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Structure of the Insecticidal Crystal Protein from *Bacillus thuringiensis* var. *kurstaki* HD-73

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Abstract

The structure of the DNA-protein complex in the insecticidal crystals produced by *Bacillus thuringiensis* var. *kurstaki* HD-73 was investigated. The role of DNA in the activation mechanism of the crystals was determined by monitoring the fate of the DNA and protein during the activation process with gut juice enzymes from spruce budworm, *Choristeneura fumiferana*. The results demonstrate that the DNA is co-processed with the protein, giving rise to novel DNA-protein complexes as intermediates. The role of the DNA appears to be mainly structural, providing a framework for the sequestering of the protein into the crystal. Based on the results obtained, a structural model for the arrangement of the DNA-protein complex is proposed. An activation model is also proposed, which accounts for the processing of the DNA and protein components of the crystals. The model offers explanations for the unusual mechanism of proteolytic activation of the Bt crystal protein.

The sunlight inactivation (photo-inactivation) of Bt crystals was studied by UV-irradiation studies. The results of these studies suggest that the photo-inactivation is due to the formation of covalent linkages between the DNA and crystal protein. UV irradiation at 302 nm for 8 hours was sufficient to cause the total loss of biological activity of the crystals. Incorporation of water-soluble antioxidants into crystal preparations inhibits the photo-inactivation reaction. Ascorbic acid was found to be an effective antioxidant and inhibited the photo-inactivation of Bt crystals. In the presence of ascorbic acid, the toxicity of the irradiated crystals did not decrease significantly under the same conditions and the retention of toxicity is estimated to be approximately one order of magnitude longer than unprotected crystals. The inactivation of crystals was inducible in the presence of a free-radical generator, suggesting that a free-radical mechanism is responsible for the photo-inactivation of the crystals.

A highly sensitive method has been developed for the determination of the C-terminal sequence of proteins. The C-terminal sequence of chymotrypsin was determined using this methodology. C-terminal peptides from ribonuclease and from the crystal protein from *Bacillus thuringiensis* var. *kurstaki* HD-73 were also isolated using this procedure.

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List of Abbreviations

APS	Ammonium persulphate
BHT	Butylatedhydroxytoluene
CAM	S-Carbaminomethyl
CAPS	3-(cyclohexylamino)-1-propane sulphonic acid
CM	Carboxymethyl
CMC	Carboxymethyl cellulose
DFP	diisopropyl fluorophosphate
EDTA	ethylene diamine tetraacetic acid
ES-MS	Electrospray Mass Spectrometry
FPLC	Fast Protein Liquid Chromatography
FPMI	Forest and Pest Management Institute
HPLC	High Performance Liquid Chromatography
kDa	kiloDaltons
PMSF	Phenylmethylsulphonylfluoride (alpha-toluenesulfonylfluoride)
SDS	Sodium dodecylsulphate
SDS-PAGE	SDS Polyacrylamide Electrophoresis
TAE	Tris-acetic acid-EDTA
TE	Tris EDTA
Tris	Tris-(hydroxymethyl)-aminomethane
VC	Vitamin C (L-ascorbic acid)
VE	Vitamin E (dl-alpha-tocopherol)
UV	Ultraviolet

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Chapter 1

General Introduction to *Bacillus thuringiensis*

1.1 Overview

The control of insect pests is a major economic concern to the agricultural and forestry industries. Generally, the control of insect pests is achieved through the application of chemical insecticides. Environmental concerns with respect to the impact of chemical spraying on ecosystems has become of major concern and as a result, much effort has been directed towards the search for more environmentally friendly alternatives to manufactured chemicals. In particular, the search for effective bioinsecticides has received much impetus, especially those which display high specificity and toxicity for the target organism and the absence of toxicity towards mammalian and avian species (Fisher and Rosner, 1959). Despite the large number of microbial products, originating from bacteria, fungi and viruses, which display toxicity towards insect pests (Miller et al., 1983), only 13 such products have been approved for use in the field by the American Environmental Protection Agency (Wilcox et al., 1986).

The most widely used and effective bioinsecticides currently in use are derived from the natural products obtained from the bacterium *Bacillus thuringiensis* (Bt). Commercial production of Bt-derived products was initiated in 1958 in the United States of America. Widespread use of these products did not begin until the 1980s at which time they also found extensive use in Canada against food crop pests. The annual world-wide expenditure of Bt products is reported to be approximately 100 million dollars (Lambert and Peferoen, 1992) and is estimated to be 1-2% of global insecticide sales. These figures are estimated to be equivalent to 2.3 Mkg of Bt crystals annually (Rowe and Margaritis, 1987). In recent years, the extensive use of Bt-based products has been employed against forest pests, namely against spruce

budworm control in Ontario and Quebec, in the control of jackpine budworm and western spruce budworm in the Western provinces, and against the hemlock looper and gypsy moth in the Atlantic provinces (Statistics Canada, 1987).

The most severe limitations of the use of Bt products as bioinsecticides arise mainly from an economic standpoint, namely the cost effectiveness of application in the field, the loss of products from plant foliage due to rain and the loss of insecticidal activity due to exposure to sunlight; photo-inactivation. Another factor of importance is the acquired resistance of insects to Bt formulations upon extensive exposure to the bioinsecticides, however this phenomenon has only been reported for the diamondback moth, *Plutella xylostella* in field studies (McGaughey and Whalon, 1992). Other insects have displayed resistance in laboratory experiments. However, as a whole, this factor appears to be less important than the observed photoinactivation of Bt formulations upon exposure to sunlight.

1.2 Biological Properties

B. thuringiensis is a gram positive soil bacterium which is easily distinguished from other closely related species *B. cereus* and *B. anthracis* by the parasporal inclusion body (crystal) which is produced during sporulation in the former. The insecticidal activity of various species of bacilli towards silkworm larvae was discovered in Japan as early as 1902 (Andrews, R.E., 1987). It was suggested that the insecticidal activity was the result of a toxin despite the fact that no identifiable toxin could be isolated from these bacterial cultures (Aoki, K. and Chigasaki, Y., 1915; Angus, C.T., 1954). It was later discovered that

crystalline inclusions were observable in these cultures (Hannay and Fitz-James, 1955; Angus, C.T., 1954) and it was soon reported that the toxic effects were associated with the crystalline inclusions (Angus, T.A., 1954). The toxic component of the crystal is known as the δ-endotoxin in order to differentiate it from other toxins which are produced by the bacterium. The biosynthesis of the crystals coincides with sporulation (Huber and Lüthy, 1981). Bipyramidal crystals, one per bacterium, are produced during sporulation and can be observed through a phase contrast microscope or by electron microscopy as shown in Figure 1.1.

Each sporulated cell contains a spore and a crystalline inclusion of about the same size; approximately 1 micron (Holmes and Monro, 1965) and accounts for approximately 30% of the total cellular protein (Dean, 1984). In the latent stage of sporulation, the crystals and spores are released from cells, which undergo lysis. The abundance of crystals produced during this phase suggests that they provide some survival value to cells. Due to their toxic nature, susceptible insects will eventually provide the nutrition for germinating spores under favourable growth conditions. In so doing, bacterial proliferation would be favoured allowing for mating and hence exchange of genetic material (Gonzalez et al., 1982).

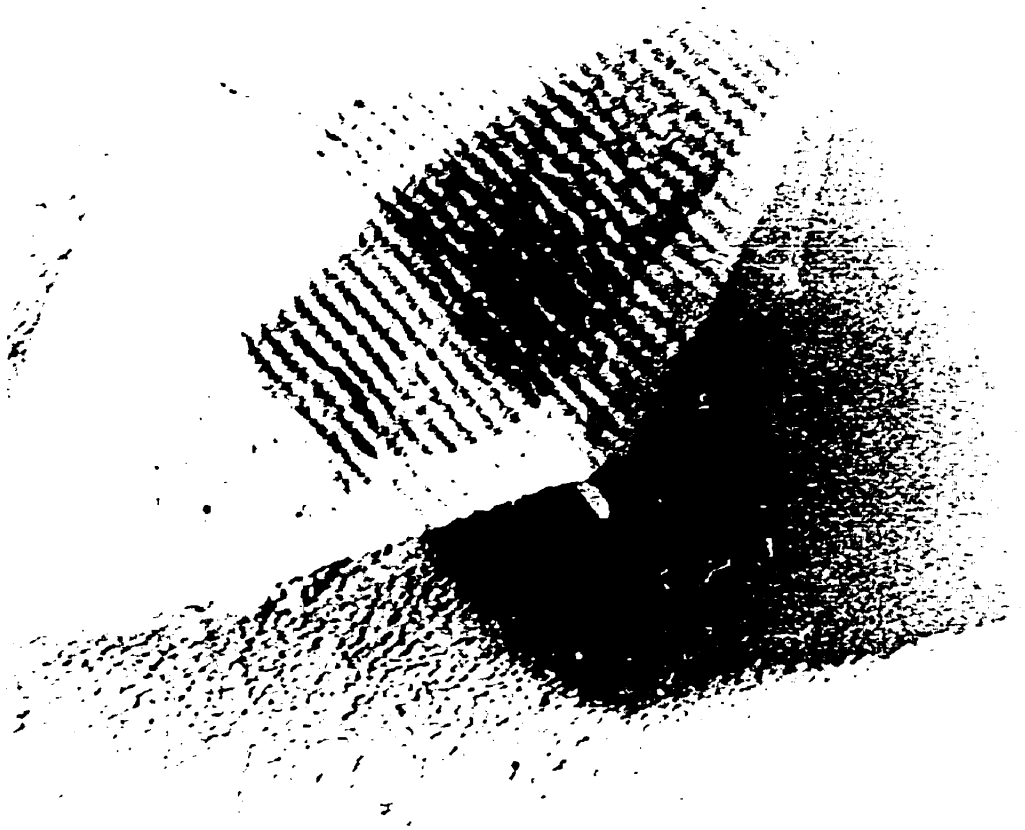


Figure 1.1 **Crystal of *Bacillus thuringiensis* subsp. *berliner* as observed under the electron microscope** (Hannay and Fitz-James, 1955)

The classification of *Bacillus thuringiensis* strains is accomplished according to H-serotypes, based on flagellar or H-antigens. Although a new classification scheme has been proposed (vide infra), the H-serotyping by flagellar agglutination has proved to be the most sensitive, specific, reliable and rapid method of identification. The serological classification can be further subdivided into varieties or subspecies based on biochemical analysis for esterase activity (de Barjac, H., 1981). Ellar et al. (1985) proposed a new scheme of classification based on the order of insects to which the proteins produced within the crystals are toxic. This scheme allows the differentiation of Bt subspecies into the following categories: (1) lepidopteran specific (moths and butterflies), (2) dipteran specific (black flies and mosquitoes), (3) coleopteran specific (beetles), (4) lepidopteran and dipteran specific and (5) no known toxic activity.

1.3 Mode of Action

There is still considerable disagreement with respect to the mode of action of the crystal protein on susceptible insects. While the molecular details of the mode of action of the crystal protein have not been elucidated, there are several features on which there is general agreement.

Upon ingestion of the crystal protein, the alkalinity of the insect gut, pH>10 (Berenbaum, M., 1980) causes cleavage of the disulphide bridges and dissolution of the crystal. Milne and Kaplan (1993), isolated a single trypsin-like enzyme from the insect gut juices from spruce budworm, *Choristeneura fumiferana*, which has been shown to be responsible for the

proteolytic processing of the crystal protein (protoxin) to the toxin, which may then exhibit its cytotoxic effects within the insect.

It is generally accepted that the target tissue for the toxin is the gut epithelium of susceptible insects. The epithelial cells undergo morphological changes upon ingestion of the crystal protein and the cells eventually rupture (Tojo, 1986). Percy and Fast (1983) reported that the toxin induced a cytoplasmic response in columnar cells within one minute after ingestion of the crystals although external symptoms were not exhibited until fifteen minutes later. One minute after ingestion, the microvilli became less consistently uniform in diameter and appeared swollen. Within five minutes of ingestion, the microvilli of some columnar cells disappeared completely and the mitochondria in these cells were swollen. Within ten to fifteen minutes, general metabolic breakdown had occurred. Knowles and Ellar (1987) have proposed a two step mechanism for the observed swelling and lysis of cells based on the leakage of intracellular markers. They propose that the δ-endotoxin binds to a specific plasma membrane protein and then generates small pores in the plasma membrane, either by inserting into the membrane or indirectly by perturbing the lipid bilayer through disruption of resident plasma membrane molecules. The net result of the created pores is colloid-osmotic lysis; a net influx of ions into the cells with a concomitant influx of water causing swelling and lysis of cells.

Several techniques have been employed in the study of the mode of action of the Bt toxins. These methods including binding assays, voltage-clamp experiments and membrane potential / conductance studies, using epithelial midguts of various susceptible insects, brush border membrane vesicles (BBMV) and/or planar lipid bilayers as model membranes.

Several important features concerning the toxic activity of Bt δ-endotoxins have been determined by these methods. In-vivo and in-vitro ligand blotting assays have confirmed that Cry toxins bind to microvillae in the midgut of susceptible insects and receptors in model membrane systems (Knowles and Ellar, 1986; Haider, et al., 1987; Lee et al., 1992; Bravo et al.. 1992; Denolf, P. et al., 1993; Vadlamudi, R.K., et al., 1993; Yi, S. et al., 1996; Lee et al., 1996c: Lee et al., 1997) Putative receptors of the Cry1Ac toxin have been identified and purified from *Manduca sexta*, tobacco hornworm (Sangadala et al., 1994), *Lymantria dispar*, gypsy moth (Valaitis et al., 1995) and BBMVs of *Heliothis virescens*, tobacco budworm (Knight et al., 1995). Aminopeptidase N (APN), a 120 kDa protein purified from brush border membrane vesicles of *Manduca sexta* (Knight et al., 1994) and of *Lymantria dispar* (Lee et al., 1996c), has been shown to be a specific receptor for the Cry1Ac toxin. Competitive binding assays have raised some controversy with respect to binding sites (Lee, M.K and Dean, D.H., 1996b) and the relationship between binding affinity and insecticidal activity (Van Rie, J. et al., 1989; Garczynski, S.F. et al., 1991; Sanchis, V. and Ellar, D.J., 1993). Other studies have found that toxicity is directly correlated to the irreversible binding (Liang, et al., 1995).

The ion-channel / pore-forming model for the mode of action of toxin, initially proposed by Knowles and Ellar (1987), has been studied extensively by means of voltage clamp experiments (Harvey and Wolfersberger, 1979) and membrane potential / conductance measurements (Schwartz, J.-L., et al., 1993; Peyronnet et al., 1997; Schwartz, J.-L. et al., 1997c). Incorporation of toxin into membranes has been shown to affect membrane

permeability to various ions (Knowles and Ellar, 1987; Sangadala et.al., 1994, Schwartz, J.-L., et al., 1993). The ion-channels that are formed, as a result of toxin incubation with membranes, were shown to be pH-dependent; forming anion-selective channels at low pH and cation-selective channels at higher pH (Schwartz, J.-L., et al., 1993). The observed pH-dependent ion-specificity has been ascribed to the titration of ionizable amino acid residues responsible for pore formation and due to conformational changes (Feng and Becktel, 1994) occurring within the toxin. Structural evidence for the mode of action of Bt toxins, obtained by mutagenesis studies, is discussed in section 1.6.

1.4 Structure, Properties and Composition of the Crystal

The parasporal crystal produced by Bt is a bipyramidal crystal of approximately 1 micron in size as viewed through the electron microscope (Figure 1.1). The structure of the intact crystal shows a well organized striated morphology resembling beads on a string. It was known for some time that disulphide linkages existed in the crystal protein however the quantification of cysteic acid (half-cystine) created some debate as to the extent of stabilization that could be achieved through disulfide linkages alone (Holmes, K.C. and Monro, R.E., 1965; Bulla. L.A. et al., 1977; Huber, H.E. et al., 1981). Bietlot et al. (1990) reported that the crystal structure is stabilized through symmetrical intermolecular disulphide bonds. An unusual structural property of the crystal protein is the apparent absence of any intramolecular disulphide linkages among the 16-18 cysteine residues in the protein. The proteinaceous crystals are insoluble at acidic and neutral pH but are readily solubilized at pH values above 9

and dissolution is greatly accelerated in the presence of reducing agents such as beta-mercaptoethanol or dithiothreitol. CryI crystals (lepidopteran specific) obtained from different subspecies of Bt have been shown to contain different protein components, and in some cases more than a single protein, however the majority of crystals are composed of a 130 kDa protein (protoxin) (Chungjatupornchai, W. et al., 1988; Höfte, H. et al., 1989).

More recently, the crystals produced by *Bacillus thuringiensis* subsp. *kurstaki* HD-73 have been shown to contain DNA as an integral component of the crystal (Bietlot, H.P., et al., 1993). This study revealed that the generation of toxin from the solubilized protoxin required the presence of the DNA. It was postulated that the DNA was mainly associated with the highly conserved C-terminal region of the protoxin and that the DNA might be involved in maintaining structural integrity of the crystal and in the generation of toxin from the protoxin (Bietlot, H.P., et al., 1993).

1.5 Structure and Properties of the Crystal Proteins

Early studies into the structure and properties of the Bt crystal protein led to several different estimates with respect to the apparent molecular weight of the primary gene product (protoxin or crystal protein). Representative literature values ranged from 200 to 1.5 kDa, the wide range was proposed to be the result of contaminating proteases (Nickerson, K.W., 1980). Further confusion may have been introduced in some cases by the fact that the crystals are occasionally composed of more than a single gene product. By the 1980s, having determined

the diversity and classification of the crystal protein genes, it was possible to deduce the expected molecular weights of the different crystal proteins. The molecular weights were found to range from 70-140 kDa with the majority of gene products having molecular weights of approximately 130 kDa (Höfte H. and Whiteley, H.R., 1989).

The protoxins possess a highly conserved C-terminal region that is enzymatically degraded upon conversion to toxin. In *Bacillus thuringiensis* var. *kurstaki* HD-73, the crystals used in the current investigations, the C-terminal region contains all of the cysteine residues found in the protoxin (Fast, P.G., 1981; Schnepf et al., 1985; Adang et al., 1985). It has been suggested that most of the disulphides play a role in host selectivity, since their reduction resulted in the loss of specificity, and that their interactions were required for the stabilization of some protoxins (Arvidson, H. et al., 1989). The sequence of the crystal protein (protoxin) for *B. thuringiensis* var. *kurstaki* HD-73 is given in Appendix 1. The crystal protein contains 1178 amino acids as deduced from the gene nucleotide sequence (Adang et al., 1985). In the region which encodes the toxic moiety (toxin), five conserved blocks can be distinguished with the exception of three crystal proteins (refer to Appendix 2). However, the primary structure of the C-terminal region, which is not required for toxicity, is the most highly conserved region of the crystal proteins (Höfte and Whiteley, 1989).

Proteolytic processing of the protoxin to toxin has been shown to occur through seven cleavages in a sequential order starting from the C-terminal region of the protoxin and proceeding towards the N-terminus. Each cleavage was shown to involve the removal of a C-terminal fragment of approximately 10 kDa. The partially processed intermediates

resulting from the 3rd through 6th cleavages were shown to accumulate before being further proteolyzed (Choma, C.T. et al., 1990a). These observations were interpreted to be the consequence of subdomain interactions in the C-terminal end of the protoxin. The sequential proteolysis is unusual in that the C-terminal region of the protoxin is mainly hydrophilic, thus any number of possible proteolytic sites could have been attacked. A proposed explanation to account for the unusual processing was that specific protein interactions, arising from the tertiary structure of the protoxin, could limit the accessibility of other proteolytic sites thereby necessitating a sequential proteolytic processing scheme involving conformational changes. There is however. no known structural motif that would account for such a series of conformational changes.

Chemical modification of the cysteine and lysine residues of the protoxin was shown to increase the solubility of the crystal protein at neutral pH values and found to occur quantitatively with cysteine residues while approximately only 40 % of the lysine residues had reacted. These results suggested that the cysteine residues are located on the surface of the crystal protein, while the lysine residues are distributed throughout the crystal protein; some being inaccessible to solvent and thus remained unreacted. Furthermore, treatment of the chemically modified protoxin with proteases still generated toxin by the same sequential proteolytic mechanism observed for the native crystal protein, and retained full insecticidal activity, indicating that the chemical modifications did not significantly affect the structure of the protein (Choma, C.T. and Kaplan, H., 1992).

1.6 Structure and Properties of the Toxin

Proteolytic processing of the protoxins produced by the various serotypes of Bt generates protease-resistant toxins that display insecticidal activity towards various orders of insects. In a manner similar to the protoxin, the molecular weights of toxins produced by the various subspecies of Bt vary considerably; from approximately 25-75 kDa depending on the encoded primary gene product (protoxin). Based on the gene nucleotide sequence, the toxin derived from *B. thuringiensis* var *kurstaki* HD-73 consists of amino acids 29-623 of the primary structure of the protoxin (Adang, M.J. et al., 1985). DNA sequencing techniques and deletion mutation studies, conducted on *Bacillus thuringiensis* var. *kurstaki* HD-1, showed that the sequences from the 645th codon to the 1176th codon were not required for toxic activity (Schnepf, H.E. et al., 1985). This determination yields an apparent molecular weight of approximately 66,000 Da for the toxin, consistent with molecular mass determinations obtained by SDS-PAGE (Fast, P.G. 1981).

Structural studies conducted on Diptera-specific and Lepidoptera-specific toxins, from *B. thuringiensis* subspecies *israelensis* and subspecies *berliner* respectively, have shown that the toxins have structural similarities with respect to the distribution of hydrophilic and hydrophobic amino acids and cross immunological reactivity (Chungjatupornchai, W. et al., 1988). Despite the different host specificities, the structural similarities suggested a similar mechanism of action. Thorne et al. (1986) reported similar results for the toxins derived from *B. thuringiensis* subspecies *kurstaki* and *israelensis*.

The calorimetric profiles for the thermal unfolding of the protoxin and toxin obtained from *B. thuringiensis* subsp. *kurstaki* HD-73 were compared by Choma et al. (Choma, C.T. et al., 1990b) in yet another structural study. While the protoxin generated a relatively simple two-step endotherm, the toxin endotherms were more complex and extended over 50°C to 80°C with two well-defined components centred near 63 and 69°C. These results were interpreted as evidence for a conformational change that occurs upon activation of the protoxin to toxin (Choma, C.T. et al., 1991). Previous investigations in denaturing conditions had suggested that the toxin was present in the active conformation (Choma, C.T. et al., 1990b), therefore it was postulated that the conformational changes observed by DSC were probably very subtle and most likely involved changes in the tertiary structure, rather than changes in the secondary structure, of the toxin. Based on the observed conformational difference of the toxic moiety in the protoxin and in the activated toxin, it was suggested that the C-terminal end of the protoxin may be responsible in modulating the biological activity of the toxin (Choma, C.T. et al. 1991).

Structural studies, performed to elucidate the secondary structure of the toxin obtained from *B. thuringiensis* subsp. *kurstaki* HD-73, were conducted by CD, IR and Raman spectroscopy, as well as predictive methods (Choma, C.T. et al., 1990c). The results indicated that the toxin was composed of three domains. Circular dichroism and infrared spectroscopy estimated the secondary structure to be 33-40% alpha helix while Raman and predictive methods indicated approximately 20% alpha-helical structure. Raman and circular dichroism spectra, as well as predictive methods yielded an estimated 32-40% beta-sheet structure while infrared spectroscopy gave a slightly lower value for the beta-sheet content. Thus all methods

were found to be in relative agreement that the toxin is highly folded and is composed largely of alpha-helical and beta-sheet structures with less than 20% random structure (Choma, C.T. et al., 1990c).

More recently, the x-ray crystal structures of two Bt toxins have been elucidated. The crystal structure of the Coleopteran-specific toxin produced by *Bacillus thuringiensis* subspecies *tenebrionis* has been reported with a resolution of 2.5 Å (Li, J. et al., 1991). The crystal structure revealed that this toxin is a novel structure composed of three domains. Domain I. which consists of a seven-helix bundle spanning from the N-terminus (residue 66) to residue 290. Domain II, which includes residues 291-500, is composed of three antiparallel β sheets surrounding a hydrophobic core. Domain III, comprising residues 501 to 644 at the C-terminus. forms a sandwich of two antiparallel β sheets. From the structure, it was proposed that the bundle of long, hydrophobic and amphiphatic helices is suited to the formation of pores in the insect membrane. while regions of the three-sheet domain, Domain II, are most likely responsible for receptor binding (Li, J. et al., 1991). Domains I and III, make contacts which cause one of the two sheets in Domain III to be almost entirely buried. The authors suggested that the determined structure could be representative of the general structure of the family of related insecticidal proteins.

The crystal structure of the Lepidopteran-specific CryIAa toxin form *Bacillus thuringiensis* var. *kurstaki* HD-1 was determined at a resolution of 2.25 Å (Grochulski et al., 1995). It also contains three domains and the structure is virtually identical to the toxin structure deduced for the Coleopteran-specific toxin. Domain I, which extends from amino

acid residues 33 to 253, contains eight helices; seven of which form a bundle structure, with the eighth being a helix located in the centre of the bundle. Domain II consists of three antiparallel β sheets and two short helices. Domain III, spanning residues 463 to 609, is a β sandwich formed by two twisted antiparallel β sheets. Superposition of the crystal structures shows a high degree of structural similarity. Sequence homologies of the domains of the two toxins were compared. Domain I shares 35% homology, Domain II, which is the most divergent, shares 23% homology, and Domain III being the most highly conserved at 41% sequence homology.

The functional significance, with respect to the mode of action of the toxin, of the different domains has been investigated by mutation studies by Dean and co-workers. There is general consensus that the functional role of Domain I involves irreversible binding of the toxins to midgut receptors (Chen et al., 1995; Hussain et al., 1996, Schwartz et al., 1997b). Similar studies suggest that Domain II is required for membrane insertion, receptor binding and toxicity (Smith, G.P. and Ellar, D.K., 1994; Lu et al., 1994; Rajamohan et al., 1995; Wu et al., 1996: Rajamohan et al., 1996a; Rajamohan et al., 1996b; Rajamohan et al., 1996c). As previously mentioned, this region has been shown to be the most variable portion of the toxin, and the results of binding and toxicity studies of various mutants indicates that the two loops were indeed important determinants for the activity and specificity of the δ-endotoxins. The results of mutational studies conducted on Domain III suggest the involvement of this domain in ion-channel formation (Chen et al., 1993; Wolfersberger et al., 1996; Schwartz et al., 1997c)

and receptor binding (Lee et al., 1992), while others have suggested that this domain serves a structural role by conferring stability to the protein (Li et al., 1991; Nishimoto et al., 1994).

1.7 Rationale

Bt has been employed as a bioinsecticide for several decades and its structure and mode of action have been extensively investigated. However, details with respect to the structural properties and activation of the protoxin, as well as the mode of action of the toxin, are still not well understood. The initial objectives of the presented research were to characterize the interaction(s) between the protoxin and DNA components in the nucleoprotein complex which make up the insecticidal crystals produced by *Bacillus thuringiensis* var. *kurstaki* HD-73. The finding that DNA is present in these crystals provided a new perspective and element of complexity with respect to Bt research. The presence of DNA might also provide potential explanations for a number of well-documented, but poorly understood, observations relating to 1) the structural and chemical properties of the crystal protein, 2) the mode of action of the toxin and 3) the photoinstability and lack of field persistence of Bt products.

Chapter 2 describes experiments conducted in an attempt to elucidate the role that DNA-protein interactions may have in the proteolytic processing of the protoxin to toxin. Although the processing of the protoxin to toxin has been actively investigated for a number of years and well characterized (Choma, 1990a), the discovery that DNA is present as an integral component of the crystalline inclusion body required that its role be investigated.

Chapters 3 and 4 describe experiments performed in an effort to determine the reasons for the observed photoinstability and inactivation of Bt products observed and reported by other groups studying this bioinsecticide (Ahmed, S.M. et al., 1973; Ignoffo, C.M. and Garcia, C., 1978; Morris, O.N., 1983; Pozgay, M., et al. 1987; Dunkle, R.L. and Shasha, B.S., 1988, 1989; Putzai. M. et al. 1991; Liu,Y.T. et al., 1993; Cokmus, C. et al., 1995; Patel, K.R. et al., 1996; Sundaram. A. et al., 1996; Sundaram, K.M.S. et al., 1996).

Experiments described in Chapter 5 were conducted in an attempt to elucidate the reaction products that may be formed as a result of UV irradiation of the Bt DNA-protein complex. Numerous studies have been conducted in an attempt to elucidate the effect of UV irradiation on field persistence and toxicity of Bt formulations and in developing photostable formulations. however the success has been somewhat limited. Knowledge of the UV-inactivation reaction mechanism and characterization of the reaction products would offer useful insight with respect to developing new formulations with increased field persistence.

Finally, Chapter 6 describes the development of a sensitive, rapid technique, which is of general applicability for the isolation of C-terminal peptides of proteins. The method described in the current investigations was employed in the determination of the C-terminal peptide sequence of the *Bacillus thuringiensis* crystal protein (protoxin). The currently accepted C-terminal sequence for this protein, is that deduced from the gene nucleotide sequence.

All of the research conducted in this thesis has been performed on crystals obtained from laboratory cultures of *Bacillus thuringiensis* var. *kurstaki* HD-73, unless specifically stated otherwise.

Chapter 2

Role of DNA in the Mechanism of Activation of the Insecticidal Crystal Protein from *Bacillus thuringiensis* var. *kurstaki* HD73

2.1.1 Introduction

The crystals purified from a variety of bacillus species including *Bacillus thuringiensis*, *Bacillus cereus* and *Bacillus sotto* are known to possess insecticidal activity towards a number of insect pests (Aoki, K. and Chigasaki, Y., 1915; Andrews, R.E. et al., 1987). Bt products have now been used for several decades as an insecticide, yet the details of the mode of action and toxicity still remain unclear.

It was recently discovered that the crystals produced by *Bacillus thuringiensis* are associated with DNA (Bietlot H.P. et al., 1993). Toxin derived from the activation of the *Bacillus thuringiensis* CryIA(c) crystal protein by bovine trypsin was shown to be resolved into two components by anion-exchange chromatography. Two forms of the toxin could be isolated: toxin associated with low molecular weight DNA (LMW-DNA toxin; 100-300 base pairs or bp) and DNA-free toxin (toxin) (Bietlot H.P. et al., 1993). Furthermore, it was demonstrated that upon solubilization of purified crystals, a single protoxin component was observed by SDS-PAGE and a single high molecular weight DNA band (HMW-DNA; approximately 20 kilobase pairs; kbp) by agarose gel electrophoresis. It was suggested that the DNA was mainly associated with the highly conserved C-terminal region of the protoxin and could be important maintaining the structural integrity of the crystal, making it essential for the generation of toxin (Bietlot, H.P., et al., 1993).

Until the discovery that DNA is associated with the protoxin, it was believed that proteolysis by insect gut protease was the only requirement for activation of the crystals, by converting the protoxin to toxin. The discovery that DNA was associated with the crystals from

Bacillus thuringiensis var. *kurstaki* raised the question as to the generality of this observation. Crystals purified from various subspecies of Bacillus thuringiensis, as well as cloned crystals, were examined for the association of DNA (Bietlot, H.P., 1993). All of the crystals tested were found to contain DNA. Also, the DNA observed in each of the crystal preparations gave a single high molecular weight band corresponding to an apparent molecular mass of approximately 20 kilobase pairs (kbp) as observed by agarose gel electrophoresis. The observation that low molecular weight DNA was found to be associated with the toxin raised the possibility that the DNA was responsible in directing the activation of the protoxin to toxin. Furthermore, the associated DNA could be responsible for the unusual sequential proteolysis observed during activation of the protoxin to toxin (Choma, C.T. et. al. 1992).

2.1.2 Rationale for the Present Investigation

The objectives of the research undertaken were 1) to determine the role, structural or functional, of the DNA during the activation of protoxin to 2) to determine how the crystal protein is interacting with the DNA and 3) to determine the fate of the DNA during the activation of the crystal protein

2.2 Experimental Procedures

2.2.1 Materials

Enzymes

Bovine pancreatic trypsin (EC 3.4.21.4) and DNase I (3.1.21.1) were purchased from Sigma Chemical Co. Gut juice from spruce budworm (*Choristoneura fumiferana*) was obtained from Dr. R.E. Milne of the FPMI (Forest Pest Management Institute) in Sault Ste. Marie.

Chemicals and Solvents

GASTROgrafin ® was obtained from Bracco Diagnostics Canada Inc. UltraPURE agarose was purchased from GIBCO BRL Life Sciences. Reagents used to prepare SDS-polyacrylamide gels were purchased from Bio-Rad Laboratories. All other reagents were obtained from standard commercial sources and were of the highest grade available.

Bacillus thuringiensis var. *kurstaki* HD-73 Crystals

The investigations described throughout this thesis were performed on crystals obtained from *Bacillus thuringiensis* var. *kurstaki* HD-73 (Cry1Ac), unless specified otherwise, which were purified according to the procedure previously described (Milne, R. et al., 1977).

I) Production and Harvest

The crystals were obtained from stored cultures of *Bacillus thuringiensis* which were kept as slants or as bacto-agar plate cultures at 4°C. Typically, a starter culture is grown in a 250 ml flask overnight. Growth cultures for the production of crystals were initiated by inoculation of 1L of sterile pepticase soy broth or other rich growth media with 1 ml of the starter culture and subsequently incubated at 28°C in a shaking chamber in baffled culture vessels. The cultures were monitored (by phase contrast microscopy) for crystal production and cell lysis periodically over the course of a week and harvested when a minimum of 50% of cells were observed to be lysed or containing the crystal. When crystals formation and cell lysis were observed, the culture medium was decanted into centrifuge bottles and centrifuged at 10 krpm for 15 minutes to recover the crystals, spores and cells which remained intact (unlysed). The culture medium was discarded and the centrifugation pellet, containing the crystals and all sedimentable material (spores, unlysed cells, cellular debris), were resuspended in 1M NaCl containing 0.01% Triton X-100 overnight to induce complete cell lysis. The resultant solution was centrifuged at 15 krpm for 15 minutes. The pellet was then resuspended in distilled water and the centrifugation repeated. The suspension was centrifuged at 15 krpm for 15 minutes and the resulting pellet was resuspended in a minimum amount of distilled water.

II) GASTROgrafin ® Purification of Crystals

The purification of crystals from the crude pellet obtained from the total cell lysates of *Bacillus thuringiensis* cultures was accomplished as previously described (Milne, R. et al., 1977). The pellet is resuspended in a minimum amount of distilled H_2O and the solution made up to be 64% GASTROgrafin ® and centrifuged at 15 krpm for 1 hour in 50 ml centrifugation tubes. This density gradient centrifugation results in three layers; the top layer contains mainly lipids and fragmented, free DNA (genomic). The intermediate layer, which is a cloudy suspension, contains the crystals and the third layer (or pellet), contains mainly cellular debris, spores, remaining unlysed cells with a small amount of crystals. (The pellets can be resubjected to GASTROgrafin centrifugation to obtain the maximum yield of crystals). The layers were carefully recovered to avoid mixing. GASTROgrafin® purified crystals were treated with DNase in the presence of 25 mM PMSF (to eliminate protease activity) at pH 7.5 in DNase buffer (40 mM Tris-HCl, 10 mM NaCl, 6 mM $MgCl_2$, 0.1 mM $CaCl_2$) for 20 minutes to remove any exogenous or nonassociated DNA. The crystals were recovered by centrifugation and washed with doubly distilled deionized water and finally recovered by centrifugation. The crystal layer is then examined for purity by phase contrast microscopy and analyzed for purity by SDS-PAGE (10%) and agarose gel electrophoresis (0.9% in 0.1M CAPS or 0.1M bicarbonate/carbonate buffer, pH 10.5).

Cloned Cry1A Proteins

Inclusion bodies containing Cry1Aa, Cry1Ab an Cry1Ac protoxins (Brousseau, R. and Masson, 1988; Masson, L. et al., 1989) cloned in *E. coli* were obtained from Dr. Luke Masson of the Biotechnology Research Institute, Montreal, Québec.

2.2.2 Method

Preparation of Solubilized Protoxin

GASTROgrafin® purified crystals were solubilized by resuspending the crystal slurry in a solution of 0.1M CAPS or 0.1M bicarbonate/carbonate buffer (pH 10.5) with 0.1% (v/v) 2-mercaptoethanol (Bietlot, H., et al., 1993). CAM-protoxin (S-carbaminomethyl-protoxin) was prepared as previously described (Choma and Kaplan, 1992) and solubilized in 0.1M CAPS or 0.1M bicarbonate/carbonate buffer (pH 10.5).

Preparation of Toxin-20-kbp DNA Complex

Purified crystals from *Bacillus thuringiensis* were solubilized in 0.1 M bicarbonate/ carbonate buffer in the presence of 10 mM EDTA (to inhibit nuclease activity). The solubilized crystals were treated with 0.05% trypsin (w/v) for 2 hours then centrifuged to remove insoluble material and dialyzed against distilled water/acetic acid (pH 5) for 24 hours with several changes of the dialysis medium. The starting material (crystal protein; protoxin) and the digest were then analyzed by SDS-PAGE and agarose gel electrophoresis.

Spruce Budworm (*Choristoneura fumiferana*) Gut Juice

Sixth instar spruce budworm larvae were forced to expectorate by teasing the mouth parts with a capillary. The gut juice collected was centrifuged at 12,000×g, and aliquots of the supernatant were stored at -20°C as described previously (Milne and Kaplan, 1993). Aliquots of the gut juice were thawed and diluted in distilled H_2O to yield the desired concentration just prior to use. Gut juice was supplied by Dr. R.E. Milne from the FPMI in Sault Ste. Marie.

Gut Juice Activation of *Bacillus thuringiensis* Crystals

The mechanism of activation of *Bacillus thuringiensis* crystals in the midgut of the spruce budworm (*Choristeneura fumiferana*) was investigated by digesting the crystal protein with gut juice enzymes for various time intervals and then analyzing the products by SDS-PAGE and agarose gel electrophoresis. *Bacillus thuringiensis* crystals were solubilized in 0.1 M CAPS buffer (pH 10.5) and incubated at 37°C in a shaking water bath with 0.1% (v/v) or 0.5% (v/v) spruce budworm gut juice. At various time intervals, 100 µl aliquots of the incubation mixture were drawn and EDTA (20 mM final concentration) and PMSF (1 % final concentration) were added to quench the reaction. The aliquots were frozen immediately upon withdrawal from the incubation mixture by immersing in liquid nitrogen. The aliquots were then analyzed by SDS-PAGE and agarose gel electrophoresis after the final time point.

Protein Quantification

For routine procedures, protoxin and toxin concentrations were estimated from the absorbance at 280 nm (Bietlot, H.P. et al., 1989a) or by Bio-Rad standard protein assay procedures (Bradford, M., 1976). For quantification of the DNA / protein ratio, protoxin and toxin were quantified by dividing the amount of each amino acid obtained from amino acid analysis by the number of residues predicted from the gene nucleotide sequence of the protoxin or toxin (Bietlot, H.P. et al., 1989a; Adang, M.J. et al., 1985).

Phosphate Analysis

Phosphate analysis was performed by the modified micro-procedure of Bartlett (Marinetti, G.V. 1965).

I) Preparation of Standards: A standard phosphate solution was prepared by dissolving 1.097 g of KH_2PO_4 in 250 ml of deionized-distilled water such that a 1:100 dilution of the stock yielded a final phosphate concentration of 10 μg/ml phosphate. Aliquots of this solution (0.1-1 ml) were lyophilized in hydrolysis tubes. Two additional tubes were prepared but lacking the phosphate standard solution (blanks).

II) Preparation of Bacillus thuringiensis and Cloned Crystal samples: Preliminary phosphate analyses were performed on crystal suspensions and solubilized protoxin to determine the approximate amount of crystals necessary for analysis (that is, an amount found to be within the linear range of the Beer-Lambert Law; 10 μg of phosphate). Various aliquots (5-1000 μl

aliquots) of crystal suspensions and solubilized protoxin were lyophilized in hydrolysis tubes and subjected to the analytical procedure with the assumption that treatment with perchloric acid was sufficient to fully oxidize and liberate total phosphate contained within the samples. Based on the results of the preliminary analyses, appropriate samples of crystals, cloned crystals and solubilized protoxin were prepared and these samples were analyzed along with the standards and blanks as described below.

After lyophilization, 0.4 ml of perchloric acid was added to each tube, hydrolyzed for 4 minutes and cooled. 4.2 ml of deionized distilled water, 0.2 ml of a 5% ammonium molybdate solution and 0.2 ml of amidol reagent (0.5g of amidol in 50 ml of 20% sodium bisulphite solution and filtered) were added to each hydrolyzed sample tube. The tubes were covered with aluminum foil and heated in a boiling water bath for 7 minutes. The color was allowed to develop for 30 minutes. Absorbance of the molybdenum blue complex was measured at 830 nm (relative to a water blank) in quartz cuvettes (1.0 cm) using a Pye Unicam PU 8800 UV-visible spectrophotometer. A standard curve was constructed using SigmaPlot 4.0 (using a 95% confidence interval).

Amino Acid Analysis

Amino acid analyses were performed on 20 µl aliquots of crystal suspensions, cloned crystals or solubilized protoxin by adding 100 µl of a 1 mM norleucine solution, 500 µl of 12 N HCl and 400 µl of distilled water; this yields a 6 N HCl solution containing 100 µM norleucine as internal standard. The solutions were then hydrolyzed for 24 hours at 110°C under vacuum.

Amino acid quantification was carried out on an Applied BioSystem Model 420 amino acid analyzer equipped with an automated PITC (Phenylisothiocyanate) precolumn derivatization system.

Quantification of DNA/Protein Ratio

The DNA/protein ratio, expressed in base pairs of DNA/molecule of protein, was calculated as the ratio of the concentrations of phosphate and protein from a minimum of two independent assays for each crystal type. The mean error in the determinations was calculated from the standard errors in the phosphate and protein concentrations. Since the DNA is double stranded, the value obtained for the amount of phosphate was halved to obtain a value expressed as base pairs of DNA/molecule of protein. Phosphate analyses were performed by Marjolaine Carrière as part of the fourth year honours project in chemistry (Carrière, M., 1994).

Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-polyacrylamide gels (10%) were prepared from materials supplied by Bio-Rad, and electrophoresis was performed according to standard procedures from the manufacturer using the MiniProtean System (Bio-Rad Laboratories). Alternatively, SDS-PAGE was performed on precast 10-15% gradient gels obtained from Pharmacia and analyzed on the Phastsystem (Pharmacia Biotech) according to standard automated procedures from the manufacturer. Protein detection was achieved by staining with Coomassie Brilliant Blue R-250.

Agarose Gel Electrophoresis

Agarose gels (0.9%) were prepared using Ultrapure agarose (Life Technologies, Inc.). Gels were prepared in 0.1 M CAPS or 0.1 M bicarbonate/carbonate buffer (pH 10.5), or 0.1 M TAE (Tris-acetic acid-EDTA, pH 8.8) and treated with ethidium bromide for detection on the DNA.

DNA Extractions

DNA was extracted from the DNA-protein complexes using a modified phenol/chloroform procedure previously described (Sambrook, J. et al., 1989; Bietlot, H.P. et al., 1993). The DNA-protein complexes were solubilized in 0.1 M CAPS or 0.1 M bicarbonate/carbonate buffer (pH10.5) and 10 mM EDTA and 1% (v/v) 2-mercaptoethanol. Extraction was accomplished by the addition of an equal volume of phenol / chloroform / isoamyl alcohol (pre-equilibrated with 0.1 M CAPS, pH 10.5) at 65°C. The aqueous phase was re-extracted in a similar manner and the extractions were pooled. The DNA was precipitated by the addition of 0.3 M sodium acetate (pH 5.2) and two volumes of ice-cold ethanol. The precipitated DNA was recovered by centrifugation (0°C, 12,000×g, 10 minutes) and washed with 70% ethanol and recentrifuged (4°C, 12,000×g, 2 minutes). The DNA was resuspended in 0.1 M TE (Tris-EDTA, pH 8.0) and analyzed by agarose gel electrophoresis as described above.

Ion-Exchange Chromatography

Samples were chromatographed using a Bio-Rad HRLC MA7Q (50×7.8mm) anion exchange column. Elution was carried out using a linear gradient of 0 to 1 M NaCl in 0.1 M CAPS buffer adjusted to pH 10.5. The flow rate was 2.5 ml/min, and the eluate was monitored by UV absorbance at 280 nm.

Reconstitution of Toxin with DNA

Toxin (0.1 mg) was incubated with salmon sperm DNA (0.01 mg and 0.1 mg) in the presence of 1 mM $MgCl_2$ in 100 mM sodium phosphate buffer (pH 7.5) and subjected to UV irradiation at 302 nm for up to 4 hours. Control samples were prepared as above but in the absence of DNA. The samples were analyzed by agarose gel (pH 10.5) and SDS-PAGE.

2.3 Results

The presence of DNA in the low molecular weight DNA-toxin complex (100-300 bp DNA) (Bietlot, H.P. et al., 1993), suggested that toxin could be generated in the absence of nuclease activity. The generation of toxin from purified crystals was attempted in the absence of nuclease activity by treating purified crystals with bovine trypsin in the presence of 10 mM EDTA. Figure 2.1 A (lane 2) shows the DNA associated with the crystal protein, the DNA extracted from the crystal protein (lane 3) and Figure 2.1 B (lane 2), the corresponding protoxin

component of the crystal before treatment with trypsin. Figure 2.1 A (lane 4) shows the DNA associated with the toxin, the DNA extracted from the toxin (Lane 5) and the corresponding toxin band shown in Figure 2.1 B (lane 3) after treatment with trypsin. The results indicate that toxin can readily be generated in the absence of nuclease activity, with the DNA associated with the crystal protein remaining intact.

The processing of the protein and DNA components during the activation process was determined by incubating purified crystals with gut juice (0.1 %) obtained from spruce budworm (*Choristoneura fumiferana*). Aliquots were withdrawn at various time intervals and the fates of the DNA and crystal protein were monitored by SDS-PAGE and agarose gel electrophoresis as shown in Figures 2.2a (agarose gel) and 2.2b (SDS-PAGE).

The earliest time point (30 seconds) shows that most of the crystal protein (Figure 2.2b) has already been proteolyzed to toxin while the DNA associated with the crystal (Figure 2.2a) has remained intact. With longer incubation periods, the DNA is slowly and continuously being processed while the crystal protein has been completely converted to toxin. The DNA processing appears to result in the accumulation of DNA in the molecular mass range of 100-300 bp and reflects the presence of the LMW-DNA toxin complex.

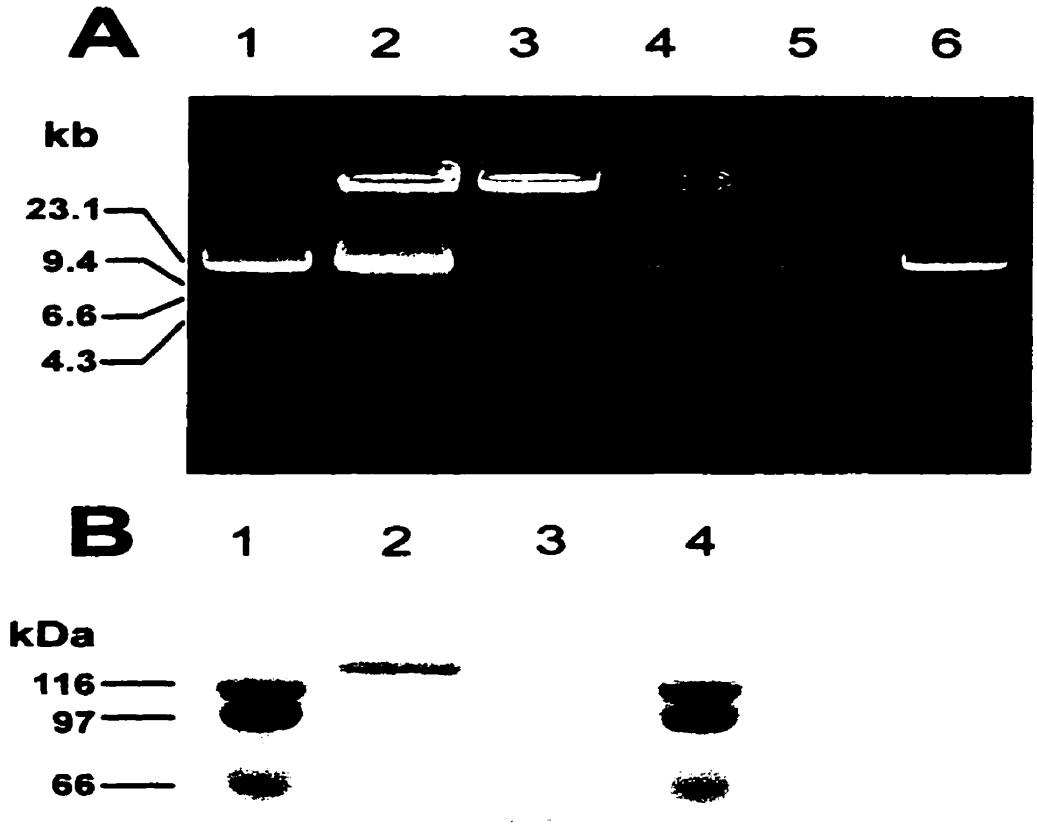


FIG. 2.1 B. thuringiensis DNA-protein complexes.

(A) Samples were subjected to electrophoresis on a a 0.9% (w/v) agarose gel in 100 mM TAE, pH 8.8 and stained with ethidium bromide. *Lane 1*, λ phage Hind III digest; *Lane 2*, protoxin-DNA; *Lane 3*, toxin-DNA; *Lane 4*, DNA extracted from protoxin-DNA; *Lane 5*, DNA extracted from toxin-DNA; *Lane 6*, λ phage Hind III digest. **(B)** Samples were subjected to SDS-PAGE and stained with Coomassie Blue. *Lane 1*, molecular mass markers; *lane 2*, protoxin-DNA; *lane 3*, toxin-DNA; *lane 4*, molecular mass markers.

The activation of the crystal protein with gut juice was also monitored by anion-exchange chromatography as shown in Figure 2.3. The vertical arrows indicate the elution of 100-300 bp-DNA-toxin (Bietlot H.P. et al., 1993) at approximately 0.8 M NaCl (9.2 minutes) and the DNA-free toxin eluting at approximately 0.3 M NaCl (3.8 minutes) respectively. In Figure 2.3 (A), the earliest time point (30 minutes), the DNA-associated toxin is the major eluting peak. With increased processing time (Figure 2.3 B-D), the DNA-associated toxin is slowly converted to the DNA-free toxin. Within 24 hours, the DNA-toxin is completely converted to the DNA-free toxin.

The chromatographic analysis of these samples was carried out by Pham Van Thong as part of the requirements for his honours research in the Biochemistry / Biotechnology program.

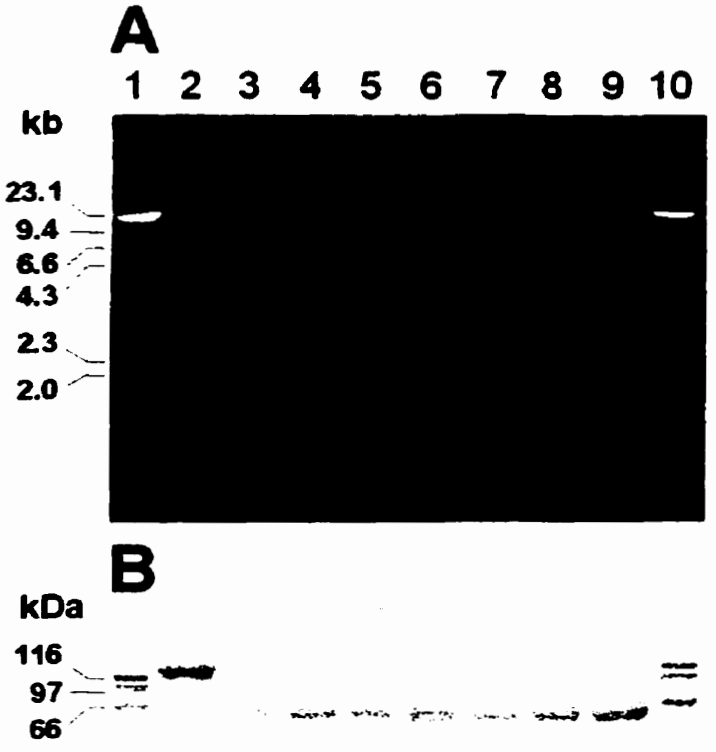


FIG. 2.2. Activation of the solubilized protoxin by spruce budworm gut juice.

Bt crystals were solubilized and treated with 0.1% gut juice and subjected to A, Agarose gel electrophoresis and stained with ethidium bromide and B, SDS-PAGE and stained with Coomassie blue. *Lanes 1 and 10* are, A, λ phage Hind III digest and B, molecular mass markers; *lane 2*, 0 time; *lane 3*, 30 sec; *lane 4*, 15 min; *lane5*, 30 min; *lane 6*, 1 h; *lane 7*, 2 h; *lane 8*, 4 h; *lane 9*, 24 h.

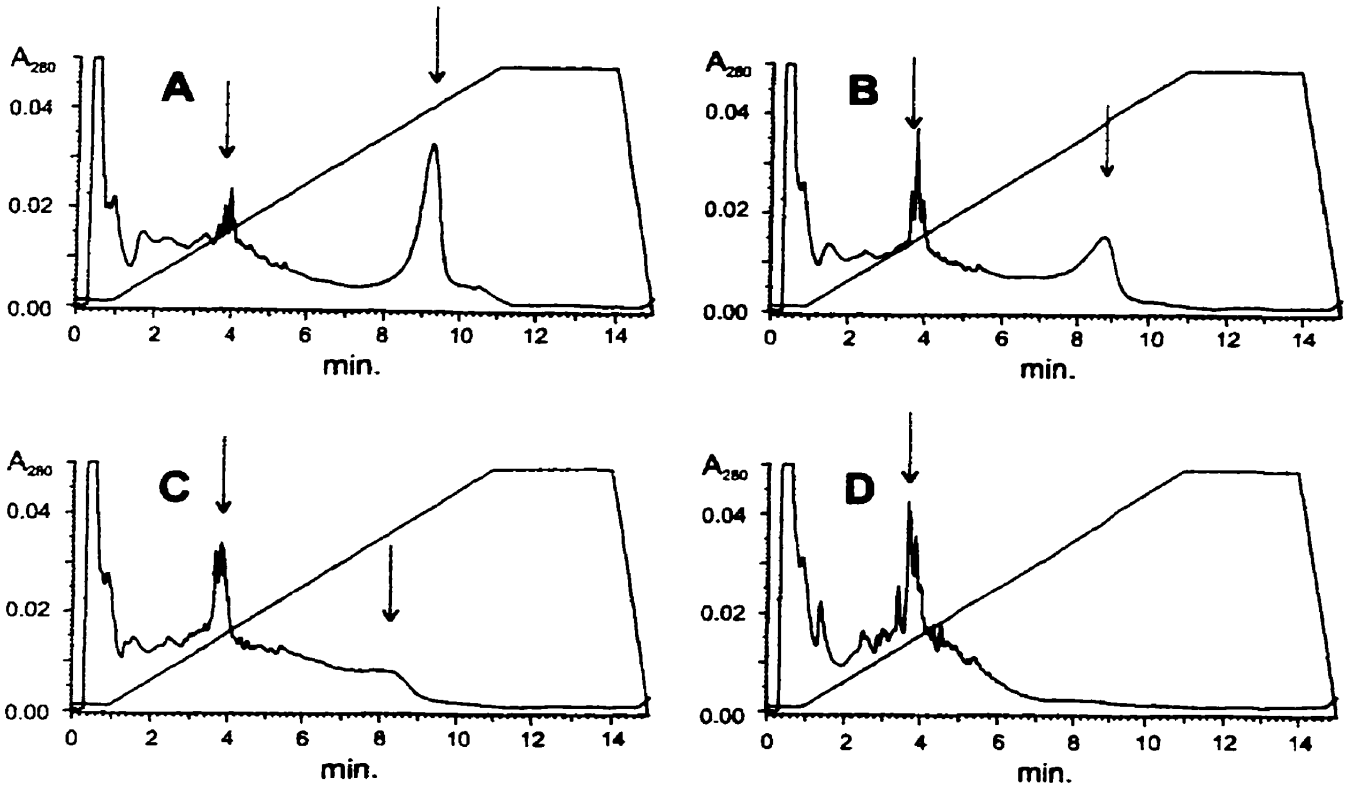
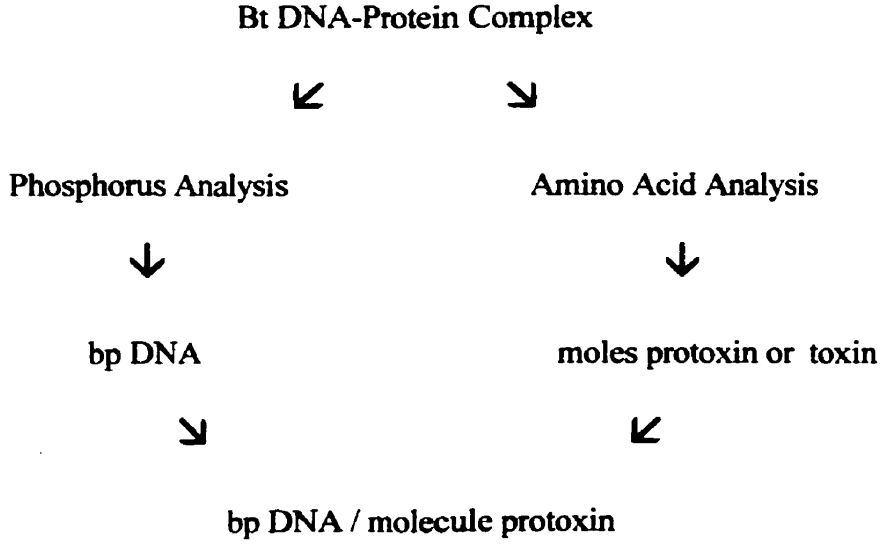


FIG. 2.3. Products of activation of the solubilized protoxin by spruce budworm gut juice.

Bt crystals were solubilized and treated with 0.5% gut juice. Aliquots were withdrawn and various time intervals and analyzed by anion exchange chromatography. Elution conditions consisted of a linear gradient 0 to 1 M NaCl in 0.1M CAPS, pH 10.5 at a flow rate of 2.5 ml/min. A, 30 min; B, 2h; C, 4 h; D, 24 h.

In an attempt to gain insight with respect to the structure of the DNA-protein complex, the relative amounts of DNA and protein were quantified. Samples tested consisted of crystals from various *Bacillus thuringiensis* subspecies, cloned crystals as well as the DNA-protein complexes (20 kbp-DNA protoxin and 20 kbp-DNA toxin) generated from the crystals from *Bacillus thuringiensis* var. *kurstaki* HD73. The strategy employed was to quantify the protein concentration by amino acid analysis, combined with the results of phosphate analysis by the modified micro-procedure of Bartlett (Marinetti, G.V. 1965) of the corresponding sample as shown in Figure 2.4.

Figure 2.4 Strategy for the Quantification of the Relative Amounts of DNA and Protein in *Bacillus thuringiensis* Crystals and Related DNA-Protein Complexes



Quantification of the amount of protein (protoxin or toxin) was based on the amino acid composition determined from the gene nucleotide sequence (Adang, M.J. et al., 1985). The samples were hydrolyzed in 6 N HCl and each amino acid was quantified by amino acid analysis. The molar amount of protein was estimated by dividing the molar quantities of each amino acid by the number of residues of the amino acid in the protoxin or toxin. Phosphorus analysis was carried out on a corresponding aliquot of the sample, and the molar quantity of phosphate used for the calculation was half of the molar phosphorus content. Table 2.1 summarizes the results obtained for the relative DNA/ protein compositions of these samples.

TABLE 2.1: Quantification of the Ratio Base Pairs DNA/Protein

Bt Sample	Bp DNA/molecule protein[a]
Bt *kurstaki* HD73 Crystal	3.5 ± 0.5
Bt *kurstaki* HD73 Crystal	3.1 ± 0.4
Bt *kenya* Crystal	4.8 ± 1.5
Bt *Sotto* Crystal	2.0 ● 0.5
Solubilized HD73 Protoxin	3.2 ± 1.1
Solubilized HD73 Protoxin	4.0 ± 1.4
Cloned E. Coli Cry1Ac Protein	2.3 ± 0.2
HD73 Toxin-20 kpb DNA	5.0 ± 1.0
	Average: 3.5 ± 1.0

[a] The values are given with the 95% confidence interval determined from the standard error in the estimate of the phosphorus content and the standard error in the estimate of the amount of protein from amino acid analysis.

These results indicate that there are from 2 to 5 nucleotide base pairs per molecule of protein. The average of all determinations yields a value of 3.5 ± 1 nucleotide base pairs per molecule of protein.

2.4 Discussion

The initial discovery that the insecticidal crystals from *B. thuringiensis* subsp. *kurstaki* HD-73 are associated with heterologous 20-kbp DNA (Bietlot, H.P., et al. 1993) raised the question as to the role of the DNA in the crystals. Subsequent analysis of a variety of crystal types, as well as cloned crystals, provides yet further evidence that the association of DNA with the insecticidal crystals is a general phenomenon and that the DNA is not artifactual but is intimately associated with the crystal protein.

The observation that an intermediate complex consisting of 20-kbp DNA-toxin can be generated by proteolytic processing with trypsin in the absence of nuclease activity, suggests that the DNA is interacting with the protein primarily through contacts made with the N-terminal region of the protein. These results show that the initial hypothesis that the DNA interacted primarily with the highly conserved C-terminal region of the protoxin is incorrect (Bietlot, H.P., et al. 1993).

In an attempt to determine the extent of the interactions, and to gain insight with respect to the structure of the DNA-protein complex, the relative amounts of protein and DNA were quantified for a variety of crystals from various subspecies of *B. thuringiensis* as well as the solubilized 20-kbp DNA-protoxin and 20-kbp DNA-toxin complexes. A previous estimate was made using the ratio of absorbances at 260 and 280 nm (Monro and Fleck, 1966) which yielded approximately 10 bp per molecule of protein (Bietlot, H.P. 1993). However, estimates made by this method assume that the absorbances of DNA and protein are not affected by interactions among the individual components, and values obtained are approximate at best. However, the

value of 10 bp / molecule of protein does indicate a large amount of protein relative to the DNA.

The strategy adopted in the present study was to quantify each component separately. Protein quantification was achieved by amino acid analysis and DNA by phosphate analysis. The data obtained by this approach gave a value of approximately 3 to 4 bp DNA per molecule of protein which indicates a very large quantity of protein relative to DNA, and suggests that only a very small portion of the protein is involved in interactions with the DNA.

Based on these results, and the results obtained from the activation mechanism, a model is proposed (Figure 2.5) for the structural arrangement of the DNA and protein components in the *B. thuringiensis* crystal protein. The model highlights a number of important structural features that are consistent with all the data that has been acquired to date. Protoxin molecules are depicted as elongated molecules surrounding a central double-stranded DNA core, with the N-terminal toxic moiety (darkened) interacting with the DNA and the C-terminal region extending outwards from the central core. The model also shows a compact arrangement of the protein molecules surrounding the DNA indicating: 1) that only a small portion of the protein molecule is interacting with the DNA and 2) that the protein is processed rapidly while the DNA remains intact in the early stages of the activation of the crystal protein. Notably, based on x-ray powder diffraction of the B. thuringiensis crystals, Holmes and Monro (1965) concluded that the protoxin was an elongated ellipsoid molecule, which provides further support for the model proposed in this investigation. Furthermore, this closely packed arrangement of the protein molecules accounts for the unusual proteolysis that is observed during the activation of the crystal protein to toxin. This unusual proteolytic mechanism

(Choma, C.T. et. al., 1990a), whereby the protein appears to be processed through a series of sequential proteolytic steps starting from the C-terminal end, may simply be the result of steric hindrance imposed on the proteases due to such a tightly packed structure. Proteases are only able to attack the peripheral portions of the C-terminal region, giving rise to the sequential proteolysis observed in the generation of toxin (Chestukhina, G.G. et al., 1982; Choma, C.T. et al., 1990a). The protoxin protects the DNA from attack by nucleases, but once its C-terminal region is removed, portions of the DNA become exposed and susceptible to nuclease attack. Nucleases rapidly process the DNA to approximately 100-bp, giving the observed 100-bp DNA-toxin complex, which accumulates and can be isolated from partially processed *B. thuringiensis* crystals. This may be indicative of higher levels of structure (quaternary) which have not yet been characterized.

The proposed model is reminiscent of the association of RNA and protein observed in the RNA-protein complex of the tobacco mosaic virus. A central helical strand of RNA is surrounded by protein subunits (Hewish, D.R. et al., 1973) forming a rod-like structure. In the *Bacillus thuringiensis* DNA-protein complex, the protein sheathes the DNA, rendering the DNA protected from nucleolytic activity. It has been reported that the structure of tobacco mosaic virus (TMV) is stabilized through the association of three nucleotides bound to each protein subunit (Stubbs, G., 1989). Based on the phosphate analyses of a large number of crystals of *Bacillus thuringiensis* subspecies and recombinant crystals from E. coli, each protein subunit (protoxin molecule) of the crystal is estimated to be associated with approximately 3 bp of DNA.

Based on the results of the DNA and protein processing observed by electrophoresis and anion-exchange chromatography, an activation scheme is also proposed for the conversion of crystal protein to toxin as shown in Figure 2.6. Upon ingestion by larvae, the crystal rapidly dissolves, due to the high alkalinity of the insect midgut, leading to the solubilized 20-kbp DNA protoxin complex. The proteases present in the gut juices of the insect quickly process the protoxin to toxin while the DNA remains intact. Gut juice nucleases then act upon the 20-kbp DNA-toxin complex and rapidly convert the 20-kbp DNA-toxin complex to the 100 bp-DNA-toxin complex which is processed more slowly to the DNA-free toxin, the final processing product. This final processing step, which occurs slowly compared to the earlier processing events, indicates that the toxin molecules still shield the DNA to nuclease attack to some degree and therefore suggests additional structural interactions which must await further experimentation.

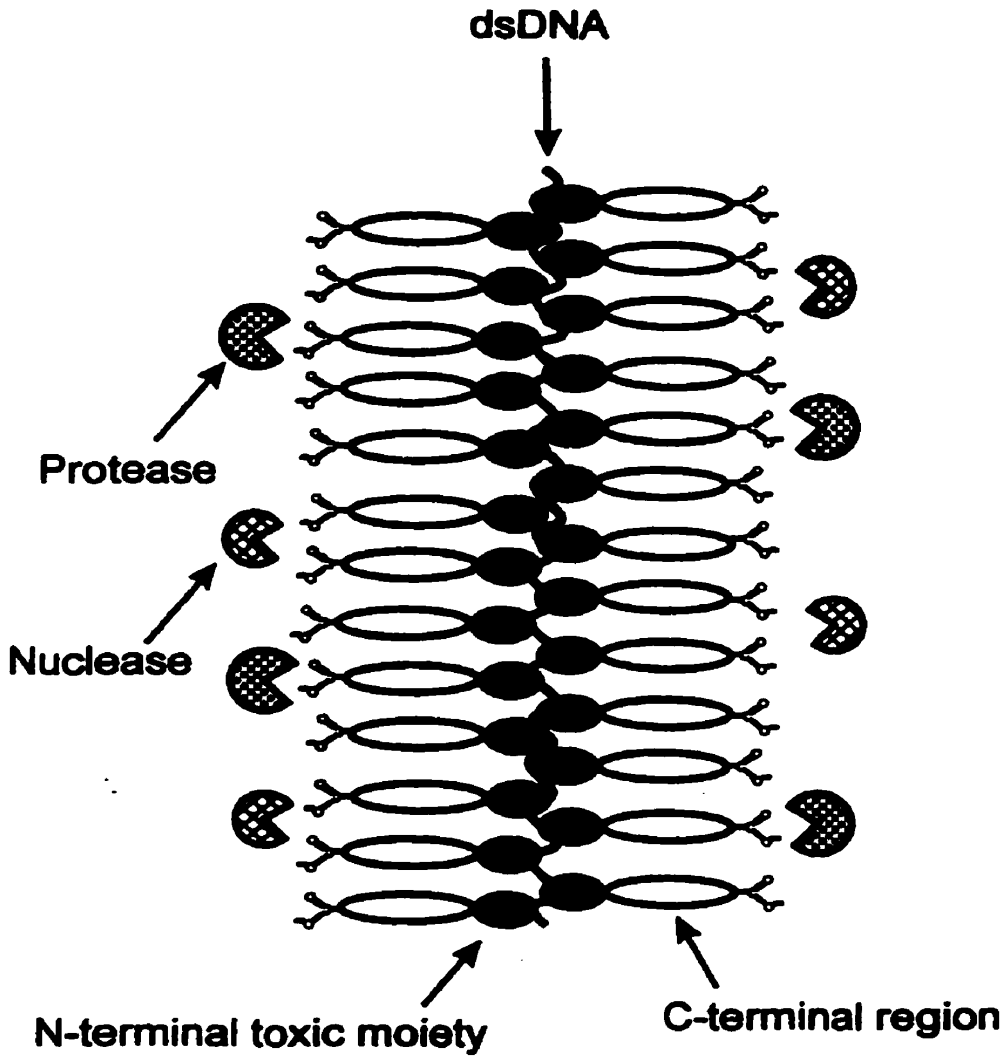


FIG. 2.5. Proposed model of the structure of the DNA-protoxin complex.

Crystal protein (protoxin) molecules surround a central dsDNA strand. The N-terminal toxic moiety interacts with three nucleotide base pairs leaving its C-terminal region extending away from the central core.

Attempts to reconstitute the DNA-toxin complexes, starting from DNA-free toxin and salmon sperm DNA, were unsuccessful. This may be the result of the experimental conditions employed however it may also be due to conformational changes in the toxin upon complete removal of the DNA (conversion of the 100 bp-DNA-toxin to DNA-free toxin). The results of DSC (Differential Scanning Calorimetry) studies of the crystal protein and toxin indicated that the toxin undergoes a conformational change during activation from protoxin to toxin (Choma et al., 1991). Compared to the protoxin, the complex endotherm observed for the toxin showed a much broader temperature profile indicating that the toxin has a substantially more thermally stable conformation than the protoxin. Therefore the toxin may bind the last vestiges of DNA quite strongly, accounting for the slow conversion to DNA-free toxin. However, once the association is disrupted, the toxin assumes a stable conformation and apparently unable to reassociate with the DNA. Hence the inability to reconstitute these complexes may indicate that reassociation of the toxin with the DNA is unfavourable as a result of a conformational change upon conversion to the DNA-free toxin, such that the reassociation is neither observed nor inducible.

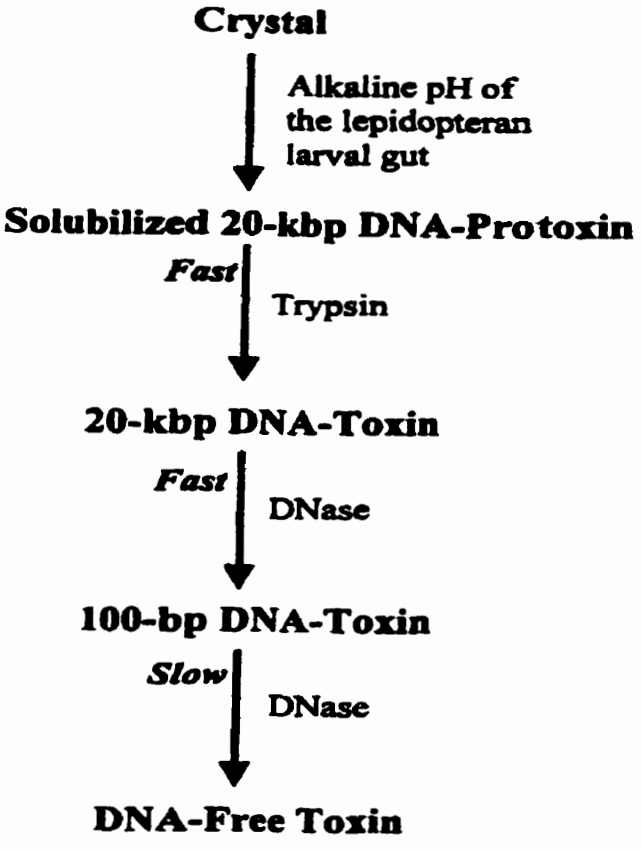


FIG. 2.6. Proposed scheme for activation of the crystal protein in the larval gut.

Activation of the crystal protein to toxin involves initial activation of the protoxin to toxin, followed by co-processing of the DNA.

2.5 Conclusion

The activation mechanism of the crystal protein from *Bacillus thuringiensis* var. *kurstaki* HD-73, involves co-processing of the DNA and protein associated with the crystal and appears to be crucially dependant on the presence of DNA (Bietlot, H. P. et al., 1993; Clairmont, F. R. et al., 1998). The role of the DNA appears to be mainly structural in nature, allowing for crystal formation and the sequestering of a large amount of protein within the prokaryotic cytoplasm. The DNA is protected by the protein from nuclease degradation, consistent with the DNA forming a central core in the complex that is completely coated by protein.

Similar nucleic acid-protein complexes are ubiquitous in viruses and allow for the formation of virion particles. The proposed structural model of the DNA-protein complex from *Bacillus thuringiensis* is reminiscent of the arrangement of the nucleic acid-protein complex found in the tobacco mosaic virus (Fraenkel-Conrat, H. and Williams, R.C., 1955; Butler, P.J. and Klug, A., 1971; Stubbs, G. (1989). Chemical analysis of the relative ratios of DNA and protein found in the crystals from *Bacillus thuringiensis* yields a similar ratio (approximately 3-4 bp / protein molecule) of nucleic acid/protein previously determined for TMV (Stubbs, G. (1989). The results obtained from phosphate and amino acid analyses confirm that only a small portion of the protein interacts with the DNA. The ratio of DNA base pairs to protein molecule calculated based on these analyses suggests that each protein molecule is associated with only 3 base pairs of DNA. A structural model, depicting the arrangement of the crystal protein and

nucleic acid in Bt crystals, has been proposed which is consistent with all the currently available experimental data.

A model, representing the sequence of processing events for the activation mechanism for the crystals of *Bacillus thuringiensis* var. *kurstaki* HD-73, has also been presented. This model, combined with the structural model, account for the unusual sequential proteolysis of the protoxin to toxin (Choma C.T. et al., 1990a). The sequential proteolysis may be postulated to arise from DNA-associated protein conformations or interactions that have not yet been characterized. The structural model shows the elongated protein molecules in a tightly packed arrangement. Such an arrangement may account for the obligatory sequential proteolysis observed in the activation of protoxin to toxin as a result of a sterically encumbered structure, and does not appear to involve conformational changes as previously suggested (Choma C.T. et al., 1990b and 1991).

Finally, the presence of DNA in the crystals may account for the existence of exogenous / endogenous chromophores reported to be in crystal preparations (Carey, R.P. et al., 1986; Pozgay, M. et al., 1987; Pusztai, M. et al., 1991) As well, the complex calorimetric endotherms observed by DSC and the non-reversibililty of the endotherms upon cooling could be the result of DNA dissociation upon heating of samples, causing the denaturation of DNA-protein complex and hence instability of the samples (Choma, C.T. et al., 1991). The DNA may also be responsible for the photoinactivation of Bt products. This topic is the subject of Chapter 3.

Chapter 3

Photo-inactivation Studies of the *Bacillus thuringiensis* Crystal Protein

3.1.1 Introduction

One of the major limitations of commercial *Bacillus thuringiensis*-based insecticidal formulations is the relatively short persistence in the field; the insecticidal activity of the formulations diminishes rapidly after spraying (Ahmed, S.M. et al., 1973; Ignoffo, C.M. and Garcia, C., 1978; Morris, O.N., 1983; Dunkle, R.L. and Shasha, B.S., 1988, 1989; Liu,Y.T. et al., 1993; Cokmus, C. et al., 1995; Patel, K.R. et al., 1996; Sundaram, A. et al., 1996; Sundaram, K.M.S. et al., 1996). The persistence of bioactivity has been shown to be greatly influenced by sunlight, rainfall and the droplet sizes of deposits as well as by heat, desiccation and pH (Leong, K.L. et al., 1980; Couch and Ignoffo, 1981; Patel, K.R. et al., 1996; Sundaram, A. et al., 1996 and references therein).

General acceptance of the economical and practical importance of the development of photostable Bt-based products has led investigators to study the effects of the environmental conditions leading to sunlight inactivation. In an attempt to prolong the field persistence of Bt formulations, investigators have tried encapsulating Bt crystals in starch matrices, with or without UV absorbing materials (sunscreens) (Raun, E.S. and Jackson, R.D., 1966; Dunkle, R.L. and Shasha, B.S., 1988; 1989); encapsulation into carboxymethylcellulose beads (Cokmus, C. et al., 1995); incorporation into clay granules (Ahmed, S.M. et al., 1973); the addition of horse radish peroxidase (Ignoffo, C.M. and Garcia, C., 1978); and the incorporation of various UV sunscreens or combinations of sunscreens (Morris, O.N., 1983; Liu,Y.T. et al., 1993; Patel, K.R. et al., 1996). Despite these attempts, no significant increases in the persistence (insecticidal activity) were observed in these field trials. Therefore the sunlight inactivation of Bt formulations remains a major problem, and

the mechanism of inactivation remains unknown (Pozgay, M. et al., 1987; Dunkle, R.L. and Shasha. B.S., 1989; Pusztai, M. et al., 1991). Reasons that have been suggested for the mechanism of photoinactivation include the destruction of tryptophan residues due to the production of singlet oxygen (Pozgay, M. et al., 1987; Pusztai, M. et al., 1991) and oxidation by peroxide radicals (Ignoffo, C.M. and Garcia, C., 1978).

The deleterious effects of UV radiation on biological systems, particularly proteins and nucleic acids, are well documented (McLaren and Shugar, 1964; Grossweiner, L.I., 1976; Grossweiner, L.I., 1984). It was recognized as early as 1936 that UV light could be used to inactivate viruses (Stanley, W.M., 1936). With the discovery that *Bacillus thuringiensis* crystals consists of DNA and protein, the possibility that the observed photoinstability is a result of photochemical crosslinking reactions occurring between the DNA and crystal protein components immediately became an attractive explanation for this phenomenon.

3.1.2 Rationale for the Present Investigation

The current studies were undertaken to determine the effect of UV irradiation on Bt crystals and the DNA-toxin complexes by monitoring the fate of the protein and DNA components after irradiation, in order to gain a better understanding of the probable cause for the photoinactivation.

3.2 Experimental Procedures

3.2.1 Materials

Preparation of *Bacillus thuringiensis* Crystals

Crystal preparations used for these studies were grown and harvested as previously described (Chapter 2) and purified by the GASTROgrafin® density centrifugation procedure (Milne, R. et al., 1977).

Solubilized Crystal Protein and DNA-Protein Complexes

Studies were undertaken using purified crystals and the DNA-protein complexes derived from the crystals obtained by the procedures previously described in Chapter 2.

UV Irradiation Instrumentation

Irradiation was accomplished using a germicidal lamp equipped with a 15 watt CX-50 BL long wave bulb producing typical peak 365 nm light (UV A- 2200$\mu W/cm^2$) and a 15 watt CX-50B medium wave bulb producing typical peak 302 nm light (UV B- 2100$\mu W/cm^2$) purchased from Spectroline. The power was measured using a Spectroline medium wave (UV B) ultraviolet meter model DM-300H. The power emitted at 302 nm was measured to be 0.6 mW/ cm^2. Measurements of natural light (the same day) at noon during a typical sunny day in late spring / early summer was measured at 0.2 mW/ cm^2 using the same meter.

3.2.2 Method

Irradiation Procedure

Irradiation experiments were carried out by exposing samples to UV-A (365 nm), or UV-B (302 nm) light for various time intervals as indicated in the results section. Samples of crystals and the derived DNA-protein complexes were irradiated by placing samples under the lamp of choice at a distance of 20 cm from the source.

Sample Preparation

Several different conditions for UV irradiation were employed. The various modes of sample preparation aimed to determine the influence of hydration and air (for the potential involvement of singlet oxygen) on the rate of inactivation. GASTROgrafin® purified crystals (or derivatives) stored in distilled water were assayed for protein concentration using the Bio-Rad Protein Assay method (Bradford, M., 1976). Equal aliquots containing approximately 100-250 μg of protein were transferred to appropriate vessels and prepared for irradiation as outlined below.

Irradiation of Air-Dried Bt Crystals

Equal aliquots containing approximately 100-250 μg of protein were pipetted onto ethanol-cleaned glass vessels over an area of approximately 2 cm^2. The aliquots of crystals or the derived DNA-protein complexes were air-dried and the crystals were irradiated for varying time intervals. The samples were recovered by washing glass plates with several washings with

distilled water. For analysis, the samples were adjusted to pH 10.5 by the addition of an appropriate aliquot of 1 M bicarbonate / carbonate or CAPS buffer prepared at pH 10.5.

Irradiation of Bt Crystals Conducted in Solution

Samples of Bt crystals (100 μg) suspended in water (pH 7) were prepared in a similar manner to the dry cross-linking samples, made up to the desired volume and transferred to the vessel for crosslinking. A small ethanol-cleaned magnetic stir bar was introduced into the sample to achieve constant stirring during irradiation. Quartz covers were introduced on the vessel, with a thin coating of vacuum grease along the perimeter of the vessel, in order to reduce evaporation during irradiation. The vessels were then mounted on a magnetic stirrer and irradiated. At predetermined time intervals, aliquots were drawn for the vessels and frozen with liquid nitrogen until all aliquots were collected. For analysis, the samples were adjusted to pH 10.5 by the addition of an appropriate aliquot of 1 M bicarbonate / carbonate or CAPS buffer prepared at pH 10.5.

Irradiation of Bt Crystals Under Reduced Pressure

Samples of Bt crystals (100 μg) were prepared as described (for solution crosslinking of samples) but transferred to glass tubes, to be sealed under vacuum, containing a small magnetic stir bar. The samples were then frozen with CO_2/ethanol and placed on a vacuum line. The samples were thawed and degassed several times prior to sealing under vacuum. Samples were mounted on a magnetic stirrer and irradiated for predetermined time intervals, at which time individual tubes were removed from under the UV lamp. The sample tubes were cracked and

the crystals recovered by centrifugation. For analysis, the samples were adjusted to pH 10.5 by the addition of an appropriate aliquot of 1 M bicarbonate / carbonate or CAPS buffer prepared at pH 10.5.

After irradiation and solubilization, the samples were analyzed by SDS-PAGE for the recovery of crystal protein. Control samples were prepared in parallel with the experimental samples to be irradiated and set aside as a nonirradiated control. Likewise, another aliquot of stored crystals from the same preparation, was set aside to determine the effect of the experimental conditions (reduced pressure), if any, on the samples.

Toxicity Assays

Toxicity assays were performed on samples prepared in the dry state in lawn assays using cultures of CF-1 cells (Gringorten, J.L., et al., 1990). 7 x 0.5 ml aliquots (approximately 200 μg protein) of a crystal suspension were centrifuged at 15 krpm for 1 minute and the supernatant discarded. The crystals were further washed with 1 ml dH_2O, centrifuged at 15 krpm for 1 minute and resuspended in 295 μl dH_2O. Samples were spread on glass plates, air dried and irradiated for 5 minutes, 30 minutes, 1, 2, 4 and 8 hours respectively. These samples were then sent to Sault Ste. Marie for toxicity assays. Each plate was rinsed with 4 x 250 μl dH_2O and volumes made up to 2 mls. Samples were then sonicated on ice (2 sec on 5 sec off) and 250 μl of each incubated in 250 μl 0.2 M CAPS at pH 10.5 in the presence of 4 mg / ml bovine trypsin overnight at room temperature. The samples were then centrifuged at 10 krpm for 10 minutes and protein concentrations were determined by Bio-Rad protein assay along with

a trypsin blank to correct for the trypsin present in the samples. Appropriate dilutions were prepared for ranging assays on CF-1 cells and for threshold response on CF-1 cells.

DNA Extractions

DNA was extracted from the DNA-protein complexes using the same procedure described in Chapter 2 (Sambrook, J. et al., 1989; Bietlot, H.P. et al., 1993).

3.3 Results

Protein Recovery after Irradiation

The total recovery of protein from air-dried Bt crystals (pH 7), prior to and after irradiation, was determined using the Bio-Rad protein assay procedure (Bradford, M. 1976) and the results are shown in Figure 3.1. The amount of crystal protein that is recovered, after irradiation at 302 nm, decreases rapidly with increased exposure. For irradiation times longer than 240 minutes (4 hours), no protein could be recovered or was below the limits of sensitivity of the assay (approximately 5 μg protein); no recoverable protein could be detected for a sample irradiated for 480 minutes. As the irradiation time is increased, the protein samples form films / crusts which are extremely insoluble and resistant to proteolytic and nucleolytic digestion.

Irradiated samples were also analyzed by SDS-PAGE as shown in Figure 3.2. After irradiation at 302 nm for 8 hours, no detectable protein can be seen on SDS-PAGE by attempting to solubilize the crystals. These results are consistent with the results obtained for the toxicity assays described above.

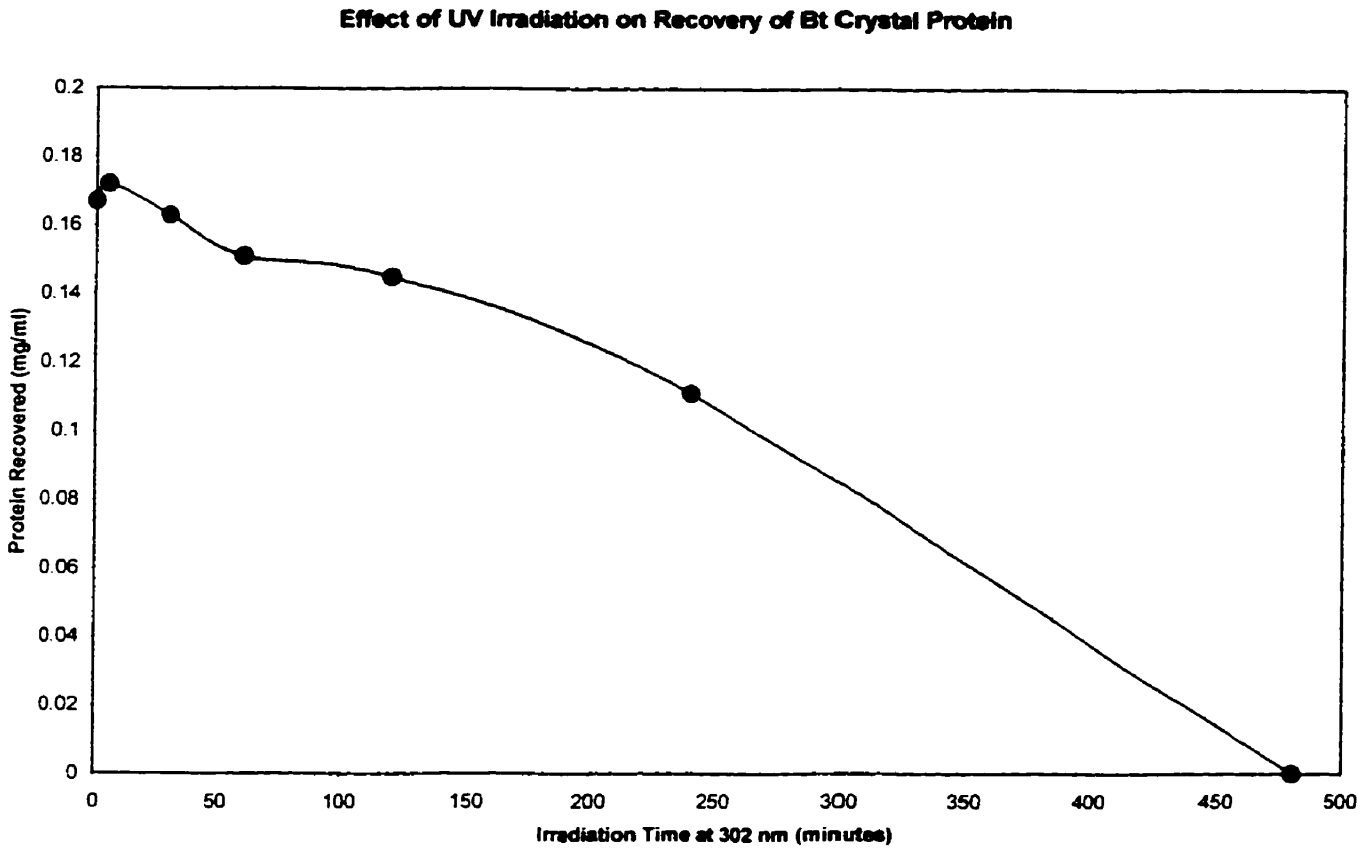


Figure 3.1 Effect of UV Irradiation at 302 nm on the Recovery of Bt Crystal Protein

Air dried Bt crystals (200 μg crystal protein) were irradiated at 302 nm for up to 480 minutes. The crystals were treated with trypsin overnight and subsequently assayed for protein recovery using the same procedure described in the experimental procedures (Bradford method).

DNA Extractability

The extractability of the DNA from irradiated crystals and control samples, was determined by agarose gel (0.9%) electrophoresis. The results are shown is Figure 3.3. The DNA associated with the crystals is not extracted effectively even after drying of the crystals samples. The DNA that is extracted even after drying does not migrate into the gel, indicating that the DNA has become aggregated in some way. Following irradiation, the DNA is not extracted even after as little as 5 minutes. As noted above, the irradiated material becomes highly insoluble and this insolubility increases rapidly with the length of exposure.

Toxicity Assays

Toxicity assays were conducted on control (nonirradiated) and irradiated crystal samples which had been irradiated for upto 240 minutes. The results of assays conducted on CF-1 cell lines from spruce budworm, *Choristeneura fumiferana*, are shown in Figure 3.4.

The threshold dose, in nanograms, required for 50 % mortality in spruce budworm (LD_{50}), was estimated on the basis of lysis of CF-1 cells. The threshold dose can be seen to increase rapidly with increased UV exposure.

Generation of Toxin From Irradiated Crystals

Attempts to generate toxin from air dried crystals, irradiated for up to 8 hours at 302nm, demonstrate that toxin cannot be generated within 8 hours irradiation, with levels decreasing significantly within the first few hours as shown in Figure 3.5.

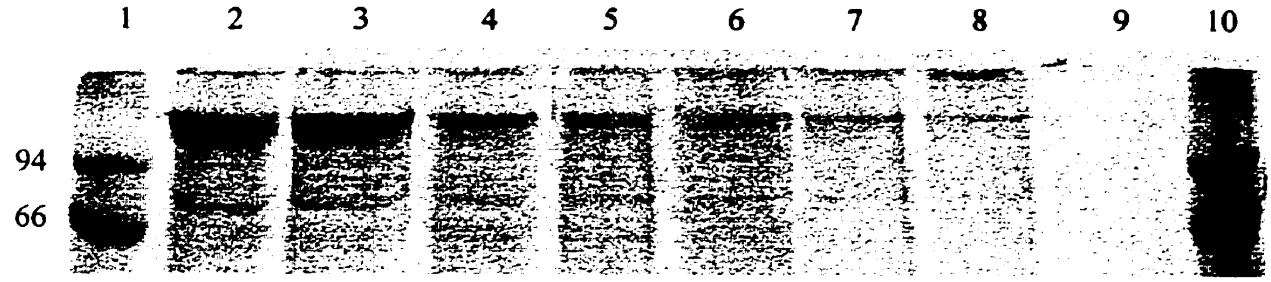


Figure 3.2 SDS-PAGE of Air-Dried Bt Crystals Irradiated at 302 nm

Air dried Bt crystals (100 μg) were irradiated at 302 nm for upto 8 hours and analyzed by SDS-PAGE for protein recovery. Lanes 1 and 10 are molecular weight markers; Lane 2, Non-irradiated, non-dried crystals; Lane 3, Non-irradiated, air dried crystals; Lane 4, 5 minutes; Lane 5, 30 minutes, Lane 6, 1 hour; Lane 7, 2 hours; Lane 8, 4 hours; Lane 9, 8 hours.

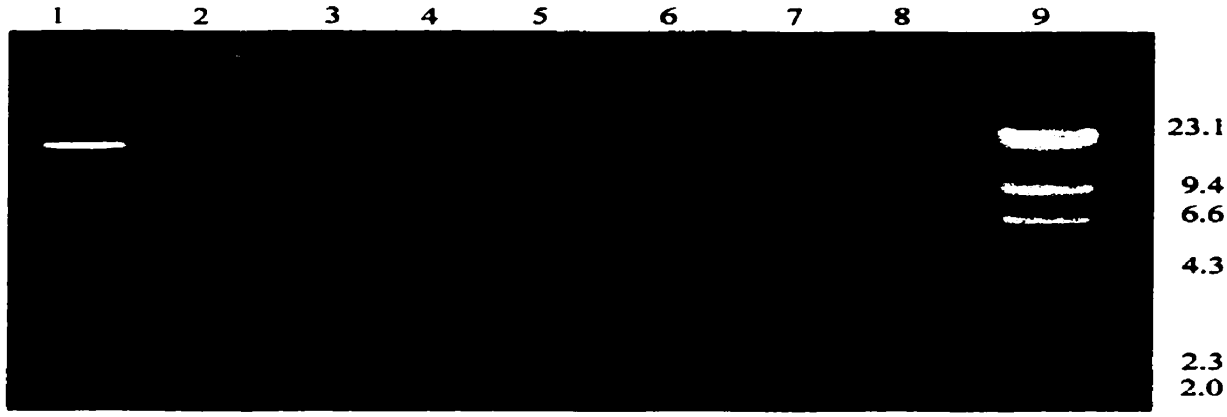


Figure 3.3 DNA Extracted from Bt Crystals After Irradiation at 302 nm

Air dried Bt crystals (100 μg) were irradiated at 302 nm for upto 8 hours and the DNA was extracted and analyzed by agarose gel electrophoresis. Lane 1, Non-irradiated, non-dried crystals; Lane 2, Non-irradiated, air dried crystals; Lane 3, 5 minutes; Lane 4, 30 minutes, Lane 5, 1 hour; Lane 6, 2 hours; Lane 7, 4 hours; Lane 8, 8 hours, Lane 9, λ-Hind III molecular weight markers.

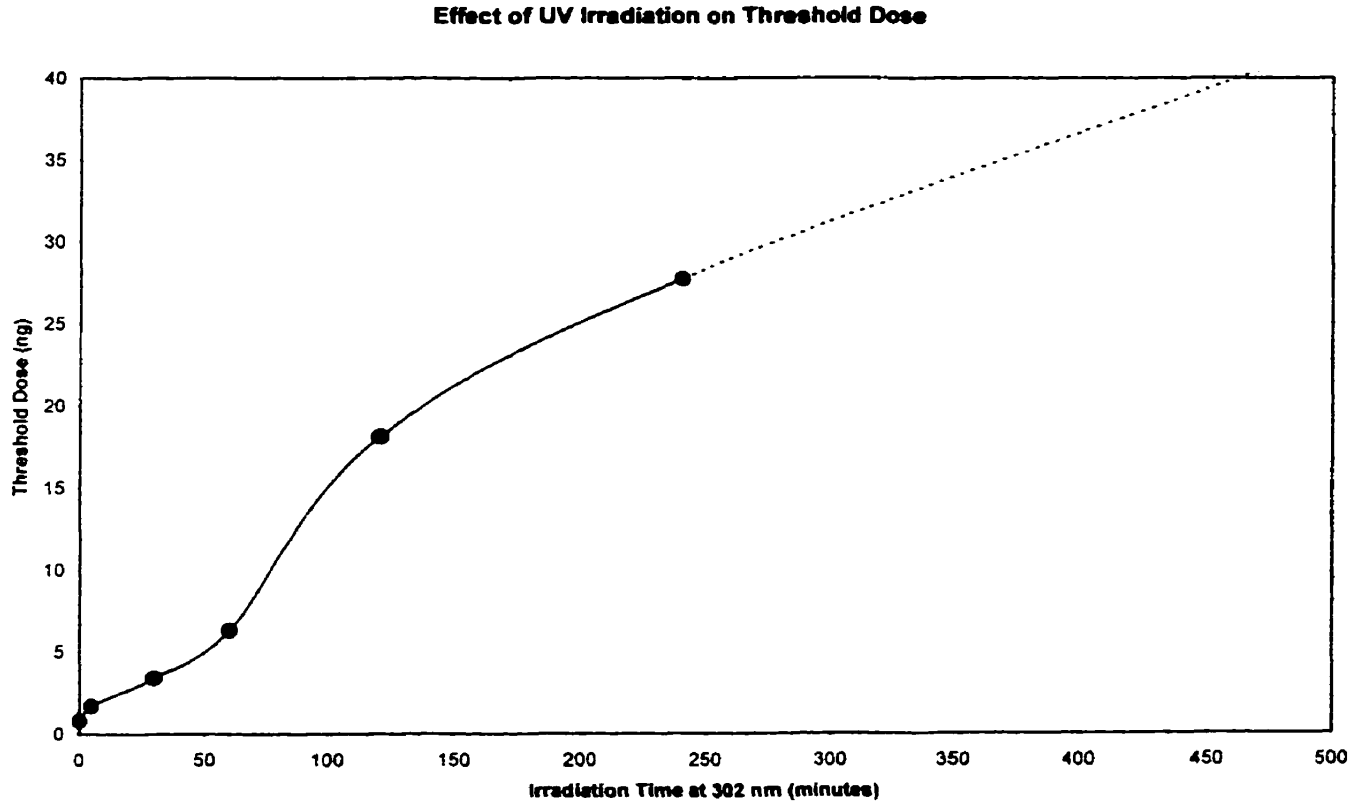


Figure 3.4 Effect of Irradiation on the Insecticidal Activity of Bt Crystals

Bt crystals (200 μg) were irradiated for up to 480 minutes at 302 nm and assayed for toxicity using CF-1 cell line lawn assays as described in the experimental procedure. The threshold dose (ng) is shown as a function of UV irradiation time.

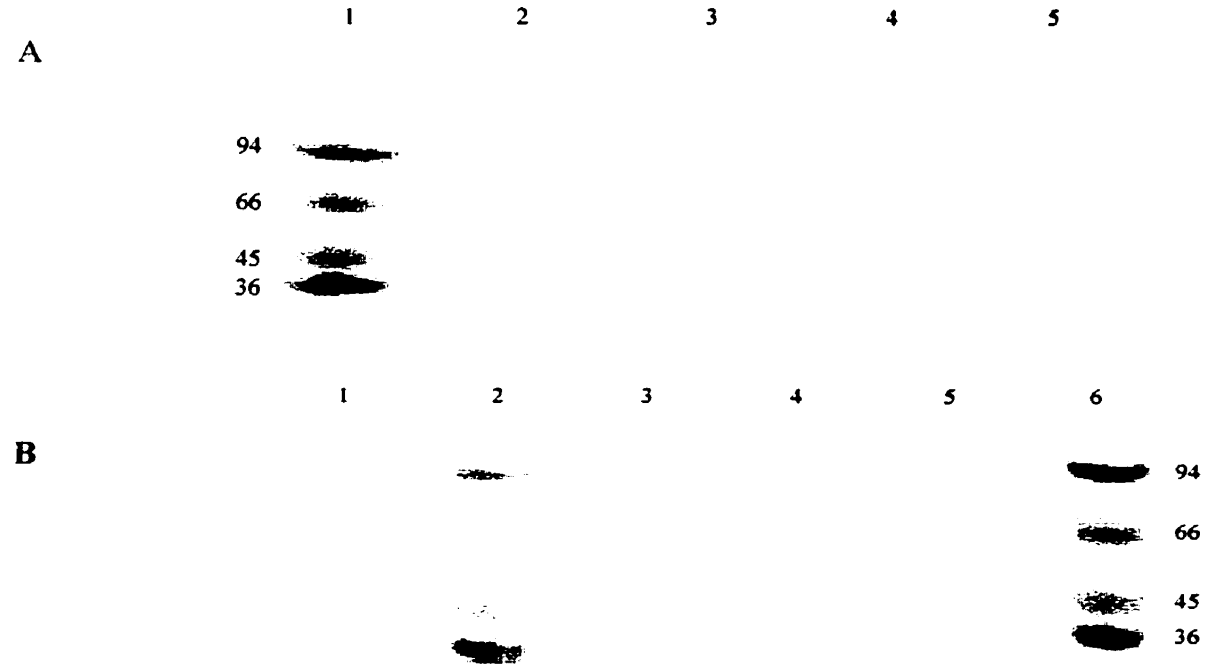


Figure 3.5 Generation of Toxin from Bt Crystals Irradiated at 302nm

The ability to generate toxin from irradiated Bt crystals was determined after irradiation at 302 nm for upto 8 hours. Irradiated crystals were treated with trypsin (1:20 trypsin to crystal protein) for 4 hours at 37°C in 0.1 M carbonate/bicarbonate buffer (pH 10.5).

Gel A: Lane 1; molecular weight markers; Lane 2, Non-irradiated, non-dried crystals; Lane 3, Non-irradiated, air dried crystals; Lane 4; 5 minutes; Lane 5, 30 minutes.

Gel B: Lane 1, 1 hour; Lane 2, molecular weight markers; Lane 3; 2 hours; Lane 4, 4 hours; Lane 5, 8 hours; Lane 6; molecular weight markers.

UV Inactivation of 20-kbp DNA-Toxin Complex and of Purified Toxin

200 μg of the 20-kbp DNA-toxin complex (prepared as described in Chapter 2) was dried on glass plates and irradiated at 302 nm for upto 8 hours and analyzed for protein recovery by SDS-PAGE analysis. The results are shown in Figure 3.6. Loss of the DNA-toxin complex is complete within approximately 4 hours, which is consistent with the loss of crystal protein within the same time interval. FPLC-purified DNA-free toxin (250 μg) was subjected to UV radiation in solution (pH 7.5) at 302 nm for upto 4 hours. SDS-PAGE analysis, shown in Figure 3.7, revealed that the toxin is apparently also affected by UV irradiation. No toxin is recovered after 4 hours irradiation.

UV Inactivation of Bovine Serum Albumin

BSA (250 μg) was subjected to UV radiation in solution at 302 nm for upto 4 hours. SDS-PAGE analysis, shown in Figure 3.8, revealed that BSA is much less affected by UV irradiation than Bt toxin, with only a small decrease in the recovery of BSA being noted after 4 hours irradiation.

UV Inactivation of Bt Crystals at 365 nm

The UV irradiation of Bt crystals (250 μg) in the dry state was also conducted at 365 nm to determine the effect of UVB radiation on the extent of inactivation. The results for inactivation at 365 nm are shown in Figure 3.9; compare with Figure 3.2 for inactivation at 302 nm. The apparent loss of crystal protein observed by SDS-PAGE analysis shows that the rate of

inactivation increases with decreasing wavelength. At 302 nm (Figure 3.2), the crystal protein cannot be recovered after 4 hours irradiation, however crystal protein can still be recovered after 8 hours irradiation at 365 nm, although a decrease in crystal protein recovery is also noted with increased irradiation time.

UV Inactivation of Bt Crystals in Solution

In order to assess the effect of hydration on the inactivation, a sample of Bt crystals (100 μg) was irradiated without prior drying. SDS-PAGE analysis was used to assess the photo-inactivation of crystals irradiated for upto 8 hours. The results are shown in Figure 3.10. The results obtained suggest that the inactivation occurs within approximately the same time interval as for the dried crystal samples. However, comparison of the control sample (neither dried nor irradiated) and the first aliquot drawn (5 min) shows little difference in the extent of recovery of the crystal protein. These results, when compared to irradiation experiments conducted in the dried state, clearly demonstrate the loss of protein recovery as a result of drying of the crystals. A significant initial decrease in the recovery of crystal protein can therefore be attributed to drying out of the Bt crystals.

UV Inactivation of Bt Crystals in Solution Under Reduced Pressure

To determine the effect of the presence of oxygen of the extent of photo-inactivation, samples of Bt crystals were delivered to individual disposable culture tubes, degassed and irradiated for upto 8 hours. The results of SDS-PAGE analysis are shown in Figure 3.11. Once again, photo-inactivation was observed, however there appears to be a slight decrease in the rate

at which the inactivation occurs. The extent of reduction of the inactivation does not appear to be significantly affected.

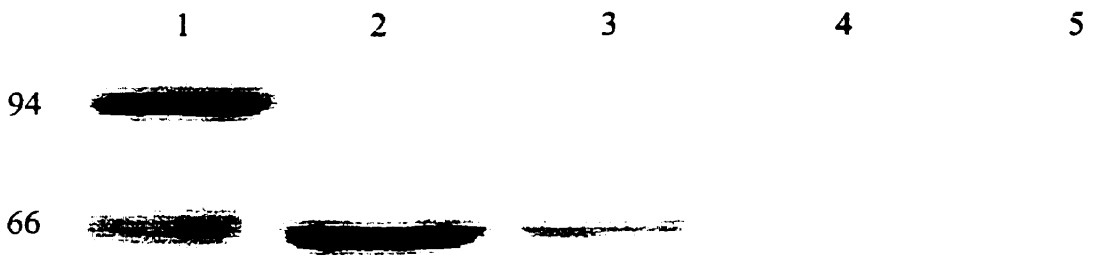


Figure 3.6 UV Inactivation of 20-kbp DNA-Toxin Complex

200 μg of 20-kbp DNA-toxin complex were air dried in glass vessels and irradiated at 302 nm for up to 8 hours. Aliquots were drawn at various time intervals and analyzed by SDS-PAGE. Lane 1, Molecular mass markers; lane 2, no irradiation, 0 hours; lane 3, 2 hour; lane 4, 4 hours; lane 5, 8 hours.

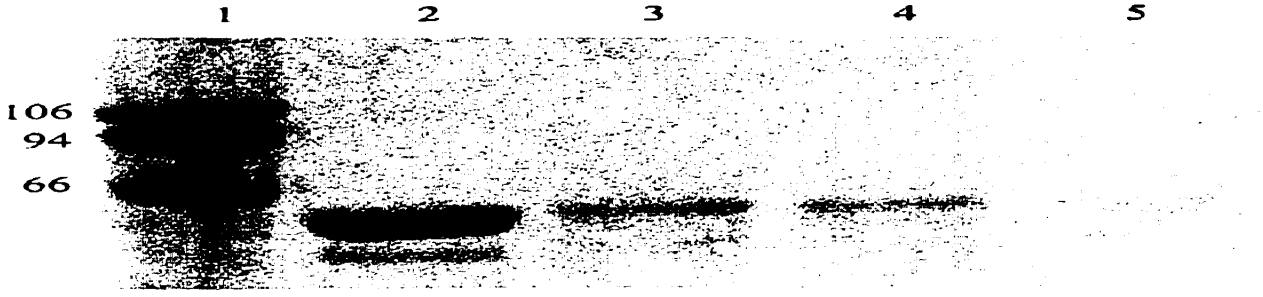


Figure 3.7 UV Inactivation of FPLC Purified Toxin

250 μg DNA-free toxin (T1) was dissolved in 50 mM phosphate buffer (pH 7.5), 20 mM EDTA, and irradiated at 302 nm for up to 4 hours. Aliquots were drawn at various time intervals and analyzed by SDS-PAGE. Lane 1, Molecular mass markers; lane 2, no irradiation, 0 hours; lane 3, 1 hour; lane 4, 2 hours; lane 5, 4 hours.

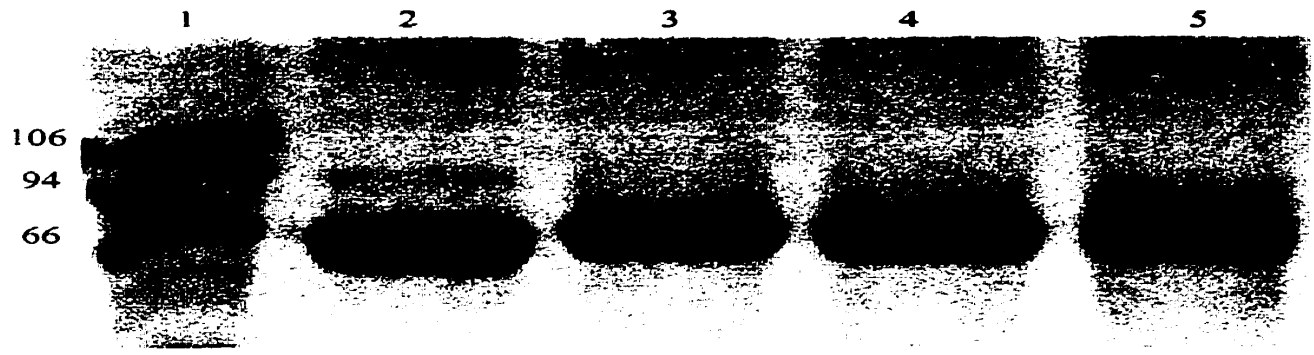


Figure 3.8 UV Inactivation of Bovine Serum Albumin

250 μg BSA was dissolved in 50 mM phosphate buffer (pH 7.5), 20 mM EDTA, and irradiated at 302 nm for up to 4 hours. Aliquots were drawn at various time intervals and analyzed by SDS-PAGE. Lane 1, Molecular mass markers; lane 2, no irradiation, 0 hours; lane 3, 1 hour; lane 4, 2 hours; lane 5, 4 hours.

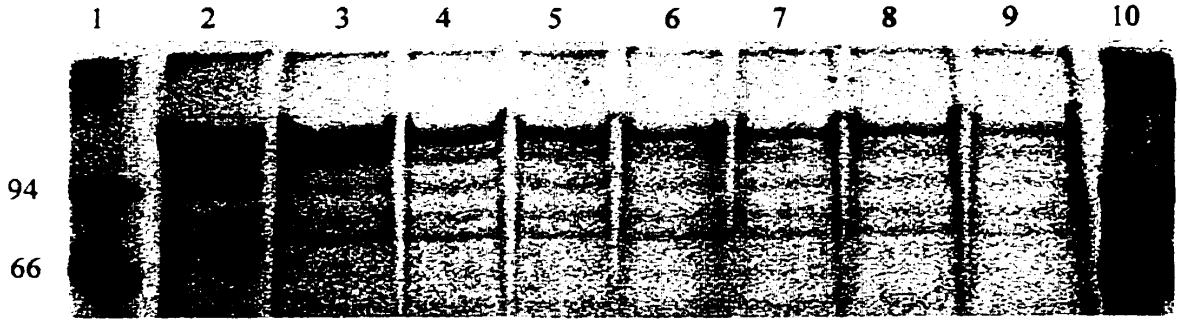


Figure 3.9 UV Inactivation of Bt Crystals at 365 nm

Air dried Bt crystals (100 μg) were irradiated at 365 nm for upto 8 hours and analyzed by SDS-PAGE for protein recovery. Lanes 1 and 10 are molecular weight markers; Lane 2, Non-irradiated, non-dried crystals; Lane 3, Non-irradiated, air dried crystals; Lane 4, 5 minutes; Lane 5, 30 minutes, Lane 6, 1 hour; Lane 7, 2 hours; Lane 8, 4 hours; Lane 9, 8 hours.

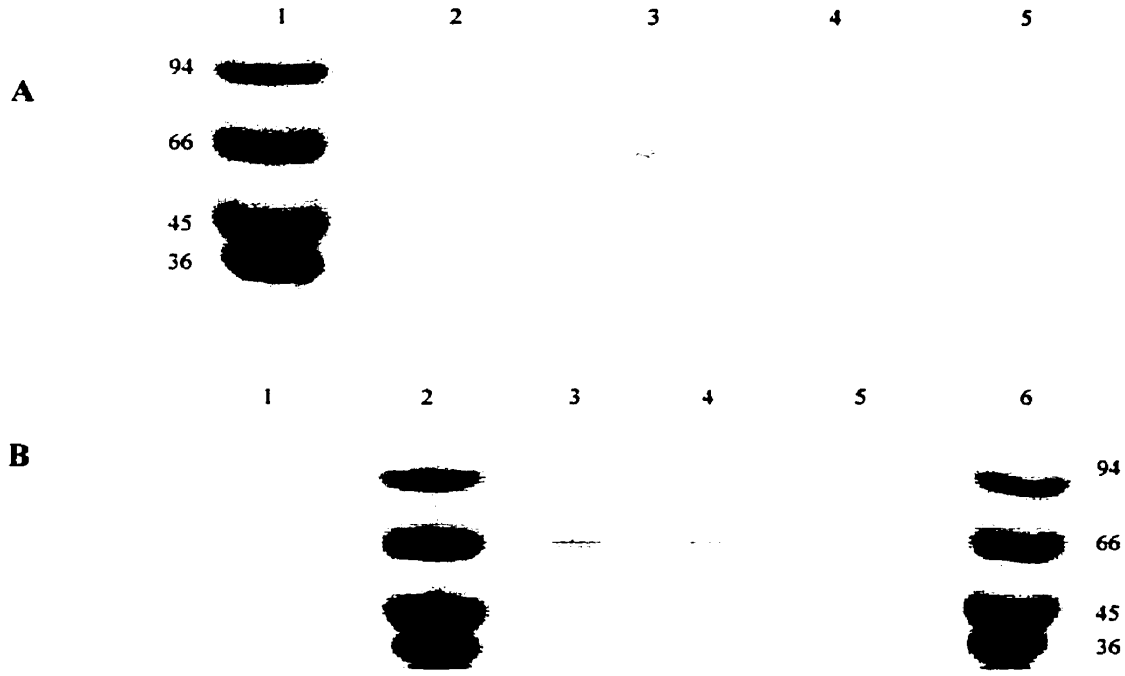


Figure 3.10 Generation of Toxin from Bt Crystals Irradiated at 365nm

The generation of toxin from Bt crystals was determined after irradiation at 365 nm for upto 8 hours. Control and irradiated crystal samples were treated with trypsin (1:20 trypsin to crystal protein) for 4 hours at 37°C in 0.1 M carbonate/bicarbonate buffer (pH 10.5).

Gel A: Lane 1; molecular weight markers; Lane 2, Non-irradiated, non-dried crystals; Lane 3, Non-irradiated, air dried crystals; Lane 4; 5 minutes; Lane 5, 30 minutes.

Gel B: Lane 1, 1 hour; Lane 2, molecular weight markers; Lane 3; 2 hours; Lane 4, 4 hours; Lane 5, 8 hours; Lane 6; molecular weight markers.

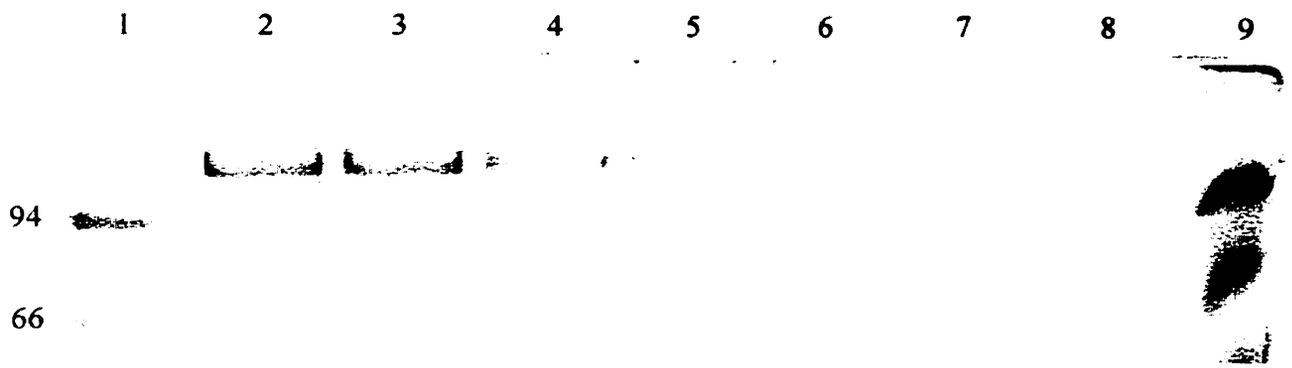


Figure 3.11 UV Inactivation of Bt Crystals in Solution

Bt crystals (100 μg) were irradiated in solution (pH 7) at 302 nm for upto 8 hours and analyzed by SDS-PAGE for protein recovery. Lanes 1 and 10 are molecular weight markers; Lane 2, Non-irradiated, non-dried crystals; Lane 3, Non-irradiated, air dried crystals; Lane 4, 5 minutes; Lane 5, 30 minutes, Lane 6, 1 hour; Lane 7, 2 hours; Lane 8, 4 hours; Lane 9, 8 hours.

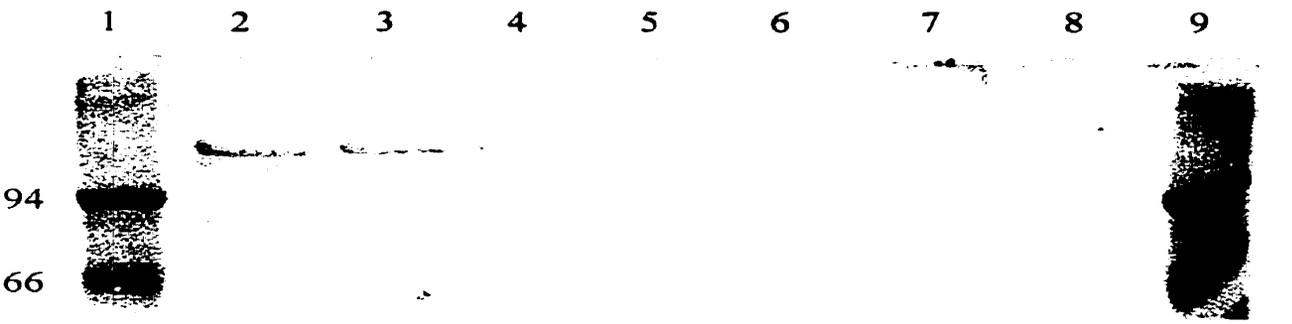


Figure 3.11 UV Inactivation of Bt Crystals in Solution Under Reduced Pressure

Bt crystals (100 μg) were irradiated in solution under reduced pressure at 302 nm for upto 8 hours and analyzed by SDS-PAGE for protein recovery. Lanes 1 and 9 are molecular weight markers; Lane 2, Non-irradiated crystals; Lane 3, 5 minutes; Lane 4, 30 minutes, Lane 5, 1 hour; Lane 6, 2 hours; Lane 7, 4 hours; Lane 8, 8 hours.

3.4 Discussion

The photo-inactivation of the Bt formulations has been recognized for some time, however none of the attempts to increase the photostability of the formulations (encapsulation, incorporation of UV sunscreens) have been successful. The photo-instability of microbial insecticides have been noted for many such products that involve the use of whole cells; the use of viruses and bacteria suffers from a lack of field persistence, in which one of the most important and uncontrollable factors is sunlight inactivation (Bedford, G.O., 1981; Ignoffo, C.M., et al., Couch,T.L. et al., 1981).

The results obtained in the current studies not only confirm the photo-instability of the Bt crystal, but also suggest that this phenomenon may be, at least partially, the result of photochemical crosslinking of the DNA and protein components which make up the crystals. The disappearance of the protein on SDS gels, as well as the accumulation of high molecular weight material in the stacking gel, and combined with the non-extractability of the DNA, suggest that covalent bonds are being formed between the crystal components as a direct result of exposure to UV radiation.

Irradiation experiments conducted on FPLC-purified, DNA-free toxin, revealed that the toxin is also sensitive to UV exposure. Control experiments, conducted on BSA (bovine serum albumin), at the same concentration and wavelength (302 nm), indicate that BSA is less sensitive to UV radiation than the Bt toxin. These results indicate that a second process, besides the proposed DNA-protein crosslinking, may also play a role in the photo-inactivation of the crystal protein. However, the sensitivity of the Bt toxin to UV radiation may also indicate that the FPLC purified toxin was not entirely free of DNA.

Irradiation experiments were also conducted in solution in order to determine the effect of desiccation of the crystals. The results obtained for irradiations conducted in solution, when compared to the inactivation experiments conducted in the dried state, indicate a significant contribution to the loss of crystal protein caused by drying of the crystals. The initial decrease in crystal protein recovery upon drying (lanes 2 and 3 of SDS-PAGE gels for experiments conducted in the dry state) and prior to irradiation, confirm earlier reports of the loss of toxicity as a result of drying of the Bt crystals (Leong, K.L. et al., 1980).

Irradiation experiments conducted in solution under reduced pressure, aimed to determine the effect of molecular oxygen (and the potential role of singlet oxygen) on the inactivation of the crystals. The results obtained from these experiments indicate that the contribution of oxidation by molecular oxygen, as a causative agent of inactivation, is less significant than anticipated. The rate of loss of crystal protein in these samples, as observed from SDS-PAGE, appears to be approximately the same as that observed for irradiation experiments conducted in solution without degassing.

Finally, irradiation experiments using 302 and 365 nm UV light were conducted to determine the extent of damage occurring at different wavelengths. The results demonstrated that the photo-induced damage is also related to the wavelength of UV light. Irradiation of crystals at 365 nm is insufficient to cause complete photoinactivation of the crystals within 8 hours, in contrast to the much more rapid inactivation observed when crystals are irradiated at 302 nm. Furthermore, Figure 3.10 A and B also demonstrates that toxin can still be generated by trypsin treatment for samples irradiated for as much as 8 hours at 365 nm. The residual absorption of the nucleotides of DNA in the UVA region, although seemingly insignificant, may

be sufficient to produce excited states in the DNA causing reactions to occur between the DNA and crystal protein. Most of the DNA-protein crosslinking experiments have been conducted using UVC radiation, particularly in the range of 254 to 266 nm, where DNA displays its largest molar absorptivity. However, in the present studies, irradiation with UVB light is also capable of causing photo-inactivation of the Bt protein-DNA complex. Pusztai et al. (1991) reported that the toxicity of Bt crystals decreases with decreasing wavelength, with the greatest losses of toxicity occurring for irradiations conducted below 380 nm. UV irradiation of Bt crystals at 254 nm (UVA) was found to occur even more rapidly than at 302 nm (data not shown), showing a direct correlation between the rate of inactivation and wavelength of irradiation; as expected, the order of inactivation increases with increasing energy of irradiation.

The insolubility of the crystals following irradiation suggested that the toxicity of *Bacillus thuringiensis* crystals might be affected by UV exposure. To test this hypothesis, samples were prepared for toxicity assays against cell cultures of the spruce budworm Choristoneura fumiferana (CF-1 cells). The results shown in Figure 3.4 clearly demonstrate that the threshold dose required for toxicity increases dramatically with increased exposure of the crystals to UV light. The results show that the threshold dose increases by approximately 30-fold within the first 4 hours when irradiated in the dry state. Threshold doses for crystals irradiated for longer periods (ie: 8 hours), could not be estimated due to the extreme insolubility of the crystals after prolonged UV exposure. Thus, the most probable explanation for the reduced toxicity of the crystal protein upon irradiation was that the protein could no longer be enzymatically converted to the toxin by protease treatment. In order to support this possibility, crystals were irradiated for various time intervals and

subsequently treated with trypsin in an attempt to generate and recover toxin (SDS-PAGE Coomassie-positive staining) from the irradiated crystals. The results indicate that the crystals are no longer susceptible to enzymatic hydrolysis after exposure to UV light for 8 hours. This is consistent with the observed toxicity data presented above. The high degree of insolubility and the inability to generate detectable quantities of toxin from the irradiated crystals after 8 hours irradiation, provide further evidence for the formation of covalent protein-DNA linkages.

3.5 Conclusion

The proteinaceous crystals produced by *Bacillus thuringiensis* have been shown to be biologically inactivated as a result of exposure to UV radiation, which was confirmed by the results of toxicity assays. The loss of activity is related to drying, the wavelength of UV radiation and length of exposure, and appears to be the result of formation of insoluble, protease-stable high molecular weight matrices. A large contribution to the reduced recovery of soluble crystal protein appears to be solely due to initial drying of the crystals.

The loss of proteolytic susceptibility of the crystal protein after irradiation may be due to the covalent crosslinking of the protein and DNA components of the crystal. Monitoring the fate of the protein and DNA components of the crystal has provided strong evidence for the formation of covalent linkages between these components, however the formation of both DNA-DNA and protein-protein products cannot be ruled out.

Chapter 4

Protection of *Bacillus thuringiensis* Crystals From UV Photo-Inactivation

4.1.1 Introduction

The deleterious effects of UV radiation on biological systems and its reported involvement in a number of pathological conditions in humans are well documented. Prolonged exposure to UV radiation has been shown to affect the growth and viability of bacterial cell cultures (Smith, K.C., 1962, Jagger et al., 1964), to be responsible for the inactivation of viruses (Stanley, W.M., 1936), for destruction of antigenicity of viruses (Kleczkowski, A., 1962) and for the induction of structural alterations of protein and lipid oxidation in cells (Kochevar, I., 1990), thereby demonstrating its lethal qualities.

Photochemical reactions induced in biologically important compounds, particularly nucleic acids and proteins, as a result of UV irradiation have been studied extensively. It is generally accepted that DNA is the most sensitive biological target for UV damage (McLaren and Shugar, 1964, Kornhauser, A., 1976), and that the photochemical reactions between DNA and proteins are a consequence of the photochemical excitation of nucleic acids. In biological systems, these excited states can initiate primary reactions among nucleic acid components, to yield various products such as thymidine dimers (Chapman, O.L., 1967), or react with DNA-associated proteins such as histones (Angelov, D. et al., 1988) and nuclear lamina proteins (Galcheva-Gargova, Z. and Dessev, G.N., 1987). Evidence for the covalent attachment of nucleic acids to proteins has been obtained by terminal labelling of the protein-linked DNA after irradiation (Galcheva-Gargova, Z. and Dessev, G.N, 1987), the retention of radioactivity (Angelov, D. et al., 1988), from gel shift assays (Buckle et al., 1991) and by mass spectrometry

(Jensen et al., 1993), however the chemical structures of these covalent compounds have yet to be elucidated.

Studies conducted on the photoaddition products of nucleic acid bases (and/or polynucleotides) with amino acids (and/or peptides or proteins) suggest the formation of free radicals in the nucleic acid components, which then react with the amino acid or protein component of the system (Smith and Alpin, 1966; Jellinek and Johns, 1970; Smith, K.C. 1970; Varghese, A.J., 1973a; Varghese, A.J., 1973b). In nucleic acids, the pyrimidines have been shown to be the most photochemically reactive of the nucleic acid bases. Thymine in DNA and uracil in RNA, have been shown to form a variety of photoproducts with different amino acids, with the proposed formation of free radicals. These topics will be addressed in Chapter 5.

UV irradiation of biological systems, particularly in the presence of photosensitizers and molecular oxygen, has also been implicated in a number of photo-oxidation reactions in macromolecules. Singlet oxygen, which can be produced during the photosensitization process from the triplet state of molecular oxygen (ground state), has been implicated in the photo-oxidation of amino acids, lipids and nucleosides and other cellular components. Photo-oxidation has been observed in virtually all classes of organisms, with effects ranging from interference with metabolic processes and reproduction, to membrane damage and mutagenesis. The photo-oxidation products of thymine and guanine in DNA and of uracil and uric acid derivatives have been identified, and the amino acids histidine, methionine, tryptophan, tyrosine and cysteine have been shown to be affected both as the free monomers and when found in peptides (Foote, C.S., 1976; Henriksen, et al., 1976a and b).

Fortunately, the ubiquitous presence of antioxidants in biological systems, the presence of lipid-soluble antioxidants in biomembranes and water-soluble antioxidants in the intracellular fluid of cells, are believed to limit the destruction of important biological molecules (Roberfroid and Calderon, 1995). The isolation of Vitamin C (ascorbic acid) in 1922 by Albert Szent-Gyorgyi was one of the first breakthroughs in our understanding of antioxidants and biological oxidation processes (Buettner, G.R. et al., 1996).

Antioxidants can be broadly defined as any molecule that protects a target against oxidative damage (Halliwell, B., 1996). They can also be coarsely divided into two main categories according to solubility; namely hydrophilic or lipophilic. These are further classified as preventive, chain-breaking, or repair antioxidants, depending on the mode of action. Vitamin C and Vitamin E are classical chain-breaking antioxidants, or radical scavengers, in that they interact directly with free radical species, suppressing the initiation and propagation steps leading to chain reactions (Niki, E., 1996). Both Vitamins C and E donate electrons, in the form of hydrogen atoms (AH to $A^{\cdot}$), to the free radical species, to form the resulting antioxidant radicals, which are stable and terminate radical propagation (Roberfroid and Calderon, 1995, Buettner, G.R. et al., 1996, Niki, E., 1996).

In-vitro studies of putative biological antioxidants have confirmed the antioxidant capabilities of both water-soluble compounds, particularly Vitamin C, as well as lipid-soluble compounds such as Vitamin E (Burton, G.W. and Ingold, K.U., 1981; Barclay, L.R.C. and Ingold, K.U., 1981; Wayner et al., 1986; Wayner et al., 1987; Singh et al., 1990; Niki, E., 1996).

Antioxidants are also used in the food industry to reduce the oxidative damage to foodstuffs (rancidity of fats and oils) and have also been incorporated into the packaging materials in order to retain freshness. The antioxidants used in the food industry, in the protection of oxidative damage include BHT (butylated hydroxytoluene) and propyl gallate, which can act as chain-breaking antioxidants as well as scavengers of initiating radicals (Halliwell and Gutteridge, 1990).

Non-biological polymers also require the addition of antioxidants to retain their physical properties and to acquire a reasonable life expectancy (Dexter, M., 1992). A number of synthetic compounds, possessing antioxidant and antiozonant activities, are used in the protection of organic polymers from UV and oxidative damage. These are used to reduce the development of colour (polyolefins), protect from aging and cracking (elastomers), and the formation of gums and deposits (fuels and lubricants).

4.1.2 Rationale for the Current Investigations

The UV-induced photo-inactivation of *Bacillus thuringiensis* crystal DNA-protein complex(es) represents yet another example of the deleterious effects of UV irradiation in biological systems. It also provides a model system to study the mechanism of UV-induced DNA-protein crosslinking. The photo-instability of *Bacillus thuringiensis* products has been previously investigated by other researchers, however no satisfactory explanation for this phenomenon has been presented. The cause of the photo-inactivation has been suggested to be

the result of photosensitized destruction of aromatic amino acid degradation by singlet oxygen (Pozgay, M. et al., 1987, Putzai, M., et al., 1991) and oxidation by peroxide radicals (Ignoffo, C.M. and Garcia, C., 1978). As mentioned in the previous chapter, attempts at increasing the field persistence of Bt formulations have had very limited success.

The current investigations aimed to determine the efficacy of water-soluble and lipid-soluble antioxidants in preventing the photo-inactivation and to gain a better understanding of the nature of the reactions responsible for the UV-induced photo-inactivation of Bt crystals.

4.2 Experimental Procedures

4.2.1 Materials

Chemicals and Enzymes

L-Ascorbic acid (Vitamin C) and Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Aldrich Chemical Company. Propyl gallate, PABA (p-aminobenzoic acid), BHA (butylated hydroxyanisole), cysteine and histidine, uric acid and EDTA were purchased from Sigma Chemical Co. BHT (butylated hydroxytoluene) was purchased from ICN Pharmaceuticals Inc. Bovine pancreatic trypsin was obtained as a lyophilized powder from Sigma.

Bacillus thuringiensis Crystals and Related DNA-Protein Complexes

Bacillus thuringiensis subsp. *kurstaki* HD-73 and the derived DNA-protein complexes were prepared as described previously.

Irradiation Instrumentation

Irradiation experiments were conducted using a germicidal lamp equipped with a 15 watt CX-50B lamp producing typical peak 302 nm light with an intensity of 2100 microW/cm^2 unless specified in the results.

4.2.2 Method

Sample Preparation

Experiments were conducted in the dry state and in solution as reported in the results. Antioxidant stock solutions (0.5-1 M) were prepared for the water-soluble compounds and the pH of the antioxidant stock solutions were adjusted just prior to addition to the Bt samples. The final concentration of antioxidant in the samples was adjusted by dilution of aliquots of the stock solutions. For lipophilic antioxidants, solutions of aqueous / organic mixtures were prepared and added to the crystal protein or derived DNA-protein complexes.

Antioxidants/ Sunscreens

Initial studies were performed on the following antioxidants and U.V. protectants: Vitamin E (dl-alpha-tocopherol), BHT, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), propyl gallate, uric acid, Vitamin C (ascorbic acid), PABA (para-aminobenzoic acid), cysteine, histidine and EDTA. Aliquots of solutions of these compounds were added to the crystals or DNA-protein complexes to achieve the desired final concentration, and the samples were left to air-dry on plates before irradiation.

Experiments performed in solution were prepared in quartz-covered vessels, to reduce evaporation, and aliquots were drawn at the predetermined time points.

Irradiation Procedure

From the results obtained in Chapter 3, it was pre-established that 8 hours irradiation was sufficient to cause total loss of crystal protein (protoxin) by SDS PAGE analysis. Therefore, for most of the studies, a maximum of 4 hours irradiation was employed. Irradiation of samples for longer intervals were only performed for specific applications, as described in the results, with irradiation times ranging form 0 to 48 hours as required.

Ammonium Persulfate Treatment of Bt Samples

Protoxin samples (pH 10.5) were stirred in the presence of ammonium persulfate (0.017%) and aliquots withdrawn at predetermined time points up to 24 hours. Two sets of samples were prepared 1) samples containing Vitamin C (0.1 M) and 2) no Vitamin C added.

Toxin Generation from Irradiated Bt Samples

Irradiated samples were incubated with trypsin (1:20 trypsin / protein (m/m)) at 37°C for 4 hours and aliquots of the digests were analyzed by SDS-PAGE (10%).

4.3 Results

Lipophilic Antioxidants

Vitamin E, BHT, Trolox

The poor solubility or insolubility of these antioxidants in aqueous solutions made it necessary to investigate the maximum solubility that could be achieved for mixtures of water-miscible organic solvents and water. Solutions of these antioxidants were prepared in organic solvents (THF, 1,4-dioxane, isopropanol or pyridine) which were combined with aqueous solutions and the solubility determined. The results of these solubility studies indicated that further studies were not feasible since preincubation of crystals in solutions containing 10% (w/v) Vitamin E (20% H_2O / 70% pyridine), 10% (w/v) BHT in isopropanol (40% H_2O / 60% isopropanol) and of 5% (w/v) Trolox in isopropanol (40% H_2O / 60 % isopropanol), gave no recovery of protein when analyzed by SDS-PAGE. These crystal solutions were noticeable flocculent indicating protein denaturation, due to the presence of the organic solvents, and thus were not investigated further. Trolox, which has a relatively low solubility in water (0.1% at saturation), was treated as a lipophilic antioxidant since it appeared to offer no protection in trials performed in aqueous solution.

Amphoteric and Hydrophilic Antioxidants

Propyl gallate and Uric Acid

The low solubility of propyl gallate (0.35g/100mls) and uric acid (0.7g/100ml) limited their use in the current application, however initial studies were performed due to their reported effectiveness as antioxidants. Protoxin samples, prepared in saturated solutions (pH 10.5) of these compounds and irradiated for up to 4 hours at 302 nm, did not show any protection when analyzed by SDS-PAGE and were not investigated further.

PABA (1g/170mls)

The relatively poor solubility of PABA (1g/170mls) in aqueous solution also made studies at pH 7.0 unfeasible. However, in the study of protoxin, 0.1 M stock solutions of PABA could be prepared when adjusted to pH 10.5. Protoxin samples prepared in 0.1 M PABA (pH 10.5) were air dried and irradiated at 302 nm up to 4 hours. The results of SDS-PAGE analysis of the irradiated samples and trypsin activation to toxin are shown in Figures 4.1 A and 4.1B respectively. The results indicate that PABA offers relatively good protection for the duration of the irradiation period.

Cysteine

Protoxin samples, prepared in a 0.1 M cysteine solution adjusted to pH 10.5, were air dried and irradiated for up to 4 hours at 302 nm. The results of SDS-PAGE analysis of protoxin recovery the irradiated samples and trypsin activation to toxin are shown in Figures 4.1 C and 4.1 D respectively.

Histidine

Protoxin samples, prepared in a 0.1 M histidine solution adjusted to pH 10.5, were air dried and irradiated for up to 4 hours at 302 nm. The results of SDS-PAGE analysis of protoxin recovery the irradiated samples and trypsin activation to toxin are shown in Figures 4.1 E and 4.1 F.

Vitamin C (Ascorbic Acid)

Crystal suspensions and protoxin samples, prepared in the presence of 0.1 M Vitamin C solution adjusted to pH 10.5, were air dried and irradiated for upto 4 hours at 302 nm. The results of SDS-PAGE analysis of protoxin recovery from the irradiated samples and trypsin activation to toxin are shown in Figures 4.1 G and 4.1 H respectively.

CAM-Protoxin

CAM-protoxin samples, prepared in a 0.1 M Vitamin C solution adjusted to pH 10.5, were air dried and irradiated for up to 4 hours at 302 nm. The results of SDS-PAGE analysis of

protoxin recovery the irradiated samples and trypsin activation to toxin are shown in Figures 4.2 A and 4.2 B. The results suggest that the presence of the disulfide linkages does not affect the rate of photo-inactivation.

Ammonium Persulfate Treatment of the Bt Crystal Protein

Crystal suspensions and protoxin samples were treated in solution at pH 10.5 with 0.017% (v/v) ammonium persulfate in a solution containing 0.1 M Vitamin C at pH 7.5 (crystal slurries) or pH 10.5 (protoxin samples). Aliquots were drawn at the indicated time intervals and analyzed by SDS-PAGE as shown in Figures 4.3 A and 4.3 B. A control experiment, consisting of the protoxin in the absence of Vitamin C, was analyzed in the same way. The results indicate that the free radical oxidation of the DNA-protein complex occurred within 1 hour in the absence of Vitamin C, while the sample containing Vitamin C was protected for up to 24 hours with some evidence of oxidation occurring 2 hours. Treatment of the crystals (pH 7) and protoxin (pH 10.5) in solutions containing 0.1% APS for 2 hours was sufficient to completely oxidize the crystals and protoxin in the absence of Vitamin C.

Photostabilization of *Bacillus thuringiensis* Preparations with Vitamin C

The efficiency and duration of Vitamin C protection were investigated for extended irradiation periods at 302 nm. Crystal slurries were prepared in the presence of 0.1% up to 1.8 % (w/v) of Vitamin C and irradiated for up to 4 hours. The duration of Vitamin C (1.8% w/v)

was also determined in dried crystal samples containing 85 μg crystal protein for extended irradiation time points of up to 48 hours. As shown in Figure 4.4, the crystal protein was protected for up to 48 hours.

Protein Recovery and Toxicity Assays of Vitamin C-Protected *Bacillus thuringiensis* Crystals

The photostabilization of crystal preparations prepared in the presence of Vitamin C, shown in the previous experiments, prompted an investigation into the toxicity of the irradiated crystals. Crystals samples were prepared in the presence of 0.1 M Vitamin C adjusted to pH 7 (+VC) or in the absence of Vitamin C (-VC) and air dried. The samples were irradiated for time intervals of up to 8 hours and sent to Sault Ste. Marie for lawn assays against CF-1 cells as described in the previous chapter. The protein recovery and toxicity of the two sets of samples are shown in Figures 4.5A and 4.5B respectively.

The toxicity of the Vitamin C-treated crystals and the total recovery of protein from these samples remained essentially constant for the duration of the experiment. In the absence of Vitamin C, both the toxicity and total protein recovery decreased rapidly over the course of 4 hours.

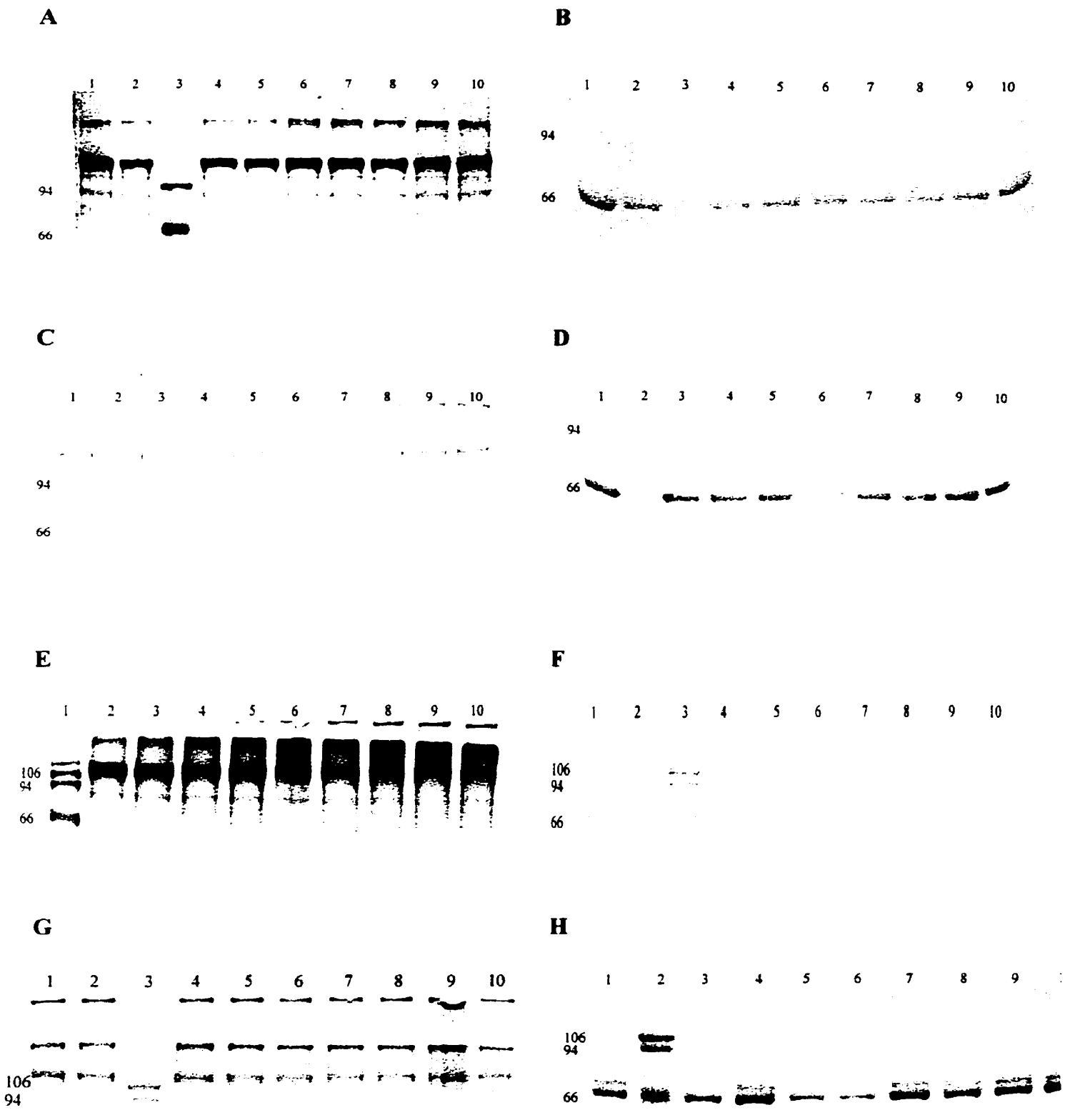

A
1 2 3 4 5 6 7 8 9 10
94
66
B
1 2 3 4 5 6 7 8 9 10
94
66
C
1 2 3 4 5 6 7 8 9 10
94
66
D
1 2 3 4 5 6 7 8 9 10
94
66
E
1 2 3 4 5 6 7 8 9 10
106
94
66
F
1 2 3 4 5 6 7 8 9 10
106
94
66
G
1 2 3 4 5 6 7 8 9 10
106
94
H
1 2 3 4 5 6 7 8 9
106
94
66

Figure 4.1 UV Protection of Bt Crystals in the Presence of Various Antioxidants

For Figures 4.1 A through J, time points were 0 hours (air dried, non-irradiated), 30 minutes, 1 hour, 1.5 hours, 2 hours, 2.5 hours, 3 hours, 3.5 hours, and 4 hours.

Crystal Protein Samples

250 µg crystal protein was air dried in the presence of 0.1 M antioxidant (pH 10.5), unless stated otherwise, and irradiated at 302nm for up to 4 hours. The crystals were resolubilized in 0.1 M solubilization buffer (0.1 M carbonate/bicarbonate buffer, 10 mM EDTA, 0.1 % (v/v) BME, pH 10.5) and analyzed by SDS-PAGE electrophoresis.

Trypsin Digests From Irradiated Crystals

100 µg resolubilized crystal protein (in 0.1 M solubilization buffer; 0.1 M carbonate/bicarbonate buffer, 10 mM EDTA, 0.1 % (v/v) BME, pH 10.5), irradiated in the presence of 0.1 M antioxidant, was treated with trypsin for 4 hours and analyzed by SDS-PAGE electrophoresis.

A **Crystal Protein Recovery From Irradiated Crystals in the Presence of PABA**

B **Toxin Generation From Irradiated Crystals in the Presence of PABA**

C **Crystal Protein Recovery From Irradiated Crystals in the Presence of Cysteine**

D **Toxin Generation From Irradiated Crystals in the Presence of Cysteine**

E **Crystal Protein Recovery From Irradiated Crystals in the Presence of Histidine**

F **Toxin Generation From Irradiated Crystals in the Presence of Histidine**

G **Crystal Protein Recovery From Irradiated Crystals in the Presence of Vitamin C**

H **Toxin Generation From Irradiated Crystals in the Presence of Vitamin C**

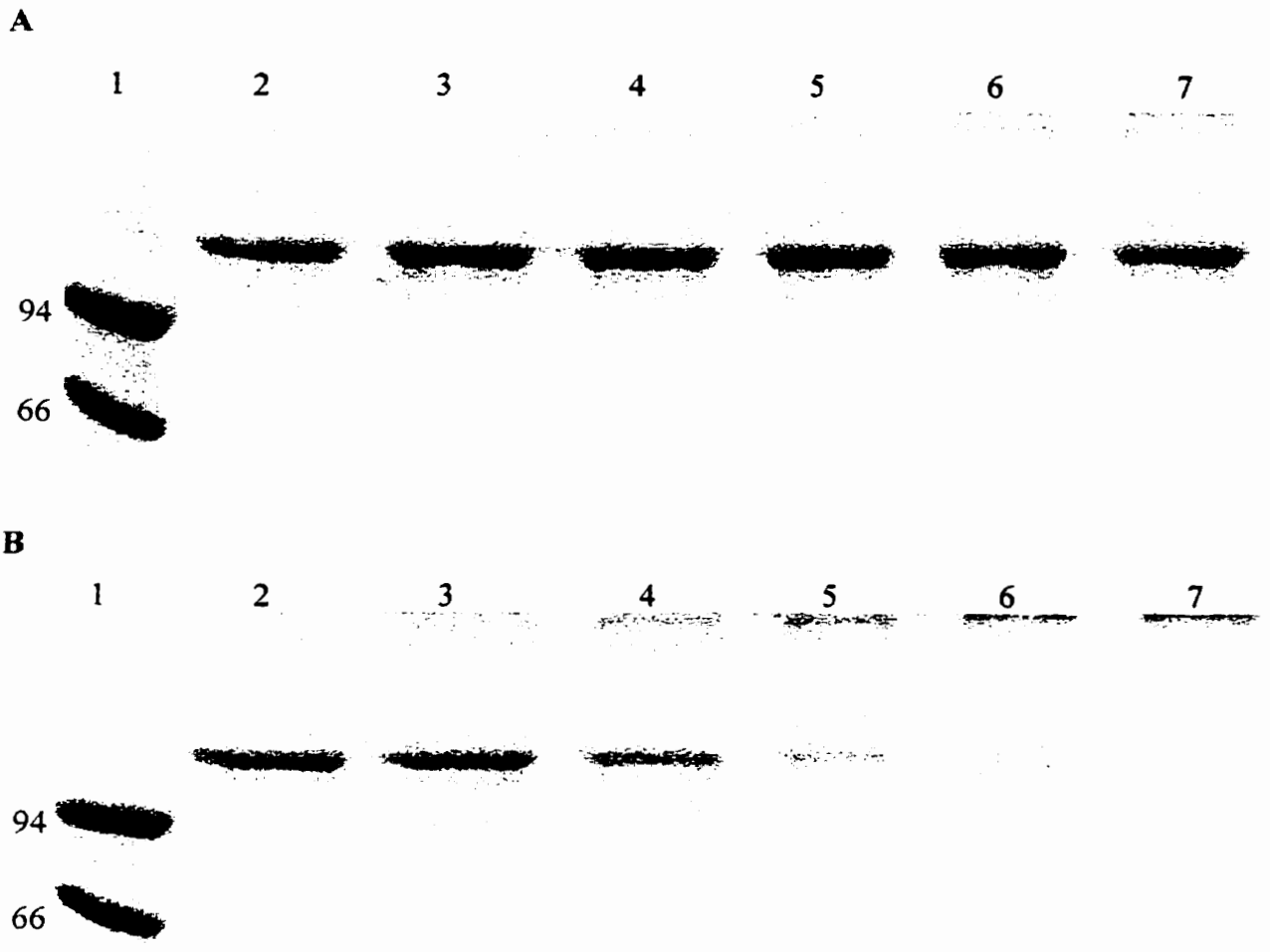


Figure 4.2 UV Protection of CAM-Protoxin in the Presence (A) and Absence (B) of Vitamin C

100 μg CAM-protoxin were irradiated in solution (0.1 M carbonate/bicarbonate buffer) in the presence (or absence) of 0.1 M Vitamin C (adjusted to pH 10.5) at 302 nm for up to 4 hours. Aliqouts of the irradiated material were withdrawn at various time points and analyzed by SDS-PAGE electrophoresis. A: With Vitamin C; B: Without Vitamin C; Lane 1, Molecular mass markers; lane 2, no irradiation (0 minutes); lane 3, 30 minutes; lane 4, 1 hour; lane 5, 2 hours; lane 6, 3 hours, lane 7, 4 hours.

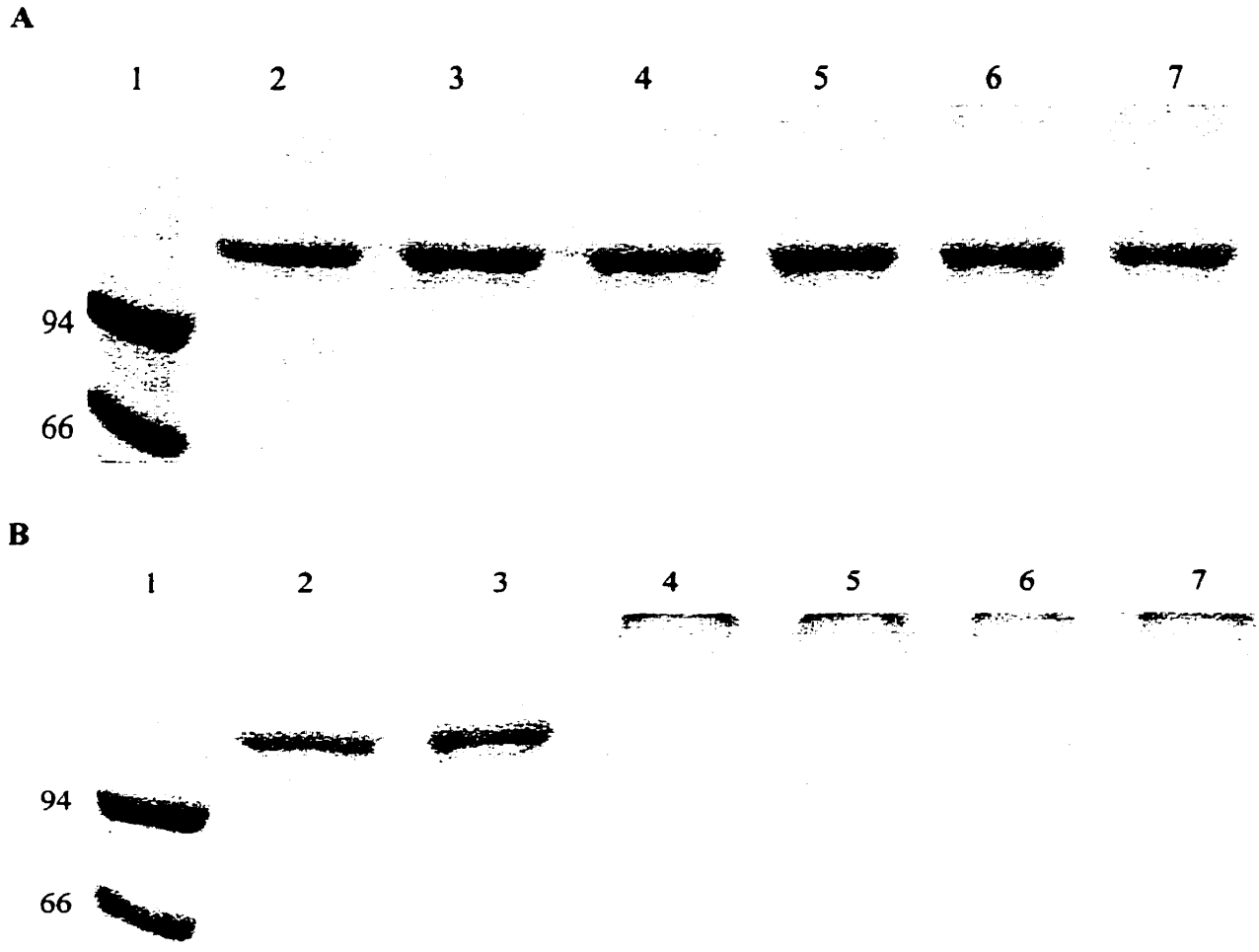


Figure 4.3 Ammonium Persulfate Treatment of the Bt Crystal Protein in the Presence (A) and Absence (B) of Vitamin C

100 μg CAM-protoxin were treated with 0.018 % APS in solution (0.1 M carbonate/bicarbonate buffer) in the presence (or absence) of 0.1 M Vitamin C (adjusted to pH 10.5). Aliqouts of the irradiated material were withdrawn at various time points and analyzed by SDS-PAGE electrophoresis. A: With Vitamin C; B: Without Vitamin C; Lane 1, Molecular mass markers; lane 2, before APS addition; lane 3, 30 minutes; lane 4, 1 hour; lane 5, 2 hours; lane 6, 3 hours, lane 7, 4 hours.

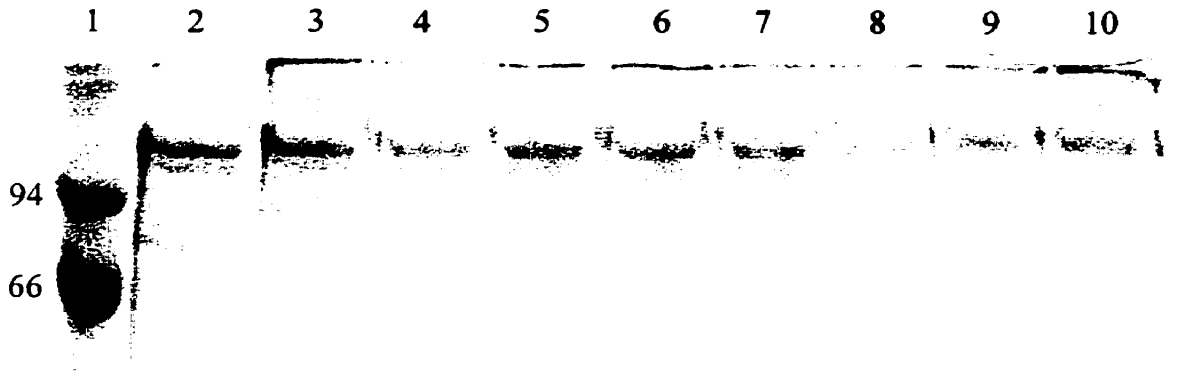
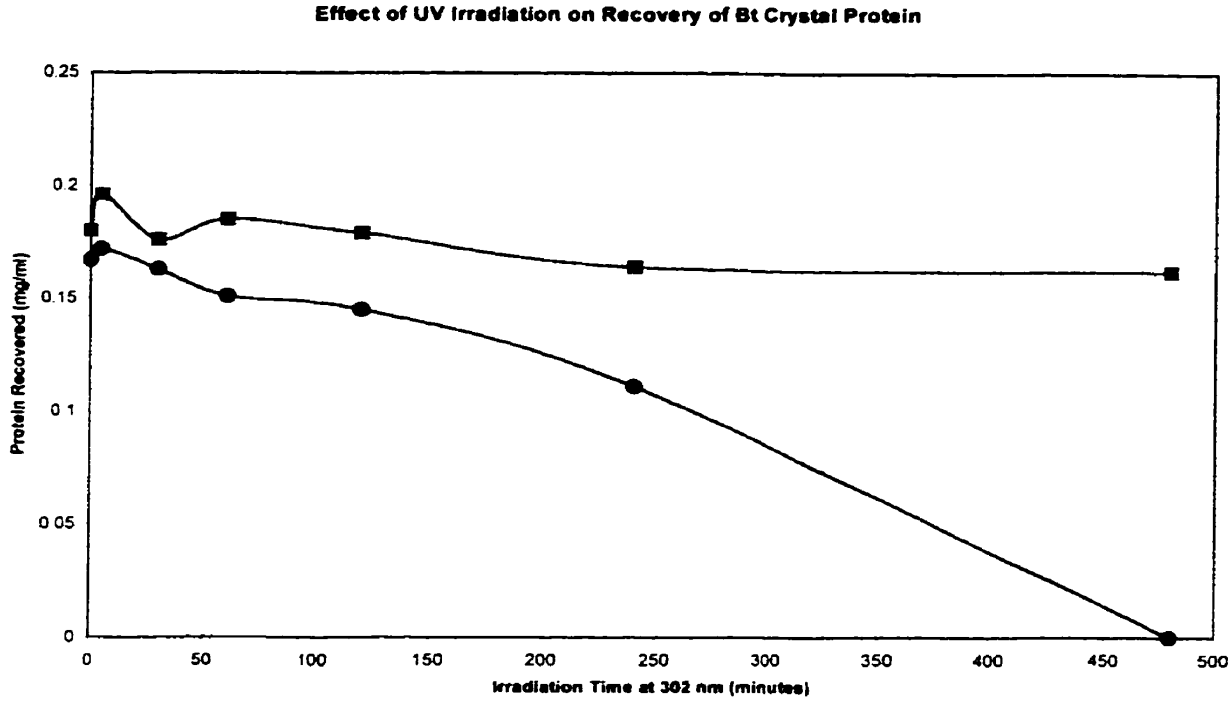


Figure 4.4 Duration of Protection of Vitamin C in the Photostabilization of *Bacillus thuringiensis* Preparations

100 µg of crystal protein were suspended in a 0.1 M solution of Vitamin C, air dried and irradiated for up to 48 hours at 302 nm. The samples were then solubilized and analyzed by SDS-PAGE for protein recovery.

Lane 1, Molecular mass markers; lane 2, non-dried, non-irradiated crystals; lane 3, air dried, non-irradiated crystals; lane 4, 2 hour; lane 5, 4 hours; lane 6, 8 hours; lane 7, 16 hours; lane 8, 24 hours; lane 9, 36 hours; lane 10, 48 hours.

A


Effect of UV Irradiation on Recovery of Bt Crystal Protein
Protein Recovered (mg/ml)
0.25
0.2
0.15
0.1
0.05
0
0
50
100
150
200
250
300
350
400
450
500
Irradiation Time at 302 nm (minutes)

B

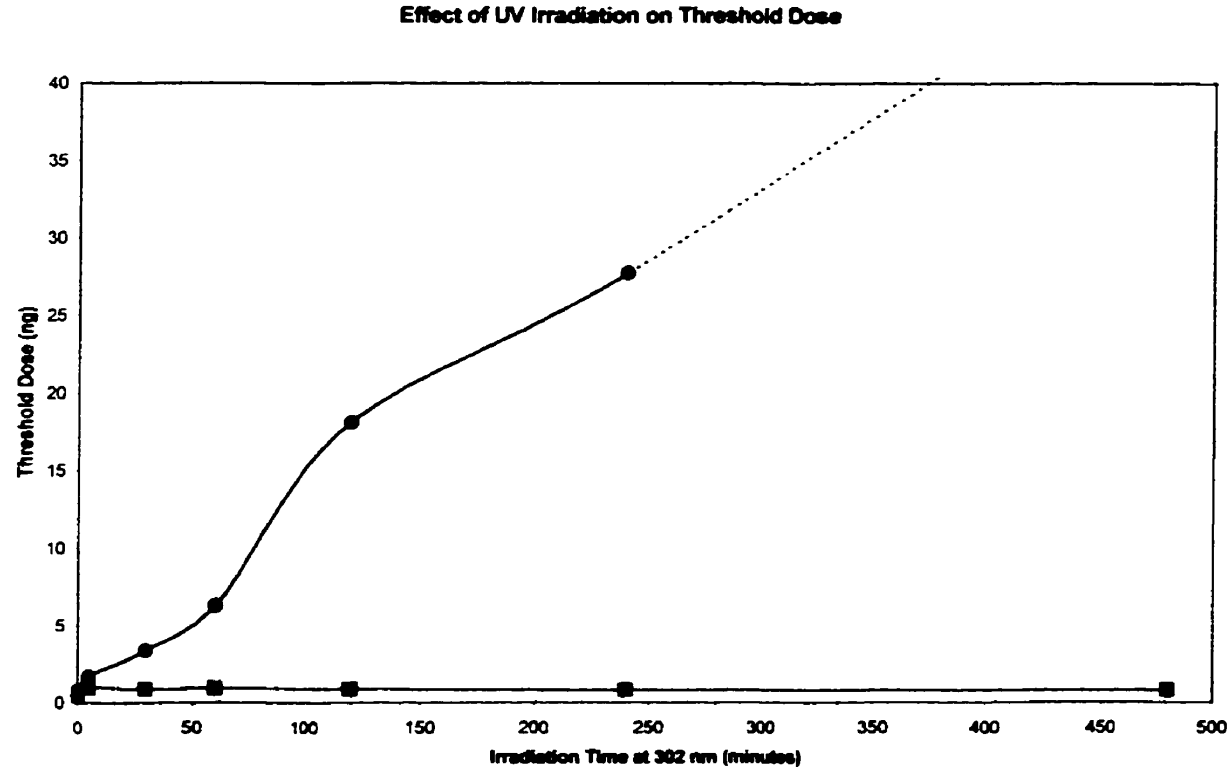

Effect of UV Irradiation on Threshold Dose
Threshold Dose (ng)
40
35
30
25
20
15
10
5
0
0
50
100
150
200
250
300
350
400
450
500
Irradiation Time at 302 nm (minutes)

Figure 4.5 Protein Recovery and Toxicity Assays of Vitamin C-Protected *Bacillus thuringiensis* Crystals

A

Air dried Bt crystals (200 μg crystal protein) were irradiated at 302 nm for up to 480 minutes. The crystals were treated with trypsin overnight and subsequently assayed for protein recovery by the Bradford method (● = absence of Vitamin C; ■ = 0.1M Vitamin C).

B

Air dried Bt crystals (200 μg) were irradiated for up to 480 minutes at 302 nm and assayed for toxicity using CF-1 cell line lawn assays as described in the experimental procedure. The threshold dose (ng) is shown as a function of UV irradiation time (● = absence of Vitamin C; ■ = 0.1M Vitamin C).

4.4 Discussion

The results of the current investigation suggest that the photo-inactivation of *Bacillus thuringiensis* crystals is the result of a UV-induced free radical reaction. Incorporation of water-soluble antioxidants, particularly Vitamin C, provided protection against photo-inactivation. Other candidate antioxidants, such as cysteine, histidine and PABA, also showed moderate to good protection. The limited solubility of PABA (0.35 g / 100 ml; H_2O) at neutral pH limits its potential use in Bt formulations. It was not surprising to find that the most efficient antioxidants were those displaying a high degree of solubility in water (Vitamin C 1g / 3ml; cysteine and histidine, freely soluble (Merck Index)), since, for an antioxidant to be effective, it must be able to gain access to the radicals which are formed as described below (Doba et al., 1985; Wayner et al., 1986; Wayner et al., 1987).

The superior protection offered by Vitamin C, relative to other water-soluble antioxidants such as cysteine for example, is most likely a consequence of its ability to undergo two successive one-electron oxidations, and thereby reducing two equivalents of free radicals per equivalent of Vitamin C. The bimolecular reactions involved in these reduction reactions, and specifically with Vitamin C as antioxidant, have been shown to be concentration dependant (Wayner et al., 1986). Furthermore the reduction potential of Vitamin C ($E^{o'}$ = • 282 mV; ascorbate, H^+ / ascorbate monoanion redox couple in water, pH 7; Buettner et al., 1996) makes the ascorbate radical a particularly good reducing agent. The concentration dependence of Vitamin C in the current investigations was shown to be a minimum of approximately 1 % Vitamin C (w/w) in protecting a 100 µg sample of the crystal protein over a period of 8 hours

(data not shown). The apparent protection of 100ug of crystal protein for a period of up to 48 hours was achieved with a 1.8 % solution of Vitamin C. It appears that the protection could last for substantially longer period s of UV irradiation at 302 nm and aboveor above up to 48 hours irradiation at 302 nm (Figure 4.4).

The protection against photo-inactivation of Bt crystals displayed by the water-soluble antioxidants appears to be simply a consequence of their ability to reach the radicals formed during UV exposure. The increased permeability of the crystals towards these compounds would allow these compounds greater access to the sites of radical initiation and to interact with the photo-induced free radicals, whether initially produced in the protein or DNA components. Numerous studies dealing with the antioxidant activity of potential antioxidants have shown that it is the accessibility of the antioxidant that governs the degree of efficacy. Indeed, Doba et al. (1985) showed that Vitamin E is an excellent antioxidant in phospholipid membranes (liposomes). In an aqueous environment, the presence of Vitamin C in the aqueous phase acted synergistically with Vitamin E in preventing oxidative damage to the lipid bilayers. When radicals were generated in the aqueous phase, Vitamin C was found to efficiently trap aqueous peroxyl radicals, but was ineffective as an antioxidant for peroxy radicals generated within the phospholipid bilayer. These authors postulated that the synergism observed between Vitamins E and C in preventing oxidative damage in the lipid bilayers is a result of the ability of Vitamin C to regenerate α-tocopherol from the α-tocopherol radical by interaction with ascorbate at the phospholipid / water interface.

The efficiency of Vitamin C in protecting the crystals and crystal protein products from photo-inactivation suggests that a free-radical reaction may be responsible for the UV-induced photo-inactivation. The reported formation and detection of free radicals in nucleic acids upon irradiation with UV light (Smith and Alpin, 1966; Jellinek and Johns, 1970; Smith, K.C. 1970; Varghese, A.J., 1973a; Varghese, A.J., 1973b), as well as the demonstrated covalent attachment of a number of proteins to polynucleotides by UV irradiation (Galcheva-Gargova, Z. and Dessev, G.N, 1987; Angelov, D. et al., 1988; Buckle et al., 1991; Jensen et al., 1993), provide precedent for the conclusions of the current investigation, that the presence of the DNA in the crystal protein may be at least partially responsible for the observed photo-inactivation of the Bt crystal protein.

In an effort to provide further evidence for a free radical mechanism, the crystal protein was treated with a free radical generator. Ammonium persulfate (0.017%) was found to produce an inactivation profile (SDS-PAGE) comparable to the UV-induced inactivation observed by irradiation at 302 nm. Vitamin C was also found to protect the crystal protein from apparent polymerization, which paralleled the findings of UV-inactivation. These results suggest a similar mechanism, a free radical mechanism, for the observed photo-inactivation.

Inactivation studies were conducted on CAM-protoxin in order to determine the involvement of the sulphydryl residues of the crystal protein in photo-inactivation due to their reported reactivity and formation of pyrimidine-cysteine photoproducts (Smith, K.C., 1970; Varghese, A.J., 1973 a and b). The photo-inactivation of CAM-protoxin shows the same SDS-PAGE profile observed for the Bt crystals. These results suggest that cysteine residues are

not directly involved in the formation of cysteine-DNA covalent linkages in the Bt DNA-crystal protein complex.

Evidence was also obtained for the participation of metal-catalyzed oxidation of Bt crystals. Photo-inactivation was partially reduced when EDTA (0.1M) was incorporated into Bt crystal slurries (data not shown). This result suggests that oxidative damage by Fenton-type reactions may also play a role in the photo-inactivation of Bt crystals, however the degree of protection was not as significant based on the decrease of crystal protein by SDS-PAGE. Metal-catalyzed oxidative (MCO) damage has been suggested to be responsible for the oxidative damage of proteins and lipoproteins, nucleic acids and lipids by the formation of reactive oxygen species (Stadtman, E.R., Oliver, C.N., 1991). While the photo-excitation of amino acids is greater in the UV-C region (200-290 nm), the photolysis of tryptophan has been shown to occur in the UV-B, with formation of N-formylkynurenine (NFK), and photosensitized oxidation of tryptophan has also been shown in the UV-A and visible regions of the electromagnetic spectrum. Near–UV excitation of NFK has been suggested to generate superoxide and hydroxyl radicals via several reaction pathways (Grossweiner, L.I., 1984).

4.5 Conclusion

Evidence obtained in the current studies suggest that the photo-inactivation of the *Bacillus thuringiensis* products occurs through a free radical process, which may be inhibited in the presence of water-soluble antioxidants. Under the current experimental conditions, Vitamin C was found to be the most effective antioxidant examined, and efficiently inhibited the loss of toxicity over an extended time for irradiations conducted at 302 nm. It is proposed that the efficiency of Vitamin C in this system is due to its high permeability within the crystals and its ability to quickly reduce the radicals which are generated as a result of UV irradiation, thereby inhibiting radical propagation

Previous attempts by other researchers in preventing the photo-inactivation of Bt formulations have been relatively unsuccessful, with little improvement in the field persistence of the formulations. The incorporation of antioxidant and sunscreens in previous trials were reported to be unsuccessful. These studies include the incorporation of Uvinul DS49 and Erio Acid Red (Morris, O.N., 1983), Congo Red, folic acid and p-aminobenzoic acid (PABA) (Dunkle, R.L. and Shasha, B.S., 1989) and melanin (Liu,Y.T. et al., 1993; Patel, K.R. et al., 1996) as potential protectants. Several dyes and UV absorbing compounds are known to act as photosensitizers and have been suggested to cause the photo-oxidation of proteins and DNA (Grossweiner, L.I., 1984). Although the notion of incorporating UV-absorbing materials into Bt preparations appears logical, previous attempts to photostabilize the preparations with these compounds may have actually induced damage instead of offering protection.

The specific nature of the covalent bonds formed in these reactions remains undetermined. As will be discussed in Chapter 5, there exist a large number of possible UV-induced photoproducts that can be formed between nucleic acid-protein complexes by irradiation with UV light. The elucidation of the nature of the radicals formed and the products obtained from these reactions might provide further insight into the nature of the photo-induced damage and the efficacy of Vitamin C as a superior antioxidant in the *Bacillus thuringiensis* system.

Chapter 5

Attempts at the Isolation and Characterization of the UV-Induced Crosslinking Products from *Bacillus thuringiensis* Crystals

5.1.1 Introduction

The mechanism of UV-induced reactions between DNA and proteins has been an active field of investigation since the late 1960s. These reactions have been studied extensively through model systems, using nitrogenous bases or short oligomers and amino acids or peptides, and many photochemical products have been characterized. The photochemical products have been informative with respect to the possible UV-induced reactions occurring in biologically important nucleic acid-protein complexes and have allowed investigators to propose reactions mechanisms based on the observed products.

As mentioned in the previous chapter, the pyrimidines have been shown to be more photochemically reactive than purines. Thymine in DNA and uracil in RNA, have been shown to form a variety of photoproducts with different amino acids, with the proposed formation of free radicals. The first evidence for the formation of UV-induced reactions between proteins and DNA was obtained from irradiation experiments performed on E. coli (Smith, K.C., 1962). The loss of DNA from irradiated cells was shown to follow a dose-dependent decrease, with the formation of DNA-protein precipitates, which could liberate free DNA upon treatment with trypsin. Evidence for the potential photochemical products between proteins and DNA was obtained from the isolation of 5-S-cysteine-6-hydrouracil from irradiation of a solution containing uracil and cysteine at 254 nm (Smith, K.C. et al., 1966). In similar experiments, a mixed photoproduct was isolated from the irradiation of thymine and cysteine for 1 hour at 254 nm (Smith, K.C., 1970). The isolated product was shown to be 5-S-cysteine-6-hydrothymidine. The mechanism of this reaction was proposed as the addition of a thiyl radical to the 5-position of a thymyl radical.

In other irradiation studies of thymine and cysteine, Varghese (1973a) demonstrated the formation of α-S-cysteinyl-5,6-dihydrothymine as one of the major photoaddition products. This product differed from the product described by Smith; the difference being attributed to differences in experimental conditions. In yet another publication, Varghese described the formation of six different thyminyl-cysteine photoproducts resulting from irradiation at wavelengths greater than 230 nm (Varghese, A.J. 1973b).

The photochemical reactions of uracil and thymine were also observed from irradiations carried out at 254 nm in the presence of cysteine, lysine, arginine, cystine and tyrosine, the most reactive of the naturally occurring amino acids in reactions with thymine and uracil (Schott, H.N. and Shetlar, M.D., 1974). In one set of experiments, various amino acids were mixed with ^{14}C-uracil or ^{14}C-thymine and irradiated. Although the reaction products were not characterized, the retention of radioactivity by the amino acids provided evidence for the formation of photoproducts. With thymine, the amino acids displaying reactivity under the experimental conditions were found to be tyrosine, tryptophan, cystine, cysteine, methionine, arginine and lysine to varying degrees. However with uracil, the amino acids histidine, proline, serine and phenylalanine were also shown to form photoproducts showing that uracil was more reactive than thymine at least for the conditions employed (Schott, H.N. et al., 1974). This was the beginning of a very intensive period of research into the mechanisms of UV-induced reactions between the nitrogenous bases of nucleic acids and amino acids. The brief introduction presented above represents only a small sample of a vast body of literature regarding UV-induced reaction products between DNA components (nitrogenous bases or oligonucleotides) with amino acids. Since then, photochemical products have been isolated and characterized for the photoaddition reaction of N-acetyl-tyrosine with thymine

and thymidine (Shaw, A.A., et al., 1992) and L-lysine and N-acetyl-L-lysine with cytosine and 5-methylcytosine (Dorwin, E.L., et al., 1988).

Attempts to characterize similar products from naturally occurring nucleic acid-protein complexes have been unsuccessful (Lin, S.-Y. and Riggs, A.D., 1974; Paradiso P.R. et al., 1979; Harrison, C.A., 1982; Hockensmith, J.W. et al., 1985; Jensen, O.N. et al., 1993). Paradiso et al. (1979) reported the isolation of a photochemical product from the UV irradiation of the gene 5 protein and fd DNA from wild type fd bacteriophage with 254 nm light using a germicidal lamp as the UV source. The gene 5 protein and fd DNA were radiolabelled in order to increase the sensitivity of detection of UV-induced products. Following irradiation, the products were purified by column chromatography and high voltage paper electrophoresis. The electrophoretic bands were subjected to amino acid analysis, which revealed the presence of a peptide consisting of amino acid residues 29-34 of the gene 5 protein. After extensive treatment with proteases, cysteic acid (cysteine 33) was the only amino acid found to be covalently linked to the fd DNA. The nucleic acid base involved in the product was suggested to be thymine based on preliminary experiments which showed that the gene 5 protein was 5 times more reactive with p(dT) oligomers compared to the other DNA bases. The molecular details of the site of covalent attachment between the base and cysteine 33 were not elucidated.

5.1.2 Rationale for the Current Investigations

The research described in this chapter was conducted in an attempt to isolate and characterize the products resulting from UV-induced reactions between the DNA and protein

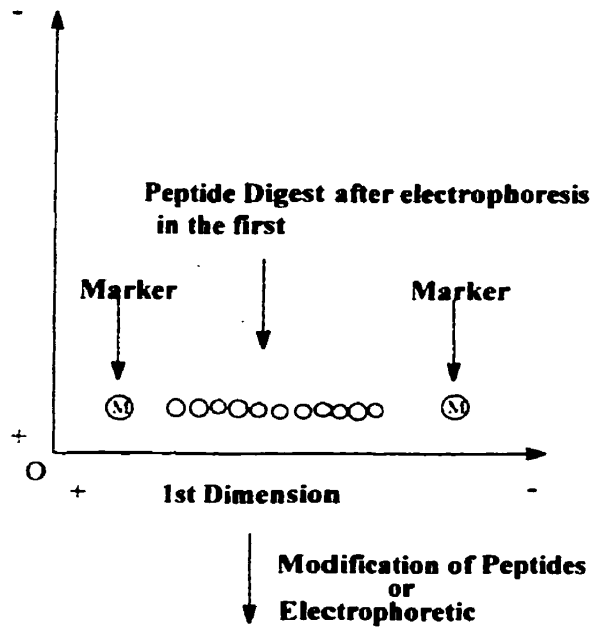
components present in the crystals of *Bacillus thuringiensis* subspecies *kurstaki* HD-73. Isolation of the reaction products would provide evidence for the site(s) of interaction between the protein and DNA components in the crystals, which is presently unknown. Also, isolating the product(s) would provide *in vivo* evidence for the reactions proposed in the literature and the elucidation or proposal of the reaction mechanism. As illustrated in the introduction, many of the reactions between DNA (or nitrogenous bases) and proteins (or amino acids) have been conducted at wavelengths which are not representative of conditions experienced at the earth's surface (Barker, R.E. Jr., 1968). In the research presented in this chapter, the conditions employed were limited to UV wavelengths that are an integral part of the solar spectrum in order to relate the results to field conditions. Therefore, in the current studies, irradiation experiments were performed at 302 or 365 nm exclusively. The inspiration for the technique devised for the isolation of the photoproducts from irradiation of the crystals from Bt was based on the photoproducts that were reported by Smith and and Varghese.

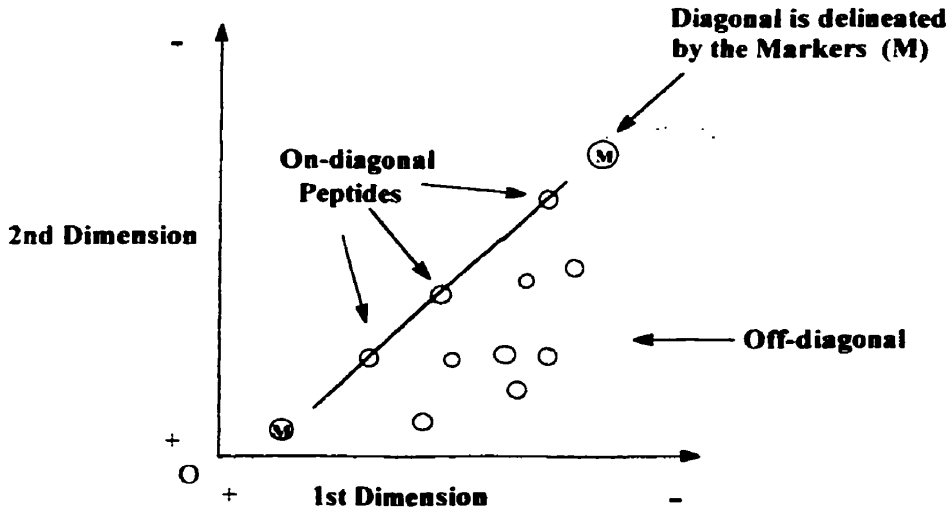
5.1.3 Introduction to 2-Dimensional High Voltage Paper Electrophoresis

2D-HPVE exploits the change in electrophoretic mobility of peptides in an electric field based on their ionization state. The ionization state of the residues can be manipulated by the pH of the solvents at which the electrophoresis is conducted, or by modifying the peptides after electrophoresis in the first dimension (refer to Figure 5.1). For example, the electrophoretic mobility of carboxylic acid-containing peptides may be manipulated by electrophoresis in the first dimension at pH 2.1, and the second dimension at pH 4.4 (Duggleby and Kaplan, 1975).

Under these conditions, the mobility of peptides containing free carboxylic acids is reduced in the second dimension (as a result of deprotonation) due to a net increase in anionic character of the peptides, and the peptides would lie off the diagonal line (off-diagonal peptides). Peptides, which do not contain free carboxylic acid functionalities in the side chains or at the carboxyl terminus, would be unaffected by this change in conditions and lie on the diagonal line (on-diagonal peptides). The diagonal line can be delineated by appropriate markers, also unaffected by the change in conditions; for example taurine (T), which remains neutral in both dimensions and choline (C), which possesses a net charge of +1 in both dimensions. Conditions can be developed, or the modification of the peptides can be achieved in such a way, for the selective isolation of the desired peptides. The induced change in electrophoretic mobility can be achieved in many different ways; two such methods have been employed in the work described in the next two chapters.

Figure 5.1
Two-dimensional HVPE





5.1.4 Strategy for the Isolation of DNA-Protein Crosslinked Products

Based on the structures of the reported photoproducts (Figure 5.2) formed as a result of UV irradiation, a two-dimensional method was devised that could be used for the isolation of phosphorylated peptides. The addition products between cysteine, lysine and tyrosine in the crystal protein, with thymidine of the DNA associated with the crystal appeared to be the most likely candidates (Smith, K.C., 1970; Varghese, A.J. 1973a and b; Dorwin, E.L., et al., 1988; Shaw, A.A., et al., 1992).

Initial treatment of the crosslinked material with proteases and RQ1 DNase (DNase I), would be expected to generate peptidyl-oligomers from the crosslinked complex. RQ1 DNase treatment yields 3'-hydroxyl oligonucleotides, leaving a phosphate group at the 5' ends of the oligonucleotides (Moore, S., 1981). The mixture is subjected to electrophoresis in the first dimension. The strip is dried and treated with alkaline phosphatase, to remove the 5'-phosphate group, making the peptides more "basic" overall (expected change in charge is +2). The treated strip is then re-subjected to electrophoresis in the second dimension, and the dephosphorylated peptides are located above the diagonal as represented in Figure 5.3.

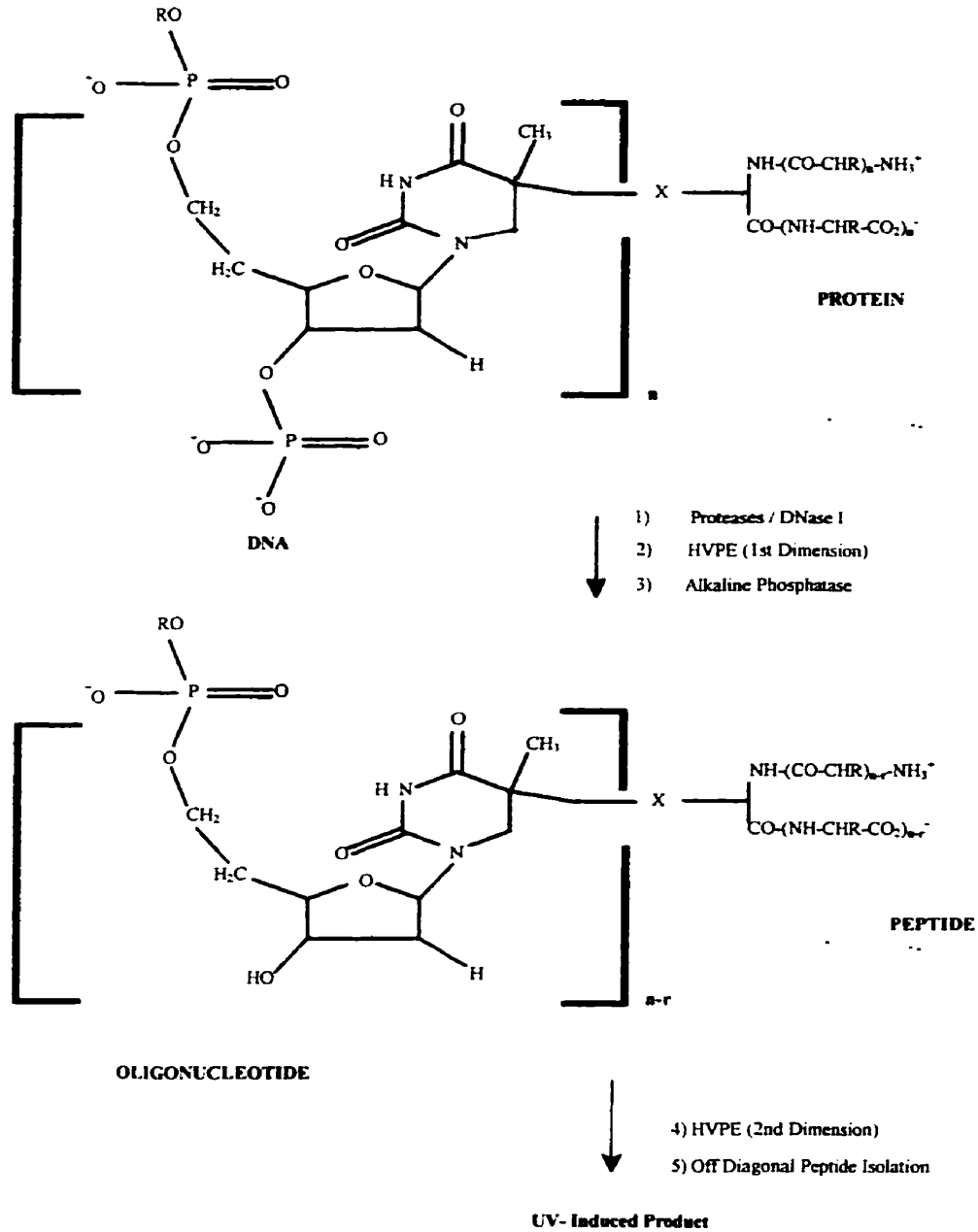
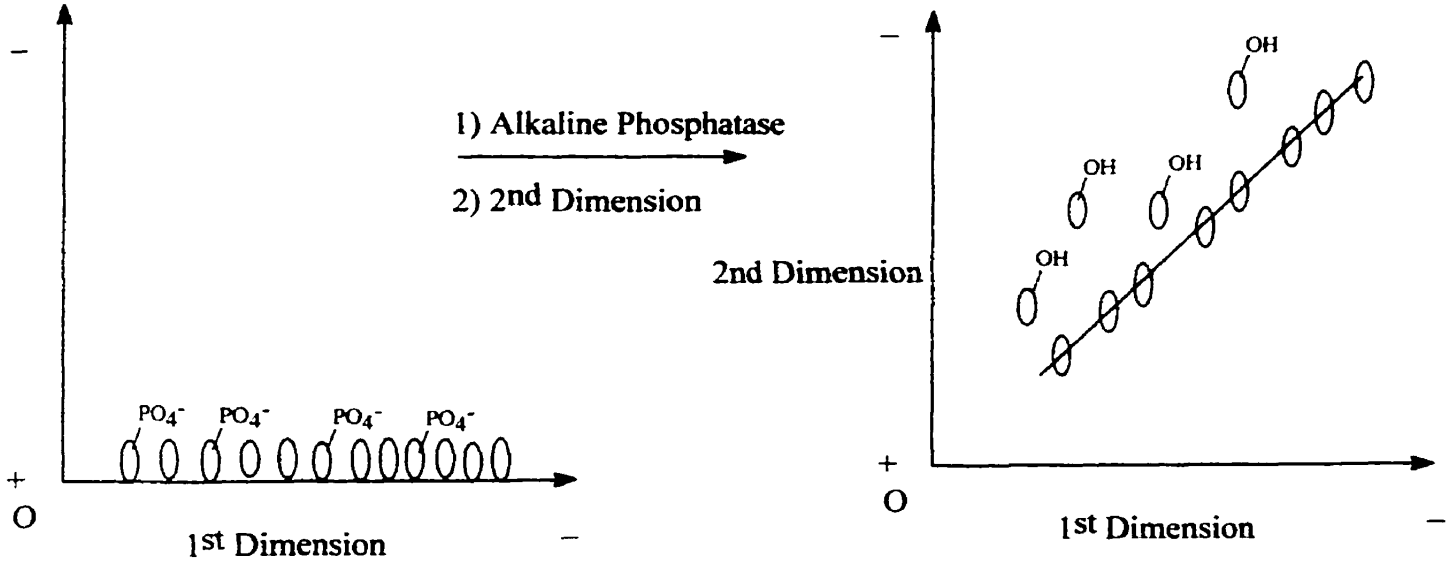


Figure 5.2 **Chemical Structures of Expected UV-Induced Products (thymidine-cysteine, thymidine-lysine and thymidine-tyrosine photoproducts) between DNA and amino acids and of treatment with alkaline digestion**

Figure 5.3
Strategy for the Isolation of Phosphorylated Peptides



UV irradiated crystals are treated extensively with proteases and nucleases to yield peptides and oligomers. The digested material is subjected to HPVE in the first dimension and treated with alkaline phosphatase. Removal of the phosphate groups from the oligomers covalently attached to peptides should allow for significant changes in electrophoretic mobility.

5.2 Experimental Procedures

5.2.1 Materials

Crystals and Crystal Protein Products

Crystals were obtained as described in previous chapters. Radioactive labelling of the crystals with ^{32}P-α-ATP and ^{3}H-(6-thymidine) were accomplished by growing *Bacillus thuringiensis* cultures (1 L) in the presence of 1 mCi of either ^{32}P-α-ATP or ^{3}H-(6-thymidine). The cultures were harvested and the crystals purified using density gradient centrifugation with Renografin as previously described.

Chemicals and Enzymes

Ninhydrin was obtained from Pierce. Trypsin and DNase I were obtained as lyophilized powders from Sigma. RQ1 and Proteinase K were purchased from Promega. Calf intestinal alkaline phosphatase was obtained from Sigma.

All other chemicals used to prepare buffers for enzymatic digestion and solvents used for High Voltage Paper Electrophoresis (HPVE) were obtained from commercial sources as the highest purity grades available.

Radioisotopes, ^{32}P-α-ATP (1 mCi) and ^{3}H-(6-thymidine) (1mCi) were obtained through Amersham Radiochemicals.

5.2.2 Method

Irradiation

All irradiations wre performed using a germicidal lamp equipped with a 15-watt BL tube CX-50B lamp providing light of 302 nm (2,100 $\mu W/cm^2$) or 365 nm (2,200 $\mu W/cm^2$) for the indicated time intervals. Sample aliquots were stored, if necessary, by freezing with liquid nitrogen and subsequent storage at -20°C.

Enzymatic Hydrolysis of Irradiated Products

Several strategies were adopted in order to prepare the samples for analysis and isolation of photoinduced reaction products. Samples were digested with various combinations of proteases (pepsin / thermolysin / trypsin / Proteinase K) and RQ1 DNase to reduce the samples to peptides and oligomers / nucleotides for HPVE.

Analysis and Recovery of Irradiation Products by 2-D HVPE

The first method adopted to isolate the cross-linked products was the diagonal electrophoresis method described earlier (vide supra). The irradiated samples were resuspended / solubilized in 5% formic acid and digested with pepsin for 24 hours. Distilled water was added to the digest, which was shelled and lyophilized. The residue was then resolubilized in ammonium bicarbonate (1%) and the pH adjusted to 9. Trypsin (1 % w/v) was added and the sample incubated

at 37°C for another 24 hours. Finally the digested samples were digested with RQ1 DNase for 24 hours at 37°C in DNase buffer containing 6 mM $MgCl_2$ and 0.1 mM $CaCl_2$.

The digested samples were then run on 3MM Whatman paper at pH 6.5 for 30 minutes at a potential difference of 60 V/cm in the first dimension and allowed to dry. Strips (5-8 cm) were cut from the first dimension electrophoresis and buffered toward the centre of the strip using pH 6.5 buffer and allowed to dry. The strip was then treated with a dilute solution of ammonia (1% ; pH 9.8) containing 10 units of alkaline phosphatase and 10 mM final concentrations of $MgCl_2$ and $CaCl_2$. The strip was then incubated for 4 hours at 37°C in a dilute ammonia (1%) atmosphere. The strip was then removed, dried, stitched onto a fresh sheet of 3MM Whatmann paper rotated 90° relative to the original electrophoresis and re-electrophoresed for 30 minutes at pH 6.5. The paper was then stained with ninhydrin for peptide detection of off-diagonal peptides.

Partial Acid Hydrolysis

Partial acid hydrolysis was performed as previously described (Light, A., 1967). Irradiated crystals (0, 2 and 4 hours) were titrated to pH 2 and dried under reduced pressure in the presence of P_2O_5. The dried crystals were resuspended in 0.1 N HCl, flame sealed under vacuum and incubated at 110°C for 48 hours. Vacuum was released and the samples dried under reduced pressure. Samples were resuspended in 500 µl pH 2.1 buffer. Aliquots (50 µl) were subjected to HVPE for 30 minutes at a voltage gradient of 60 V/cm. Similarly, aliquots (50 µl) were subjected to electrophoresis at a potential gradient of 40 V/cm at pH 6.5.

Radiolabelling of Bt Crystals

Radioactive labelling of the crystals with ^{32}P-α-ATP and ^{3}H-(6-thymidine) was accomplished by adding 1 mCi of either ^{32}P-α-ATP or ^{3}H-(6-thymidine) to Bt cultures (1 L) in the log phase and during sporulation. The cultures were harvested and the crystals purified using density gradient centrifugation with Renografin as previously described.

5.3 Results

Two-Dimensional High Voltage Paper Electrophoresis

Isolation of the UV-induced DNA-protein crosslinked products was attempted by several methods. The first method attempted was the diagonal electrophoresis procedure that was devised specifically for this purpose. The method relies on the differential migration of peptides in an electric field as a result of enzymatic modification of the peptides previously electrophoresed in the first dimension. In the current example, the peptides were expected to form covalent linkages with nucleotides arising from the DNA associated with the crystals. The method was applied to non-irradiated crystals and crystals irradiated for different lengths of time (up to 24 hours). The irradiated crystals were subsequently treated with various combinations of proteolytic enzymes followed by nuclease treatment with RQ1 DNase and subjected to electrophoresis in the first dimension. Treatment with RQ1 DNase was expected to hydrolyze the DNA to nucleotides possessing a 3' – hydroxyl group and a free phosphate at the 5' position of the deoxyribose ring. The choice for modification of the peptide containing the covalently linked oligonucleotide was the

enzymatic removal of the phosphate group using alkaline phosphatase. This modification was expected to increase the basicity of these peptides such that, after electrophoresis in the second dimension. the peptides would be located above the diagonal line formed by peptides which were unaffected by treatment with alkaline phosphatase treatment.

In order to verify the feasibility of this method, the experimental conditions were first attempted on model compounds; phosphorylated amino acids and model phosphorylated proteins, β-casein and phosvitin. Samples of phosphotyrosine, phosphoserine and phosphothreonine were subjected to the experimental protocol excluding the digestion procedures with proteases. The phospho-amino acids were subjected to HVPE at pH 6.5 in the first dimension and re-electrophoresed at the same pH after cutting, digestion with alkaline phosphatase and stitching as described above. These compounds were found to produce off-diagonal spots as predicted and could easily be recovered from the electrophoresis paper for further analysis. Similarly, off-diagonal peptides were obtained when β-casein and phosvitin were subjected to the experimental conditions. The remarkable efficacy of the experimental method on model compounds (phosphorylated amino acids as well as β-casein and phosvitin; refer to Figure 5.4) was very encouraging.

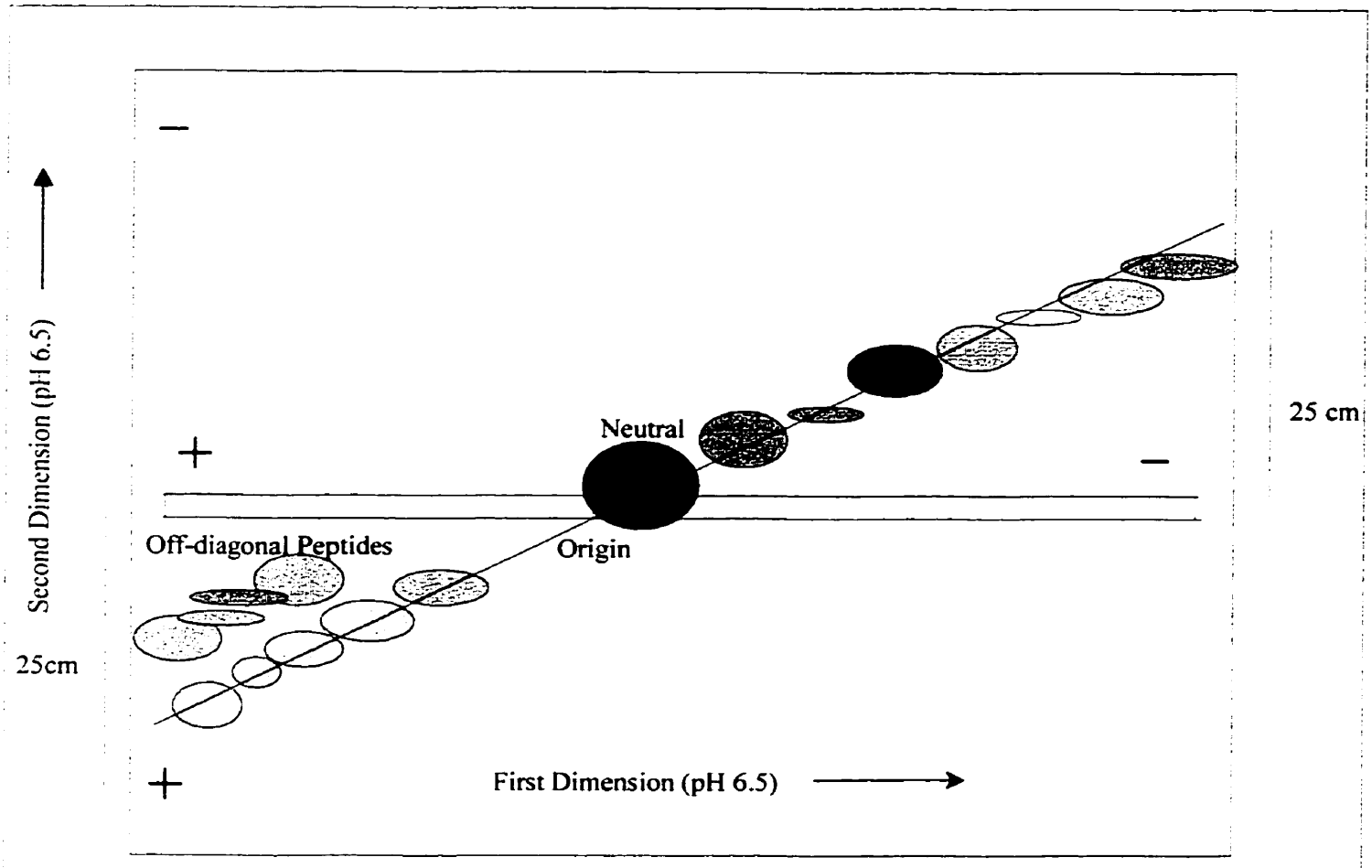


Figure 5.4 Two-Dimensional Electrophoresis of Pepsin-Digested Casein Treated with Alkaline Phosphatase

Off-diagonal peptides could be isolated from the electrophoretogram. The electrophoretic behaviour (slower in the second dimension) was as Predicted based on the removal of phosphate groups.

Attempts at isolation of putative photoproducts from irradiated Bt crystals by diagonal electrophoresis (2D-HPVE) were disappointing in that only minor amounts of off-diagonal, ninhydrin-positive material was ever observed after electrophoresis in the second dimension. Varying the duration of irradiation and the digestion conditions (treatment with different combinations of proteolytic enzymes) did not improve the results. In all cases, the digests remained mainly insoluble. These observations were confirmed by the fact that the soluble portion of the enzymatic digests contained relatively little ninhydrin positive material on the electrophoretogram, with most of the material remaining embedded in the paper at the origin of application. Streaking material originating from the origin of application, which stained positive with ninhydrin, was not considered as potentially useful due to variations in the electrophoretic profile. The lack of success is currently attributed to 1) a lack of sensitivity of the method and 2) the inability to adequately degrade the crosslinked material despite considerable enzymatic treatment. This second point is consistent with the results of Chapter 2 and 3, which confirm the non-digestibility of the UV irradiated crystals after even relatively short periods of irradiation. However, the lack of success may also be the result of other factors as discussed below.

Evidence obtained from the degradation pattern obtained when the DNA associated with the crystals is treated with restriction enzymes, as well as the partial sequences obtained for cloned fragments of the DNA, suggest that the DNA is heterogeneous (Dr. Hans Shemthaner; unpublished results). Furthermore, chemical analysis of the DNA/protein ratio for the DNA-crystal protein complexes suggests that only a very small portion of the protein is interacting with the DNA (Clairmont et al., 1998). With this in mind, it is quite possible that UV irradiation produces a wide distribution of products and in quantities well below the levels of detection obtainable by ninhydrin

staining. In theory, only a very small amount of crosslinking between the protein and DNA components would be required to cause the large apparent increase in molecular mass of the UV-irradiated material observed in the experiments described in earlier chapters. These results led to attempts by other methods of analysis which are presented below.

A second strategy was to subject the crosslinked crystals to partial acid hydrolysis. Conditions employed for partial acid hydrolysis have been reported to produce selective cleavage of polypeptides at aspartic acid residues (Light, A., 1967). The results obtained for the hydrolysis of non-irradiated crystals and irradiated crystals (2 and 4 hours), produced similar peptide patterns upon ninhydrin staining and phosphate staining of chromatograms subjected to HVPE in one dimension at pH 2.1 and pH 6.5. The apparent lack of success by this method led to yet another strategy aimed to increase the sensitivity of the diagonal procedure.

In an attempt to increase the sensitivity of detection by diagonal electrophoresis, Bt crystals were grown in the presence of ^{32}P-α-ATP and ^{3}H-(6-thymidine). Analysis of irradiated crystals by HVPE obtained from cultures grown in the presence of ^{32}P-α-ATP showed very low levels of incorporation of the label. The low levels of radioactivity in the electrophoretograms were ascribed to low levels of incorporation of the label into the crystals and losses due to the length of the procedure, requiring approximately two weeks from growth of the crystals to analysis by HVPE. Monitoring of the distribution of the radiolabel during the purification procedure of the crystals indicated that most of the incorporated radiolabel appeared to be located in the spores that are produced along with the crystals during sporulation. Unfortunately, application of the diagonal procedure on the radiolabelled crystals was still unsuccessful in detecting the crosslinked products

and again showed a large quantity of material at the origin despite extensive treatment with proteases and nucleases.

In a final attempt, Bt crystals were obtained from cultures grown in a medium containing 1 mCi ^{3}H-(6-H-thymidine) since thymine has been shown to be the most photo-reactive of the nitrogenous bases in DNA. Liquid scintillation counting of aliquots of the growth medium, the layers obtained by Renografin purification, as well as from each step of the harvesting and purification procedure gave the following isotope distribution: 89% unincorporated, 0.3 % in the "top-scum" layer (cellular debris, partially synthesized protein and DNA), 8.1 % in the spores and only 0.4 % incorporation in the crystals. The low levels of incorporation suggested that further investigations in this area would not be successful.

5.4 Discussion

Nucleic acid (RNA and DNA)-protein interactions are responsible for directing many of the essential processes in cells. Replication of genetic material requires numerous interactions between the genetic material (DNA or RNA) with several enzymes (polymerases, gyrases, helicases, integrases). Protein synthesis is directed by DNA-protein interactions to synthesize mRNA (transcription) and ultimately RNA-protein interactions (translation) to complete the process. To fully describe how these processes occur, it is necessary to understand and characterize the interactions between nucleic acids and proteins at the molecular level.

The interactions between nucleic acids and proteins have be categorized in terms of their functional and structural roles and by the degree of specificity observed in the complexes that are

formed (Neidle, S., 1994). Regulatory proteins, such as those responsible for the process of transcription, tend to form complexes with duplex DNA and generally exhibit a high degree of specificity. DNA cleavage proteins such as nucleases (eg: DNase I) generally display low specificity while restriction enzymes (eg: EcoRI) are tend to be highly specific for particular DNA sequences. Processing proteins such as DNA and RNA polymerases, structural proteins (eg: histones) and repair proteins must also form intimate contacts with nucleic acids in order to fulfill their roles.

Evidence for the covalent attachment of protein and nucleic acids subjected to UV irradiation is well documented and has been used as an important analytical tool in the elucidation of the sites and mode of interaction in DNA-protein complexes ((Lin, S.-Y. and Riggs, A.D., 1974; Paradiso P.R. et al., 1979; Harrison, C.A., 1982; Hockensmith, J.W. et al., 1986; Jensen, O.N. et al., 1993). Most often, ultraviolet crosslinking of proteins and nucleic acids is accomplished by irradiation at short wavelengths ($\leq$254 nm), using conventional germicidal UV lamps, and more recently using lasers which can deliver pulses of high energy monochromatic radiation in the nanosecond or picosecond range (Harrison, C.A., 1982; Budowsky, E.I., et al., 1986; Angelov, D., et al., 1988; Buckle, M., et al., 1991; Pashev, I.G., et al., 1991; Jensen, O.N. et al., 1993; Harrison, C.A., 1982). Even so, the yield of covalently linked DNA-protein complexes is generally low (<20 %).

UV-induced crosslinking of the protein and DNA components represent only one possible explanation for the observed photoinstability of *Bacillus thuringiensis* products. Several other probable explanations (vide infra) can be invoked to account for the observed photoinactivation of *Bacillus thuringiensis* products.

Initial formation of the radicals may result from the absorption of radiant energy, as outlined above, but may also be the result of secondary reactions that are initiated by other chemical species such as metal ions. Fenton chemistry, production of radicals through Fe^{2+} and Fe^{3+} catalyzed decomposition of hydrogen peroxide which yield the hydroxyl radical ($OH^{\cdot}$) and superoxide anion ($O_2^{\cdot-}$) respectively, has been shown to be a major source of free radicals in biological systems and a number of possible mechanisms have been proposed (Stadtman, E.R and Oliver, C.N., 1991).

Photolysis and photo-oxidation studies of amino acids have shown that a number of transformations can occur and depend on the source of radiation, solvent conditions as well as the length of exposure. One such study involved the irradiation of aqueous solutions of cystine with various light sources (Forbes W.F. and Savige, W.E., 1962). The results of this study showed that at least nineteen different products were possible of which ten were characterized.

Likewise, independent studies regarding the photoproducts of DNA and products derived by other free radical reactions have been conducted. Fisher and Johns (Fisher G.J. and Johns, H.E., 1970) studied the ultraviolet photochemistry of thymine, orotic acid, uracil and thymidine in aqueous solutions. The net result of these studies was the identification of a number of photodimers resulting from irradiations at 265 nm at 24°C. At least fourteen different products were observed. With this in mind, the possibility of characterising the products obtained from the Bacillus thuringiensis system appeared to be a formidable task.

The diagonal electrophoresis method devised for the isolation of the crosslinked products was unsuccessful. The lack of success is attributed to the lack of sensitivity of detection, the non-digestibility of the crystals and potentially to a low yield and unfavourable distribution of products.

Just the same, the method was shown to be extremely successful in the identification of phosphorylated peptides and is a new procedure that may be potentially useful it in own right.

5.5 Conclusion

The identification of the sites of interaction and crosslinking between the DNA and protein components will require further investigation. One possible explanation, for the limited success in the identification of DNA-protein crosslinked products in the current studies, may be the composition of the DNA associated with the Bt crystals. A reduced number of specific DNA-protein contacts, as a result of the heterogeneity of the DNA, could have limited the yield of any specific product. However one of the greatest difficulties is the lack of digestibility of irradiated crystals towards proteases and nucleases.

Despite the lack of success by the approaches taken in the current studies, other potential strategies for the identification of the sites of DNA-protein interactions in the Bt crystals can be proposed. Perhaps the best route to identify the sites of interaction would involve chemical labelling of the toxin in the presence and absence of the DNA. The DNA-toxin complex could be reacted with ^{14}C-MeI by nonaqueous chemical modification (Taralp and Kaplan, 1997), and compared to the same chemical modification of the DNA-free toxin; analogous to chemical / enzymatic "footprinting". Comparison of the results with the labelling of DNA-free should provide information with respect to the sites protected from modification; the sites of interaction with the DNA.

As a second possibility, the crystals could be grown in the presence of a radiolabelled amino acid, rather than a DNA constituent, which may be more feasible overall. ^{14}C-arginine or lysine, for example, might be selected based on the high occurrence of these amino acids in many DNA binding proteins (Freemont, P.S., et al., 1991). The use of radioisotopes is however fairly expensive, and based on the limited incorporation of the radioisotopes into the DNA associated with the crystals, other alternatives may be more feasible until further evidence is obtained with respect to the regions of the toxin which are involved in the DNA-protein interactions.

Chapter 6

Determination of the C-terminal Sequence(s) of Proteins: Application to the C-terminal Sequence of *Bacillus thuringiensis* Crystal Protein

6.1.1 Introduction

The complete characterization of proteins requires the determination of the primary, secondary, tertiary and quaternary (if existing) structures. Knowledge of the primary structure is the most basic requirement as it determines the remaining levels of organization of the polypeptide. The development of N-terminal sequencing by Edman, was a major development in sequencing strategies and provided an efficient method for determining the amino acid sequence of peptides (Edman, P. and Begg, G., 1967). Before the development of DNA sequencers, the primary structures of proteins were determined by enzymatic degradation or chemical cleavage of the polypeptide chain, with subsequent alignment of peptides sequences. However, this approach was labor intensive and unambiguous assignment was rendered difficult at times due to missing sequences (Creighton, T.E., 1993c). The current methodology for N-terminal sequence determination is quite successful and sequences in the range of 40 - 70 amino acids can now be determined on automated sequenators (Ward, C.W., 1986). The major problem associated with N-terminal sequencing is the occurrence of natural N-terminal blocking groups such as formyl (f-Met), acetyl and pyroglutamyl groups (Ward, C.W., 1986). In such cases, the Edman N-terminal sequencing methodologies cannot be easily used without further modification of the protein.

Currently, the primary structures of proteins are deduced from the gene nucleotide sequences encoding the proteins (Creighton, T.E., 1993a). This approach allows for the determination of the primary structure of any protein regardless of any posttranslational modifications. However, the determination of the primary structure of a protein from the

gene nucleotide sequence still requires that the amino acid sequence of small portions of the polypeptide be determined by conventional sequencing techniques in order to synthesize DNA probes for isolation of the gene. The gene can be located by means of hybridization of a cDNA probe synthesized on the basis of the partial protein sequence (Creighton, T.E., 1993b). The main difficulty with primary structure determination through gene sequence analysis is the inability to predict the final primary structure of the mature polypeptide. This problem arises as a result post-translational modifications of the primary gene product. Proteolytic processing of the precursor polypeptide (primary gene product), to yield the mature form of the protein, produces modifications which are not evident in the sequence determined by DNA sequencing methods. Ultimately, N-terminal and C-terminal sequencing strategies must be performed to obtain the sequence of a mature polypeptide, and therefore, these methods remain crucial to the analysis of proteins. With the growing availability of proteins obtained by recombinant DNA technology (Jackson, D., et al., 1972; Cohen, S., et al., 1973) and PCR (Mullis K., et.al., 1986), the use of rapid and reliable C-terminal sequencing strategies has become increasingly important.

Unfortunately, no reliable methodology for the determination of the C-terminal amino acid sequence of proteins has been developed that approaches the generality, sensitivity and reliability of the Edman degradation procedure. The currently available methods have limitations and none are of general applicability (Lundblad, R.L. and Noyes, C.M., 1995). A number of methods have been devised for the determination of the C-terminal sequences or the identification of C-terminal amino acids in a variety of proteins, however the success of individual methods is generally highly sequence

dependant due to limitations arising from side reactions, low yields or lack of sensitivity. These strategies are usually based on selective modification of the C-terminal carboxyl group of proteins, coupled with an isolation procedure (IEC, RP-HPLC, CE, HVPE) which exploits the particular modification (Ward, C.W., 1986). One of the more successful chemical modification techniques involves carbodiimide coupling of the C-terminal carboxyl group (Duggleby and Kaplan, 1975). These authors isolated C-terminal peptides from the α- and β-chains of human hemoglobin by amidation of the activated carboxyl groups with ethanolamine followed by enzymatic digestion with thermolysin. The digest was subjected to two-dimensional (diagonal) electrophoresis at pH 2.1 and pH 4.4 respectively. This isolation procedure exploits the fact that the electrophoretic mobility of the chemically modified peptides with a blocked C-terminus will not be affected and will lie along a diagonal line (Duggleby and Kaplan, 1975). Peptide detection was achieved using ninhydrin staining. The only limitation to this method lies in the sensitivity of detection, which requires that relatively large amounts of protein (in the order of 0.5 - 1 μmole protein) be used due to losses in the isolation procedure.

A similar approach, modified to increase the sensitivity of the method, was successful in isolating the C-terminal peptides from the Bt toxin generated from the crystals of *Bacillus thuringiensis* var *kurstaki* HD-73 (Bietlot et al., 1989b). The C-terminal labelling reaction was conducted using undiluted ^{14}C-methylamine, in order to maximize the incorporation, followed by the addition of excess methylamine to complete the derivatization procedure. Furthermore, the toxin was suspected to have a C-terminal lysine residue as a result of trypsin activation, so the toxin was ^{14}C-acetylated prior to the application of the C-terminal diagonal procedure. The method was successful in isolating

and identifying a C-terminal peptide containing two amino acid residues, glutamic acid, and lysine, the C-terminal end-group (Bietlot, H.P., et al., 1989b). The increased sensitivity of this method was achieved by using radiolabelling as a means of detection, which eliminates unnecessary losses during the isolation procedure; the peptides are recovered directly from the electrophoretogram without losses due to staining, which is required for detection.

The current emphasis in research and development is the production of automated C-terminal sequencing systems analogous to those developed for the Edman procedure (Bailey, J.M., et al., 1993; Bailey, J.M., et al., 1994; Bailey, J.M., et al., 1995). However, the difficulties related to lack of performance with certain amino acid compositions remain to be solved. Recently, attempts to improve the C-terminal sequence methodology have been made with approaches that rely on the enzymatic (Dal Degan, F., et al., 1992; Patterson, D.H., et al., 1995; Bonetto, V., et al., 1997) or chemical degradation (Tsugita, A., et al., 1992; Shenoy, N.R., et al., 1993; Takamoto, K., et al., 1995; Thiede, B., et al., 1997; Bilan, M., et al., 1997) of the C-terminus, followed by sequencing of the released fragments from the N-terminus by Edman degradation, or by direct determination of the sequence of the C-terminal fragment or amino acids by tandem mass spectroscopy. The majority of these studies have been performed on peptides and proteins with relatively short sequences, and none have been successfully applied to the determination of unknown C-termini.

6.1.2 Rationale for the Current Investigation

The work described in this chapter was undertaken to provide a more sensitive and rapid method for the identification of peptides derived from the C-terminal, which is easily performed, and of general applicability. The procedure was developed for application to the identification of the C-terminus of the protoxin and toxin produced by *Bacillus thuringiensis* and the cleavage sites of the protoxin during the activation of the protoxin to toxin. Two well-characterized proteins, bovine pancreatic ribonuclease, which contains a single C-terminus, and bovine α-chymotrypsin, which contains three polypeptide chains, were used as test proteins.

6.1.3 Strategy for the Isolation of C-terminal Peptides

The methodology involves the formation of radiolabelled methyl esters of all carboxyl residues including the C-terminal carboxyl group with ^{14}C-methyl iodide followed by enzymatic cleavage of the polypeptide. C-terminal peptide isolation is achieved through peptide purification by two-dimensional high voltage paper electrophoresis (2D-HPVE) (Duggleby and Kaplan, 1975).

The derivitization of the carboxyl groups via a non-aqueous chemical modification procedure (Taralp, A. and Kaplan, H., 1997) with radiolabelled reagent increases the sensitivity of detection of diagonal peptides from the electrophoretogram obtained after two-dimensional electrophoresis and autoradiography. The labelling procedure, which is accomplished in-vacuo, greatly increases the amount of radiolabel which is incorporated into the protein, increasing the ease of detection by

autoradiography. The strategy developed for the isolation of C-terminal peptides is outlined in Figure 6.1 and the isolation by 2-D HPVE is shown in Figure 6.2.

Figure 6.1

Strategy For Isolation of C-terminal Peptides

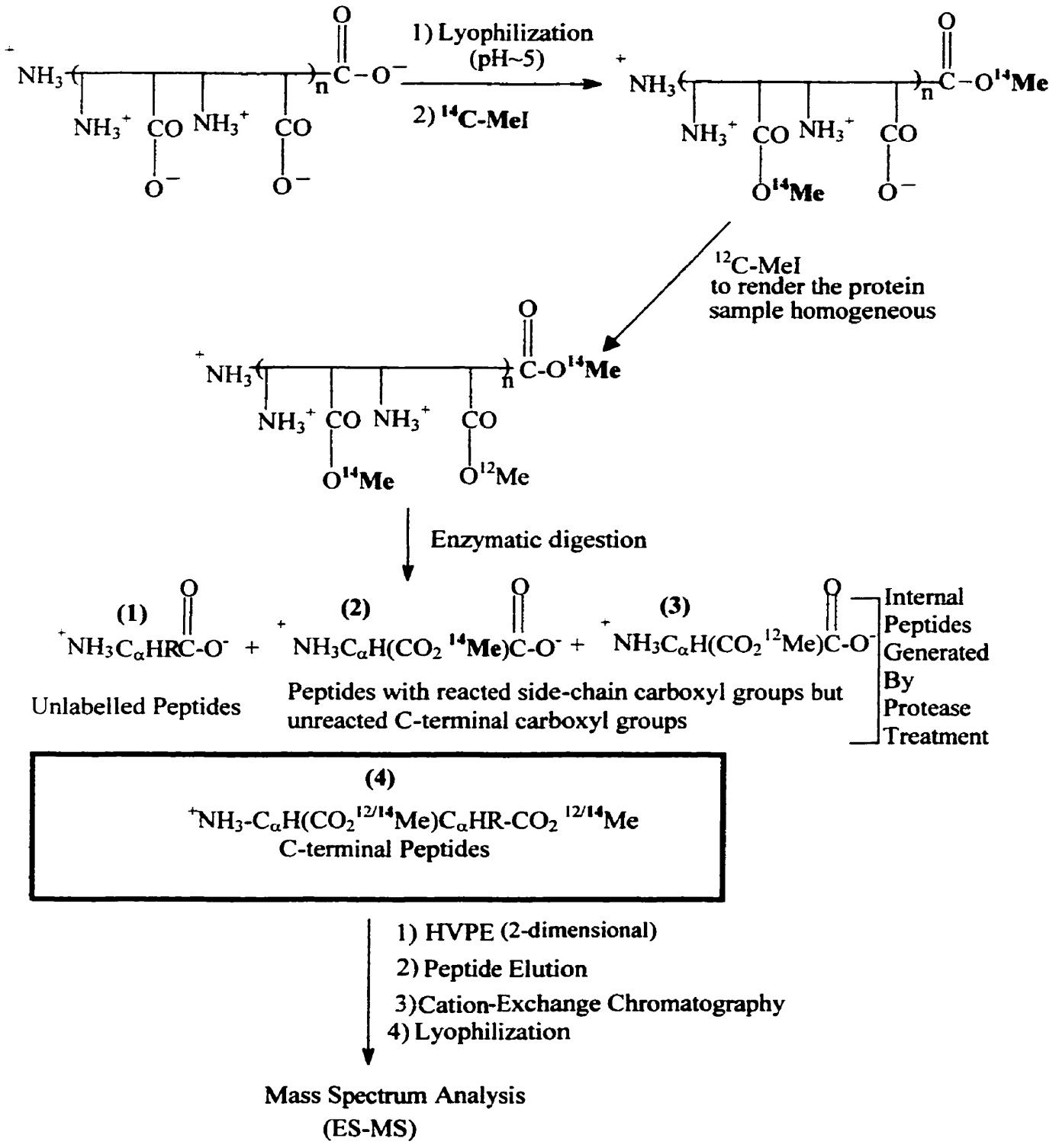
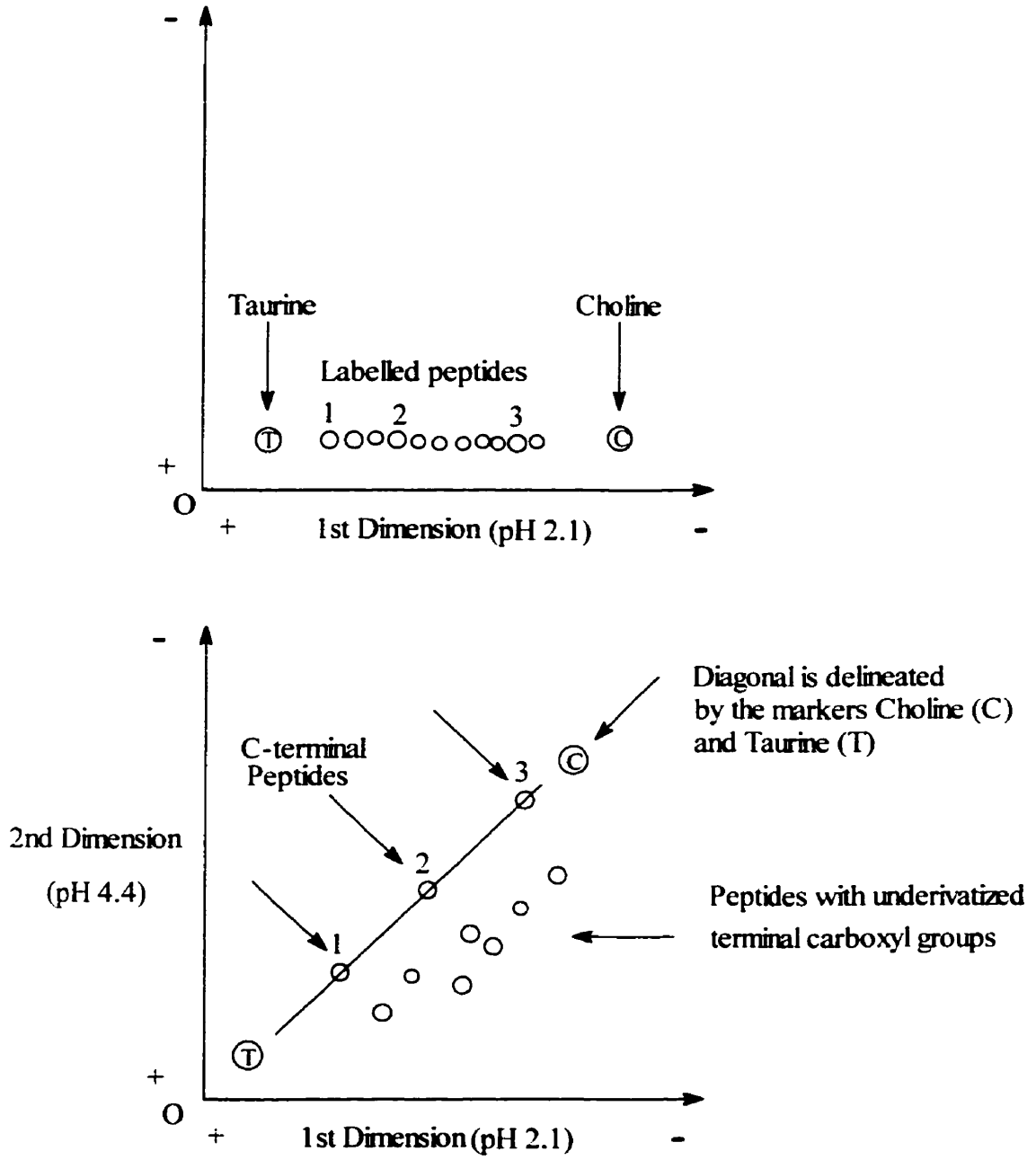


Figure 6.2

Two-dimensional HVPE For C-terminal Peptide Isolation



6.2 Experimental Procedures

6.2.1 Materials

Enzymes (Proteins)

Bovine α-chymotrypsin (EC 3.4.21.1) and pancreatic ribonuclease A (EC 3.1.27.5) were obtained from Sigma and used without further purification. Leu-Enkephalin and all other peptides were purchased from Sigma. *Bacillus thuringiensis* crystal protein was obtained and purified from crystal preparations as described in Chapter 1. CAM-protoxin (S-carbaminomethyl derivative) was prepared as previously described (Choma et al., 1992).

Chemicals and Solvents

^{14}C-CH_3I (1 mCi) was purchased from Amersham Chemicals. The working stock was obtained by resuspending the radiolabelled reagent in anhydrous octane to yield a specific radioactivity of 0.5 mCi/ml. The stock solution was stored at -4°C.

6.2.2 Method

6.2.2.1

Initial Development of the C-terminal Isolation Procedure

Preparation of Standards

Standard methyl esters were prepared for analysis by ES-MS in order to verify the utility of the devised method, to determine the expected fragmentation patterns for peptidyl methyl esters, and to develop the analytical conditions to be employed for the analysis of peptides to be isolated from the test (α-chymotrypsin and ribonuclease) and unknown (Bt crystal protein) proteins.

Standards were prepared for Leu-Enkephalin (Tyr-Gly-Gly-Phe-Leu), Phe-Gly-Gly, tetra-Gly and tri-Ala. The methyl esters were prepared by dissolving the peptides (1-2 μmoles) in cold anhydrous methanol (1.5 ml) with the addition of concentrated HCl (12.5 μl) (Means and Feeney, 1971). The reaction mixture was mixed on a wrist shaker at room temperature for 24 hours and the progress of reaction was monitored by HPVE (pH 4.4, 30 min, 2 kV) on small aliquots of the reaction mixture. The mixture was dried under a stream of nitrogen and purified by HVPE (same conditions) with a guide strip being stained with ninhydrin to locate the peptides. The esterified peptides were eluted from the paper in a vertical chromatographic elution chamber and the eluates were lyophilized overnight. The standards were sent for analysis by ES-MS.

Protein Sample Preparation

α-Chymotrypsin and ribonuclease were dissolved in distilled H_2O to a concentration of 20 mg/ml. The pH of the solution was adjusted to pH 4, 4.5 or 5 with dilute HCl and the final pH adjustments were monitored using a pH meter equipped with a calomel electrode. Equal aliquots of the proteins were added to individual disposable culture tubes and immediately frozen by immersing the tubes in liquid nitrogen. The frozen samples were lyophilized overnight and the tubes containing the lyophilized proteins were transferred into larger Pyrex screw-top test tubes for subsequent chemical modification under non-aqueous conditions.

Non-aqueous Labelling Reaction

^{14}C-Methyl iodide (50 μCi), dissolved in anhydrous octane, was added to each tube which were simultaneously immersed in liquid nitrogen to freeze out the radioactive labelling reagent along the inside of the screw-cap tubes. The tubes were quickly flame-sealed under vacuum and allowed to react at 80° C in a heating block for 24 hours. The tubes were then scored and vacuum released by applying a heated glass rod to the scored tubes. To ensure complete esterification, the procedure was repeated with ^{12}C-CH_3I under identical conditions for another 24 hours. The tubes were then scored and vacuum released.

Enzymatic Digestion of the Labelled Protein

The ^{14}C-methylated protein samples were transferred to round bottom flasks by resuspending the lyophilized protein samples in a total of 10 mls pH 2.1 buffer. Pepsin was added to yield a protein:pepsin ratio of 10:1 with the enzymatic digestion was allowed to proceed for 12 hours at room temperature at which time the digestion was terminated by lyophilization.

High Voltage Paper Electrophoresis (HPVE) of the Labelled Proteins

1st Dimension

The lyophilisates were resuspended in 2 mls of pH 2.1 buffer and 100 µl of the digestion mixture was applied to 3MM Whatman paper along with 30 µl of a mixture of ^{14}C-choline and ^{14}C-taurine as markers (to delineate the diagonal in the second dimension). The samples were subjected to high voltage paper electrophoresis (HPVE) in the first dimension at pH 2.1 with a voltage gradient of 40 V/cm for 30 minutes. The paper was removed from the electrophoresis chamber and allowed to air dry.

2nd Dimension

Paper strips containing the samples subjected to electrophoresis in the first dimension were cut and stitched (at 90° with respect to the origin from the first dimension) onto a fresh sheet of 3MM Whatman paper. The sample was then subjected

to HVPE in the second dimension at pH 4.4 at 40 V/cm for 30 minutes. Once again, the paper was removed from the electrophoresis chamber and allowed to air dry.

Autoradiography

After drying, the papers were marked with radioactive ink and autoradiographed with Fuji x-ray film for upto 72 hours. The films were developed and used to locate the radioactive peptides on the paper by aligning the film with the radioactive ink spots.

Peptide Recovery

The radioactive peptides located on the diagonal line delineated by ^{14}C-taurine (origin) and ^{14}C-choline (extremity) (refer to Figure 6.2) were cut from the electrophoretogram. Elution was carried out by eluting the cuttings over CMC (carboxymethyl cellulose) columns (0.5 cm x 3 cm), rinsing the column with 3 mls of dH_2O, and eluting from the column with pH 2.1 buffer. The elution buffer was removed by lyophilization.

Analysis of the On-Diagonal Peptides

The lyophilized on-diagonal peptides were analyzed by ES-MS by Dr. Pierre Thibault at the Institute of Biological Sciences (IBS) at the National Research Council of Canada (Ottawa, Ontario). The analyses were performed on a Perkin-Elmer PE SCIEX API 300 Electrospray Mass Spectrometer.

6.2.2.2

Application to the Crystal Protein from *Bacillus thuringiensis* var *kurstaki* HD 73

In order to minimize the incorporation of the radioactive reagent into the crystal protein, the crystal protein was chemically modified to the S-carbaminomethyl derivative by the method of Choma et al. (1992). The derivatized protein was extensively dialyzed in distilled water / HCl (pH 4). The precipitated protein was quantified, by dissolving a small aliquot of the protein at pH 10.5 in bicarbonate/carbonate buffer, using the standard Bio-Rad Protein Assay method. The pH of the protein suspension was adjusted to pH 4.5 for reaction using dilute HCl. Aliquots containing 2 mg of protein were transferred to disposable culture tubes and lyophilized overnight. The lyophilized pellets were then subjected to the same experimental procedure described for α-chymotrypsin and ribonuclease.

6.3 Results

Standards

The ES-MS spectrum for Leu-Enkephalin-OMe is shown in Figure 6.3. The sequence deduced from the ES-MS spectrum yielded the sequence Tyr-Gly-Gly-Phe-Leu, consistent with the known sequence for this pentapeptide (Table 6.1). The MS-MS spectrum for the molecular ion peak (570.4) is shown in Figure 6.4.

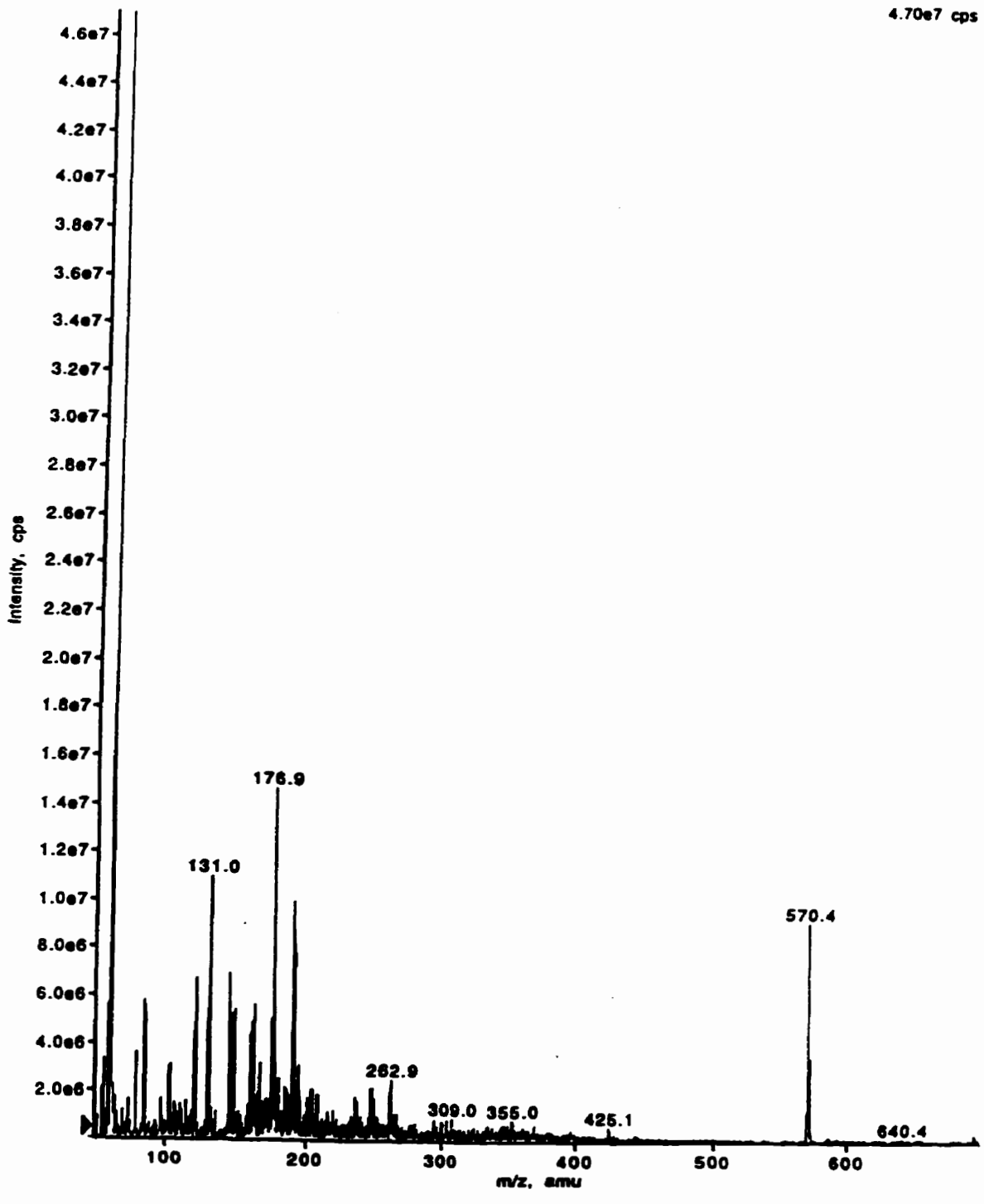


Figure 6.3 ES-MS Spectrum of Leu-Enkephalin-OMe

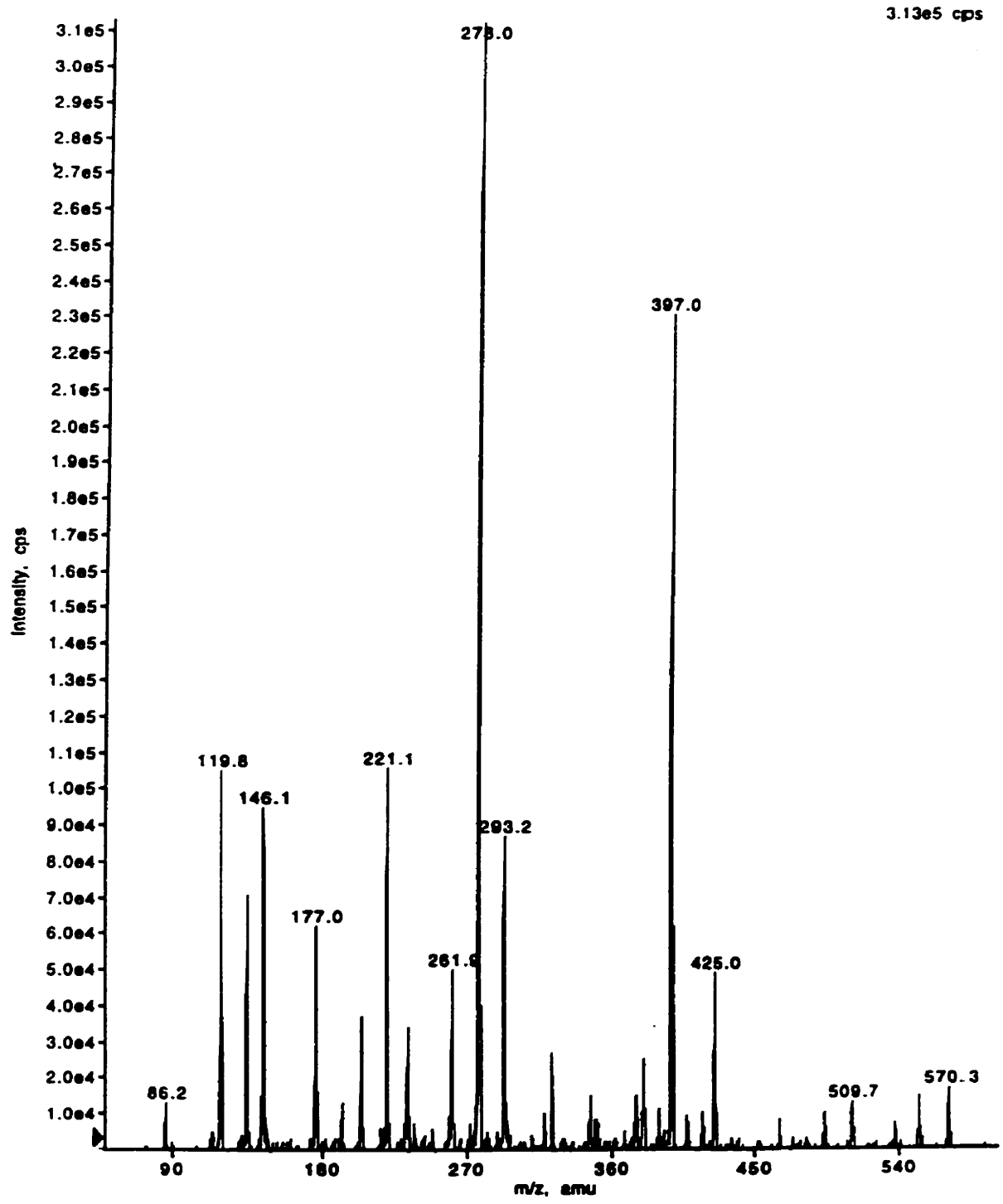


Figure 6.4 MS-MS Spectrum of the Molecular Ion Peak (m/z 570.4) of Leu-Enkephalin-OMe

Table 6.1 **Amino acid composition deduced from the MS-MS spectrum of the molecular ion peak (570.4) of Leu-Enkephalin-OMe**

Protein Composition and Molecular Weight

	Amino Acid		Number	% residues
A	Ala	Alanine	0	0.00
B	---	Undefined	0	0.00
C	Cys	Cysteine	0	0.00
D	Asp	Aspartic Acid	0	0.00
E	Glu	Glutamic Acid	0	0.00
F	Phe	Phenylalanine	1	20.00
G	Gly	Glycine	2	40.00
H	His	Histidine	0	0.00
I	Ile	Isoleucine	0	0.00
J	---	Undefined	0	0.00
K	Lys	Lysine	0	0.00
L	Leu	Leucine	1	20.00
M	Met	Methionine	0	0.00
N	Asn	Asparagine	0	0.00
O	---	Undefined	0	0.00
P	Pro	Proline	0	0.00
Q	Gln	Glutamine	0	0.00
R	Arg	Arginine	0	0.00
S	Ser	Serine	0	0.00
T	Thr	Threonine	0	0.00
U	---	Undefined	0	0.00
V	Val	Valine	0	0.00
W	Trp	Tryptophan	0	0.00
X	---	Undefined	0	0.00
Y	Tyr	Tyrosine	1	20.00
Z	---	Undefined	0	0.00

Average Mass = 569.66
Monoisotopic Mass = 569.28
(Computed Without Disulfide Bonds)

α-Chymotrypsin

The diagonal electrophoretogram for α-chymotrypsin is shown in Figure 6.5. The on-diagonal peptides can be observed along the diagonal line delineated by ^{14}C-taurine and ^{14}C-choline. Five peptides were eluted and sent for ES-MS analysis.

Ribonuclease

The diagonal electrophoretogram for ribonuclease is shown in Figure 6.6. Two on-diagonal peptides can be observed along the diagonal line delineated by ^{14}C-taurine and ^{14}C-choline. Both peptides were recovered and sent for ES-MS analysis.

CAM-Protoxin

The diagonal electrophoretogram for CAM-protoxin, prepared from *Bacillus thuringiensis* var *kurstaki* HD-73 is shown in Figure 6.7. Several on-diagonal peptides can be seen along the diagonal line (delineated by ^{14}C-taurine and ^{14}C-choline). The most prominent peptides, R-1 and R-2, were recovered as previously described and sent for ES-MS analysis.

Figure 6.5 Two Dimensional (Diagonal) High Voltage Paper Electrophoresis Of α-Chymotrypsin

Pepsin-treated ^{14}C-OMe-α-Chymotrypsin (1 mg) was subjected to two-dimensional electrophoresis (pH 2.1; pH 4.4) and autoradiographed for 48 hours. The on-diagonal peptides (C-1 through C-4) were recovered, purified on CMC-cellulose and sent for ES-MS analysis.

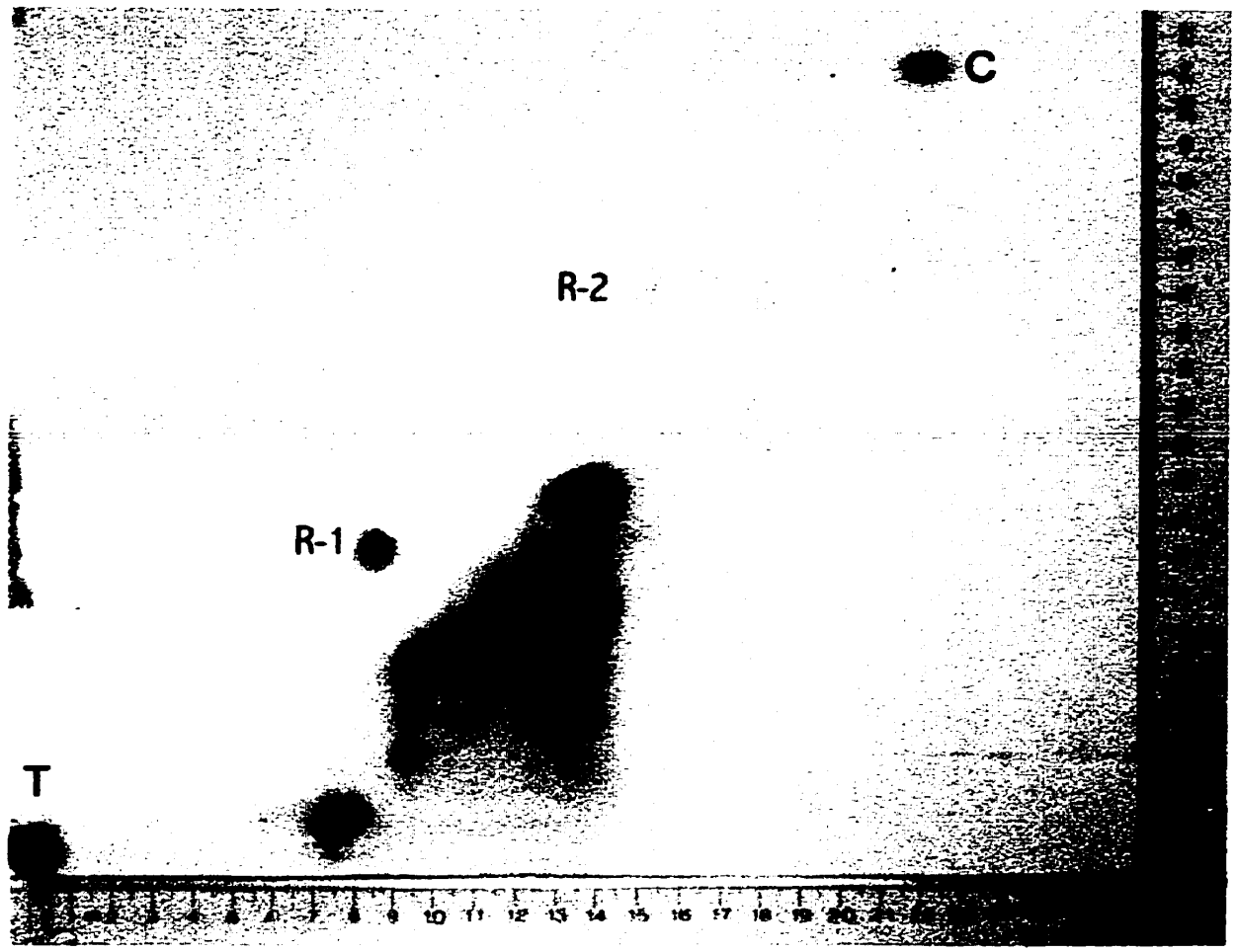


Figure 6.6 Two Dimensional (Diagonal) High Voltage Paper Electrophoresis Of Ribonuclease A

Pepsin-digested ^{14}C-OMe-Ribonuclease A (1 mg) was subjected to two-dimensional electrophoresis (pH 2.1; pH 4.4) and autoradiographed for 48 hours. The on-diagonal peptides (R-1 and R-2) were recovered, purified on CMC-cellulose and sent for ES-MS analysis.

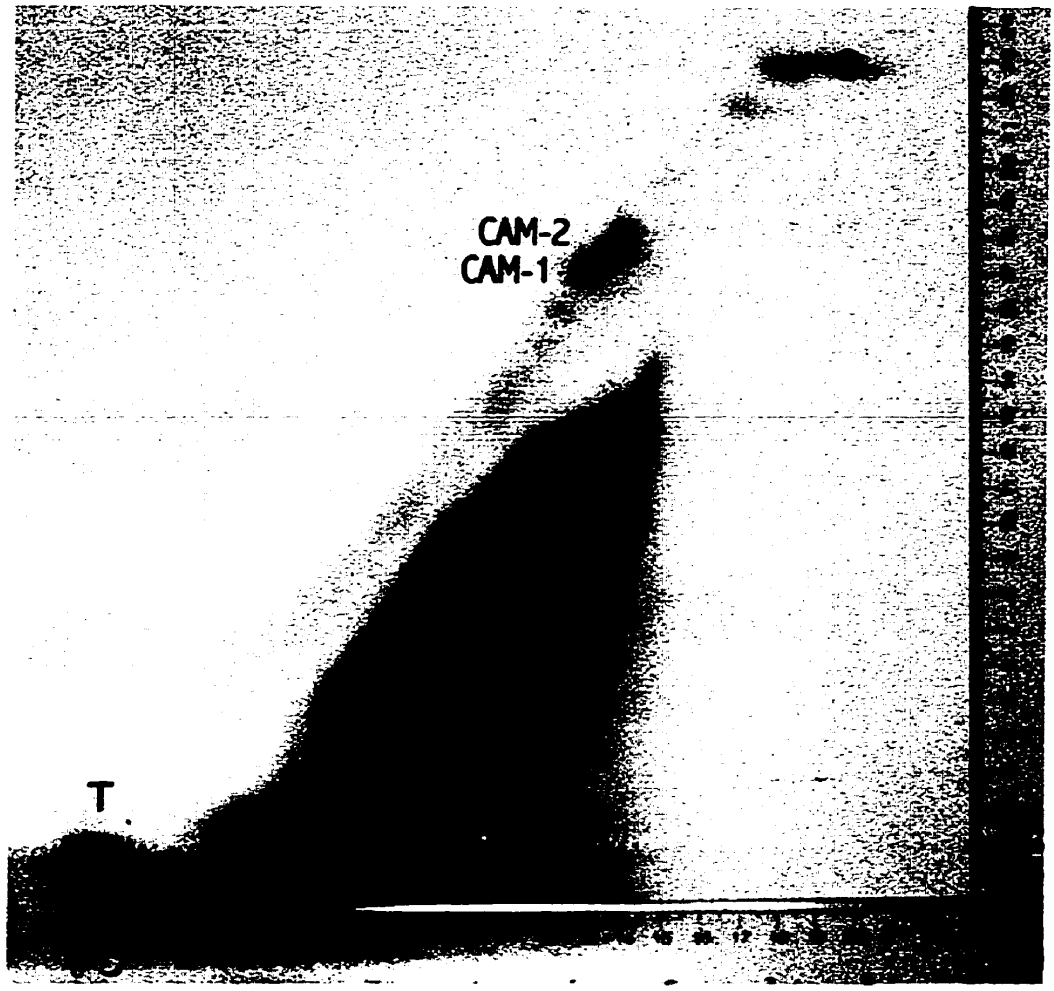


Figure 6.7 Two Dimensional (Diagonal) High Voltage Paper Electrophoresis Of Bt CAM-protoxin (S-carbaminomethylated)

Pepsin-digested ^{14}C-OMe-(S-carbaminomethylated) Bt protoxin (0.5 mg) was subjected to two-dimensional electrophoresis (pH 2.1; pH 4.4) and autoradiographed for 5 days. The on-diagonal peptides (C-1 through C-4) were recovered, purified on CMC-cellulose and sent for ES-MS analysis.

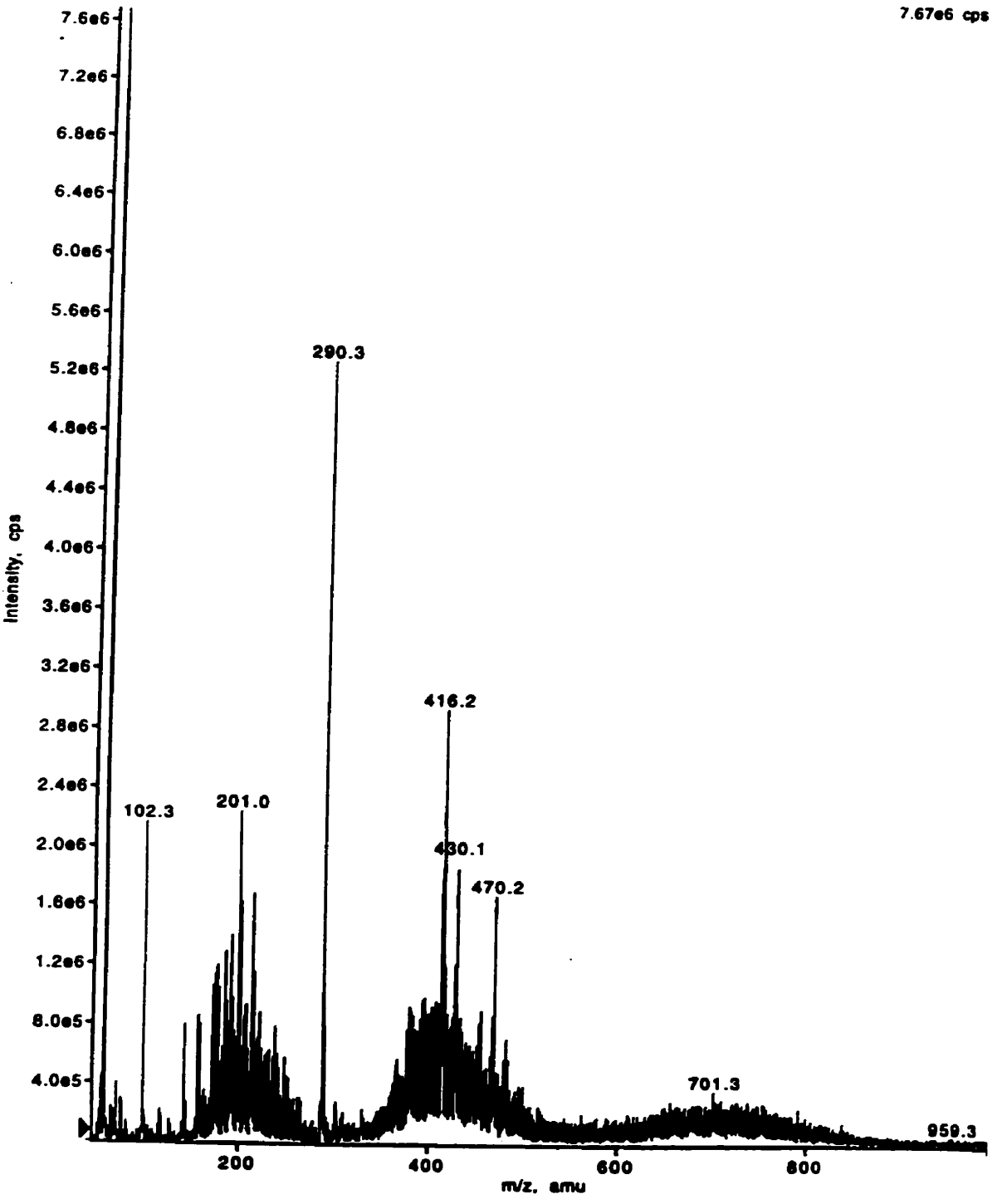


Figure 6.8 ES-MS of the Diagonal Peptide C-4 of α-Chymotrypsin (Figure 6.5)

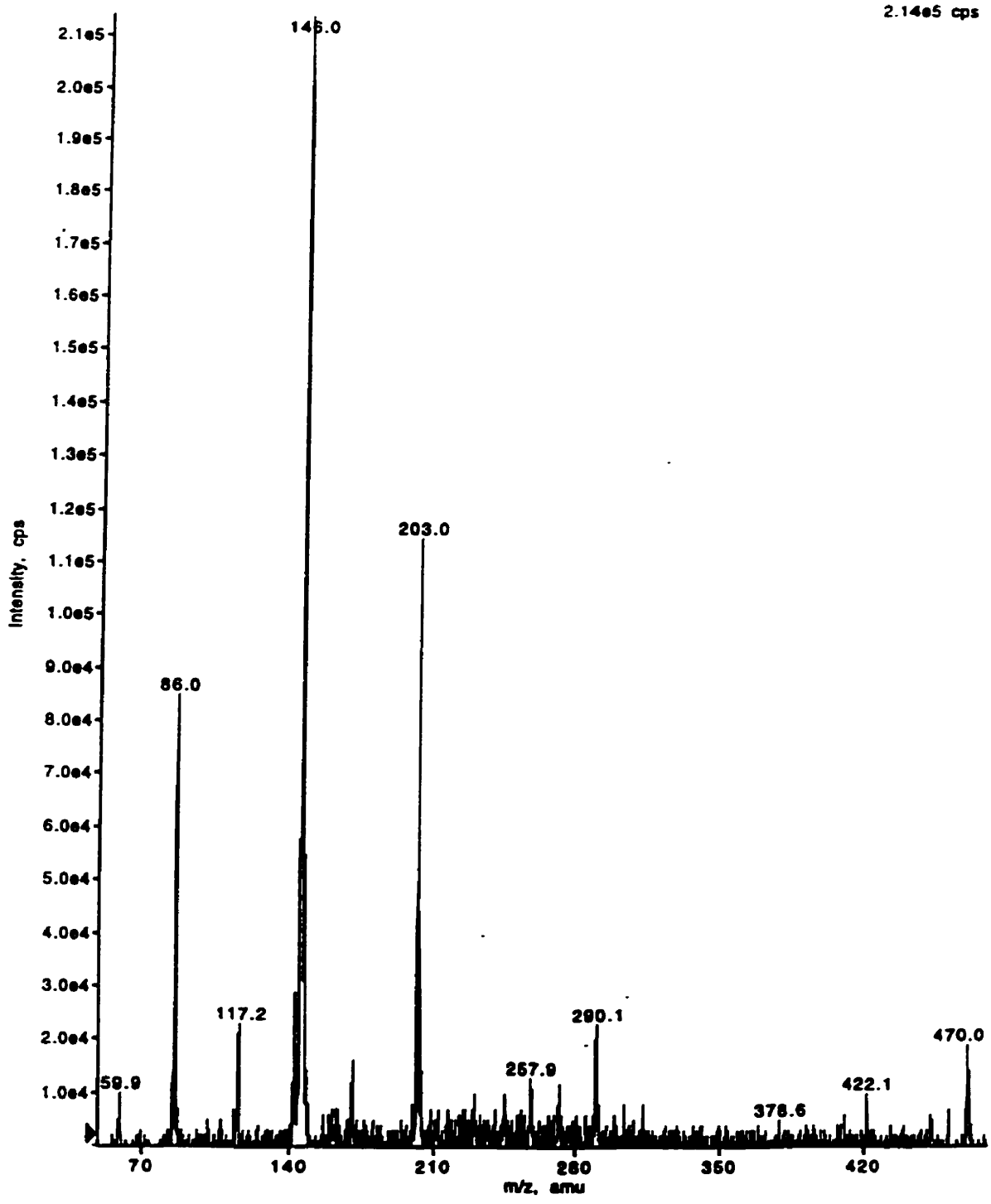


Figure 6.9 MS-MS spectrum (m/z 470) of peptide C-4 of α-Chymotrypsin

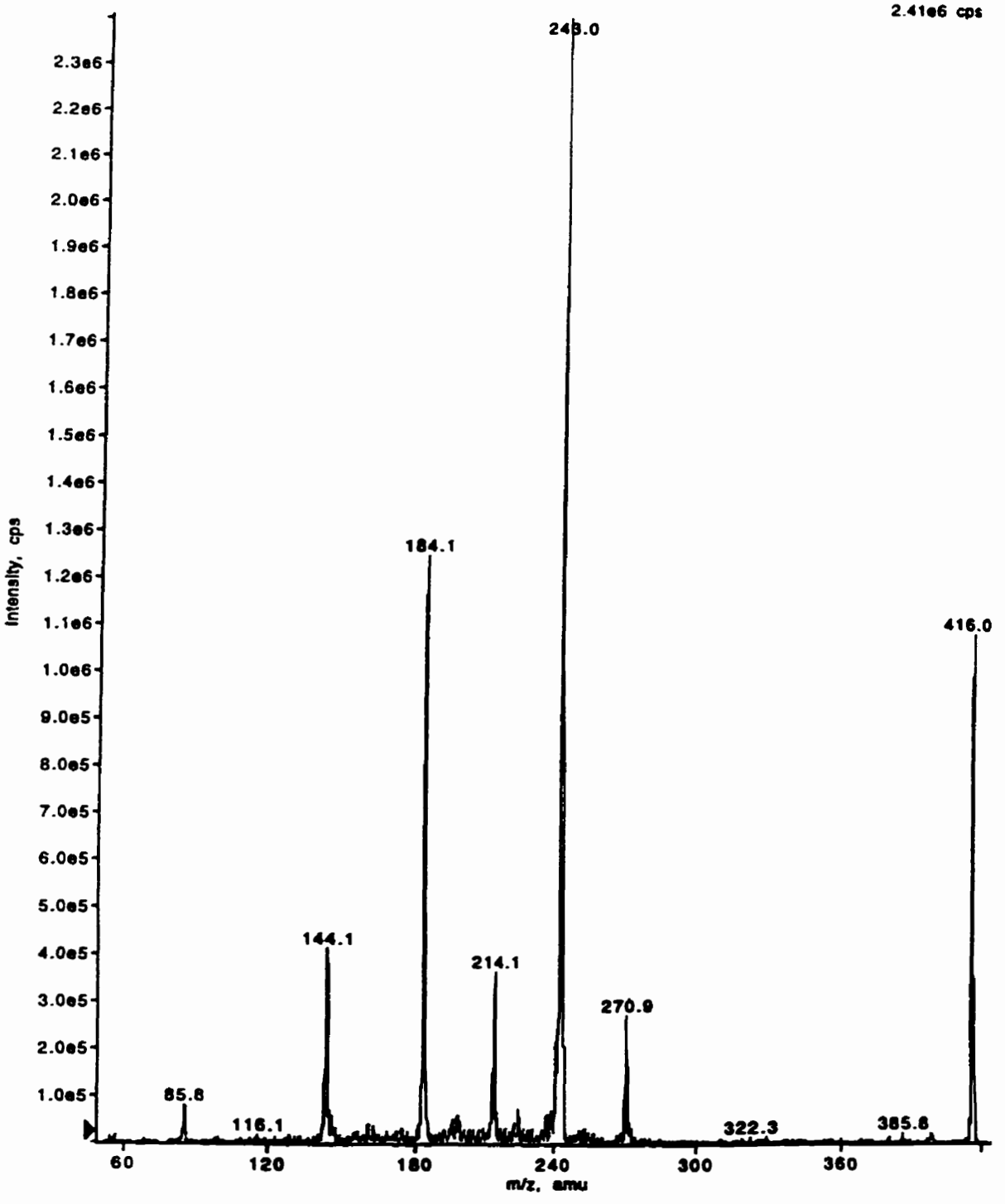


Figure 6.10 MS-MS spectrum of the ion peak (m/z 416) of peptide C-4 of α-Chymotrypsin

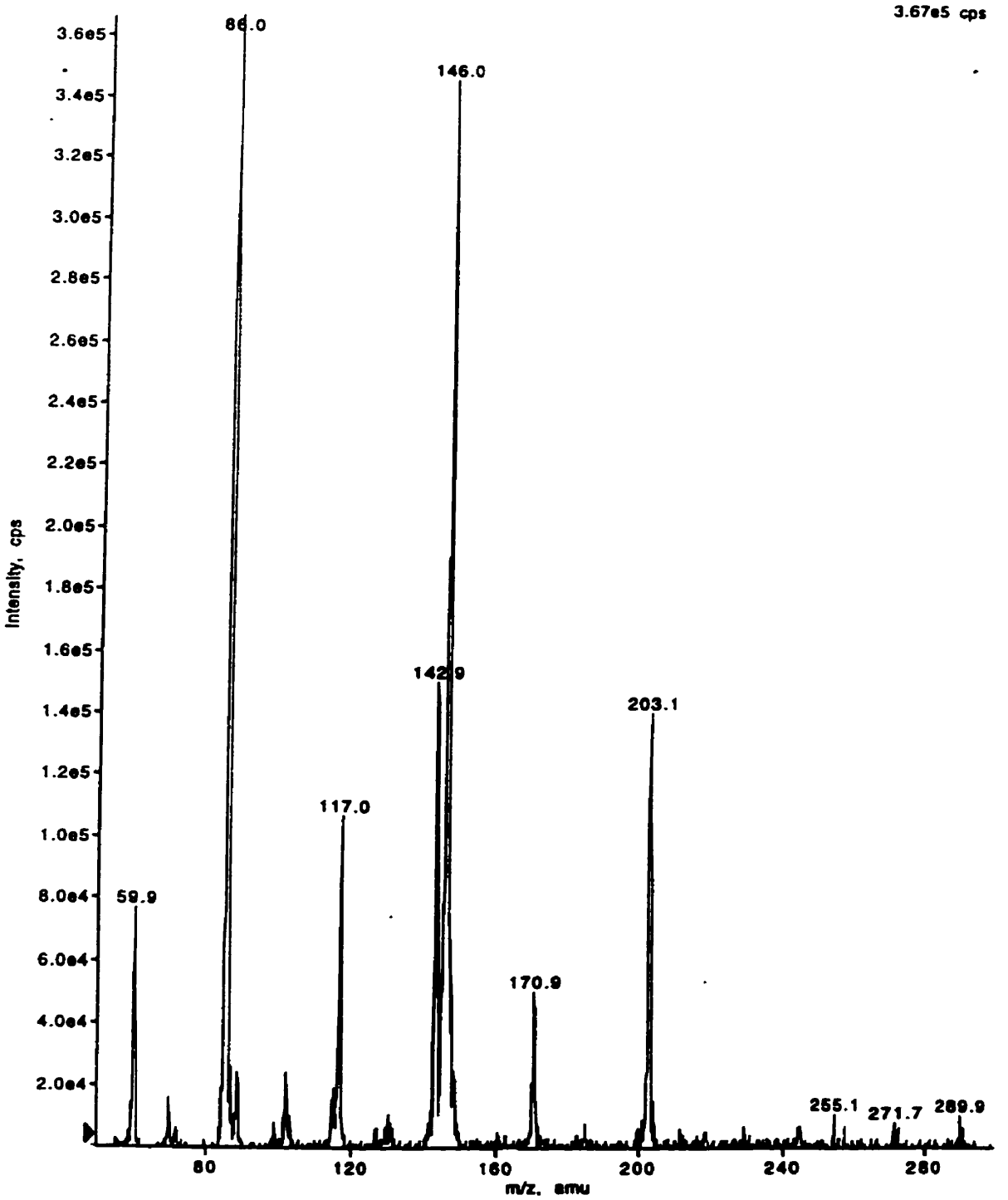


Figure 6.11 MS-MS spectrum of ion peak (m/z 290) of peptide C-4 of α-Chymotrypsin

The results of the ES-MS and MS-MS spectra of the peptide C-4 recovered from the 2-D electrophoretogram of α-chymotrypsin yield the sequence Ser-Gly-Leu, which corresponds to C-terminal sequence of chain 1 of the polypeptide (amino acids 11 through 13). These results clearly demonstrate that the method developed for the identification of C-terminal sequences is operational and can be used to elucidate the C-terminal sequence(s) of proteins.

Although several other peptides were isolated, the results obtained by ES-MS analysis did not produce spectra that could be interpreted as a result of unidentifiable cleavage patterns. Similarly, the results of the ES-MS analysis of the C-terminal peptides recovered for ribonuclease A (R-1 and R-2) and the Bt crystal protein (CAM-protoxin (S-carbaminomethylated); CAM-1 and CAM-2) could not be used to determine the C-terminal sequence of the peptides. The difficulty appears to be the result of the C-terminal sequences of the proteins, which contain acidic side chains and are therefore also derivatized to the methyl esters as discussed below.

Significantly, the intensity of the on-diagonal (C-terminal) peptides is highly variable. This is indicative of multiple sites of methylation within single peptides. The stronger signals for various peptides (R-1, CAM-1 and CAM-2) observed in the autoradiograms of ribonuclease and CAM-protoxin, and the inability to determine the sequences of these peptides, provide evidence for the incorporation of multiple methyl groups in the form of methyl esters (of acidic residues) and potential incorporation at His residues (methylation at nitrogen).

The C-terminal sequence of ribonuclease A (from amino acids 110-124; **Glu**-Gly-Asn-Pro-Tyr-Val-Pro-Val-**His**-Phe-**Asp**-Ala-Ser-Val; Dayhoff, M.O., 1972) and the

accepted C-terminal sequence of the Bt crystal protein deduced from the gene nucleotide sequence (from amino acids 1167-1176; **Asp**-Ser-Val-**Glu**-Leu-Leu-Leu-Met-**Glu-Glu**) ; Adang, M.J., et al., 1985), show that the C-termini of these proteins contain several acidic amino acid side chains (bold) which should also be converted to the methyl ester derivatives using the current methodology. The methylation pattern obtained in these peptides may vary, making analysis by the current MS method unfeasible for proteins containing acidic residues at the C-terminus, due to the algorithm employed in sequence determination. The unambiguous assignment of the cleavage pattern obtained from multiply methylated peptides, and hence the elucidation of the C-terminal sequences, would have to be accomplished by an algorithm that can analyze the total number of available methylation sites and the fragmentation patterns which would ensue.

6.4 Discussion

The development of C-terminal sequencing strategies is presently an intensive area of research. The need for sensitive methods in the characterization of proteins, particularly due to the demands imposed by biotechnology, is of obvious importance. Although a number of automated methods are being developed by several manufacturers, none of these methodologies has been shown to be of general applicability. Furthermore, none of these methods has been successful in identifying the C-terminal sequence of proteins, for which the C-terminus is unknown, by direct modification of the C-terminal carboxyl group.

The procedure developed for the isolation of C-terminal peptides presented in the current study is a variation of the general C-terminal methodology developed by Duggleby and Kaplan (1975). The current investigations were performed in order to increase the sensitivity of the method by 1) modifying the radioactive labelling procedure to increase the incorporation of the label (and thus sensitivity of the method) and 2) to combine the procedure with mass spectrometric analysis of the C-terminal peptides. The chemical labelling procedure was accomplished by non-aqueous modification of the proteins with ^{14}C-MeI (Taralp and Kaplan, 1997) to produce the methyl esters of the carboxyl groups of the proteins, and the C-terminal peptides isolated using two-dimensional HVPE. The current procedure represents an increase in sensitivity of almost two orders of magnitude, greatly due to the high degree of incorporation of label and the elimination of losses during the isolation procedure, but also due to the small quantity of starting material required for analysis by ES-MS. In this work, the entire procedure could easily be carried out on as little as 2 mg (25 nmoles) of protein (CAM-protoxin), with the isolation of C-terminal peptides from a total of 0.5 mg (6 nmoles) of the enzymatic digest. The results indicate that the procedure could be carried out on substantially less material, but would require longer exposure to the X-ray film for detection of the labelled peptides. Combining the method with ES-MS has also facilitated the analysis of the C-terminal peptides since it eliminates the need for conventional amino acid sequence analysis.

Overall, the method can be readily performed, with only minor technical difficulties (flame sealing vessels under vacuum, handling of radioactive methyl iodide) which can become routine with practice. The simplicity of the procedure (ie; the absence

of sophisticated equipment) renders the makes the methodology accessible to small laboratories and the entire procedure, excluding autoradiography, can be performed within approximately 5 days.

A concern in applying the method to the crystal protein from *Bacillus thringiensis* was the insolubility of the protein at the pH values (4 -5) required for the labelling reaction. Despite the insolubility, treatment of the derivatized crystal protein with pepsin was sufficient to liberate C-terminal peptides.

6.5 Conclusion

A sensitive method for the determination of the C-terminal sequence of proteins was developed and successfully employed in the determination of the C-terminal sequence of chain 1 of α-chymotrypsin. The method involves the selective isolation of C-terminal peptides from proteins using two-dimensional HVPE for initial isolation of the peptides, followed by sequence analysis by ES-MS. The method appears to be of general applicability with a minimum increase in sensitivity estimated at 10- to 100-fold over previous methodologies (6 nanomoles versus 40 nanomoles) (Bietlot, H.P., et al., 1989b). This increase in sensitivity is the result of the increased incorporation of the radiolabelled reagent. In the current investigation, the methodology permitted the isolation of picomolar quantities of C-terminal peptides; based on a 10% yield of C-terminal peptides.

Although the C-terminal sequences of ribonuclease and the crystal protein from *Bacillus thuringiensis* var *kurstaki* HD-73 have not be determined at this time, further investigations are being undertaken to overcome the technical difficulties associated with the final analysis of recovered C-terminal peptides.

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Claims to Original Research

1) A novel procedure for the isolation and identification of the C-terminal sequences in proteins has been developed.

2) The protection of *Bacillus thuringiensis var kurstaki* HD-73 crystals from sunlight inactivation has been demonstrated.

3) DNA-protein complexes, derived from *Bacillus thuringiensis var kurstaki* HD-73 crystals, have been prepared and characterized.

4) A novel method for the identification and isolation of phosphorylated peptides has been developed.

Publications arising from this thesis

Bietlot, H.P., Scherthaner, J.P, Milne, R.E., **Clairmont, F.R.**, Bhella, R.S., Kaplan, H. (1993). Evidence That the Cry1A Crystal Protein from *Bacillus thuringiensis* Is Associated with DNA. *Jour. Biol. Chem.* **268**, 8240-8245.

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Notes added in proof

Validation of the C-terminal Methodology

The amino acid composition of a putative C-terminal peptide from bovine pancreatic ribonuclease has been obtained using the isolation procedure described in Chapter 6. This work was carried out by Nicolas Stewart from the Department of Chemistry, during ongoing work in the development of the procedure. The amino acid composition obtained was Ala $_{1.0}$, Asp $_{0.77}$, Ser $_{0.86}$, and Val $_{1.0}$, consistent with the C-terminal sequence, Asp-Ala-Ser-Val, for this protein. Only 1 mg (50 nmol) of protein was required for this determination, and 1 nanomole of peptide was isolated from the two-dimensional electrophoretogram of the esterified protein. This data validates the methodology developed for isolating C-terminal peptides from proteins. To date, other C-terminal sequencing methodologies have only been successful in obtaining the C-terminal sequences of small polypeptides of known sequence. Still, several technical aspects are currently being addressed to facilitate the analysis of the peptides by mass spectrometry and to increase the sensitivity to picomolar quantities of peptide.

The remaining difficulties that must be solved are technical aspects involving the analysis of peptides isolated by the current procedure. The major difficulty is the inability to distinguish C-terminal peptides from contaminant signals (possibly arising from the paper used in the isolation procedure) in the initial ES-MS spectra. Also, the limited information with respect to the isolated peptides (MW and extent of derivatization / methylation) has rendered the analysis more difficult.

The current algorithm, used to search for sequences based on the molecular masses of the peptides, does not account for possible chemical modifications (esterification / methylation) of various side chains in the peptides. In particular, the masses of methylated derivatives of Asp, Glu and possibly Met and His, must be included in the algorithms used to deduce the sequence from the fragmentation pattern. Previously, the sequence of a diagonal peptide from α-chymotrypsin was obtained by mass spectrometry which corresponded to the sequence of the C-terminal peptide of chain 1 (Ser-Gly-Leu; amino acids 11 through 13) of the polypeptide. This peptide was

mass spectrometry which corresponded to the sequence of the C-terminal peptide of chain 1 (Ser-Gly-Leu; amino acids 11 through 13) of the polypeptide. This peptide was isolated and easily identified from MS-MS spectra. The ease of analysis may have been fortuitous in that the sequence contains residues that contain intact / unmodified R-groups. Putative peptides obtained from the eletrophoretograms of chymotrypsin and the Bt crystal protein, did not produce signals above background by amino acid analysis (background 100 pmol). A number of possible reasons for these difficulties and possible improvements and strategies are discussed below.

The identification of C-terminal peptides by ES-MS is one of the limitations of the current methodology. An additional piece of information that can be easily determined, and should be helpful in the analysis of signals obtained by ES-MS, is the approximate molecular mass of the isolated peptides based on their electrophoretic mobilities. This information could simplify the selection of signals obtained by ES-MS that should be analyzed by MS-MS for cleavage patterns and sequence determination. Some of the unsuccessful MS-MS analyses were conducted on components with molecular masses much greater than that expected for any peptide obtained based on the electrophoretic mobilities. Thus a preliminary molecular mass estimate could facilitate the selection of appropriate, potentially C-terminus ES-MS signals.

A final strategy is being investigated that should allow for positive identification of C-terminal peptides over possible contaminants. These studies are being carried out by Nicolas Stewart, a fellow graduate student in Dr. Kaplan's group. The strategy involves a double labelling method that will allow unambiguous identification of C-terminal peptides in the mass spectra. After initial ^{14}C-trace labelling, the second labelling is to be carried out using an equimolar mixture of CH_3 and CD_3-iodomethane. The ^{14}C-label is used to select peptides from the diagonal procedure on paper, while the CD_3-label will provide a characteristic pattern by ES-MS to identify C-terminal peptide signals derived from the methylated protein sample. A pattern of mass peaks differing in increments of 3 mass units would be obtained, with increments of three, six and nine, revealing monomethylated, dimethylated and trimethylated derivatives respectively. Thus, using this labelling strategy, the mass peak pattern would provide further evidence of the C-terminal origin of the mass peaks from the initial ES-MS spectra. The ability to

distinguish C-terminal peptides from contaminating materials would be solved unless the contaminants were already present in the starting material and were reactive towards iodomethane.

This new labelling strategy also appears to resolve the earlier problems related to the ES-MS and MS-MS analysis of multiply labelled C-terminal peptides (peptides containing carboxyl side chains mainly). Peptides that have been reacted at more than one site will provide molecular mass patterns that will also indicate the number of reactive sites in the peptide. Now, the ability to account for the number of sites of incorporation of the label (multiply labelled peptides), caused by differential labelling patterns in various peptides, will provide a confirmation of the C-terminal origin of isolated peptides. This additional labelling step will also provide a built-in check for the purity of the initial protein preparations used in the analyses.

Further studies using the aforementioned strategies are being conducted to successfully combine the diagonal isolation procedure with mass spectrometry. These strategies appear to provide a successful route towards this goal.

Amino Acid Composition of the Bt Crystal Protein from *Bacillus thuringiensis* subsp. *kurstaki* HD-73 (Pustell Sequence Analysis Programs: International Biotechnologies,Inc)

Non-polar:		Number	Percent
Ala	A	66	5.603
Val	V	84	7.131
Leu	L	94	7.980
Ile	I	74	6.282
Pro	P	53	4.499
Met	M	10	.849
Phe	F	54	4.584
Trp	W	19	1.613

Polar:		Number	Percent
Gly	G	82	6.961
Ser	S	91	7.725
Thr	T	67	5.688
Cys	C	16	1.358
Tyr	Y	57	4.839
Asn	N	85	7.216
Gln	Q	45	3.820

Acidic:		Number	Percent
Asp	D	61	5.178
Glu	E	91	7.725

Basic:		Number	Percent
Lys	K	34	2.886
Arg	R	73	6.197
His	H	22	1.868

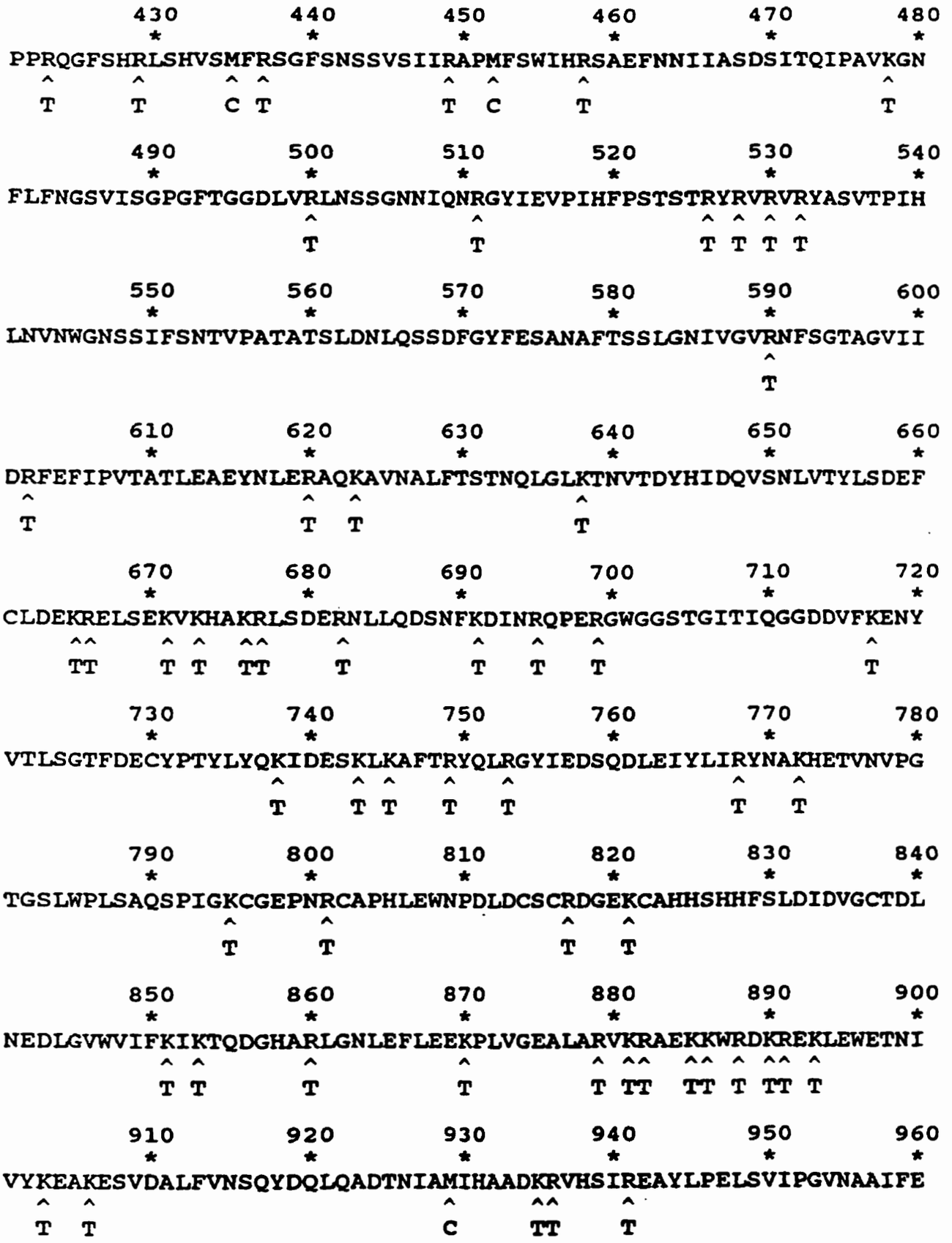
Total AAs = 1178
Calculated Molecular Weight = 133304.900
Estimated pI = 5.71

Appendix 2

Amino acid sequence of the CryIA(c) crystal protein deduced from the gene nucleotide sequence of *Bacillus thuringiensis* var. *kurstaki* HD-73 (Adang, M.J. et al., 1985), **including trypsin and cyanogen bromide cleavage sites** (Pustell Sequence Analysis Programs: International Biotechnologies,Inc)

C = CNBr T = Trypsin

```
          10         20         30         40         50         60
          *          *          *          *          *          *
MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEFVPGAGFVLGL
^                              ^
C                              T

          70         80         90        100        110        120
          *          *          *          *          *          *
VDIIWGIFGPSQWDAFLVQIEQLINQRIEEFARNQAISRLEGLSNLYQIYAESFREWEAD
                               ^     ^     ^                ^
                               T     T     T                T

         130        140        150        160        170        180
          *          *          *          *          *          *
PTNPALREEMRIQFNDMNSALTTAIPLFAVQNYQVPLLSVYVQAANLHLSVLRDVSVFGQ
       ^  ^^      ^                                       ^
       T  CT      C                                       T

         190        200        210        220        230        240
          *          *          *          *          *          *
RWGFDAATINSRYNDLTRLIGNYTDYAVRWYNTGLERVWGPDSRDWVRYNQFRRELTLTV
^           ^      ^           ^        ^      ^    ^    ^^
T           T      T           T        T      T    T    TT

         250        260        270        280        290        300
          *          *          *          *          *          *
LDIVALFPNYDSRRYPIRTVSQLTREIYTNPVLENFDGSFRGSAQGIERSIRSPHLMDIL
             ^^    ^       ^                  ^        ^   ^    ^
             TT    T       T                  T        T   T    C

         310        320        330        340        350        360
          *          *          *          *          *          *
NSITIYTDAHRGYYYWSGHQIMASPVGFSGPEFTFPLYGTMGNAAPQQRIVAQLGQGVYR
          ^          ^                     ^        ^          ^
          T          C                     C        T          T

         370        380        390        400        410        420
          *          *          *          *          *          *
TLSSTLYRRPFNIGINNQQLSVLDGTEFAYGTSSNLPSAVYRKSGTVDSLDEIPPQNNNV
       ^^                                   ^^
       TT                                   TT
```


430 440 450 460 470 480
PPRQGFSHRLSHVSMFRSGFSNSSVSIIRAPMFSWIHRSAEFNNIIASDSITQIPAVKGN
T T C T T C T T
490 500 510 520 530 540
FLFNGSVISGPGFTGGDLVRLNSSGNNIQNRGYIEVPIHFPSTSTRYRVRVRYASVTPIH
T T T T T T T T
550 560 570 580 590 600
LNVNWGNSSIFSNTVPATATSLDNLQSSDFGYFESANAFTSSLGNIVGVRNFSGTAGVII
T
610 620 630 640 650 660
DRFEFIPVTATLEAEYNLERAQKAVNALFTSTNQLGLKTNVTDYHIDQVSNLVTYLSDEF
T T T T T
670 680 690 700 710 720
CLDEKRELSEKVKHAKRLSDERNLLQDSNFKDINRQPERGWGGSTGITIQGGDDVFKENY
TT T T TT T T T T T
730 740 750 760 770 780
VTLSGTFDECYPTYLYQKIDESKLKAFTRYQLRGYIEDSQDLEIYLIRYNAKHETVNVPG
T T T T T T T T T T
790 800 810 820 830 840
TGSLWPLSAQSPIGKCGEPNRCAPHLEWNPDLDCSCRDGEKCAHHSHHFSLDIDVGCTDL
T T T T T T
850 860 870 880 890 900
NEDLGVWVIFKIKTQDGHARLGNLEFLEEKPLVGEALARVKRAEKKWRDKREKLEWETNI
T T T T T T TT TT T TT T T
910 920 930 940 950 960
VYKEAKESVDALFVNSQYDQLQADTNIAMIHAADKRVHSIREAYLPELSVIPGVNAAIFE
T T C TT T

```
         970         980         990        1000        1010        1020
          *           *           *           *           *           *
ELEGRIFTAFSLYDARNVIKNGDFNNGLSCWNVKGHVDVEEQNNQRSVLVVPEWEAEVSQ
     ^           ^   ^               ^           ^
     T           T   T               T           T

        1030        1040        1050        1060        1070        1080
          *           *           *           *           *           *
EVRVCPGRGYILRVTAYKEGYGEGCVTIHEIENNTDELKFSNCVEEEIYPNNTVTCNDYT
   ^   ^   ^   ^                       ^
   T   T   T   T                       T

        1090        1100        1110        1120        1130        1140
          *           *           *           *           *           *
VNQEEYGGAYTSRNRGYNEAPSVPADYASVYEEKSYTDGRRENPCEFNRGYRDYTPLPVG
             ^ ^                     ^     ^^        ^   ^
             T T                     T     TT        T   T

        1150        1160        1170
          *           *           *
YVTKELEYFPETDKVWIEIGETEGTFIVDSVELLLMEE
   ^           ^                   ^
   T           T                   C
```

Cleavage Fragments: **********

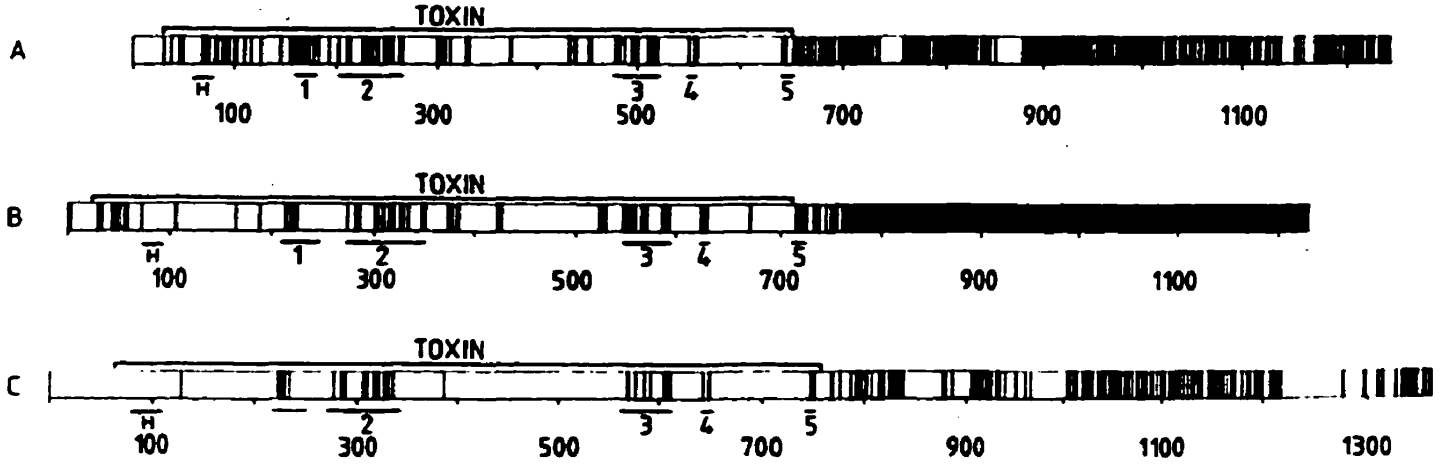
CNBr:	From	To	Mol. Wt.	Residues	pI
	453	929	53682.090	477	5.99
	930	1176	28144.360	247	5.30
	138	297	18538.980	160	11.52
	1	130	14753.840	130	4.74
	342	435	10286.870	94	11.85
	298	322	3018.220	25	5.92
	323	341	2005.170	19	3.81
	436	452	1856.020	17	12.21
	131	137	923.020	7	7.00
	1177	1178	276.240	2	3.81

Trypsin:	From	To	Mol. Wt.	Residues	pI
	17651	1115	6567.248	*****	7.00
	533	590	6140.349	58	5.32
	17480	1155	4674.189	*****	7.00
	312	349	4214.521	38	7.00
	1060	1093	3910.930	34	4.50
	370	402	3573.730	33	5.32
	822	851	3408.610	30	5.51
	907	935	3208.290	29	4.72
	639	665	3162.230	27	4.72

Appendix 3

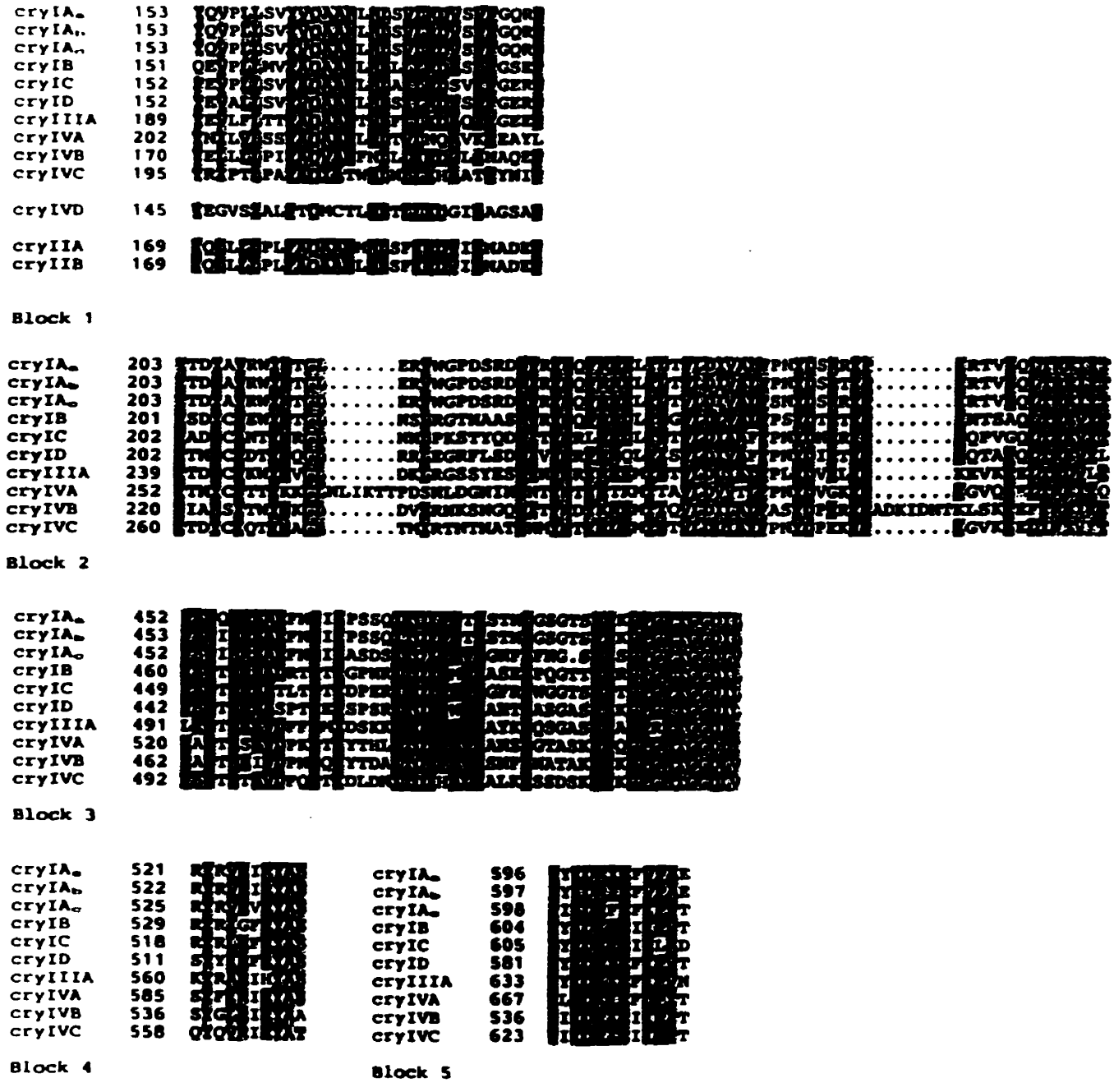
Sequence alignment of the Bt Cry1A(a), Cry1A(b) and Cry1A(c) crystal proteins.

Conserved sequences are indicated by vertical lines (Höfte and Whiteley, 1989).



Appendix 4

Conserved Amino Acid Sequence Blocks for All Crystal Proteins Encoded by Cry Genes (except CryII and CryIVD) (Höfte and Whiteley, 1989).



Appendix 5

Full list of *Bacillus thuringiensis* delta-endotoxin genes
(http://epunix.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/toxins.html)

Name	Original	Accession	Reference	Year	Journal	Coding Region
cry1Aa1	cryIA(a)	M11250	Schnepf et al	1985	JBC 260 6264-6272	527-4054
cry1Aa2	cryIA(a)	M10917	Shibano et al	1985	Gene 34 243-251	153->2955
cry1Aa3	cryIA(a)	D00348	Shimizu et al	1988	ABC 52 1565-1573	73-3603
cry1Aa4	cryIA(a)	X13535	Masson et al	1989	NAR 17 446-446	1-3528
cry1Aa5	cryIA(a)	D17518	Udayasuriyan et al	1994	BBB 58 830-835	81-3611
cry1Ab1	cryIA(b)	M13898	Wabiko et al	1986	DNA 5 305-314	142-3606
cry1Ab2	cryIA(b)	M12661	Thorne et al	1986	J Bact 166 801-811	155-3625
cry1Ab3	cryIA(b)	M15271	Geiser et al	1986	Gene 48 109-118	156-3623
cry1Ab4	cryIA(b)	D00117	Kondo et al	1987	ABC 51 455-463	163-3630
cry1Ab5	cryIA(b)	X04698	Hofte et al	1986	EJB 161 273-280	141-3605
cry1Ab6	cryIA(b)	M37263	Hefford et al	1987	J Biotech 6 307-322	73-3540
cry1Ab7	cryIA(b)	X13233	Haider & Ellar	1988	NAR 16 10927-10927	1-3465
cry1Ab8	cryIA(b)	M16463	Oeda et al	1987	Gene 53 113-119	157-3624
cry1Ab9	cryIA(b)	X54939	Chak & Jen	1993	PNSCRC 17 7-14	73-3540
cry1Ac1	cryIA(c)	M11068	Adang et al	1985	Gene 36 289-300	388-3921
cry1Ac2	cryIA(c)	M35524	Von Tersch et al	1991	AEM 57 349-358	239-3772
cry1Ac3	cryIA(c)	X54159	Dardenne et al	1990	NAR 18 5546-5546	339->2192
cry1Ac4	cryIA(c)	M73249	Payne et al	1991	USP 4990332	1-3537
cry1Ac5	cryIA(c)	M73248	Payne et al	1992	USP 5135867	1-3534
cry1Ad	cryIA(d)	M73250	Payne & Sick	1993	USP 5246852	1-3537
cry1Ae	cryIA(e)	M65252	Lee & Aronson	1991	J Bact 173 6635-6638	81-3623

			Whiteley	1988	2723-2724	1-3684
cry1Bb	ET5	L32020	Donovan & Tan	1994	USP 5322687	67-3753
cry1Bc	cryIb(c)	Z46442	Bishop et al	1994	unpublished	141-3839
cry1Ca1	cryIC	X07518	Honee et al	1988	NAR 16	47-3613
cry1Ca1	cryIC	X07518	Honee et al	1988	6240-6240	47-3613
cry1Ca2	cryIC	X13620	Sanchis et al	1989	Mol Micro 3 229-238	241->2711
cry1Ca3	cryIC	M73251	Payne & Sick	1993	USP 5246852	1-3570
cry1Cb	cryIC(b)	M97880	Kalman et al	1993	AEM 59 1131-1137	296-3823
cry1Da	cryID	X54160	Hofte et al	1990	NAR 18 5545-5545	264-3758
cry1Db	prtB	Z22511	Lambert	1993	unpublished	241-3720
cry1Ea1	cryIE	X53985	Visser et al	1990	J Bact 172 6783-6788	130-3642
cry1Ea2	cryIE	X56144	Bosse et al	1990	NAR 18 7443-7443	1-3516
cry1Ea3	cryIE	M73252	Payne & Sick	1991	USP 5039523	1-3516
cry1Eb	cryIE(b)	M73253	Payne & Sick	1993	USP 5206166	1-3522
cry1Fa1	cryIF	M63897	Chambers et al	1991	J Bact 173 3966-3976	478-3999
cry1Fa2	cryIF	M73254	Payne & Sick	1993	USP 5188960	1-3525
cry1Fb	prtD	Z22512	Lambert	1993	unpublished	483-4004
cry1G	prtA	Z22510	Lambert	1993	unpublished	67-3564
cry1H	prtC	Z22513	Lambert	1993	unpublished	530-4045
cry1Ia1	cryV	X62821	Tailor et al	1992	Mol Micro 6 1211-1217	355-2511
cry1Ia2	cryV	M98544	Gleave et al	1993	AEM 59 1683-1687	1-2160
cry1Ia3	cryV	L36338	Shin et al	1994	unpublished	279-2438
cry1Ib	cryV	U07642	Shin et al	1994	unpublished	237-2393
cry1Ja	ET4	L32019	Donovan & Tan	1994	USP 5322687	99-3519
cry1Jb	ET1	U31633	Von Tersch	1994	USP 5356623	177-3686
cry1K		U28801	Koo	1995	unpublished	
cry2Aa1	cryIIA	M31738	Donovan et al	1989	JBC 264 4740-4740	156-2054
cry2Aa2	cryIIA	M23723	Widner & Whiteley	1989	J Bact 171 965-974	1840-3741
cry2Ab1	cryIIB	M23724	Widner & Whiteley	1989	J Bact 171 965-974	1-1899
cry2Ab2	cryIIB	X55416	Dankocsik et al	1990	Mol Micro 4 2087-2094	874-2775
cry2Ac	cryIIC	X57252	Wu et al	1991	FEMS 81 31-36	2125-3990
cry3A1	cryIIIA	M22472	Herrnstadt et al	1987	Gene 57 37-46	25-1956
cry3A2	cryIIIA	J02978	Sekar et al	1987	PNAS 84 7036-7040	241-2175
cry3A3	cryIIIA	Y00420	Hofte et al	1987	NAR 15 7183-7183	566-2497
cry3A4	cryIIIA	M30503	McPherson et al	1988	Bio/technology	201-2135

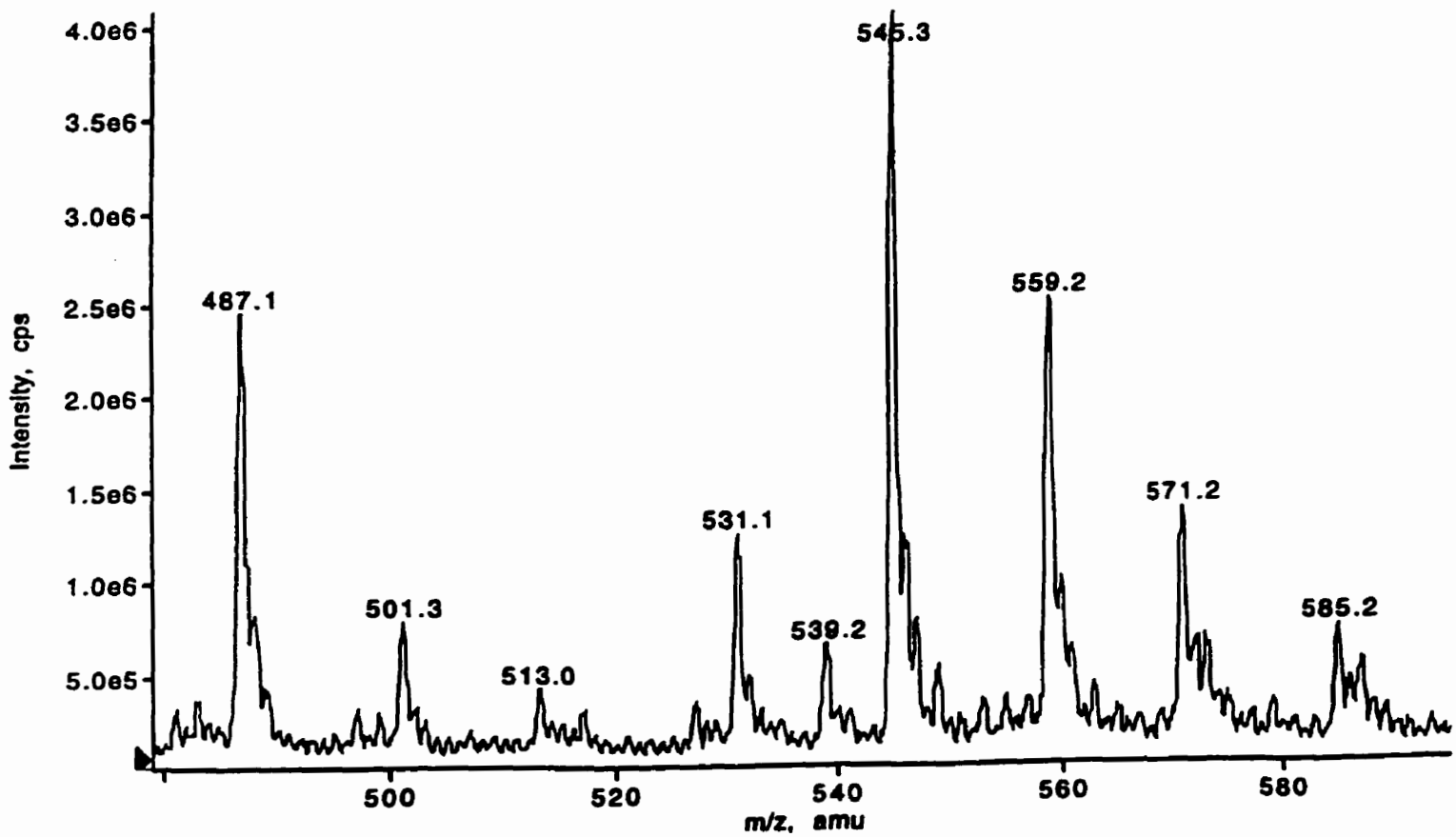
cry3A4	cryIIIA	M30503	McPherson et al	1988	6 61-66	201-2135
cry3A5	cryIIIA	M37207	Donovan et al	1988	MGG 214 365-372	569-2503
cry3A6	cryIIIA	U10985	Adams et al	1994	Mol Micro 14 381-389	569-2503
cry3Ba1	cryIIIB	X17123	Sick et al	1990	NAR 18 1305-1305	25->1977
cry3Ba2	cryIIIB	A07234	Peferoen et al	1990	EP 0382990	342-2297
cry3Bb	cryIIIBb	M89794	Donovan et al	1992	AEM 58 3921-3927	202-2157
cry3C	cryIIID	X59797	Lambert et al	1992	Gene 110 131-132	232-2178
cry4A1	cryIVA	Y00423	Ward & Ellar	1987	NAR 15 7195-7195	1-3540
cry4A2	cryIVA	D00248	Sen et al	1988	ABC 52 873-878	393-3935
cry4B1	cryIVB	X07423	Chungjatpornchai et al	1988	EJB 173 9-16	157-3564
cry4B2	cryIVB	X07082	Tungpradubkul et al	1988	NAR 16 1637-1638	151-3558
cry4B3	cryIVB	M20242	Yamamoto et al	1988	Gene 66 107-120	526-3933
cry4B4	cryIVB	D00247	Sen et al	1988	ABC 52 873-878	461-3868
cry5Aa	cryVA(a)	L07025	Sick et al	1994	USP 5281530	1->4155
cry5Ab	cryVA(b)	L07026	Narva et al	1991	EP 0462721	1->3867
cry5B	PS86Q3	U19725	Payne & Michaels	1995	USP 5427786	1->3735
cry6A	cryVIA	L07022	Narva et al	1993	USP 5236843	1->1425
cry6B	cryVIB	L07024	Narva et al	1991	EP 0462721	1->1185
cry7Aa	cryIIIC	M64478	Lambert et al	1992	AEM 58 2536-2542	184-3597
cry7Ab1	cryIIICb	U04367	Payne & Fu	1994	USP 5286486	1->3414
cry7Ab2	cryIIICc	U04368	Payne & Fu	1994	USP 5286486	1->3414
cry8A	cryIIIE	U04364	Foncerrada et al	1992	EP 0498537	1->3471
cry8B	cryIIIG	U04365	Michaels et al	1993	WO 93/15206	1->3507
cry8C	cryIIIF	U04366	Ogiwara et al.	1995	Curr Micro 30 227-235	1-3447
cry9A1	cryIG	X58120	Smulevitch et al	1991	FEBS 293 25-28	522-3989
cry9A2	cryIG	X58534	Gleave et al	1992	JGM 138 55-62	385->3837
cry9B	cryIX	X75019	Shevelev et al	1993	FEBS 336 79-82	26-3488
cry9C	cryIH	Z37527	Lambert et al	1994	WO 94/05771	2096-5569
cry10A	cryIVC	M12662	Thorne et al	1986	J Bact 166 801-811	941-2965
cry11A1	cryIVD	M31737	Donovan et al	1988	J Bact 170	41-1969

cry11A1	cryIVD	M31737	Donovan et al	1988	4732-4738	41-1969	
cry11A2	cryIVD	M22860	Adams et al	1989	J Bact 171 521-530		cry11BJeg80X
cry12A	cryVB	L07027	Narva et al	1991	EP 0462721	1->3771	
cry13A	cryVC	L07023	Schnepf et al	1992	WO 92/19739	1-2409	
cry14A	cryVD	U13955	Payne & Narva	1994	WO 94/16079	1-3558	
cry15A	34kDa	M76442	Brown & Whiteley	1992	J Bact 174 549-557	1036-2055	
cyt1A1	cytA	X03182	Waalwijk et al	1985	NAR 13 8207-8217	140-886	
cyt1A2	cytA	X04338	Ward & Ellar	1986	JMB 191 1-11	509-1255	
cyt1A3	cytA	Y00135	Earp & Ellar	1987	NAR 15 3619-3619	36-782	
cyt1A4	cytA	M35968	Galjart et al	1987	Curr Micro 16 171-177	67-816	
cyt2A	cytB	Z14147	Koni & Ellar	1993	JMB 229 319-327	270-1046	

Last Updated 6th July 1995

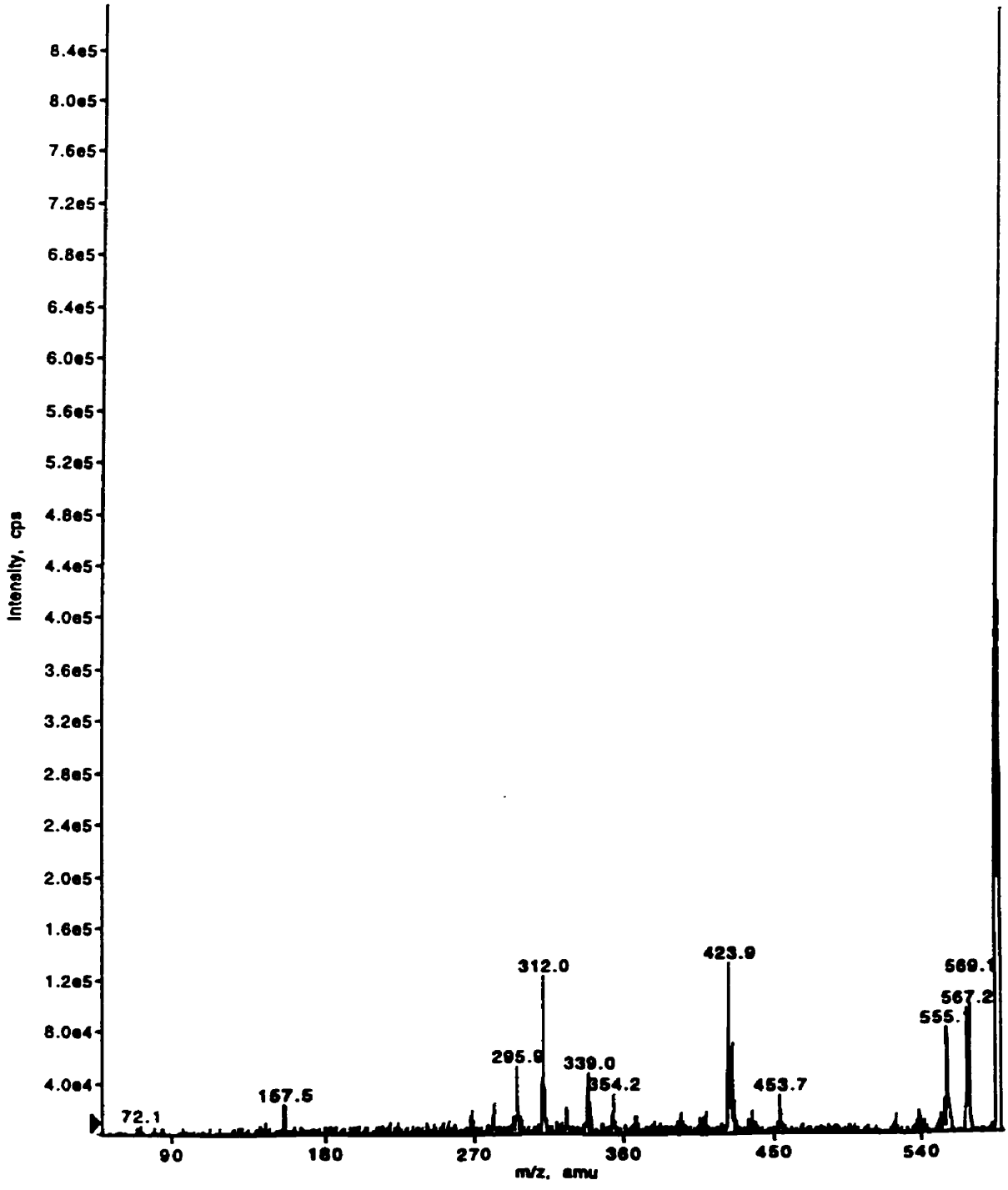
Appendix 6

ES-MS Spectrum of Peptide R-1 from Ribonuclease A



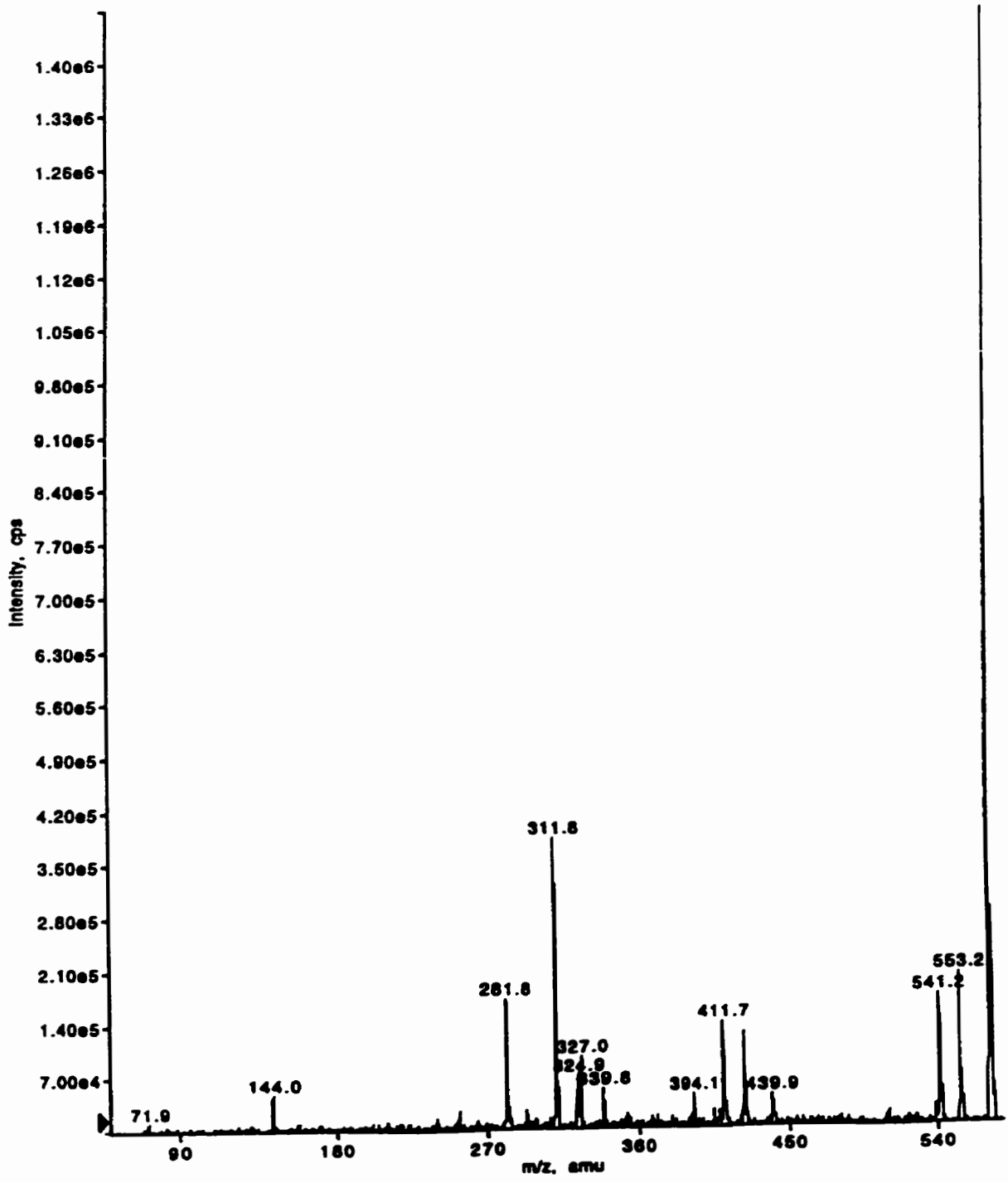
Appendix 7

MS-MS Spectrum of Peptide R-1 (m/z 585.2) from Ribonuclease A



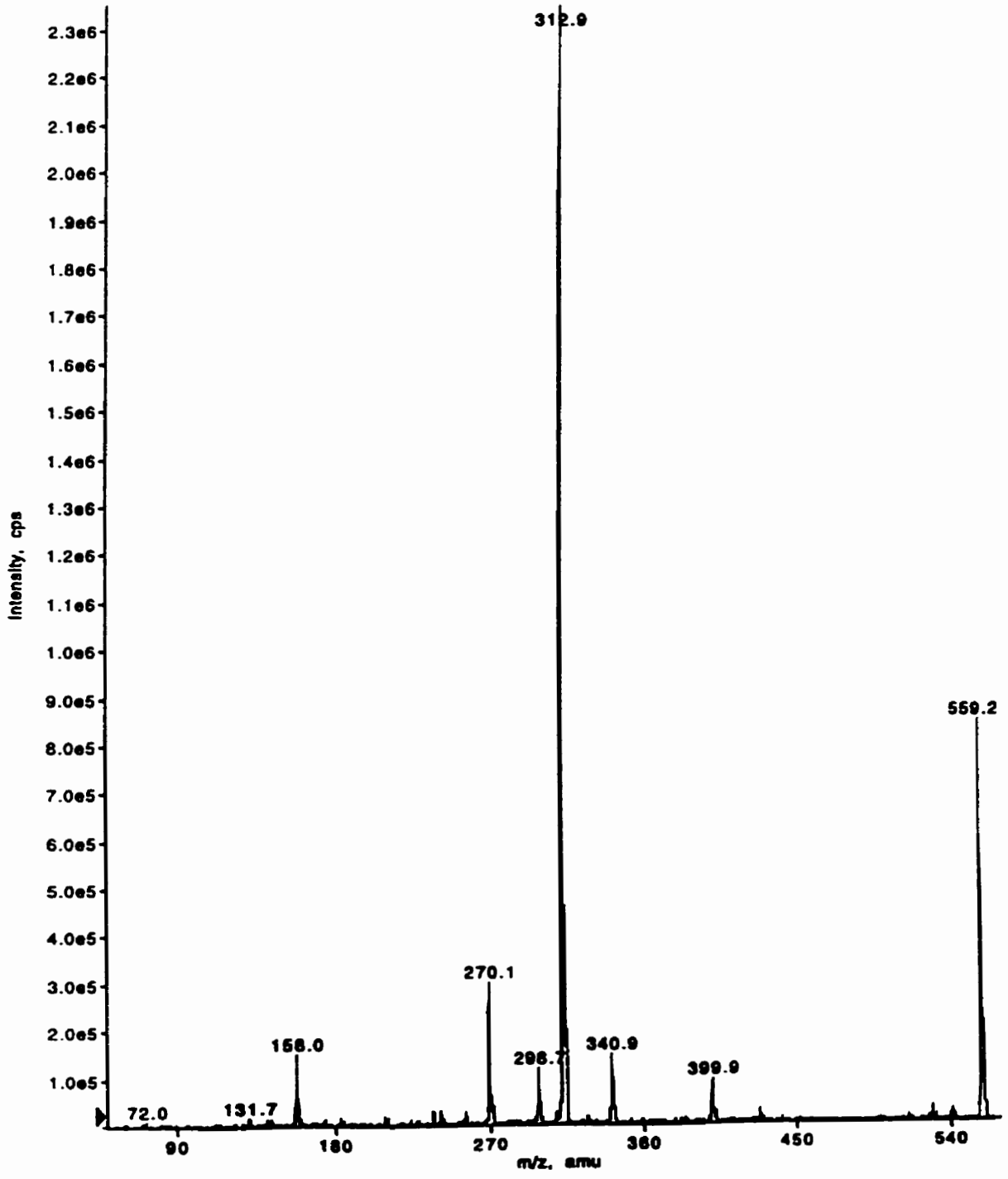
Appendix 8

MS-MS Spectrum of Peptide R-1 (m/z 571.2) from Ribonuclease A



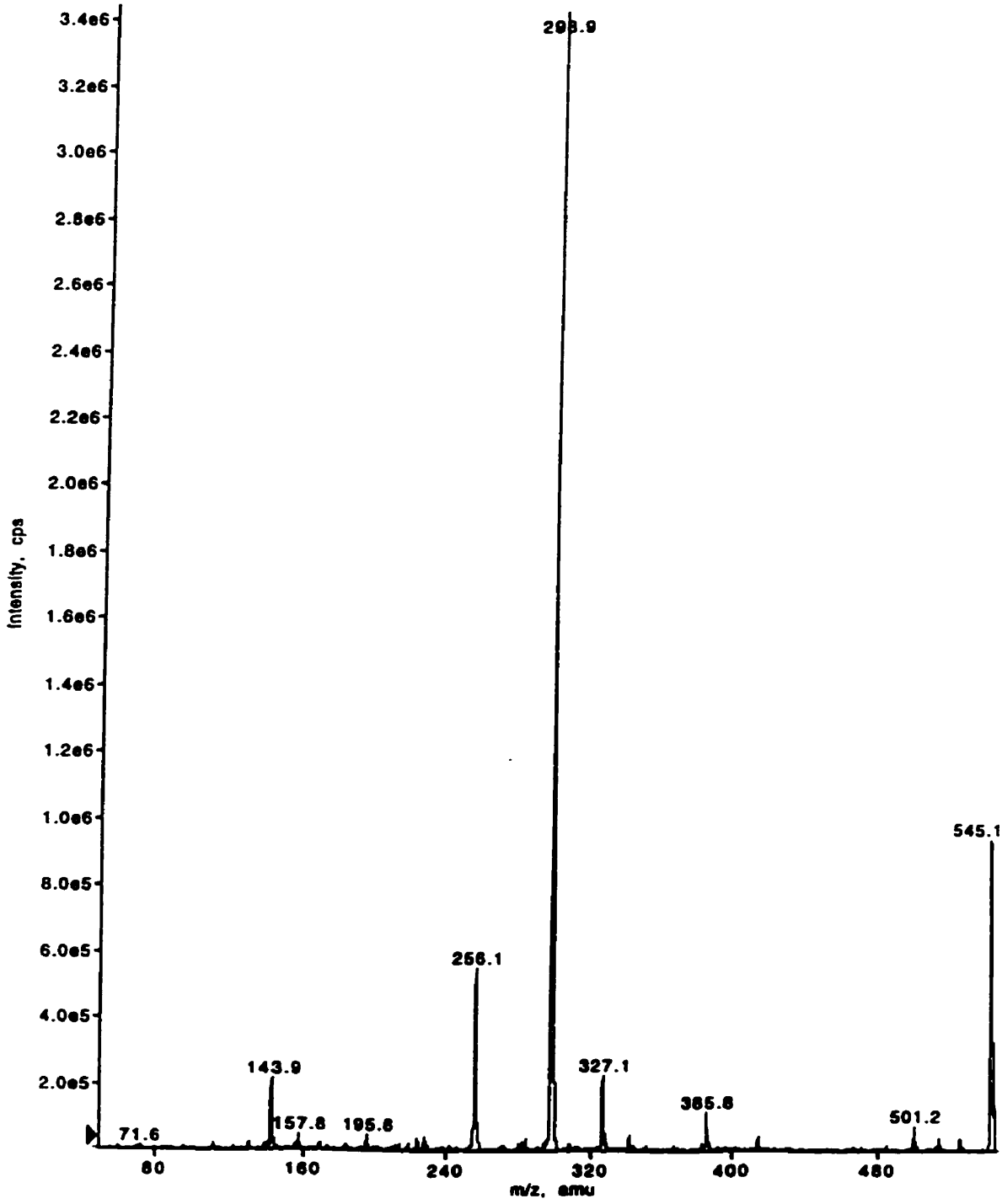
Appendix 9

MS-MS Spectrum of Peptide R-1 (m/z 559.2) from Ribonuclease A



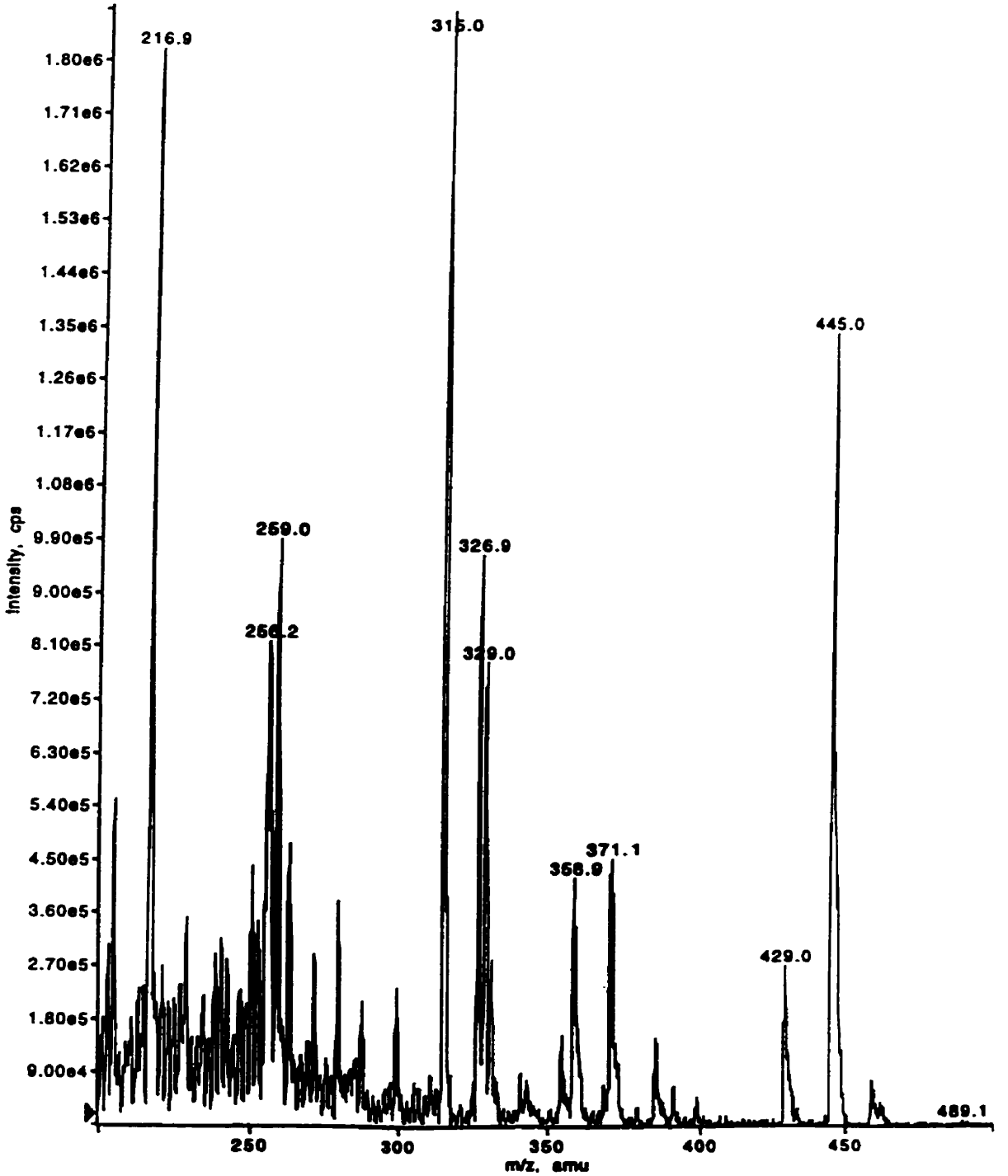
Appendix 10

MS-MS Spectrum of Peptide R-1 (m/z 545.2) from Ribonuclease A



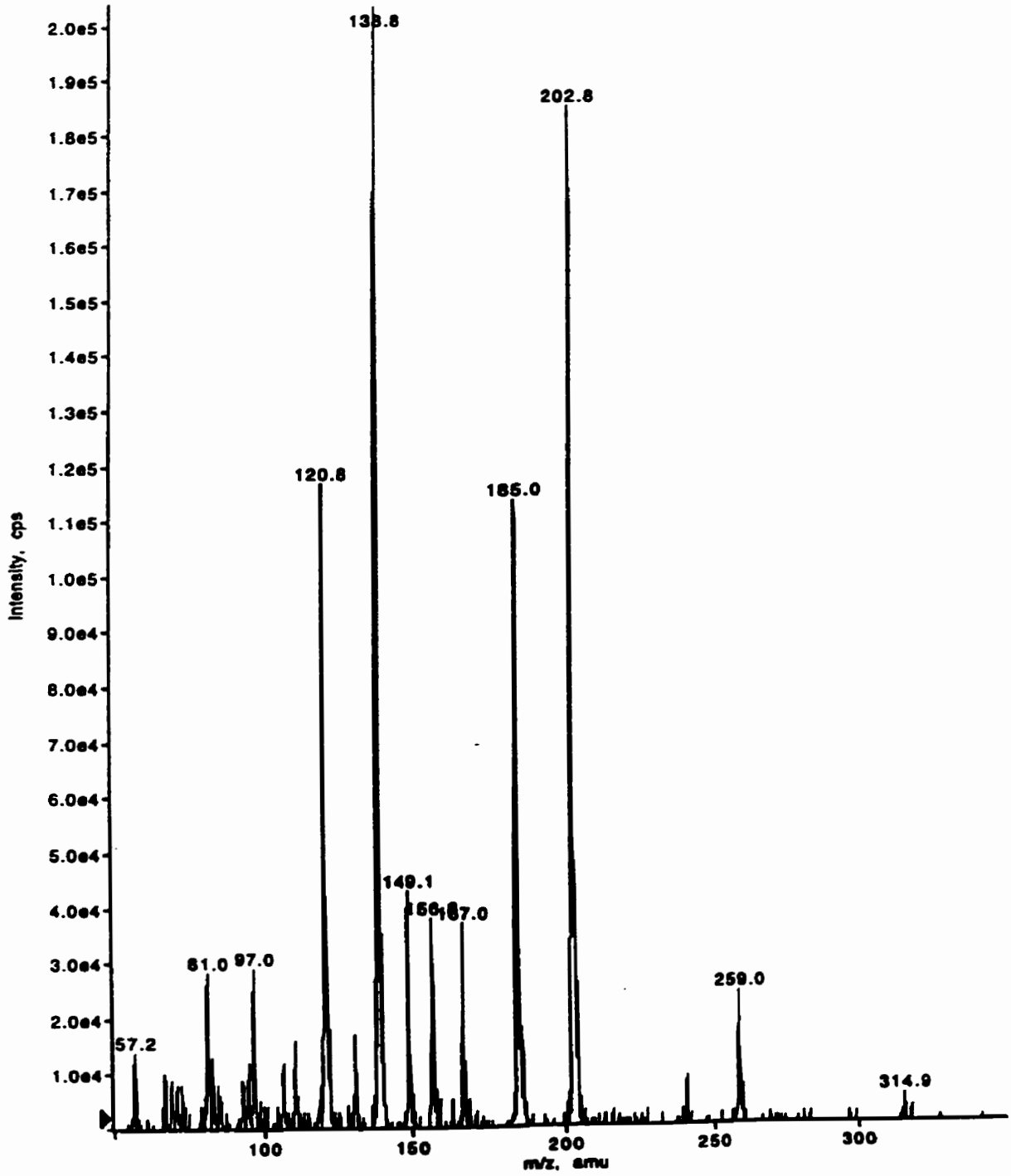
Appendix 11

ES-MS Spectrum of Peptide R-2 from Ribonuclease A



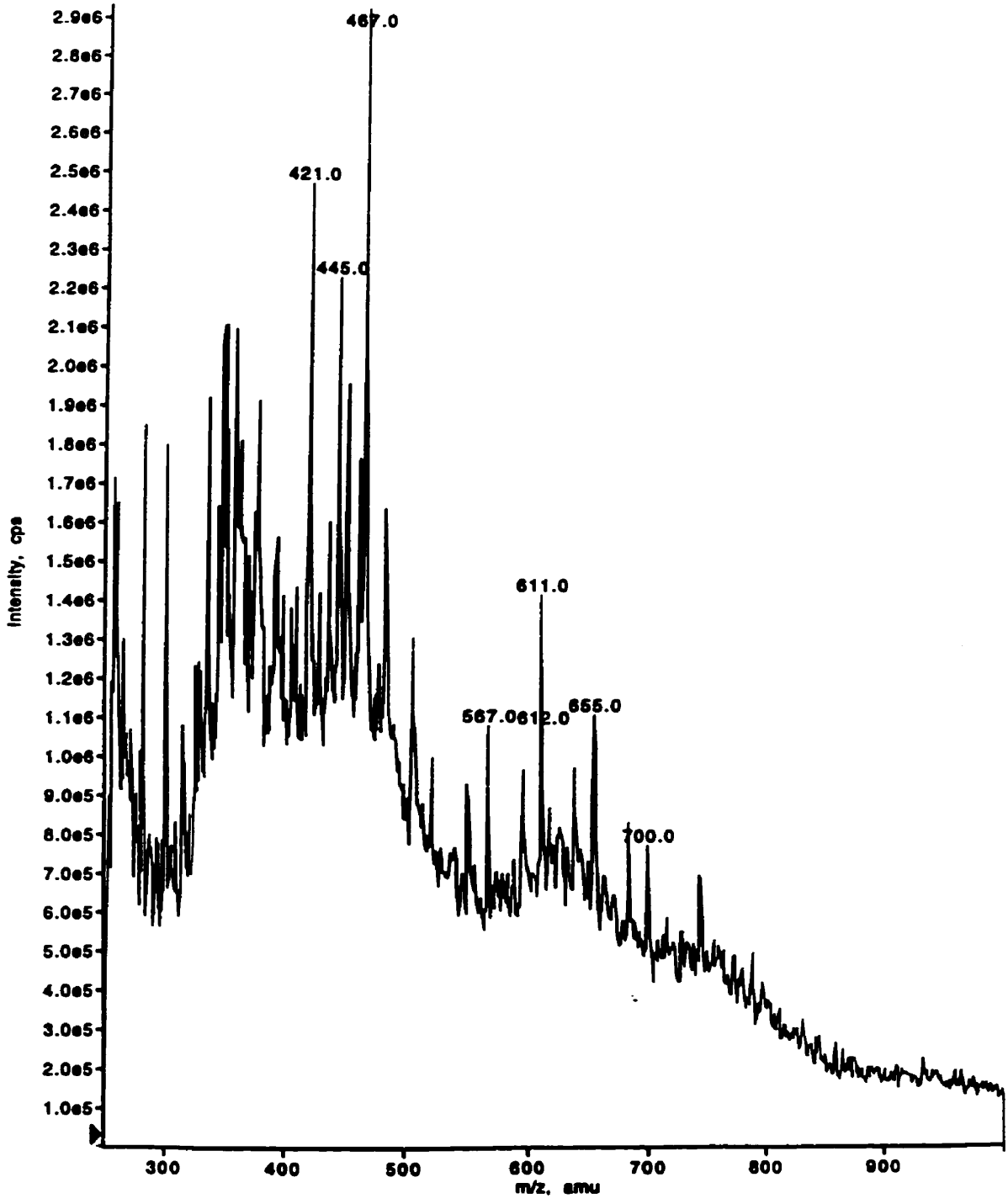
Appendix 12

MS-MS Spectrum of Peptide R-2 (m/z 315) from Ribonuclease A



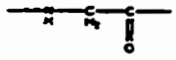
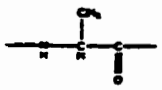
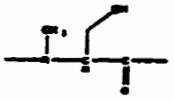
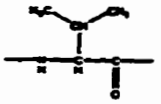
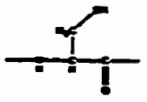
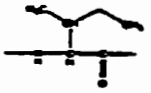
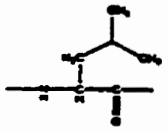
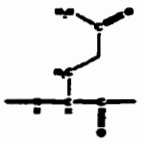
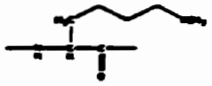
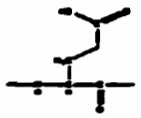
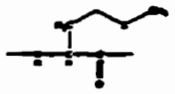
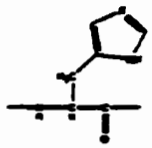
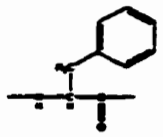
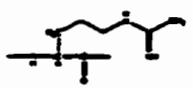
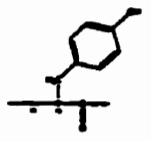
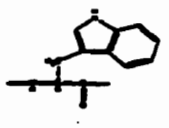
Appendix 13

ES-MS Spectrum of Peptide CAM-1 Bt Crystal Protein



Appendix 14

Table of Common Amino Acid Residues and Masses

Amino Acid	Structure	Monoisotopic Mass	Average Mass
Gly, G		57.02146	57.0520
Ala, A		71.03711	71.0788
Ser, S		87.03203	87.0782
Pro, P		97.05279	97.1167
Val, V		99.06847	99.1326
Thr, T		101.04768	101.1051
Cys, C		103.00919	103.1448
Ile, I		113.08406	113.1595
Leu, L		113.08406	113.1595
Asn, N		114.04293	114.1039
Asp, D		115.02694	115.0886
Gln, Q		128.05858	128.1308
Lys, K		128.09496	128.1742
Glu, E		129.04259	129.1155
Met, M		131.04049	131.1986
His, H		137.05891	137.1412
Phe, F		147.06841	147.1766
Arg, R		156.10111	156.1876
Tyr, Y		163.06333	163.1760
Trp, W		186.07931	186.2133