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CHARACTERIZATION OF THE INSECTICIDAL PROTEIN FROM *BACILLUS THURINGIENSIS*: THE IMPORTANCE OF DNA-PROTEIN INTERACTIONS

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ABSTRACT

Many strains of *Bacillus thuringiensis* produce a crystalline inclusion during sporulation which is toxic to insect larvae. The major component of crystals toxic to lepidopteran larvae is a 130-kDa protein, the protoxin. Following ingestion by susceptible insect larvae, protoxin is proteolysed by larval gut proteinases to yield a 58-70 kDa toxic fragment, toxin. A procedure was developed to prepare purified toxin for chemical characterization. Toxin generated by bovine trypsin was shown to be composed of the amino acid residues that span position 29-623 of the protoxin.

The results obtained from competitive labelling experiments on the protoxin show that the functional groups of the lysine and tyrosine residues do not exhibit regular titration behaviour over the pH range of 7 to 10. These results indicate that the majority of these groups are not free in solution but are involved in inter and intra molecular interactions.

During purification by ion exchange chromatography of the bovine generated toxin, it was discovered that the toxin could be separated into two components. One component (T2) was DNA-associated toxin, and the other was the DNA-free toxin (T1). Only one major protoxin component was observed, and it was found to be associated with DNA. The DNA from the T2 toxin varied in size from 100 to 300 base pairs, whereas the crystal and the solubilized protoxin contain 20-kilobase DNA as the major DNA component. DNase treatment converted the T2 toxin to the DNA-free T1 toxin. In contrast, the DNA in the crystal and the solubilized protoxin was resistant to DNase digestion and was not dissociated from the protein by 1.5 M NaCl. The protoxin and DNA appeared to elute as a complex with a molecular mass of greater than 2×10^6 Da on gel-filtration chromatography. No toxin was generated from the protoxin with trypsin after extensive digestion of the protoxin with DNase or dissociation of the DNA by succinylation of the lysine residues. It is proposed that DNA binds to the carboxyl terminal half of the crystal protein and is essential for maintaining the conformational integrity required for crystal formation and generation of toxin.

DEDICATION

This thesis is dedicated to my wife

Ramona

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LIST OF ABBREVIATIONS

BRI	Biotechnology Research Institute
CAM	carbaminomethyl
CAPS	3-(cyclohexylamino)-1-propane-sulphonic acid
CM	carboxymethyl
DFP	Diisopropyl fluorophosphate
Dnp	2,4-dinitrophenyl
Dnp-F	1-fluoro-2,4-dinitrobenzene
EDTA	Ethylenediaminetetraacetic acid
FAB-MS	fast atom bombardment mass spectrometry
FPLC	fast protein liquid chromatography
FPMI	Forest Pest Management Institute
GuHCl	Guanidinium hydrochloride
HPLC	high performance liquid chromatography
kDa	kilodalton
N.R.C.	National Research Council
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SCAM	succinyl-carboxymethyl
TAE	tris-acetic acid-EDTA
TCA	trichloroacetic acid
TE	tris-EDTA
Tris	tris(hydroxymethyl)aminomethane
μ_{ser}	mobility relative to serine

CHAPTER 1

GENERAL INTRODUCTION

1.1 Overview

The control of insect pests is a major problem affecting the economics of the agricultural and forestry industries. This control is largely achieved by the liberal application of chemical insecticides. Worldwide, the annual expenditure for chemical pesticides is estimated to be well over four billion dollars (Wilcox *et al.*, 1986). The widespread use of these insecticides has awakened concerns over the dumping of manufactured chemicals into the ecosystem. As a result, there is a demand for safer approaches to the control of insect pests. The use of naturally occurring insecticides is particularly attractive because of their environmental safety (Fisher and Rosner 1959), high degree of host specificity and lack of toxicity to other organisms, in particular mammalian and avian species. Over one thousand micro-organisms or microbial products, including viruses, bacteria and fungi, are known to be effective against insects, and yet as of 1986 only 13 microbial organisms were approved by the American Environmental Protection Agency (EPA) for field use (Wilcox *et al.*, 1986).

The limited target specificity of biological insecticides gives rise to the possibility of tailoring insecticidal formulations for specific insect pests while leaving

beneficial insects and other organisms undisturbed. With the recent advances in biotechnology, in particular the use of recombinant DNA techniques, the promise of making biological insecticides more economically attractive and of producing new insecticides with novel host specificities has further served to generate interest in the field of bioinsecticides.

The most successful and widely used bioinsecticide products are those based on the bacterium *Bacillus thuringiensis*. Commercial production of *B. thuringiensis* insecticides began in the United States in 1958, but its use did not become widespread until the early 1980s at which time they were also deployed in Canada. In both Canada and the United States, *B. thuringiensis* is extensively used against food crop insects. In recent years, it has also been widely used in Canada against forest pests, being employed in spruce budworm control operations in Ontario and Quebec, in the control of jackpine budworm and western spruce budworm in the Western provinces, and against hemlock looper and gypsy moth in the Atlantic provinces (Statistics Canada, 1987).

The use of *B. thuringiensis* as a bioinsecticide has been limited by problems encountered with cost-effective field application of the formulations, removal of the insecticide from foliage by rain, and sunlight inactivation of the insecticidal activity. Insect resistance has only been reported for the diamondback moth (*Plutella xylostella*) in the

field (McGaughey and Whalon, 1992). Various other insects have been shown to develop resistance in the laboratory. The effort in resistance management for the *B. thuringiensis* susceptible insects, has many strategies by which the spread of resistance can be controlled. Understanding the factors which affect the efficacy of *B. thuringiensis* requires knowledge of the structure of the insecticidal element as well as an understanding of the mechanism of action. This knowledge will aid in the development of an improved insecticide.

1.2 Biological Properties

B. thuringiensis is a gram positive soil bacterium distinguishable from the closely related species *Bacillus cereus* and *Bacillus anthracis* by the large parasporal inclusion that appears during sporulation. The active component of crystal is known as the delta-endotoxin, in order to distinguish it from the other toxins produced by this organism. Unlike other sporulation-dependent proteins, the crystal protein is accumulated in large amounts, as a crystalline inclusion body.

By 1987, 28 subspecies of *B. thuringiensis* had been identified (Himeno, 1987a). Several methods have been used to classify strains of *B. thuringiensis* into serotype, subspecies

(subsp.) or varieties (var.). The most commonly used classification system is based on flagellar antigens (Himeno *et al.*, 1987b). Further classification is achieved by comparing the biochemical differences of these subspecies within a serotype. Within the lepidopteran specific *B. thuringiensis* proteins different subspecies are toxic to the larvae of many different insects; and sometimes individual strains within the same subspecies, have a characteristic spectrum of insect toxicities (Dulmage, 1978). This classification scheme suffers some shortcomings in that isolates which are classified as being identical show very different toxicities. Ellar, *et al.* (1985) proposed a new classification scheme based on the order of insects to which the isolate is toxic. This system separates the different subspecies of *B. thuringiensis* into: (1) lepidopteran specific (i.e. moths and butterflies), (2) dipteran specific (black flies and mosquitoes), (3) coleopteran specific (beetles), (4) lepidopteran and dipteran specific and (5) no known insecticidal activity.

The biosynthesis of the entomocidal crystal takes place concomitant with sporulation (Huber and Lüthy, 1981). The crystal grows to approximately the same size as the spore (1μ in length) and constitutes 30% of the cellular protein (Dean, 1984). In the last phase of sporulation, the crystal, which is generally bipyramidal in shape, is released into the

environment together with the spore when the cell lyses. The prominence of the crystal within *B. thuringiensis* cells suggests that the crystal confers substantial survival value to individual cells. It is known that spores of *B. thuringiensis* germinate slowly, and the natural concentration of the bacterium in the soil is low (Aronson *et al.*, 1986). An insect host provides an excellent nutritional environment for bacterial proliferation and sporulation, thereby permitting the bacteria to reach the high concentrations required for cell mating and the exchange of genetic material (Gonzalez *et al.*, 1982).

1.3 History of *B. thuringiensis*

The organism was first isolated in 1901 by Ishiwata (Ishiwata, 1901) from the larvae of diseased silkworms (*Bombyx mori*) and was subsequently named *Bacillus sotto* or, in the current nomenclature, *B. thuringiensis* subsp. *sotto*. The name *B. thuringiensis* dates from 1911 when Berliner isolated a crystal containing organism from diseased flour moth larvae in the German province of Thuringia. Attempts were made in the following decades to utilize *B. thuringiensis* as an insecticide, but so little was known about the source and specificity of the insecticidal action that the organism could not be efficiently exploited. Little interest was shown in

B. thuringiensis until 1954, when Angus showed that the crystalline inclusion body was responsible for the insecticidal action (Angus, 1954). In 1956, Angus also showed that the crystal protein was the major component of the crystal (Angus, 1956). Steinhous (1951) demonstrated that *B. thuringiensis* could reduce the population of alfalfa caterpillars which led to the first commercial preparation of B. thuringiensis available for testing by entomologists by 1958 (Hall, 1963 and Heimpel, and Angus, 1963). The first standard for *B. thuringiensis* toxicity was developed in France in 1965. The more widely used standard, was developed in the United States, in 1973 and is based on the "spore-crystal" complex of *B. thuringiensis* subsp. *kurstaki* HD-1-S-1971 (Reichelderfer, 1985). The efforts of Howard Dulmage to systematically catalogue the various *B. thuringiensis* species led to considerable information on strain specificity and to a comprehensive set of *B. thuringiensis* strains, the HD collection (Dulmage, 1981). With the establishment of the cabbage looper (*Tricholplusia ni*) as the industry-wide test organism, and the agreement on *B. thuringiensis* subsp. *kurstaki* HD-1 as the industrial bioinsecticide standard (Dulmage, 1970), information on the specificity and toxicity of *B. thuringiensis* could be evaluated in a more systematic manner.

1.4 Mode of Action

B. thuringiensis δ-endotoxin is pathogenic primarily to insects in the larval stages of development. In susceptible lepidopteran insect larvae, the midgut pH is highly alkaline (between pH 9 and 10), while the hemolymph pH is approximately neutral. It is believed that the alkaline pH of the gut aids the insect in the breakdown of tannin and other plant material which remain insoluble at lower pH values (Milne *et al.*, 1990).

The high pH of the lepidopteran midgut dissolves the parasporal crystal. The protoxin is cleaved by insect gut trypsin-like proteinase to yeild a toxin with an apparent molecular mass of 65-66 kDa toxin (Fast, 1981; Fast, 1983). Several factors, such as pH and proteinases in the insect gut have been proposed to explain insect specificity (Jaquet *et al.*, 1987, Haider *et al.*, 1986, Haider and Ellar, 1987). Recently, the binding of toxin to lipid bilayers (Schwartz *et al.*, 1993) or specific receptors on the plasma membrane of midgut epithelium cells of target insects has been investigated as a basis for specificity (Almond and Dean 1993; Lee *et al.*, 1992 and Ge. *et al.*, 1989).

The physiological changes which occur in the insect larvae are well documented (Bravo *et al.*, 1992). Within minutes of ingestion, the insect gut and mouth become

paralysed, the pH of the midgut drops and the pH of the hemolymph rises (Heimpel and Angus, 1959). Within one hour of crystal ingestion, the microvilli in the midgut have swollen (Fast *et al.*, 1978), and within four hours the midgut cells have separated from the basement membrane and are totally destroyed (Fast and Donaghue, 1971; Greigo *et al.*, 1980; Oron *et al.*, 1985 and Bravo. *et al.*, 1992). The specific binding of toxin to receptors forms pores in the epithelium; ions and small molecules then equilibrate across the membrane, leading to cell lysis (Knowles and Ellar, 1987). Changes in the endoplasmic reticulum and mitochondria, disruption of ion and glucose transport and oxygen uptake, and loss of ATP from midgut cells, all contribute towards total body paralysis and death (Aronson *et al.*, 1986; Himeno *et al.*, 1987b). Investigations into the mode of action of the crystal protein suggest that the mechanism of action of the toxin involves the inhibition of active potassium transport across the midgut epithelium (Griego *et al.*, 1979; Harvey and Wolfersberger, 1979, and Schwab and Culver 1990).

1.5 Molecular Genetics

To date, 14 distinct crystal protein genes in

B. thuringiensis have been identified. Thirteen of these genes code for a family of related insecticidal proteins, also refered to as Cry proteins, crystal proteins, protoxin and δ-endotoxin. These 13 cry genes have been divided into four major classes based on gene homology, protein structural similarities and insect specificities (Höfte and Whitely, 1989). CryI proteins are toxic only to Lepidoptera, CryII proteins are toxic to both Lepidoptera and Diptera, CryIII proteins are toxic to Coleopteran, and CryIV proteins are specifically toxic to Diptera. The proteins characterized in the present study, the protoxin and toxin from *B. thuringiensis* subsp. *kurstaki* HD-73, belong to the CryI class.

B. thuringiensis bacteria harbour between two and 17 plasmids, depending on the subspecies (Gonzalez *et al.*, 1982, Lereclus *et al.*, 1982). Curing bacteria of their plasmids results in the loss of crystal production, indicating that the cry genes are located on plasmids (Gonzalez and Carlton, 1980). When plasmid DNA from the industrial standard strain *B. thuringiensis* subsp. *kurstaki* HD-1 was digested with the restriction enzyme HindIII, three different DNA fragments with lengths of 4.5, 5.3 and 6.6 kilobases were found to contain protoxin genes (Kronstad and Whiteley, 1986). These genes have recently been reclassified as the cryIA(a), cryIA(b) and cryIA(c) genes, and code for protoxin proteins of 1176, 1155 and 1179 amino acids respectively (Höfte and Whiteley, 1989).

Only the cryIA(c) gene is found in *B. thuringiensis* subsp. *kurstaki* HD-73 (Adang *et al.*, 1985), but multiple heterogeneous copies of the protoxin gene have been identified in many *B. thuringiensis* subspecies. As the toxicities of products from a given class of genes are very similar, the advantages of gene diversity remain somewhat obscure. However, it is possible that the heterogeneous copies of the protoxin gene are regulated differently within the bacterial cell, or that the various gene products of a particular subspecies act in a synergistic manner and thus contribute to the insect host spectrum of a *B. thuringiensis* subspecies.

When the cryIA gene sequences are aligned and gaps are introduced to maximize homology, 85% overall homology between the three sequences becomes evident (Adang *et al.*, 1987). The differences that do exist among the gene types are not randomly distributed, but rather are clustered in several distinct regions (Adang *et al.*, 1987). The amino terminal 280 amino acids of all three gene types are 98% conserved. The next 350 amino acids encompass the hypervariable region, where homology falls to approximately 60%. This region can be further divided in two. In the case of the cryIA(c) gene type, the sequence between amino acid residues 280-460 shows some variance with the cryIA(a) and cryIA(b) gene types, while the region between residues 460 and 640 shows pronounced heterogeneity (Geiser *et al.*, 1986; Andrews *et al.*, 1985).

The coding for the carboxyl terminal half of the protoxin molecule by the three genes again shows strong conservation of the amino acid sequence, although the preservation of the sequence is less striking than that observed for the first 280 amino terminal residues.

Deletion studies of protoxin genes from *B. thuringiensis* cry gene sequences has led to the identification of two distinct functional regions in the protoxin molecule. Similar studies with the protoxin genes from *B. thuringiensis* subsp. *berliner* revealed that only the amino-terminal segment encoding the first 630 amino acids was required to produce a protein with insecticidal activity comparable to that of the native protoxin (Lilley *et al.*, 1980). The protein encoded by this gene fragment had a molecular mass of 68 kDa, which corresponds to the size of protease-activated toxin. Further deletion studies on other protoxin genes confirmed that in all cases, the toxin is derived from the amino terminal half of the protoxin genes. In contrast, the function of the conserved carboxyl terminal half of the protoxin molecule remains unclear, but the fact that it is highly conserved indicated that it plays an important role. In *B. thuringiensis* subsp. *kurstaki* HD-73 for example, the carboxyl terminal half contains 14 of the 16 cysteine residues, and 31 of the 34 lysine residues (Adang *et al.*, 1985). Although no role has yet been proposed for the lysine residues, the abundance of

cysteine residues suggests that the carboxyl terminal region plays a structural role in crystal stabilization through the formation of disulphide bonds between protoxin molecules (Nickerson, 1980; Huber *et al.*, 1981; Bietlot *et al.*, 1990c). As the amino terminal region alone is sufficient for toxic activity, it has been postulated that the carboxyl terminal region, in addition to its role in crystal formation, serves to protect the toxic moiety or to mediate binding to insect gut epithelial cell receptors (Aronson *et al.*, 1986).

The functional domains in the toxin portion of the molecule (amino acids 1-630) were determined by Lee *et al.*, (1992) and Ge *et al.*, (1989). When the hypervariable region (amino acid 332-612) is exchanged between the various cryIA genes, the specificities of the proteins towards their insect targets are transferred accordingly. The binding region was determined to lie between amino acids 332-450 (Lee *et al.*, 1992) for the cryIA(a) and cryIA(c) proteins. This region has two predicted hydrophilic peaks which superimpose on two predicted ß-sheets at positions around 403 and 423 in the whole protein.

1.6 Structure and Properties of the Protein Crystal

The terms "crystalline inclusion" or "protein crystal" come from the bipyramidal shape which is usually observed when

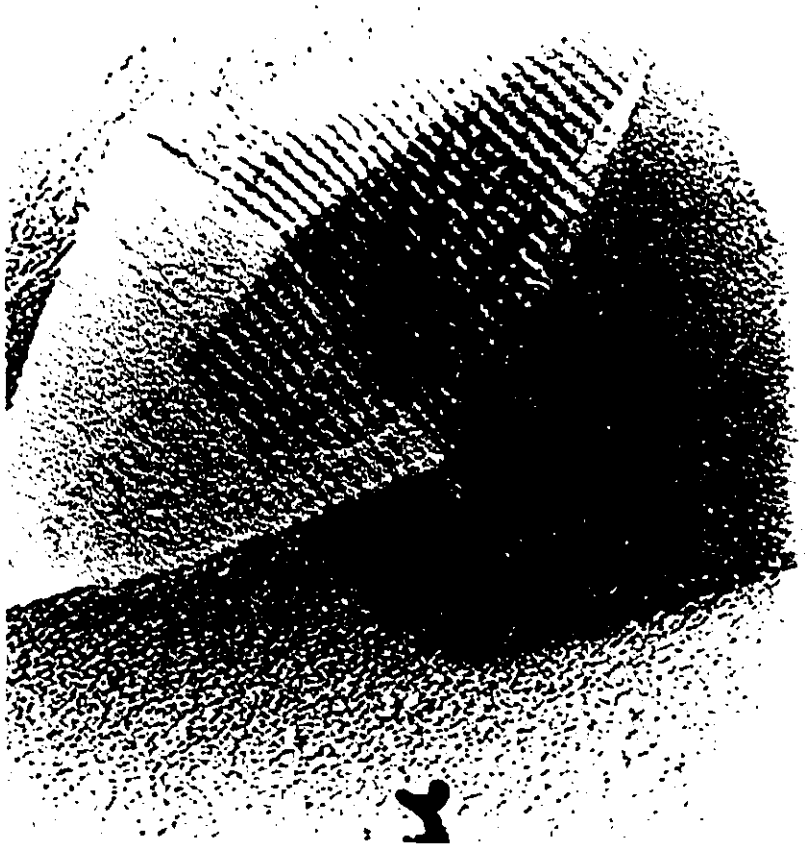


Figure 1

Crystals of *Bacillus thuringiensis* subsp. *berliner* viewed under the electron microscope (Hannay and Fitz-James 1955).

the protein is viewed through a phase contrast or electron microscope (Figure 1). Crystals from various subspecies of *B. thuringiensis* differ in the number of protein components, but in most crystal types the predominant protein is the protoxin or crystal protein. Some lepidopteran-specific strains also contain proteins which are active against insects of different orders. *B. thuringiensis* subsp. *kurstaki* HD-1 a CryIA protein, contains a protein which is active against mosquitoes and blackflies (classified as CryIII proteins). Unlike most lepidopteran-specific subspecies of *B. thuringiensis*, the crystals from subspecies *israelensis* are composed of several proteins, some of which are toxic towards Dipteran larvae, and others which are lytic towards mammalian cells (Thomas and Ellar, 1983).

The bipyramidal crystals are composed of protoxin molecules packed into a crystalline array held together by a network of disulphide bonds. These disulfide bridges are rather unusual in that all 16-18 cysteine residues form symmetrical interchain disulfide linkages (Bietlot *et al.*, 1990). Interchain disulfide bridges are relatively uncommon and symmetrical interchain bridge are rare (Schultz and Schirmer 1988). The protein crystal is insoluble at neutral pH but becomes more soluble as the network of disulphide bridges is broken at pH above 9. Holmes and Monroe (1965) and

Huber *et al.*, (1981) reported that the repeating subunit in crystals from a number of *B. thuringiensis* isolates were dimers of the protoxin molecule. A study into the solubilization of the crystal by Nagamatsu *et al.*, (1984) provided strong evidence that the crystal from *B. thuringiensis* subsp. *dendrolimus* is composed of a single subunit of molecular mass 145 kDa. Small angle X-ray powder diffraction patterns have been obtained from crystal isolates of *B. thuringiensis* subsp. *tenebrionis*. These patterns indicated that each unit cell of the crystal contains only one protoxin molecule (Li *et al.*, 1988). To date, none of the Lepidopteran-specific protoxins have been recrystallized to yield X-ray diffraction quality crystals.

1.7 Structure and Properties of the crystal protein (protoxin)

Studies of the *B. thuringiensis* crystals during the 1960's and 1970's led to a range of estimates for the molecular mass of the principal crystal protein component. The situation was complicated by the presence of contaminating bacterial proteinases which acted on the crystal protein during the solubilization process and led to the accumulation of protein fragments (Kaplan *et al.*, 1986). By the early 1980s it was established that the primary component of Lepidopteran-

specific crystals was a protein with a molecular mass of approximately 130 kDa, and that a minor fraction of protein from each crystal type had a molecular mass of approximately 68 kDa (Tyrell *et al.*, 1981). The larger protein, the protoxin, was found to be non-toxic towards cultured cells of the spruce budworm (*Choristeneura fufimerana*), whereas the smaller protein, the toxin, was very toxic (Huber and Lüthy, 1981). Protoxin could be converted into the smaller protein by the action of either purified mammalian proteinases or insect gut juice (Lilley *et al.*, 1980). From these observations, Bulla *et al.*, (1981) concluded that the crystals of *B. thuringiensis* are composed of protoxin molecules which are proteolyzed into smaller toxin molecules within the insect gut. Andrews *et al.*, (1985) established that the protoxin was stoichiometrically converted to toxin by the action of proteinases. The molecular mass of the protease-resistant, Lepidopteran-specific toxin molecule was shown to vary from approximately 58 to 70 kDa, depending on the subspecies from which the crystal were obtained and the proteinases used to accomplish the activation of protoxin to toxin (Höfte and Whiteley, 1989).

Raman spectroscopic studies gave the first secondary structure analysis of the crystal protein from *B. thuringiensis* subsp. *kurstaki* HD-73 (Carey *et al.*, 1986). It was reported that the crystal protein was approximately 54%

unordered structure, 25% α-helix and 21%-ß sheet. These results are in good agreement with the secondary structure predictions based on the gene sequence carried out by Whitely and Schnepf (1986).

Yamamoto and Iizuka *et al.*, (1983) reported that the protoxins from *B. thuringiensis* subspecies *kurstaki* HD-1 and HD-73 were initially degraded into approximately six fragments by random proteolytic cleavages. However, Chestukhina *et al.*, (1982) concluded that the carboxyl terminal region of the protoxin is composed of one 35-kDa domain and two 15-kDa domains which are cleaved in a stepwise fashion. The data obtained by Choma *et al.*, (1990), demonstrated that the activation processes is highly unusual. They showed that this processes is not a random process but that it occurs in seven specific cleavages starting at the carboxyl terminus of the protoxin and proceeds towards that amino terminal region. At each step, carboxyl terminal fragments of approximately 10 kDa are produced and rapidly degraded into small peptides (Choma *et al.*, 1990).

Choma and Kaplan (1990) showed that the protoxin from *B thuringiensis* subsp *kurstaki* HD-73 regained its native biologically active conformation after treatment with 8 M urea and 6 N GuHCL. The results obtained from limited proteolysis experiments allowed them to follow the unfolding and refolding of the protoxin and toxin. They concluded that the unfolding

and folding of the amino terminal half of the protoxin molecule was essentially independent of the carboxyl terminal half of the molecule.

1.8 Structure and Properties of the toxin

The X-ray crystal structure of the CryIC coleopteran-specific toxin has recently been published (Li *et al.*, 1991). Much of the CryIC toxin is homologous to the CryIA toxin and it is likely that the coleopteran and lepidopteran toxins have the same overall structural motif. This molecule was shown to be composed of three domains, the first is composed of a 7 helix bundle, the second is three sheets of ß-sheet structure and the third is a ß sandwich. Choma *et al.*, (1990b) have shown that the protease resistant toxic fragment from *B. thuringiensis* subsp. *kurstaki* HD-73, a CryIA(c) protein, is composed of three domains with apparent molecular masses of 33, 26 and 10 kDa. These are very close to the sizes of the domains in the CryIC toxin indicating that the structural motif of the two toxins are very likely simmilar.

The secondary structure of the B. thuringiensis subsp. *kurstaki* HD-73 toxin was estimated by Raman, infrared, and circular dichroism spectroscopy as well as by predictive methods (Choma *et al.*, 1990b). The results from all of these methods were consistent and led to the conclusion the toxin is

a highly folded protein with approximately 70% of the residues involved in forming α-helix and ß-sheet structures.

When the proteolytic sensitivity of the isolated toxin was compared to that of the molecule generated from the protoxin in the presence of denaturant (either urea or GuHCl), a small increase in the protease sensitivity of the molecule generated in the presence of urea or GuHCl was observed (Choma and Kaplan, 1990). This result was interpreted to be a minor conformational change in the toxic moiety during the activation process. This was consistent with the X-ray (Li *et al.*, 1991) structure for the coleopteran toxin which had the carboxyl terminal buried between two domains of the protein.

1.9 Rational for the Present Study

The biological activity of proteins is determined by the precise spacial arrangement of its constituent amino acids. As such, the elucidation of structure-function relationships in proteins represents some of the most important challenges facing biological sciences at this time. Despite the economic importance of *B. thuringiensis* very little direct structural studies have been carried out on the crystal protein. Most of the structural information available has been deduced from the nucleotide sequences of the various genes. Point mutations, deletion and rearrangement studies have helped unravel the

functional roles played by specific regions of the protein. Perhaps the primary reason for the lack of direct studies on the protein is the difficulty in working with this molecule. These include the size and low solubility of this protein at neutral pH as well as obtaining reasonable quantities of spore free crystals.

The work carried out for this thesis represents the continuation of a master's thesis entitled **"Characterization of the Insecticidal Crystal Protein from *B. thuringiensis*."** although a great deal of work has been carried out on the gene(s) coding for the insecticidal protein, very little was known about the protoxin and toxin proteins when my graduate studies was started. The focus of this thesis was to elucidate the factors which govern the structure-function relationships in this family of proteins.

The generation of stable, defined toxin from protoxin was achieved and the toxin characterized in cooperation with other graduate students and research groups at the National Research Council of Canada and the Forest Pest Management Institute. During the course of protein purification of the toxin and protoxin, it was discovered that these proteins are associated with DNA. Experiments were carried out to determine 1) the nature of this association and 2) the role that DNA plays in the structure and function of the *B. thuringiensis* insecticidal protein.

CHAPTER 2

Characterization of the trypsin generated toxin from *B. thuringiensis*

2.1 INTRODUCTION

Many different protocols for the generation and purification of toxin from protoxin have been reported (Chestukhina *et al.*, 1982, Nagamatsu *et al.*, 1984, Andrews *et al.*, 1985, and Aronson and Arvidson, 1987). These procedures vary considerably and use *B. thuringiensis* strains which are known to contain more than one gene coding for the protoxin (Yamamoto *et al.*, 1988). Toxin is typically generated by solubilizing the crystals at high pH, usually in the presence of reducing agents, and by treatment with larval gut-juice enzymes or exogenous proteinases. After proteolysis, several chromatographic steps combined with other standard methods of purification have been used for the purification of the toxin (Chestukhina *et al.*, 1982, Nagamatsu *et al.*, 1984, Andrews *et al.*, 1985, and Aronson and Arvidson, 1987). For the CryIA proteins, toxic fragments of different sizes and properties have been reported (Chestukhina *et al.*, 1982, Nagamatsu *et al.*, 1984).

Bovine trypsin has been widely used *in vitro* to generate toxin from crystal protein. In the case of the spruce budworm, a trypsin-like proteinase has been shown to be responsible for the proteolytic processing of the protoxin (Milne and Kaplan, 1993). Nagamatsu *et al.*, (1984) reported isoleucine 29 of the protoxin as the amino terminal cleavage

site in *B. thuringiensis* subsp. *dendrolimus*. In contrast, Chestukhina *et al.*, (1982) reported that no amino terminal cleavage occurred during activation. In the case of the dual specificity of lepidopteran/dipteran toxins, the exact position of the amino and carboxyl termini have been shown to be critical in determining the specificity of the toxin. Haider and Ellar, (1987) showed that for *B. thuringiensis* subsp. *aizawai*, the toxin generated by lepidopteran enzymes retain lepidopteran activity. This same toxin can be further processed by dipteran enzymes to produce a dipteran specific toxin. Using gene deletion experiments Höfte *et al.*(1986), showed that toxicity was lost if the first 37 amino acids were removed from the protoxin but not if the first 29 amino acids were removed. The exact position of the carboxyl cleavage site has not been reported and has only been approximated from the apparent molecular mass. The only clear consensus which was present when this work started was that the toxin was derived from the amino terminal half of the protoxin. In order to define the toxin chemically it was necessary to determine the amino and carboxyl termini cleavage sites of the protoxin.

The determination of carboxyl terminal sequences of proteins has lagged behind the developments for amino terminal sequence determination. Various methods and approaches for carboxyl terminal sequence determination have been developed (Ward, 1986, Jones, 1986) but no single reliable and sensitive method has emerged. One such method is a technique using

trypsin digestion and ion-exchange HPLC to generate and separate carboxyl terminal peptides lacking basic residues (Kawasaki *et al.*, 1987). This method depends on a favourable distribution of lysine and arginine residues in the carboxyl terminal region of the protein. Another method, fast atom bombardment mass spectrometry (FAB-MS), has a high sensitivity and appears to have general applicability but its use is restricted due to the complexity of the data analysis and availability of the instrumentation (Marino *et al.*, 1981 and Rose *et al.*, 1988). Baily *et al.*, (1992) have used a method based on the derivatization of the carboxyl terminal amino acid to a thiohydantoin using diphenyl phosphoroisothiocyanatidate (DPP-ITC) and pyridine. This method involves the sequential cleavage from the carboxyl terminus of the protein in a manner analogous to the sequential cleavage from the amino terminus used in the Edman degradation. The results using model peptides are promising, but the methodology is not generally applicable to proteins and cannot be used when proline residues are present. Although several procedures for carboxyl terminal sequence determination have proven to be very useful, all current approaches have their drawbacks and limitations. These include a lack of applicability to some carboxyl terminal sequences or the requirement for highly specialized equipment which is not generally available.

Duggelby and Kaplan (1975) described a diagonal electrophoretic procedure for the isolation of carboxyl terminal peptides which is applicable to all proteins regardless of the carboxyl terminal sequence. This methodology lacks the sensitivity needed for the small amounts of protein available in many cases. In this procedure only carboxyl terminal peptides lie on a diagonal line after two dimensional electrophoresis and these peptides can be isolated and sequenced. The problem with application of this procedure to the toxin from *B. thuringiensis* is that the detection and isolation of the carboxyl terminal peptide(s) entails significant losses and the sensitivity is limited by the ninhydrin staining procedure. To this end, a modified procedure was developed in order to increase the sensitivity of this methodology.

2.2 MATERIALS AND METHODS

All experiments that were not exclusively performed by the candidate have been noted in this section in order to acknowledge the contribution of my fellow workers.

2.2.1 Materials

Ribonuclease A, DNase I, α-chymotrypsin, thermolysin, trypsin, pepsin, and 1-ethyl-3-(3-dimethylaminoproyl)-carbodiimide were obtained from Sigma (St. Louis, MO. USA). [^{14}C]Taurine (115 mCi/mmol), [^{14}C]choline (40 mCi/mmol), [^{14}C]methylamine (56 mCi/mmol), [^{14}C]acetic anhydride (9.2 mCi/mmol) were obtained from Amersham Corp. (Oakville, Ontario, Canada). Autoradiography was carried out using Fujii X-ray film. RENOGRAFIN-76/GASTROgrafin was obtained from Squibb Canada, Montreal, PQ. All dialysis tubing was purchased from Spectrum, Los Angeles, California, USA. Sephadex G-200 was obtained from Pharmacia, Montreal, Canada Other chemicals were high purity preparations obtained from commercial sources.

The *Bacillus thuringiensis* subsp. *kurstaki* HD-73 strain used in all experiments carried out for this thesis was obtained from stock cultures maintained on nutrient agar slants and stored at 4°C at the Forest Pest Management Institute (FPMI), Sault Ste. Marie, Ontario, Canada.

The solvent systems used for electrophoresis were: pH 2.1 buffer, formic acid:acetic acid:water (1:4:45 by volume) and pH 4.4 buffer pyridine:acetic acid:water (6:10:1200 by volume).

2.2.2 Growth of *B. thuringiensis*

B. thuringiensis subsp. *kurstaki* HD-73 was grown in half-strength trypticase soy broth (1 l of medium in a 4 l baffle flask) at 28°C with vigorous shaking. After 72 h the culture was examined microscopically to assess the degree of lysis. When 90% or more cells appeared lysed, the crude spore crystal mixture was pelleted and washed with 1 M NaCl, 0.01% (v/v) Triton X-100 and 10 mM EDTA. The mixture was then sonicated in distilled water for 20 min and washed as described above.

2.2.3 Purification of *B. thuringiensis* Crystal Inclusion Bodies

Purification of crystals followed the discontinuous gradient method developed by Milne *et al.*, (1977) using RENOGRAFIN-76 or GASTROgrafin. After separation, the purity of the crystals was checked by phase contrast microscopy and the purification procedure repeated as necessary. The purified crystals were washed twice in distilled water and stored at 4°C in 0.01% NaN_3 and 10 mM EDTA.

2.2.4 Preparation of Toxin

Trypsin (5 mg) was added to a suspension of 100 mg of crystals in 5 ml of 0.1 M CAPS buffer, pH 10.5, and the suspension stirred for 20 h at 20°C. Undissolved crystals were removed by centrifugation at 10,000 x g at 4°C for 20 min. Ammonium sulphate (40% w/v) was added to the supernatant and stirred at 4°C for 15 min and then centrifuged. The precipitated protein was suspended in distilled water and thoroughly dialysed against distilled water (3 x 4 litre) at 4°C using 50 kDa cut-off dialysis tubing. The precipitated protein was collected by centrifugation and was stored at 4°C in distilled water.

2.2.5 Column Chromatography of the *B. thuringiensis* Toxin

The molecular mass of the toxin was estimated using gel filtration chromatography on a Sephadex G-200 column equilibrated in 0.1 M CAPS, pH 10.5. Column calibration was carried out in accordance with the manufacture's instructions using molecular mass markers; phosphorylase (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and carbonic anhydrase (30 kDa) at pH 7.0 in a 0.1 M phosphate buffer.

2.2.6 Amino Terminal Identification

(i) **Dansylation.** A sample of toxin (0.5 mg) was allowed to react with dansyl chloride as described by Gray (1967). The dansyl derivative was identified by two dimensional high-voltage paper electrophoresis at pH 4.4 and 2.1.

(ii) **Isolation of Amino terminal peptide.** The isolation procedure is based on the fact that after acetylation of the toxin and enzymic digestion, only peptides derived from the amino terminus will be neutral at pH 2.1 and therefore can be readily isolated. Toxin (11 mg) was acetylated with [^{14}C]acetic anhydride (Kaplan *et al.*, 1982) and digested with pepsin as described above. The neutral peptide from pH 2.1 electrophoresis was further purified by paper electrophoresis at pH 3.5 and located by autoradiography. The amino acid composition of the isolated peptide was determined by amino acid analysis.

2.2.7 Amino terminal Sequence Determination

Automated gas-phase sequencing was performed on an Applied Biosystems 474A protein sequencing system, using a modification of the method originally described by Edman and Begg (1967). Toxin (approximately 0.5 nmol) from an SDS/polyacrylamide gel was electroblotted onto a polyvinylidene difluoride membrane (Matsudira, 1987). Using

the protocol suggested by the manufacturer (Bio-Rad), the first twenty amino acids were sequenced by D. Watson, Division of Biological Sciences, N.R.C., Ottawa, Ontario, Canada.

2.2.8 SDS-PAGE

Electrophoresis was carried out on a Pharmacia Phast system with preformed gels and other materials supplied by Pharmacia. Samples were dissolved in sample buffer (8 M urea, 2.5% (w/v) SDS, 5% (v/v) ß-mercaptoethanol and 10 mM Tris-HCl, pH 8.3), placed in a boiling water bath for 5 min, and then applied to 10-15% gradient gels.

2.2.9 Cell Bioassays

The biological activity of the purified toxin was determined by the Forest Pest Management Institute in Sault Ste. Marie, Ontario, Canada using CF-1 insect cells in a lawn assay as described by Gringorten *et al.*, (1990) (Appendix 3).

2.2.10 Amino Acid Analysis

Hydrolyses of the protein or peptides were carried out in 6 M HCl containing 0.1 M phenol at 110°C *in vacuo* for 16, 24 and 48 h. Tryptophan was determined by hydrolysis of the protein or peptide in 4 M methanesulphonic acid (Simpson *et*

al., 1976) at 110°C for 24 h. Serine and threonine were determined by extrapolation to zero time. Leucine, isoleucine, methionine and valine were determined from the 48 h hydrolysate. All other amino acids were determined as the average of the 24 and 48 h hydrolysate. A Technicon TSM amino acid analyzer with a ninhydrin detection system was used for quantification.

2.2.11 Carboxyl-terminal ^{14}C-Labelling Procedure

The procedure for the isolation of carboxyl terminal peptides from α-chymotrypsin is typical: α-chymotrypsin (1 mg, 40 nmol) was dissolved in 1.0 ml of acidified (pH 3.0) 8 M urea. After 30 min the pH was raised to pH 4.75 with 1 M KOH, and 12.5 μCi of methylamine (sp. act. 56 mCi/mmol) in 100 μl of water was added. The pH was readjusted to 4.75 and 100 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide was added. The pH was maintained at 4.75 for 1 h and then 1 ml of 20% (w/w) unlabelled methylamine adjusted to pH 4.75 was added followed by 250 mg of the carbodiimide. After 1 h the sample was dialysed (3 x 4 litre) against 0.5% (w/v) ammonium bicarbonate. Thermolysin (0.1 mg) was added to the sample and left to digest for 6 h and then freeze dried.

The procedure for the *B. thuringiensis* toxin was slightly modified from that of the test proteins. As it was suspected that the toxin may have a carboxyl terminal lysine residue,

the toxin (6 mg) was ^{14}C-acetylated (Kaplan *et al.*, 1982) prior to the application of the carboxyl terminal diagonal procedure described above in order to increase the sensitivity of the detection. Digestion in this case was carried out in 10% formic acid with pepsin (0.06 mg).

2.2.12 Carboxyl Terminal Peptide Isolation

The freeze-dried enzymatic digest was dissolved in a minimum volume of pH 2.1 buffer (100-500 μl), containing 10^5 cpm of [^{14}C]taurine and [^{14}C]choline, and spotted along a 2-cm band of Whatmann 3MM paper. Dansylarginine was spotted over the same band as an internal fluorescent marker. Electrophoresis was carried out at pH 2.1 for 40 min at a voltage gradient of 40 V/cm. After being dried, a strip running from the origin to 10 cm past the dansylarginine marker was cut out. The [^{14}C]choline ran slightly ahead of this marker and its presence and that of [^{14}C]taurine near the origin could be verified with a radiation monitor. This strip was stitched onto a sheet of Whatmann 3MM paper and was run at right angles to the original direction at pH 4.4 for 40 min at 60 V/cm.

The carboxyl terminal peptides were detected by autoradiography (3 days). The [^{14}C]taurine (net charge = 0 at pH 2.1 and 4.4) and [^{14}C]choline (net charge = +1 at pH 2.1 and 4.4) delineate the diagonal on which the carboxyl terminal

peptides lie. The radioactive spots on the diagonal line that pinpoint the carboxyl-terminal peptides were cut out, placed on a small Büchner funnel, and eluted with 500 µl of 1% acetic acid. Because the precise location of each peptide was known, they could be excised with no loss of material and no contamination from other peptides.

2.3 RESULTS

2.3.1 Characterization of the Trypsin Generated Toxin

Toxin was generated from RENOGRAPHIN purified crystals by treatment of the crystals with bovine trypsin (TPCK treated) at pH 10.5 and incubation overnight. A fully toxic (Appendix 3) electrophoretically pure protein precipitated upon the addition of 40 % (W/V) ammonium sulphate (figure 2). The apparent molecular mass of the protein was 67 kDa on SDS-PAGE. This agreed with the apparent molecular mass of 66 kDa obtained by gel filtration chromatography on a Sephadex G-200 column.

2.3.2 Determination of the Amino Terminal Cleavage Site on the Protoxin

The amino terminal of the toxin was identified by several methods. The results are summarized in table one. In all cases, the amino terminal amino acid of the toxin was found to be isoleucine. The amino acid composition of the amino terminal peptide isolated from a pepsin digest of the toxin was Thr,Glu,Gly,Ile. This analysis matched the sequence IETG starting at position 29 of the amino acid sequence deduced

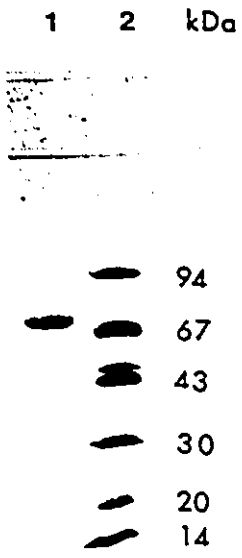


Figure 2

SDS-PAGE of the Toxin from *Bacillus thuringiensis* subsp. *kurstaki* HD-73 Generated with Bovine Trypsin. Lane 1, Bovine trypsin generated toxin from *B. thuringiensis* subsp. *kurstaki* HD-73. Lane 2, is a molecular mass standard.

Table 1

Determination of the of the N-terminal of the Toxin From *B. thuringiensis* subsp. *kurstaki* HD-73 Generated with Bovine Trypsin.

Method of determination	N-terminal Composition/sequence[§] Obtained
Dansylation[◆]	I
Isolation of N-terminal Peptide	IETG
Gas-phase sequencer*	IETGYTPIDISLSTQFLLS

[§] Adang *et al.*, (1985).
[◆] Determined by using the method of Gray (1967).
* Determined by Dr. D. Watson, NRC.

from the cryIA(c) gene. Amino terminal sequencing of the toxin on a gas phase sequencer (carried out by Dr. D. Watson, NRC Ottawa, Ontario, Canada) confirmed the position of the amino terminal of the toxin as being isoleucine 29 of the protoxin. The sequence IETGYTPIDISLSLTQFLLS was obtained and corresponded exactly to that predicted by the gene nucleotide sequence (Adang. *et al.*, 1985).

2.3.3 Determination of Carboxyl Terminal Cleavage Site

The underlying principle behind the procedure for the selective isolation of carboxyl terminal peptides is that the digestion of a protein in which all the carboxyl groups have been chemically modified will generate peptides in which only those derived from the carboxyl terminus will not contain a free carboxyl group. Therefore, only peptides derived from the carboxyl terminal will have the same mobility on high voltage paper electrophoresis at pH 2.1 and pH 4.4. They will lie on a diagonal line and be selectively separated from all other peptides. This will hold true for all carboxyl terminal peptides regardless of their composition.

The results obtained for α-chymotrypsin are illustrated in figure 3. The diagonal line is delineated by [^{14}C]taurine, which is neutral at pH 2.1 and 4.4, and [^{14}C]choline, which carries a charge of +1 at both pH values. The [^{14}C]choline is slightly overloaded and has streaked, but the diagonal line is

nevertheless quite distinct. There are five peptides (CT-1 through to CT-5) that lie on the diagonal line. Each of these peptides was carefully excised and eluted from the paper with dilute acetic acid. After lyophilization, the peptides were hydrolysed and the results of amino acid analysis are presented in table two. The amino acid analysis of CT-4 corresponds to a dipeptide from the carboxyl terminal of the B chain of α-chymotrypsin. The carboxyl terminal of the C chain was identified as CT-3. CT-1, CT-2 and CT-5 are all carboxyl terminal peptides derived from the A chain of α-chymotrypsin.

The carboxyl terminal electrophoretogram for ribonuclease A is shown in figure 4. There are two radioactive spots on the diagonal. The spot with the greatest electrophoretic mobility, R-2, was found to be a valine residue (table 2) which corresponds to the known carboxyl terminus of ribonuclease A. A second spot (R-1) was observed in the electrophoretogram . The amino acid analysis of this peptide did not give a composition which could be matched to any sequence in ribonuclease A. An SDS-PAGE gel of the starting stock of ribonuclease A revealed a contaminant of apparent molecular mass 15,000 Da. No further characterization of this contaminating protein was carried out.

The carboxyl terminal electrophoretogram of the *B.thuringiensis* subsp. *kurstaki* HD-73 trypsin generated toxin

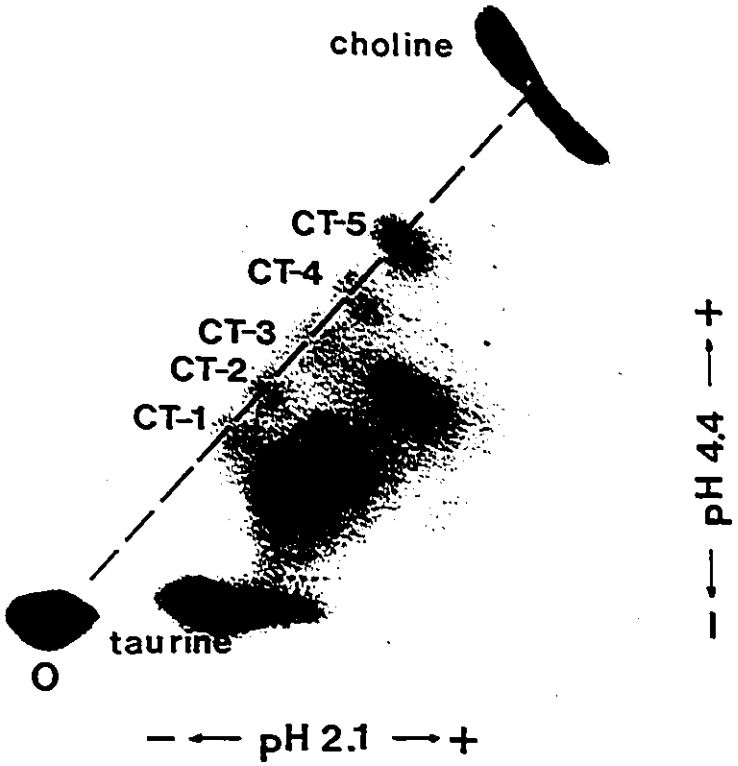


Figure 3

Carboxyl Terminal Autoradiogram from α-Chymotrypsin. Autoradiogram of the two-dimensional electrophoretogram obtained from a thermolysin digest of α-chymotrypsin. The broken line defines the diagonal delineated by [^{14}C]taurine and [^{14}C]choline. The peptides on the diagonal were isolated directly from the chromatogram by elution with dilute acetic acid.

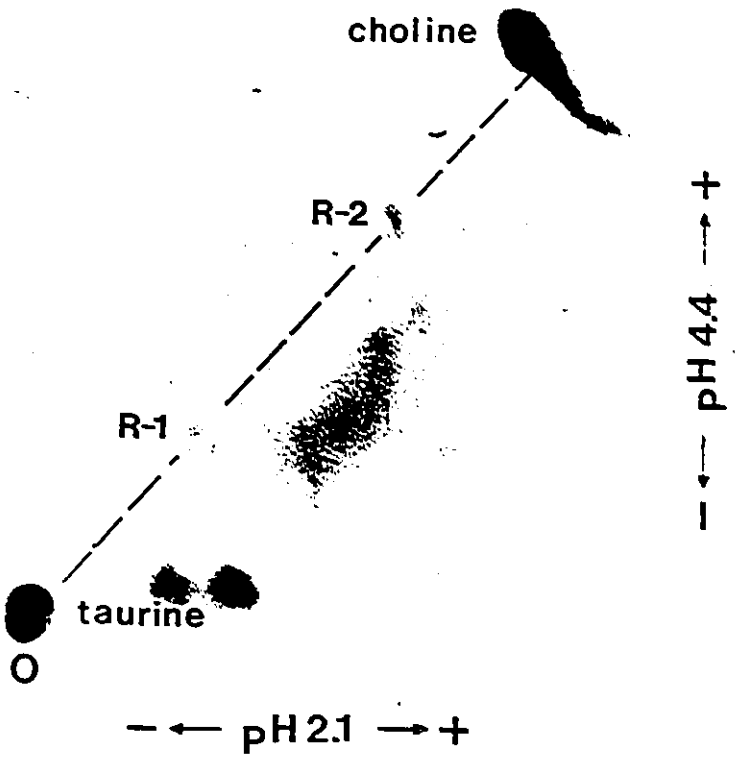


Figure 4

Carboxyl Terminal Autoradiogram from Ribonuclease A. Autoradiogram of the two-dimensional electrophoretogram obtained from a thermolysin digest of ribonuclease A. The broken line defines the diagonal delineated by [^{14}C]taurine and [^{14}C]choline. The peptides on the diagonal were isolated directly from the chromatogram by elution with dilute acetic acid.

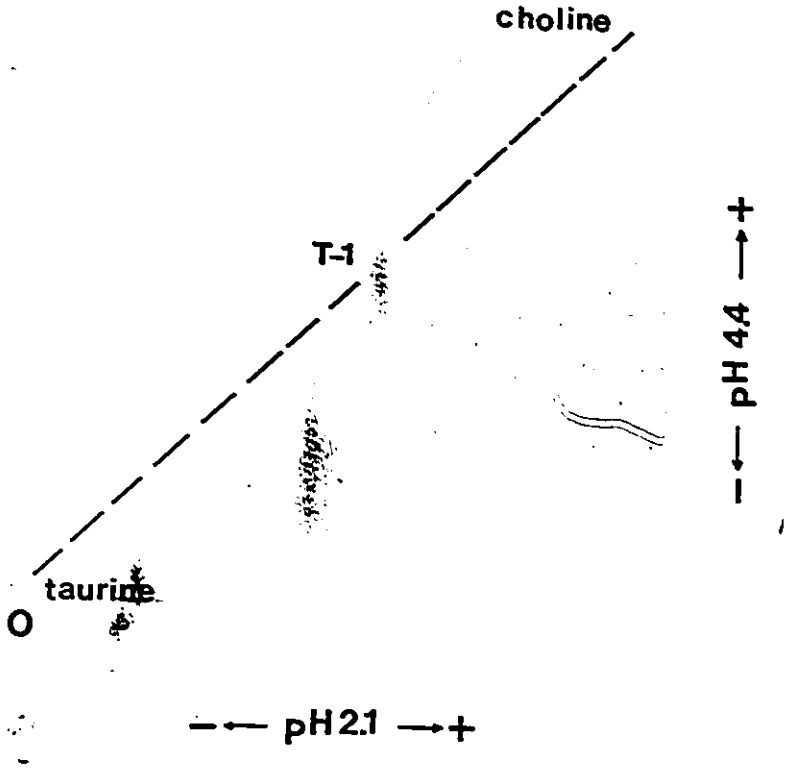


Figure 5

Carboxyl Terminal Autoradiogram from B. thuringiensis Toxin. Autoradiogram of the two-dimensional electrophoretogram obtained from a pepsin digest of ^{14}C-acetylated toxin from *B. thuringiensis* generated with bovine trypsin. The broken line defines the diagonal delineated by [^{14}C]taurine and [^{14}C]choline. The peptides on the diagonal were isolated directly from the chromatogram by elution with dilute acetic acid.

Table 2

Amino Acid Composition of Carboxyl Terminal Peptides from α-Chymotrypsin, Ribonuclease A and *B.thuringiensis* subsp. *kurstaki* HD-73 Toxin Generated with Bovine Trypsin.

	Composition of the isolated peptides				Carboxy-terminal sequences of the proteins
CT-1	Ser (1.30)	Gly (1.00)	Val (0.90)	Leu (2.00)	Val-Leu-Ser-Gly-Leu*
CT-2	Ser (1.20)	Gly (1.00)	Leu (2.00)		Leu-Ser-Gly-Leu*
CT-3	Asp (0.55)	Ala (1.67)	Leu (1.00)		Leu-Ala-Ala-Asn*
CT-4	Tyr (0.61)	Arg (1.00)			Arg-Tyr*
CT-5	Leu (1.00)				Leu*
R-1	Ala (1.51)	Thr (3.00)	Leu (1.10)	Arg (1.85)	
R-2	Val (1.00)				Val*
T-1	Glu (1.00)	Lys (1.00)			Gln-Lys[1]

* Dayhoft 1972
[1] Adang et al., (1985).

(figure 5) gave a single radioactive spot lying on the diagonal line. The electrophoretic mobility (μ_{ect}=1.3 at pH 2.1) corresponded to that of a dipeptide with a charge of +1. Amino acid analysis of this peptide (table 2) gave a composition of one glutamic acid and one lysine. This corresponds to the sequence Glu-Lys at position 622 and 623 of the cryIA(c) gene deduced amino acid sequence (Adang *et al.*, 1985).

2.3.4 Amino Acid Composition of the Toxin

The isolated and purified toxin contains 595 amino acid residues which corresponds to a protein spanning residues 29 to 623 of the protoxin. The amino acid composition of the toxin (table 3) is in excellent agreement with that predicted by the gene nucleotide sequence (Adang. *et al.*, 1985). The molecular mass of 66.7 kDa calculated from the amino acid composition, is also in excellent agreement with the molecular masses determined by SDS-PAGE and gel filtration.

Table 3

Amino Acid Analysis of the *B. thuringiensis* subsp. *kurstaki* HD-73 Toxin Generated with Bovine Trypsin.

Amino acid	Predicted number of residues[1] (Ile 29 to Lys 623)	Observed number of residues*
Ala	37	38.6
Arg	43	44.5
Asx	67	67.2
Cys[§]	0	0
Glx	54	57.8
Gly	43	43.2
His	9	9.4
Ile	45	37.5
Leu	48	50.9
Lys	3	2.7
Met	7	5.7
Phe	36	33.4
Pro	30	28.4
Ser	60	61.3
Thr	36	37.3
Trp[#]	10	9.7
Tyr	27	27.9
Val	39	39.3

* Calculated as mol/595mol; average of two determinations with the estimated error less than 7% for all amino acids.
§ Determined as cysteic acid after performic acid oxidation.
# Determined by hydrolysis in methane sulphonic acid.
[1] From the predicted sequence Adang *et al.*, (1985)

2.4 DISCUSSION

2.4.1 Characterization of the Trypsin Generated Toxin.

Lecadet and Martouret (1967) have shown that enzymatic hydrolysis is required to activate the protoxin. The term "activation" is loosely defined as the enzymatic process which converts the ~130 kDa protoxin to the biologically active toxin of apparent molecular mass ~50-70 kDa. This activation process can be accomplished *in vitro* by endogenous proteinases (Chestukhina *et al.*, 1982), digestive enzymes from the insect gut (Lüthy 1980), or with a variety of bacterial, plant or mammalian enzymes (Faust *et al.*, 1967). The endogenous proteinases have been identified as serine-, sulfhydryl-, amino-, and metallo-proteinases (Chestukhina *et al.*, 1982, and Andrews *et al.*, 1985). The lepidopteran larvae which show susceptibility to the δ-endotoxin have, in general, high alkaline midgut digestive systems with characteristically high pH optima for their trypsin-like and chymotrypsin-like digestive enzymes (Applebaum, 1985., Eguchi and Kuriyama, 1983. and Houseman *et al.*, 1988). The products of these enzymatic digestion have not been characterized as to the enzymatic cleavage sites. Since a supply of purified insect enzymes was not available, bovine trypsin was used in our studies to activate the protoxin.

Tryptic digestion of protoxin crystals which are free of spores and contaminating endogenous proteinases results in a fully toxic toxin preparation which can be purified to electrophoretic homogeneity by a rapid and straightforward procedure. Precipitation of the toxin with ammonium sulphate, followed by extensive dialysis against water using 50 kDa cut-off dialysis tubing, gave a toxin preparation which was free of contaminating peptides and residual trypsin. The recovery of toxin prepared by this procedure was shown to be 60-70% of the theoretical maximum, and the protein was highly toxic towards spruce budworm larvae (Bietlot *et al.*, 1989).

Toxin preparations were shown to be homogeneous by several criteria. On both SDS and isoelectric focusing gels, toxin samples consistently yielded only a single band (Bietlot *et al.*, 1989). HPLC fractionation resulted in a single large peak being separated from several other components which were present in trace amounts (Bietlot *et al.*, 1989). The purified toxin was also shown to be a single polypeptide chain with only one carboxyl and one amino terminus.

The apparent molecular mass of toxin estimated from reducing and non reducing SDS gels was 67 kDa (Bietlot *et al.*, 1989). This apparent molecular mass agreed with the value obtained by gel filtration chromatography using Sephedex G-200. The molecular mass calculated from the amino acid analysis was 66.7 kDa. These values are in general agreement with the size of proteinase-stable toxins generated from

protoxin isolated from other strains of *B.thuringiensis*: 63 kDa from *kurstaki* HD-263 (Aronson and Arvidson, 1987), 58 kDa toxin from *dendrolimus* (Nagamastsu *et al.*, 1984), 68 kDa from *kurstaki* LB1 (Andrews *et al.*, 1985), and 65-80 kDa from several other subspecies of *B. thuringiensis* (Chestukhina *et al.*, 1982).

The identification of the bond between arginine-28 and isoleucine-29 as the amino terminal tryptic cleavage site of the protoxin was accomplished by three methods; isolation of the amino terminal amino acid, isolation of the acetylated amino terminal peptide, and by gas phase sequencing of the isolated toxin. Each method gave isoleucine-29 of the protoxin as the amino terminal of the isolated toxin. The identification of arginine 28 as the amino terminal of the toxin is in agreement with the amino terminal cleavage site determined by Nagamatsu *et al.*, (1984) for *B. thuringiensis* subsp. *dendrolimus*. Aronson and Arvidson (1987) also identified an isoleucine residue as the amino terminal of *B. thuringiensis* subsp. *kurstaki* HD-263. Chestukhina *et al.*, (1982) reported that no amino terminal processing by trypsin occurred during the activation process in four subspecies of B. thuringiensis. In all of these investigations, including the present study, the amino terminal half of the protoxin molecule was found to be resistant to tryptic digestion.

The difference in amino acid composition between the toxin and the protoxin suggests an unusual distribution of

certain amino acid residue in the protoxin (see appendix two for gene nucleotide sequence). Only three of the 34 lysine residues in the protoxin are present in the isolated toxin and none of the 16 cysteine present in the protoxin are contained in this fragment. It is interesting to note that the studies which report processing of the amino terminal also report no cysteine residues in the toxic fragment (Nagamatsu *et al.*, 1984).

2.4.2 Isolation of Carboxyl Termini of Proteins

The methodology developed for the isolation of carboxyl terminal peptides relies on establishing conditions where peptides with blocked carboxyl terminals will migrate equal distances under two different sets of conditions whereas those that have free carboxyl termini will not. This is achieved by working over a pH range (pH 2 to 4) where only carboxyl groups will ionize. Due to electroosmosis, it is necessary to include markers to delineate a diagonal line on which all carboxyl terminal peptides will lie. The use of radioactive compounds for the amidation reaction and to delineate the diagonal line has eliminated the losses involved in the ninhydrin detection procedure and has increased the sensitivity by a order of magnitude. The results obtained with the two standard proteins demonstrate that the methodology can be used to isolate the carboxyl terminal from

single chain proteins or from proteins with multiple polypeptide chains.

The carboxyl terminal cleavage site has not been previously determined for any *B. thuringiensis* toxic fragment (Aronson *et al.*, 1986, Milne *et al.*, 1990). Our amino acid analysis of the isolated toxin (table 3) determined that the toxin contained three lysine residues per molecule, making lysine at position 623 of the protoxin a likely site for the final carboxyl cleavage site. The determination of lysine 623 as the carboxyl terminal of the *B. thuringiensis* toxin generated with bovine trypsin represents the first report of a defined carboxyl terminal of an isolated toxic fragment.

The one variable in this methodology is the choice of digestive enzyme. In the case of α-chymotrypsin, the enzyme used produced several peptides from the same carboxyl terminal with a corresponding loss in potential yield. In the cases where the gene-derived amino acid sequence is known, it will be possible to use this information to choose the most appropriate enzyme and increase the possibility of obtaining high yields. In the cases where the protein is not soluble in dilute alkali (i.e. the *B. thuringiensis* toxin), acidic proteinases can be used to carry out the digestion. The proteins can also be denatured prior to digestion to increase the yield of the carboxyl terminal peptide.

This carboxyl terminal diagonal procedure is applicable to all proteins regardless of the carboxyl terminal

composition or number of polypeptide chains in the protein. It is only limited by the sensitivity of the detection method. With the modifications described here, carboxyl terminal peptides can be isolated from as little as 40 nmol of protein. As yield of carboxyl terminal peptides usually vary from 1 to 10% this corresponds to the range of 400 pmol to 4 nmol of peptide. These amounts are more than sufficient for sequencing by automated gas phase sequencer currently in use. Greater sensitivity could be achieved by using a higher specific activity of ^{14}C-label in the amidation step or by using more sensitive film with enhancers for detection. Another possibility is to cut out the diagonal line between the diagonal markers, elute the peptides lying on the diagonal line, and separate them by reverse-phase HPLC using UV absorbance for detection. This variation has the added advantage of detecting naturally blocked carboxyl terminal of proteins as they will not incorporate any radioactive label.

The methodology developed can be used for the determination of the carboxyl terminal sequences on relatively small amounts of protein (i.e. 40 nmol.) regardless of composition. It could also be useful in the verification of carboxyl terminal sequences predicted by gene nucleotide sequence. The large number of cloned gene products being produced has increased the need for carboxyl terminal sequence determination in order to define post-synthetic processing of proteins and to confirm the correct placement of initiation

and termination codons. Carboxyl terminal determination should also be useful in the design of oligonucleotide probes for the screening of cDNA libraries.

CHAPTER 3

Competitive Labelling of the *B. thuringiensis* Crystal Protein

3.1 INTRODUCTION

The study of functional groups in a protein that are involved in inter and intra molecular interactions is not easily accomplished. One approach which can be used to study these interactions is the method of competitive labelling (Kaplan *et al.*, 1971).

Competitive labelling is a technique for determining the chemical properties of a class of functional groups in a protein. Since the pK and reactivity of each functional group reflects it's micro-environment, the technique can be used to study structural and functional aspects of proteins. Chemical reactivity is an intrinsic property that can be influenced by several factors, including medium effects, steric constraints and micro-environment (Young and Kaplan, 1989). For example, if a group is involved in hydrogen bonding, ionic interaction, or is buried in the interior of a protein or complex, its chemical properties will differ from those of the same group interacting freely with the bulk solvent (Young and Kaplan, 1989).

The method of competitive labelling employs a trace label to ensure that the protein is in large excess over the modifying reagent and hence, the ratio of functional group to modifying reagent is even greater. The functional groups on

the protein must then compete for reagent. Unlike many conventional chemical modification procedures in which the perturbation of the native structure cannot *a priori* be ruled out, the low levels of derivatization used during the trace labelling procedure ensures that the chemical properties of the functional group reflect the properties of the group in the native structure of the protein. In order to provide a scale of reference against which the functional group's reactivity can be compared, an internal standard can be introduced into the reaction mixture.

3.2 MATERIALS AND METHODS

3.2.1 Materials

N-acetyl-L-lysine and N-acetyl-L-tyrosine were obtained from Sigma (St. Louis, MO, USA). 1-fluoro-2,4-dinitrobenzene (Dnp-F) (16.6 Ci/mmol) and [^{14}C] Dnp-F (12 mCi/mmol) were obtained from Amersham Corp. (Oakville, Ontario, Canada). Poropak Q was supplied by Waters Associates (Mississauga, Ontario, Canada). Sephacryl S-300 was obtained from Pharmacia Canada (Montreal, PQ.). Other chemicals were high purity preparations obtained from commercial sources.

The *Bacillus thuringiensis* subsp. *kurstaki* HD-1 strain used in all experiments carried out in this thesis was obtained from stock cultures maintained on nutrient agar slants and stored at 4°C at the Forest Pest Management Institute (FPMI), Sault Ste. Marie, Ontario, Canada.

3.2.2 Growth of *B. thuringiensis*

B. thuringiensis subsp *kurstaki* HD-1 was grown and purified as described in section 2.2.2. and 2.2.3.

3.2.3 Preparation of Soluble Protoxin for Competitive Labelling

Purified *B. thuringiensis* subsp. *kurstaki* HD-1 crystals (25 mg) were solubilized by incubation in 5 ml of 0.1 M CAPS, 2% (v/v) ß-mercaptoethanol at pH 10.0. Any insoluble material was removed by centrifugation before the solubilized protoxin was applied to a Sephacryl S-300 column (120X1 cm) and eluted with 50 mM Tris, 1 mM EDTA, 0.1% (v/v) ß-mercaptoethanol at pH 8.0. The flow rate was 15 ml/hour and 3 ml fractions were collected. The pooled fractions containing the protoxin were dialysed against 0.1 M KCl, 5 mM N-methylmorpholine, 5 mM sodium borate at pH 8.0. No reducing agent was present for the labelling as it would act as a compeating nucleophile. Homogeneity of the protoxin peak was verified by SDS-PAGE.

3.2.4 Protoxin Quantification for Competitive Labelling

A measured aliquot (500 µl) of the protoxin stock solution at pH 8.0 was removed and 50.0 nmol of norleucine was added. Samples were hydrolysed in 6 N HCl at 110°C *in vacuo* for 24 h and each amino acid was quantified by amino acid analysis. The amount of protoxin was determined by dividing the moles of each amino acid by the number of residues of the amino acid in the protoxin. The results from each amino acid except serine, threonine, proline and tryptophan were averaged

to give the quantity of protoxin per unit volume.

3.2.5 Preparation of [^{14}C]DNP-Derivatives for Competitive Labelling

O-[^{14}C]-DNP-tyrosine, N$^{\alpha}$[^{14}C]-DNP-lysine and [^{14}C]-DNP-alanylalanine were prepared by reacting alanylalanine and the N-acetyl derivatives of these amino acids with [^{14}C]Dnp-F as described previously (Hefford, *et al.*, 1985). The purification procedure was the same as that described by Hefford, *et al.*, (1985) except that the final purification step was carried out by C18 reverse phase HPLC. The [^{14}C]DNP-alanylalanine was eluted using the following solvents: 0.01 N HCl-17% acetonitrile for 6 minutes; 0.01 N HCl-25% acetonitrile for 4 minutes followed by 0.01 N HCl-60% acetonitrile for 30 minutes. The [^{14}C]-DNP-residues were eluted isocratically with 0.01 N HCl-60% acetonitrile.

3.2.6 Competitive Labelling of the Crystal protein (Protoxin)

An aliquot (1.0 ml) of a stock solution containing 0.113 mg/ml (3.57×10^{-7}M) of protoxin and alanylalanine (7.50×10^{-5}M) in 5 mM N-methylmorpholine and 5 mM sodium borate were equilibrated at 25°C in a water bath. The pH was adjusted to the desired value between 7 and 10 using either 1 N NaOH or 1 N HCl. An aliquot (50 μl) of acetonitrile

containing [^{3}H]Dnp-F (20.8 nmol, Sp. act. 16.6 Ci/mmol) was added with vigorous stirring and the reaction allowed to proceed 18 h in the dark. Concentrated HCl was added to bring the pH to 2.0.

The following was added to each sample: an aliquot (1.0 ml) of a 25% acetone solution containing 2500 dpm of [^{14}C]DNP-alanylalanine and each of the [^{14}C]-DNP-residues and 0.03 mg of each unlabelled DNP-derivative as carrier. The acetone was removed by evaporation and the samples were hydrolysed in 6 N HCl for 18 h at 110°C *in vacuo*.

3.2.7 Purification of [^{3}H]/[^{14}C]DNP-Derivatives

The [^{3}H]/[^{14}C]DNP-derivatives were isolated and purified as described previously (Hefford, *et al.*, 1985), with the only change being that the final purification step was carried out by C18 reverse phase HPLC as described in section 3.2.5.

3.2.8 Liquid Scintillation Counting

Samples were dissolved in 0.1 ml of 0.01 N HCl and added to 10 ml of Aquasol-2 (Dupont, Canada). Scintillation counting was carried out on a programmable LKB 1215 Rack Beta scintillation counter equipped with automatic quench correction and a disintegrations per minute converter.

3.3 RESULTS

The method of competitive labelling (Kaplan *et al.*, 1971, Young and Kaplan, 1989) was used on the solubilized protoxin from *B.thuringiensis* subsp. *kurstaki* HD-1 to determine the reactivities and pKa's of the lysine and tyrosine functional groups. These experiments were carried out at pH values between 7.0 and 10.0 with alanylalanine added as an internal standard. The reactivity of the side chains of the lysine and tyrosine residues of the solubilized protoxin, relative to the α-amino group of alanylalanine, was determined by reacting with a trace amount of [^{3}H]Dnp-F. Under these conditions the functional groups in the protein compete for the label, and the amount of [^{3}H]-label incorporated is dependent on the reactivity of the functional group at the pH value of the labelling. In order to determine the relative reactivities of the functional groups, it is necessary to determine the incorporation of the [^{3}]H-label into each group. This was achieved by adding equal amounts of N$^{\varepsilon}$[^{14}C]DNP-lysine, O-[^{14}C]DNP-tyrosine and [^{14}C]DNP-alanylalanine to each reaction mixture, extracting the [^{3}H]/[^{14}C]DNP-alanylalanine and then hydrolysing the mixture in 6 N HCl. Each of the [^{3}H]/[^{14}C]DNP-derivatives was purified by HPLC and its ^{3}H/^{14}C-ratio quantified by scintillation counting. This ratio was corrected for the amount of each residue present in the

protoxin (Table 4).

The data was analyzed using the following expression (Young and Kaplan 1989):

$$\alpha_x r = \alpha_s \, (^3H/^{14}C)x/(^3H/^{14}C)s$$

In the above expression, α_x is the degree of ionization of the functional group under study, α_s is the degree of ionization of the internal standard, alanylalanine (pK = 8.31) at 25°C, r is the pH-independent second order velocity constant for the reaction of the functional group under study relative to the amino group of alanylalanine, and $(^3H/^{14}C)x$ and $(^3H/^{14}C)s$ are the radioactivity ratios determined by scintillation counting for the functional group and alanylalanine. If the groups under study titrate normally over the entire pH range employed, a plot of $\alpha_x r$ versus pH will give a titration curve with pK = pH at the inflection point and a limiting value of $\alpha_x r = r$.

The reactivity data obtained over the pH range 7-10 for the tyrosine and lysine side-chains of the protoxin from *B. thuringiensis* subsp. *kurstaki* HD-1 is shown in Figures 6 and 7. The tyrosine and lysine residues show a general upward trend in reactivity but do not show regular titration behaviour that can be fitted to a titration curve. Table 5 compares the reactivities of the lysine and tyrosine functional groups of the protoxin with the corresponding groups in concanavalin A (Jackson and Young, 1986), IgA, IgG and IgM (Kaplan *et al.*, 1980), VIP and glucagon (Hefford *et al.*, 1985) relative to the amino group of alanylalanine.

Table 4

Number of Residues in the Solubilized Protoxin from *B. thuringiensis* subsp. *kurstaki* HD-1

Amino Acid	Number of residues
Lysine	33
Tyrosine	52

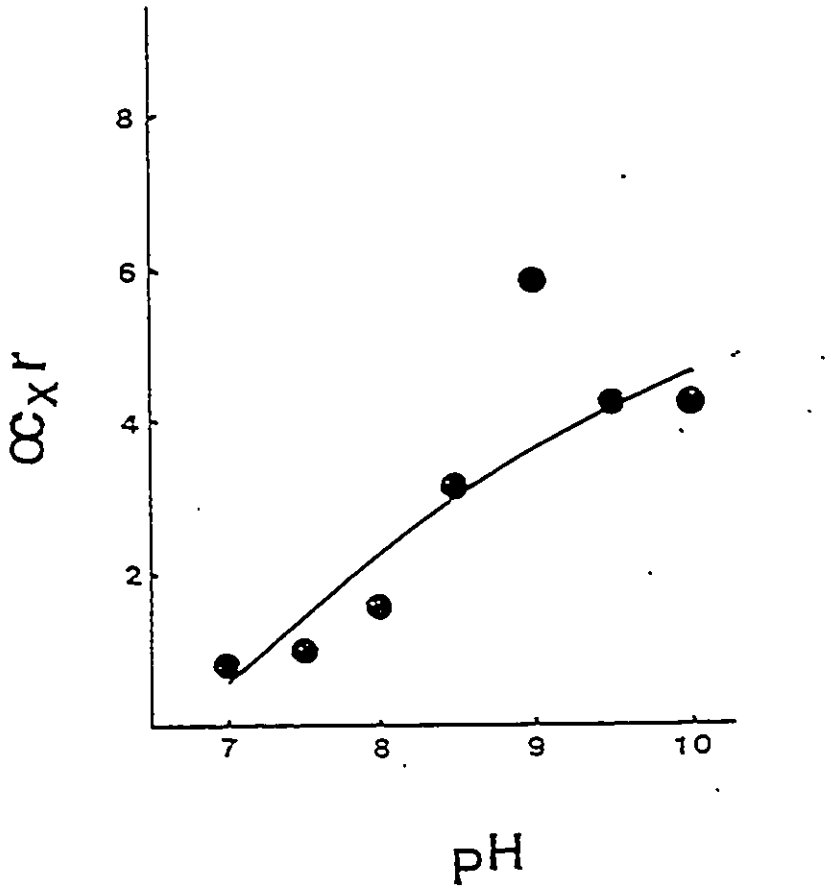


Figure 6

Reactivity-pH profile for the ε-amino groups of the lysine residues in the solubilized protoxin from *B. thuringiensis* subsp. *kurstaki* HD-1. Solvent conditions were 0.1 N NaCl, 5mM N-methylmorpholine, 5mM sodium borate at 25°C.

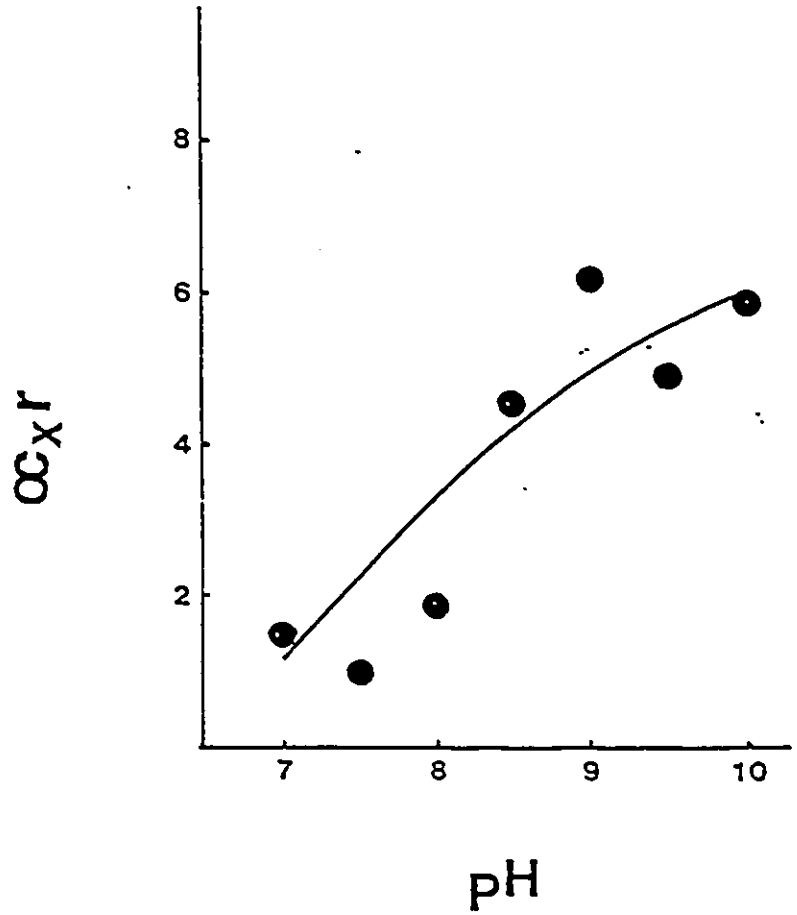


Figure 7

Reactivity-pH profile for the hydroxyl groups of the tyrosine residues in the solubilized protoxin from *B. thuringiensis* subsp. *kurstaki* HD-1. Solvent conditions were 0.1 N NaCl, 5mM N-methylmorpholine, 5mM sodium borate at 25°C.

Table 5

Comparision of the pH independent Reactivities of Amino Acid Side Chains in *B. thuringiensis* and other proteins*

Protein	Residue	
	Lysine	Tyrosine
Cancanavalin A[b]	8.0	4.0
VIP[c]	2.4	-
Glucagon[c]	0.338	-
IgA[a,d]	25	10.4
IgM[a,d]	10	10.5
IgG[a,d]	10.5	9.8
Protoxin	6.0	4.0

* Reactivity relative to the α-amino group of alanylalanine.
[a] Value calculated on the basis of a reactivity of 25.4 ± 0.5 for alanylalanine relative to L-ß-imidazolylactic acid (Hefford *et al.*, 1985).
[b] Jackson and Young, 1986.
[c] Hefford *et al.*, 1985.
[d] Kaplan *et al.*, 1980.

3.4 DISCUSSION

Competitive Labelling has been applied to a wide variety of proteins (Young and Kaplan, 1989) and it has been observed that in some cases the reactivity-pH profiles fit continuous titration curves whereas in others, discontinuities or irregular behaviour is observed. *A priori* there is no reason why a functional group in a protein should fit a regular titration curve. A continuous titration curve will be observed with groups which are exposed on the surface of the protein and remain in the same environment over the whole pH range employed, that is, there are no conformational changes which alter the nature of the equilibria which determine the reactivity of the group in question. Groups which are buried or interacting with other structural features e.g. salt bridge or hydrogen bonding usually do not exhibit normal titration and/or reactivity behaviour (Young and Kaplan 1989).

The data obtained represents the average reactivity for all the lysine and tyrosine residues relative to reactivity of the amino terminal of the internal standard, alanylalanine. The reactivities for the lysine and tyrosine residues of various proteins are given in table 5. In comparison to the reactivities observed for concanavalin A, IgA, IgG and IgM, the reactivity of the lysine and tyrosine in the protoxin are not unusually reactive. Furthermore, the reactivity data

obtained for the lysine and tyrosine side chains does not fit a regular titration curve. This indicates that the functional groups of lysine and tyrosine are not behaving as free functional groups in solution but that they are interacting with other groups or feature in a pH dependent manner.

CHAPTER 4

Evidence that the *B. thuringiensis* insecticidal proteins are associated with DNA

4.1 Introduction

During the course of characterization of the *B. thuringiensis* toxin it was discovered that the lepidopteran-specific insecticidal proteins are associated with DNA. When this research was initiated, it was generally accepted that the crystal was entirely proteinaceous in nature and that neither nucleic acid nor any other molecule had a structural or functional role. The only study which investigated the possibility of nucleic acid association with the crystal (Bulla *et al.*, (1977) found that no DNA or RNA was present in *B. thuringiensis* preparations. Our first inclination on detecting the presence of nucleic acid bound to the crystal protein was that the observed association was a non-specific adsorption of the nucleic acid to the crystalline inclusion and to the protoxin. The last series of experiments performed for this thesis were aimed at characterizing the association of DNA with the insecticidal protein from *B. thuringiensis* and at determining the role of this association in the structure-function relationships in the protoxin and toxin.

4.2 Materials and Methods

4.2.1 Materials

RQ1 DNase was obtained from Promega, (Montreal, PQ., Canada). RENOGRAFIN-76/GASTROgrafin was obtained from Squibb Canada, (Montreal, PQ.). All dialysis tubing was purchased from Spectrum (Los Angeles, California, USA). Poropak Q was supplied by Waters Associates (Mississauga, Ontario, Canada). Other chemicals were high purity preparations obtained from commercial sources.

The *Bacillus thuringiensis* subsp. *kurstaki* HD-73 and HD-1 strains used in all experiments carried out in this thesis were obtained from stock cultures maintained on nutrient agar slants and stored at 4°C at the Forest Pest Management Institute (FPMI), Sault Ste. Marie, Ontario, Canada. Purified crystal preparations of *B. thuringiensis* subsp. *kurstaki* HD-1, *kurstaki* HD-73, *galleria, tolworthi, alesti and entomocidus* were generously donated by R. Milne of the FPMI.

4.2.2 Growth and purification of *B. thuringiensis crystal inclusion bodies*

B. thuringiensis subsp. *kurstaki* HD-73 and/or HD-1 were grown and purified as described in section 2.2.2 and 2.2.3.

4.2.3 Cloned CryIA Gene Products Produced in *E.coli*

The cryIA(a), (b), (c) genes cloned into *E.coli* were provided by Drs. Masson and Brousseau of the BRI (McLinden *et al.*, 1985, Brousseau and Masson, 1988, Masson *et al.*, 1989). These bacteria were grown in trypticase-soy broth (30 g/l) and yeast extract (10 g/l) at 32°C with constant vigorous shaking. After 24 h the cells were pelleted and washed with 25 mM Tris-HCl, 100 mM NaCl, and 1 mM EDTA at pH 8.0. The cells were re-suspended in the same buffer and treated with lysozyme (2% w/w), deoxycholate (0.2% w/w), and DNase I and RNase (50 μg/ml) for 1 hour. The inclusion bodies were then purified by the RENOGRAFIN density gradient described above.

4.2.4 Diisopropyl Fluorophosphate (DFP) Treatment of the Crystal Inclusion Bodies

Crystals were treated with diisopropyl fluorophoshate (DFP) (Naughton *et al.*, 1960) when they were first harvested and when they were solubilized. Typically the protein was treated with 1μl of DFP per milligram of protein.

4.2.5 Preparation of Carbaminomethyl Cysteine (CAM) Protoxin

Typically, 10 mg of *B. thuringiensis* subsp. *kurstaki*

HD-73 protein was dissolved at 20°C in 10 ml of 0.1 M tris, 0.1 M CAPS containing 0.01% (v/v) ß-mercaptoethanol. To help solubilize the protoxin, the pH of the solution was made 10.5. After 5 min, the pH was lowered to 8.6 and maintained throughout the reaction with the addition of base. To carbaminomethylate the cysteine residues, 250 mg of iodoacetamide was added directly to the reduced protein. The reaction was stopped by the addition of ß-mercaptoethanol followed by extensive dialysis against distilled water at pH 4.5 in dialysis tubing with a molecular weight cut-off 12-14 kDa. The samples were stored in distilled water at 4°C.

4.2.6 Preparation of Toxin

Trypsin (5 mg) was added to a suspension of crystals (100 mg) in 5 ml of 0.1 M CAPS buffer, pH 10.5, and the suspension stirred for 20 h at 20°C. Undissolved crystals were removed by centrifugation at 10,000 x g at 4°C for 20 min. The supernatant was then dialysed against distilled water (3 x 4 litre) at pH 4.5 at 4°C using 50 kDa cut-off dialysis tubing. The samples were stored at 4°C in distilled water.

4.2.7 Ion Exchange Chromatography of *B. thuringiensis* Toxin and CAM-protoxin

Toxin was purified on a Pharmacia FPLC equipped with a Mono Q HR 10/10 anion exchanger. Elution was carried out at room temperature with a 0 to 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 detected by UV absorbance at 280 nm. CAM-protoxin was purified by HPLC on a Bio-Rad MA7Q column (50 x 7.8 mm). Elution was also carried out at room temperature with a 0 to 1 M NaCl gradient in 0.1 M CAPS pH 10.5. The flow rate was 1.5 ml/min and the protein detected by absorbance at 280 nm. All materials collected were thoroughly dialysed against distilled water. Acetic acid was added to pH 5 and the precipitated protein was removed and stored in distilled water at 4°C.

4.2.8 Extraction of DNA from Protoxin and T2 Toxin

Renografin purified crystals were solubilized in CAPS buffer at pH 10.5 in the presence of 1% (v/v) ß-mercaptoethanol. The extraction of the DNA from the protoxin was achieved by the addition of an equal volume of phenol-chloroform at 65°C previously equilibrated in 0.1 M CAPS at pH 10.5. The aqueous phase was then re-extracted with an equal volume of chloroform, and the DNA was precipitated by

the addition of two volumes of 0.3 M sodium acetate at pH 5.5 and two volumes of ethanol. The DNA was then run on a TAE agarose gel (0.8% w/v). DNA was extracted from the T2 toxin with phenol-chloroform in the same fashion as the crystal protein except that the phenol-chloroform was equilibrated in TE buffer at pH 8.0 at room temperature. The samples were then run on agarose gels as described above (Bietlot *et al.*, 1993).

4.2.9 DNase and RNase Digestion of the Extracted DNA

Isolated DNA was treated with DNase 1 and ribonuclease A under standard conditions as described in Sambrook *et al.*, (1989). This work was performed by Dr. Schernthaner.

4.2.10 Fluorometric DNA Detection

The nature of the nucleic acid associated with the crystal protein was determined by Dr. Schernthaner (Bietlot *et al.*, 1993) using the DNA specific dye bisbenzimidazole (Hoechst 33258) according to the procedure of Labarca and Paigen (1980).

4.2.11 Quantification of the DNA

The amount of DNA present in FPLC purified CAM protoxin was estimated using OD 260/280 nm (Monroe and Fleck, 1966) at pH 10.5. The extinction coeficients used were 0.05 $M^{-1}cm^{-1}$ and 0.028 $M^{-1}cm^{-1}$ at 260 and 280 nm respectively (Sambrook *et al.*, 1989). Protein quantification was carried out using the BIO-RAD protein quantification kit.

4.2.12 Succinylation of CAM-Protoxin

CAM-protoxin (25 mg) was succinylated in the absence of denaturants as previously described to give SCAM-protoxin Choma and Kaplan (1992). The SCAM-protoxin was divided into two equal portions in order to demonstrate that SCAM-protoxin renatured after treatment with 8 M urea. As a control, one portion was dialysed extensively against water adjusted to pH 8.5 by the addition of NH_3 (Figure 18 A). The other portion (Figure 18 B) was made 8 M in urea at pH 4 and allowed to stir for 30 min. The pH of the solution was then raised to 8.5 and left stirring for another 30 min before being dialysed against distilled water-NH_3 (1 w/v) at pH 8.5.

A second 25 mg aliquot of CAM-protoxin was dissolved in 8 M urea at pH 4. This solution was left stirring for 30 min before the pH was raised to 9 by making the solution

1% in $NaHCO_3$. This sample was then divided into two equal portions in order to demonstrate the effect of succinylating buried amino groups on the binding of DNA. One portion (Figure 18 C) was succinylated by the addition of succinic anhydride and then dialysed against distilled water-NH_3 (1% w/v), pH 8.5. As a control, the other portion (Figure 18 D) was dialysed against distilled water-NH_3 (1% v/w), pH 8.5. A final dialysis of all four samples was carried out against distilled water and the precipitated protein removed by centrifugation. Equal amounts of the precipitated proteins were then digested with trypsin (0.001% w/w) at pH 10.5 as described previously (Choma *et al.*, 1990).

4.2.13 DNase Sensitivity of CAM-Protoxin

Three samples of CAM-protoxin (2 mg) were dissolved in 2 ml of buffer (40 mM tris pH 9.5, 10 mM $MgCl_2$ and 2 mM spermidine) at 25°C. The first sample (Figures 19 A and 19 B) was used as a control and had no DNase added. To the second sample (Figures 19 C and 19 D), 2% (w/w) of Sigma DNase I was added and the incubation was continued for up to 3 days. The third sample (Figures 19 E and 19 F) contained 10 mM EDTA which was added prior to the addition of the DNase and digested in the same conditions as described above. All samples were stirred for the duration of the experiment. At each time point, two aliquot were removed, one to determine

the protoxin content and the other to be digested with 0.1% (w/w) trypsin for 1 h.

4.2.14 SDS-PAGE

SDS-PAGE was carried out as previously described.

4.2.15 Toxicity Assay

The biological activity of the T1 and T2 toxins was determined by the Forest Pest Management Institute of Saute Ste Marie using CF-1 insect cells in a lawn assay (Gringorten *et al.*, 1990).

4.2.16 Microscopy

B. thuringiensis subsp. *kurstaki* HD-1 was grown for 24 h at 30°C in half-strength trypticase soy broth (100 ml in 300 ml baffled flasks). Freshly grown culture was incubated for 30 min at room temperature with ethidium bromide at 1 ug/ml in TAE buffer. Cells were observed by R. Milne with phase contrast and fluorescence using a 100x objective and oil immersion (Bietlot *et al.*, (1993).

4.3 RESULTS

4.3.1 Association of DNA with *B. thuringiensis* Proteins.

Cells from sporulating *B. thuringiensis* subsp. *kurstaki* HD-1 cultures incubated with ethidium bromide showed a shifting pattern of nucleic acid distribution within the bacterium. Just prior to sporulation, nucleic acid was observed to condense in the region of spore formation. After spore formation the fluorescence from this region vanishes and appears in the region where the crystalline inclusion body is assembled (Figure 8).

The elution profile of trypsin-generated toxin on a Mono Q quaternary ammonium anion exchanger is shown in Figure 9. Two major peaks elute, one at 0.3 M NaCl (T1) and the other 0.9 M NaCl (T2). The relative proportions of T1 and T2 obtained depended on the source of the trypsin, the amount used and the length of treatment. Overnight treatment of the crystal protein with high concentrations (5% w/w) of trypsin yielded mostly T1 whereas shorter treatments of a few hours yielded mostly T2. Both these peaks gave a protein band with an apparent molecular mass of 67 kDa (figure 10) and had equal cytolytic activities toward CF-1 cells. The spectrum of T2 (Figure 11) showed that it had an absorption at 260 nm which was not present in the T1 fraction.

The absorption at 260 nm indicated the presence of nucleic acid associated with the T2 toxin. This possibly was supported by the observation that incubation of T2 with DFP treated DNase I (Sigma and Promega RQ1) yielded T1. Further evidence for the presence of DNA was obtained by a positive reaction with the DNA specific dye, Hoechst 33258, with T2 but not with T1. Extraction of T2 with phenol-chloroform at pH 8 showed the presence of nucleic acid fragments of 100-300 bp (Figure 12, lane 3) on ethidium bromide treated agarose gels. The susceptibility of the isolated nucleic acid to DNase I but not to RNase A (figure 13) again confirmed the presence of DNA.

CAM-protoxin was prepared by derivatization of the sulfhydryl groups of the reduced crystal protein with iodoacetamide (Choma and Kaplan, 1992). The elution profile of the CAM-protoxin on an anion exchanger using UV absorption at 260 nm and 280 nm shows only one peak (Figure 14). As in the case of the T2 toxin fraction, the absorbance was greater at 260 nm than at 280 nm indicative of the presence of nucleic acid. Incubation of the CAM-protoxin with DNase for less than 1 hour did not alter its HPLC elution profile. Similarly, attempts to extract the nucleic acid with phenol-chloroform at pH 8 and room temperature were unsuccessful. However, extraction at pH 10.5 and 65°C with phenol-chloroform yielded a major 20-kb fragment along with a tail of lower molecular weight fragments down to a 100 bp in length (Figure 12, lane

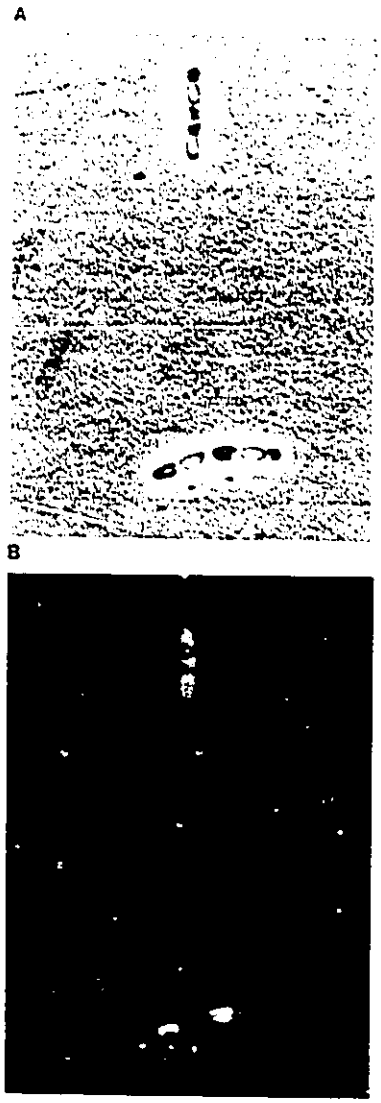
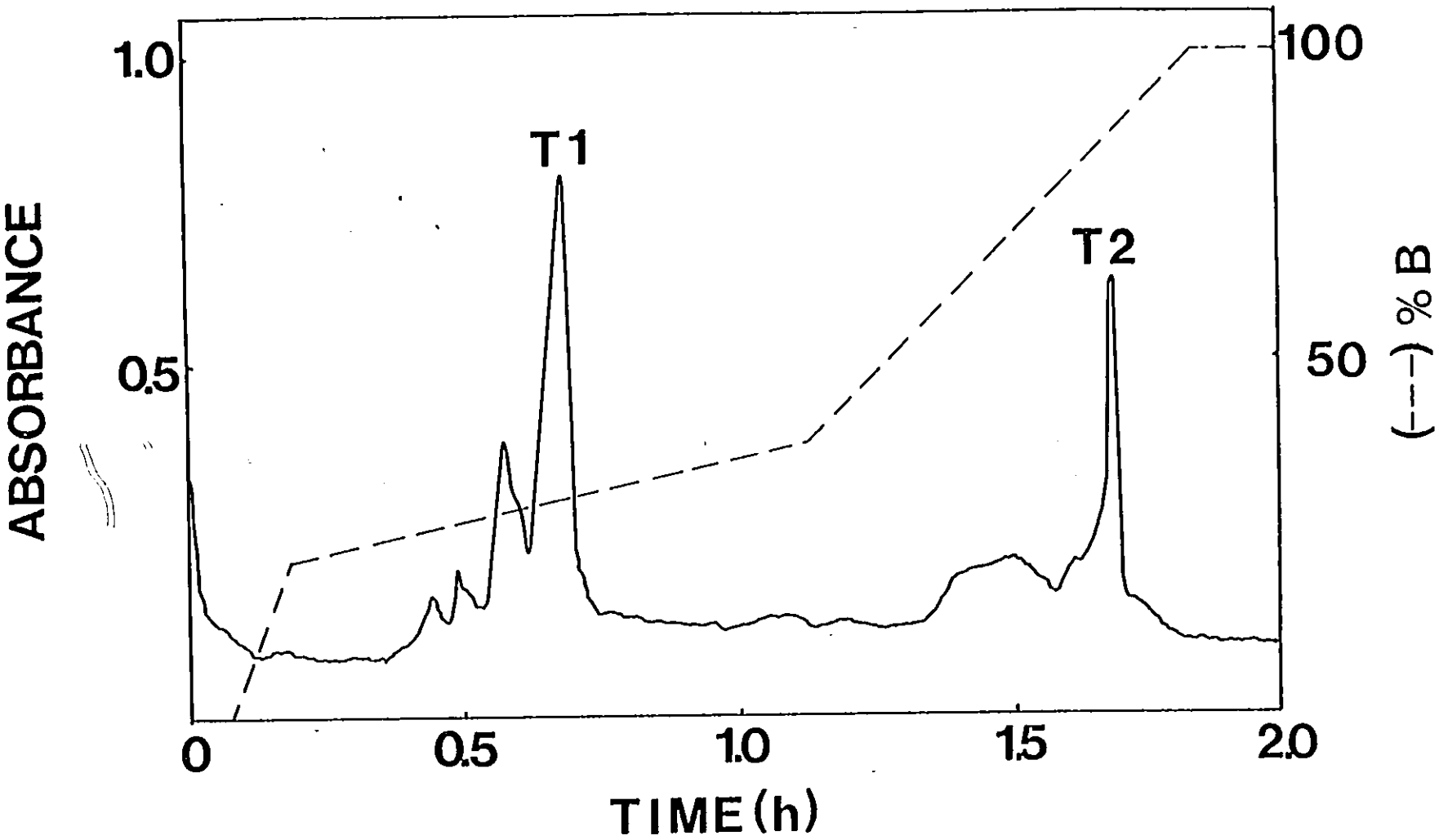


Figure 8

Phase Contrast and Fluorescence Microscopy of *B. thuringiensis* subsp. *kurstaki* HD-1. The cultures shown consists of mature un-lysed cells at stage VII (Bulla *et al.*, 1980) at 5000X magnification. **A.** Phase contrast : the four lighted areas are mature spores and the adjacent dark areas are regions of crystal formation. **B.** Fluorescence observed after incubation of cells with ethidium bromide. The cells were photographed under fluorescence conditions first to record maximum fluorescence then under phase contrast in an effort to match the two views.

Figure 9

FPLC Elution Profile of CryIA(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 to 1.0 M NaCl gradient in 0.1 M CAPS at 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.


ABSORBANCE
1.0
0.5
0
0.5
1.0
1.5
2.0
TIME (h)
T1
T2
100
50
(---) % B

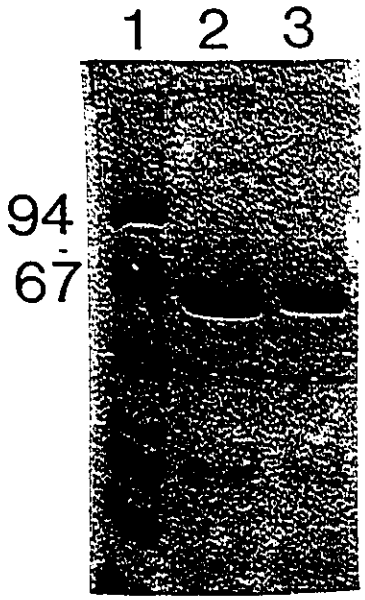
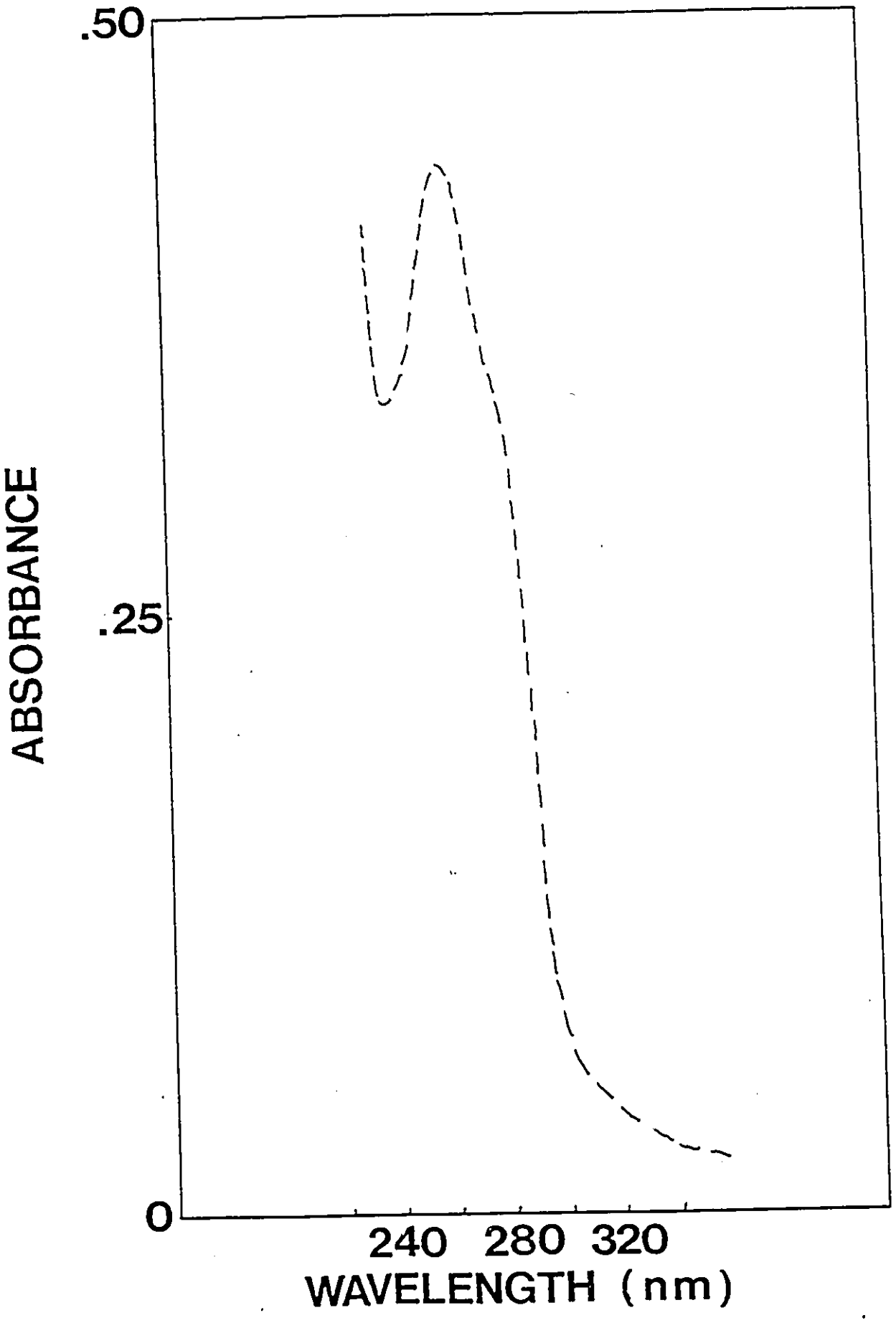


Figure 10
SDS-PAGE of T1 and T2. Lane 1, molecular mass standard, lane 2, T1 toxin, Lane 3, T2 toxin.

Figure 11

Spectrum of T2 Toxin. T2 toxin (0.26 mg/ml) was dissolved in distilled water and scanned from 350 nm to 220nm at a rate of 0.2 nm/sec at a band with of 2nm on a Pye-Unicam spectrophotometer.


.50
.25
0
ABSORBANCE
240 280 320
WAVELENGTH (nm)

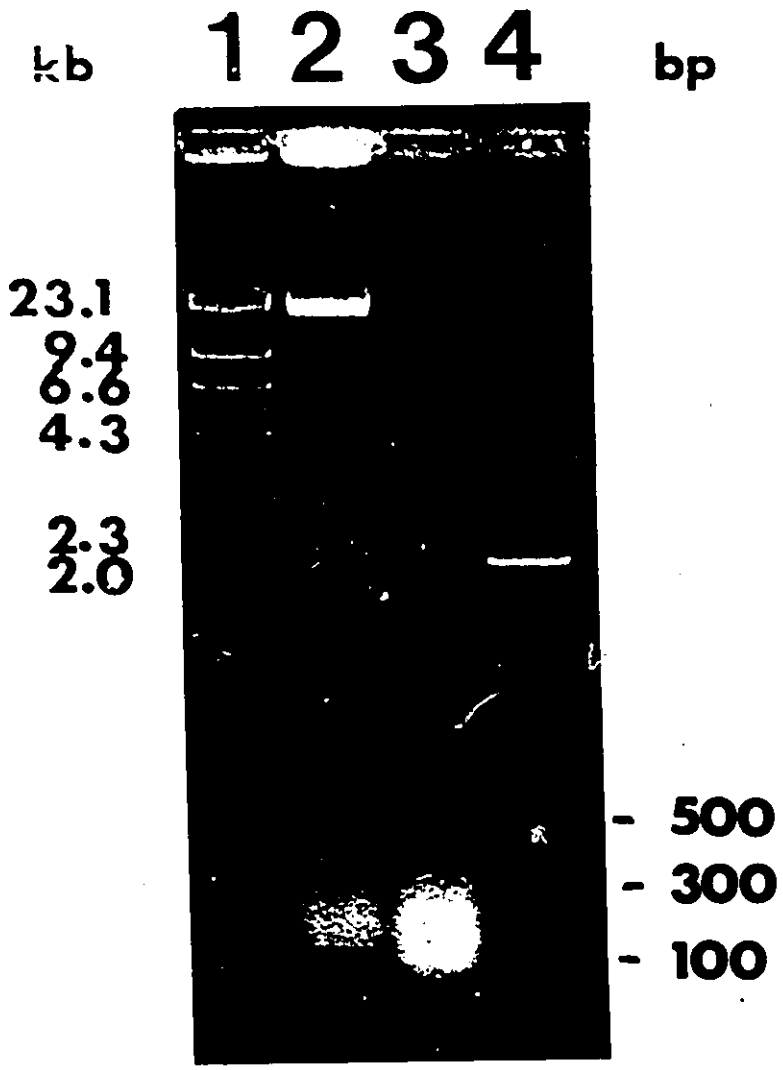


Figure 12

DNA Associated with *B. thuringiensis* Proteins. DNA was subjected to electrophoresis on 0.8% agarose gel in TAE. The DNA was stained with ethidium bromide and the gel photographed. Lane 1, phage λ-HindIII digest; lane 2, DNA isolated from the *B. thuringiensis* crystal protein; lane 3, DNA isolated from the T2 toxin; lane 4, 100 bp DNA ladder.

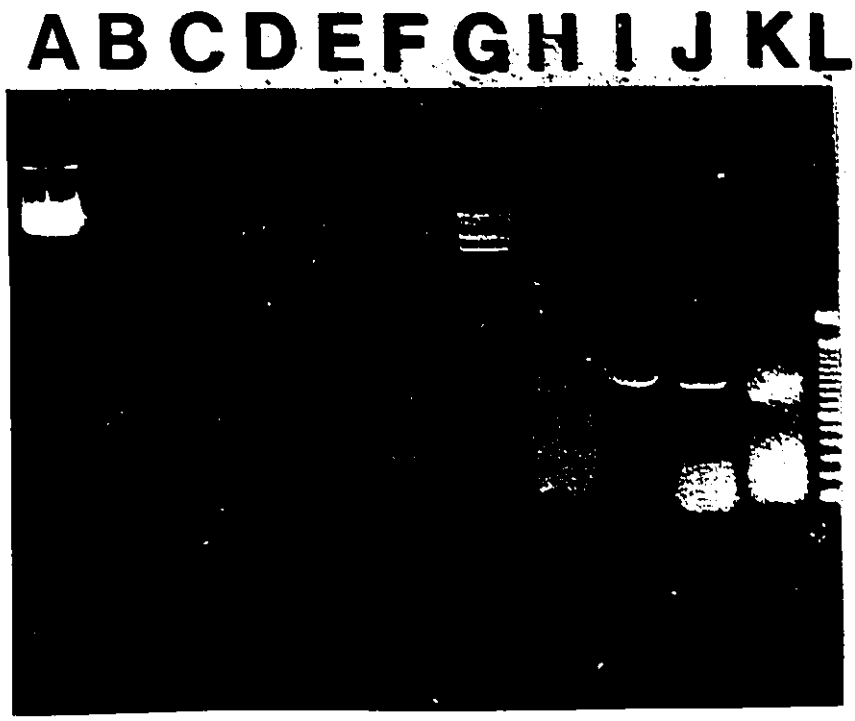
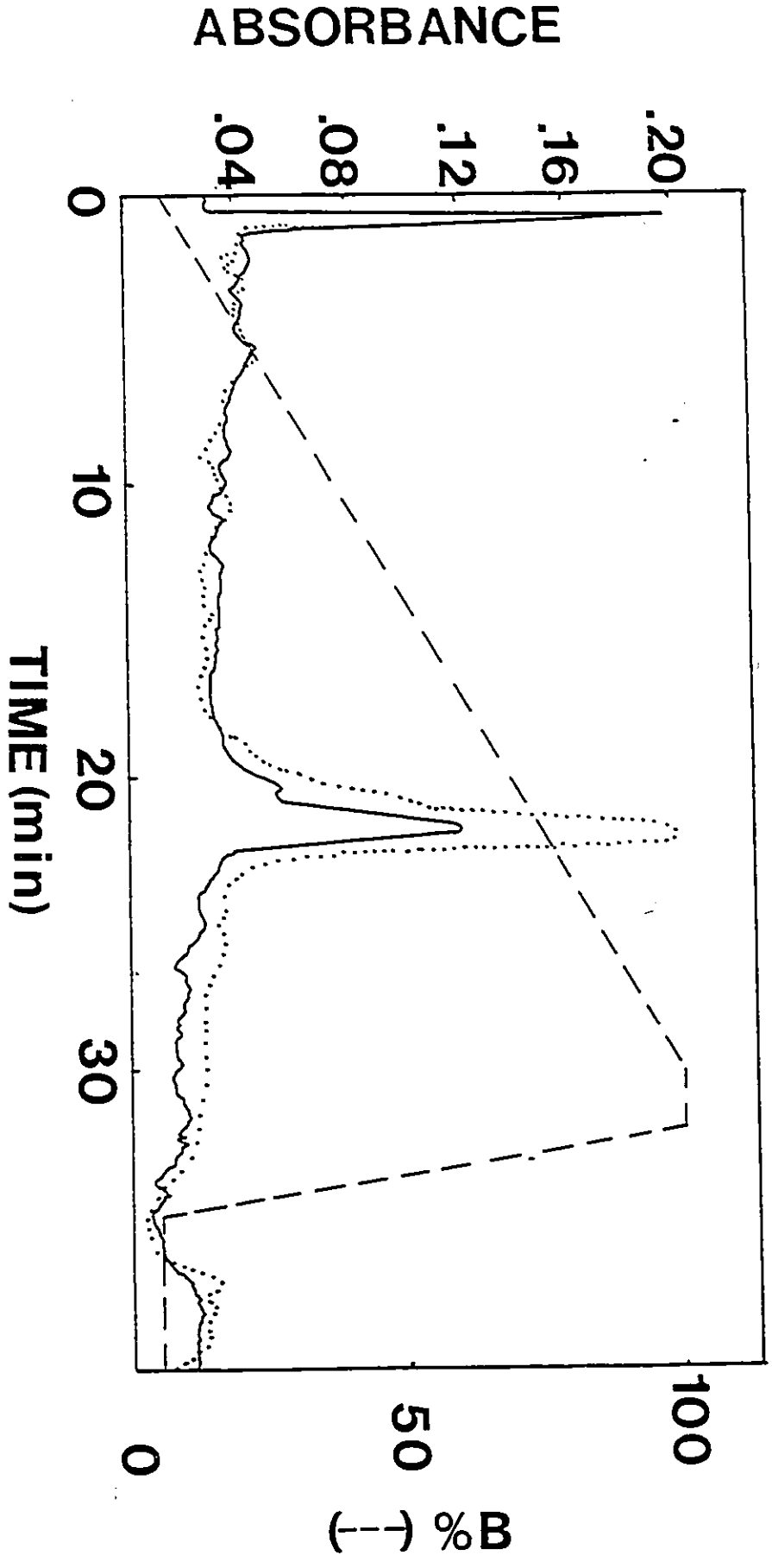


Figure 13

DNase and RNase Susceptability of the DNA Isolated From the T2 toxin. DNA was subjected to electrophoresis on 0.8% agarose gel in TAE. lane A, Oat genomic DNA; lane B, Oat DNA treated with RQ 1 DNase; lane C, DNA purified from the T2 Toxin; lane D, DNA purified from T2 toxin treated with RQ 1 DNase; lane E, T2 toxin treated with RQ 1 DNase; lane F, T2 toxin; Lane G, phage λ-HindIII digest; lane H, total oat seed RNA; lane I, total oat seed RNA treated with RNase A (DNase free); lane J, DNA isolated from T2 toxin; lane K, DNA isolated from T2 toxin treated with RNase A (DNase free); lane L, 100 base pair DNA latter.

Figure 14

HPLC Elution Profile of CAM-Protoxin. CAM-protoxin was eluted on a Bio-Rad MA7Q column (50 X 7.8 mm). Elution was also carried out at room temperature with a 0 to 1 M NaCl gradient in 0.1 M CAPS pH 10.5. The flow rate was 1.5 ml/min and the protein detected at 260 nm (·····) and 280 nm (———).


ABSORBANCE
.04
.08
.12
.16
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0
10
20
30
TIME (min)
0
50
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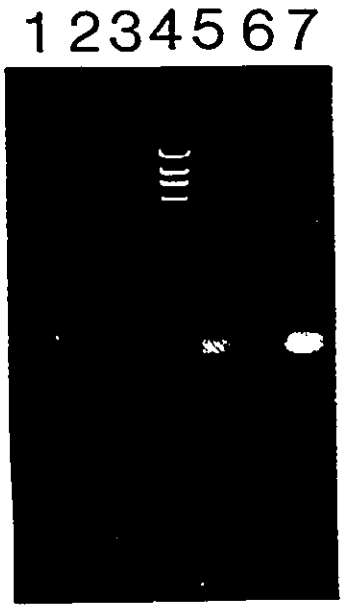

Figure 15

DNase and RNase Susceptability of the DNA Isolated From the Protoxin. DNA was subjected to electrophoresis on 0.8% agarose gel in TAE. Lane 1, DNA isolated from crystal protein treated with RQ 1 DNase; lane 2, DNA isolated from crystal protein; lane 3, DNA isolated from crystal protein treated with RNase A; lane 4, phage λ-HindIII digest; lane 5, Bacterial DNA isolated from *E. coli*; lane 6, Bacterial DNA isolated from *E. coli* treated with RNase A; lane 7, Bacterial DNA isolated from *E. coli* treated with RQ 1 DNase.

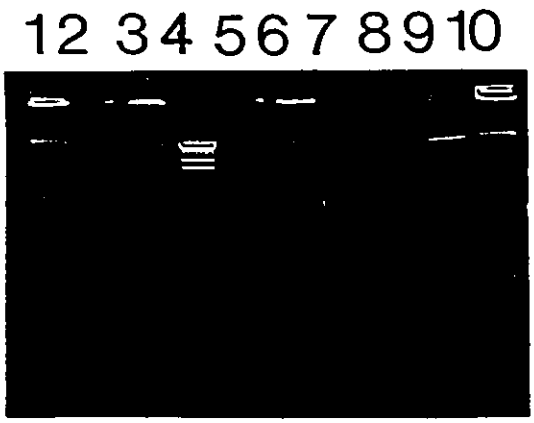

Figure 16

DNA associated with different B. thuringiensis subspecies. Solubilized purified crystal *B. thuringiensis* preparations were run on an agarose gel at pH 10.5. Lane 1, CryIA(c), lane 2, CryIA(b), lane 3, CryIA(a), lane 4, phage λ-HindIII digest, lane 5, subsp. *alesti*, lane 6, *entomocidus*, lane 7, subsp. *tolworthi*, lane 8, subsp. *galleria*, lane 9, subsp. *kurstaki* HD-73 and lane 10, subsp. *kurstaki* HD-1.

2). Digestion of the isolated fragment by DNase but not RNase (figure 15) and a positive reaction with the Hoechst dye confirmed the presence of DNA. The isolated 20-kb DNA fragment yielded a smear of smaller DNA fragments when digested with the restriction endonuclease Eco RI and Bam HI.

A 20-kb DNA fragment(s) could also be isolated from the intact crystals by extraction with phenol-chloroform at pH 10.5 and 65°C. A fragment(s) of the same size was also isolated after treatment of purified crystals with RQ1 DNase (10 units/ml in 20 mM Tris, 5mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4) for 15 min at 37°C. When RQ1 treated crystals were solubilized in 0.1 M CAPS buffer at pH 10.5 and applied to a Sephacryl S-400 (70 cm X 2.5 cm) column, a major peak with an apparent molecular mass of greater than 2 X 10^6 daltons elutes at the void volume. This peak contains the 130-kDa protoxin and the 20-kb DNA fragment(s). Solubilization of the crystals in CAPS buffer containing 1.5 M NaCl and fractionation on a Sephacryl S-400 column with the same buffer, did not cause the dissociation of the 20-kb DNA and the 130-kDa protoxin as they still co-eluted at the void volume.

Purified lepidopteran-specific crystals from various subspecies were obtained from the Forest Pest Management Institute. These crystal proteins were also tested for the presence of DNA. These preparations contained various gene types from the lepidopteran cryIA gene family. The cloned gene products CryIA(a) (b) and (c) produced in *E. coli* were

also tested for association of the cloned insecticidal protein product with DNA. It was found that all these proteins were associated with DNA (figure 16). The amount of DNA present in FPLC purified *B. thuringiensis* subsp. *kurstaki* HD-73 CAM protoxin was estimated using OD 260/280. On average, 10 bp were found per molecule of protoxin.

4.3.2 Sites of interaction of the DNA with the protein

In order to determine whether an interaction between positive and negative charges was involved in the apparent association of the protoxin and DNA, negative charges were introduced on the lysine residues by succinylation. CAM-protoxin was reacted with succinic anhydride in the presence of 8 M urea. The urea was dialysed out under basic conditions to permit renaturation of the DNA and protein and then the protein was precipitated. The precipitated protein did not contain the DNA (figure 17, lane 3) indicating that the DNA did not re-associate with the protoxin succinylated in the presence of denaturant. In contrast, CAM protoxin succinylated under native conditions (figure 17, lane 2) , and CAM protoxin treated with 8 M urea and renatured (figure 17, lane 4) all co-precipitated with DNA. Most of the fluorescence remains trapped in the well indicative of a large protein-DNA complex. Toxin was not generated from the DNA-free succinylated protoxin (figure 18C). However, toxin could be

generated from SCAM-protoxin prepared in the absence of urea (figure 18A), from SCAM-protoxin treated with 8 M urea and renatured (figure 18B) and from CAM-protoxin treated with urea and then renatured (figure 18D). The banding pattern observed in each of these gels is characteristic of the sequential proteolysis which occurs during activation (Choma, *et al.*, 1990) and is indicative of the fact that the unfolded proteins have refolded to their native conformation. The toxicities and fluorescence spectrum of the chemically modified protoxins have been reported by Choma and Kaplan, (1990) and Choma and Kaplan (1991). The extent to which the lysine residues were derivatized under these conditions (figure 18C) was not quantified.

4.3.3 Enzymatic Hydrolysis of the DNA

Short periods (20 minutes to 6 hours) of treatment of the protoxin with DNase (0.01 to 2% w/w) did not yield any detectable amount of DNA-free protoxin. Treatment with large amounts (2% w/w) of DNase for longer periods of time (72 hours) gave no recovery of any *B. thuringiensis* protein. Under these conditions the amount of protoxin remaining decreased with time until no protoxin remained (figure 19C). Treatment of the DNase with the serine proteinase inhibitor, diisopropyl fluorophosphate (DFP), did not inhibit this apparent proteinase activity. The amount of toxin generated

by trypsin varied inversely with the length of incubation of the protoxin with DNase (figure 19D). As noted above the DNA-free toxin was unaffected by incubation with DNase. When the protoxin was incubated with DNase and 10 mM EDTA to inactivate the DNase, the amount of protoxin again decreased with time but, in contrast, proteinase stable toxin was produced (figures 19E and 19F). Highly purified DNase from another source, Promega RQ1 DNase, was also treated with DFP and gave the same results as the Sigma DNase.

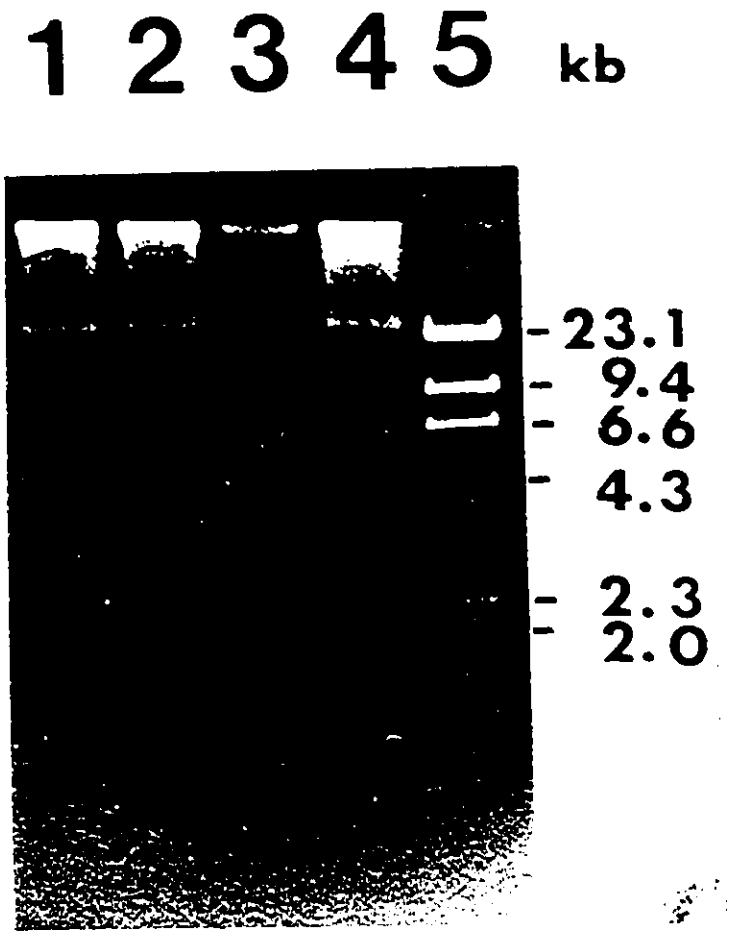

Figure 17

Effect of Succinylation of CAM Protoxin on DNA Association. Crystal protein was subjected to electrophoresis on a 0.6% (w/v) agarose gel in 40 mM CAPS, pH 10.5, 1mM EDTA. The DNA was stained with ethidium bromide and the gel photographed. Lane 1, SCAM protoxin; Lane 2, SCAM protoxin treated with 8 M urea and renatured; Lane 3, CAM protoxin succinylated in 8 M urea; Lane 4, CAM protoxin treated with 8 M urea and renatured; Lane 5, phage λ-HindIII digest.

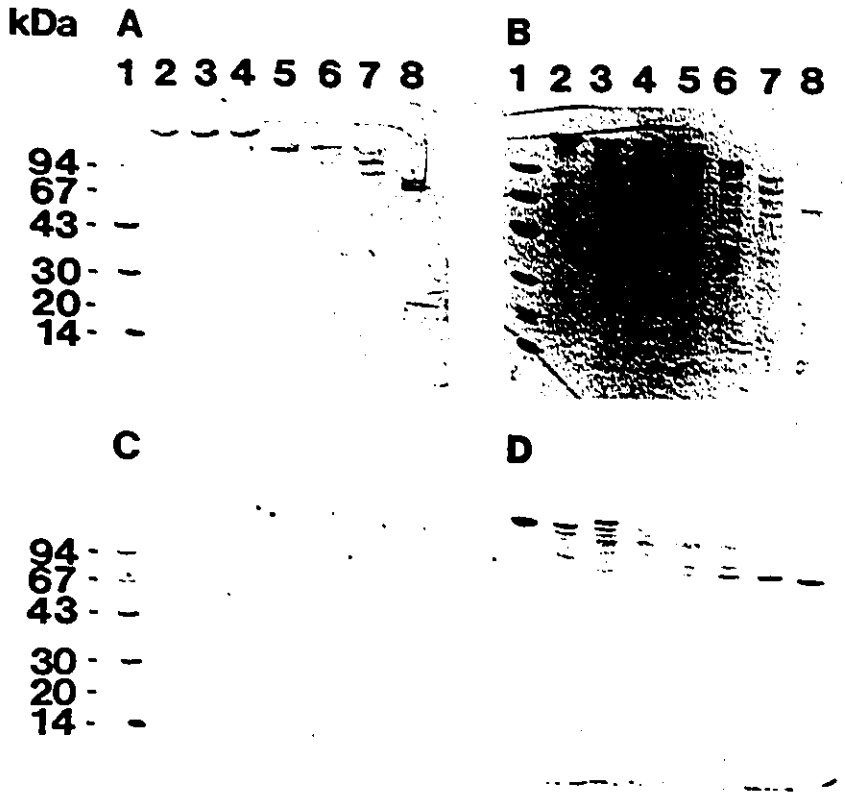

Figure 18

Effect of Succininylation of CAM-Protoxin on Generation of Toxin. Protoxins were incubated with bovine trypsin (0.001% W/W). Aliquotes were taken at various time intervals and run on SDS-PAGE: A (SCAM-protoxin); B (SCAM-protoxin treated with 8 M urea); C (CAM-protoxin succinylated in 8 M urea); D (CAM-protoxin treated with 8 M urea). Gels A, B and D show intermediate products obtained as a result of the sequential proteolysis observed during activation of the protoxin to toxin . For gels A, B and C lane 1 is a molecular mass standard, and lanes 2 to 8 are time points at 0. 5, 10, 15, 30, 45 and 60 minutes. For gel D, lanes 1 to 8 are time points at 0, 1, 5, 10, 15, 30, 45 and 60 minutes.

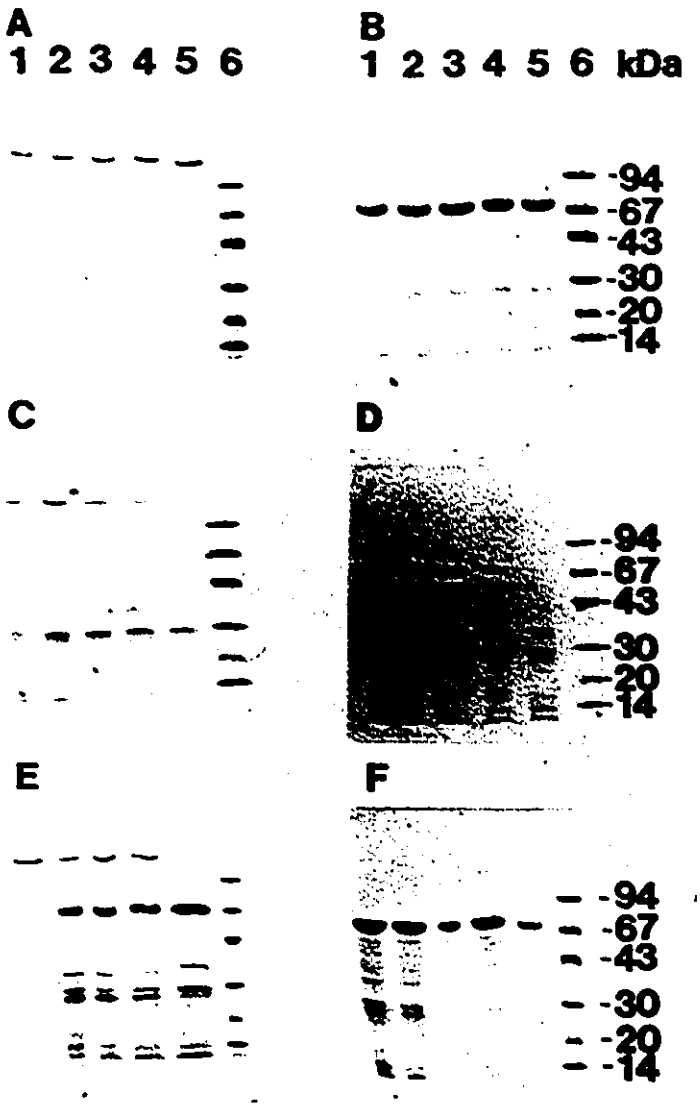

Figure 19

Effect of DNase Treatment of CAM-Protoxin on the Generation of Toxin. Protoxins were incubated in the presence or absence of DNase for various lengths of time and then treated with bovine trypsin. Aliquotes were run on SDS-PAGE. Lane 6 is a molecular mass standard and lanes 1 to 5 are time points at 0, 8, 24, 48 and 72 hours. Gel A is the untreated CAM-protoxin and Gel B shows the corresponding samples after treatment with bovine trypsin (1% W/W) for one hour. Gel C is the CAM-protoxin incubated with DNase (2% W/W) and Gel D shows corresponding samples after treatment with trypsin. Gel E is CAM-protoxin incubated with DNase and 10 mM EDTA and Gel F shows the corresponding samples after treatment with bovine trypsin.

4.4 DISCUSSION

Experiments were carried to establish the specificity of the association of DNA with the insecticidal crystal protein and to determine if this observed association was an isolated phenomenon or occurred in the insecticidal proteins from other *B. thuringiensis* subspecies. The second series of experiments that were carried out explored the role that the DNA plays in the structure-function of the *B. thuringiensis* insecticidal protein.

4.4.1 Discovery that DNA is associated with the insecticidal proteins

The discovery that the insecticidal proteins from *B. thuringiensis* were associated with DNA was totally unexpected. It occurred during attempts to purify protoxin and toxin directly by FPLC or HPLC chromatography in order to eliminate intermediate purification steps. Two major peaks were always obtained when toxin was purified by ion exchange chromatography on FPLC. Upon further investigation it was found that the protein in the peak eluting at 0.9 M salt, (T2) had the same apparent molecular mass on SDS-PAGE as the protein in the peak eluting at 0.3 M salt (T1) and that the

toxicities of the material in the two peaks was identical. The difference between these two seemingly identical components was uncovered by running the spectrum of each. It was found that T2 had a higher absorbance at 260 nm than at 280 nm which is indicative of the presence of nucleic acid. Nucleic acids was also found to be associated with the protoxin. The nucleic acid was shown to be DNA and it was found to be tightly associated with the insecticidal protein.

4.4.1.1 Evidence for the Specific association of the Protein Crystal with DNA

The major questions that arose regarding the observed association of the crystal protein with DNA were: 1) What is the specificity of this interaction? and 2) Is the DNA required for insecticidal activity? All the evidence obtained in the present study is consistent with the conclusion that the association is not artifactual and that there is indeed a specific interaction between the protoxin and DNA. Furthermore, the evidence indicates that the association of the nucleic acid with the *B. thuringiensis* insecticidal protein plays a role in determining the structure of the protein which is ultimately responsible for the generation of toxin and it's biological activity.

4.4.1.2 Nature and quantification of the Nucleic Acid

The nature of the nucleic acid associated with the insecticidal protein was investigated to determine if it was DNA or RNA. Sensitivity to DNase I but not to RNase A digestion as well as a positive reaction with the DNA specific dye, Hoechst 33258, led to the conclusion that DNA was associated with the insecticidal protein. Although RNA is probably present in the initial purification steps no RNA was detected in the purified crystals or in the solubilized protoxin. This observation is consistent with a specific interaction between the crystal protein and the DNA.

The amount of nucleic acid associated with the protoxin was estimated using A_{280} and A_{260} at pH 10.5 and was found to be 10 bp per molecule of protein. This represents a rough approximation of the amount of DNA present per molecule of protein. The DNA associated with the protoxin is electrophoretically homogeneous but not chemically homogeneous. The extinction coefficients of the protein and of the DNA used in this determination were average literature values (Sambrook *et al.*, 1989) determined at pH 7 and not at pH 10.5 where the reading were obtained. It is not known to what extent these extinction coefficients at pH 10.5 differ from the values at pH 7. The error in determining the amount of DNA present in a protein-DNA complex using this

spectroscopic method increases as the ratio of DNA to protein decreases and as the pH of the solution increases (Munro and Fleck, 1966).

The quantification of DNA present in the *B. thuringiensis* crystal remains undetermined at this time. It will have to be done on multiple samples from multiple batches grown under different conditions. The amount of DNA associated with the protoxin must be quantified precisely. This can only be done by amino acid analysis and phosphorous analysis on purified protein samples and will have to await further study.

4.4.1.3 Strength of the Association

Treatment of the crystals with up to 1.5 M salt during the isolation procedure does not remove the nucleic acid from the protein. As salt bridges are influenced by the concentration of salt in solution it was expected that treatment with high salt concentration could disrupt salt bridges between the DNA and the protein. Treatment of the crystal protein with denaturants also did not disrupt the protein DNA association.

It proved very difficult to separate the DNA from the protoxin. The usual phenol chloroform extraction procedures led to a precipitation of the protoxin-DNA complex and no release of the DNA. Bulla *et al.*, (1977) did not detect any nucleic acid associated with the crystal protein by the

diphenylamine reaction. However, their procedure involved precipitation of the protein with trichloroacetic acid and assumed that any DNA would remain in the supernatant. The results obtained showed that the DNA is strongly bound to the crystal protein and that it is not easily dissociated. In light of this observation, it is likely that Bulla *et al.*, (1977) did not detect DNA in the supernatant because it was removed along with the precipitated protein.

The DNA was released from the protein by increasing the pH and temperature of the extraction buffer. The high pH and elevated temperature helped to solubilize the protoxin and allowed the DNA to be extracted. The fact that the DNA is not highly susceptible to DNase I digestion when it is complexed with the protein indicates that the DNA is protected from enzymatic digestion. In contrast, the DNA from the toxin-DNA complex (T2) could easily be dissociated by phenol-chloroform and was sensitive to DNase digestion than DNA associated with the protoxin.

It was observed that carbaminomethyl-protoxin and T2 toxin co-purify with the DNA on ion exchange chromatography. The absorption spectrum for both the CAM-protoxin and the trypsin generated toxin showed a higher absorption at 260 nm than at 280 nm. The absorption at 260 nm is indicative of the presence of nucleic acid in the sample. This purified protoxin had an apparent molecular mass of 130 kDa on SDS-PAGE, however, F. Clairmont (Bietlot *et al.*, 1993) showed that the

protein-DNA complex had an apparent molecular mass greater than 2 X 10^6 dalton on gel-filtration chromatography and again it co-purified with the DNA (Bietlot *et al.*, 1993) The most plausible explanation for the huge difference in apparent molecular masses obtained by these two methods is that the protein is dissociated from the DNA during the preparation of the sample for SDS-PAGE.

4.4.1.4 Generality of the Association of DNA with Crystal Proteins

The nucleic acid was found to be associated with the crystal proteins from a number of *B.thuringiensis* subspecies including; *kurstaki* HD-73, *galleria*, *kurstaki* HD-1, *tolworthi*, *alesti* and *entomocidus* as well as from the inclusion bodies isolated from *E. coli*. In *B. thuringiensis*, the crystal is deposited after the spore is formed. The DNA which is present at this point has no survival value for the bacteria. In contrast to the production of crystal protein in *B. thuringiensis*, the production of the crystal protein in *E.coli* occurs continuously throughout the growth of the bacterium and hence the inclusion of DNA into the inclusion body is DNA which has potential survival value. In spite of these differences, the insecticidal protein produced by *E. coli* was also found to be associated with DNA. It therefore appears that DNA is required as the crystal protein is being formed.

Furthermore, as the inclusion bodies are treated with nuclease (both DNase and RNase) during the isolation procedure (Masson *et al.*, 1989) the survival of the DNA provides further support for the conclusion that the DNA is not simply adsorbed to the surface of the protein crystals.

Mattes (1927) reported in a detailed developmental study on *B. thuringiensis* that chromatic substances were present in the region of crystal formation and called this region the "kernhaltiges Zelläquivalent" or "nucleus-containing cell-equivalent". These results were obtained and interpreted before the structure of DNA was known. Mattes' interpretation can now be re-evaluated in light of the results presented in this thesis. Mattes used Giemsa to detect the presence of chromatic material and this experiment was repeated using ethidium bromide to confirm the presence of nucleic acid in the region where the protein crystal is formed.

4.4.1.5 Characterization of the DNA

The DNA associated with the protoxin appeared to be relatively homogeneous in size with the major component being a 20-kb fragment. The isolated 20-kb fragment was digested with restriction endonuclease in an attempt to isolate fragments of manageable size for sequencing. The restriction endonuclease used, Eco RI and Bam HI, recognize the sequence GAATTC and GGATCC respectively. The fact that no banding

pattern was observed indicates that the *B. thuringiensis* DNA is composed of many different 20-kb fragments. Whether the protoxin recognizes structural features common to all of these 20 kb fragments or whether it recognizes specific nucleotide sequences or structural feature common to all DNA molecules will have to await further investigation. The origin of the DNA was not investigated. The fact that the DNA fragment observed with the crystal, solubilized protoxin or from the FPLC purified protoxin is 20 kbp represents what was experimentally observed. It is not known if the size of the fragment is representative of the fragment that is incorporated in the crystal or if it results from shearing of the DNA.

The fact that both the DNA-free toxin (T1) and the toxin-DNA (T2) complex are equally toxic to CF-1 cells indicated that the DNA probably does not play a direct role in the toxicity mechanism of the insecticidal protein. The role played by the DNA in the T2 toxin remains unclear. The fact that DNA can be easily extracted from the toxin and that it can be enzymatically removed without loss of biological activity suggests that the DNA-toxin is an intermediate, likely the penultimate, in the pathway from protoxin to DNA-free toxin.

4.4.2 Role of the DNA in the Crystal Protein

The toxic moiety of the protoxin appears to be resistant to proteolytic degradation by proteinase (Bietlot *et al.*, 1989, Bulla *et al.*, 1977, Chestukhina *et al.*, 1982, Coma *et al.*, 1990). It was therefore surprising to observe that DNase treatment of CAM-protoxin resulted in the apparent proteolysis of the entire molecule including the proteinase resistant toxic moiety. In contrast, the DNA-toxin complex (T2) and the DNA-free toxin (T1) are not significantly proteolysed when treated with DNase that is slightly contaminated with proteinase, thus indicating that the contaminating proteinases present, like all other known basic proteinases, are not able to proteolyse the toxin. Inactivation of the nuclease with EDTA resulted in the generation of toxin from protoxin and confirmed the presence of proteinase in the DNase preparations. It was perhaps somewhat fortuitous that traces of proteinase activity were present as this alerted us to the possibility that the structural integrity of the protoxin was dependent on the presence of DNA. This possibility was further confirmed by the failure to generate toxin when the DNA was removed by the introduction of a large number of negative charges into the carboxyl terminal half of the molecule by succinylation. The most straightforward explanation of these observations is that the DNA is required for the conformational integrity of the

carboxyl terminal half of the protoxin which, in turn, is essential for maintaining the structural integrity of the amino terminal toxic moiety required for the generation of toxin. The possibility that our DNase preparation contained one or more novel proteolytic enzymes capable of completely degrading the protoxin cannot be unequivocally ruled out. However, these same enzymes would have to be present and co-purify with the DNase from bovine pancreas, *E. coli* and the spruce budworm gut juices(data not presented)

4.4.3 Characterization of the Protein-DNA Interaction

Binding sites comprise relatively little of the structure of most proteins and often are just a small patch on the protein surface; small localized alterations of the protein, therefore can produce large changes in its affinity for ligands (Creighton, 1993).

Intermolecular forces will determine how the insecticidal protein will interacts with the DNA. The interaction between proteins and DNA are far more complex than protein small ligand interactions. The forces between proteins and nucleic acids can be classified into four types; salt bridges, hydrogen bonds, hydrophobic effects and base staking.

Electrostatic forces are not very structure-specific but contribute substantially to the overall free energy of association (Blackburn and Gaits, 1990). Hydrogen bonds are

dipolar, do not contribute significantly to the free energy of association, but do determine the specificity of the protein-nucleic acid interaction. Hydrophobic forces are due to changes in solvation of the complex and are sensitive to the conformation of each of the components. Base stacking are due to hydrophobic interactions. In any protein DNA interaction, the combined effects of these four types of forces will determine the strength of the association. This thesis does not specifically quantify or identify which of these forces are involved in the association of the *B. thuringiensis* insecticidal protein with DNA.

The carboxyl terminal half of the protoxin contains almost all of the lysine residues of the protoxin. Choma and Kaplan (1992) have reported that only 35% of the lysine residues in the carbaminomethlyated protoxin (CAM) could be derivatized with succinic anhydride under native conditions. The resulting protoxin (SCAM) was characterized by limited proteolysis experiments and toxicity assays (Choma and Kaplan, 1991). It had the same toxicity towards spruce budworm larvae and had the same characteristics as the CAM protoxin. They concluded that the relative difficulty in modifying the majority of the lysine residues may be attributed in part to the formation of salt bridges or to the fact that the lysine residues within the protoxin's molecular structure are not accessible to the modifying reagent in solution. This

conclusion is supported by the results obtained from competitive labelling experiments (chapter 3) on the *B. thuringiensis* subsp. *kurstaki* HD-1 protoxin. The competitive labelling results taken in conjunction with the inability to react the lysine residues under native conditions (Choma and Kaplan 1992) supports the conclusion that the a majority of the lysine residues are not free in solution but are interacting with other groups.

The unusually low reactivity of the ε-amino group of lysine residues of histones in chromatin was described by Malchy and Kaplan (1974). They concluded that the majority of the ε-amino groups of histones in chromatin were buried and that this supported the hypothesis that these groups formed salt linkages with the phosphates of the DNA. The data obtained in the competitive labelling experiments on the protoxin is consistent with the hypothesis that most of the ε-amino groups of the lysine residues of the protoxin are not free in solution, possibly involved in salt linkages with DNA.

4.4.4 Effect of Modification of the Lysine Residues

The possibility of salt linkages between the ε-amino group of the lysine residue and the DNA was further investigated. This was accomplished by converting the positively charged lysine residues to negatively charged residues by succinylation in the presence of denaturant. The

chemical modification resulted in the dissociation of the DNA from the protoxin. The resulting succinylated protoxin was completely proteolysed by trypsin. The simplest explanation of these results is that the introduction of a large number of negative charges in the protoxin caused the dissociation of DNA from the protein rendering the prctein proteinase sensitive and the complete degradation of the insecticidal protein. It is not known whether the introduction of these negative charges caused the disruption of any salt linkages between the DNA and the protoxin or caused the protein to fold in an improper conformation causing the DNA to be released. However, it is clear from figure 18 and 19 that when the DNA is released from the protoxin, this leads to the complete proteolysis of the protoxin and toxin.

Salt linkages will occur between the negatively charged phosphate groups and either the ϵ-amino groups of lysine, the guanidium group of arginine, or the protonated imidazole of histidine.

It is possible that there are salt linkages between arginine and histidine side chains and the DNA. We can not rule out this possibility, however it is clear that modification of the lysine groups by succinilation cause the removal of the DNA. The modification of arginine sidechains occurs at a pH of 12-12.5. Prolonged exposure of the *B. thuringiensis* protein to this elevated pH results in a loss of all insecticidal properties. The imidazole side-chain is a

relatively weak nucleophile. It is not possible to modify these residues without extensive modifications of other side-chains within the protein. The contribution of these side chains in the protein-DNA complex using complete chemical modification cannot be estimated. The physico-chemical properties of these individual side chains will have to be determined in order to evaluate their contribution to the binding of the *B. thuringiensis* protein and DNA.

4.4.5 Biological Properties of the Protein-DNA complex

Hydrolysis of the DNA does not appear to be essential for the generation of toxin and its fate depends on the particular conditions used for activation. When the proteinase activity greatly exceeds the DNase activity, a mixture of DNA-free toxin (T1) and toxin associated with DNA (T2) is generated. However, when the reverse is the case, little or no toxin is generated. The bovine pancreatic trypsin normally used for the *in vitro* activation of the protoxin is not nuclease free but toxin is generated because of the high ratio of proteolytic to nucleolytic activity. The shorter the time of activation, the greater the ratio of T2 to T1 that was observed. The T2 toxin is much less soluble than T1 toxin. It has often been observed that different toxin preparations had vastly different solubilities and that this probably arose from variations in the relative amount of T2 and T1 generated.

In contrast to the DNA associated with the protoxin which appears to be homogeneous in terms of size, the DNA present in the T2 fraction is small and heterogeneous. The DNA associated with the protoxin is only slowly degraded by nuclease indicating that it is protected by the protein. It therefore seems likely that, during activation, proteolysis occurs at exposed sites in the carboxyl terminal region and is followed by fragmentation of the nucleic acid by any nuclease present. This would account for the small size of the DNA associated with the T2 toxin.

The objective of this thesis was to elucidate structure-function relationships in the crystal protein from *B. thuringiensis* and the toxin which is derived from it. The crystal proteins are unrelated to any known class of proteins and have several unusual features (Li, 1991, Höfte and Whitely, 1989, Choma *et al.*, 1990, and Bietlot *et al.*,1990). The discovery that the *B. thuringiensis* insecticidal proteins were associated with DNA makes this class of proteins appear even more unusual. Further study of the *B. thuringiensis* proteins will provide the opportunity to obtain new insight into structure-function relationships and contribute to the understanding of the process of protein folding. In particular, the interaction of the protoxin with DNA will be of general interest to protein chemists and biochemists. The elucidation of the role of DNA association with the

B. thuringiensis crystal protein will also be of interest to companies and researchers involved in the commercial application of *B. thuringiensis* as this discovery opens up new avenues of research into the stability and host specificity of the B. thuringiensis insecticide.

4.4.6 Alternate hypothesis for the association of DNA with the *B. thuringiensis* protein

As mentioned in section 4.4.2, the discovery that DNA was associated with the *B. thuringiensis* insecticidal protein was unexpected. It was first thought that this association was artifactual and resulted from improper isolation of the crystal from the fermentation media. Adsorption of DNA to the surface of the crystal during the isolation procedure and adsorption of DNA to the protein while the crystal is being solubilized and/or proteolytically processed were two possible explanations for this observed association. These explanations were dismissed when it became apparent that no matter what precautions were taken (i.e. repeated washes, treatment with salt, urea or nuclease), the DNA was not removed or processed from the crystal, the solubilized protoxin or from the FPLC purified protoxin. It was concluded that the observed association was not due to random adsorption of DNA to the surface of the crystal or from adsorption during the solubilization procedure.

The possibility of occlusion of DNA molecules within the matrix of the crystal as the crystal is deposited within the bacterial cell was also considered. Fast (1971) reported that the crystal could be swollen with 8 M urea with no loss of toxicity when the urea is removed. This swelling of the crystal could be monitored under the phase contrast microscope and the results were interpreted as an opening up of the crystal structure. Treatment of the crystal with urea did not result in the removal of the DNA. Treatment of the solubilized protoxin with salt or DNase also did not result in the removal of the DNA indicating that the association of DNA with the insecticidal protein was not a simple adsorption of the DNA which is occluded within the matrix of the crystal.

In contrast to the situation in the *B. thuringiensis*, where the crystal is deposited after the spore is formed, *E. coli* is an actively growing culture and it is doubtful that there is any extraneous DNA in the cell. This is further evidence that the association of the DNA to the protoxin is not the result of simple trapping of the DNA within the matrix of the crystal.

It became necessary to determine if DNA was associated with all the protein molecules. It was concluded that DNA was associated with the whole protein crystal since no DNA-free protoxin was ever observed. If the DNA was tightly associated (and protected from enzymatic digestion) with the surface of the crystal or if the DNA is a site of nucleation around which

the crystal is formed (the grain of sand around which the pearl is formed) then, there would be two distinct populations of protoxin molecules, one DNA bound, and one DNA free. At no time did we observe two populations of protoxin.

We concluded that the DNA was tightly associated with the all the protein molecules within the crystal. This conclusion was based on the fact that the DNA could only be removed from the protein DNA complex under extreme conditions. The importance of the DNA association with the protein and the role that the DNA plays in the insecticidal activity of the crystal were then investigated. It was not known if the DNA had any role in the *B. thuringiensis* protein or if it was merely tightly associated. The results obtained are unequivocal, when the DNA is removed, the protein is enzymatically degraded. The most probable hypothesis to explain these results is that the DNA is an integral part of the structure of the insecticidal protein.

CHAPTER 5

Re-Examining the *B. thuringiensis* Insecticidal Protein

5.1 INTRODUCTION

The insecticidal crystal protein produced by *B. thuringiensis* has many unusual characteristics and properties. The discovery that DNA is an essential structural component of the protein was unexpected and may help in understanding some of the puzzling phenomenon and unexplained observations associated with this protein. This finding therefore calls for a re-evaluation of the current views on the structure-function relationships in the crystal protein.

5.2 Role of the Carboxyl Terminal Region of the Protoxin

Currently, very little is known about the functional role of the carboxyl terminal half of the protoxin molecule. The fact that the carboxyl terminal of the protoxin sequence is highly conserved (Höfte and Whitely 1989, Brousseau and Masson, 1988) is an indication that this region of the protoxin plays an important role in the structure and function of protoxin. Höfte and Whitely (1989) have speculated on the function of the carboxyl terminal region, and have proposed that this region is conserved because it is essential for the co-crystallization of different gene products. A possible functional role of this region which should now be considered,

is that the conservation in the amino acid sequence which is observed in this region also reflects the structural requirements for DNA binding.

5.3 Activation Process

The proteolytic processing which removes the carboxyl-terminal of the protoxin to produce the toxic moiety occurs by seven specific cleavages in an obligatory ordered sequence starting at the carboxyl terminus and proceeding towards the amino terminal region (Choma *et al.*, 1990). To our knowledge there is no precedent in the literature for the type of protein structure or protein folding process that would give rise to such an unusual proteolytic mechanism. The proteolytic pattern observed (Choma *et al.*, 1990) was attributed to a highly unusual domain structure of the protoxin. This sequential proteolysis observed was postulated to be due to the structural constraints imposed on the protoxin by the requirements for the formation of a regular crystal lattice stabilized by the formation of disulfide bridges. Given the finding that DNA is strongly associated with the carboxyl terminal, it is reasonable to postulate that the unusual sequential proteolysis is the result of the interaction of the DNA with the protoxin. A plausible mechanism which could account for this activation process is a sequential alternating cleavage of protein and DNA.

5.4 Photoinstability of the *B. thuringiensis* Insecticidal Protein

The discovery that DNA is associated with the crystal protein sheds a new light on the problems associated with the use of B. thuringiensis as a cost-effective insecticide. Commercial preparations of *B. thuringiensis* sprayed in the field have a half life of 1.5 to 2 days (Morris, 1983, Milne *et al.*, 1990 and Andrews *et al.*, 1987). The loss of insecticidal activity was also shown to occur in purified *B. thuringiensis* crystals when exposed to direct sunlight for 4.5 hours (Morris, 1983). It has been shown (Morris, 1983) that U.V. light of wavelength 300 to 400 nm is responsible for the loss of toxicity. This finding was surprising. Pure proteins containing no cofactors or prosthetic groups, have no major electronic transition to the red of 290 nm and sunlight at the earth's surface has zero intensity to the blue of 300 nm (Grossweiner, 1976, 1884). Thus, direct photochemical damage to the protein by direct absorption of a photon is an extremely improbable event. Pozsgay et al., (1987) and Pusztai *et al.*, (1991) have proposed a photosensitization mechanism involving the presence of exogenous, and possibly endogenous, chromophores which causes the formation of singlet oxygen upon irradiation by light to account for the loss of biological activity.

The singlet oxygen was thought to cause the destruction of amino acid side chains, primarily the side chains of tryptophan. Methionine, histidine, and tyrosine side-chains were also investigated as potential targets for destruction by singlet oxygen. The results of amino acid analysis on irradiated crystals showed that the loss of biological activity occurred much more rapidly than the destruction of the side-chains of tryptophan (Bietlot, 1990 and Pusztai *et al.*, 1991). The presence of DNA in the crystal raises the possibility of a protein-DNA cross-linking which may render the insecticidal protein non-toxic.

Ultraviolet light-induced cross-linking of proteins has been demonstrated for many DNA/protein complexes (Grossweiner, 1984). Cross linkage was also shown to occur between thymine in the DNA and the side chains of lysine and cysteine residues (Smith, 1970, Harison *et al.*, 1982 and Dorwin *et al.*, 1988). The cross linkage of cysteine to cytosine (Smith, 1970 and Shetlar, 1980) and cysteine to adenine and guanine was also shown to occur in model compounds (Shetlar, 1980). As the carboxyl half of the protoxin contains most of the lysine and cysteine residues, it is reasonable to expect any cross-linking to occur in this region.

CHAPTER 6

Conclusions

In 1954 Angus showed that the insecticidal activity of *B. thuringiensis* resided in the insecticidal crystal produced during sporulation. Very little work had been carried out directly on the protein when this work was started and most of the information available on the structure of the protein was deduced from the gene nucleotide sequence. Given the commercial interest in this protein, it was surprising to see that so little structure-function work had been carried out on the protein. Poorly defined preparations of toxin had resulted in a body of literature which is inconclusive and often contradictory (Fast 1982, Andrews *et al.*, 1987).

The work presented in this thesis has been carried out on the lepidopteran-specific insecticidal crystal protein from *B. thuringiensis* subsp. *kurstaki* HD-73. The first two series of experiments were carried out to produce a chemically defined toxin. A procedure which yields a fully delineated toxin molecule was described. The toxin generated by bovine trypsin activation spans positions 29-623 of the gene nucleotide sequence. The molecular mass of the isolated fragment was determined to be 67 kDa and the amino acid composition was in good agreement with the predicted amino acid content deduced from the gene nucleotide sequence.

The discovery that the B. thuringiensis insecticidal protein is associated with DNA was totally unexpected. Far more questions were raised than were ever considered when this

work was undertaken and tantalizing potential explanations can now be put forward for the many unusual characteristics of this protein. The work presented in this thesis has provided evidence that DNA is an integral structural component of the crystal and that it has an important role in determining the structure and insecticidal properties of this protein. Association of the protoxin with DNA is essential for insecticidal activity as DNA-free toxin can only be generated from the protoxin-DNA complex. A more complete understanding of the role of the DNA in the structure-function of the insecticidal protein will require further characterization of the DNA, determination of the sites of interaction, its role in the photo stability of this protein and its role in the processing of the protoxin to the toxin.

CHAPTER 7

Claims to original research

- Development of an improved methodology for the isolation of carboxyl terminal peptides.

- Determination of the bovine trypsin generated toxin from *B. thuringiensis* subsp. *kurstaki* HD-73 crystal protein comprises residues 29-623 of the protoxin.

- Evidence obtained that most of the lysine and tyrosine residues of the protoxin are involved in inter- and intra-molecular interactions.

- Discovery that DNA is associated with the crystal protein from *B. thuringiensis*.

- Demonstration that DNA is probably an essential structural component of the protoxin required for the generation of toxin.

CHAPTER 8

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Appendix 1

Publications Arising From This Thesis

Bietlot, H.P., Carey, P.R. Choma, C.T. Kaplan, H., Lessard, T. and Pozsgay, M.(1989). Facile Preparation and Characterization of the Toxin from *Bacillus thuringiensis* var. *kurstaki*. Biochem. J. **260**, 87-91.

Bietlot, H.P., Carey, P.R., Pozsgay, M. and Kaplan, H.(1990) Isolation of Carboxyl-Terminal Peptides from Proteins by Diagonal Electrophoresis: Application to the Entomocidal Toxin from *Bacillus thuringiensis*. Anal. Biochem. **181**, 212-215.

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.

Puplications Arising From the Master's Thesis

Kaplan, H., Oda, G., **Bietlot, H.**, Carey, P., Pozsgay, M. and Fast, P.(1986). Selective Isolation of Free or Blocked Amino-Terminal Peptides from Digest by High Performance Liquid Chromatography: Application to the Crystal Protein of *Bacillus thuringiensis*. Biochem. Cell Biol. **64**, 1226-1233.

Bietlot, H.P., Vishnubhatla, I., Carey, P., Pozsgay, M. and Kaplan, H.(1990). Characterization of the Cysteine Residues and Disulfide Linkages in the Protein Crystal of *Bacillus thuringiensis* subps. *kurstaki* and *entomocidus*. Biochem. J. **267**, 309-315.

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Appendix 2

Amino Acid sequences of the cryIA(a), (b), (c) deduce from the gene nucleotide sequences

```
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CRYIAC    MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEFVPGAGFVLGL
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CRYIAB    PTNPALREEMRIQFNDMNSALTTAIPLFAVQNYQVPLLSVYVQAANLHLSVLRDVSVFGQ
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consens   PTNPALREEMRIQFNDMNSALTTAIPLFAVQNYQVPLLSVYVQAANLHLSVLRDVSVFGQ
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                 190       200       210       220       230       240
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consens   RWGFDAATINSRYNDLTRLIGNYTDYAVRWYNTGLERVWGPDSRDWVRYNQFRRELTLTV
                 190       200       210       220       230       240

                 250       260       270       280       290       300
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          ****.********.*********.*********.*********.*********.*********.*
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                 250       260       270       280       290       300
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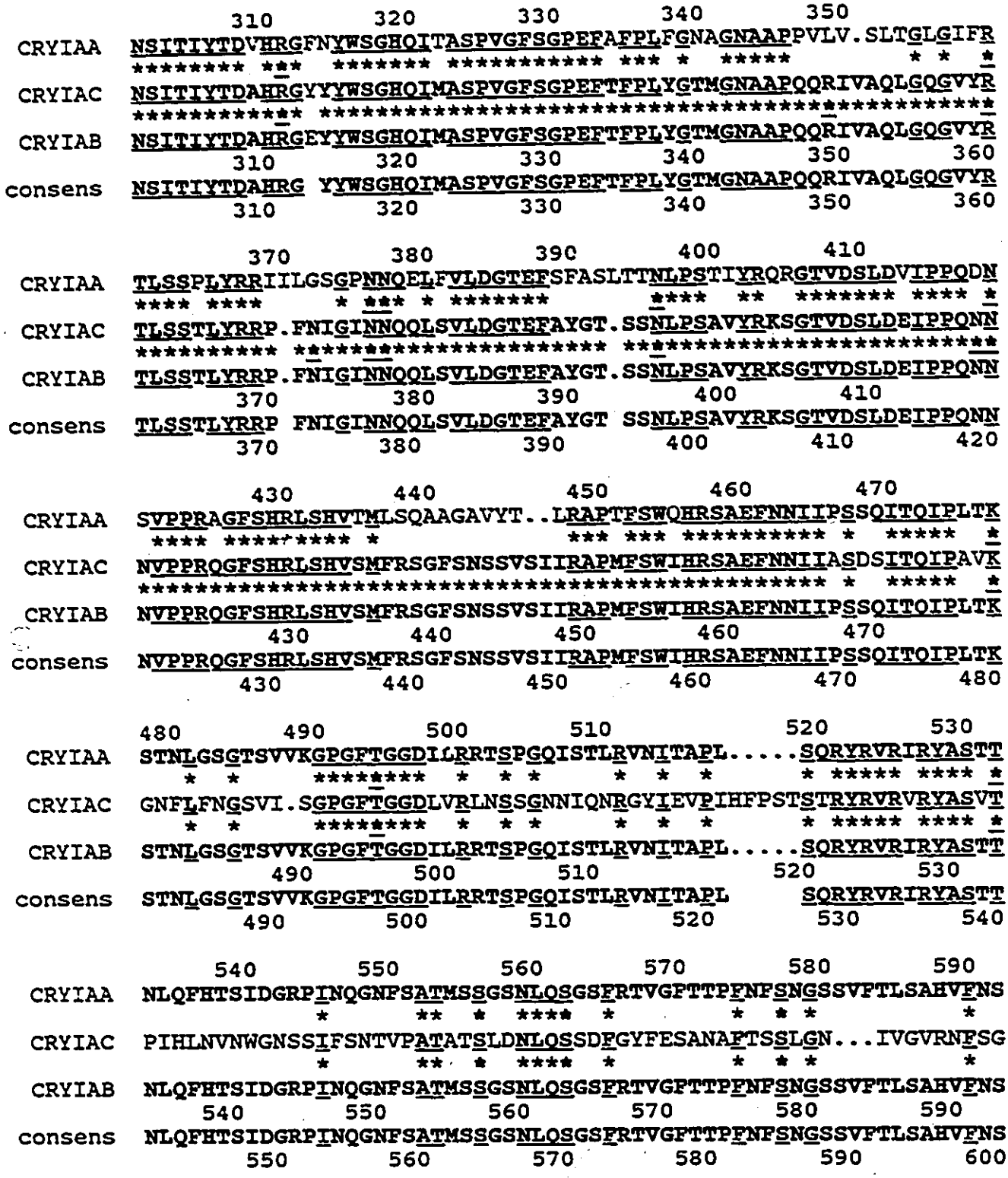
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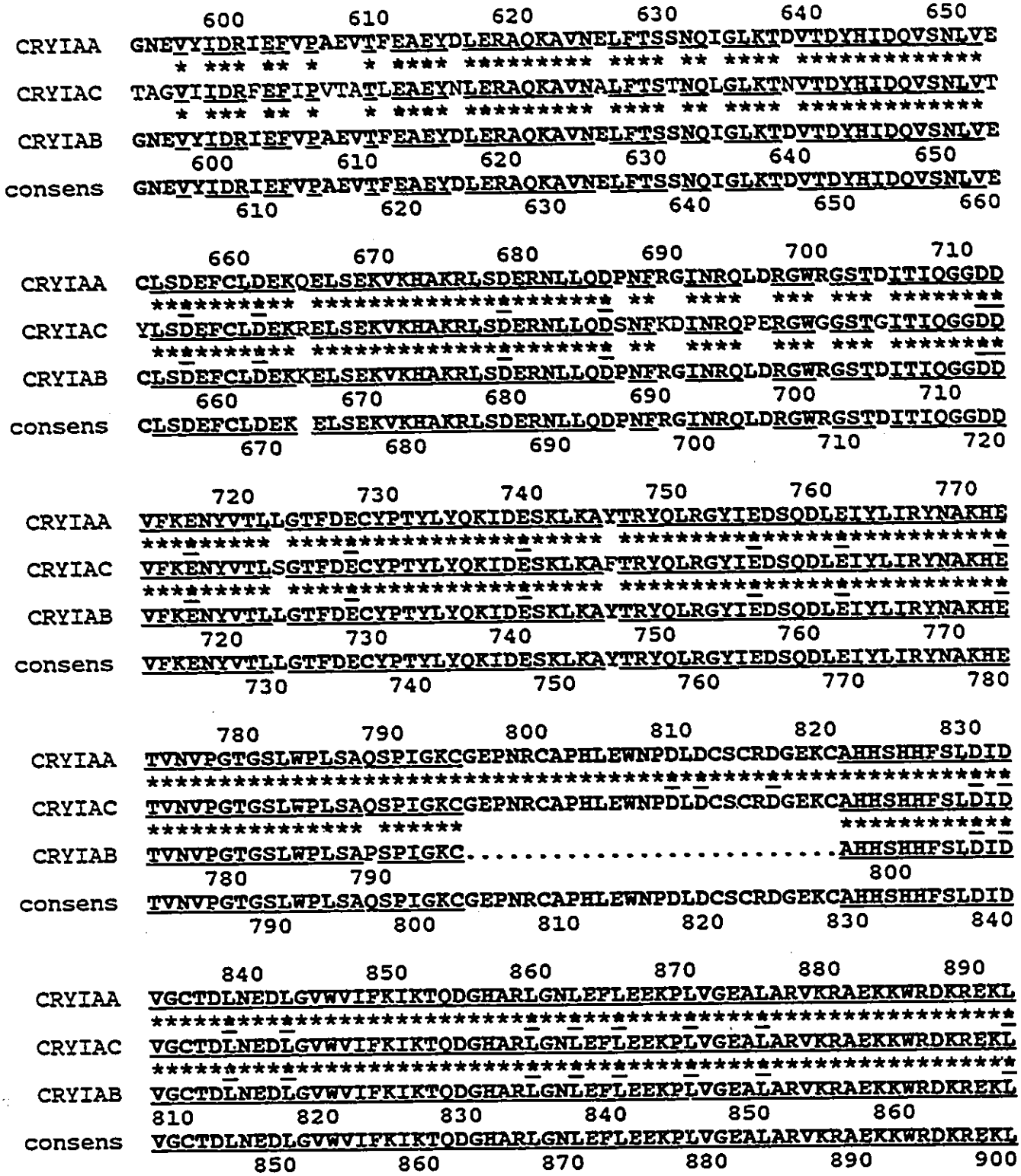
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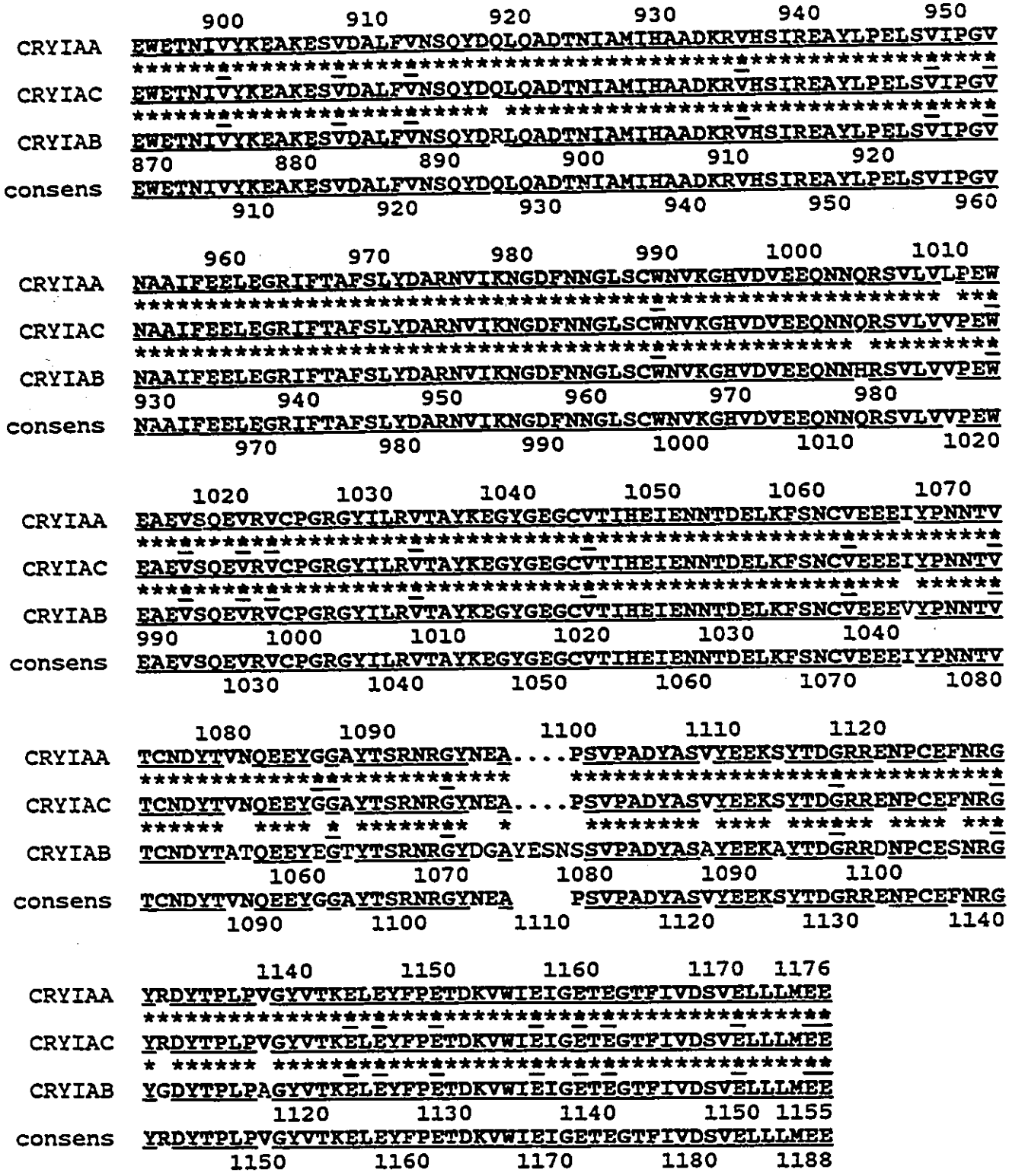
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Appendix 3

Toxicity of *B. thuringiensis* subsp *kurstaki* HD-73 toxin to CF-1 cells

Toxin	Thereshold level(ng/µl)[a]
HD-73	0.32-0.16

[a] Threshold level lies between the given values