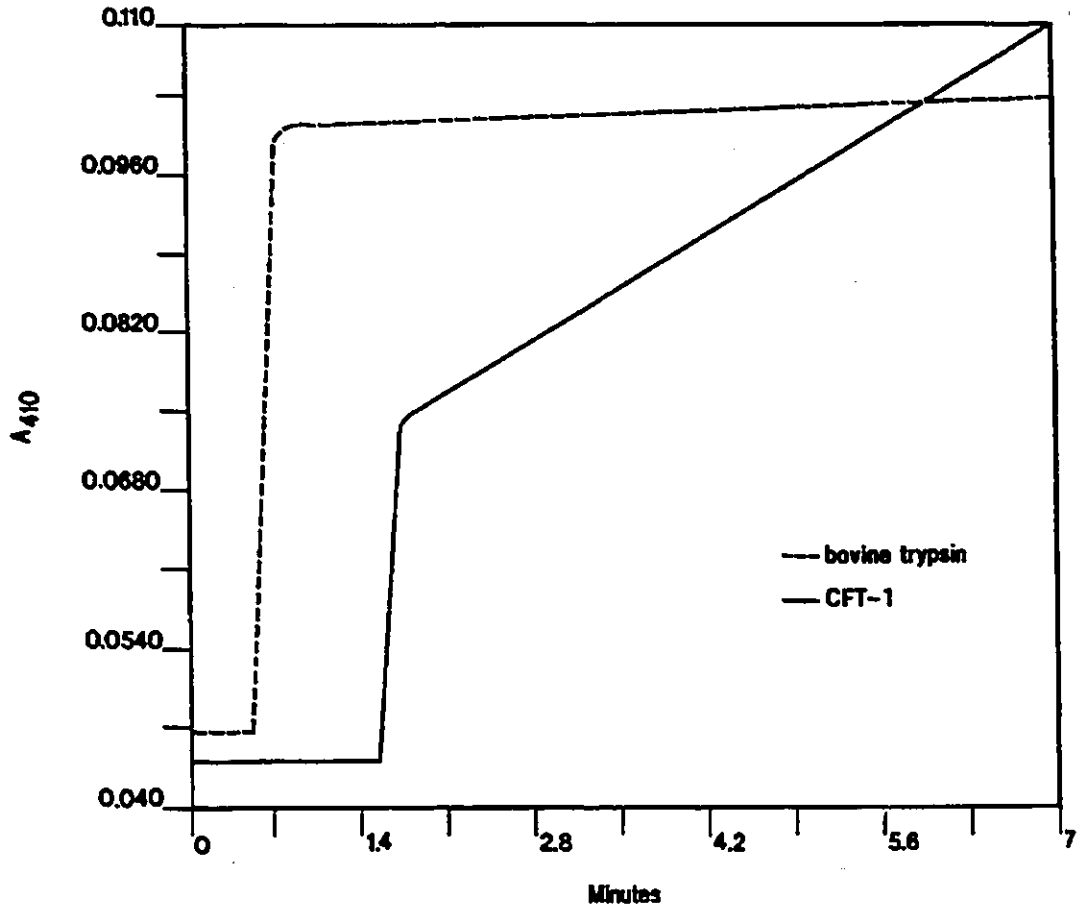

Figure 11. Comparison of the burst kinetics for bovine trypsin and purified CFT-1. The burst titrant was NPGB and the reaction was carried out at 25°C. From the traces, the burst for bovine trypsin represents 73 µg of active enzyme and for CFT-1, 40 µg active enzyme, based on an average molecular weight for both enzymes of 24 kDa

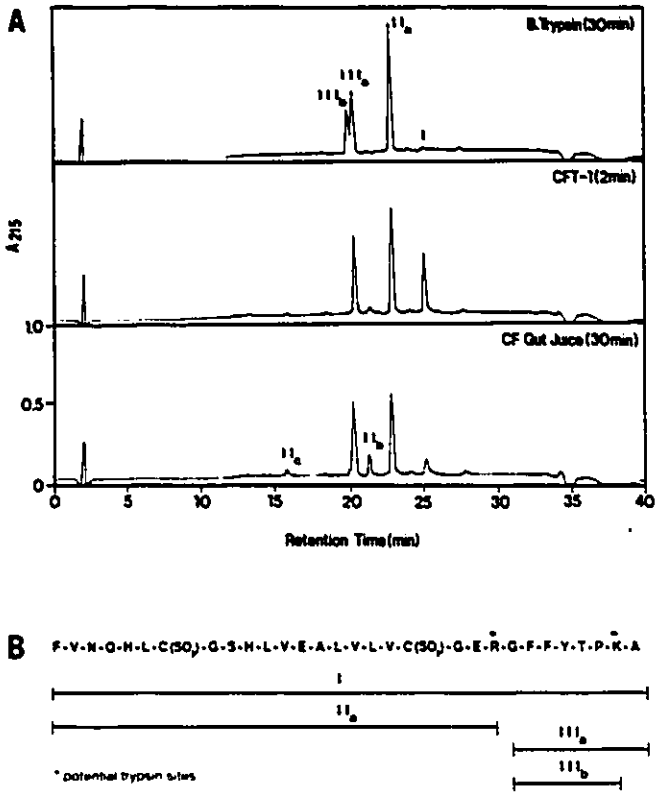

Figure 12. A; HPLC chromatograms showing the peptides generated by limited proteolytic digestion of the insulin B-chain with bovine trypsin, CFT-1 and gut juice.
Traces were chosen from the time course of each enzyme to reflect similar levels of activity. B; sequence of the insulin B-chain showing the potential peptides generated from trypsin specific hydrolysis.

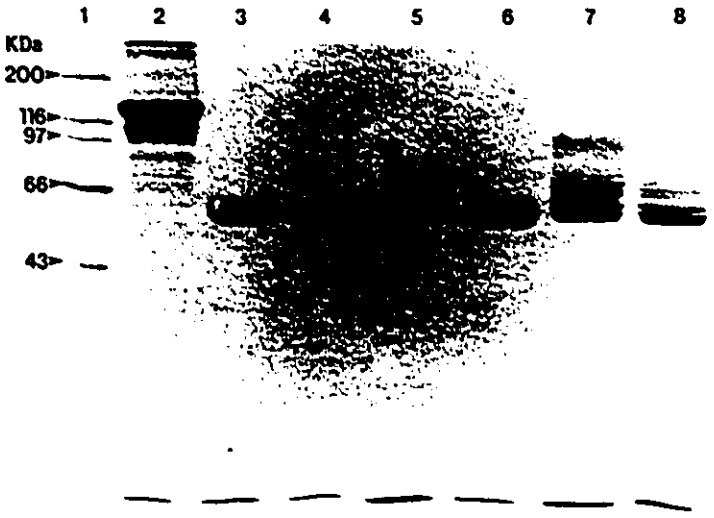

Figure 13. 15% SDS PAGE gel, Coomassie blue stained. Comparison of the proteolytic activation of the CrylA(c) protoxin at 1 mg/ml with neat gut juice and the purified CFT-1 enzyme.

Lane 1, molecular weight markers; lane 2, CrylA(c) protoxin; lanes 3 and 5 protoxin digested for 18 H with 0.75 and 0.075µg CFT-1/ml ; lanes 4 and 6 protoxin digested for 18 H with 0.3 and 0.03µl gut juice/ml ; lane 7, protoxin digested with CFT-1 at 0.075µg/ml for 90 min; lane 8, protoxin digested with gut juice at 0.075µl/ml for 15 min.

proteolysis of the δ-endotoxin for 90 min. with purified CFT-1 (lane 7) and 15 min. with gut juice (lane 8), shows that the higher molecular weight, intermediate products of digestion are similar.

DISCUSSION

Serine proteases appear to be responsible for all the endoprotease activity in spruce budworm gut juice since DFP treatment will completely abolish the conversion of Bt δ-endotoxin to toxin. This activity is associated with a single protein band with an apparent molecular mass of 25 kDa. The major activity in this band is trypsin-like in its specificity. The specificity for the minor cleavage seen in the peptide digestions has yet to be determined . While this band appeared to be homogeneous in terms of molecular mass and proteolytic specificity, it appears from the ion exchange chromatography (Figures 8 and 9) that there is trypsin-like activity associated with several components. The CFT-1 component comprises 70% of this activity and appears to be a stable component. It is not clear whether these other activities are isoenzymes or intermediates in the processing of a proCFT-1. Either of these possibilities could account for the apparently higher rate of activation of δ-endotoxin by the neat gut juice.

Mammalian serine proteases have a highly conserved active site sequence, asp-ser*-gly-gly (Naughton *et al.* 1960), and active site triad consisting of the residues serine-195, aspartic acid-102 and histidine-57. Furthermore a common amino terminus (isoleucine-16) is generated on activation. In comparison, CFT-1 yields the same series of radiolabeled peptides as the mammalian proteases after acid hydrolysis (Figure 7) and therefore probably has the sequence asp-ser*-gly-gly, in the immediate vicinity of the active site serine. The observation that the active site peptides from CFT-1, obtained by enzymatic digestion, are different, indicates that the sequence homology with mammalian proteases is lost beyond the immediate vicinity of the active site serine. Homology with the mammalian enzymes was also observed in the N-terminal region

(Table 6) where the first four residues and pro-13 and leu-19 from the amino terminus are identical. Catalysis by CFT-1 is dependent on a single ionization with a pK_a of approximately 8. Mammalian serine proteases show a very similar pH profile for V_{max} and this ionization with CFT-1 is likely the active site triad histidine residue. No information is given for CFT-1, that demonstrates an aspartic acid residue is involved in an active site triad. However, it appears likely that CFT-1 has the same active site triad as the mammalian serine proteases and is probably derived from an inactive precursor by proteolytic activation.

The high pH dependence for maximal hydrolysis of BAEE is consistent with other known lepidopteran gut enzymes (see introduction). The reader should note that comparisons between bovine trypsin and CFT-1 were made under conditions (pH~8), and with substrates that were established for the mammalian enzyme. Hydrolysis of the p-nitroanilide substrates with neat gut juice and digestions of the CryIA(c) protoxin with purified CFT-1 were carried out at pH 10, consistent with the pH activity profile of these gut enzymes and necessary for the solubility of the δ -endotoxin products.

It is common practice in biochemical studies to activate the Bt δ-endotoxin *in vitro* with bovine trypsin (Bietlot *et al.*, 1989). However, it has been our experience that the toxin produced by the insect gut juice enzymes can differ significantly in molecular weight. This study shows that the major activity in spruce budworm gut juice is a trypsin-like enzyme. Digestion of the δ-endotoxin with purified CFT-1 produces a toxin with an apparent molecular mass of 60 kDa (Figure 13) while bovine trypsin yields a 66-67 kDa product (Choma *et al.*, 1990). Therefore, while bovine trypsin and CFT-1 show similarities in terms of their specificity towards small substrates, there are likely significant differences in the rates and specificities with which they attack large proteins.

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CHAPTER 4

A PROTEIN COMPLEX FROM *CHORISTONEURA FUMIFERANA* GUT JUICE INVOLVED IN THE PRECIPITATION OF δ-ENDOTOXIN FROM *BACILLUS THURINGIENSIS*

The sciences do not try to explain, they hardly even try to interpret, they mainly make models. By a model is meant a mathematical construct which, with the addition of verbal interpretations , describes observed phenomena. The justification of such a mathematical construct is solely and precisely that it is expected to work.

John von Neumann

INTRODUCTION

The δ-endotoxins produced by *Bacillus thuringiensis* (Bt) have been shown to be pathogenic to at least 3 orders of insects, including Lepidoptera, Diptera and Coleoptera (Höfte and Whiteley, 1989). These toxins are produced during sporulation of the bacterium and accumulate in the sporangium as insoluble crystalline protoxins (Bechtel and Bulla, 1976). Activation of the toxin in the lepidopteran gut is thought to be a two step process where high alkaline conditions (pH 9.5-10.5) in the gut initially solubilize the crystal protein, then proteases convert the 130 kDa protoxin to a 60-70 kDa toxin (Tojo and Aizawa, 1983).

The enzymatic activation of Bt δ-endotoxin *in vitro* with commercially available mammalian trypsins has been widely employed as a routine procedure for the generation of toxin used in structure-activity studies (Bietlot *et al.*, 1989; Ogiwara *et al.*, 1992; Almond and Dean, 1993; Schwartz *et al.*, 1993). This trypsin activation has been shown to occur by sequential proteolytic cleavages starting at the C-terminus of the 130 kDa protoxin and proceeding in the N-terminal direction, until a proteolytically stable 55-65 kDa toxin remains (Chestukhina *et al.*, 1982; Choma *et al.*, 1990,). However, in the insect gut the generation of toxic activity may not be as straightforward, since several enzymes in the gut-juice of susceptible larvae have been implicated either directly or indirectly in the action of toxin. Recently, alkaline phosphatases have been shown to interact with toxin (English and Readdy, 1989), leucine aminopeptidase has been identified as a possible binding protein for one toxin (Knight *et al.*, 1994; Sangadala *et al.*, 1994) and DNases have been implicated in the processing of protoxin to toxin (Bietlot *et al.*, 1993). These enzymes, as well as other components present in the insect gut, could affect the ultimate yield of toxin and/or the expression of toxicity.

We have previously reported the purification and characterization of a trypsin-like enzyme from *Choristoneura fumiferana* (spruce budworm) gut-juice responsible for the activation of toxin (Milne and Kaplan, 1993). These studies were carried out using the substrate CrylA(c) protein from cultures of Bt subsp. *kurstaki* HD-73. In subsequent studies using the CrylA(a) protein from Bt subsp. *sotto*, we noted that the recovery of toxin after gut-juice activation was dependent on the concentration of the gut-juice. Higher levels of gut-juice resulted in lower yields of soluble toxin. We now report the purification and characterization of the gut-juice fraction responsible for the apparent loss of toxin during activation.

MATERIALS AND METHODS

Chemicals

Bovine trypsin type XIII, diisopropyl fluorophosphate (DFP), Nα-benzoyl-DL- arginine p-nitroanilide (BAPNA), N-acetyl-ala-ala-ala p-nitroaniline (ATAPNA), leucine p-nitroanilide (LPNA), p-nitrophenyl phosphate (p-NPP), deoxyribose nucleic acid (DNA), 3-(cyclohexylamino)-propanesulfonic acid (CAPS), dithiothreitol (DTT), Triton-x-100 and ethylenediamine tetraacetic acid (EDTA) were purchased from Sigma Chemical Co.; Polyamide sheets (5x5 cm) by Schleicher & Schuell from Pierce Chemical Co. and Gel Bond, Sea Kem and Sea plaque agarose from FMC Bioproducts. All electrophoresis reagents were supplied by BioRad Laboratories. Nutrient agar and trypticase soy broth was supplied by DIFCO Laboratories and 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) from Calbiochem Corporation.

Insect gut-juice

Larvae from the spruce budworm, *C. fumiferana* (Tortricidae), forest tent caterpillar, *Malacosoma disstria* (Lasiocampidae), gypsy moth, *Lymantria dispar* and white marked tussock moth, *Orgyia leucostigma* (Lymantriidae), reared on artificial diet, and field-collected larvae of *C. fumiferana* were used for gut-juice extraction. Gut-juice was collected from final instar larvae as previously described for spruce budworm using a micro-capillary tube and stored at -20°C (Milne and Kaplan, 1993). Gut-juice from *Bombyx mori* (Bombycidae) was similarly collected from 5th instar larvae fed on fresh mulberry leaves. Care was taken to ensure that haemolymph did not contaminate the gut-juice samples.

C. fumiferana gut-juice was also treated with 10 µl DFP/ml gut-juice (c. 55 mM final concentration of DFP) to inactivate serine proteases, as described previously (Milne and Kaplan, 1993).

Insect bioassays

Toxicity assays for LD_{50} determinations were carried out using 4th instar *B. mori* larvae and *per os* microinjection as described previously (van Frankenhuyzen *et al.*, 1991).

Bt cultures

Bt subsps. *sotto, kurstaki* HD-1 and *kurstaki* HD-73 were grown from nutrient agar slants maintained at FPMI. The *sotto* isolate used was from a slant originally obtained from Ishiwata (Angus, 1956) and is designated in house as *sotto i.* Both HD-1 and HD-73 were grown in 1/2 strength trypticase soy broth as described previously (Milne and Kaplan, 1993). *Sotto* was grown

on nutrient agar plates for 72 h at 30°C or until judged to be maximally lysed. Spores and crystals were harvested by scraping and washing the surface with 0.1M Tris, pH 7 buffer containing 1 M NaCl, 0.01% Triton X-100 and 0.01M EDTA. After centrifugation, pellets were washed 2× in the same buffer and then stored at 4°C. *Sotto* crystals were purified using discontinuous Renografin gradients, as previously described (Milne *et al.*, 1977).

HD-1 and HD-73 toxins were prepared using bovine trypsin activation as previously described (Bietlot *et al.*, 1989). Although HD-1 has been reported to contain P2 toxin (Nicholls *et al.*, 1989) a previous study (van Frankenhuyzen et al. 1991) indicated little or no P2 activity in the culture used here. The preparation of *sotto* toxin is described below.

Sotto δ-endotoxin activation and purification

Crystals (approx. 2 mg/ml) were solubilized from a crude spore-crystal suspension using 0.1M CAPS, pH 10.5, containing 4 mg/ml DTT and then incubated 1 h at 25°C. After centrifugation the supernatant was removed and $(NH_4)_2SO_4$ added to 50% saturation. Precipitation of protein was carried out for 1 h on ice, and centrifugation was repeated. The pellet containing protoxin was resuspended in 0.1M CAPS, pH 10.5, and then digested with 1 mg/ml bovine trypsin for 18 h at 25°C. The activated toxin solution was filtered using a 0.22 μ Millex filter (Millipore). Chromatography was carried out using a DEAE 5PW (75 x 7.5 mm) column (BioRad) equilibrated with 0.1M CAPS, pH 9.75, at 1 ml/min with elution using a linear NaCl gradient from 0 to 0.4 M over 25 min. Toxin activity was identified by applying 100 μl of column fractions to the surface of c. 10 cm^2 of a mulberry leaf-disc and allowing 5 newly molted 4^{th} instar *B. mori* larvae to feed *ab libitum*.

Agarose gel diffusion

The following method was developed in collaboration with Dr. A. Pang at the Forest Pest Management Institute. Gel diffusion was carried out using 1% agarose (Sea Kem) in barbital buffer pH 8.6. Trypsin-activated toxins from HD-1, HD-73 and *sotto* (1 mg/ml), or activated and column purified *sotto* toxin (0.25 mg/ml) at 10 µl/well and gut-juice preparations at 10 µl/well were allowed to diffuse 18-96 h in a moist box at 25°C. Precipitation was normally observed as an opaque line after as little as 12 h incubation. The gel was washed overnight in phosphate buffered saline (PBS), pH 7.3, with two changes of buffer, and then with water for 4 h. The gel was then dried and stained with Coomassie blue R-250 (CBB). In addition, 25 µl *C. fumiferana* gut-juice (diluted 1:1 in 0.1M CAPS, pH 10) was pretreated for 1 h at room temperature prior to diffusion against HD-1 activated toxin with one of the following: 5 µl 0.1M EDTA, 10µl 100 mM 1,10 phenanthroline, 10µl 10% SDS or 5 min in a boiling water bath.

HD-1 toxin was used only in the first diffusion test (see Results Fig. 14) and HD-73 toxin was only used in the comparison with *sotto* toxin vs column fractions (see Results, Fig. 17).

A modification of the above diffusion test was used to identify the precipitating protein separated by native gel electrophoresis. Activated soluble toxin (from *sotto*) was incorporated in 1% agarose (Sea Plaque low temperature gelling) at 0.5 mg/ml and layered on Gel Bond (at approx. 2 ml/ 15 cm^2). An anion-exchange purified fraction of the toxin precipitating protein (see conditions below) was electrophoresed in a 10% native acrylamide gel (10 µl/well). A lane from the acrylamide gel containing the toxin precipitating protein was overlayed on the agarose/toxin gel and incubated at 25°C in a moist box. After 24 h the gels were separated. Both agarose and acrylamide gels were washed in 100 ml 0.1 M CAPS buffer pH 10.5 for 30 min to remove soluble toxin and then stained with Coomassie blue R-250 (the agarose gel was dried before staining). In this toxin-overlayed-agarose-diffusion test, the region of toxin precipitation in the agarose gel was

compared to an original Coomassie blue R-250 stained portion of the native acrylamide gel and to the acrylamide gel slice that was in contact with the agarose.

Toxin-overlayed-agarose-diffusion was repeated using DFP-treated gut-juice separated by SDS-PAGE. First the DFP-treated gut-juice was separated by anion-exchange chromatography (see conditions below). Then DFP-treated neat gut-juice (diluted 1:1 in 0.1 M CAPS buffer, pH 10.5) and DFP-treated column fractions (fraction #14 and 15 contained c. 100 µg/ml protein) were diluted 1:1 in SDS sample buffer and then boiled for 1 min. These samples (1 µl each) were electrophoresed on 12.5 % Phast SDS gels (three identical gels were prepared). After electrophoresis, one acrylamide gel was stained using a standard silver stain (BioRad). The second acrylamide gel was washed for 1 h in 100 ml 0.1M CAPS buffer, pH 10.5, with 4 changes of buffer. After being washed the gel was overlayed with an agarose/toxin gel (described above) and incubated 24 h at room temperature. Following incubation, the gels were separated and both gels were washed for 1 h in buffer with 4 changes. The acrylamide gel was stained with Coomassie blue R-250 and the agarose gel was similarly stained after drying. Following electrophoresis the third acrylamide gel was immediately stained with Coomassie blue R-250.

High performance liquid chromatography

C. fumiferana gut-juice (0.5 ml) was thawed and clarified by centrifugation. Ammonium sulfate was added to 50% saturation and the sample allowed to flocculate 18 h at 4°C. After centrifugation at 13.25 K RPM for 10 min, the pellet was resuspended in 0.5 ml 0.1 M CAPS, pH 10, and centrifugation was repeated to clarify. The supernatant was filtered at 0.22µ and 500 µl was injected on an SW 300 (300 x 8 mm) glass size exclusion column (Waters). Elution was carried out at 0.5 ml/min in 0.1 M Tris, pH 7, and 1 min fractions were collected. The fraction showing the maximum precipitation of toxin in the agarose diffusion test was rechromatographed

on a DEAE-5 PW (75 x 7.5m) column (BioRad). A pooled sample from four size exclusion runs (total 2 ml) was injected on the column and washed with 10 column volumes of 0.01M Tris, pH 7. Gradient elution was performed by a linear increase from 0-90% of 2M NaCl in the running buffer over 25 min. Gut-juice treated with DFP to inactivate serine proteases was treated identically.

Electrophoresis

SDS PAGE was carried out using either the Mini gel (BioRad) or the Phast system (Pharmacia) following the supplier's methods. Samples that contained gut-juice fractions were routinely prepared with sample buffer, which contained 1 mM AEBSF and 0.01 M EDTA. Native PAGE was carried out using 12% precast gels (BioRad) running in Tris-glycine buffer as recommended by the supplier. Samples were applied at 10 µl/well. Phast gels were run in 2 dimensions according to the manufacturer's instructions, except that the first dimension gel was not removed from the acetate backing. An anion exchange purified fraction of *C. fumiferana* gut-juice responsible for precipitating toxin was mixed with the SDS-PAGE sample buffer (not boiled) and then 1 µl samples were immediately electrophoresed in the 12.5% first dimension gel. A lane was then excised and floated in 1ml additional sample buffer and boiled for 1 min. Electrophoresis in the second dimension was also carried out in a 12.5% gel. Silver staining of 2-D Phast gels was adapted from the procedure outlined by BioRad Laboratories.

Blotting and sequencing

Samples separated by SDS-PAGE were electrophoretically transferred using a Trans-Blot SD (BioRad) apparatus to a PVDF (BioRad) membrane (prepared per supplier's instruction). Recommended transfer buffers included Towbin-buffer (Towbin et al., 1979) and 0.01 M CAPS,

pH 10, both with and without methanol. Passive blots were also performed with the above mentioned buffers for 24-72 h and with the native gel running buffer. Sequencing was performed in an Applied Biosystems pulsed liquid sequencer model 473A with microgradient PTH analysis by Queen's University Core Facility.

Enzyme assays

Alkaline phosphatase activity was measured using the chromogenic substrate p-NPP as follows. One tablet p-NPP was dissolved in 0.1M CAPS, pH 10.5, and 10 µl of each column fraction was added to 990 µl substrate. The rate of increase in A_{410} was monitored using a Beckman DU7 spectrophotometer and 6-cell transport. Trypsin and leucine aminopeptidase initial rates were assayed at pH 10.5 in 0.1M CAPS using BAPNA and LPNA, respectively, according to procedures reported by Christeller *et al.* (1989). Elastase-like activity was measured using a microtitre plate (Titertek Multiscan). Assay conditions were as follows: 10 µl of column fractions were incubated with 50 µl of 5 mM ATAPNA in 0.1M CAPS pH10.5. The change in A_{410} was recorded after 18 h incubation at room temperature. DNase activity was measured as an increase in A_{260} resulting from the digestion of DNA, following standard assay methods suggested by the supplier of DNase 1, with the following changes. Reaction buffer for gut-juice DNase included 0.01M CAPS pH 10 instead of sodium acetate (pH 5). All activities were normalized (peak activity = 1) to show where peak activities appeared with respect to the chromatographic profile and the precipitating reaction.

Carboxypeptidase activity was determined qualitatively by digesting Dns-gly-phe (carboxypeptidase-A) or Dns-gly arg (carboxypeptidase-B) and fluorescence detection of the generated Dns-gly after thin-layer chromatography in 1.5% formic acid on 5x5 cm polyamide sheets. Digestions of the dansyl peptides were carried out at 0.1 mM substrate in 10 µl of column

fractions for 2 h at room temperature. Then 1 µl was spotted and chromatographed on a polyamide sheet. This procedure enabled identification of carboxypeptidase activities in the complex mixture of proteins unresolved by HPLC.

Dns-gly-phe and Dns-gly-arg were prepared from their respective dipeptides according to the method of Gray and Hartley (1963), purified by high voltage paper electrophoresis and quantified by amino acid analysis of phenylalanine and arginine following hydrolysis.

Proteolysis of toxin

Sotto protoxin at 1 mg/ml in 0.1 M CAPS, pH 10.5, was incubated with 25 µl gut-juice for 3 h at 25°C and the suspension was stored 18 h at 4°C. The sample was centrifuged and the pellet resolubilized by addition of 0.25 ml of 1M KSCN. The solution was diluted to 1.0 ml with 0.1M Tris, pH 7. An identical sample was prepared using bovine trypsin at 1 mg/ml and, although no pellet was formed, KSCN was added to the reaction mixture to give 0.25 M. The resolubilized gut-juice-activated sample was chromatographed on the SW 300 column and compared to the trypsin-activated sample. Column peaks were further analyzed by SDS-PAGE analysis and larval bioassay.

RESULTS

Agarose diffusion test

Figure 14A and B shows the diffusion of various gut-juices against the protoxin and toxin derived from HD-1. Preliminary diffusion tests (not shown) revealed that extract alone from the artificial diet used to rear the larvae did not give a precipitin line with toxin. Although, in the one

diffusion test shown (Fig. 14A), protoxin was used, it is likely that the gut enzymes would have activated a portion of protoxin to toxin resulting in similar patterns. The undigested protoxin is likely responsible for the strong staining surrounding the centre well. A small difference in precipitation patterns was observed between the protoxin and toxin for *B. mori* and *O. leucostigma* gut-juices. Gut-juice from both field-collected and lab-reared *C. fumiferana* larvae gave precipitin lines showing that precipitation was not unique to the laboratory reared larvae. All other gut-juices gave precipitin lines for both protoxin and toxin, with *B. mori* appearing to give the overall weakest response. Pretreatment of gut-juice with either 28 mM 1,10-phenanthroline, 0.02 M EDTA or boiling had no effect on the precipitation reaction nor did the direct incorporation of 0.1 M EDTA into the agarose gel. Only the pretreatment of gut-juice with 2.8% SDS significantly reduced the precipitation (data not shown). Figures 14C and D show the dilution of *C. fumiferana* gut-juice vs HD-1 toxin and dilution of HD-1 toxin vs *C. fumiferana* gut-juice. The precipitin line became very weak after toxin was diluted four fold. However gut-juice diluted 32 fold continued to produce precipitation.

Fractionation of gut-juice proteins

C. fumiferana gut-juice was fractionated by size exclusion chromatography (Fig. 15) and the precipitating factor(s) was found in a fraction eluting just after the void volume. The precipitating fraction was then subjected to anion-exchange chromatography (Fig. 16). Again, the precipitating factor(s) eluted in a single fraction (at 0.9-1.0 M NaCl) and gave a UV spectrum with maximum absorbance at 280 nm (not shown), indicative of protein comprising the major component. The column fractions causing precipitation were identified by diffusion of the fractions against toxins (Fig. 17). *Sotto* and HD-73 toxins were compared, but only the *sotto* toxin yielded the readily

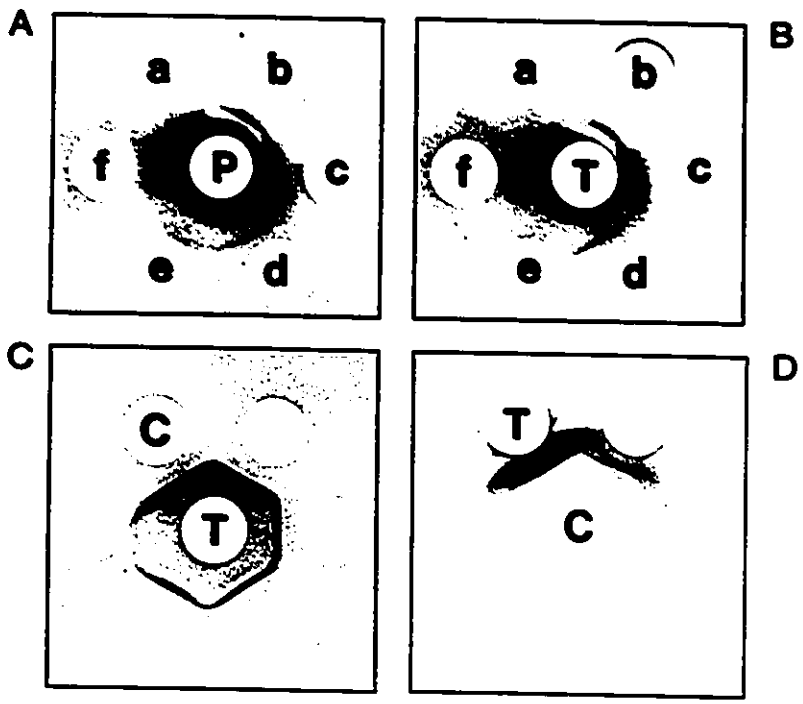

Figure 14. Agarose diffusion tests showing gut-juice from different lepidopteran larvae causing precipitation of protoxin and toxin from HD-1:
A) centre well (P) contained HD-1 protoxin; outer wells contained gut-juice from (a) *L. dispar*, (b) *C. fumiferana* (field-collected), (c) *C. fumiferana* (lab-reared), (d) *M. disstria*, (e) *O. leucostigma*, (f) *B. mori*; B) centre well (T) contained HD-1 toxin; outer wells contained gut-juices (same as A); C) shows stock lab-reared *C. fumiferana* gut-juice (C) and 2-fold serial dilutions of the gut-juice vs HD-1 toxin (T); D) shows stock (C) and 2-fold serial dilution of T vs C.

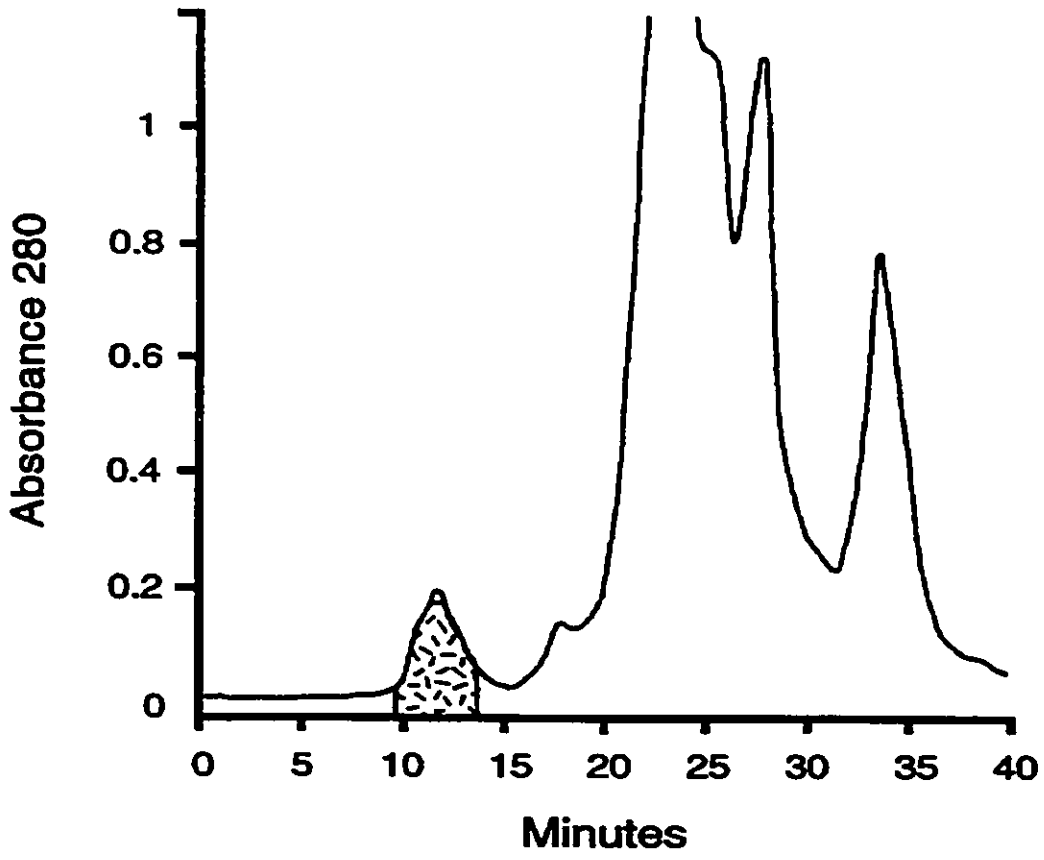

Figure 15. HPLC size exclusion chromatography of gut-juice from *C. fumiferana* using an SW 300 column.
Injection was 500 µl gut-juice, and elution was carried out with 0.1 M Tris pH 7.0 at 0.5 ml/min. One minute fractions were collected and the precipitating fraction identified by the agarose diffusion test. Cross hatching indicates fractions causing precipitation.

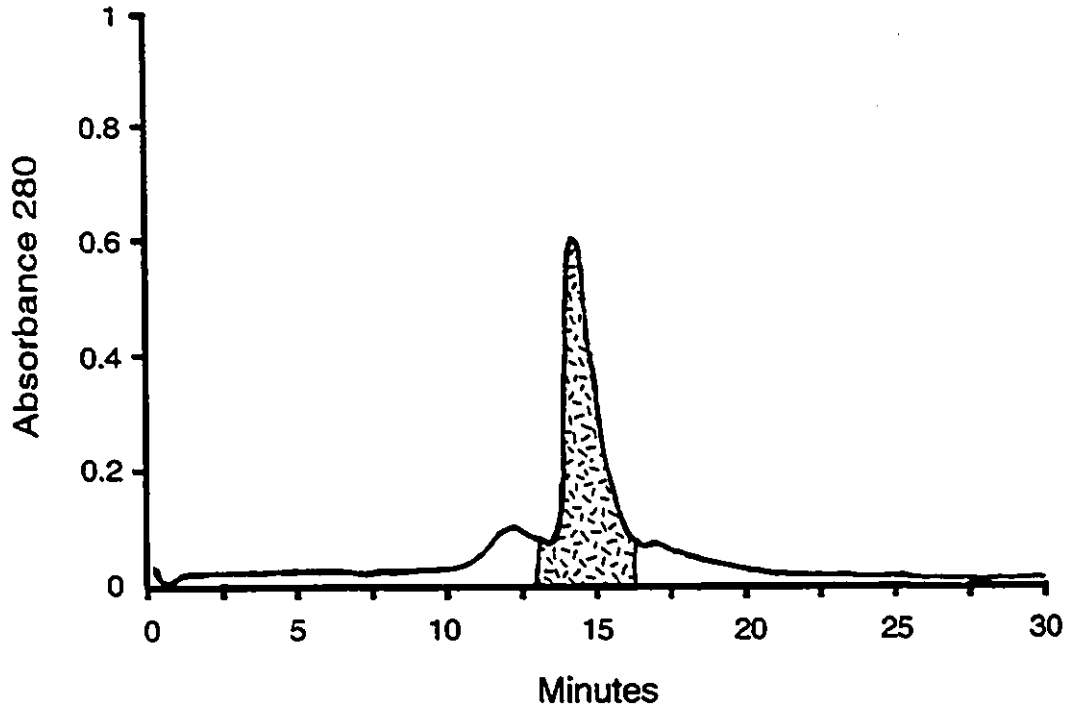

Figure 16. HPLC anion exchange chromatography of the precipitating fractions from the size exclusion chromatography, using a DEAE 5 PW column.
Injection was 2 ml of pooled the precipitating fractions. The column was then washed in running buffer followed by elution with a gradient of 0-90% 2 M NaCl beginning at 0 time for 25 min. Cross hatching indicates fractions causing precipitation.

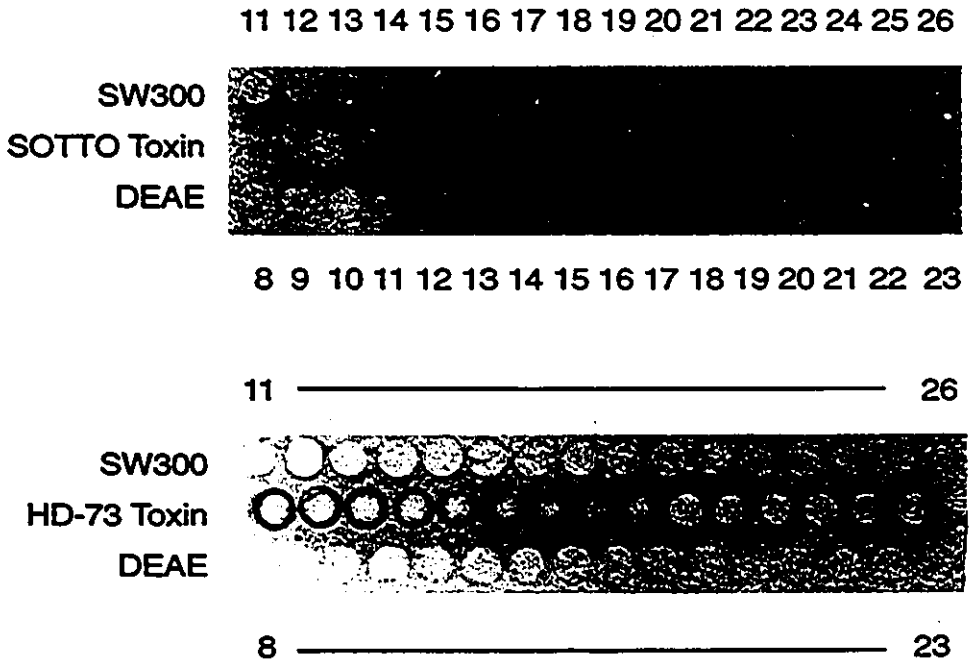

Figure 17. Agarose diffusion test showing diffusion of gut-juice fractions from the size exclusion and ion-exchange HPLC chromatography against toxin
: A) centre wells contained *sotto* toxin; upper row contained fractions 11 to 26 from size exclusion chromatography; lower row contained fractions 8 to 23 from DEAE chromatography; B) centre well contained HD-73 toxin; upper and lower wells same as (A).

visible precipitate. The size exclusion and anion-exchange separations were repeated with DFP-inhibited gut-juice with no apparent change in elution or precipitation patterns (data not shown).

The column fractions from size exclusion and ion exchange chromatography were subjected to SDS-PAGE analysis (Fig. 18). Proteins with apparent molecular masses > 200,000 Da are present in both fractionations, but their elution patterns did not correspond with the precipitin reaction. The column fractions, that gave the strongest precipitin reaction contained a major protein component with an apparent molecular mass of approximately 75 kDa on SDS mini gels (Fig. 18a, lanes 11, 12 and 13; Fig. 18b, lanes 14, 15 and 16). This protein has been designated as the 75 kDa toxin precipitating protein (TPP-75). However, the elution pattern of a lower molecular weight component (TPP-27) with an apparent molecular mass of 27 kDa also appeared to coincide with precipitin reaction. When the samples were treated with, but not boiled in, the sample buffer prior to electrophoresis, the low molecular weight component at 27 kDa was not present (Fig. 18b, lanes 15B boiled vs 15NB not boiled). When a lane containing the TPP-75 (not boiled) band was excised from the Phast gel, boiled and subjected to electrophoresis in a second dimension, the low molecular weight component was again observed (Fig. 19). It therefore appeared that TPP-27 was a component of TPP-75 and was released or generated by boiling the sample. The samples prepared for Phast gel analysis (Fig. 19) were typically less contaminated with the higher molecular weight proteins (>200 kDa) than were samples prepared for the mini gels (Fig 18), because less starting material was used in the chromatography steps.

The apparent molecular masses of the TPP proteins are different in the two dimensional system from those listed in Fig. 18 (75 vs 97 kDa and 27 vs 35 kDa), because a different gel system was used (Pharmacia Phast System compared to the BioRad mini gel). This difference was consistently observed. It has been reported previously that the Phast gel system can give rise

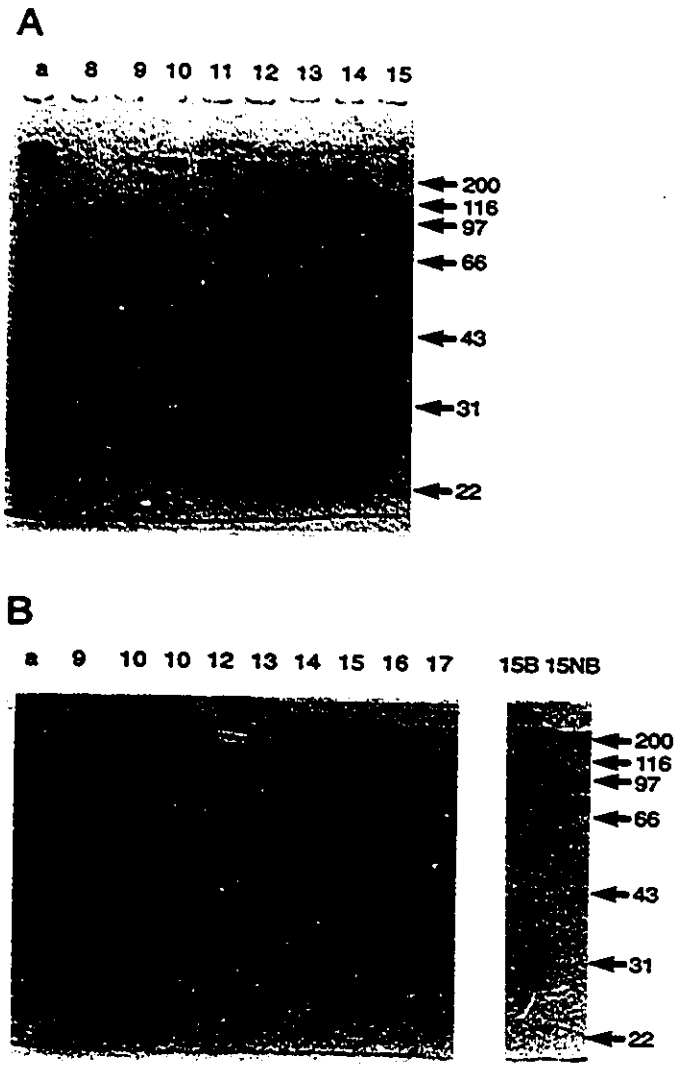

Figure 18. 12% SDS PAGE gels showing the electrophoresis of gut-juice fractions from HPLC chromatography, responsible for the precipitation of toxin.
(A) size exclusion fractions corresponding to Fig. 15; lane a = whole gut-juice, lanes 8-15 represent respective column fractions (B) anion exchange fractions corresponding to Fig. 16; lane a = pooled fraction from size exclusion chromatography, lanes 9-17 represent respective column fractions. Lane 15B shows the fraction boiled in sample buffer (same as lane 15) and lane 15NB shows the fraction in sample buffer but not boiled. Gels were stained with Coomassie blue R 250. Marker proteins; myosin 200 kDa, β-galactosidase 116 kDa, phosphorylase b 97 kDa, bovine serum albumin 66 kDa, ovalbumin 43 kDa, carbonic anhydrase 31 kDa, soya bean trypsin inhibitor 22 kDa.

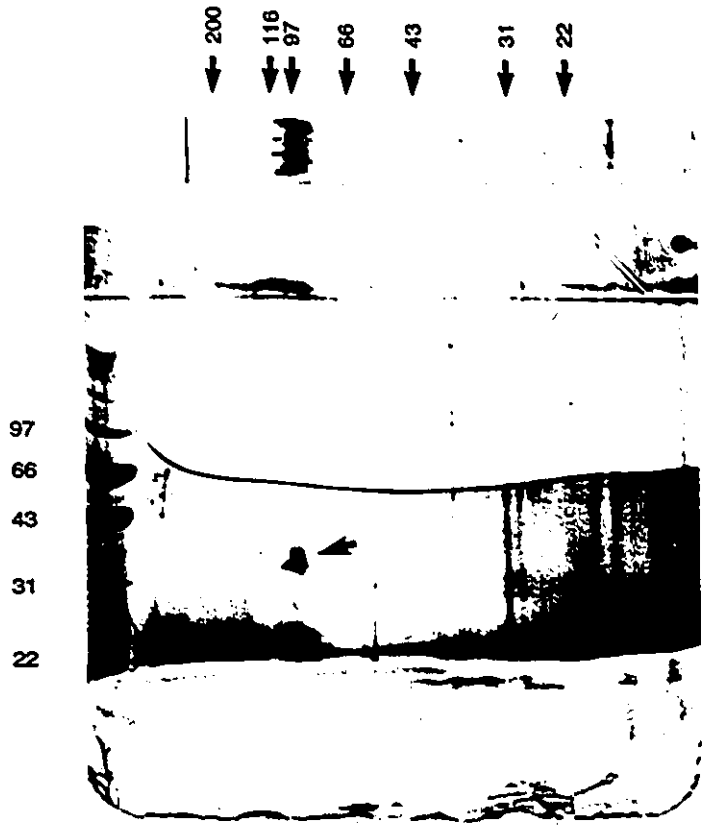

Figure 19. 2-D gel electrophoresis of the TPP-75 fraction from the anion exchange chromatography.
Upper gel shows an excised lane containing TPP-75 (not boiled) electrophoresed in the 1st dimension using a 12.5% SDS Phast gel. This excised gel was then boiled in additional sample buffer and applied to the lower gel for the 2nd dimension electrophoresis using a 12.5% SDS Phast gel. Marker proteins, see legend for Fig. 18. Arrow indicates the lower molecular weight component released after boiling.

to different apparent molecular masses than those obtained with the conventional homogeneous slab gels (Choma *et al.*, 1990).

Fractions from the size exclusion column containing TPP-75 were analyzed by native gel electrophoresis (Fig. 20, lane A). The gel was blotted to agarose containing soluble *sotto* toxin and a precipitate formed in the agarose gel (lane C), corresponding to the location of the stained band on the native gel. Stained material, which does not enter the separating gel, is visible but is not involved in the precipitation reaction and is likely a high molecular weight contaminant also observed on the SDS gels. The agarose/toxin gel (lane C) shows a region of protein concentration that aligns with a major band from the Coomassie-stained gel (lane A). More interesting was the apparent increased staining in the same region of the acrylamide gel (lane B) after blotting to the agarose/toxin gel. We have interpreted this increased staining in lane B to be due to a more rapid diffusion of toxin from the agarose gel into the acrylamide gel. A minor diffusion of the gut-juice protein must occur in the opposite direction (acrylamide to agarose), yielding the coincident region of precipitated protein in the agarose gel.

The blotting of toxin-incorporated agarose was repeated for gut-juice that was treated with DFP. Neat gut-juice and anion-exchange column fractions were electrophoresed in 12.5% Phast SDS gels. Fig. 21A shows the silver stained pattern and indicates that column fractions beginning at #14 (lane 4) begin to show a similar profile to the first dimension gel in Fig. 19. This gel (Fig. 21A) was overstained to indicate the absence of minor components in the column fractions. The acrylamide gel that was immediately stained with Coomassie blue R-250 (Fig. 21C) shows that very little protein is evident in lanes #1-7, but the five high molecular weight markers are easily identified. The acrylamide gel that was incubated in contact with the toxin/agarose gel and then washed (Fig 21B), shows an increased Coomassie blue R-250 staining in the region of protein noted in Fig. 21A, but only one of the molecular weight markers (97 kDa) and a contaminating band (c. 150 kDa) remained visible after the repeated washings. Of note is the band in Fig. 21B,

lane 1, representing whole gut-juice, which stains similar to the fractions (#14 and 15, lanes 4 and 5) purified by chromatography. The gels in Figs. 21A and 21B were run simultaneously on the Phast system and can be compared directly. The gel in Fig. 21C was run subsequently and the dye front appears to have migrated slightly farther. This migration difference does not obscure the dramatic difference in staining between Figs. 21B and 21C. A band observed in Fig. 21B, lanes 4 and 5 just below the c. 97 kDa band also shows a slight increase in staining and may be indicative of minor gut-juice components which also have an affinity for toxin or breakdown products of the major precipitating band.

The agarose/toxin gel that was incubated in contact with the SDS gel (not shown) and then washed similarly did not reveal a coincident pattern of precipitated protein as was observed in the native PAGE agarose/toxin gel described above.

PVDF blotting and N-terminal sequencing

N-terminal sequencing results for the proteins involved in toxin precipitation (TPP-75 and TPP-27) are summarized in Table 8. Blotting to a PVDF membrane from the SDS PAGE gels was only successful for the 27 kDa band (TPP-27). Several attempts were made to transfer the 75 kDa band (TPP-75) using both passive and electrophoretic transfer techniques in low and high pH buffers, but they were unsuccessful. However, transfer of TPP-75 was achieved after separation in 12% native PAGE gels (similar to Fig. 20A), followed by passive blotting with the native gel

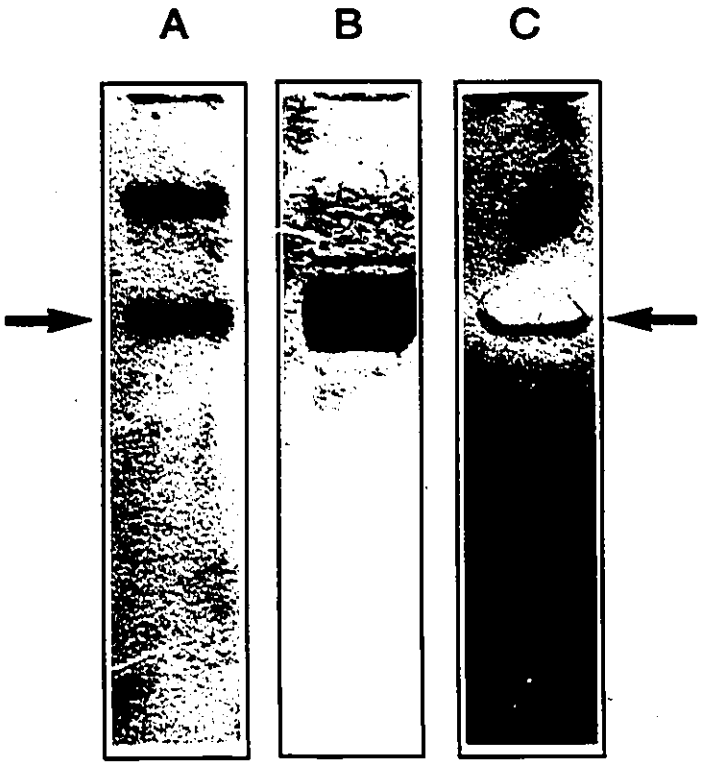

Figure 20. Native electrophoresis of TPP-75 followed by blotting to agarose with *sotto* toxin incorporated.
Lane A, native PAGE; Lane B, native acrylamide gel after blotting; Lane C, agarose gel after blotting. All gels were stained with Coomassie blue. Arrow on the right indicates the region of precipitation of toxin and the arrow on the left shows the corresponding protein band in the native gel.

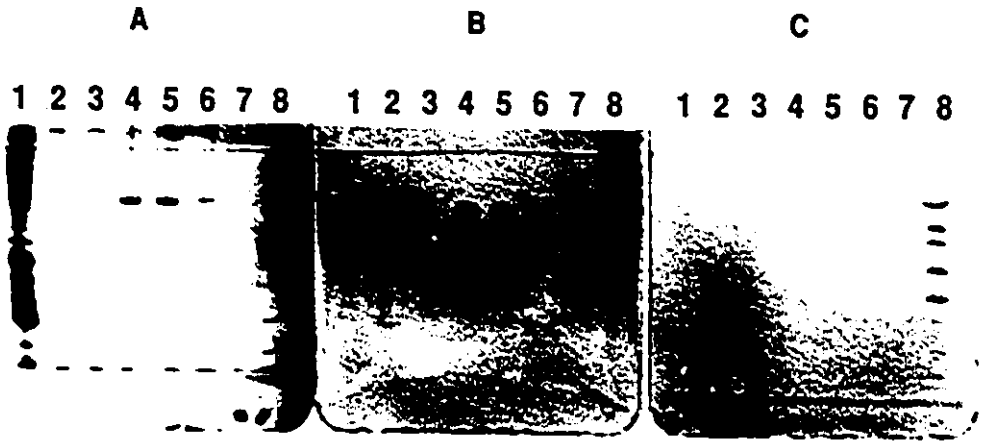

Figure 21. 12.5% Phast gel SDS-PAGE of anion-exchange column fractions containing TPP-75, followed by blotting to agarose with *sotto* toxin incorporated.
Lane 1= *C. fumiferana* gut juice, lanes 2-7 = fractions from anion-exchange similar to fractions 12-17, Fig. 16B, lane 8 = high molecular weight markers. (A) after electrophoresis the gel was stained with silver stain. (B) after electrophoresis the gel was washed in buffer then incubated in contact with an agarose/toxin gel and again washed in buffer before staining with Coomassie blue R-250. (C) after electrophoresis the gel was stained with Coomassie blue R-250. Marker proteins; myosin 200 kDa, β-galactosidase 116 kDa, phosphorylase b 97 kDa, bovine serum albumin 66 kDa, ovalbumin 43 kDa

buffer to a PVDF membrane. The sequences obtained were compared to known proteins in the data banks using the GCG programs (Devereux *et al.*, 1984). No matches were found at the 60% similarity level.

Table 8. N- terminal amino acid sequences obtained for TPP-75 and TPP-27

TPP-75	LSVTDDAFAGYTTQNPDL
TPP-27	FLAALGENPT

Enzyme activities in gut-juice

The gut-juice fractions from size exclusion chromatography (Fig. 15) were characterized with regard to their enzymatic activities. Fig. 22 shows the normalized activities (substrate in brackets) of alkaline phosphatase (p-NPP), DNase (DNA), trypsin (BAPNA), and leucine aminopeptidase (LPNA). The carboxypeptidase activities were only determined qualitatively but their profiles (not shown) appeared similar to that of the trypsin-like activity. Carboxypeptidase A (Dns-F) and B (Dns-R) activity and trypsin-like activity eluted in the large peak at 24 min (Fig. 15), far from the precipitating fraction. Only the peak of elastase-like (ATAPNA) activity coincided with those fractions which precipitated the toxin in the agarose diffusion test. Similarly, the protein peak from the anion-exchange chromatography, which precipitated the toxin, also gave a corresponding peak of elastase-like activity (not shown).

Proteolysis of *sotto* toxin

Size exclusion chromatography of trypsin-activated toxin, solubilized with KSCN, resulted in a single major toxin peak at 22.5 min (Fig. 23a). The resolubilized precipitate of *sotto* protoxin

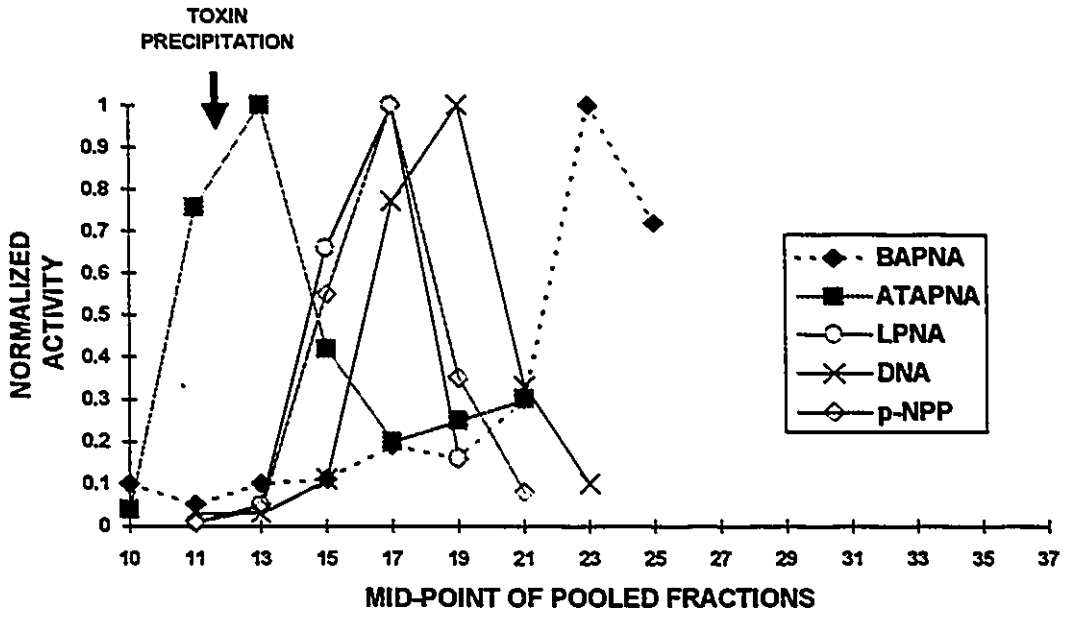

Figure 22. Enzyme activities in *C. fumiferana* gut-juice fractions separated by HPLC size exclusion chromatography (see Fig. 15), according to the substrates hydrolyzed.
Rates of hydrolysis were normalized in order to compare regions of peak activity. Arrow indicates the peak region of toxin precipitation corresponding to the SW 300 fractions in Fig. 17.

activated with gut-juice gave two major peaks (Fig. 23b). Only the second peak at 23 min in Fig. 23b corresponded to the bovine trypsin-generated toxin (22.5 min, Fig. 23a). Differences in retention times of less than 1 min were not considered significant. From these chromatograms it was determined that c. 85% of the gut-juice-activated toxin, relative to that of the trypsin-activated toxin, was recovered in the precipitate. The single peak from trypsin activation and two peaks from gut-juice activation were subjected to SDS PAGE analysis (differences in the apparent amount of protein recovered from the size exclusion fractions for trypsin-generated and gut-juice-generated toxins were due to peak elutions distributed over more than one fraction). Figure 24, lane 1, shows that trypsin-generated toxin yields a single 60 kDa band, whereas only a portion of the precipitated gut-juice-generated toxin remains at 60 kDa (lane 2). One of the column-separated toxin fragments (lane 4) appears to have undergone proteolysis and its molecular weight was reduced by approximately 5 kDa (measurements were taken at the upper boundary of the stained bands). However, this lower MW toxin fragment eluted from the column with a broad peak at 16 min. This shorter retention time during gel exclusion chromatography suggested the 55 kDa fragment would have a much higher molecular weight. This aberrant behavior may have been due to an aggregation of the toxin, or an interaction with the column matrix.

In a force feed assay using *B. mori* larvae, the trypsin-activated toxin peak at 22.5 min had an LD_{50} of 0.9 ng/larva as compared to a previously reported LD_{50} of 2 ng/larva for a recombinant Cry1A(a) toxin (van Frankenhuyzen *et al.*, 1993). The gut-juice-activated toxin peak at 23 min had an LD_{50} of 1.0 ng/larva, and the toxin peak at 16 min had virtually no toxic activity, with an LD_{50} >> 100 ng/larva. In a separate test the doublet toxin (Fig. 24, lane 3) derived from the precipitate was blotted to a PVDF membrane and sequenced. Both toxin fragments yielded the same N-terminal sequence, I E T G Y, consistent with the published sequence beginning at position 29 (Shibano *et al.*, 1985).

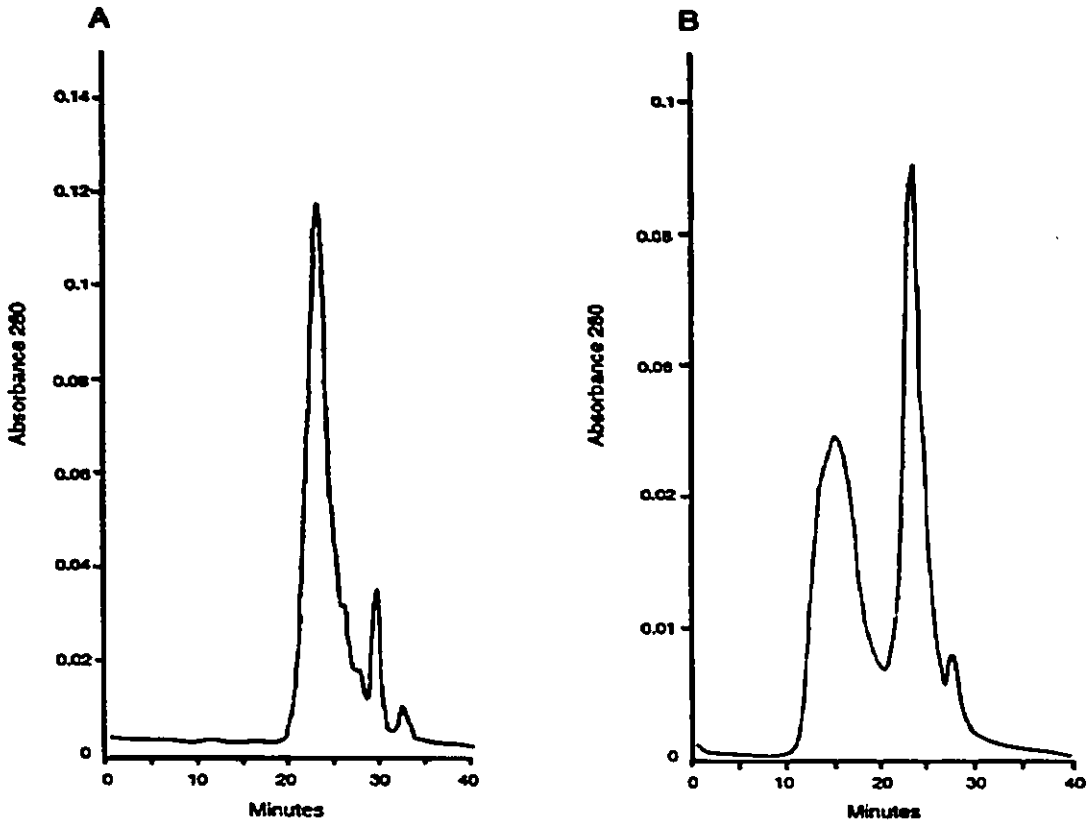

Figure 23. HPLC size exclusion chromatography of *sotto* protoxin (1 mg/ml) activated with either bovine trypsin or *C. fumiferana* gut-juice.
A.) 500 µl injection of *sotto* toxin activated with bovine trypsin. B.) 500 µl injection of *sotto* toxin activated with *C. fumiferana* gut-juice (pellet resuspended in 1 M KSCN). Column conditions; elution buffer was 0.1 M Tris, pH 7, with flow at 0.5 ml/min.

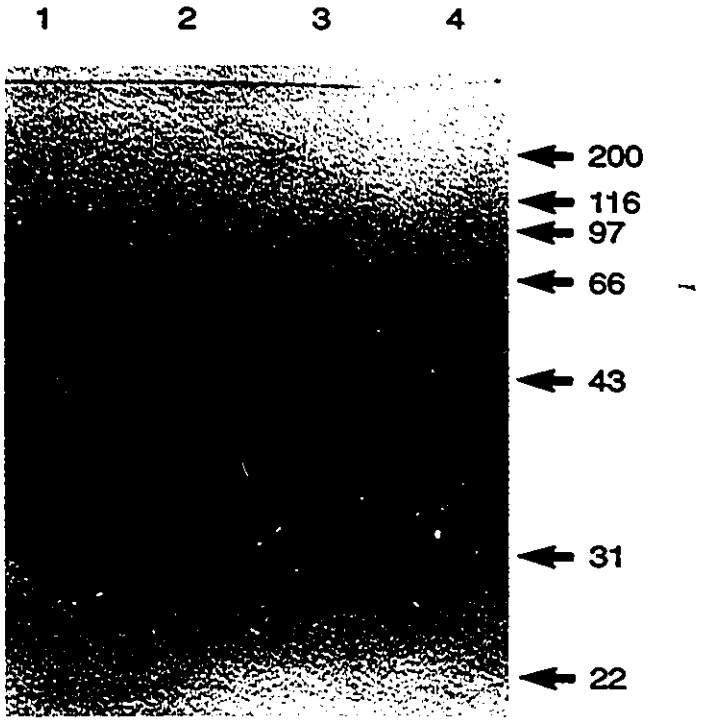

Figure 24. 10% SDS PAGE analysis of *sotto* toxin peaks from HPLC size exclusion chromatography(see Fig. 23).
Lane 1, peak at 22.5 min (Fig. 23A); Lane 2, peak at 23 min, ;Lane 3, mixture of equal volumes of peaks at 23 and 16 min (Fig 23 B) Lane 4 peak at 16 min (Fig. 23B). All samples were 10 µ l/well. Gel was stained with Coomassie blue R 250. Marker proteins, see legend for Fig. 5.(Fig 10B);

DISCUSSION

The recovery of soluble toxin from enzyme activated crystals (Table 3, Chapter 2) is dependent on the origin of the activating system. When a high concentration of gut-juice from *C. fumiferana* is used, the total soluble protein recovered is low. This low recovery of protein is due to precipitation of the activated toxin molecule. None of the previously identified proteins/enzymes, which interact with toxin (see Introduction), coincided with the TPP-75 fraction. Evidence has been obtained for a 75 kDa protein complex, as an anionic component in *C. fumiferana* gut-juice, responsible for the precipitation of the δ-endotoxin from *Bt sotto*. A 27 kDa protein can be generated from this complex, but we have been unable to identify the remaining component(s) of this complex. The 75 kDa complex enters a 12% gel but, on dissociation of the complex by boiling in SDS sample buffer, only the 27 kDa migrates and the other components remain in the stacking gel (Fig. 19). Furthermore, we were unable to blot the 75 kDa protein to PVDF membrane from SDS gels for sequencing. It appears that when unfolded, one of the components of the complex interacted strongly with the acrylamide and prevented recovery. This poor recovery was partially resolved by native gel electrophoresis of the TPP-75 complex, allowing both blotting to a PVDF membrane for sequencing and migration of the complex into a toxin/agarose gel to show coincident precipitation.

We have taken advantage of the poor mobility once the TPP-75 complex has migrated into the acrylamide matrix. After electrophoresis of TPP-75 in SDS-PAGE (Fig. 21), the gel was washed extensively. Nevertheless, sufficient TPP-75 protein remained in the gel to react with the toxin and cause a dramatic increase in protein staining. This increased staining was interpreted as an accumulation of toxin in the acrylamide due to a high affinity for the TPP-75 complex.

TPP-75 fractionated by size exclusion and anion-exchange chromatographies showed a

very low level of elastase-like activity. This low level of activity is associated with a region of the size exclusion chromatogram that represents less than 1% of the total chromatographic material measured at A_{280}. This type-1 elastase-like activity in *C. fumiferana* gut-juice was not observed previously (Milne and Kaplan, 1993). An extended assay of 18 h was required to measure activity in the size exclusion fractions. The incubation was shortened to 4 h for the anion-exchange fractions (not shown), indicating a concentration of the activity after ion-exchange. This type-1 elastase-like activity is not to be confused with a type-2 activity reported by Christeller *et al.* (1992) for a major cationic component found in several lepidopteran guts.

Given the apparent molecular mass of 75 kDa for TPP-75, its associated serine protease-like activity, and the dissociation of a lower molecular weight component on boiling in SDS sample buffer, we suspect that this protein is a complex, that contains an elastase-like component. Of note is that the substrate used in this study, which is diagnostic for elastase-like activity, does not differentiate between proteolytic and amidolytic activities and therefore additional investigation will be required to further characterize the TPP-75 protein complex.

A search of the GenBank data bases using the GCG program (Devereux *et al.*, 1984) did not result in sequence matches greater than 60% for either TPP-75 or TPP-27. Although, protease inhibitors were included in the SDS-PAGE sample buffer when TPP-27 was generated for sequencing, we cannot rule out proteolysis at the boiling stage. We suspect that TPP-27 dissociates from the TPP-75 complex during boiling, as a result of denaturation of the complex. However, the difference in the N-terminal sequences between TPP-75 and TPP-27 may be due to proteolysis by enzymes (Inagaki et al., 1992) present during the preparation of fractions for sequencing. Alternatively, the two sequences could represent two different proteins in the complex.

We have shown that this precipitation event is dependent on the concentration of toxin, and that gut-juice can be diluted several fold before losing the ability to precipitate the toxin. This dependence on toxin concentration is possibly due to the inability of the test presented here to

detect low levels of precipitate. The inhibition of serine proteases by DFP, or metalloproteinases by 1,10-phenanthroline, does not affect the precipitation reaction. Similarly, neither the inclusion of EDTA in the agarose substrate nor boiling of the gut-juice inhibit the reaction. The pretreament of gut-juice with SDS resulted in little or no precipitation, which may be due to the general dissociating effects of SDS on proteins. Of note is that after SDS is washed from the PAGE gel (Fig. 21) containing TPP-75, the precipitation of toxin is observed.

The precipitation in the agarose diffusion test often appeared to give more than one precipitin line (Fig. 14). This does not appear to be due to multiple toxin types associated with the HD-1 preparation. Purified *sotto* toxin also yielded secondary precipitin lines (Fig 17). These secondary precipitin lines may in part reflect the two fractions separated by size exclusion chromatography (Fig. 23b). We have focused on the major precipitation event, but acknowledge that several proteins in the gut milieu may react with toxins in a similar manner, resulting in precipitation.

In..ıbation of toxin with gut-juice results in the precipitation of virtually all the toxin. From the SDS gel profile of neat gut-juice proteins, it is evident that very little TPP-75 is present. Yet gut-juice diluted 32 fold continued to precipitate toxin in the diffusion test. Due to the low level of gut-juice protein required to precipitate the toxin, we have been unable to show quantitatively the amount of TPP-75 required to precipitate a given concentration of toxin. Similarly, TPP-75 did not appear as a separate peak near the void volume in the size exclusion chromatogram (Fig. 23b) due to its low level. Nevertheless, the precipitated toxin can be fractionated by size exclusion chromatography to reveal two components before SDS-PAGE analysis . One component (23 min peak, Fig. 23b) appears to be the same MW as toxin that has not been precipitated (22.5 min peak, Fig. 23a). The second component (16 min peak, Fig. 23b) has undergone major proteolysis. This fraction of toxin has lost its activity in assays against *B. mori*. In spite of the pretreatment of the precipitated toxin with a chaotropic agent (1 M KSCN), a portion of the toxin appears to be strongly

aggregated.

The appearance of toxin doublets during SDS PAGE analysis has been reported by other investigators (Knowles *et al.*, 1991; Inagaki *et al.*, 1992; Ogiwara *et al.*, 1992; Oppert *et al.*, 1994) . Some of the reported doublets are likely just the result of incomplete digestion of the protoxin, or minor processing at the N-terminus (Ogiwara *et al.*, 1992). However, from our observation that the N-terminus of toxin remained unchanged, it is possible that many of the observed doublets in samples containing the CrylA(a) protein are the result of an enzymatic activity cleaving the C-terminus, which eventually causes inactivation of the toxin. We propose that major enzymatic modification is not involved in the initial precipitation of toxin, because approximately half the precipitated sample appears to have undergone little or no hydrolysis, and the inhibition of major proteases does not interfere with the reaction. The co-precipitation of toxin and gut enzymes could, however, lead to an effective increase in the concentration of both enzyme and substrate, after which subsequent proteolysis would result in the lower molecular weight form of the toxin.

We have shown that precipitation of toxin with gut-juice occurs for both HD-1 [containing CrylA(a), (b), and(c) proteins] (Höfte and Whiteley, 1989) and *sotto* [CrylA(a)] (Pang and Mathieson, 1991), but not for HD-73 [CrylA(c)] (Höfte and Whiteley, 1989). Only the C-terminal half of these three toxins differ appreciably in their amino acid sequence. Since no precipitation was observed for the CrylA(c) protein from Bt-HD-73, but precipitation was observed for both HD-1 and *sotto,* we speculate that this reaction may be specific for the C-terminal portion of the CrylA(a) protein. It has been suggested that carbohydrate either attached to gut proteins (Sangadala *et al.*, 1994) or toxin (Bhattacharya *et al.*, 1993) or in solution (Knowles *et al.*, 1991), may play a role in the interaction of toxin with the larval gut proteins. Less than 1% of the mass of toxin may be composed of carbohydrate (Bhattacharya *et al.*, 1993). Therefore, it is unlikely that the loss of carbohydrate would account for the doublet observed in the precipitated toxin (Fig. 24, lane 3). We have not determined whether carbohydrate could be involved in the precipitation of a

specific toxin. Again, further investigation would be required to determine whether the primary amino acid sequence of the toxin C-terminal is solely responsible for the specificity of this reaction.

Oppert *et al.* (1994) have recently suggested that Bt resistant *Plodia interpunctella* larvae show an altered proteolysis profile with regards to midgut proteins acting on δ-endotoxin. They compared gut-juice from susceptible and resistant larvae by normalizing protease levels using BAPNA as substrate, but the difference in δ-endotoxin digestion patterns between susceptible and resistant larvae could not be accounted for by the trypsin-like activity alone.

Nevertheless, their conclusion was that an altered proteolytic processing may play a role in those larval resistance mechanisms that cannot be explained by differences in receptor-mediated binding. From our observations, the altered proteolytic processing may be mediated by toxin precipitating proteins in the gut. Interestingly, of the insects we tested, representing 4 different families, *B. mori* showed the least ability to precipitate toxin (Fig. 14a) and has previously been shown to exhibit the greatest susceptibility to the CrylA(a) toxin (van Frankenhuyzen *et al.*, 1993).

Yunovitz *et al.* (1986) have reported that toxin which has precipitated after dialysis does not affect mid gut cells when the peritrophic membrane is intact. We hypothesize that in several larval species the CrylA(a) toxin is sequestered by the TPP-75 complex, resulting in either physically limiting the movement of precipitated toxin through the semipermeable peritrophic membrane (Wolfersberger *et al.*, 1986), or eventual detoxification by proteolysis of the toxin. The overall effect would be to decrease the available active CrylA(a) toxin in those larva with TPP-75 present in their gut-juice, while other CrylA toxins would remain unsequestered. The present evidence suggests that toxin specificity is not determined solely by interaction with receptor proteins, but that interactions with the soluble endogenous proteins/enzymes in the gut-juice also play a critical role.

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CHAPTER 5

DNA ASSOCIATED WITH THE δ-ENDOTOXIN

"Yet if there really is a complete unified theory, it would also presumably determine our actions. And so the theory itself would determine the outcome of our search for it! And why should it determine that we come to the right conclusions from the evidence? Might it not equally well determine that we draw the wrong conclusion? Or no conclusion at all?"

Stephen Hawking... in, A Brief History of Time (1988)

INTRODUCTION

Little or no mention is made in the recent literature of the significance of DNA in preparations of δ-endotoxin (Bulla et al., 1980; Dean, 1984; Aronson et al., 1986). It is likely that early reports of the lack of nucleic acids associated with this protein (Bulla et al., 1977; Nickerson, 1980; Fast, 1981) interrupted any further investigation. However some very early studies did suggest the presence of DNA. Mattes (1927) first observed a concentration of "Giemsa-stained" material in the region of the sporangium beside the spore. He interpreted this material as a secondary nucleus or "nucleus-containing cell equivalent". Hannay and Fitz-James (1955) provided a chemical analysis of the crystalline inclusion which on the basis of nitrogen and phosphorus analysis suggested a small amount of nucleic acid in their preparation which was considered contamination. Alternatively they suggested a virus type particle may be involved. Nevertheless a quote from their discussion indicates they were not totally convinced. - *"It is probable that undue emphasis has been placed on the presence of an amount of phosphorus which is less than that usually found in protein preparations but is nevertheless a point which requires resolving".*

During the course of study to determine the digestive enzymes responsible for the activation of δ-endotoxin it became apparent that the poor recovery of purified toxin from anion exchange chromatography was not always due to the direct precipitation of the toxin by gut juice. Furthermore it was observed that the activation of Cry1A$_{(c)}$-type toxins with mammalian trypsin often resulted in two chromatographic species of toxin. Purified toxin, as determined by SDS-PAGE analysis, normally eluted from the anion exchange column at 0.3 M salt concentration. A second toxin peak was observed to elute during the washing phase at 0.8-0.9 M salt. Further investigation showed that the second toxin peak was associated with DNA.

Evidence for the *in vitro* association of DNA with toxin and its significance was primarily the work of Dr. H. Bietlot and was presented in detail in his doctoral thesis (Bietlot, 1993) and

published (Bietlot et al., 1993). A separate study was undertaken to determine the *in vivo* origin of the DNA that was associated with the toxin and whether or not the DNA influenced the activity of toxin. Details of both the tissue culture bioassays and the fluorescence photomicrography are presented here.

METHODS

Preparation of toxin

Toxin was generated from solubilized protoxin by a 24 h incubation with 5% (w/w) bovine trypsin as described by Bietlot et al (1989). Shorter incubations were carried out for 3 h with 0.1% trypsin.

Ion-exchange chromatography

Toxin was purified on a Pharmacia LKB Biotechnology fast protein liquid chromatography system equipped with a Mono Q HR 10/10 anion exchanger. Elution was carried out at room temperature with a 0-1 M NaCl gradient in 0.1 M CAPS, pH 10.5 . The flow rate of the system was 0.2 ml/min and the proteins were detected by UV absorbance at 280 nm. All materials collected were thoroughly dialyzed against distilled water. Acetic acid was added to pH 5 and the precipitated protein was removed and stored in distilled water at 4^{o}C. Protein concentrations were determined by dye-binding (Bradford 1976).

Cell bioassay

CF-1 cells were incorporated in an agarose "lawn" as described by Gringorten et al.,

(1990). Column fractions were diluted in 0.1 M CAPS buffer, pH 10.5 and one microlitre aliquots of each dilution were spotted on the lawn and allowed to incubate 1 h. The cells were stained with Trypan blue and destained before recording. Threshold values were defined as the dose that gave the last visible response from the dilution series.

Fluorescence Photomicrography

B. thuringiensis subsp. *kurstaki* was grown on nutrient agar plates at 30°C. At stage VII (see Chapter 1, Fig. 3) the culture showed cells which had sporulated and crystals had formed. The cells were scraped and washed in physiological saline. Ethidium bromide (EtBr) dye was prepared at 1µg/ml in TEA buffer. The dye was added to cells at a 1:1 ratio (V:V)and was incubated at least 15 min before observation. A very thin slide (i.e.; 1 small drop) was prepared and a cover slip added, then observed with a Leitz-Diaplan microscope. Photography was carried out using Ektachrome p800/1600 (pushed to 1600). The exposure meter was set to auto, BF, cal = 1, ASA = 1600. A view was chosen, focused and exposed for EtBr, then exposed for the phase contrast view.

RESULTS

Ion-exchange purification of toxin

The elution of the trypsin-generated toxin from the Mono Q column is shown Fig 25. Two major peaks elute , one at 0.3 M salt (T1) and the other at 0.9 M salt (T2). The relative proportions

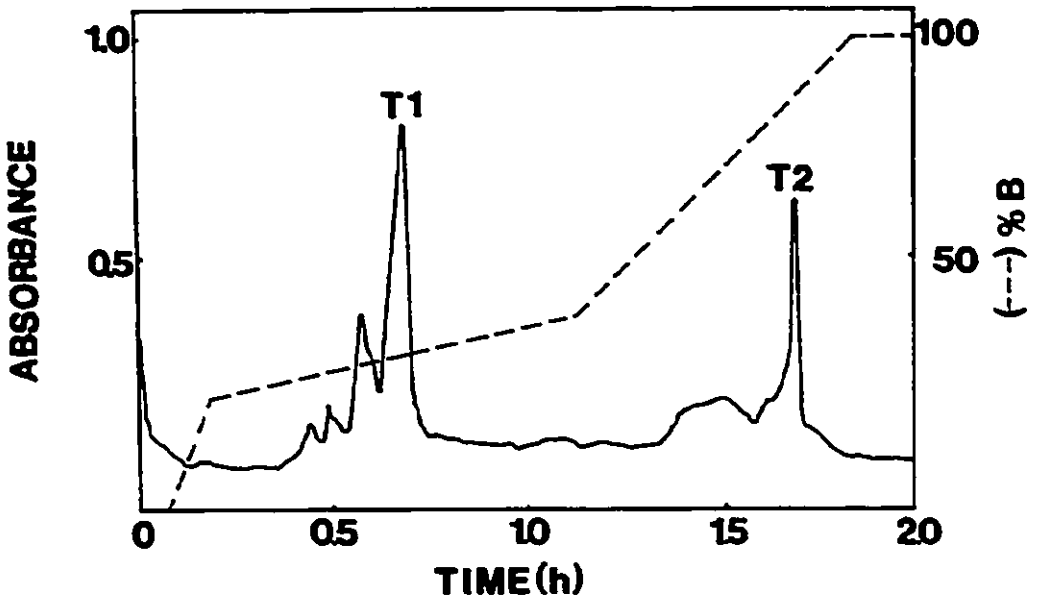

Figure 25. FPLC elution profile of CrylA(c) toxin.
Toxin generated with bovine trypsin was run on a Pharmacia FPLC equipped with a Mono Q HR 10/10 anion exchanger. Elution was carried out at room temperature with a 0-1 M NaCl gradient in 0.1 M CAPS, pH 10.5. The flow rate was 0.2 ml/min, and the proteins were detected by UV absorbance at 280 nm.

of T1 and T2 obtained depended on the source of trypsin, the amount used and the length of treatment.

Cell bioassay

Based on dye-binding protein concentrations the threshold values for T1 and T2 were 0.3 and 0.5 ng/µl respectively.

Fluorescence Photomicrography

The upper view in Fig. 26 shows the phase contrast image of the sporulated cell. The spore is visible as a phase bright ovoid structure and the crystal appears dark and angular. In comparison the lower EtBr view does not clearly show spore structures, but a bright region of fluorescence appears where the crystals are forming.

DISCUSSION

Interestingly Lüthy and Ettlinger (1967) reported the dissolution of a spore crystal mixture of *B. fibourgensis* (a strain which causes Milky Disease) with alkali (at pH 12.0 and pH 12.5 or 0.01 and 0.05 N NaOH respectively). They compared the absorbance spectrum before and after the parasporal crystals dissolved. Their results are shown (Fig. 27A) and compared to the result reported by Bietlot (1993). The UV spectrum for the total crystal solubilization (Fig 27A) compares favorably to the fraction of toxin protein that elutes only at the high molar salt condition (T2 in Fig. 27B). Clearly these preparations of toxin indicate a 280-260 nm shift suggesting the presence of nucleic acids. The origins of this nucleic acid appears to be from the sporangium where the crystal

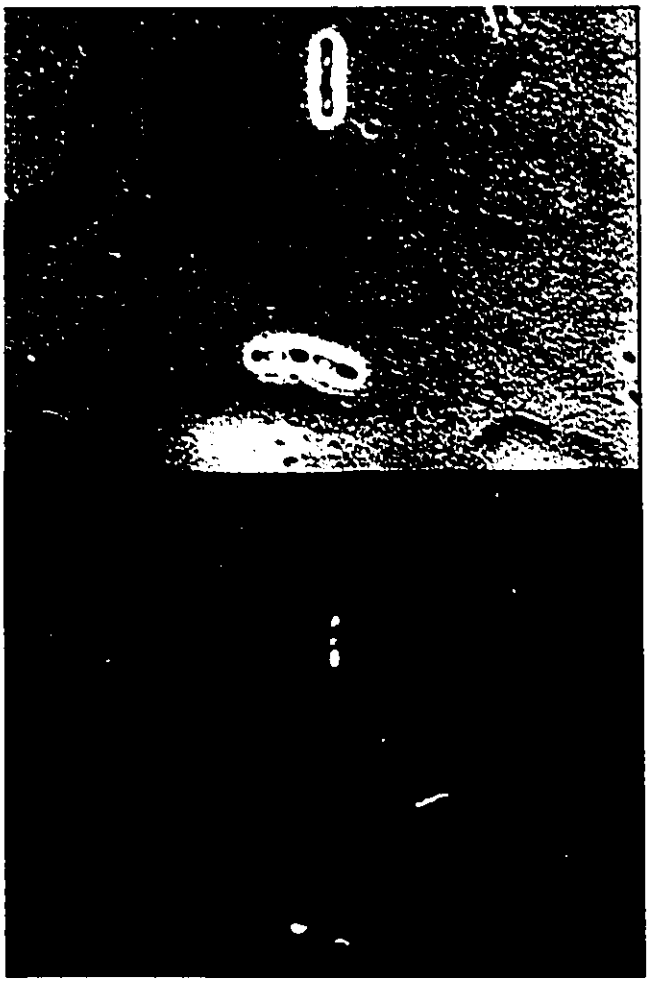

Figure 26. Phase-contrast and fluorescence microscopy of B. thuringiensis subsp. kurstaki HD-1.
The culture shown consists of mature unlysed cells at stage VII (magnification X 5000). A, Phase contrast view. The four light areas are mature spores and the adjacent dark areas are the regions of crystal formation. B, Fluorescence view , observed after incubation of the cells with Ethidium bromide. The cells were photographed under fluorescence conditions first to record the maximum fluorescence then under phase-contrast conditions in an effort to match the two views.

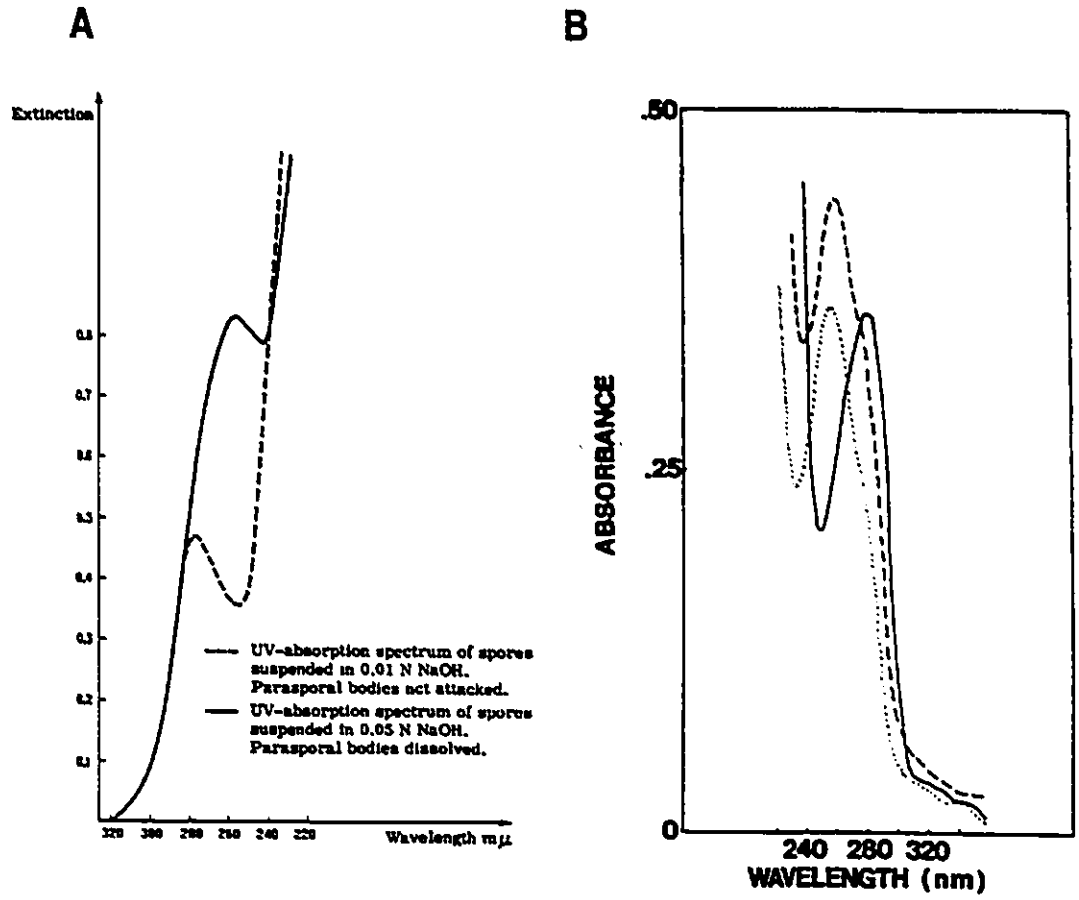

Figure 27. UV absorbance profiles of crystal protein.
A, The displacement of the UV-absorption spectrum by the dissolved substance of the parasporal bodies of *Bacillus fibourgensis.* (taken from Lüthy and Ettlinger, 1967). B, Spectrum of T2 toxin. T2 toxin (0.26 mg/ml) was dissolved in distilled water and scanned (taken from Bietlot, 1993). —, T1; —, T2; ....., difference spectrum of T2 and T1 with T1 in the reference position.

is formed. The uptake of EtBr in the cell, at the time of crystal formation, indicates both a temporal and spatial association of the DNA and the δ-endotoxin. Further investigation would be required to determine if this association is a prerequisite for the formation of the crystalline structure. The idea that some material in the sporangium serves as a nucleus for the protein to begin crystallization was first suggested by Mattes (1927).

The cell lawn assay was chosen to assess activity, as it was reasoned that no further chemical or enzymatic activity would act on the chromatographically separated fractions. The two forms of toxin, T1 and T2, appear to be equally toxic when assayed with the cultured insect cells. It is likely that the DNA association with toxin does not interfere with the expression of activity. Of concern though is that the recovery of toxin may be underestimated if only the T1 fraction is collected from the ion-exchange chromatography.

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CHAPTER 6

CONCLUSIONS

The huge technical power of mankind has as yet, failed to cope with the problem of the biological regulation of numbers.

J. de Wilde ..... Introductory remarks at the Proceedings of the International Colloquium on Insect Pathology and Microbial Control Wageningen (1966)

The major proteolytic enzyme in the midgut of the spruce budworm larva responsible for the activation of δ-endotoxin has been identified as CFT-1, a trypsin-like enzyme. This enzyme exhibited typical trypsin-like characteristics in terms of size, cationic charge, substrate specificity and sequence homology. Of note in the comparison with bovine trypsin was the higher pK_a and the difference in deacylation when the substrate NPGB was used in the burst kinetic analysis. More specifically, this enzyme showed a high pH dependence for catalysis which indicated it was well suited for activity both in the high pH environment of the midgut and also the high pH of buffers used during *in vitro* activation of toxin. In this case activation was referred to as the proteolytic degradation of the 130 kDa protoxin to yield a 60 kDa active toxin. This does not take into account the individual proteolytic events occurring between the starting material at 130 kDa and the final product at 60 kDa. Although it was shown that the purified enzyme and the neat gut juice appeared to effect the same pattern of cleavages during activation these events were not specifically studied.

The data reported for the activation of a Cry1A_c - type toxin with CFT-1 would lead one to believe that the activation of δ-endotoxin is carried out with little difficulty. However observations made during this study suggested that not all toxins were activated in the same manner. These and other observations also made by Drs. H. Bietlot and H. Kaplan led to a re-investigation of the digestion events occurring during the proteolytic processing beginning with the 130 kDa protoxin. It now appears that not only are proteases active in the processing of toxin but also nucleases, leading to the discovery of DNA associated with the toxin (Bietlot et al. 1993). This DNA appears to be of cellular origin and condenses in the same region of the sporangium that the crystal forms.

The routine activation of Cry1A_a type toxins with gut juice led to the irregular recoveries of protein which could not be explained by known mechanisms. These losses of protein were not due to the DNA-toxin phenomenon observed by Bietlot et al. (1993). Further investigation led to

the discovery of a gut juice fraction which preferentially precipitated the toxin. This fraction was subsequently shown to be a 75 kDa anionic protein and was purified and partially characterized. Enzyme activities were investigated, in light of the recent reports of aminopeptidase and alkaline phosphatase proteins reacting with the toxin. However none of the reported enzyme activities were associated with the precipitation event. Only a very low level of elastase-like activity co-chromatographed with this protein.

The implications of a toxin-precipitating protein with enzymatic activity were shown from a practical point, that is, the enzymatic effect of deactivating toxin. However, more interesting are the questions: "What is the mechanism of toxin precipitation with this gut juice protein?" , "Does the toxin-precipitating protein provide a tolerance mechanism that pre-exists in the larval gut?" or "Can the toxin-precipitating protein be induced with exposure to toxin?". Throughout this study it has been suggested that the protein from gut juice precipitates the toxin. Given the propensity for toxin to precipitate under a variety of conditions (Fast, 1981) it is equally possible that it is a characteristic of toxin that precipitates the gut juice protein. An intriguing hypothesis is that the toxin-precipitating protein is involved in the aggregation or concentration of toxin that has been proposed to be necessary for the ultimate pore formation in the susceptible cell membranes (Maddrell et al., 1988; Gill et al., 1992; Walters et al. 1994; Liebig et al., 1995).

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APPENDIX A

CLAIMS TO ORIGINAL RESEARCH

1. Purification and characterization of the serine protease in spruce budworm responsible for the activation of Bt δ-endotoxin.
2. A blotting technique using purified crystals embedded in agarose to identify electrophoretically separated gut juice proteins which activate the toxin.
3. Recognize that some losses of toxin during activation are due to a gut juice component in the enzyme activation system.
3. Purification and characterization of a specific toxin-precipitating protein.
4. A "Western-type" blotting technique using SDS PAGE-separated gut juice to identify the toxin precipitating protein
5. The toxin-overlaid-agarose-diffusion method using native PAGE separated gut juice proteins for identifying the toxin-precipitating protein.
6. Evidence for the *in vivo* association of DNA and toxin and show that the DNA/toxin retains activity.

APPENDIX B

PUBLICATIONS

Bietlot H. P., Schernthaner J. P., **Milne R. E.**, Clairmont F.R., Bhella R.S. and Kaplan, H. (1993) Evidence that the CryIA crystal protein from *Bacillus thuringiensis* is associated with DNA. *J. Biol. Chem.* **268**, 8240-8245.

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